March 2012 Volume 95 Supplement ISSN 0301-4797

ELSEVIER

Journal of Environmental Management

Environmental Risks and Problems, Strategies to reduce them through Biotechnology and Engineering

Guest Editors: Isabel Sastre-Conde, Héctor Poggi-Varaldo, M. Carmen Lobo, J. Luis Sanz, Hervé Macarie

Govern de les Illes Balears
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MINISTERIO DE ECONOMÍA Y COMPETITIVIDAD

Journal of

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The Journal of Environmental Management is a journal for the publication of peer reviewed, original research for all aspects of management and the managed use of the environment, both natural and man-made. Critical review articles are also welcome; submission of these is strongly encouraged.

As governments and the general public become more keenly aware of the critical issues arising from man's use of his environment, this journal provides a forum for the discussion of environmental problems around the world and for the presentation of management results. It is aimed not only at the environmental manager, but at anyone concerned with the sustainable use of environmental resources.

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DE ECONOMÍA
Y COMPETITIVIDAD

ELSEVIER

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Publication information: *Journal of Environmental Management* (ISSN 0301-4797). For 2012, volumes 93-110 (18 issues) are scheduled for publication. Subscription prices are available upon request from the Publisher or from the Elsevier Customer Service Department nearest you or from this journal's website (http://www.elsevier.com/locate/jenvman). Further information is available on this journal and other Elsevier products through Elsevier's website (http://www.elsevier.com). Subscriptions are accepted on a prepaid basis only and are entered on a calendar year basis. Issues are sent by standard mail (surface within Europe, air delivery outside Europe). Priority rates are available upon request. Claims for missing issues should be made within six months of the date of dispatch.

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USA mailing notice: *Journal of Environmental Management* (ISSN 0301-4797) is published by Elsevier Ltd. (The Boulevard, Langford Lane, Kidlington, Oxford OX5 1GB, UK). Periodical postage paid at Rahway NJ and additional mailing offices.

USA POSTMASTER: Send change of address to *Journal of Environmental Management*, Elsevier Customer Service Department, 3251 Riverport Lane, Maryland Heights, MO 63043, USA.

AIRFREIGHT AND MAILING in USA by Mercury International Limited, 365, Blair Road, Avenel, NJ 07001.

Printed by Henry Ling Ltd., The Dorset Press, Dorchester, UK

♾ The paper used in this publication meets the requirements of ANSI/NISO Z39.48-1992 (Permanence of Paper)

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Journal of Environmental Management 95 (2012) S1–S2

Contents lists available at SciVerse ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Editorial

Preface

Sustainable development should become the basis for the life of future generations as opposed to over-exploitation of non-renewable energy and material resources and the shortening of life cycles. Here, the synergistic interaction of Environmental Biotechnology and Environmental Engineering should be present as an intelligent tool, which must be used with responsibility, in remediation strategies on the true pollution risks and potentials of the environment. Therefore, Environmental Biotechnology and Environmental Engineering are two faces of a modern, valuable, and indispensable scientific and technical coin.

The growing significance and awareness of environmental problems, caused especially by use of fossil resources in connection with industrial pathways of production, depletion of finite natural resources, mismanagement of renewable resources, etc., have led to the development of both disciplines. They have their own historical roots, i.e., one has blossomed from Biotechnology and the other has grown from the old Civil and Sanitary Engineering. Yet, they have developed into full fledged branches of knowledge and specialization, and at the same time they complement each other.

Regarding Environmental Biotechnology, its contributions span from environmentally-friendly and cost effective "end-of-the-pipe" solutions to environmental pollution and problems (bioremediation of soils and aquifers, biological waste treatment), to the development of sustainable alternatives for their prevention and alleviation, such as the replacement of fossil fuels by biohydrogen and methane from wastes and futuristic "biorefineries". Biotechnology has the potential of a reduction of operational and investment costs for the design and operation of more sustainable processes based on microbes and other living organisms as agents. Yet, so far the sustainability of technical processes is more the exception than the rule. In this regard, Environmental Biotechnology is a serious candidate to provide substantial advances in the near future.

On the other hand, Environmental Engineering has developed several significant fields of research and applications (everything seems to matter in Environmental Engineering; natural sciences as well as social sciences are as significant to the practice of environmental engineering as engineering skills); some of them partially overlap with Environmental Biotechnology (for instance, biological waste treatment), whereas other subjects are original and cover environmental issues that Environmental Biotechnology cannot, and have proved to be of use to other branches of knowledge. With respect to this, we would like to highlight a significant contribution of Environmental Engineering that has transcended to other fields of Engineering and Technology: sound Environmental Engineering has designed the imprescindible framework of System Engineering Analysis applied to environmental issues, also known as Life Cycle Analysis (LCA) and other denominations. The contemporary history of industry and technology has sadly taught us that new technological solutions and new processes derived from Environmental Biotechnology (and from other fields of knowledge) should be examined under the light of LCA and environmental impact analysis before attempting their implementation. Very often, a precipitated and irreflexive application of a new product or process has led to adverse impacts on health and the environment that have become technical, ethical and economic burdens on modern societies.

There were several international and regional events dealing with Biotechnology but no international event was devoted to Environmental Biotechnology. At most, Environmental Biotechnology has one or two sessions in a Biotechnology Congress. On the other hand, most regional Environmental Engineering events showed a strong commercial component that negatively competed with the exchange of advanced knowledge and the formation of research networks. Moreover, Environmental Biotechnology and Environmental Engineering are two dynamic motors with a strong interaction and the scientific community could obtain several advantages from their joint diffusion. In short, there was a need for an event dedicated to both disciplines. This way in 2004, "The First International Meeting on Environmental Biotechnology and Engineering" (1IMEBE) was born in Mexico City, Mexico, guided by the concern of Dr. Poggi-Varaldo and a group of pioneering biotechnologies in Mexico led by Dr. Fernando Esparza-García and Professor Elvira Ríos-Leal, accompanied by a constellation of international scientists such as Dr. Isabel Sastre-Conde from Spain, Dr. Hervé Macarie from France, Dr. Franco Cecchi from Italy, Dr. Irene Watson-Craik from Scotland, and others, who had identified a gap in the diffusion of both Environmental Biotechnology and Environmental Engineering. This was particularly true for developing countries, although the situation in developed countries was not much better. From that moment on, all the Organization's activities have been guided toward an International Meeting on Environmental Biotechnology and Engineering that has significantly grown and matured. Its outreach has been multiplied by a factor of 10 compared to that of the 1st IMEBE in the 3IMEBE (Third International Meeting on Environmental Biotechnology and Engineering, source from the present work). Especially in view of the current global situation of the planet, considering the trade and technology imbalance between the North and South, such imbalances especially among developing countries and those in developing countries, which have diverse implications for the environment and ecological diversity in these countries.

0301-4797/$ – see front matter
doi:10.1016/j.jenvman.2011.12.016

We conclude this preface with two parting recommendations: Enjoy and profit from the knowledge condensed in this work and please be actively involved in the exciting adventure of forging the coming 4IMEBE. Where we can draw up a work plan among all participant colleagues, that must too aim to achieve a same goal prepared jointly, that is, a planet of solidarity, whose wealth and strength are at the very heart of its diversity, thus healthiest. Thus, among all must make blurring limits of the imaginary line built up by economy among countries, when uniquely are true its environmental disasters due to the direct effect of our collective way of life.

Thanks on behalf of each editor to all authors of this special issue, whom contributed with the 3IMEBE (http://www.uibcongres.org/imgdb/archivo_doc7256.pdf), and the different members of JEM involved in "Journal of Environmental Management Vol 95/S (2012)" by their important collaborative role in this work.

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Journal of Environmental Management 95 (2012) S3–S8

Contents lists available at ScienceDirect

Journal of Environmental Management

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Physico-chemical and biological studies on water from Aries River (Romania)

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ARTICLE INFO

Article history:
Received 1 September 2009
Received in revised form
3 April 2011
Accepted 27 April 2011
Available online 18 May 2011

Keywords:
Water
Pollution
Heavy metals
Stable isotopes
Diatoms

ABSTRACT

Our work was focused on physico-chemical and biological characteristics of Aries River, one of the largest rivers from Romania. Water samples were collected from 11 sites along Aries River course. We have measured de ^{18}O and D isotopic composition of Aries River water in these locations and correlated these data with the isotopic composition of aquatic plants and with the pollution degree. Some ions from Aries River water were also analyzed: NO_3^-, NO_2^-, PO_4^{3-} Cu^{2+}, Fe^{3+}. Analysis of diatom communities has been performed in order to quantify the level of water pollution of Aries River. All physico-chemical analyses revealed that the most polluted site is Abrud; the source of pollution is most probably the mining enterprise from Rosia Montana. Water isotope content increases from upstream to downstream of the locations analyzed. The structure of diatom communities is strongly influenced by the different pollution sources from this area: mine waters, industrial waters, waste products, land cleaning, tourism etc. The water eutrophication increases from upstream of Campeni to downstream of Campia Turzii.

1. Introduction

Toxic substances can enter lakes, streams, rivers and other water bodies and they get dissolved or lie suspended in water or get deposited on the bed. This results in the pollution of water, affecting aquatic ecosystems. Water pollution has many sources. Organic wastes are produced by animals and humans, and include such things as fecal matter, crop debris (Stoate et al., 2009), yard clippings, food wastes, rubber, plastic, wood, and disposable diapers (Finnveden et al., 2009). Minerals, such as iron, copper, chromium, platinum, nickel, zinc, and tin, can be discharged into streams and lakes as a result of various mining activities (Gray, 1998), oil and gas technology (Fakhru'l-Razi et al., 2009). Nutrients, like phosphorus and nitrogen, support the growth of algae and other plants forming the lower levels of the food chain. However, excessive levels of nutrients from sources such as fertilizer can cause eutrophication, i.e. the overgrowth of aquatic vegetation.

The European Water Framework Directive-WFD (EC, 2000) establishes a framework for the protection of groundwater, inland surface waters, estuarine waters, and coastal waters. The WFD constitutes a new view of water resources management in Europe, based mainly upon ecological elements; its final objective is achieving at least 'good ecological quality status' for all water bodies by 2015 (Borja et al., 2006). The DPSIR framework (Driving forces - Pressure - State - Impact - Response) aims at analyzing the cause–effect relationship between interacting components of complex social, economic and environmental systems and at organizing the information flow between its parts (Kristensen, 2004). In agreement with the DPSIR framework we aimed to evaluate the level of water pollution of Aries River, to find '*driving forces*' (economic sectors, human activities) through '*pressures*' (emissions, waste) to '*states*' (physical, chemical and biological) and '*impacts*' on ecosystems, human health and functions and eventually leading to political '*responses*' (prioritization, target setting, indicators). In order to achieve this aim, several physico-chemical and biological analyses were performed. The stable isotope ratio of oxygen ($^{18}O/^{16}O$) and hydrogen ($^{2}H/^{1}H$ or D/H) from water and the stable isotope ratio of carbon ($^{13}C/^{12}C$) from aquatic plants as well as the concentration of several ions such as NO_3^-, NO_2^-, PO_4^{3-} Cu^{2+}, Fe^{3+} have been investigated and also the distribution of diatom taxa in Aries River.

2. Material and methods

The physico-chemical analyses and distribution of diatom taxa were carried out on water samples collected from eleven sample sites, as follows (odd number–upstream; even number-downstream of the collecting site): P1,2-Campeni; P3,4-Baia de Aries; P5,6-Salciua; P7,8-Turda; P9,10-Campia Turzii; and P11-the site where the Abrud River flows into the Aries. Samples have been collected during 2006–2007.

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doi:10.1016/j.jenvman.2011.04.017

Some physico-chemical characteristics of water were analyzed: pH, Eh, conductivity and O_2 concentration. The physico-chemical parameters were accomplished using a multiparameter 340. Determination of NO_3^-, NO_2^-, PO_4^{3-} $Cu^{2+}$and Fe^{3+} concentrations has been done using an ionic analyzer.

The isotopic content of water samples is usually expressed in delta (δ) values defined as the relative deviation from the adopted standard representing mean isotopic of the global ocean (SMOW): $\delta_{S/R} = (R_{sample}/R_{standard} - 1) \times 10^3‰$, where R_{sample} and $R_{standard}$ stands for the isotope ratio in the sample and the standard ($^2R = D/H$ and $^{18}R = {}^{18}O/{}^{16}O$).

The isotopic content of plant material is expressed in δ values defined as the relative deviation from the PDB standard representing ^{13}C content of a powdered specimen of *Belemnitella americana* from Peedee formation of South Carolina.

The isotopic ratio of oxygen in CO_2 was measured using the isotope ratio mass spectrometer (IRMS) type Delta V Advantage designed by Thermo Finnigan. The δD values were measured with a home-made mass spectrometer type SMAD-1 on hydrogen gas (Berdea et al., 1992). The precision of the measurement was ±0.01‰ for $\delta^{18}O$ and 0.5‰ for δD.

For isotopic analysis of $\delta^{13}C$ in plant, the plants were dried at 80 °C, 24 h and then were ground in a mortar to pass through a 40-mesh screen (0.425 mm). Conversion of the C from plant material in CO_2 for isotopic analysis was accomplished by dry combustion in excess of oxygen (Cuna et al., 2003). Purified CO_2 was analyzed with IRMS Delta V Advantage. The standard deviation for replicate combustions of the same sample was ±0.03‰.

Diatom determination was achieved by using a Nikon Eclipse E 400 microscope.

3. Results and discussion

3.1. Variation of the ^{18}O and D values in Aries River

$\delta^{18}O$ and δD of meteoric waters (precipitation, atmospheric water, vapor) are strongly correlated. If δD is plotted versus $\delta^{18}O$, the data cluster along a straight line: $\delta D = 8\delta^{18}O + 10$ ‰. This line is referred to as the Global Meteoric Water Line (GMWL). Water in river may originate from many sources and because of this its isotopic composition can vary. Seasonal isotopic variations will be observed in rivers in which surface run-off dominates the discharge, whereas small isotopic variations will be observed in rivers with only a single groundwater source. The factors controlling the isotopic composition of precipitation are: temperature, latitude, altitude, amount, and a seasonal variation (Sharp, 2007).

Plants are generally depleted in ^{13}C compared to the source CO_2 needed for the photosynthesis. The isotopic fractionation associated with carboxylations depends on the enzyme involved and thus on the photosynthetic pathway (C3, C4 and CAM) (Brugnoli and Farquhar, 2000). Isotopic variations are more pronounced among C3 plants (−35 to −22‰) than among C4 species (−20 to −8‰). Carbon isotope composition in plants in the aquatic environment is extremely variable, with values of $\delta^{13}C$ between −10 and −50‰ (Hemminga and Mateo, 1996; Gu et al., 1999). These large variations reflect changes in the carbon source for photosynthesis, plant physiological and ecological features, as well as environmental changes such as temperature, pH, salinity and substrate concentration. Despite the large variations in isotopic composition, most plants of the aquatic habitat possess the C3 photosynthetic pathway (Keeley and Sandquist, 1992).

The spatial and temporal variation of $\delta^{18}O$ and δD values of the water samples along the Aries River are shown in Table 1. The $\delta^{18}O$ and δD values are the result of mixing of the groundwater and of the precipitation. From Campeni to Salciua, the $\delta^{18}O$ and δD values show small variations, especially in September: from −10.25‰ to −10.05‰ for oxygen, and from −70.7‰ to −74.4‰ for D in September 2006; from −9.99‰ to −10.22‰ for oxygen and from −68.70‰ to −68.40‰ for deuterium in September 2007. It is possible that predominant groundwater sources contribute at the river in the upper course of Aries. The isotopic values are larger at Turda and Campia Turzii sites: about −68‰ for deuterium and about −9‰ for oxygen. There is an altitude effect that marks this difference between sites. $\delta^{18}O$ and δD values become higher with increasing altitude because it is colder at higher elevation. Many environmental parameters change with the seasons. Seasonal changes in temperature clearly affect isotopic composition of precipitation. The area of Aries River is a direct run-off dominates system, so the seasonality of $\delta^{18}O$ and δD in precipitation has influence on the $\delta^{18}O$ and δD values of the river waters. There is an isotopic pulse for both ^{18}O and D in March, with lower values (−11.88‰ for ^{18}O and −81.20‰ for D at Turda). The waters originating from melted ice and snow supply the river with lower levels of having $\delta^{18}O$ and δD. Generally, waters that are depleted of $\delta^{18}O$ and δD originate from snow melt (Ahmad et al., 2003). The mean temperature in March (indicate value) is consistent with conditions leading to melting snow and explains this isotopic pulse. In Fig. 1 it is shown the seasonal effect on the $\delta^{18}O$ and δD values and in Fig. 2 it is shown how the experimental data for ^{18}O and D fall on the GMWL. The water samples from Aries River fall on, or close, to the GMWL indicating that these waters are of meteoric. Some scattering of $\delta^{18}O$ (as example −12.86‰ for the water collected at Baia de Aries site) could be correlated with the pollution of the river.

3.2. $\delta^{13}C$ variations in aquatic plants of the Aries River

The $\delta^{13}C$ values of aquatic plants were investigated to determine factors that affect the variability of the $\delta^{13}C$. The $\delta^{13}C$ values varied

Table 1
Spatial and temporal variation of the $\delta^{18}O$ and δD values of the water of Aries River.

No	Site	Sept. 2006		October 2006		March 2007		June 2007		Sept. 2007	
		$\delta^{18}O$ (‰)	δD (‰)	$\Delta^{18}O$ (‰)	δD (‰)	$\Delta^{18}O$ (‰)	δD (‰)	$\delta^{18}O$ (‰)	δD (‰)	$\delta^{18}O$ (‰)	δD (‰)
1	Abrud	−9.45	−64.4	−10.64	−71.89	−10.51	−70.2	−10.00	−71.9	−9.37	−68.4
2	Campeni upstream	−10.25	−70.7	−10.74	−72.74	−11.25	−73.6	−10.80	−72.8	−9.99	−68.7
3	Campeni downstream	−10.85	−70.3	−10.74	−76.59	−11.63	−76.3	−11.31	−72.40	−9.95	−68.2
4	Baia de Aries upstream	−11.14	−70.9	−11.78	−71.12	−11.50	−73.3	−10.08	−75.50	−9.97	−68.6
5	Baia de Aries downstream	−10.09	−73.8	−10.43	−70.86	−12.26	−72.7	−10.43	−73.20	−10.05	−68.7
6	Salciua upstream	−10.76	−72.2	−11.52	−71.90	−11.03	−72.8	−10.85	−74.50	−9.89	−68.8
7	Salciua downstream	−10.05	−74.4	−11.50	−72.10	−12.86	−74.6	−10.67	−74.60	−10.22	−68.4
8	Turda upstream	−10.12	−68.1	−11.07	−68.84	−11.88	−74.59	−10.06	−70.2	−9.80	−68.5
9	Turda downstream	−10.40	−66.1	−10.48	−71.18	−11.12	−75.9	−10.83	−70.8	−9.71	−68.4
10	Campia Turzii upstream	−10.61	−69.8	−9.70	−70.83	−11.60	−81.2	−10.69	−70.5	−9.53	−67.8
11	Campia Turzii downstream	−10.17	−68.5	−12.45	−68.61	−11.77	−78.8	−10.70	−75.1	−9.40	−67.2

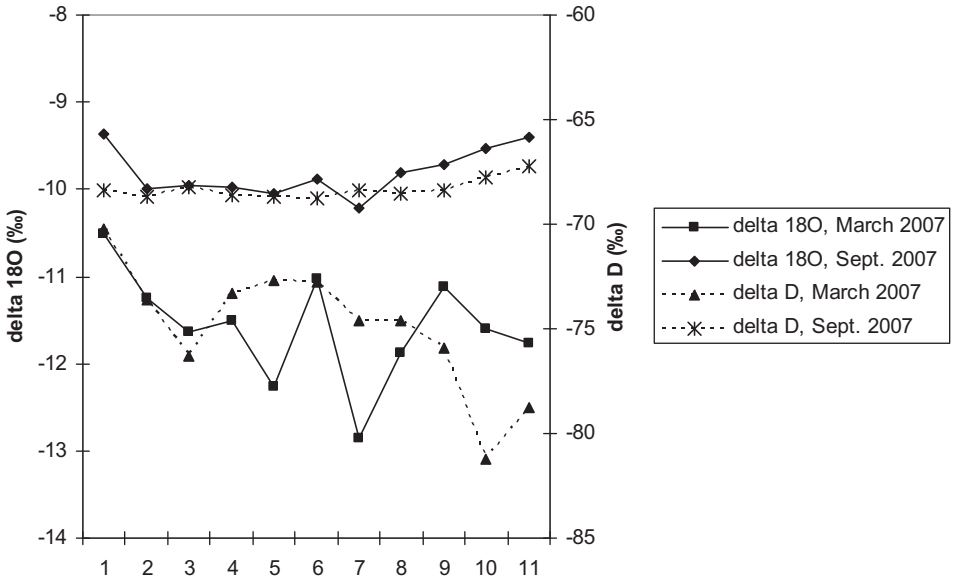

Fig. 1. The variation of the $\delta^{18}O$ and δD values in March 2007 and September 2007.

from −28.6‰ (Campia Turzii) to −32.60‰ (Campeni) and were significantly correlated with site of sampling. The lower isotopic values was found for the plants collected on the upper course of the Aries River, and the $\delta^{13}C$ values have increased toward the Campia Turzii site. In moving waters, one mechanism that leads to such increasing is due to the diffusion of CO_2 in the water, and in essence the thickness of the boundary layer around the plant which in turn can be related to water velocity (Finlay et al., 1999). Low turbulence of the water resulted in more positive $\delta^{13}C$ values at Turda and Campia Turzii sites due to greater diffusion resistance. High flow of the water conducts at Abrud, Campeni, Baia de Aries and Salciua to small boundary layer, and fast diffusion, and high ^{13}C discrimination due to large CO_2 pool available relative to plant requirements. Slow flow of the water at Turda and Campia Turzii results in the larger boundary layer, and slow diffusion, and less ^{13}C discrimination due to plant CO_2 requirements coming closer to available CO_2 pool. The trend of this variation is shown in Fig. 3.

Other physico-chemical analyses of water are presented in Table 2. One can notice the difference between the P11 (the site where the Abrud River flows into the Aries) where the pH = 4.5 and the Eh is positive and all the other sampling sites, where the pH is alkaline (>7.5), and the redox potential (Eh) is negative. Nutrients content in Aries water was also investigated and is shown in Table 3. The higher concentration of NO_3^- was observed in the P11 site (Abrud), 8.67 mg/l and in P6 site (downstream of Campia Turzii), 8.42 mg/l. The indices of water quality in lakes and rivers from Romania are listed in STAS 4706/88 (a compilation of parameters used in Romania to determine quality indices). According to this STAS, the concentration of NO_3^- should be 10–30 mg/l, thus the concentrations found are in admissible limits. The highest concentration of NO_2^- was detected in P10 site, 0.96 mg/l. According to STAS 4706/88, the concentration of NO_2^- should be 1–3 mg/l. Samples collected from P5 and P6 sites contain the highest concentrations of PO_4^{3-} (2.85 mg/l, upstream and 2.35 mg/l, downstream). In STAS 4706/88 there are no indications about the admissible limits of PO_4^{3-}. Nutrients formation in the river water is either the result of synthesis and distruction of organic materials or indirect inflows of polluted municipal, agricultural and stock breeding waste waters (Finnveden et al., 2009). In the last decades the impact of the used fertilizers which trough the precipitations enter the rivers increased significantly. In stream orthophosphate concentrations can also be produced through mobilization of sediment bound phosphorus in anoxic water column and/or sediment conditions, sediment in surface run-off from areas having had

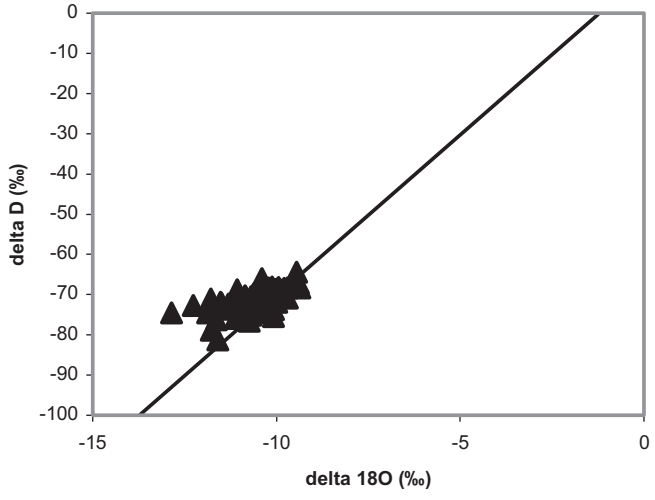

Fig. 2. $\delta^{18}O$ and δD values in water of Aries River.

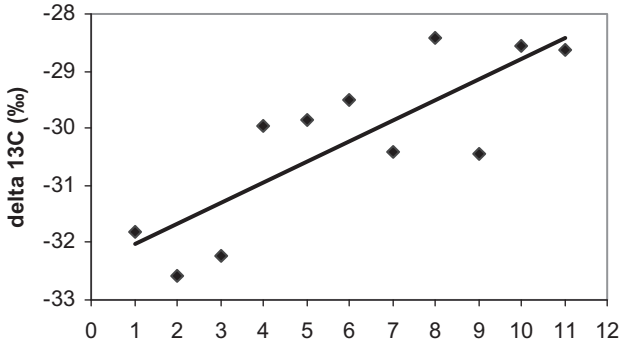

Fig. 3. The trend of the $\delta^{13}C$ values in the aquatic plant sampled in the Aries River.

Table 2
Results of the physico-chemical analyses carried out in water of Aries River.

Sampling site	pH	Eh (mV)	Coductivity (μS/cm)	O_2 mg/l	Temperature °C
P1	7.96	−62	140	11.30	8
P2	7.94	−62	144	11.00	8
P3	7.5	−40	194	10.60	9
P4	7.85	−55	195	10.75	8.7
P5	7.80	−53	180	10.40	8.6
P6	7.85	−60	222	9.85	8.5
P7	8.60	−105	421	11.33	8.3
P8	8.40	−88	492	10.65	8.6
P9	8.50	−94	580	10.75	9.5
P10	8.60	−100	666	10.80	9.7
P11	4.45	+145	920	10.30	8.3

surface applied phosphorus and groundwater from phosphorus saturated soils (Tian et al., 1993).

In natural aquatic ecosystems, metallic compounds occur in low concentrations, normally at nanogram to microgram per liter level. We have detected low concentrations of Cu^{2+} as 0–0.46 μg/l (Table 3) and according to STAS 4706/88, the admissible limits of Cu^{2+} could be 0.05 mg/l. Fe^{3+} was detected in high concentrations especially in P11, P7 and P10 sites (440–1072 μg/l) and according to STAS 4706/88, the admissible limits of Fe^{3+} could be 0.3–1.0 mg/l. In fact, P11 site is the most polluted site as regard the physico-chemical parameters. The pollution source might be the mining enterprise in Rosia Montana (Kraft et al., 2006). Heavy metals may come from natural sources, leached from rocks and soils according to their geochemical mobility or come from anthropogenic sources, as the result of human land occupation and industrial pollution. Although trace metals at low concentrations are essential to life, at high concentrations, may become hazardous (Espinoza-Quiñones et al., 2005).

3.3. Diatom communities analysis

In several European countries, as well as in America and Australia, the algae represent the main group of organisms used in the monitoring of rivers (Stevenson et al., 1996; Prygiel and Coste, 2000; Potapova and Charles, 2007). The evaluation of water quality from natural or influenced by human activities based on aquatic organisms agrees to the actual legal rules (EU Frame Directive 60/2000). Diatoms (*Bacillariophyta*) represent the dominant algal group developing in streams, an additional reason for their use in the water quality evaluation in running waters (Patrick, 1997).

Our study established marked differences in the number of diatom species in the different sampling sites probably due to various environmental changes, especially caused by human impact present in the catchment area of the Aries River. The most severe impact forms are the inflowing acidic mine waters, the outflows of decantation ponds or the sterile waste dumps of the mining areas (Bucium Poieni-Rosia Montana-Abrud-Baia de Aries) located in Aries River catchment basin. In the sampling site P8 there have been identified 101 diatom taxa. The second highest number of taxa (over 60 diatom taxa) was identified in sampling site P1, located upstream Campeni, the lowest one-a single taxa, in sampling site P2, located downstream Campeni, just below the confluence with the Abrud (tributary of the Aries). In the Abrud (sampling site P8) no diatoms could be found, due to its high degree of acidic mine water pollution, drainage waters of sterile wastes and decantation ponds located in the mining area of Bucium Poieni-Rosia Montana-Abrud (Fig. 4). Environmental conditions causing the occurrence of high number of diatoms on the upper course of the Aries River (sampling site P1) are those natural, as well as human pollution sources like tourism, wood clearings, and waste waters of various origins conducing surely to the eutrophication of the river. This statement is sustained by the frequency in the diatom community of several eutrophic elements like *Amphipleura pellucida*, various species of *Amphora, Cymbella* and *Navicula*. The dominant species in this community are the indifferent ones (*Achnanthes minutissima, Fragilaria capucina, F. ulna* etc.) with high relative abundance values (over 85%). The characteristic diatoms for clear, mountain rivers, expected to be frequent here (*Diatoma hyemalis, Fragilaria arcus* and *Meridion circulare*), are very scarce indeed. The acidic mine waters, very low pH, heavy metals, and suspensions of various nature, caused the total lack of living diatoms in the Aries River in sampling site P2, located just below their confluence, where only few frustules of *Achnanthes minutissima* could be detected, brought possibly from the upstream community. In sampling site P3-upstream of Baia de Aries, the restoration of the diatom community could be observed; the dominant elements being the cosmopolitan ones, but exhibiting relative abundance values less than 80%. Downstream the mining area of Baia de Aries (sampling site P4), due to the same kind of mine waters, of household wastes and of gravel pits from the river bad, the number of diatom species is drastically diminished again (only 5). Toward downstream, as the inflow of clear waters of the tributaries (at Posaga and Ocolis), causes the dispersion of pollutants, the regeneration of the diatom communities took place. Therefore, in sampling site P5-upstream of Turda, 45 diatom taxa could be detected and downstream the town (sampling site P6) almost 60 taxa. These communities are equally dominated by the same cosmopolitan elements, intermingled with several eutrophic forms. New feature of these communities (sampling sites P5 and P6) is the occurrence of the diatoms indicating critical saprobity levels (β-α, α- or α-polysaprobic taxa): Amphora veneta, Navicula accomoda, N. cuspidata, N. goeppertiana, Nitzschia capitellata, N. filiformis and

Table 3
Nutrients and metals concentrations in water of Aries River.

Sampling site	NO_3 (mg/l)	NO_2 (mg/l)	PO_4 (mg/l)	Cu (μg/l)	Fe (μg/l)
P1	0.443	0	2.29	0	166
P2	3.54	0	0.97	0	59
P3	0	0.096	0.96	0	142
P4	0	0	0.64	0	43
P5	0	0.032	2.85	0.15	440
P6	8.42	0.064	2.32	0.32	57
P7	0	0	2.25	0	540
P8	0	0	0.03	0.13	384
P9	0	0	0.42	0.46	300
P10	4.87	0.96	0.49	0	476
P11	8.67	0	0.2	0.31	1072

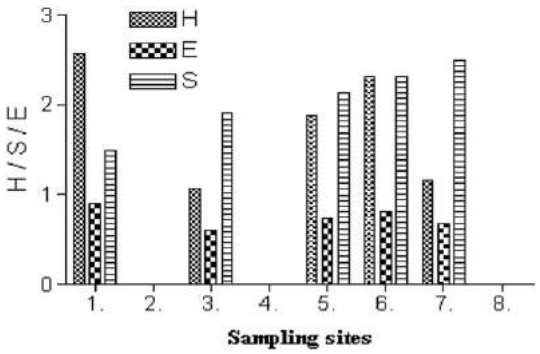

Fig. 4. The values of Shannon-Wiener diversity (H), equitability (E) and the index of saprobity (S) in the sampling sites of the Aries River.

some others. The appearance of these elements might be explained by the involvement of new types of pollutants in this zone, household garbage heaps on the river side, agricultural wastes etc. The industrial pollution in the area of the towns Turda and Campia Turzii involved once more the fall of the diatom taxa (to 44 in sampling site P7, downstream of Campia Turzii). The dominant diatoms indicate critical saprobity levels (the *Nitzschia* and *Surirella* species already mentioned above). Our data agree with previous findings concerning the same river (Momeu and Péterfi, 2007; Momeu et al., 2007). The values of Shannon-Wiener diversity and those of equitability (Fig. 4) show generally the same tendencies as concerning the degree of benthic diatom community organization. These indices have not been computed for sampling sites P2, P4 and P8, due to the low number of species detected there. The index of saprobity (Fig. 4) exhibit growing tendency from upstream toward downstream in the Aries River; its water quality could be included in class I–II upstream Campeni, being namely oligosaprobic, clear or very slightly polluted. In sampling site P3 according to the value of the saprobity index the water belong to class II, β-mesosaprobic or moderately polluted. The river water in sampling site P5 could be included in class II-III, β-α- mesosaprobic level with moderate to strong pollution, but in sampling sites P6 and P7 it could be referred to quality class III-IV, β-α- and α-mesosaprobic levels, namely being strongly polluted (Momeu and Péterfi, 2007).

In agreement with the recommendations of the DPSIR framework the results of this study indicate that several responses are needed to develop a right management of rivers from Romania. Some physico-chemical methods such as filtration, chemical precipitation, ion-exchange, and membrane systems have been used for the last four decades (Shiao-Shing et al., 2007), so such methods could be also used for water purification. As a promising solution, bioremediation i.e. the use of biocomponents for environmental remediation, is a potentially effective, safe, and environment-friendly method. Since early 1970s several works have demonstrated that aquatic macrophytes can be used to remove metals by surface adsorption and/or absorption and incorporate them into their own system or store them in a bound form (Garg et al., 1997). New technologies relying on enzymatic mechanisms that can be applied in various ways, such as biocatalyst-containing foams and an enzymatic sponge, for environmental as well as personal exterior decontamination could be also employed (Simo et al., 2008). In the last few years, reliable biological methods have been used, including the use of microorganisms (fungi, algae, bacteria), plants (live or dead) and biopolymers (Singh and Gadi, 2009).

4. Conclusions

The $\delta^{18}O$ and δD values of the water from Aries River confirm the meteoric provenance of these waters. Some scattering of $\delta^{18}O$ values could be correlated with pollution at the site of collection.

The $\delta^{13}C$ values measured on the plant material varied largely. This variation was correlated with the site of sampling. The mechanism that leads to such variation is the diffusion of CO_2 in the water that is related with the water velocity. The $\delta^{13}C$ values are not influenced by pollution.

According to the STAS 4706/88 the concentrations of NO_3^-, NO_2^-, PO_4^{3-} $Cu^{2+}$and Fe^{3+} in Aries River are in admissible limits.

The structure of benthic diatom communities in the Aries River is strongly affected by the various pollutants detected in the investigated area.

The qualitative and quantitative structure of the diatom communities confirm previous studies showing that the drastic changes in the number of species is related to the presence, these changes occur in both values of diversity and equitability. The level of trophicity and saprobity grows from upstream toward downstream.

Acknowledgement

Financial support of this work was provided by CEEX project no. 61/2005, financed by Ministry of Education and Research from Romania.

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Journal of Environmental Management 95 (2012) S9–S14

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Heavy metal content in sediments along the Calore river: Relationships with physical–chemical characteristics

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ARTICLE INFO

Article history:
Received 1 September 2009
Received in revised form
21 January 2011
Accepted 23 February 2011
Available online 5 May 2011

Keywords:
Trace metals
Organic carbon
Campania
Sediments
Pollution
Rivers

ABSTRACT

In the present study, trace metals contents (V, Cr, Mn, Fe, Ni, Cu, Zn, Cd and Pb) and physico–chemical parameters (nitrogen, organic and inorganic carbon, pH and particle size) in sediments samples along the Calore river were analyzed in two seasons.

Sediment samples were collected in ten sites upstream and downstream of the city of Benevento and its industrial area, the confluence of Sabato and Tammaro tributaries, and the confluence of Calore and Volturno rivers. The highest contents of trace metals were found, generally, in the sites immediately downstream of industrial area and of Benevento city. The sites on the Tammaro and Sabato also showed relatively high contents of Ni and, only for Sabato sites, of Cr, and Fe. With the exception of Cd, the heavy metal contents were highest in the last site of Calore river, which therefore is a source of pollution to the Volturno river. Besides the sites downstream of Benevento city showed the higher pH values and also the highest contents of fine particles size and organic matter. Positive correlations among trace metals, organic substance, particle size sediments were found.

The data obtained in this study were analyzed with reference to Interim Sediment Quality Guidelines and indicated moderate-to-high pollution by some trace metals (V, Cr, Mn, Fe, Ni, Cu).

1. Introduction

Trace elements, especially the so called 'heavy metals', are among the most common environmental pollutants and their occurrence in waters and biota indicate the presence of natural or anthropogenic contamination. The main sources of metals in aquatic systems are the weathering of soils and rocks and anthropogenic activities (drainage of land and alterations in land use), whereby industrial and urban wastes are discharged into water bodies and so disturb the equilibrium between the metals in sediment/soils and ground water or surface water (Yu et al., 2001; Klavins et al., 2000; Filgueiras et al., 2004).

Throughout the hydrological cycle, far less than 1% of pollutants remain dissolved in water whereas over 99% are stored in sediments, that therefore are the major sinks and carriers for contaminants in aquatic environments (Filgueiras et al., 2004). Analysis of sediments is of common use to estimate the health state of water bodies and to determine the index of pollution of drainage basins (Bryan and Langston, 1992; Bubb and Lester, 1994; Darkalakis and O'Connor, 1995). Because of the tendency of pollutants to accumulate in sediments, the living community associated with sediments is particularly prone to harmful effects of pollution (Tam and Wong, 2000; Samecka-Cymerman and Kempers, 2001; Baldantoni et al., 2004). The concentration of pollutants stored in sediments, however, are affected by sediments mineralogy, dimension and distribution. Trace elements are adsorbed by organic substances like carbohydrates, and minerals like Fe and Mn oxides (Filgueiras et al., 2004). The adsorption capacity increases with decreasing particle sizes (Jain, 2004; Filgueiras et al., 2004). The overall process is reversible and dependent on pH and redox potential, hence the absorbed trace metals can be released again in the water body (Chen et al., 1996; Kashem and Singh, 2001).

Heavy metals of anthropogenic origin are generally introduced into the environment as inorganic complexes or hydrated ions, which can easily bind to the surface of sediment particles by relatively weak physical and chemical bonds. As a consequence, heavy metals are found predominantly as a labile extractable fraction in sediments (Jain et al., 2008). The presence and distribution of heavy metal in aquatic systems, have been investigated quite extensively in the last decade, but unfortunately essentially in water bodies of northern Europe (Fytianos and Lourantou, 2004; Chandra Sekhar et al., 2003; Singh et al., 2005; Jain et al., 2008; Venugopal et al., 2009), includingnorthern Italy rivers (Camusso et al., 1999; Pettine et al., 1994, 1996; Viganò et al., 1999, 2003; Farkas et al.,

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doi:10.1016/j.jenvman.2011.02.013

2007). Very few data, however, are presently available on the topic as concerns southern Italy rivers (Bartoli et al., 2009; Isidori et al., 2004; Parrella et al., 2003).

The aim of this study was to evaluate trace metal contents (V, Cr, Mn, Fe, Ni, Cu, Zn, Cd and Pb) and possible relationships with physical–chemical properties (particle size, pH, nitrogen, organic and inorganic carbon) in sediments samples along the Calore river, its affluents Tammaro and Sabato, and as well as its contribution to the Volturno, the most important river of Campania Region (South-Italy). The "Calore" river was a navigable river before 1958 when water deviation to Puglia region greatly reduced its flow. Urban and industrial waste, in addition, as well as unlawful dumping and quarries have highly increased the concentration of pollutants.

2. Material and methods

2.1. Study area

The "Calore" river (118,50 km) risen in the "Acellica" Mount, in the group of the "Picentini" mountains (Campania), flows into the Volturno river, near "Amorosi". Its tributaries are "Ufita", "Sabato" and "Tammaro" before crossing Benevento (a city of ~70.000 inhabitans).

Agriculture, handcraft and industrial activities are the main sources of river pollution along its drainage basin.

2.2. Sample collection and preparation

Fig. 1 shows the 10 sites selected for sampling: C1 (upriver of Benevento, near agricultural fields); T (Tammaro tributary, immediately upstream confluence with Calore river); C2 (upstream of Benevento city and downstream of the industrial area); C3 (Calore river, downstream confluence of Tammaro); S (Sabato tributary, downstream of urban city and immediately upstream confluence with Calore river); C4 (Calore river, downstream confluence of Sabato); C5 (Calore river, downstream site C4); C6 (Calore river, immediately upstream confluence with Volturno); V1 and V2 (Volturno river, respectively upstream and downstream confluence of Calore).

2.2.1. Sample collection and analytical methods

Sediment samples were collected in late Autumn 2004 (at the end of the summer dry season) and in late Spring 2005 (after winter floods). At each sampling site, five sediment cores were collected by a snapper (∅ 5 cm) in the layer 0–15 cm and mixed to form the site sediment sample.

Before transferring the sampled sediment in the special containers, after decantation, the above water has been recovered and used for the immediate pH determination with an electronic pH meter (HI 8424, HANNA Instruments, Sarmeola di Rubano PD, Italy).

A representative portion (500 g) of each sample was used for the determination of coarse sand, fine sand, silt and clay composition in according to USPRA classification (U.S. Public Roads Administration) (Hiller, 1982). A second portion of each sample, oven-dried at 75 °C until constant weight, was sieved (pore diameter 2 mm and nylon sieves) and ground to a fine powder by a Fritsch Germany pulverisette 6 with an agate pocket, to prevent trace element contamination.

The pH of the sediment was measured by shaking an aliquot of sediment in distilled water (10 g of dry sediment in 25 ml of water) for 10 min. The suspension was left to stand for 10 min. The pH of the supernatant was measured with an electronic pH meter (HI 8424, HANNA Instruments, Sarmeola di Rubano PD, Italy).

Total carbon (TC), inorganic carbon (IC) and nitrogen analyses were carried out in triplicate on powdered sediment aliquot by an NCS Analyzer (Carlo Erba NA 1500).

Inorganic C was evaluated by treating samples at 550 °C for 2 h before combustion in the NCS Analyzer. Organic carbon (OC) was determined as difference between total carbon and inorganic carbon (OC = TC − IC).

Trace element (V, Cr, Mn, Fe, Ni, Cu, Zn, Cd and Pb) analyses were carried out in triplicate by atomic absorption spectrometry (SpectrAA 20 Varian) and quantified using standard solutions (STD

Fig. 1. Map of sampling sites. Description in the text.

Table 1
Physical and chemical characteristics of sediment sampled in the different sites along the Calore river. The pH of the water column above the sediments (pH_{H2O}) was, also, reported. Standard deviation is in parenthesis. The site names are as in Fig. 1

Sites	pH_{sed}	pH_{H2O}	OC (mg/g)	OC/IC	C/N	Coarse sand (% d.w.)	Fine sand (% d.w.)	Silt and Clay (% d.w.)
C1	8.0 (0.1)	8.16 (0.1)	43.2 (0.6)	2.75 (0.14)	65.18 (0.4)	38.8	51.0	10.2
T	7.6 (0.1)	7.73 (0.1)	43.1 (0.9)	2.49 (0.11)	60.89 (0.6)	68.7	24.2	7.1
C2	7.1 (0.1)	7.56 (0.1)	70.4 (0.6)	3.67 (0.15)	50.08 (0.4)	34.2	36.7	29.1
C3	8.0 (0.1)	8.26 (0.1)	42.5 (0.5)	2.45 (0.15)	55.33 (0.9)	59.6	30.3	10.1
S	7.7 (0.1)	7.66 (0.1)	41.5 (0.3)	3.11 (0.21)	51.46 (1.0)	21.2	70.7	8.1
C4	8.0 (0.1)	8.20 (0.1)	123.2 (1.1)	6.33 (0.23)	52.11 (1.0)	20.2	55.5	24.3
C5	8.0 (0.1)	8.00 (0.1)	119.6 (1.5)	6.10 (0.11)	49.66 (1.3)	15.6	34.0	50.4
V1	7.9 (0.1)	8.00 (0.1)	31.1 (0.3)	2.27 (0.11)	45.93 (1.1)	46.2	43.0	10.8
C6	8.1 (0.1)	8.15 (0.1)	41.2 (0.4)	2.75 (0.12)	41.36 (1.0)	48.9	38.1	13.0
V2	7.9 (0.1)	8.00 (0.1)	38.2 (0.8)	2.50 (0.13)	42.93 (0. 8)	42.3	49.0	8.7

Analyticals, Carlo Erba). Aliquots of the powdered sediment samples (250 mg) were mineralized in a Milestone Microwave Laboratory Systems (Ethos 900), endowed with temperature control, by a combination of hydrofluoric and nitric acid (HF 50%:HNO_3 65% = 1:2). After digestion the solutions were diluted by deionized water to a final volume of 50 ml. Nikel, Cr, Pb, Cu, V and Cd concentrations were measured by graphite furnace AAS and the Fe, Zn and Mn concentrations by flame AAS (Baldantoni et al., 2004; Pagnotta and Pettine, 2005).

Accuracy was checked by concurrent analysis of standard reference materials by the Resource Technology Corporation, Laramie, WY; the recovery ranged from 90 to 100%.

2.3. Statistics

Data all measures were performed in triplicate for each sample ± SD.

The correlations were determined using the simple Pearson correlation coefficient. The significance of differences was tested by one-way analysis of variance (ANOVA) followed by Tukey test (MINITAB INC 13).

3. Results and discussion

Table 1 shows physico–chemical properties of sediments sampled along the Calore river. The pH in bioleaching process of contaminated sediment depends on the buffering capacity of the sediment. When pH reaches a certain value, metals will start being released from sediments (Chen and Lin, 2001). In this study the sediments displayed analkaline pH between 7.1 and 8.1 (Table 1) and, in this range, there was no release of metals except for Mn, as reported by Chen and Lin (2001). The lower pH (7.1) was determined in sediment taken immediately downstream of the industrial area (C2), an intermediate value (7.6–7.7) in the tributaries

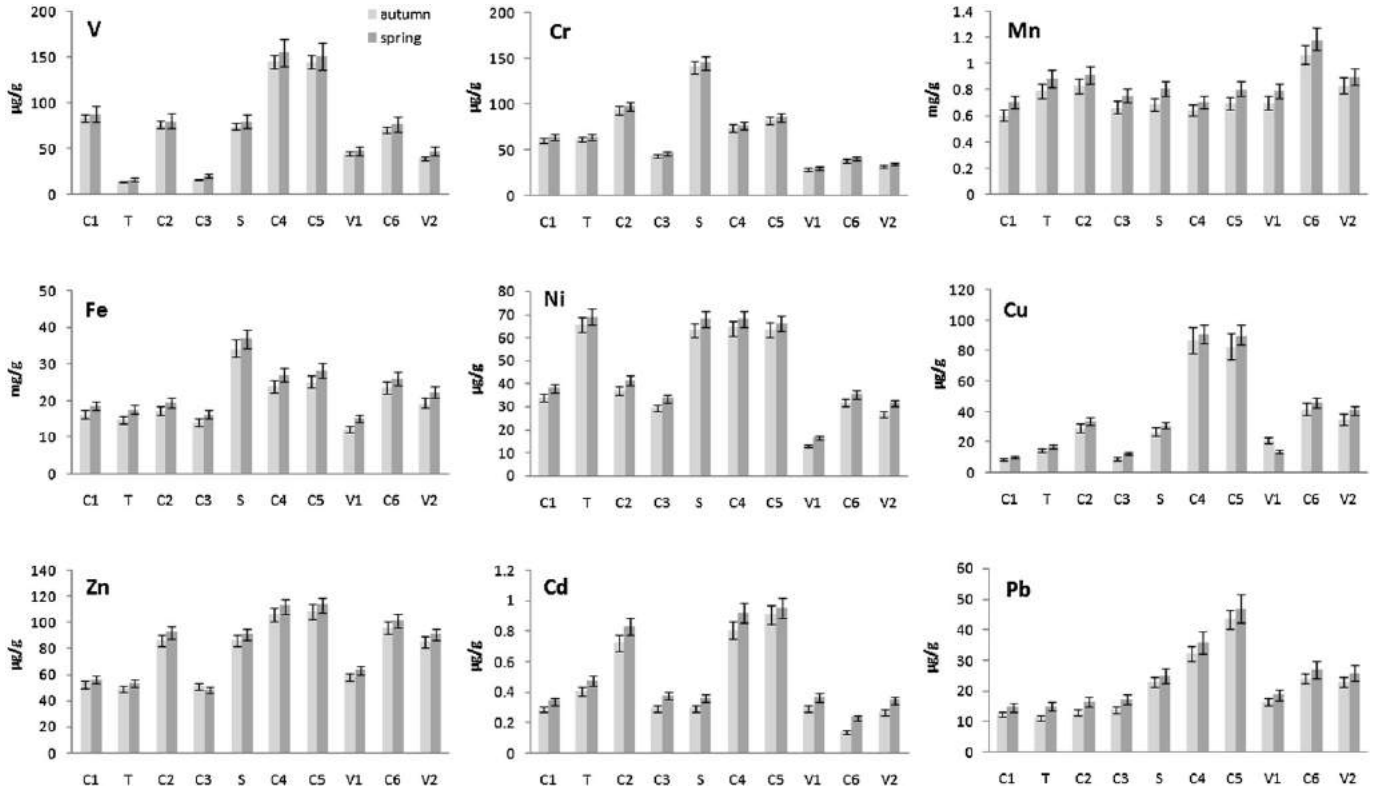

Fig. 2. Trace elements in sediments (μg/g d.w.) collected in 10 sampling sites. The names ofsites are as in Fig. 1.

Table 2
Comparison of heavy metals in sediments of different rivers of the world.

Rivers	Cd (μg/g)	Cr (μg/g)	Cu (μg/g)	Fe (μg/g)	Mn (μg/g)	Ni (μg/g)	Pb (μg/g)	Zn (μg/g)	References
Ganga	2.55	–	–	–	–	–	25.6	36.1	Sakai et al. (1986)
Genesse	–	–	10.8	–	–	23	40.0	69.0	Subramanian et al. (1987)
Ganga	–	–	21.0	–	–	–	25.0	46.0	Subramanian et al. (1987)
Olona	0.32	16	20	13,980	240	10	56	90	Dalmiglio et al. (2005)
Astura	0.305	74	32.1	15,576	255	31	122	480	Ceradini et al., 2005
Guadaia	3.0	38	25	25,000	477	37	20	51	Gonzalez et al. (2000)
Lambro	2.10	–	90	–	–	161	98.5	305	Viganò et al. (2003)
Yamuna	9.5	–	22.2	–	–	–	60.0	59.2	Jain (2004)
Gomti	2.42	8.15	5.0	2660	148.13	15.7	40.33	41.67	Singh et al. (2005)
Lambro	3.70	–	187	–	–	82	63.8	645	Farkas et al. (2009)
Piacenza	1.25	–	54.6			90.8	55.5	202	Farkas et al. (2009)

Tammaro and Sabato (T, S), and the higher values (8.0–8.1) in sediments collected in the Calore river (C1, C3, C4, C5, C6).

As is well known, metals are not permanently bound to the sediment, but can be remobilization from chemical and biological agents both in the sediments and in the water column above (Stone and Droppo, 1994; Filgueiras et al., 2004). For these reasons, measurements on the water column above the sediments were made. They showed values between 7.56 and 8.26, not significantly different from pH sediment (Table 1).

It is well-established that granulometry and also organic matter contents are important factors affecting the distribution of trace metals (Farkas et al., 2009; Jain et al., 2005). Fine-grained sediments tend to have relatively higher metal contents, due in part to the high specific surface of particles. This enrichment is mainly due to surface adsorption to the mineral component and coatings of organic matter (Rubio et al., 2000).

In this study coarse and fine sand (Table 1) were found to be dominant in most of the sites (~90% on average) with respect to silt and clay (~10% on average) and this may be attributed to continuous deposition of alluvium on the riverbed of the Calore. A slightly divergent situation was found in immediately downstream of the industrial area and Benevento city sites (C2, C4 and C5), silt and clay accounted for about 29%, 24.3% and 50.4% of sediment, respectively. A higher percentage of fine particle size in the sediment results in a more loose fabric, higher porosity and permeability, and more easy transportation of the sediment downstream.

The organic carbon content (Table 1), which was found, varied between 31.1 and 123.2 mg/g d.w. and, in particular, C2 site resulted significantly lower ($p < 0.001$) than C4 and C5 sites and higher than the others ($p < 0.001$). This significant increase the organic carbon, C2 than in C1 and C4 than C3, is probably originated from the release of industrial and civil wastewater.

Significantly higher OC/IC ratio in C2 ($p < 0.01$) and in particular in C4 and C5 ($p < 0.001$) pointed out a higher OC content than IC, probably due to direct industrial and civil wastewater. On the contrary, the C/N ratio, which decreased from C1 to V2, could suggests a progressive increase in N contents, probably due to leaching from extensively cultivated soil (Parrella et al., 2003).

Fig. 2 shows the contents of trace metals analyzed in the sediments.

The metal concentrations determined in sediment sampled in autumn showed similar values as those samples in spring.

The significant higher values of all assayed metals, except Mn, were observed in the C4 and C5 sites ($p < 0.01$) compared to C3, the upstream site of Benevento city. These sites showed also the highest values of OC, particle fine size and alkaline pH_{sed} (Table 1). The C2 site, downstream of the industrial area, showed Cd, Cr, Cu and Zn higher values ($p < 0.01$), compared to the upriver site (C1). Nickel, instead, was high in T and S sites apart from the C4 and C5 ones ($p < 0.001$). The S site, with the lowest OC, was, also, characterized by relatively high content of Cr and Fe ($p < 0.01$). Mn, instead, had the highest values in the C6 site. Sediments in the V1 site showed the significant lowest values ($p < 0.01$) than C6 site (immediately upstream confluence with Volturno) for the all the metals except for Cd.

These data were compared with metal concentrations reported by other workers on some of the major rivers of the world (Table 2). In particular it was evidenced that the highest concentrations

Table 3
Correlation between heavy metals and physical-chemical characteristics of sediments sampled in the different sites along the Calore river.

	Cr	Pb	Ni	Zn	Cu	V	Cd	Fe	Mn	pH_{sed}	pH_{H2O}	OC	IC	N tot
Cr														
Pb	*													
Ni	***	***												
Zn	**	***	**											
Cu	NS	***	***	***										
V	**	***	***	***	***									
Cd	**	***	***	***	***	***								
Fe	***	***	***	***	***	***	*							
Mn	*(−)	NS	NS	*	NS	NS	**(−)	NS						
pH_{sed}	***(−)	**	NS	NS	NS	NS	*(−)	NS	NS					
pH_{H2O}	***(−)	NS	**(−)	NS	NS	NS	NS	NS	NS	NS				
OC	**	***	***	***	***	***	***	*	*(−)	NS	NS			
IC	**	**	*	***	***	***	***	NS	NS	***(−)	**(−)	***		
N tot	**	***	**	***	***	***	***	*	NS	**(−)	NS	***	***	
Coarse sand	**(−)	***(−)	*(−)	***(−)	***(−)	***(−)	***(−)	***(−)	NS	NS	NS	***(−)	***(−)	***(−)
Fine sand	NS	NS	NS	NS	NS	*	NS	**	*(−)	*	NS	NS	**(−)	NS
Silt and clay	*	***	***	***	***	***	***	**	NS	NS	NS	***	***	***

NS = not significantly.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Table 4
Metal pair ratio (M/Fe) for heavy metal content in sediments.

Sites	Cr ($\times10^{-3}$)	Pb ($\times10^{-3}$)	Ni ($\times10^{-3}$)	Zn ($\times10^{-3}$)	Cu ($\times10^{-3}$)	V ($\times10^{-3}$)	Cd ($\times10^{-3}$)
C1	3.68	0.77	2.08	3.22	0.52	5.10	0.02
T	4.13	0.77	4.42	3.33	0.99	0.91	0.03
C2	5.41	0.76	2.14	5.02	1.72	4.45	0.04
C3	3.09	1.00	2.10	3.63	0.65	1.17	0.02
S	4.09	0.73	1.85	2.51	0.79	2.19	0.01
C4	3.08	1.35	2.69	4.45	3.65	6.10	0.03
C5	3.01	1.60	2.43	3.98	3.05	5.34	0.03
V1	2.31	1.24	1.05	4.78	1.74	3.64	0.02
C6	1.59	1.02	1.34	4.04	1.76	3.00	0.01
V2	1.61	1.18	1.38	4.38	1.81	2.06	0.01

occurred in this study (C2, C4, C5, S e T) were: (a) lower than Po and Lambro rivers; (b) higher than Alona and Astura rivers, except for Pb and Zn; (c) generally higher than Ganga, Genesse, Guadaia, Yamuna and Gomiti rivers, except for Cd (Table 2).

In the present study all heavy metals assayed, except Mn, were correlated to each other, OC, IC, N_{tot} and the fine fraction (silt and clay) (Table 1). Positive correlations with OC (Table 3) suggest that binding to organic substance has a major role in metal ion adsorption to sediments. It has been reported, in fact, that the charge of the metal ions is a major factor affecting absorption to sediment particles (Gaw and Chen, 1998; Rule, 1986).

In order to facilitate comparison and integration of data relative to heavy metal assessment, a number of specific "indexes" have been introduced. For example Farkas et al. (2009) and Jain et al. (2008) used the Index of geoaccumulation (Igeo) to determine the quantitative extent of metal pollution in the middle stretch of River Po (Italy) and the basin of River Narmada (India) respectively. Others (Jain, 2004; Singh et al., 2005) use a comparison of metal concentration ratios in the sediments as a quick and practical method for tracing heavy metal enrichment. In natural river systems, elements as well as metals exist together in relative proportions to each other. These ratios are dependent on a large number of processes in a geochemical cycle including weathering, transport and deposition. The ratios of trace metals to conservative elements may reveal geochemical imbalances due to elevated trace metal concentrations normally attributed to anthropogenic activities.

Table 4 reports trace metal to iron ratios in the different sampling sites selected for this study. The metal pair ratios clearly reflect maximum enrichment of lead, copper and vanadium at site C4 and C5; nickel showed enrichment at site T followed by C4 and C5; chromium showed maximum enrichment at site C2 followed by T and S; zinc showed enrichment at site C2, C4, C5, C6, V1 and V2, cadmium showed enrichment at site C2, C4, C5 and T. The highest values were found in the sediments collected to downstream of the industrial area, of the city and its two tributaries, which highlights the major contribution of the anthropogenic origin of trace metals. The values were similar to Jain et al. (2005).

In contrast to marine sediments, there is no legal specify in Italy that defines the limits for trace metals in river sediments. The data obtained in this study, therefore, were analyzed with reference to Interim sediment quality guidelines (ISQG) (Anzecc and Armcanz, 2000; McCauley et al., 2000; McCready et al., 2006). Effects range-low (ERL) and effects range-median (ERM) guidelines (Long et al., 1995) were re-named ISQG-Low and ISQG-High guidelines, respectively (Anzecc and Armcanz, 2000). These values correspond to the lower 10th percentile (ERL) and 50th percentile (ERM) of chemical concentrations associated with adverse biological effects in field studies and laboratory bioassays, in a large database compiled from studies across all three coastlines of North America (Long et al., 1995). These guidelines distinguish three ranges of concentrations of sediment-associated contaminants, the first rarely associated with adverse effects (<ERL), the second occasionally (≥ERLs and <ERMs), and the third frequently (≥ERMs). Under this perspective, it was observed that: (a) Cd, Pb and Zn occurred below the ISQG-low limit value (1.5 μg/g, 50 μg/g and 200 μg/g respectively) in all sites; (b) Cr was above the ISQG-low limit (80 μg/g) only in C2 and S, and close to limit in C4 and C5 sites; (c) Cu was above the ISQG-low limit (65 μg/g) only in C4 and C5 sites; (d) Nickel was above the ISQG-low limit (21 μg/g) in all sites except V1, (d) Ni was above the ISQG-High limit (52 μg/g) only in T, S, C4 and C5 sites.

In conclusion, total amounts of trace metals in ten sampling points distributed along the Calore river course, with exception of Cd, Pb and Zn, indicate moderate-to-high pollution by them, according to the ISQG criteria. Positive correlations among trace metals, organic substance, particle size sediments indicate that they are probably discharged from the same pollution source. So, the river sites down the industrial area and the urban area of the city were significantly more polluted than upstream sites. In addition the two tributaries Tammaro and Sabato can be considered sources of pollution of the "Calore", and on turn the "Calore" resulted a source of pollution for the "Volturno" river for all the elements tested except Cd.

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Journal of Environmental Management 95 (2012) S15–S18

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

DNA-based diagnostic tests for *Salmonella* strains targeting *hilA*, *agfA*, *spvC* and *sef* genes

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ARTICLE INFO

Article history:
Received 2 September 2009
Received in revised form
11 April 2010
Accepted 18 July 2010
Available online 10 November 2010

Keywords:
Molecular diagnosis
PCR
Salmonella strains

ABSTRACT

The goal of this study was to evaluate the suitability of the *hilA*, *agfA*, *spvC* and *sef genes* amplification by PCR as a method for detection of *Salmonella* strains. Twenty nine isolates of *Salmonella* spp. including 6 different serotypes were analyzed in this study. The bacteria were isolated between 2005 and 2007 and serotyped at the Clinical Hospital of Infectious Disease, Cluj-Napoca. Ten non-*Salmonella* strains were also tested by the same procedure. We used a direct PCR technique, DNA extraction had been skipped and the bacterial cell wall denaturated in the first step of the reaction. All *Salmonella* strains gave positive results by the PCR amplification of *hilA* gene. The utilization of the *sef*, and *spvC* genes or *spvC* and *agfA* genes in a multiplex PCR provides a valuable diagnostic tool for *Salmonella enteritidis* strains.

1. Introduction

Salmonellae are invasive enteropathogens of humans and animals. In humans, *Salmonella* is the etiological agent of gastroenteritis and typhoid fever. One common feature among all *Salmonella* spp. is that they display enhanced survival in non-host environments, including soil and water (Winfield and Groisman, 2003). This fits into the cyclic lifestyle that has been proposed for *Salmonella* spp., consisting of passage through a host into the environment and back into a new host (Winfield and Groisman, 2003). The eradication of *Salmonella* isolates from the environment is practically impossible. Therefore the development of control measures is necessary (White et al., 2003). Control of infection depends on the availability of rapid methods and precise diagnostic tests.

Clinical diagnosis of the salmonelloses is often difficult because the symptoms closely resemble other diarrhoeal diseases (Keusch, 2002). *Salmonella* can be isolated and characterized using standard bacteriologic media. Conventional culture methods require 5–7 days for presumptive results. Serological techniques may be used for epidemiological characterization. Additional biochemical and serologic tests are needed to identify specific serotypes. Conventional methods of isolation of *Salmonella* strains are therefore laborious and require substantial manpower (Van der Zee and Huis in't Veld, 2000). Molecular testing has been most successful in areas for which conventional microbiological techniques do not exist, are too slow or are too expensive (Jungkind, 2001). Polymerase chain reaction (PCR) is the best known and most successfully implemented nucleic acid detection technology to date (Nissen and Sloots, 2002).

The goal of this study was to evaluate the suitability of the, *hilA*, *agfA*, *spvC* and *sef* genes amplification by PCR as a method for detection of *Salmonella* strains.

2. Materials and methods

2.1. Bacterial strains

Twenty nine isolates of *Salmonella* spp including 6 different serotypes were analyzed in this study (Table 1). Ten non-*Salmonella* strains were also tested by the same procedure. The bacteria were isolated between 2005 and 2007 and identified by biochemical and serological tests (microagglutination, tube agglutination, and rapid whole-blood plate agglutination assays) at the Clinical Hospital of Infectious Disease, Cluj-Napoca, Romania (Gast and Beard, 1990).

2.2. Preparation of DNA samples

For selectivity tests, *Salmonella* or non-*Salmonella* strains were grown aerobically without shaking at 37 °C for 16 h in Luria–Bertani medium. Five to six colonies were suspended in ultra pure water to enhance the cell wall destruction. The optical density

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doi:10.1016/j.jenvman.2010.07.027

Table 1
Bacteria strains used in PCR.

Bacteriological isolation	No. of strains
S. typhimurium	5
S. choleraesuis	2
S. dublin	2
S. enteriditis	15
S. gallinarum	2
S. infantis	3
Klebsiella pneumoniae	4
Escherichia coli	5
Shigella dysenteriae	1

Table 2
Primers for PCR amplification.

Gene target	Location or function	Sequence 5′–3′ (forward/reverse)	Amplicon size (bp)	Reference
spvC	Virulence plasmid	TATGATGGGGCGGAAATACC /GCGTTTACTGTTCCGTTGC	700	This study
agfA	Aggregative fimbriae	TCCGGCCCGGACTCAACG /CAGCGCGGCGTTATACCG	261	Doran et al. (1993)
sefC	Fimbrial protein	TGGGGACAAATATACCAGTGC /CTATTTGCCCTCTTGCTTGC	1100	This study
misL	SPI3	GACGTTGATAGTCTGCCATCCAG /CAATGCCGCCAGTCTCCGTGC	986	Soto et al. (2006)
hilA	SP1	GCGAGATTGTGAGTAAAAACACC /CTGCCCGGAGATATAATAATCG	413	This study

at 600 nm of the suspension (OD600) was 0.4. Three μl of this suspension were used in the reaction. Using this technique we can skip the expensive DNA extraction and the self contamination of workers is minimized. The PCR primers used in the experiment are presented in Table 2.

The primers were designed according to the sequences found at NCBI, accession numbers: U25352 for the *hilA* gene; DQ115388 for *spvC* gene and L11010 for *sefC* gene.

2.3. PCR protocol

A typical 25-μl PCR mixture contained 2.5 μl 10 × PCR reaction buffer, 25 pmol of each primer, 200 μM concentrations of each dNTP, 2 μl $MgCl_2$ 25 mM (2 mM final concentration) 0.75 U of *Taq* polymerase, and 3 μl bacterial suspension. PCR was performed in a Thermocycler, (MJ Research). The parameters for amplification were as follows: initial denaturation at 94 °C for 4 min, 30 cycles of: 1 min each at 94 °C, 1 min at 63 °C, 1 min at 72 °C and a final extension step at 72 °C for 10 min. Amplicons have been separated on 1.5% agarose gel, stained with ethidium bromide.

3. Results

All *Salmonella* strains amplified a 413-bp fragment with the set of primers for *hilA* gene (Fig. 1). Non-*Salmonella* strains did not amplify and no nonspecific products were amplified. For the *Salmonella enteritidis* strains an amplification of a 261 bp fragment of the *agfA* gene was obtained. However, the same result could not be achieved for the *Salmonella choleraesuis* strains (Fig. 1).

SpvC and *sef* genes amplification products were obtained at *S enteritidis*; *agfA* gene product was obtained only at *S. enteritidis* (Fig. 2). Amplification products for *misL* gene were obtained at all *Salmonella* species that we analyzed (Fig. 3).

For discrimination between *S. enteritidis* and *S. choleraesuis* we performed a multiplex PCR. Co amplification of *sef* and *spvC* or *spvC* and *agfA* allowed us to see the difference between these two species (Fig. 4).

4. Discussion

Serovar, *S. enteritidis* is a model for the study of fimbriae as a virulence factor, by binding to specific host receptors; fimbriae mediate bacterial colonization and/or optimal toxin delivery. Fimbriae are proteinaceous filamentous structures present on the surface of many members of the *Enterobacteriaceae*, including the genus *Salmonella* (Thorns, 1995). Increasing evidence suggests that bacterial fimbriae play an important role in infection (Naughton et al., 2001; De Buck et al., 2003), although their exact role in the pathogenesis of *Salmonella* is still controversial (Rajashekara et al., 2000). *S. enteritidis* produces several fimbrial types (Bäumler and Heffron, 1995). Its genome contains many putative fimbrial operons: agf, bcf, fim, lpf, pef, saf, sef, stb, stc, std, ste, stf, sth, sti (Porwolik and McClelland, 2003); however, expression of fimbrial proteins encoded by these operons has been demonstrated for a few of them. It has been shown that *S. enteritidis* elaborates fimbriae designated SEF17 (encoded by the agf operon) (Collinson et al., 1996) that mediate fibronectin binding (Collinson et al., 1991). SEF17 fimbriae are composed mainly of a fimbrin and are highly stable structures. SEF14 fimbriae are encoded by the sef operon (Clouthier et al., 1993). Diarrheagenic *Escherichia coli* strains

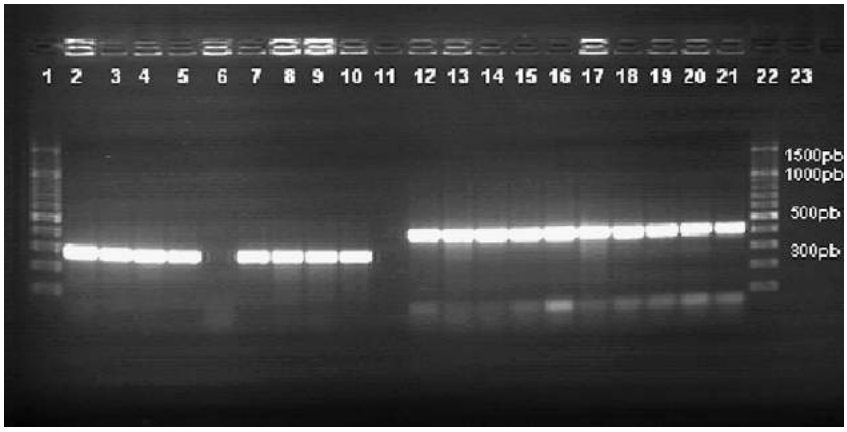

Fig. 1. Agarose gel electrophoresis of PCR products after amplification of *agfA and hilA* genes. Lanes: 1, 22 – molecular weight marker; 2–5 and 7–10 – different strains of *S. enteritidis* (*agfA* gene products); 6 and 11 – *S. choleraesuis* (*agfA* gene products), 12–15 – *S. enteritidis* (hilA gene products): 16–18 – *S. typhimurium* (*hilA* gene products); 19–21 – *S. choleraesuis* (*hilA* gene products); 23-negative control.

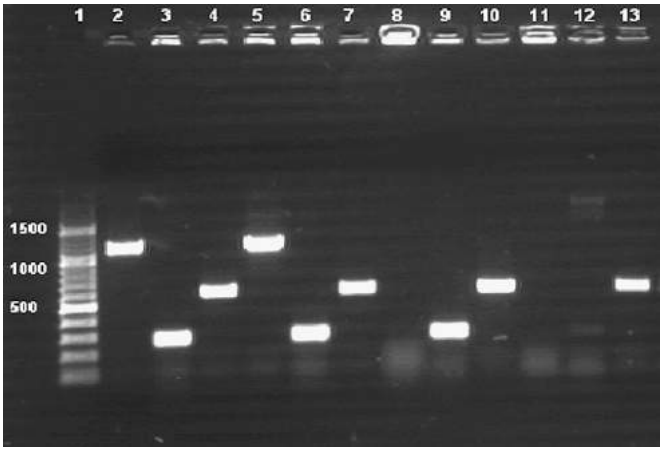

Fig. 2. Agarose gel electrophoresis of PCR products after amplification of *sef*, *agfA* and *spvC* genes. Lanes: 1 – molecular weight marker; 2, 5, – different strains of *S. enteritidis* (*sef* gene products); 3, 6, 9 – different strains of *S. enteritidis* (*agfA* gene products); 4, 7, 10 – different strains of *S. enteritidis* (*SpvC* gene products); 8 – *S. typhimurium* (*sef* gene products); 11–13 – *S. choleraesuis* (*sef*, *agfA* and *SpvC* gene products).

produce thin, aggregative fimbriae that are biochemically and serologically related to those of SEF17 (Collinson et al., 1992). However, the degree of DNA sequence dissimilarity between the respective fimbrin genes is sufficient that *agfA*-based nucleotide probes hybridize only to *Salmonella* DNA, thereby providing a valuable, genus-specific diagnostic for *Salmonella* spp. (Doran et al., 1993).

Many *Salmonella* serovars harbour virulence (V) plasmids with variable size, depending on the serovar (Fierer and Guiney, 2001). All V plasmids share a highly conserved 8 kb region with five genes designated spvRABCD (*Salmonella* plasmid virulence) (Paesold et al., 2002). The spv region appears to promote rapid growth and survival of *Salmonella* within the host cells, being important for systemic infection in experimental animals.

Many of virulence factors are clustered within *Salmonella* pathogenicity islands (SPIs) (Fierer and Guiney, 2001) of which SPI-1 and SPI-2 have been the most intensively studied (Galán, 2001; Hensel, 2000). Several studies have reported that the expression

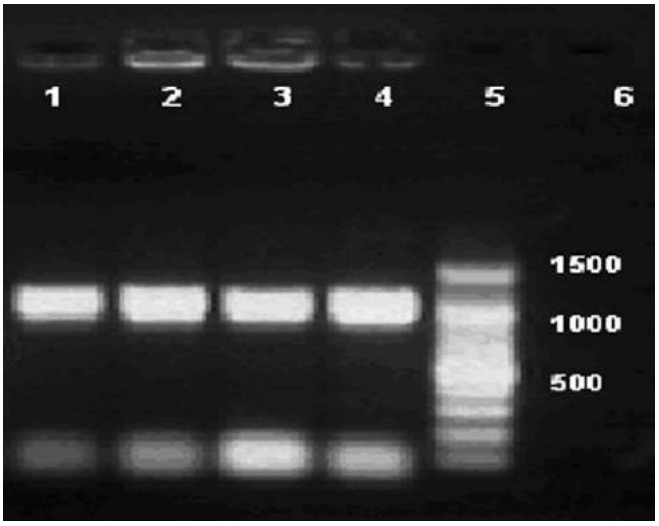

Fig. 3. Agarose gel electrophoresis of PCR products after amplification of *misL* gene. Lanes: 1–*S. enteritidis*; 2 – *S. Dublin*; 3 – *S. infantis*; 4 – *S. choleraesuis*; 5 – molecular weight marker; 6 – negative control (no template).

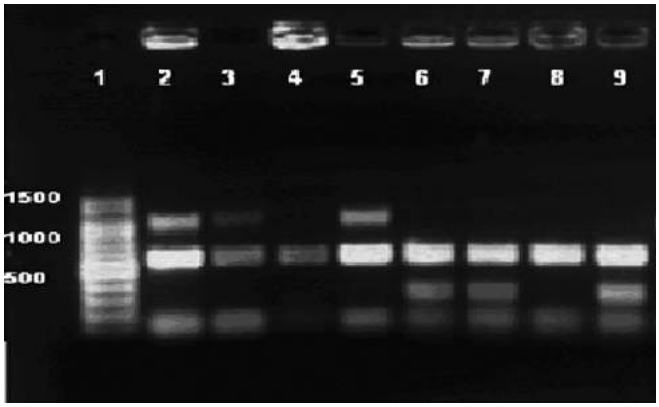

Fig. 4. Agarose gel electrophoresis of PCR products after multiplex amplification of *sef* and *spvC* genes (lanes 2–5), *spvC* and *agfA* (lanes 6–9). Lanes: 1 – molecular weight marker; 2, 3, 5, 6, 7, 9 – different strains of *S. enteritidis*; 4, 8 – *S. choleraesuis*.

of pathogenicity island genes is coordinated with that of genes contributing to motility (Ellermeier and Slauch, 2003). This connection between virulence gene expression and motility probably reflects a need for the pathogen to coordinate its physical mobility with the expression of genes involved in niche invasion and adaptation. Moreover, mobility is known to be required for *Salmonella* virulence (Schmitt et al., 2001; Merrell et al., 2002). Genetic studies have identified regulators that are specific to particular virulence genes. These include the HilA protein that regulates transcription of the SPI-1 island genes (Boddicker et al., 2003).

Several PCR methods to detect *Salmonella* spp. in human samples, food and water have been developed with the aim of improving diagnosis of the infection or contamination (Pathmanathan et al., 2003; Sanchez-Jimenez and Cardona-Castro, 2004).

The goal of this study was to evaluate the suitability of the *hilA*, *agfA*, *spvC* and *sef*, genes amplification by PCR as a method for detection of *Salmonella* strains. We have chosen these genes because they play an important role in the pathogenicity and virulence of *Salmonellae*. We have performed a multiplex PCR which allowed us to see the difference between *S. enteritidis* and *S. choleraesuis*. Our results confirmed yet again that PCR amplification of *hilA* gene with primers that are specific for *Salmonella* is a promising technique for diagnosing *Salmonellae*. The multiplex PCR (using two sets of primers pairs, which were targeted for the *sef* and *spvC* or *spvC* and *agfA* genes) allows correct identification of *S. enteritidis* and *S. choleraesuis*.

Acknowledgements

This work was supported by CNCSIS grant 1349/2006 from the Ministry of Education and Research, Romania.

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Journal of Environmental Management 95 (2012) S19–S24

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Evaluating the response of two high yielding Indian rice cultivars against ambient and elevated levels of ozone by using open top chambers

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ARTICLE INFO

Article history:
Received 29 September 2009
Received in revised form
16 June 2011
Accepted 24 June 2011
Available online 23 July 2011

Keywords:
Ozone pollution
Indian rice
Open top chamber
Growth
Yield
Proteome analysis

ABSTRACT

A continuous increase in the background level of tropospheric ozone (O_3) has become a major challenge for present and future agricultural productivity at worldwide. Present study was designed to assess the impact of ambient (present) and elevated (future) concentrations of O_3 on two cultivars of Indian rice (*Oryza sativa* L. cvs Malviya dhan 36 and Shivani). Shoot and root lengths, number of leaves and total leaf area were severely affected by both ambient and elevated concentrations of O_3. Photosynthetic rate, stomatal conductance and photosynthetic efficiency (F_v/F_m) were also reduced by O_3 with more drastic effects under elevated levels of O_3. Leaf proteome showed reduction of some major proteins due to O_3. Pollen viability, viable florets plant^{-1} and economic yield also showed significant negative impact under O_3-exposure in both the test cultivars. The experimental findings depict that both the cultivars of rice demonstrate differential response against O_3, and it may help the plant breeders in selection of resistant cultivars for the area having higher concentrations of O_3.

1. Introduction

Tropospheric ozone (O_3) has long been recognized as a major threat to global agriculture (Booker et al., 2009; Cho et al., 2011). This secondary air pollutant is normally produced by photochemical reactions, involving volatile organic compounds and nitrogen oxides (NO_x), under bright sun light. According to Vingarzan (2004), the mean global concentrations of tropospheric O_3 will rise by 0.5–2% annually; unless the levels of primary pollutants are reduced.

Rice is cultivated in around 95 countries at worldwide; and provides food for more than 50% of global population (IRRI, 2002). Yonekura et al. (2005) reported that the yield of Japanese rice is reduced by 5–10% under ambient O_3-exposure; and this loss might increase up to 25–35% by 2050. Ainsworth (2008) calculated 14% yield loss in rice, exposed to 62 ppb of O_3, in a meta-analysis of 12 peer-reviewed studies published between 1980 and 2007. Researchers have also found that, not only yield but other major growth parameters in plants were severely affected by O_3 too (Ainsworth, 2008; Rai and Agrawal, 2008; Sarkar and Agrawal, 2010a). Rai and Agrawal (2008) observed significant reductions in photosynthetic rate, stomatal conductance and total chlorophyll; which resulted in 11–15% yield loss in two cultivars of Indian rice under ambient O_3-exposure at Varanasi, India. Singh et al. (2005) have also reported major loss in total biomass of *Beta vulgaris* L. plants under ambient O_3 concentrations in Allahabad, India. Recently, Sawada and Kohno (2010) have noticed dose dependent yield reduction in 12 Indian and Japanese cultivars of rice under elevated O_3-exposure. Plant reproductive parameters, *viz.* flowers, pollen viability, fruit set, etc., also respond poorly under O_3-exposure (Ollerenshaw and Lyons, 1999; Black et al., 2000; Sarkar and Agrawal, 2010a). Some researchers reported that O_3 causes severe damage in plant proteome too, by inhibiting the expression of several photosynthetic and primary metabolism related proteins; and indicated this as a major cause behind the reduced productivity in crop plants under O_3-exposure (Cho et al., 2008; Sarkar and Agrawal, 2010b; Sarkar et al., 2010).

Keeping the above points in mind, this investigation was designed to evaluate the impact of O_3 on two Indian rice cultivars by assessing certain growth, reproductive, physiological, molecular and yield parameters. The findings might help in screening of rice cultivars for the area experiencing high concentrations of O_3.

2. Materials and methods

2.1. Rice cultivation at experimental site

The experiment was carried out at the Agricultural Research Farm, Banaras Hindu University, India. The site (25° 14′ N, 82° 03′ E) was

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doi:10.1016/j.jenvman.2011.06.049

located at 76.1 m above mean sea level. Soil of the study site was typical sandy loam with pH 7.2. Two high yielding cultivars of Indian rice (*Oryza sativa* L. cultivars Malviya dhan 36 and Shivani) were selected for the experiment. At the age of 21 days, seedlings were transplanted in different experimental setups. Recommended dose of fertilizers (120, 60 and 60 kg ha^{-1} N, P and K as urea, single super phosphate and muriate of potash, respectively) were used for present study.

2.2. Ozone monitoring at experimental site

Ozone monitoring was done on a 12 h day^{-1} basis (6:00–18:00 h) at the experimental site and its concentrations were measured by using automatic O_3-analyzer (Model APOA 370, HORIBA Ltd., Japan).

2.3. Experimental design

Rice plants were exposed to two elevated levels of O_3 by using open top chambers (OTCs) as performed by Sarkar and Agrawal (2010a). Experimental OTCs were divided as: charcoal filtered air (FC), non – filtered air (NFC), non – filtered air + 10 ppb O_3 (NFC+) and non – filtered air + 20 ppb O_3 (NFC++). By this design, the rice plants were exposed to four different levels of O_3: (i) nearly no O_3 in FC, (ii) ambient level of O_3 in NFC, (iii) ambient + 10 ppb O_3 in NFC+, and (iv) ambient + 20 ppb O_3 in NFC++. Open plots (OPs, $n = 3$) were also maintained to monitor the effects of chamber enclosures. The treatments were performed in a complete randomized manner. O_3-exposure was done by O_3 generators (Model Systrocom, India) daily at the peak O_3 period (from 10:00 h to 15:00 h) of local time.

2.4. Growth response analysis

To determine various growth and biomass parameters, five monoliths ($10 \times 10 \times 20$ cm^3) containing intact roots were carefully collected at random from each chamber and also from open plots at 25, 50 and 75 days after transplant (DAT). Growth parameters like root and shoot length, leaf area and number of leaves were recorded. Leaf area was measured using portable leaf area meter (Model LI – 3100, LI – COR, Inc. USA).

2.5. Reproductive response analysis

Fertile florets $plant^{-1}$ and pollen viability were assessed as reproductive parameters. For counting of fertile florets, the tagged spikelets were periodically evaluated until the seed set; and pollen viability was scored with 2% aceto-carmine solution as described earlier by Sarkar and Agrawal (2010a).

2.6. Photosynthetic pigment and photosynthetic efficiency analysis

Total chlorophyll and carotenoids were measured according to the methods given by Machlachlan and Zalik (1963) and Duxbury and Yentsch (1956). Chlorophyll fluorescence was measured by using portable Plant Efficiency Analyzer (Model MK2 9414, Hansatech

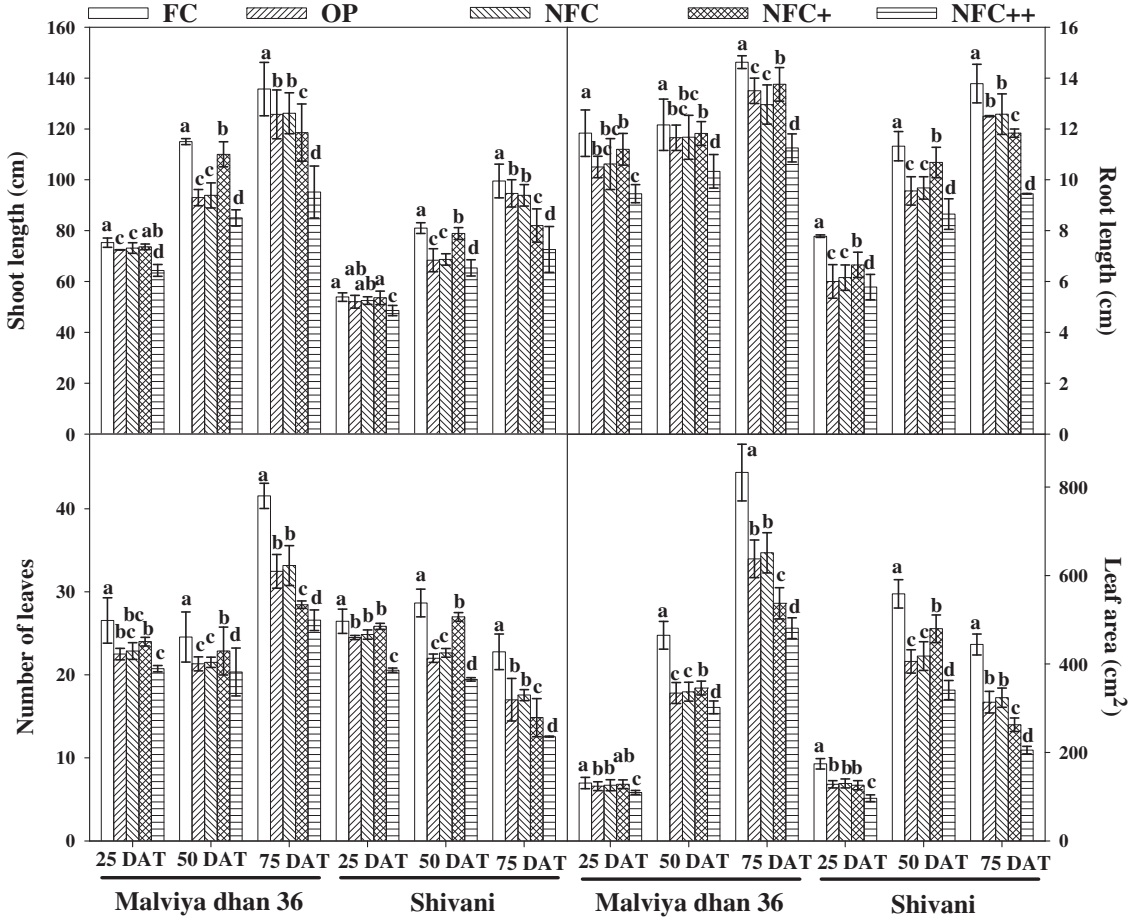

Fig. 1. Effect of O_3 on shoot height, root length, number of leaves and leaf area of two different rice cultivars at different stages of growth. Values are mean ± SE. Bars showing different letters indicate significant differences among each group of bars according to Duncan's test at $p < 0.05$.

Instrument Ltd., UK). Initial fluorescence (F_o) and maximum fluorescence (F_m) were measured to find variable fluorescence (F_v) and F_v/F_m ratio. Photosynthetic rate (Ps) and stomatal conductance (gs) were quantified with the help of portable photosynthetic system (Model LI-6200, LI-COR, USA). The measurements were made on the third fully expanded mature leaves from the top of each plant on cloud free days between 08.00 and 10.00 h.

2.7. Leaf proteome analysis through 1 − DGE (one dimensional gel electrophoresis)

Comparative analysis of leaf proteome from both O_3-exposed and unexposed rice plants was performed 11.5% SDS PAGE as described earlier by Sarkar et al. (2010).

2.8. Yield parameters

Rice plants, at maturity, were harvested to assess different yield attributes. Ten plants from each treatment were sampled; and weight of grains m^{-2} and harvest index (HI) were recorded.

2.9. Statistical analysis

Observed data were subjected to one and two way analysis of variance (ANOVA) for assessing the significance of quantitative changes in different parameters due to various treatment and treatment with cultivars. Duncan's multiple range test was performed as post hoc on parameters subjected to ANOVA test. The

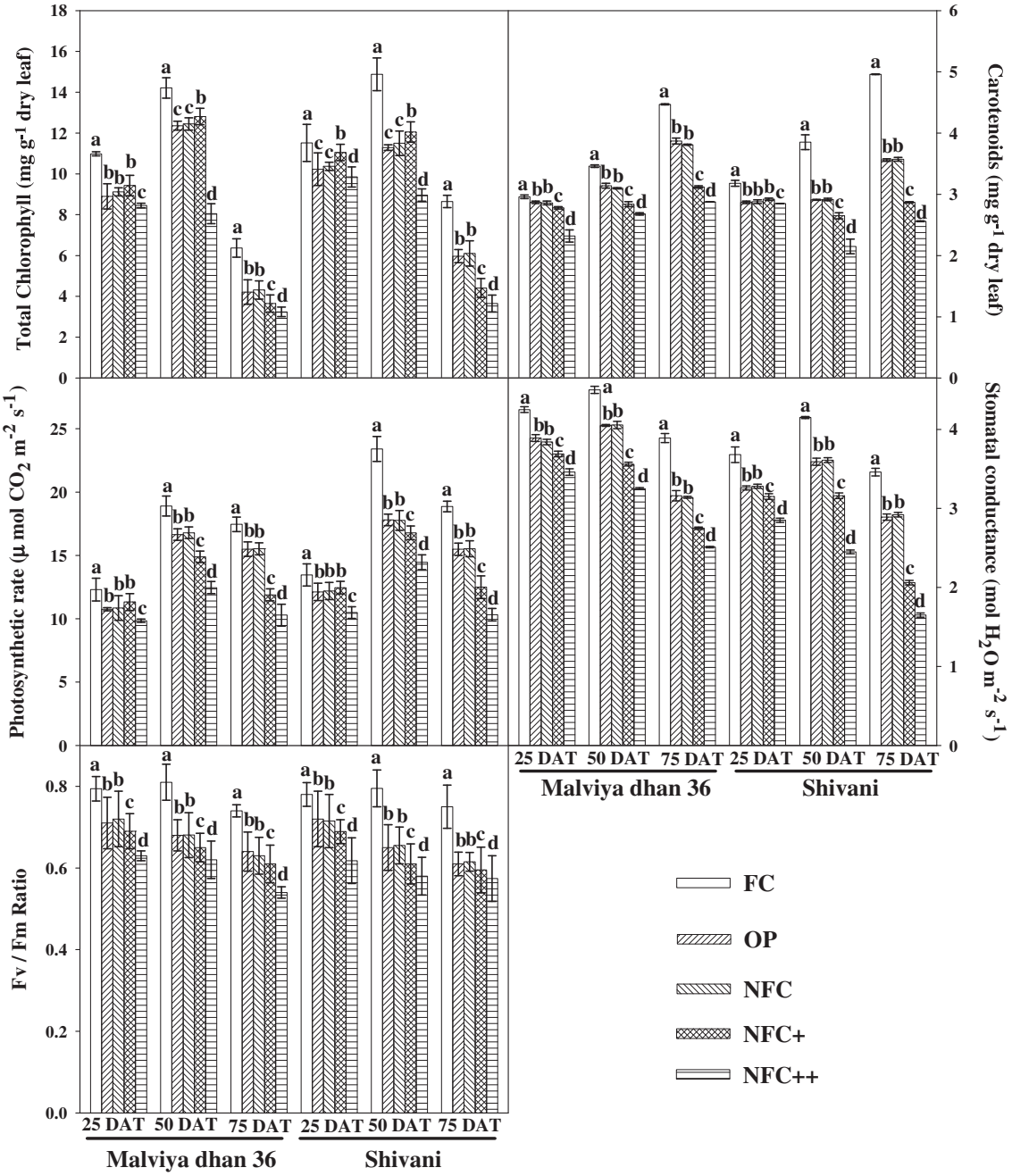

Fig. 2. Effect of O_3 on total chlorophyll, carotenoids, photosynthetic rate stomatal conductance and F_v/F_m ratio of two different rice cultivars at different stages of growth. Values are mean ± SE. Bars showing different letters indicate significant differences among each group of bars according to Duncan's test at $p < 0.05$.

statistical analyses were performed using SPSS software (SPSS Inc., version 16.0).

3. Results and discussion

Present experiment was designed to evaluate the impact of ambient and elevated concentrations of O_3 on two high yielding Indian rice cultivars; on growth, photosynthetic, reproductive, molecular and yield parameters; under near natural conditions using OTCs. Use of activated charcoal filters in FCs reduced the O_3 concentration by 92.5%. During the experimental period, mean monthly O_3 concentrations were 42.7 ppb in June, 07; 41.3 ppb in July, 07; 44.7 ppb in August, 07; 58.2 ppb in September, 07 and 59.9 ppb in October, 07.

In general, O_3 severely affects the growth and development of plants. Shi et al. (2009) reported significant reductions in plant height and leaves in four rice cultivars under elevated O_3 levels at Xiaoji town, China. Sarkar and Agrawal (2010a) also found 39–62% reduction in plant height, 54–60% in total number of leaves under ambient + 20 ppm elevated O_3 in two wheat cultivars at Varanasi, India. Agrawal et al. (2005) also reported significant reductions in shoot and root lengths, total leaf area and biomass of mung bean plants under ambient levels of O_3 at Allahabad, India. Present results also followed similar trend at post O_3-exposure (Fig. 1). At 75 DAT; Malviya dhan 36 showed 7, 12.6 and 30%, and Shivani showed 5.7, 17.6 and 27% reductions in shoot length at NFC, NFC+ and NFC++, as compared to FCs, respectively (Fig. 1). Total number of leaves was also reduced by 20, 31.5 and 36% in Malviya dhan 36; and 23, 34.8 and 44.9% in Shivani, in 75 DAT at NFC, NFC+ and NFC++, as compared to FCs, respectively (Fig. 1). Other growth parameters like root length and total leaf area also reduced similarly at post O_3-fumigation (Fig. 1). Miller et al. (1999), in their study with *Arabidopsis*, reported that O_3 can induce several senescence associated genes (SAGs) too. In the present study the decreasing trend in healthy leaves at post O_3-exposure, might be an effect of SAGs activity in rice; which lead to early senescence (Fig. 1). However, at lower level of elevated O_3 (NFC+), it showed hormetic effect on both the rice cultivars by stimulating some growth parameters at the early stage (Fig. 1). Ishii et al. (2004) also found early induction in the growth of rice plants under O_3-exposure with lower elevation.

As an initial effect on photosynthesis, O_3 initiates the destruction of total chlorophyll by preventing the synthesis of this pigment (Castagna et al., 2001). Rai and Agrawal (2008) reported 23–27% reduction in total chlorophyll in rice under ambient concentration of O_3. Results of the present study also revealed reduction in chlorophyll by 18, 31 and 41% in Malviya dhan 36, and 26, 38 and 44% in Shivani, at NFCs, NFC+ and NFC++, as compared to FCs, respectively (Fig. 2). Carotenoids also reduced significantly at post O_3-exposure (Fig. 2). According to Lichtenthaler (1987), carotenoids are important photo-protective compounds that prevent photo-oxidative damage of chlorophyll. Rai and Agrawal (2008) also reported a 41–44% reduction in carotenoids under ambient O_3. However, Rainieri et al. (2001) suggested that any reduction in plant pigments may serve as an adaptation against O_3-stress; as the reduced number of light harvesting antennae complex may protects PSII from further photo-inhibition.

Ainsworth (2008) and Rai and Agrawal (2008) reported reduced photosynthetic rate (Ps) and stomatal conductance (gs) in rice under ambient and elevated O_3-exposure. Present study also showed significant reduction in Ps in both the cultivars (Fig. 2). Reduction in photosynthesis at post O_3-exposure can be correlated with the reduced amount in photosynthetic pigments too. Even, significant reduction in gs was also observed during the present

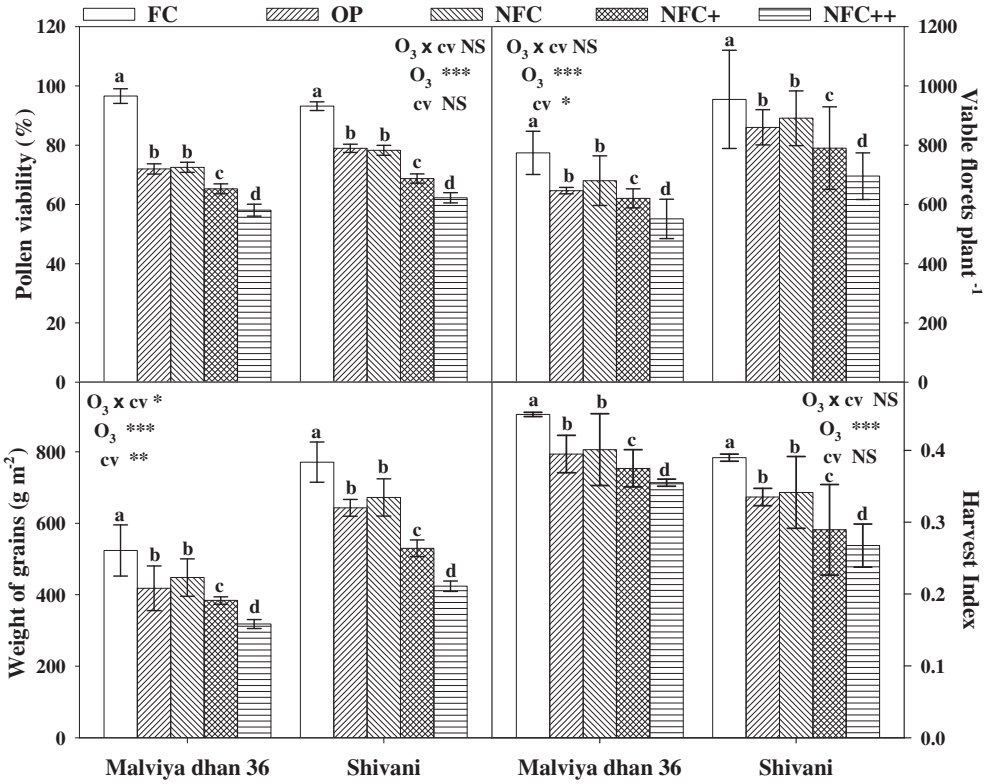

Fig. 3. Effect of O_3 on various reproductive and yield parameters of two different cultivars of rice. Values are mean ± SE. Bars showing different letters indicate significant differences among each group of bars according to Duncan's test at $p < 0.05$. Results of two way ANOVA shown as $O_3 \times$ cv, O_3 and cv. Level of significance $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ and NS: not significant.

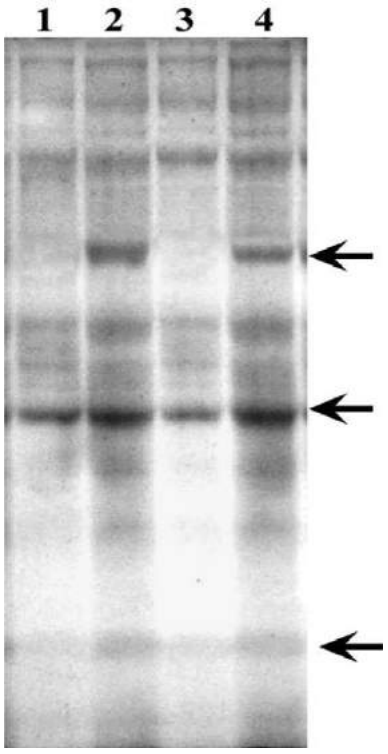

Fig. 4. 1 − DGE analysis of O_3-exposed (NFC++) and unexposed (FC) leaf proteome of two Rice cultivars. (1: Protein from O_3-exposed leaves of cultivar Shivani; 2: Protein from unexposed leaves of cultivar Shivani; 3: Protein from O_3-exposed leaves of cultivar Malviya dhan 36; 4: Protein from unexposed leaves of cultivar Malviya dhan 36). Arrows indicate the position of differential response in proteins.

study in both the cultivars under O_3-exposure (Fig. 2). F_v/F_m ratio indicates toward the photochemical efficacy of PSII, and any decrease in this ratio might serve as a reliable indication of O_3-induced photo-inhibition in plants. In the present experiment, F_v/F_m decreased by 15, 18 and 27% in Malviya dhan 36, and 18, 21 and 24% in Shivani at NFCs, NFC+ and NFC++, as compared to FCs, respectively (Fig. 2). Rai and Agrawal (2008) also found a significant reduction in F_v/F_m in rice under ambient O_3-exposure.

Ozone has also been recognized as a potent inhibitor of reproductive structures in plants (Black et al., 2000). Schoene et al. (2004) found that O_3 affected the development of pollen by inhibiting starch accumulation in pollen grains in perennial ryegrass (*Lolium perenne* L.). Sarkar and Agrawal (2010a) also reported significant reduction in pollen viability and viable florets in wheat plants under O_3-exposure. In present study, pollen viability was affected by 25, 32 and 40% in Malviya dhan 36, and 16, 26 and 33% in Shivani at NFCs, NFC+ and NFC++, as compared to FCs, respectively (Fig. 3). Viable florets also followed the similar trend of reduction. Two ways ANOVA showed that in both the parameters, O_3 was the main determining factor (Fig. 3).

In any crop plant, yield is the ultimate interest of human society; and studies have shown that O_3 can cause severe damage to the grain and fruit yield of diverse crop plants (Ainsworth, 2008; Booker et al., 2009). Liu et al. (2009) showed that the relative yield loss in rice from 1990 to 1995 was 1.1–5.8% and would reach 10.8% in 2020 in Chongqing, China. Sawada and Kohno (2010) also reported significant yield loss under both ambient and elevated levels of O_3-exposure in 12 rice cultivars. Present results also responded similarly; as yield (g m^{-2}) was reduced by 15, 27 and 39% in Malviya dhan 36, and 13, 31 and 45% in Shivani at NFCs, NFC+ and NFC++, as compared to FCs, respectively (Fig. 3). Two way ANOVA also showed that yield was significantly varied due to O_3 ($p < 0.001$), cultivar ($p < 0.01$) and O_3 × cultivar ($p < 0.05$). Harvest index (HI) reflects the partitioning of photosynthates between grains and above ground biomass. Present results also showed significant reductions in HI due to O_3-exposure in both the rice cultivars (Fig. 3).

O_3-exposure also induced injuries in rice leaves, and the analysis of leaf proteome of O_3-exposed and unexposed leaves showed some definite changes at three points, i.e. at 54 kDa, 35 kDa and 15.7 kDa (Fig. 4). While comparing the results with earlier studies (Cho et al., 2008; Sarkar et al., 2010), it might be concluded that the large subunit (LSU) and small subunits (SSU) of RuBisCO were adversely affected with some other photosynthetic and energy metabolism proteins in both the rice cultivars at post O_3-exposure.

4. Conclusion

Present study clearly depicts that ambient as well as elevated levels of O_3 adversely affected the growth and yield of both the rice cultivars. Reductions of plant height, leaf area, total chlorophyll, photosynthetic rate, stomatal conductance, and chlorophyll fluorescence kinetics; increasing number of non − viable florets and pollens, and inhibition in the expression of major proteins are the fundamental manifestations of O_3-stress. Yield is the major interest of human society for retaining 'food security', and O_3 severely affected this parameter in both the test cultivars. However, cultivar Shivani showed significantly higher reduction in yield than cultivar Malviya dhan 36 under elevated levels of O_3. This clearly pointed toward the differential cultivar response of rice against O_3, and might be used for selecting suitable cultivars for an area experiencing higher concentrations of O_3.

Acknowledgments

The authors gratefully acknowledge the receipt of financial assistance in form of a research project (Scheme number: 24/292/06/EMR-II) funded by the Council for Scientific and Industrial Research (CSIR), New Delhi, Government of India. Authors also express their gratefulness to Prof. María Isabel Sastre Conde, Chairperson of the 3 IMEBE for giving the opportunity to present the findings of this paper during the conference and also for fruitful suggestions for preparing this manuscript.

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Journal of Environmental Management 95 (2012) S25–S30

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Effect of methyl parathion on nitrous oxide production: A laboratory study

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ARTICLE INFO

Article history:
Received 23 November 2009
Received in revised form
14 November 2010
Accepted 4 January 2011
Available online 4 February 2011

Keywords:
Contaminated soil
Methyl parathion
nirK gene
Nitrous oxide
TGGE

ABSTRACT

We investigated the diversity of a denitrifying gene (*nirK*) and the emission of CO_2 and N_2O, in a "chinampa" soil contaminated with methyl parathion. Soil at 40% of water holding capacity was spiked with methyl parathion at four concentrations (i.e. 0, 0.7, 1.47 and 4.27 g kg^{-1} dry soil), while emission of N_2O and CO_2 and *nirK* diversity was determined after 0, 1, 14, 30, 60 and 90 days. The emission of N_2O on a daily base and the cumulative emission of CO_2 was not affected by the different concentrations of methyl parathion applied to soil. The diversity of the *nirK* gene, determined by using temperature gradient gel electrophoresis (TGGE), decreased with increased methyl parathion application. It was found that methyl parathion had effect on the emissions of N_2O and CO_2, and reduced the diversity of the *nirK* gene. Consequently, the reduced diversity of the *nirK* gene could affect the emission of N_2O.

1. Introduction

Denitrification is a microbial process by which the nitrogen is returned to the atmosphere. The oxidized nitrogen compounds are used as alternative electron acceptors for energy production when oxygen is limited or absent (Delorme et al., 2003; Jones et al., 2008). This process is of great importance in agriculture, waste treatment and climate change. Interest in the denitrification process has increased as it is often to most important source of nitrous oxide (N_2O), an important greenhouse gas. N_2O contributes to changes in global atmospheric properties, essentially the greenhouse effect and ozone depletion (Bol et al., 2003; Zhang et al., 2008). Rochette et al. (2004) reported that agriculture contributed to nearly 70% of the annual emission of N_2O, mostly through microbial transformations of nitrogen *via* nitrification and denitrification. Although at a lower concentration in atmosphere than CO_2, the global warming potential of N_2O is approximately 296 times higher than of CO_2 (IPPC, 2001). As such, N_2O accounts for approximately 6% of the anthropogenically derived greenhouse effect (IPPC, 2001).

Contamination of soil from pesticides is a result of their bulk handling at the farm or following their application to the field. Synthetic organophosphorous compounds, such as methyl parathion, have been used extensively in certain parts of Mexico. Different bacteria, such as *Serratia* sp. strain DS001, *Bacillus* sp., *Pseudoaminobacter* sp., *Achromobacter* sp., *Brucella* sp., *Ochrobactrum* sp., *Flavobacterium balustinum, Pseudomonas* sp. A3, have been isolated from soil that can degrade methyl parathion (Sreenivasulu and Aparna, 2001; Zhang et al., 2005; Pakala et al., 2007). Methyl parathion can be used by some of these bacteria as carbon and phosphorous source (Ramanathan and Lalithakumari, 1999; Ortiz-Hernández et al., 2001). Soil characteristics, such as salinity and organic carbon content are known to affect the dissipation of methyl parathion from soil (Suter et al., 2002). Biodegradation of methyl parathion starts with a hydrolysis and two compounds are formed, i.e. 4-nitrophenol and dialkyl thiophosphate (DATP) (Singh and Walker, 2006) and two enzymes, i.e. methyl parathion hydrolase (MPH) and organophosphorous hydrolase (OPH), are involved (Singh, 2009).

Methyl parathion has been applied extensively to plants cultivated in the so called "chinampa" (from Náhuatl or Aztec, chinamitl, bulrush or cattail stalks lattice for hydroponics cultivation) found in Xochimilco (Mexico City, Mexico) (CICOPLAFEST, 2004). Little is known about the effect of pesticides on the soil microbial communities, although it has been observed that the microbial diversity decreased (Girvan et al., 2005). Pesticides might affect microbial activity and thus soil processes, such as emissions of N_2O (Spokas et al., 2006). For instance, it has been reported that chloropicrin increased the production of N_2O from soils (Spokas et al., 2005).

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doi:10.1016/j.jenvman.2011.01.002

Ecological studies have for nearly 15 years focused on the phylogenetic diversity of bacteria in the environment (Cheneby et al., 2000; Throbäck et al., 2004). Bacterial composition based on 16S rRNA analysis provides valuable information, but the functional importance of each of the identified organisms remains largely unknown. A relative new approach is to analyze the genes encoding for important functions in an ecosystem to understand the microbial ecology and biogeochemistry of an environment (Throbäck et al., 2004). Functional genes that encode for the enzymes involved in the denitrification process (i.e. nitrite, nitric oxide and nitrous oxide reductases) can be investigated by using targeting conserved regions. The *nirK* gene encodes nitrous oxide reductase (Nir) and is determinant in the emission of N_2O and N_2 ratio. It is always present in denitrifier microorganisms and could be used to study the molecular ecology of denitrifying bacteria.

The objective of this study was to investigate the diversity of the denitrification process related to gene *nirK* and the production of N_2O and CO_2 in a chinampa soil of Mexico City contaminated with methyl parathion. Cultivation in the chinampa soil is unique as it is based on the traditional Aztec agricultural technique and little is known about how methyl parathion might affect emissions of N_2O and CO_2, and *nirK* gene.

2. Materials and methods

2.1. Sampling site

The sampling site, i.e. a "chinampa" in Xochimilco N19°15.812′ and W 99° 04.10', is situated in the South of Mexico City at an altitude of 2240 m. The sampling site of 4500 m^2 was divided in three equal plots. On 30th November 2007, the 0–15 cm layer of each plot was sampled 20 times and the soil of each plot was pooled so that three soil samples were obtained ($n = 3$). The pH and the electric conductivity (EC) in the saturated extract of studied soil were 7.8 and 1.3 dS m^{-1} (low salinity), respectively. The clay loam soil had an organic C content of 16 g kg^{-1}, total N 7 g kg^{-1} and a particle size distribution of 260 g kg^{-1} sand, 440 g kg^{-1} silt and 300 g kg^{-1} clay.

2.2. Treatments and experimental design

The soil was taken to the laboratory and treated as follows. The samples, of approximately 7 kg, were kept in drums at 4 °C under aerobic conditions for a week. In each of the aerobic experimental units (microcosm), 500 g of soil at 40% water holding capacity (WHC) was added to a 1600 ml flask and amended with three different concentrations of methyl parathion, i.e. at 0.7 g kg^{-1} (treatment 1), 1.47 g kg^{-1} (treatment 2) or 4.27 g kg^{-1} (treatment 3). These concentrations cover the range used by the farmers in the field. Soil without pesticide served as control. Each treatment applied to soil of each plot was done in triplicate. As such, a total of 9 microcosms was used for each treatment. For the 4 treatments, we obtained a total of 36 microcosms. Each microcosm contained a vessel with 1 M sodium hydroxide (NaOH) solution to trap the CO_2 evolved. The 1600 ml-glass bottles were closed air-tight with a valve allowing gas interchange so that aerobic condition was maintained. The closed jars were incubated at 20 °C for 90 days. Jars were opened every 3 days to maintain aerobic conditions. Aerobic conditions prevail in the field.

After 0, 1, 14, 30, 60 and 90 days, emission of N_2O and CO_2 were determined. The CO_2 trapped in the 1 M NaOH was determined by titration with 0.1 M HCl (Jenkinson and Powlson, 1976; Amato, 1983) while N_2O was separated from the other gasses in a Porapak Q column and measured with a Fisher chromatograph fitted with a TCD detector.

2.3. Direct DNA extraction of soil and PCR amplification of nirK

Extraction of DNA from soil (0.25 g) was done with Ultra Clean soil DNA isolation kit (MoBio, Carlsbad, CA, USA). The quality and the size of the soil DNAs were checked by electrophoresis on 1% agarose gels. DNA was quantified using a BioPhotometer (Eppendorf, Hamburg, Germany) at 260 nm. Three replicates were used for DNA evaluation in each microcosm.

The *nirK* gene was amplified from 50 ng DNA extracted from soil with a total volume of 25 μl using the primers 1f (5′-GGMATGGTKCCSTGGCA-3′) with a clamp sequence (5′-CGCC-CGCCGCGCGCGGCGGGCGGGGCGGGGGCAC GGGGGG-3′) and 5r (5′-GCCTCGATCAGRTTRTGGTT-3′) (Throbäck et al., 2004) under the following conditions: one cycle of 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 58 °C for 45 s and 72 °C for 1 min; one cycle of 72 °C for 10 min and one final of 4 °C for 90 min. The final reaction mixture was: 1x *Taq* Pol buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs, 100 pmol of each primer, 200 ng of DNA and 2U of *Taq* Pol (GoFlex *Taq* Pol A, Promega), in a final volume of 25 μl.

2.4. Temperature gradient gel electrophoresis (TGGE)

Aliquots of 20 μl were separated by electrophoresis on a native 8% acrylamide-bisacrylamide gel with 8 M urea, and 1.25 TAE at 60 V for 13.5 h with temperature gradient (56–66 °C), temperature rate 0.8 °C h^{-1}, using a D-Code system (Bio-Rad Laboratories Inc.). Gels were stained with 1:10000 (v/v) SYBR Gold for 1 h followed by UV transillumination. Images were documented with the Gel Doc 2000 System (Bio-Rad) and digital pictures were analyzed with the Quantity One software (Bio-Rad Laboratories Inc.).

The range-weighted richness (Rr) is a mathematical value used to describe the total diversity of the sample analyzed, according to the following formula: Rr = Total number of bands × [(Lb − Sb)/100], where Lb is the longest band in terms of base pairs (bp), while Sb is the shortest one (Rojas-Oropeza et al., 2010).

2.5. Sequencing and computer analysis

Selected DNA-band were cut and purified with Qiagen II Kit for DNA sequencing. The nucleotide sequences of the DNA bands were determined by automated DNA sequencing using the dideoxy chain-termination method and the ABI model 373A sequencer stretch (Applied Biosystems, Instituto de Fisiología Celular, UNAM). Each sequence was compared with sequences available in databases (GenBank, Blast, NCBI). A selection of denitrifying bacteria from soil was included for the tree analysis (Functional Gene Pipeline, http://fungene.cme.msu.edu/). The absence of chimerical sequences was checked with the Pintail program (http://www.bioinformatics-toolkit.org/) (Kevin et al., 2005). Derived nucleotide sequences of *nirK* were aligned with nucleotide sequences of equivalent length using the ClustalW Multiple alignment software of BioEdit Sequence Alignment Editor, version 7.0.9.0. The tree analysis was performed with the software Phylip 3.67 and Treeview. Distance matrix analyses were done with the Jukes and Cantor correction (Jukes and Cantor, 1969). The tree was reconstructed using the neighbor-joining method (Saitou and Nei, 1987) and the tree topology was determined by bootstrap analysis using 100 replicates.

2.6. Nucleotide sequence accession numbers

Sequences obtained in this study were deposited in Genbank under accession numbers HQ292060–HQ292064.

2.7. Statistical analyses

Results were analyzed using the General Linear Model of the univariate type with SPSS 13.0.

3. Results

3.1. Emissions of N_2O and CO_2

Emission of N_2O was most accentuated in the first day for all treatments and changes thereafter were small or non-existent (Fig. 1). The emission of N_2O was significantly higher in the microcosm amended with the highest amount of methyl parathion (treatment 3) than in the control and in the microcosm amended with low concentrations of pesticides (treatments 1 and 2) ($P < 0.05$).

Emission of CO_2 was most accentuated in the first day for all treatments and changes thereafter were small (Fig. 2). The emission of CO_2 was significantly higher in the unamended control soil than in soil amended with the lowest amount of methyl parathion (treatment 1), but lower than in soil amended with the highest amounts of the pesticide (treatment 3) than in the unamended control soil ($P < 0.05$). The ratio between N_2O and CO_2 produced ranged from 1/8 and 1/12 and the higher production of N_2O was correlated to a higher production of CO_2.

3.2. TGGE study

In the control treatment, the TGGE analyses of partial *nirK* genes gave only a few bands (Fig. 3). Two dominating dense bands were found in the middle of the gel (X3 and X7). Similar patterns were obtained in soil amended with the lowest amount of the pesticide (treatment 1) (Fig. 4, supplementary material). This indicated a similar microbial diversity in the control treatment and treatment 1. Patterns in treatments 2 and 3 were similar, but less bands were found than in the control treatment and treatment 1 (Fig. 5, supplementary material; Fig. 6). It appears therefore, that the microbial diversity was lower in treatments 2 and 3 than in treatment 1 and the control. In treatments 2 and 3, the intensity of bands X3 and X7 decreased after 30 days suggesting a disappearance of these Operational Taxonomic Units (OTUs).

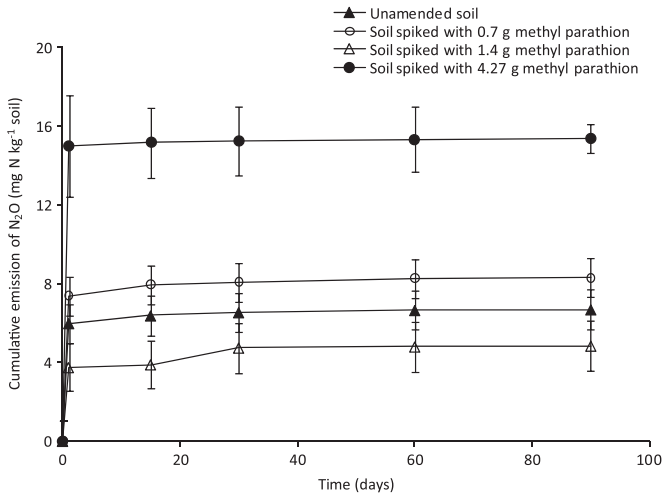

Fig. 1. Emission of N_2O (mg N kg^{-1} dry soil) from unamended soil (▲), and soil amended with 0.7 g methyl parathion kg^{-1} soil (○), 1.47 g methyl parathion kg^{-1} soil (△) or 4.27 g methyl parathion kg^{-1} soil (●) incubated aerobically for 90 days. Bars are ± one standard deviation.

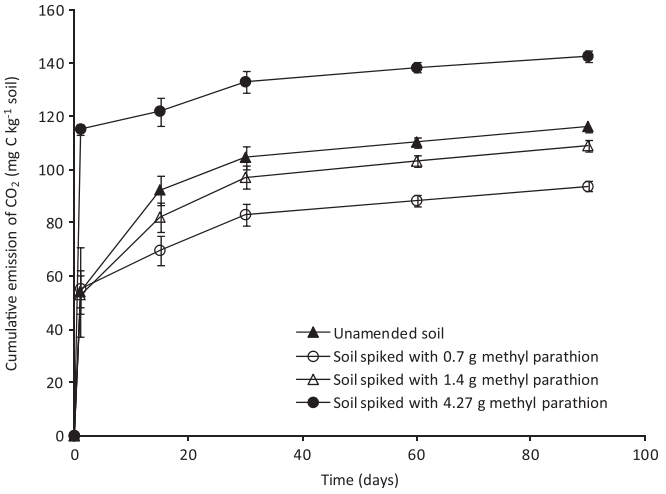

Fig. 2. Emission of CO_2 (mg C kg^{-1} dry soil) from unamended soil (▲), and soil amended with 0.7 g methyl parathion kg^{-1} soil (○), 1.47 g methyl parathion kg^{-1} soil (△) or 4.27 g methyl parathion kg^{-1} soil (●) incubated aerobically for 90 days. Bars are ± one standard deviation.

3.3. Comparison of range-weighted richness (Rr)

The range-weighted richness (Rr) of the unamended control soil and soil amended with methyl parathion (treatments 1, 2 and 3) was 3.61 ± 1.95, 3.77 ± 1.66, 0.87 ± 0.31 and 0.78 ± 0.08, respectively. This confirmed the similarity between the control and treatment 1, and between the treatments 2 and 3.

3.4. Phylogenetic analysis

At least five isolates from each of the four TGGE types were selected for *nirK* gene sequencing. No chimeras were detected for X1 OUT sequence. Similarity percent was too low to obtain accurate results for X2, X3, X6 and X7 OUT sequences. No reliable sequences were available in these cases, because all the most similar

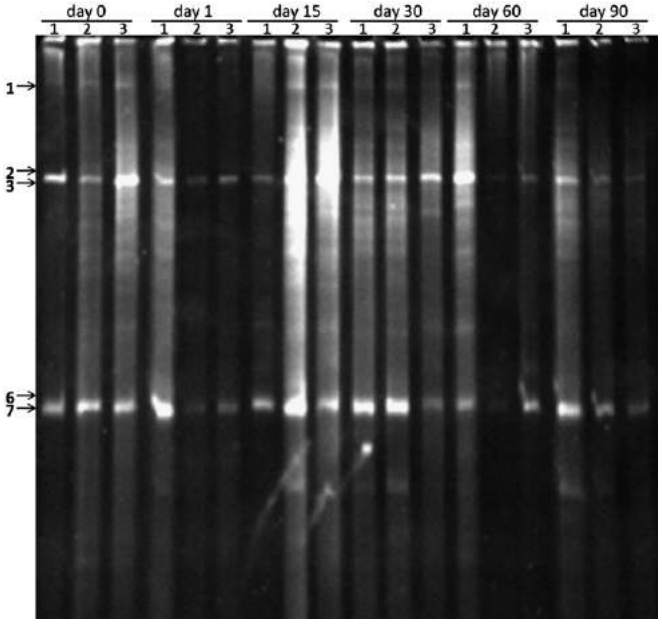

Fig. 3. TGGE profile of the unamended control soil incubated aerobically for 90 days (8% acrylamide-bisacrylamide gel, 8 M urea).

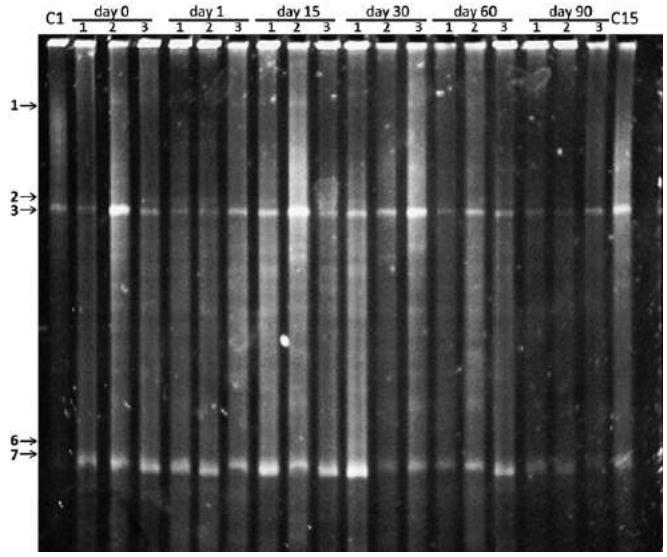

Fig. 4. TGGE profiles of soil amended with 0.7 g methyl parathion kg^{-1} soil (treatment 1) incubated aerobically for 90 days (8% acrylamide-bisacrylamide gel, 8 M urea) (lane C1 and lane C15 correspond to the profile of unamended control soil).

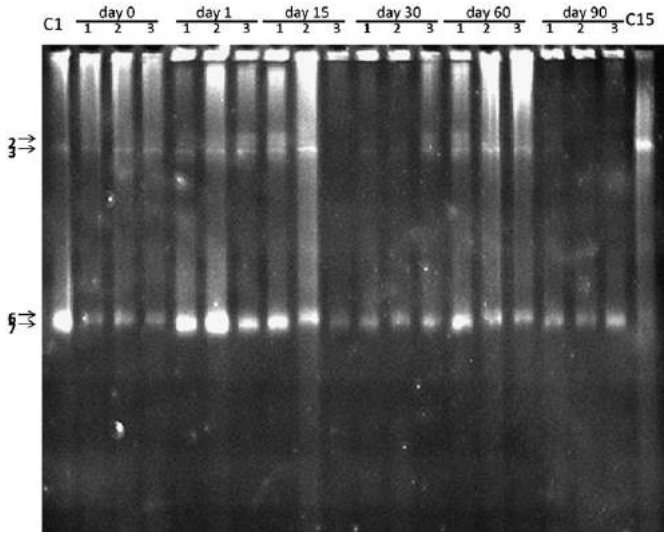

Fig. 6. TGGE profiles of soil amended with 4.27 g methyl parathion kg^{-1} soil (treatment 3) incubated aerobically for 90 days (8% acrylamide-bisacrylamide gel, 8 M urea) (lane C1 and lane C15 correspond to the profile of unamended control soil).

sequences were from uncultured origin. Approximately 450 nucleotides were sequenced from each amplified *nirK* and used to generate a phylogenetic tree with *nosZ* gene of *Marinobacter* sp. as outgroup (Fig. 7). The five isolates were associated with Bacteria. The X1, X3 and X6 isolates were not related to known denitrifying strains, although *Rhizobium* sp. was close to them, and they were similar to each other. The X2 isolate was related to an uncultured clone of *nirK* gene, but not to other isolates or to known denitrifying bacteria. The X7 isolate was related to *Pseudomonas fluorescens* Pf5, but not to the other isolates. The two dense bands dominating in the middle of the gel, X3 and X7, had a high sequence divergence for distance-based phylogenetic inference.

4. Discussion

It is a fact that constructing microcosms can distort microbial community composition, richness and soil structure (Hughes et al., 2008). In this study, the alterations due to abiotic and biotic factors were the same for each treatment. It is therefore assumed that the comparison between the treatments is valid (Philippot et al., 2002).

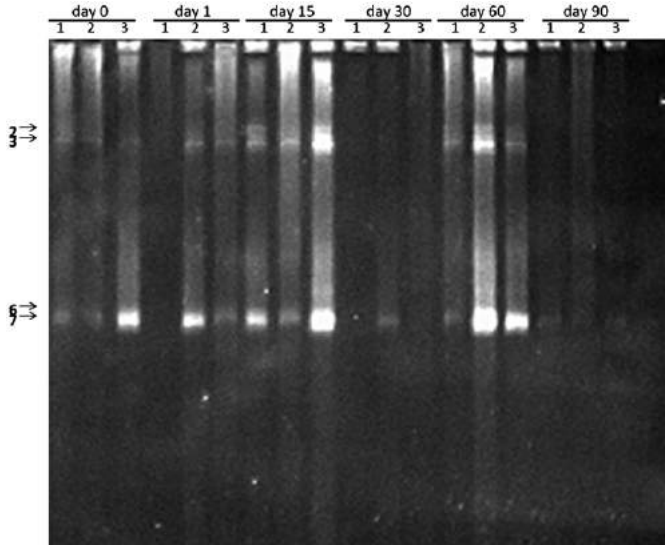

Fig. 5. TGGE profiles of soil amended with 1.47 g methyl parathion kg^{-1} soil (treatment 2) incubated aerobically for 90 days (8% acrylamide-bisacrylamide gel, 8 M urea).

The major emission of N_2O and CO_2 was produced in the first 24 h. This might be related to a sudden and rapid mineralization of organic matter as a result of manipulating the soil, addition of a substrate, e.g. methyl parathion and changes in soil water content. Emission of N_2O is mainly the result of nitrification, denitrification or denitrification–nitrification processes (Wrage et al., 2001). Denitrification is the process that normally contributes most to emissions of N_2O. Although incubation conditions were aerobic, anaerobic micro-sites could not be excluded. In these anaerobic micro-sites, denitrification is induced increasing emissions of N_2O (Zumft, 1997). It has to be remembered that aerobic denitrification might also have contributed to emission of N_2O (Kong et al., 2006). Application of methyl parathion to soil often increases emission of CO_2 as it is readily mineralized (Ragnarsdottir, 2000). It might be that the specific soil characteristics permit the mineralization of methyl parathion in this experiment.

In the phylogenetic tree, X7 OTUs were related to *nirK* of *Pseudomonas fluorescens* Pf5. Strains of this genus are known to denitrify under aerobic conditions (Kong et al., 2006). X7 was found in all four treatments and might have contributed to the emission of N_2O.

The band pattern in the control treatment suggests a representative structure of the denitrifying microbial population. The X1, X2, X3, X6 and X7 OTUs were found in the three plots, as well as a great number of bands between X1 and X2, and X3 and X6. The range-weighted richness (Rr) was always lower than 10. This indicated a low Rr and as such a low bacterial diversity (Marzorati et al., 2008). However, it must be remembered that Marzorati et al. (2008) used the 16S gene, which can give higher values.

Similar patterns were obtained for the control and treatment 1. Application of 0.7 g methyl parathion kg^{-1} soil did not affect the denitrifying diversity and its functional potential (as expressed by the emission of N_2O). Therefore the functional stability (defined here as the resistance and resilience of short-term denitrification to pesticide perturbation) was preserved in these conditions (Griffiths et al., 2004). The patterns for the treatment 2 and 3 were similar, confirmed by values of Rr.

However, N_2O emissions in the microcosm of treatment 3 were significantly higher than in the other treatments. Rates of

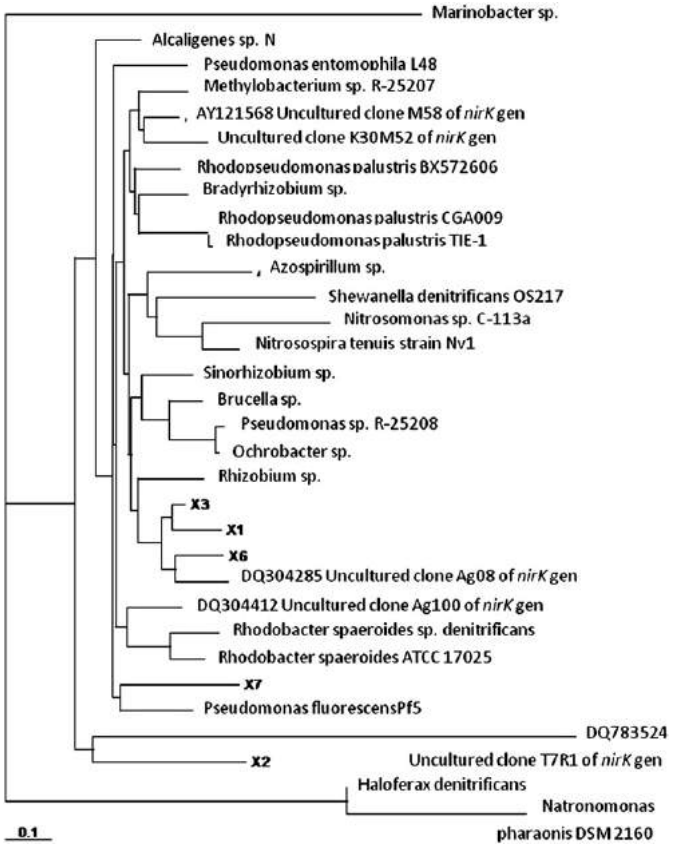

Fig. 7. Phylogenetic relationship of *nirK* gene. Phylogenetic distances were determined by neighbor-joining analysis. *nosZ* gene of *Marinobacter* sp. as outgroup. The isolates numbers are indicated in bold.

phenotypic change, involving either genetic or plastic change, are particularly high within anthropogenic contexts (Hoffmann and Willi, 2008). In Xochimilco, soil use is intensive for the production of vegetables, extensive use of pesticides with frequent irrigation with wastewaters characterized by a high salt content, organic matter and microorganisms. A 'plastic' change, which is defined as a change in phenotypic expression but not in the genotype, might have occurred as a result of environmental change due to the application of methyl parathion (Hoffmann and Willi, 2008). However, the soil functional stability might be strong enough to resist anthropogenic pressure without a probably 'plastic' change. More investigations with RNA are recommended to study this hypothetic 'plastic' change.

Sáez et al. (2003, 2006) reported that the N_2O release from *Xanthobacter autotrophicus* CECT 7064 and *Paracoccus denitrificans* strain ATCC 19367 (denitrifying bacteria) was strongly inhibited by several pesticides (methyl parathion, aldrin, etc.). The result of this observed inhibition may be related to the smaller microbial diversity noted in the patterns of treatments 2 and 3 as a great number of bands between X3 and X6 disappeared. The toxicity of methyl parathion might lead to a shift in microbial community structure tending toward a significant loss in functional diversity. Pampulha et al. (2007) observed that the widespread use of the herbicide glufosinate might have negative effects on soil microorganisms and their activities. Nevertheless it is difficult to determine which enzyme was inhibited and affected the emission of N_2O. It might be that hydroxylamine oxidoreductase (*hao* gene) in the nitrification process or the nitrous oxide reductase (*nos*Z gene) in the denitrification process was inhibited. Even so, TGGE analysis of the nitrifying and denitrifying communities might be very important in order to evaluate the nitrous oxide production.

The X3 and X7 OTUs are always present in treatments 2 and 3, with a tendency to disappear. These bands appear to represent the dominant denitrifying bacteria. Therefore the genetic pool of bacterial community was not characterized by a significant reduction, but the active fraction responding in the physicochemical assay of treatment 3 changed suggesting a reduction in the potential for a complete reduction to N_2 (Gómez et al., 2004). As such, a 'plastic' change could be observed in treatments 2 and 3. Concentration effects were also observed in previous studies (Min et al., 2001; Chen et al., 2003). Pampulha and Oliveira (2006) reported that the magnitude of these effects was dependent on the assayed concentrations of the herbicide mixture.

In our study, five isolates could be associated with Bacteria. Monophyletic grouping at the family, order, class, and phylum levels were not supported by the data, with the exception of the 2 archaeal species (Jones et al., 2008). It is difficult to identify each OTUs in Bacteria. Furthermore four of them are related to an uncultured clone of the *nirK* gene, which represents an obstacle to study the microbial ecology. The phylogenetic tree revealed a high diversity of the denitrifying bacteria. Horizontal gene transfer is the most likely explanation for this widespread ability to denitrify (Throbäck et al., 2004).

5. Conclusions

Methyl parathion had effect on the emissions of N_2O and CO_2. The diversity of the *nirK* gene as determined with the thermal gradient gel electrophoresis (TGGE) decreased when increasing methyl parathion application. Consequently, the reduced diversity of the *nirK* gene could affect the emission of N_2O in the Chinampa soil.

Acknowledgments

We thank CONACyT (60725) and DGAPA-PAPIIT (IN222507) for financial support, S. Márquez, U. Ruiz and S. Lucario for technical support, G. Sánchez Nahuacatl for bibliographical support.

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Journal of Environmental Management 95 (2012) S31–S36

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Advances in validating SALTIRSOIL at plot scale: First results

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ARTICLE INFO

Article history:
Received 1 September 2009
Received in revised form
21 February 2011
Accepted 15 March 2011
Available online 8 April 2011

Keywords:
Soil salinity
Irrigation
Agricultural modelling
Global sensitivity analysis

ABSTRACT

SALTIRSOIL (SALTs in IRrigated SOILs) is a model for the medium to long term simulation of soil salinity in irrigated, well-drained lands. Once the algorithms were verified, the objective of our study was to validate SALTIRSOIL under one of several water quality and management scenarios in Mediterranean agriculture. Because drip and surface are the most common irrigation systems in irrigated agriculture in Valencia (Spain), the validation was performed with climate, soil, irrigation water (composition and management) and crop (species and management) information from an experimental plot surface irrigated with well water and planted with watermelon that has been monitored since the late spring of 2007. To carry out the validation, first we performed a global sensitivity analysis (GSA). Second, we compared simulated soil saturation extract composition against measured data. According to the GSA, SALTIRSOIL calculations of soil salinity seem to be most affected by climate (rainfall and evapotranspiration) with 60% of explained soil salinity variance, water salinity with 26% of explained variance, and then irrigation with 4%. According to the closeness of the first comparisons between predictions and measurements, SALTIRSOIL does not seem to be affected by any systematic error, and as a consequence, neither inclusion of new parameters nor calibration of the others already included would be needed at least for surface irrigation. The validation of SALTIRSOIL continues under other water quality and irrigation management scenarios.

1. Introduction

Soil salt build-up is the main threat to the sustainability of irrigated agriculture in the world. It originates in a wrong irrigation management. The use of validated soil salinity models able to simulate the soil salt build-up is essential to devise sustainable irrigation management practices against salinization. SALTIRSOIL (Visconti et al., 2006) is a new deterministic, steady-state, capacity-type and chemical equilibrium model developed to simulate the build-up of the major inorganic ions (sodium, potassium, calcium, magnesium, chloride, sulphate, nitrate), alkalinity, pH and electrical conductivity in the soil solution of irrigated well-drained fields. SALTIRSOIL calculates the average concentration factor of the soil solution with regards to the irrigation water at field capacity, or at saturation, given climate, irrigation and soil information. Then it applies an equilibrium model to calculate the final composition of the soil solution at equilibrium with calcite, gypsum and carbon dioxide. A working version of the SALTIRSOIL model is downloadable from the website http://www.uv.es/fervisre/saltirsoil.html.

For the assessment of environmental models, sensitivity analysis (SA) is a task as important as the validation itself (Shelly et al., 2000). The objective of the SA is to evaluate the importance of the input variables on the model output. The SA is usually performed by changing one input variable at a time within a given range while maintaining the other variables unchanged and measuring the variability in the model output. In the present work, we have changed to Global SA (GSA) using the Factors' Prioritisation Setting by Saltelli et al. (2004) and performed the SA by means of a more appropriate Monte Carlo experiment (Sobol, 1994). For a suitable model output for the GSA, we have chosen the total soluble salts in the saturation extract (TSS_{se}) expressed as the sum of the major inorganic ions in $mmol_C\ L^{-1}$. Despite its importance, the SA of soil models is usually not reported: to our knowledge, it has been

Abbreviations: ETa, Actual evapotranspiration; Alk, Alkalinity; ETc, Crop evapotranspiration; EC, EC_{25}, Electrical conductivity at 25 °C; GSA, Global sensitivity analysis; SA, Sensitivity analysis; SRC, Standard regression coefficient; TSS_{se}, Total soluble salts in saturation extract, for other abbreviations the reader is referred to Table 1.

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doi:10.1016/j.jenvman.2011.03.020

applied to soil models of organic matter decomposition (Xenakis et al., 2008), hydrology (Rocha et al., 2006) and erosion (Weill and Sparovek, 2008), but not to a soil salinity model.

Our objectives in this study were i) to evaluate the importance of the input variables of SALTIRSOIL on the output variable TSS_{se} by means of a GSA, ii) to simulate the water balance and soil salinity of a plot furrow irrigated and planted with watermelon, and iii) to compare the simulated soil salinity to measurements.

2. Materials and methods

2.1. Sensitivity analysis

The GSA was done using a Monte Carlo experiment. The seventeen input variables included in the GSA are shown in Table 1 with their means and standard deviations used. Means and standard deviations were selected so that the maximum and minimum values include the values usually found in most Mediterranean agricultural environments. A normal distribution of $N = 250$ data was generated independently for each one of the variables to produce the Monte Carlo sample input matrix used in SALTIRSOIL (see supplementary data Table SD1 and SD2).

2.2. Study area

The experimental plot studied in this research is located in the lower *Palància* river basin in Valencia Community near the Mediterranean coast of Spain. The climate is characterised by high evapotranspiration (1000 mm yr^{-1}) and low rainfall (500 mm yr^{-1}). Irrigation is applied using drip or surface systems. The electrical conductivity of the irrigation water varies from low-medium (1 dS m^{-1}) to high (4 dS m^{-1}). The experimental plot has been cultivated to grow vegetables since a drainage system was installed. It was surveyed and monitored since the late spring till autumn of 2007. The soil texture (USDA) is a clay loam with soil organic matter values up to 3% in the arable layer and calcium carbonate equivalent around 15%. In 2007, it was planted with watermelon and furrow irrigated with salty water (4.2 dS m^{-1}) from a nearby well.

2.3. Simulations

Because a soil salinity appraisal is usually based on a saturated paste analysis (USSL Staff, 1954), the saturated soil paste and extract composition in the experimental plot was simulated in SALTIRSOIL using the information shown in Boxes 1 and 2. The information for the soil solution concentration factor calculation is shown in Box 1, while the information for the calculation of the final composition in equilibrium with CO_2, calcite and gypsum is shown in Box 2.

2.4. Plot sampling and soil analyses

The experimental plot was sampled during the spring-summer season of 2007 at two points: one near the irrigation water inlet to the plot (Point 1) and another point opposite this downgradient (Point 2). Each point was sampled at three depths: 0–10, 10–30 and 30–60 cm. Soil and water samples were analysed according to the methods described in Box 3.

A weighted average, soil saturation extract composition was calculated in each one of the points using Equation (1), where P is the average value of the property and P_{0-10}, P_{10-30} and P_{30-60} are its values in the samples from the 0–10, 10–30, and 30–60 soil layers, respectively.

$$P = (P_{0-10} + 2\,P_{10-30} + 3\,P_{30-60})/6 \quad [1]$$

3. Results and discussion

3.1. Sensitivity analysis

SALTIRSOIL was batch-run, and TSS_{se} was computed for each one of the 250 simulations in the Monte Carlo sample input matrix (see supplementary data Table SD3). Next, the product–moment correlation coefficients among each pair of input variables and the output variable TSS_{se} was calculated. The largest correlation coefficient among input variables was 0.24. Because the input variables were also normally distributed, they could be considered independent from each other, which is a desirable property in an SA (Saltelli et al., 2004).

The output TSS_{se} was moderately correlated with the input TSS_{iw} ($r = 0.43$). On average, TSS_{se} was 38% higher than TSS_{iw}. The next statistically significant ($p < 0.001$) correlated input variables with TSS_{se} were the average crop coefficient ($r = 0.41$) and the annual rainfall ($r = -0.39$).

The regression model of the output variable TSS_{se} using the seventeen input variables was computed (Table 2). The coefficient of determination for the linear model was $R^2 = 0.78$. Therefore, SALTIRSOIL can be considered a linear model, and the GSA can be carried

Table 1
Statistical summary of the variables used in the sensitivity analysis.

Variable	Definition	Mean	St. Dev.	Max.	Min.
R/mm yr^{-1}	Rainfall	450	120	719	88
ET_0/mm yr^{-1}	Reference evapotranspiration	1200	150	1537	780
Rf/day yr^{-1}	Number of days of rainfall per year	70	20	121	18
Clay/g $(100g)^{-1}$	Soil clay percentage	36	11	70	0
Sand/g $(100g)^{-1}$	Soil sand percentage	25	7	42	6
SP/g $(100g)^{-1}$	Stone percentage	15	5	28	3
CCE/g $(100g)^{-1}$	Calcium carbonate equivalent	50	12	85	18
SOM/g $(100g)^{-1}$	Soil organic matter	2.0	0.8	4.5	0.2
Gypsum/g $(100g)^{-1}$	Gypsum	0.40	0.15	0.76	0.01
$logpCO_2$	Log of carbon dioxide partial pressure	−3.00	0.20	−2.42	−3.57
SD/cm	Soil depth	100	10	130	70
Kcb	Annual mean basal crop coefficient[a]	0.8	0.2	1.25	0.19
SS (%)	Shaded soil	74	9	100	50
I/mm yr^{-1}	Annual irrigation	700	100	1001	443
If/day yr^{-1}	Number of days of irrigation per year	40	10	71	10
WS (%)	Wetted soil	70	10	98	44
TSS_{iw}/$mmol_C$ L^{-1}	Total soluble salts in irrigation water[b]	99.4	18.0	152.6	54.7

[a] For a definition of basal crop coefficient see footnote a to Box 1.
[b] Calculated as the sum of main inorganic ions in $mmol_C$ L^{-1}.

Box 1. Input information for the soil solution concentration factor calculation in 2007.

Weather from Benavites SIAR station:
- ❖ Rainfall of 637 mm yr^{-1}
- ❖ Reference evapotranspiration of 1050 mm yr^{-1} calculated according to Penman–Monteith (Allen et al., 1998)

Irrigation:
- ❖ Application
  - ➢ Method: furrows 154 m long
  - ➢ Wetted soil: 20%
  - ➢ Quantity and frequency (number of days a month):

Month	April	May	June	July	August
mm	28	41	49	55	44
N days	1	2	2	2	2

- ❖ Water quality:

EC_{25}	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	NO_3^-	SO_4^{2-}	Alk	pH
4.23	14.7	0.3	8.0	6.8	24.8	4.1	6.4	3.8	7.95

All ions in mmol L^{-1}, alkalinity (Alk) in $mmol_C$ L^{-1} and electrical conductivity (EC_{25}) in dS m^{-1}

Crop:
- ❖ Species: watermelon (*Citrullus lanatus*) with cabbage foot
- ❖ Basal crop coefficients (Kcb)[a] (Allen et al., 1998):

Stage	Initial	Development	Mid season	Late season
Duration/day	31	41	47	26
Crop coefficient	0.15	0.575	1.00	0.85

- ❖ Growing season: from 1st April until 24th August (145 days)
- ❖ Maximum shaded soil (SS): 35%
- ❖ Maximum rooting depth (SD): 60 cm
- ❖ Water uptake pattern within the rooting depth: 40:30:20:10[b]

Soil:
- ❖ Physical properties
  - ➢ Stone percentage (SP): <5%
  - ➢ Texture (USDA): 35–43 and 33–38 clay-sand in Point 1 and 2, respectively
- ❖ Chemical properties
  - ➢ Calcium carbonate equivalent (CCE): 11% in Point 1 and 16% in Point 2.
  - ➢ Gypsum: <0.5%
  - ➢ Soil organic matter (SOM): 3% and 3.5% in the top 10 cm of Point 1 and 2, respectively

Drainage:
- ❖ Pipeline 60 cm deep with a 9 m spacing

[a] The basal crop coefficient is defined as the quotient of crop evapotranspiration to reference evapotranspiration (ET_c/ET_0) for a non-stressed crop (neither water nor nutrient lacking, no salt-stressed, etc), and with no soil evaporation.

[b] Percentage of soil water taken by the plant roots from each quarter of the rooting depth from top to bottom.

Box 2. Input information to the chemical equilibrium calculation.

Chemical equilibrium constants:
- ❖ Ion association constants: Lindsay (1979)
- ❖ Calcite solubility product (pKs): 8.29
- ❖ Gypsum solubility product (pKs): 4.62

CO_2 partial pressure in equilibrium with the solution in the saturated soil paste after 4 h: 9.5 10^{-4} atm

out on the regression coefficients (Saltelli et al., 2004). The magnitude of the standardised regression coefficient (SRC) is a reliable measure of the importance of an input variable on the output, and the sign of the SRC (β_i) or non standardised regression coefficient (B) is a measure of the direction of that influence. Each SRC was squared (β_i^2) and divided by the summation of all the squared SRCs to obtain a parameter ($\beta_i^2/\Sigma\beta_i^2$) useful for measuring the relative influence of each input variable on the output in terms of variance. The input variables can be arranged as follows from the greatest to the least influence on the output: R > TSS_{iw} > Kcb >> I ≈ CCE ≈ ET_0 > SD > WS > clay > fR > SOM > SS > fl > sand > gypsum > SP > logpCO_2.

The annual rainfall accounts for 37% of the variance of the output. Following rainfall is TSS_{iw} that accounts for 26% of the variance. Next are the variables related to the crop evapotranspiration such as the crop coefficient with 21%, the reference evapotranspiration with 3.1% and wetted soil (1.5%). Then there is the annual irrigation with 3.7%, and the variables related to the soil water holding capacity such as CCE, soil depth and clay with 3.3, 2.1 and 1.1% of explained variance, respectively. The rest of the input variables explain less than 0.8% of variance each and less than 1.9% of the whole.

In terms of direction of influence, the TSS_{se} increases when TSS_{iw} increases because in well-drained soils, salts are only supplied with the irrigation water. With regard to the soil water balance variables, the soil water inputs, such as rainfall and irrigation, influence TSS_{se} negatively while the soil water outputs, i.e., the evapotranspiration, influence TSS_{se} positively. As expected, TSS_{se} decreases as more water enters the soil either as rainfall or irrigation. However, while rainfall has a profound influence on TSS_{se}, the influence of irrigation is significantly less. While irrigation provides salts to the soil and also washes them out, rainfall only washes out the salts. The significant rainfall amount in the simulations carried out in this SA (98% with R > 210 mm yr^{-1}, Table 1) decreased the importance irrigation had on soil salinity.

In the simulations, the CCE influences the TSS_{se} negatively because according to the pedotransfer functions developed for SALTIRSOIL (Visconti et al., 2011), the CCE significantly decreases the water amount the soil holds at field capacity but not at saturation. Then as the CCE increases, the difference between the water amount held at saturation and at field capacity also increases giving rise to more salt dilution in the soil saturation extract. In the simulations, just like with the CCE, as the clay fraction increases, the difference between the water content at saturation and at field capacity also increases, thus diluting the salts in the saturation extract with regard to field capacity.

Given these results, the soil water balance inputs, mainly rainfall and secondarily irrigation, and outputs (crop evapotranspiration accounted for by the crop coefficient, reference evapotranspiration and wetted soil) are the most important factors affecting the calculated TSS_{se}. After rainfall, the irrigation water salinity summarized by TSS_{iw} is the second most important factor. The other main variables to affect soil salinity are the properties that influence the

Box 3. Plot sampling and Sample analyses.

Soil samples were air-dried, ground and sieved to pass through a 2 mm-mesh sieve.

Saturated soil pastes were prepared by adding deionized water to the soil according to the method described by Rhoades (1996) with the only exception that sodium hexametaphosphate solution was not added to the saturation extract once collected. Soil saturation extracts were analysed for electrical conductivity (EC_{25}), sodium, potassium, calcium, magnesium, sulphate, chloride, nitrate, alkalinity and pH. Determination of EC_{25}, pH and alkalinity was performed within 2 h of extract collection. EC_{25} was measured with a Crison (Crison Instruments SA, Barcelona, Spain) microCM 2201 conductivity meter with a temperature probe, and pH was measured with a Crison GLP22 pHmeter. Alkalinity was determined by potentiometric titration with 20 mN sulphuric acid standardized every week. Simultaneously, an aliquot of the soil saturation extract was diluted with deionized water for the determination of main ions. This determination was performed by ion chromatography in the diluted extracts filtered through 0.45 μm pore filters to remove particulate material, within four days of extract collection.

Irrigation water was sampled three times during the growing season and analysed with the same methods used with the soil extracts.

Texture, soil organic matter and calcium carbonate equivalent were determined according to the Spanish Ministry of Agriculture official methods (MAPA, 1994).

soil water holding capacity. This is characterised by both intensity (CCE and clay percentages) and quantity (soil depth) properties. The other variables are of much less importance and thus not important enough to consider in a likely calibration process.

Rainfall and irrigation water salinity together explain at least 60% of the variance on the simulated soil salinity, and both were reliably determined for an essay plot, so a comparison of measurements and SALTIRSOIL calculations was conducted.

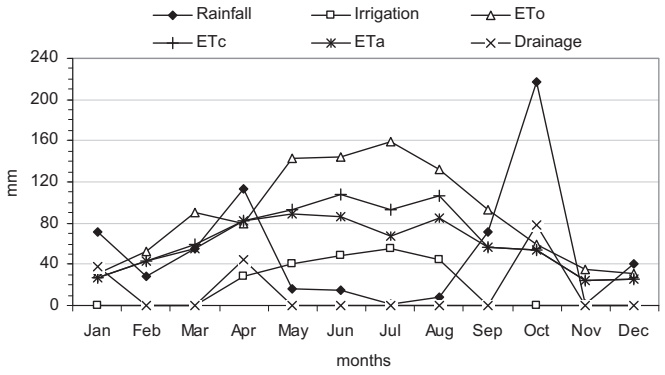

Fig. 1. Monthly soil water balance in 2007.

3.2. Modelling the properties of saturated pastes and extracts

The soil water balance simulated by SALTIRSOIL in the experimental plot is shown in Fig. 1. Crop evapotranspiration during the growing period was 481 mm, and actual evapotranspiration was 409 mm; therefore, there was a certain water stress. In contrast, the irrigation amount (217 mm yr^{-1}) and the annual rainfall (637 mm yr^{-1}, Box 1) totalled to 854 mm, which resulted in 160 mm yr^{-1} of drainage, half of it at the end of the growing season (October).

The soil saturation extract composition in Point 1 and 2 was calculated with SALTIRSOIL, called Sim.1 and Sim.2, respectively, and compared to the observed compositions (Fig. 2). The calculated values of TSS_{se} in Point 1 and Point 2 differed only in the second significant figure: 65.3 $mmol_C\ L^{-1}$ and 66.1 $mmol_C\ L^{-1}$, respectively. This difference is due to the clay fraction that changes from one point to the other. However, it is a very small difference because this textural fraction is more than 20 times less important than the annual rainfall or TSS_{iw} on the TSS_{se} calculation as known from the GSA. In Point 1, a TSS_{se} value of 41.1 $mmol_C\ L^{-1}$ was observed while the observed value in Point 2 was 62.0 $mmol_C\ L^{-1}$. The TSS point is observed above the diagonal line in the Point 1 simulation (Fig. 2a) and almost on the diagonal line in the Point 2 simulation (Fig. 2c). The error in the calculation of TSS_{se} in Point 1 is due to the error in the calculation of chloride, sodium, calcium, magnesium and

Table 2
Summary of the linear regression analysis of TSS_{se} with the seventeen input variables.[a]

Non standardised coefficients			Standardised coefficients			t	Sig.	95% confidence interval for B	
Variable	*B*	Std. error	β_i	β_i^2	$100\ \beta_i^2/\Sigma\beta_i^2$			Lím. Inf.	Lím. Sup.
Constant	62.360	32.877	–	–	–	1.90	0.059	−2.4	127.1
R	**−0.198**	**0.011**	**−0.587**	**0.344**	**36.6**	**−17.72**	**<0.001**	**−0.2**	**−0.2**
ET_0	**0.046**	**0.009**	**0.170**	**0.029**	**3.1**	**5.35**	**<0.001**	**0.0**	**0.1**
fR	0.170	0.065	0.084	0.007	0.8	2.62	0.009	0.0	0.3
clay	**−0.371**	**0.118**	**−0.101**	**0.010**	**1.1**	**−3.14**	**0.002**	**−0.6**	**−0.1**
sand	0.200	0.184	0.035	0.001	0.1	1.09	0.278	−0.2	0.6
SP	−0.045	0.257	−0.006	0.000	0.0	−0.17	0.861	−0.6	0.5
CCE	**−0.597**	**0.106**	**−0.177**	**0.031**	**3.3**	**−5.63**	**<0.001**	**−0.8**	**−0.4**
SOM	3.821	1.599	0.076	0.006	0.6	2.39	0.018	0.7	7.0
gypsum	2.987	8.638	0.011	0.000	0.0	0.35	0.730	−14.0	20.0
$logpCO_2$	−0.126	6.538	−0.001	0.000	0.0	−0.02	0.985	−13.0	12.8
SD	**−0.569**	**0.127**	**−0.141**	**0.020**	**2.1**	**−4.48**	**<0.001**	**−0.8**	**−0.3**
I	**−0.076**	**0.013**	**−0.187**	**0.035**	**3.7**	**−5.82**	**<0.001**	**−0.1**	**−0.1**
fI	0.158	0.126	0.039	0.002	0.2	1.25	0.212	−0.1	0.4
WS	**0.479**	**0.128**	**0.119**	**0.014**	**1.5**	**3.75**	**<0.001**	**0.2**	**0.7**
Kc	**89.590**	**6.600**	**0.444**	**0.197**	**20.9**	**13.57**	**<0.001**	**76.6**	**102.6**
SS	0.192	0.144	0.043	0.002	0.2	1.34	0.182	−0.1	0.5
TSS_{iw}	**1.109**	**0.074**	**0.493**	**0.243**	**25.8**	**14.98**	**<0.001**	**1.0**	**1.3**
Total	–	–	–	0.941	100	–	–	–	–

[a] The variables that account for more than 1% of variation in the output are written in bold face.

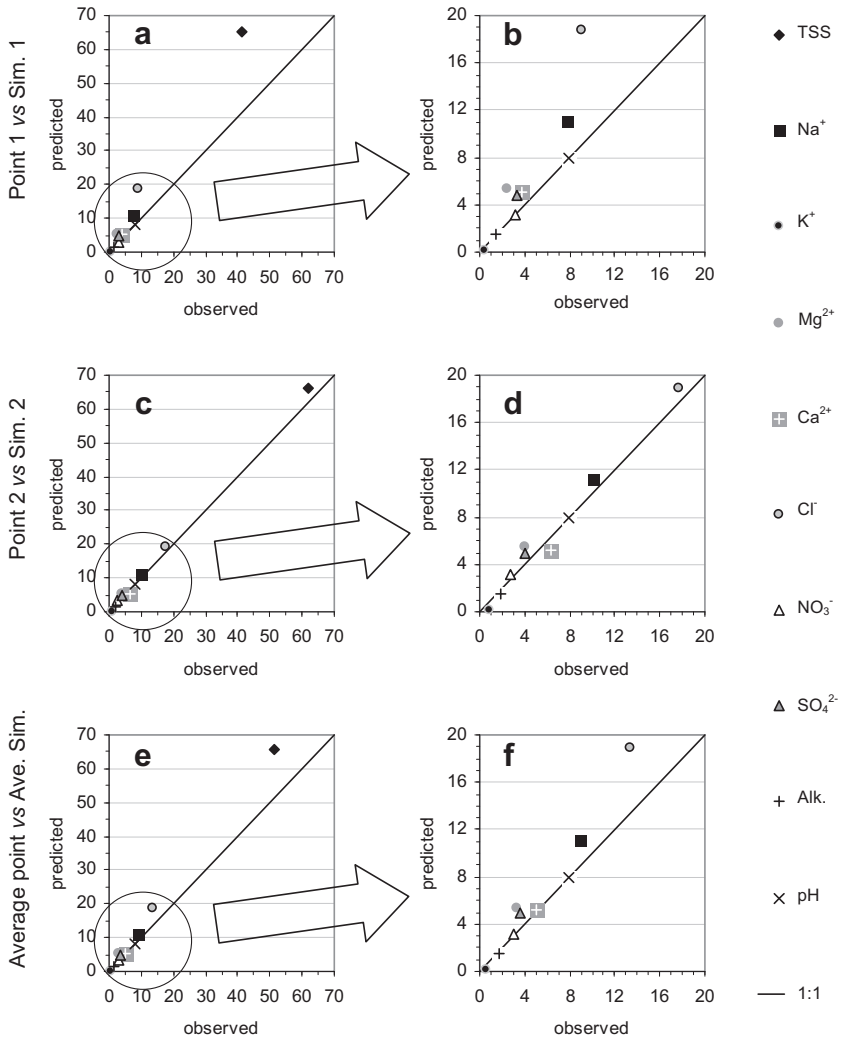

Fig. 2. Scatter plots of predicted versus observed values in 2007. All parameters are for the saturation extract except pH, which is for the saturated paste, all ions in mmol L^{-1}, alkalinity (Alk) and total soluble salts (TSS) in $mmol_C\ L^{-1}$.

sulphate, which are the most abundant ions in the irrigation water (Fig. 2b and d) and thus dominate the TSS value.

An alkalinity (Alk) of 1.50 $mmol_C\ L^{-1}$ was calculated in Points 1 and 2, 2% higher than the alkalinity observed in Point 1 (1.47 $mmol_C\ L^{-1}$) and 27% less than the alkalinity observed in Point 2 (1.90 $mmol_C\ L^{-1}$). The saturated paste pH value (pH_{sp}) simulated in both points was 7.91, which is very close to the observed values of 7.90 and 7.86 in Points 1 and 2, respectively. As the alkalinity, and hence pH, is more dependent on the carbon dioxide partial pressure in the saturated paste than on TSS_{iw} (Visconti et al., 2010), both parameters are among the most accurately calculated properties.

In general, surface irrigation practice suffers from a lack of homogeneity in water application. In the case of furrow irrigation, the further the soil is from the water inlet, the less that the water infiltrates into it. Less irrigation water means more TSS_{se} as was revealed by the GSA (Table 2). This situation explains why the soil near the water inlet (Point 1) has less TSS_{se} than the soil at the opposite point downgradient in the field (Point 2, 150 m from Point 1). The average saturation extract composition of Points 1 and 2 was calculated and compared to the plot average simulation (Ave. Sim.) (Fig. 2e and f). The simulated value of TSS_{se} is 65.7 $mmol_C\ L^{-1}$, whereas the observed one is 51.5 $mmol_C\ L^{-1}$ (Fig. 2e). In Fig. 2f, it can be observed how the overestimation of chloride and magnesium, and to a lesser extent sodium and sulphate, explains the overestimation of the saturation extract salinity. Nevertheless, the relative concentrations of the entire set of ions have been calculated accurately.

Conversely, potassium with a value of 0.2 mmol L^{-1} is far from the average observed value of 0.6 mmol L^{-1}. This is because potassium is supplied by fertilizers and organic amendments in addition to irrigation water. Surprisingly, nitrate with a calculated value of 3.1 mmol L^{-1} is very close to the observed value of 3.0 mmol L^{-1}. Thus the processes generating and eliminating the nitrate ion probably have compensated for each other in this plot.

4. Conclusions

The SA of SALTIRSOIL was successfully performed by means of a Monte Carlo experiment in the framework of the Global SA Factors' Prioritisation Setting. It was shown that SALTIRSOIL can be considered a linear model for the simulation of soil salinity on the basis of the seventeen input variables selected for the SA. As a consequence, the relative magnitude and direction of influence of every variable could be reliably assessed based on the regression coefficients obtained in the multiple regression analysis. The soil water balance variables, mainly rainfall and then evapotranspiration, appear to be the most important variables for the soil salinity

calculation in SALTIRSOIL accounting for 60% of the variance in soil salinity, closely followed by the irrigation water salinity (26%) and further by the irrigation and the soil water holding capacity.

The average saturation extract composition and pH of soil from an experimental plot planted with watermelon and furrow irrigated with well water in an area under risk of salinization was simulated. Calculated and observed concentrations of major inorganic ions and hence total soluble salts in the saturation extract are similar. Further validations are needed with other irrigation systems and water supplies.

Acknowledgements

This work has been done in the framework of projects CGL2006-13233-CO2-01 and CGL2006-13233-CO2-02. The authors acknowledge the Ministerio de Educación y Ciencia from Spain their financial support. F. Visconti also thanks the Generalitat Valenciana for its financial support through a grant in the framework of project GV 0461/2006. We thank the three anonymous reviewers and managing guest editor for their valuable indications to improve the article.

Appendix. Supplementary material

Supplementary data associated with the article can be found in online version, at doi:10.1016/j.jenvman.2011.03.020.

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Journal of Environmental Management 95 (2012) S37–S41

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Rhizospheric bacteria alleviate salt-produced stress in sunflower

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ARTICLE INFO

Article history:
Received 8 September 2009
Received in revised form
24 April 2010
Accepted 12 July 2010
Available online 3 August 2010

Keywords:
Rhizobacteria
IAA
Siderophores
K^+/Na^+ ratio
Salt stress
Sunflower

ABSTRACT

The effect of isolate *Pseudomonas fluorescens* biotype F and *P. fluorescens* CECT 378^T inoculation on fresh weight and ions accumulation was studied in sunflower plants grown in sand:peat substrate with addition of 100 mM NaCl. The inoculation resulted in an increase in fresh weight of more than 10% in salt treatments and in an accumulation of less Na^+ and more K^+ in plant tissues in all cases. The bacterial inoculants favoured the K^+/Na^+ ratio in all plant parts and in the case of the isolate CECT 378^T conducted to 66% increment in leaves, 34% in stems and 16% in roots, while the effect of isolate inoculation was (only) more evident in leaves and stems with 30% and 26%, respectively. Both strains were found to produce indoleacetic acid and siderophores in *in-vitro* tests, thus the production of indoles was highly dependent on the exogenous tryptophan in the medium. The results suggest that salt stress in sunflower plants was alleviated partially by the inoculation with strains that produce indoles and siderophores, having also a positive effect on the K^+/Na^+ ratio in the shoot. Moreover, those plants were characterized with better-developed roots.

1. Introduction

Many processes in natural soils in arid regions frequently produce saline soils. In these conditions, an inadequate water regime can increase the salinity and represents a significant problem in most regions. The direct effect of soil salinity on physical–chemical and biological properties renders these soils unsuitable for both soil microbial processes and growth of the crop plants involving osmotic and ionic stress (Munns, 2002; Benlloch-Gonzalez et al., 2005). The inhibition of growth by salinity occurs in all glicophytes, but the grade of tolerance and the rate of growth reduction by high concentrations of salt vary widely among different plant species. Salt tolerance in glicophytes has been related with their ability to avoid the accumulation of Na^+ in their tissues and to remain a high K^+/Na^+ ratio in the shoot. Both mechanisms enable the plants to maintain cell growth and avoid Na^+ accumulation. This effect has been already described in many species such as wheat (Poustini and Siosemardeh, 2004), sunflower and barley (Greenway and Munns, 1980). The role of K^+ in the plant growth is also well known. An adequate K^+ status of the plant favours the cellular hydration, the turgidity and the cell elongation (Hsiao and Läuchli, 1986)

The application of bioinoculants like arbuscular-mycorrhizal fungus, and/or plant-growth-promoting rhizobacteria such as *Azospirillum*, *Agrobacteria*, *Pseudomonas* and several *Bacillus* species is an environment-friendly, energy efficient and economically viable approach for reclaiming soils and increasing biomass production (Mayak et al., 2004; Rabie and Almadini, 2005). The inoculation of bacterial strains producing exo-polysaccharides enabled the plants to withstand the initial effects of salts and the osmotic stresses but it also benefited the inoculated plants in terms of a better exploitation of the soil nutrients and through providing an increased extent of rhizodeposits in the soil for gearing up of the soil microbial activities (Ashraf et al., 2006). Microbial populations are known to affect the mobility and availability of elements to the plant releasing chelating agents, acidification, phosphate solubilization, and redox changes (Abou-Shanab et al., 2003). Specially, some plant-growth-promoting bacteria (PGPR) associated with plant roots also may exert some beneficial effects on plant growth and nutrition through a number of mechanisms such as N_2 fixation, production of phytohormones and siderophores, and transformation of nutrient elements when they are either applied to seeds or incorporated into the soil (Glick, 1995; Glick et al., 1998). Moreover, some rhizobacteria can exude compounds, such as antibiotics, phosphate solubilization, indoleacetic acid (IAA), siderophores, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, which increase bioavailability and facilitate root absorption of

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0301-4797/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jenvman.2010.07.019

nutrients, such as Fe (Crowley et al., 1991) or non-essential elements, such as Cd and Pb (Salt et al., 1995), and enhancing the tolerance of host plants by promoting plant growth (Duffy and Défago, 1999; Shilev et al., 2001). IAA produced by rhizobacteria is believed to play an important role as a phytohormone, influencing many cellular plant processes, such as the cell elongation. It has been well documented that the biosynthesis of auxins with their excretion into soil makes a major contribution to the bacterial plant-growth-promoting effect (Lambrecht et al., 2000). Also, fluorescent pseudomonads are known to produce siderophores, the pyoverdines which are available in both homologous and heterologous uptake systems (Sharma and Johri, 2003). They are low molecular weight iron chelators which are released under iron limited conditions in the surroundings, possess high binding affinity and specificity for iron (III), and facilitate its transport into the bacterial cell (Schalk et al., 2001). All these bacterial characteristics support the symbiotic interactions in the rhizosphere zone for mutual benefits of plants and microbes.

The aim of the present work was to study the plant-growth promotion properties of *Pseudomonas fluorescens* biotype F and *P. fluorescens* CECT 378^T, as well as their capability to alleviate salt-produced stress in sunflower plants.

This work was conducted during 2008 in the Departments of Microbiology and Agronomy, University of Córdoba, Spain.

2. Material and methods

2.1. Properties of bacteria

In this study, two different fluorescent pseudomonads were used: *P. fluorescens* biotype F (isolate) and *P. fluorescens* CECT 378^T. The first one was formerly isolated from heavy metal contaminated soil (Shilev et al., 2001), while the second one was purchased from the Spanish Type Culture Collection (CECT). They were maintained at 4 °C on nutrient agar tubes and refreshed immediately before each experiment. The tolerance of bacteria to elevated concentrations of NaCl was studied in Nutrient broth (NB) medium containing different salt concentrations from 0 to 100 mM. Aliquots of 20 μl of medium with cells in late exponential phase were introduced into the flasks to final volume of 50 ml and placed on rotary shaker. Twenty-four hours later the growth was evaluated measuring optical density at 550 nm.

2.1.1. Quantification of IAA production

Both isolates were propagated overnight in liquid Bacto-Pseudomonas F (BPF) (Belimov et al., 2005). Equal aliquots were transferred into 20 ml of the same medium supplemented with the following concentrations of L-tryptophan (taken from a filter-sterilized 4 mg ml^{-1} stock solution prepared in warm water; Sigma): 0, 100, 300, 500, 700 and 900 μg ml^{-1}. After incubation for 24 h, the density of each culture was measured spectrophotometrically at 550 nm, and then the bacterial cells were removed from the culture medium by centrifugation (5500 rpm, 10 min). A 0.2 ml sample of the supernatant was mixed vigorously with 0.8 ml of Salkowski's reagent (150 ml of concentrated H_2SO_4, 250 ml of distilled H_2O, 7.5 ml of 0.5 M $FeCl_3 \cdot 6H_2O$, Gordon and Weber, 1951) and left for reaction at room temperature for 20 min before measuring the absorbance at 535 nm. The concentration of IAA in each culture medium was determined generating a standard curve for samples containing IAA.

2.1.2. Determination of siderophore production

The production of siderophores was determined by the modification made of Pérez-Miranda et al., 2007, of the method of Schwyn and Neilands, 1987.

2.2. Growth chamber experiment

Helianthus annuus L. cv. Sungro 393 seeds were purchased from Eurosemillas S.A., Córdoba. Seeds germination and growth chamber conditions are described in Quintero et al. (2007). The germinated seeds were placed in 2 l pots filled with river sand:blond peat mixture 2:1 in growth chamber. All pots were watered on daily basis with a standard nutrient solution with the following composition: 2.5 mM $Ca(NO_3)_2$, 5 mM KCl, 0.25 mM $Ca(H_2PO_4)_2$, 1 mM $MgSO_4$, 12.5 μM H_3BO_3, 1 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.25 μM $CuSO_4$, 0.2 μM $(NH_4)_6Mo_7O_{24}$ and 10 μM Fe-ethylene-diamine-di-*o*-hydroxyphenylacetic acid. The plants were watered with the above solution during the first seven days, thereafter half of the treatments received 100 mM NaCl during 4 weeks. Since sowing the pots had been inoculated with the rhizospheric bacteria *P. fluorescens* biotype F and *P. fluorescens* CECT 378^T, separately, with 10^7 cfu (colony forming units) per 1 g of substrate, once each two days. Firstly, the bacteria were grown separately in liquid BPF medium till late exponential growth phase. The inoculum needed of each culture was centrifuged at 6000 rpm for 10 min. The obtained pellets were washed first in 20 mM $MgCl_2$ to remove ions and after that in distilled water. In this way, six treatments were arranged: control, control with *P. fluorescens* biotype F, control with *P. fluorescens* CECT 378^T, and the same treatments with supplementation of NaCl to the nutrient solution. The plants never showed dehydration symptoms under those conditions during the experiment.

2.2.1. Determination of Na^+ and K^+

Roots were individually washed for 5 min in 150 ml of a cold 5 mM $CaSO_4$ solution (5 °C) to allow exchange of the cell walls contents. Shoots were washed in deionised water (Milli-Q). Then, roots, leaves and stems from each plant were weighed independently and placed in plastic vials. The vials were closed and frozen at −20 °C. Na^+ and K^+ content were determined by atomic absorption spectrophotometry Varian AA240FS after extraction with 10% acetic acid solution.

3. Theory and calculations

3.1. Properties of bacteria

Study of the tolerance of *P. fluorescens* biotype F and *P. fluorescens* CECT 378^T in liquid media (NB) showed differences between both strains (Fig. 1). The population density of isolate CECT 378^T increased from 0 to 10 mM NaCl with effective concentration (EC_{50})

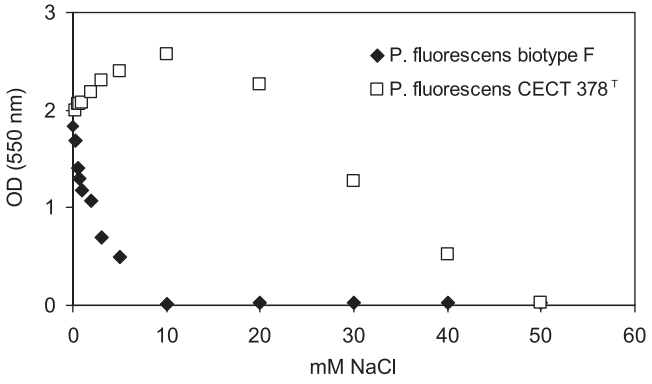

Fig. 1. Growth of *P. fluorescens* biotype F and *P. fluorescens* CECT 378^T in liquid BPF medium supplemented with different NaCl concentrations. The results represent the average of three replicates, while the standard errors were found less than 5%.

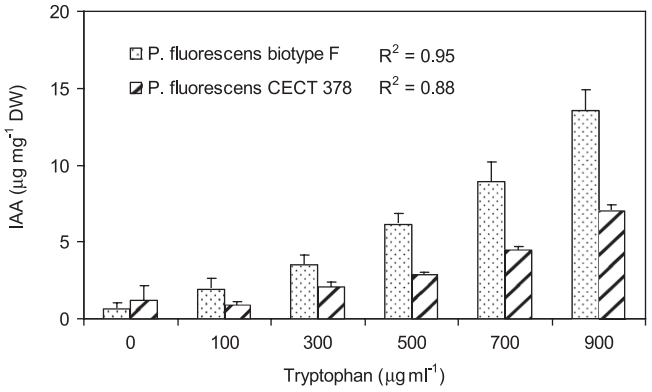

Fig. 2. IAA production of *P. fluorescens* biotype F and *P. fluorescens* CECT 378^T in liquid BPF medium supplemented or not with different concentrations of L-tryptophan, after 24 h of cultivation on rotary shaker. Bars represent the mean of three replicates ± the standard error.

that provokes a reduction of 50% of the population at 34 mM and lethal concentration at 50 mM, while the other isolate was less tolerant showing a constant decreasing curve with lethal concentration at 10 mM.

The production of IAA with tryptophan in medium was higher in *P. fluorescens* biotype F population than in that of isolate CECT 378^T (Fig. 2). In all cases, the production of IAA was correlated with the tryptophan concentration in the media. This means that exogenous tryptophan is required in most cases, although it seems that is more necessary for the first isolate.

The production of siderophores by microorganisms is often related with pathogen suppression of plant-growth-promoting microbes (Kloepper et al., 1989) and iron acquisition. In our study, the colour change of overlaid medium surrounding both bacteria inoculations from blue to orange (as reported for bacteria that produce hydroxamates; Meyer and Stintzi, 1998) was observed in a period of 30 min after the overlaid application (data not shown). Further quantification of siderophores produced by these strains is currently underway at our laboratories.

3.2. Growth chamber experiment

Sunflower plants had an optimal growth in treatments watered with standard nutrient solution without NaCl (Table 1) and no significant differences in fresh weight (FW) were found as consequence of bacterial inoculations. On the other hand, the salt concentration was found significant for the plants and cause important decline of their FW. This effect was highly marked in the case of leaves and stems, while in all studied plant parts the bacterial inoculants resulted in a higher biomass production. This was significant for *P. fluorescens* biotype F for leaves, stems and roots compared with the corresponding control (increment

Table 1
Effect of salinity and two bacteria on leaves, stems and roots fresh weight of sunflower plants. The results represent the average ± the standard error.

Treatments	Fresh weight (g)		
	Leaves	Stems	Roots
Control	19.6 ± 0.1	20.3 ± 0.5	9.9 ± 0.9
Control + *P. fluorescens* biotype F	19.9 ± 0.4	19.1 ± 0.7	10.7 ± 0.4
Control + *P. fluorescens* CECT 378^T	19.5 ± 0.6	19.3 ± 0.8	9.5 ± 0.6
100 mM NaCl	8.4 ± 0.2	8.8 ± 0.3	5.4 ± 0.4
100 mM NaCl + *P. fluorescens* biotype F	9.6 ± 0.3	9.8 ± 0.3	6.2 ± 0.2
100 mM NaCl + *P. fluorescens* CECT 378^T	9.1 ± 0.2	9.2 ± 0.4	6.3 ± 0.2

Table 2
Effect of salinity, *P. fluorescens* biotype F and *P. fluorescens* CECT 378^T on Na^+ concentration in leaves, stems and roots of sunflower plants grown in salt treatments. The plants were grown during 5 weeks in pots with sand:peat mixture and watered with standard nutrient solution supplemented with 100 mM NaCl. The results represent the average ± the standard error.

Treatments	Na^+ mM g^{-1} FW		
	Leaves	Stems	Roots
100 mM NaCl	38.4 ± 3.9	92.5 ± 7.3	98.4 ± 2.1
100 mM NaCl + *P. fluorescens* biotype F	28.9 ± 4.8	82.3 ± 7.9	101.1 ± 3.9
100 mM NaCl + *P. fluorescens* CECT 378^T	23.7 ± 5.8	79.5 ± 10.6	90.7 ± 1.9

between 11 and 15%), as well as for *P. fluorescens* CECT 378^T for roots (16%, Table 1).

The internal content of Na^+ and K^+ was measured in leaves, stems and roots of plants grown in presence or not of 100 mM NaCl (Table 2 and Fig. 3). When NaCl was used to induce salt stress, Na^+ content in root and stem was higher than in leaves (Table 2). On the other hand, the bacterial inoculants inhibited significantly Na^+ accumulation in leaves. This effect was more marked with CECT 378^T than with the Biotype F. Thus, *P. fluorescens* CECT 378^T contributed to the reduction of Na^+ content with a 38.2% in leaves, a 14% in stems and a 8% in roots, while the effect of *P. fluorescens* biotype F was 24.6%, 11% and 0%, respectively.

The accumulation of K^+ was greater in shoot than root, in all cases (Fig. 3). This effect was favoured by salinity. However, salinity inhibited the accumulation of K^+ in the root. The bacterial

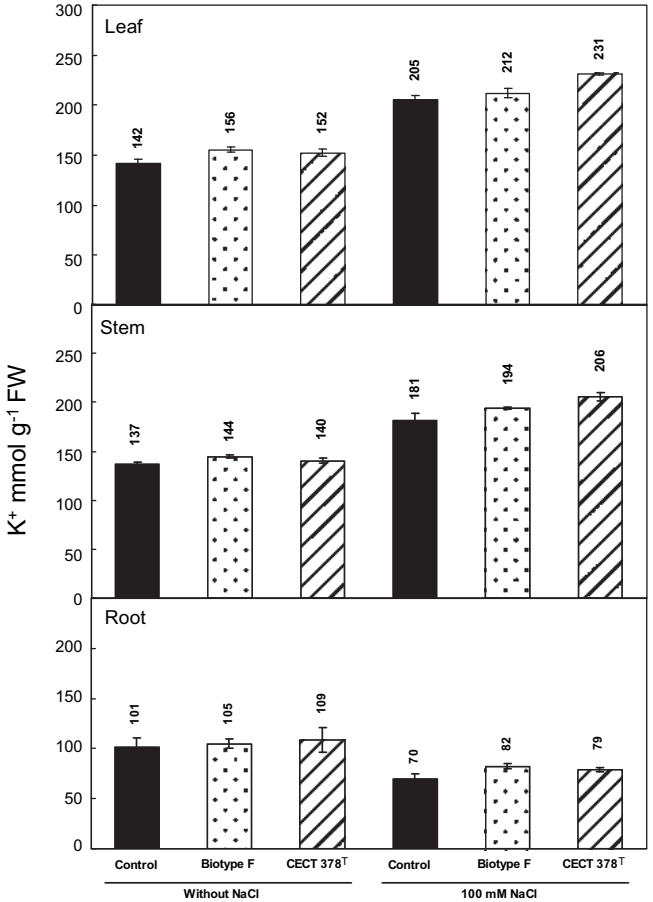

Fig. 3. Effect of salinity, *P. fluorescens* biotype F and *P. fluorescens* CECT 378^T on K^+ concentration in leaves, stems and roots of sunflower plants. The plants were grown during 5 weeks in pots with sand:peat mixture and watered with standard nutrient solution supplemented or not with 100 mM NaCl. The bars represent the average ± the standard error.

inoculants favoured significantly the accumulation of K^+ in the shoot in all cases, although this effect was more marked in the saline treatment. However, in the root the inoculants favoured significantly the accumulation of K^+ only in the saline treatment. Considering both inoculants the CECT 378^T was more efficient in the promotion of the K^+ accumulation in the shoot.

In Fig. 4 it is represented the relation of K^+/Na^+ in leaves, stems and roots of sunflower plants. In our study this relation decreases in next order: leaves > stems > roots notifying that only in roots the values are lower than 1. Both bacterial inoculants contribute to significant increment of this relation in all parts, notifying that it was higher in case of isolate CECT 378^T. This inoculation conducted to 66% increment in leaves, 34% in stems and 16% in roots, while the *P. fluorescens* biotype F incremented just in leaves and stems with 30% and 26%.

4. Discussion

Saline conditions are known to reduce the growth of plants (Greenway and Munns, 1980). In this study a high concentration of salt (100 mM NaCl) in the irrigation solution prompted a significant reduction of growth (Table 1). However, when plants under saline conditions were treated with a suspension of *P. fluorescens* biotype F or *P. fluorescens* CECT 378^T, it was observed a lower level of growth reduction and higher fresh weight values than in untreated plants. This clearly demonstrates that the use of strains can partly alleviate some of the negative effects of salt stress (Mayak et al., 2004). It is known that sunflower plants avoid the accumulation of Na^+ in the leaves (Ashraf and O'Leary, 1996). This accumulation is the balance between the total Na^+ translocation to the shoot, via the xylem, and the Na^+ recirculation from shoot to root, via the phloem that occur in sunflower plants (Lessani and Marschner, 1978; Quintero et al., 1998). In this study, the treatments with a suspension of *P. fluorescens* biotype F or *P. fluorescens* CECT 378^T prompted the Na^+ excluder character of these plants. However, it is known that a high K^+/Na^+ ratio in the shoot is more important for many species than simply maintaining a low concentration of Na^+ (Maathuis and Amtmann, 1999). It is also widely accepted that the differences in salt tolerance in Triticeae in both *Triticum* (Gorham et al., 1991) and *Hordeum* (Garthwaite et al., 2005) genus, are associated with the capacity of remaining a high K^+/Na^+ ratio in the shoot. In this study, both bacterial treatments favoured this effect, mainly in the leaves (Fig. 4).

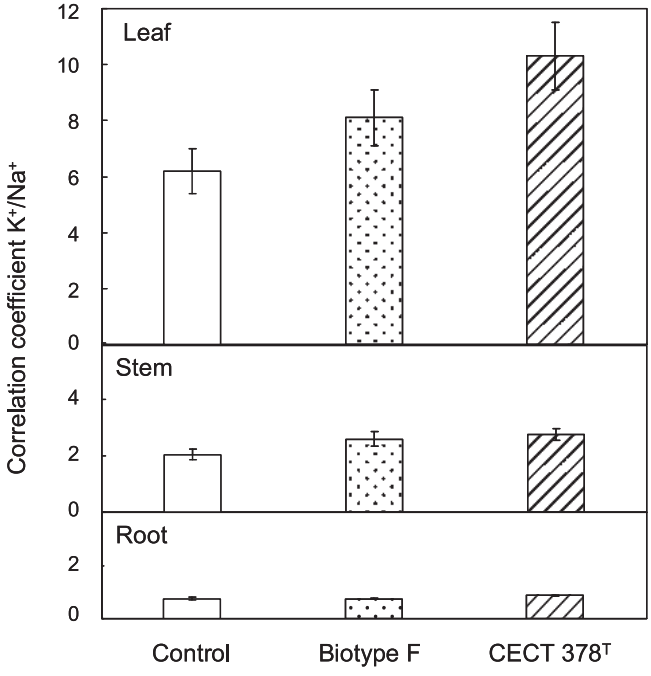

Fig. 4. Effect of salinity, *P. fluorescens* biotype F and *P. fluorescens* CECT 378^T on the relation K^+/Na^+ in leaves, stems and roots of sunflower plants. The plants were grown during 5 weeks in pots with sand:peat mixture and watered with standard nutrient solution supplemented with 100 mM NaCl. The bars represent the average ± the standard error.

In summary, the inoculation of both bacterial suspensions decreased the levels of accumulated Na^+ in all plant parts considerably, thus K^+ content in them increased, as well as the biomass of corresponding plants. Probably, this is due to the stimulation of plant root growth by IAA (Table 1), better iron status because of siderophores production, and may be due to the capability to utilize ACC through ACC-deaminase, thus decreasing ethylene production that conducts to enhanced root length (Patten and Glick, 2002). In this sense, our further investigations are directed to the determination if both strains contain ACC-deaminase, quantify the siderophores production and identification of other mechanisms involved in the bacterial plant-growth-promoting effect in abiotic stress conditions.

Acknowledgments

This work was partially supported by the Fund "Science Investigations" of the Ministry of Education and Science (Bulgaria) under Grant "Post-Doc-7".

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Journal of Environmental Management 95 (2012) S42–S49

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Heavy metals, salts and organic residues in old solid urban waste landfills and surface waters in their discharge areas: Determinants for restoring their impact

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ARTICLE INFO

Article history:
Received 30 September 2009
Received in revised form
16 June 2011
Accepted 24 June 2011
Available online 20 July 2011

Keywords:
Anthrosols
Soil pollution
Water pollution
Salinity
Heavy metals
Anions
Organic pollutants
Plants biodiversity
Nematodes

ABSTRACT

This study was designed to determine the state of polluted soils in the main landfills of the Community of Madrid (central Spain), as part of a continuous assessment of the impacts of urban solid waste (USW) landfills that were capped with a layer of soil 20 years ago. Our analysis of this problem has been highly conditioned by the constant re-use of many of the USW landfills, since they have never been the target of any specific restoration plan. Our periodical analysis of cover soils and soils from discharge areas of the landfills indicates soil pollution has worsened over the years. Here, we examined heavy metal, salts, and organic compounds in soil and surface water samples taken from 15 landfills in the Madrid region. Impacts of the landfill soil covers on nematode and plant diversity were also evaluated. These analyses continue to reveal the presence of heavy metals (Zn, Cu, Cr, Ni, Pb, Cd) in soils, and salts (sulphates, chlorides and nitrates) in soils and surface waters. In addition, non-agricultural organic compounds, mainly aromatic and aliphatic hydrocarbons, often appeared in very high concentrations, and high levels of insecticides such as gamma-HCH (lindane) were also detected in soils. Around 50% of the water samples collected showed chemical demand of oxygen (CDO) values in excess of 150 mg/l. Traces of phenolic compounds were detected in some landfills, some of which exhibited high levels of 2-chlorophenol and pentachlorophenol. All these factors are conditioning both the revegetation of the landfill systems and the remediation of their slopes and terrestrial ecosystems arising in their discharge areas.

This work updates the current situation and discusses risks for the health of the ecosystems, humans, domestic animals and wildlife living close to these landfills.

1. Introduction

Municipal solid waste landfills often represent a major environmental problem due to their proximity to inhabited areas. According to Chiemchaisri et al. (2007) for example, in 2004 there were 425 disposal sites (95 landfills; 330 open dumps) in Thailand such that for many years over 60% of solid waste disposal in Thailand involved open dumping. Recent literature in this area has been abundant, with emphasis on publications from Asian countries (Esakku et al., 2005; Fan et al., 2006; Nagendran et al., 2006; Xiaoli et al., 2007); eastern and southern Europe (Eitminavièiûtë and Matusevièiûtë, 2005; Zupančič et al., 2009; Businelli et al., 2009; Mari et al., 2009) but also with significant contributions from other regions (Øygard et al., 2004; Slack et al., 2004; Herwijnen et al. 2007; Östman et al., 2006; MacDonald et al., 2008; Schenato et al., 2008). In contrast, few research efforts have been devoted to landfills in arid or semi-arid environments (Illera et al., 2000; Al-Yaqout and Hamoda, 2003).

The risks of soil pollution for ecosystems in periurban areas are especially linked to the pollution effects of old mixed solid waste landfills (industrial and urban solids). It is possible that a significant role may be attributed to effects arising from these waste materials such as the physical medium, the movement of contaminants due to flow forces, and new interactions occurring in the system due mainly to salinity, heavy metal toxicity and organic contaminants. Around 20 years ago, a large number of the Madrid region's urban solid waste landfills were sealed. At different times over these past twenty years, the landfill soils and the discharge areas of the sealed tips have been analyzed. Since then, we have constantly been assessing the presence of contaminating residues both in landfill soils and in affected surrounding ecosystems. Hernández et al. (1998a, b), Pastor et al. (1993b) and Pastor and Hernández (2002)

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doi:10.1016/j.jenvman.2011.06.048

Table 1
Current uses (2008) of the 15 landfills.

Landfills	Lat.	Long.	Discharge areas	Current use of the landfill	No. slopes
Gneiss & granite					
Colmenar Viejo	40° 39′N	3° 45′W	Creek & wet grassland	Cattle grazing	3
S. Lorenzo	40° 35′N	4° 8′W	Creek & ash-trees	Nomad shepherding; Reforestation	3
El Escorial	40° 35 ′N	4° 7′W	Creek & wet grassland	Cattle grazing	4
Arkoses					
Móstoles	40° 20′N	3° 52′W	Creek & wetland	Cereal crops and shepherding	3
Villaviciosa	40° 21′N	3° 54′W	Wetland	Leisure	1
Navalcarnero	40° 17′N	4° 17′W	Cereal crops	Sheep grazing; Housing	–
El Álamo	40° 13′N	3° 59′W	Wetland	Restoration with herbaceous covers	2
Limestone					
Pinto 1	40° 14′N	3° 41′W	Cereal crops	Reforestation	5
Pinto 2	40° 14′N	3° 42′W	Cereal crops	In disuse	4
Pinto 3	40° 14′N	3° 42′W	Road	Re-used; Use controlled at present.	4
La Poveda	40° 17′N	3° 26′W	Creek	Re-used for rubble disposal	2
Arganda del Rey	40° 18′N	3° 26′W	Creek	Uncontrolled; new spillages of rubble	2
Mejorada de campo	40° 23′N	3° 29′W	River	Nomad shepherding. Reforestation	1
Alcalá de Henares	40° 29′N	3° 22′W	River	Reforestation	1
Gypsum					
Aranjuez	40° 1′N	3° 38′W	Creek	Crumbling observed after sealing	1

have described the physical and chemical condition of these soils, taking into account the substrate underlying the landfills that were capped with soils from their respective surroundings. These past reports have also described the main plant species growing during the process of revegetation of the tips in the first few years. The aim of the present paper was to describe some of the most significant chemical determinants for the design of measures to restore the impacts of these landfills. The goal of the "road-map" we propose is to identify the heavy metals, salinity and organic compounds responsible for the main detrimental effects produced, mainly on soils and surface waters, but also on plants and soil organisms.

2. Materials and methods

We examined 15 old urban (and industrial) solid waste landfills near the major cities and towns of the province of Madrid. These landfills were sealed around twenty years ago, simply by capping with soil. The main characteristics of the waste landfills can be found in Pastor and Hernández (2002 and 2007).

At each of these 15 landfill sites, 4 to 6 soil samples (0–10 cm) were collected depending on their size to give a total of 75 samples. Control samples were taken from the topsoil layer (0–15 cm) of surrounding grazing lands (reference ecosystems).

A spade was used to obtain an average sample of topsoil in each zone that was subsequently used for physical-chemical determinations (MAPA, 1982; Hernández and Pastor, 1989; Pastor and Hernández, 2007). Determination was made of pseudototal contents of Al, heavy metals and trace elements (Cr, Ni, Pb, Cd, Cu, Zn, Fe, Mn), as well as pH, soil anions and EC. Metals in the soil were determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES). For these determinations, the soil samples were ground in an agate mortar and acid digested using a 4:1 mixture of HNO_3 and $HClO_4$. Soil anions were analyzed by ion chromatography.

Table 2
Heavy metal contents (mg/kg) recorded for the soil covers (mean values and standard deviation) of landfills overlying the main representative substrates of the Madrid area five years after capping.

Soil heavy metals	Granites and gneiss	Arkoses	Limestones
Zn	125.5 ± 69.8	83.5 ± 146.0	57.5 ± 10.5
Cu	8.7 ± 19.4	150.9 ± 730.2	13.0 ± 11.0
Pb	7.7 ± 5.6	72.8 ± 297.0	28.5 ± 3.5
Ni	22.2 ± 3.6	15.7 ± 8.1	22.5 ± 3.5
Cr	0.0 ± 0.0	4.4 ± 4.5	0.0 ± 0.0
Cd	0.0 ± 0.0	0.3 ± 2.1	0.0 ± 0.0
Co	0.0 ± 0.0	1. 5 ± 2.3	0.0 ± 0.0

The organic pollutants determined were: total hydrocarbons by infrared spectrophotometry (UNE 77307); chlorinated insecticides and polychlorinated biphenyls by gas chromatography (ISO 10382), PAHs by gas chromatography (ISO 18287), and phenols by gas chromatography (U.S. E.P.A 3550B, U.S. E.P.A 3650B and U.S. E.P.A 8401).

Water samples were also collected from the main discharge zones of the landfills. These samples were obtained at random points across the wetland and stored in dark glass containers to determine organic and inorganic compounds.

For the determination of organic pollutants in water, organic compounds (lipophilic) were extracted from the water sample by retention on a cartridge or a disk containing the organic phase octadecylsilyl chemically bound to a solid matrix. Retained compounds were subsequently eluted using a small amount of dichloromethane, separated by gas chromatography and identified by mass spectrometry.

In a separate study conducted only on the arkosic landfills, a further 91 soil samples were obtained in spring from 1 m^2 -quadrants set up in semi-natural grasslands and agricultural lands abandoned in the last 20 years (55 sites) and in 36 urban waste landfill sites sealed with a 30 cm layer of soil. After inventoring the plant species present in these plots, the soil samples (maximum depth 10 cm) were subjected to nematode counts using previously described methods (Hernández and Pastor, 1989). Samples were kept at 4 °C until processing. Nematodes were extracted from 100 ml by the sugar centrifugation method as described in Hernandez and Pastor (1989). After counting the total number of nematodes, 100 nematodes per sample were randomly selected and identified to the genus level if possible with the help of an inverted compound microscope. Identified taxa were classified into fungivore, bacteriovore, omnivore, predator or plant feeding trophic groups on the basis of either reported feeding habits or stoma and oesophageal morphology (Yeates et al., 1993; Urcelai et al., 2000).

3. Results and discussion

3.1. Main characteristics of the landfills

Table 1 shows the geographical coordinates of each of the 15 landfills and their current uses. Mean soil heavy metal contents and

Table 3
pH and heavy metal contents (mg/kg) recorded for the soil covers of landfills in the Madrid area 18–20 years after capping (2007–08).

Landfills	pH	Al	Fe	Mn	Zn	Cu	Cr	Ni	Pb	Cd
Colmenar Viejo	6.3	33588	22598	293	68	14	3.7	5.6	13.9	0
San Lorenzo	7.3	29819	21304	218	185	40	12	6.0	223	0
El Escorial	7.2	29067	77315	446	693	139	23	13	182	0
Móstoles	5.7	23886	12188	241	38	8.8	2.7	3.1	9.4	0
Villaviciosa	7.5	26751	15479	213	93.3	36.1	1.3	2.9	24.8	0
Navalcarnero	6.5	8744	5019	202	12.4	4.3	1.6	1.1	2.7	0
El Álamo	7.2	8711	4149	93	26	8.0	1.9	1.5	4.4	0
Pinto 1	7.3	27366	16794	217	39	8.5	0.3	3.4	7.0	0
Pinto 2	7.6	31327	20261	204	39	8.2	0.4	3.4	4.7	0
Pinto 3	7.9	42483	25938	631	144	55	40	5.0	51	0
La Poveda	7.6	28985	19731	225	50	28	2.8	9.1	13.4	0
Arganda	8.0	29684	15488	193	110	27	5.9	7.4	60	0
Mejorada del campo	7.7	70090	39706	366	148	29	8.1	15	20	0
Alcalá	7.6	51800	32384	288	130	23	12	15	13.4	0
Aranjuez	7.7	71420	37512	256	86	69	2.2	15	11.6	0
Reference levels					140	36	100	35	50	1

their corresponding standard deviations are provided by geological substrate in Table 2. The plant cover characteristics of the waste landfills can be found in Pastor and Hernández (2002).

The residues deposited in the landfills examined here are of a heterogeneous nature (urban, industrial and inert solids) and have undergone no previous treatment. The edaphic landfill material has never exceeded 40 cm. The scant layer of landfill soil and the steep slopes have meant that much soil has been lost over the years through erosion, leaving many un-decomposed residues in the cracks produced by surface run-off. The characteristics of the slopes do not only affect plant colonization in these systems but also the ecosystems of the discharge areas. Even where there is only one slope, its run-off sometimes fans out, and thus differently affects the biodiversity of the damp ground in different zones of the discharge area. Also, considerable variation in soil variables exists on a given slope (Pastor and Hernández, 2002, 2007). Since our first analyses, differences have also arisen in relation to the landfill material originating from the different substrates. Research into this problem is strongly conditioned by the constant re-use of many of the landfills, given that no particular recovery plan was envisaged for any of them. This has led to the deposition, after the first sealing, not only of inert residues but also residues of manifest chemical composition. This aggravates the problems of many tips and poses great difficulty when trying to restore their impacts. In the following sections we describe some of the findings on which these assertions are based.

Table 4
Anion contents (mg/kg) and electrical conductivity (μS/cm) recorded for the soil covers of the landfills 18–20 years after capping (2008).

Landfills	F	Cl	NO_2	NO_3	PO_4	SO_4	EC
Colmenar Viejo	0.7	53.8	1.4	51.7	22.1	64.3	244
San Lorenzo	0.4	9.7	2.6	544.3	16.7	46.1	669
El Escorial	0.4	15.1	3.7	783.1	13.4	73.3	606
Móstoles	0.8	65.3	1.0	64.9	30.7	852	160
Villaviciosa	0.3	9.6	6.1	74.5	45.8	19.3	392
Navalcarnero	0.7	4.9	1.3	167.5	9.8	17.7	101
El Alamo	0.3	10.8	2.7	13.6	34.9	14.8	106
Pinto 1	3.6	9.8	5.3	9.3	5.1	17.2	190
Pinto 2	4.6	5.8	8.2	7.3	0.0	17.2	184
Pinto 3	1.6	30.9	3.0	409.1	8.3	676.9	759
La Poveda	2.9	10.8	4.4	70.8	5.3	774	645
Arganda	1.4	64.4	5.6	19795	2.5	3164	1947
Mejorada del Campo	4.9	55.0	2.3	298.5	6.3	1195	1248
Alcalá	0.9	43.9	8.4	443.3	28.3	102	650
Aranjuez	1.3	20.3	2.7	189.4	3.8	4065	2440
Reference levels	>12	>55	–	>100	>45	>150	>350

3.2. Identifying pollutants in the topsoil layer

The landfill soils and the soils from the discharge areas have been periodically assessed over the last 20 years, with attention mainly paid to inorganic chemical compounds. Tables 3 and 4 show the maximum levels of heavy metals recorded for each landfill. These factors compromise the biotic components of the affected ecosystems. Tables 2 and 3 show the soil variables indicating contamination produced by residues from the tips; in 5 of the landfills these exceed the benchmark levels for heavy metals. Johansen and Carlson (1976) found that among the heavy metals, Fe were high in all soils examined followed by Zn. We also detected Zn in highest quantities in our landfills. Concentrations of Cr, Ni, Cu, Cd and Pb were low. Xiaoli et al. (2007) studied the characteristics, distribution, and mobility of heavy metals in a landfill. The refuse was characterized as containing high concentrations of heavy metals and a relatively high pH. Zn demonstrated the greatest mobility compared to other heavy metals, whereas Cd was well retained in the landfill. Zn was also found at higher concentrations than the remaining heavy metals. In the dumpsite examined by Esakku et al. (2005), total metal contents in descending order were Fe, Cu, Zn, Cr, Mn, Pb, Ni and Cd.

In a semi-arid environment, Illera et al. (2000) observed considerably increased levels of Cd, Cu, Pb and Zn as a consequence of the high contents and/or high availability of these metals in mixed solid waste (MSW) landfills. According to these authors Zn, followed at some distance by Cu, was the most abundant heavy metal. In arid regions, the generation of leachate from MSW landfills has long been neglected on the assumption that only minimal leachate could be formed in the absence of precipitation (Al-Yaqout and Hamoda, 2003). The latter authors found that leachates collected from both active and old (closed) landfills were severely contaminated with organic compounds, heavy metals and salts. The metals we found in our 15 landfills mainly in semi-arid environments in descending order were Fe, Al, Zn, Mn, Cu, Pb, Cr, Ni and Cd. Leachate Zn contents in the study by Al-Yaqout and Hamoda were similar to those detected here while Cu levels were higher.

Heavy metals absorbed by plants can be harmful to the animals that consume them, both domestic and wild. Sánchez-Chardi and Nadal (2007) quantified the bioaccumulation of metals (Pb, Hg, Cd, Fe, Mg, Zn, Cu, Mn, Mo, and Cr) from the landfill of Garraf (Barcelona, Spain) in the greater white-toothed shrew, *Crocidura russula* (Insectivora, Mammalia). These authors observed substantial amounts of potentially toxic metals (Pb and Cd concentrations of up to 59.71 and 56.57 $\mu g\ g^{-1}$ DW respectively in the kidneys). The genotoxic actions of these metals suggested detrimental effects also

Table 5
Organic pollutants (mg/kg) recorded in the old urban landfills in 2008.

Landfill	Phenolic compounds	Chlorinated Insecticides	Hydrocarbons	PCBs	PAHs
Colmenar Viejo	Phenol	Lindane, Hexachlorebencene			
San Lorenzo					
El Escorial			**58.21**	**0.054**	Benzo(a)pyrene
				0.027	Dibenzo(a,h)anthracene
Mostoles		Lindane			
Villaviciosa		Lindane			
Navalcarnero		Lindane			
El Alamo	**Phenol (0.033)**				
Pinto 1[a]		p,p'-DDD			Benzo(a)pyrene
Pinto 2	**2-chlorophenol**	Hexachlorebencene			Benzo(a)pyrene
Pinto 3	**2,4,5-trichlorophenol, 2,4,6-trichlorophenol, 2,4-dichlorophenol, Pentachlorophenol**	Lindane; Hexachlorebencene	**62.34**		
La Poveda		Lindane			**Benzo(a)pyrene**
Arganda	Phenol, Cresols	Lindane (gamma-HCH)		**0.038**	
Mejorada	2-chlorophenol, **Pentachlorophenol**, Phenol, Cresols		**59.7**	**0.01**	
Alcala de H.	**Pentachlorophenol**, Cresols				
Aranjuez	Phenol			**0.027**	
Refer. Levels		0.01 (Lindane)	50.0	0.01	

Higher contents are represented in bold.
[a] Chlorinated hydrocarbons: 1,4-dichlorobencene, 1,2,4-trichlorobencene, 1,4-dichlorobencene.

on biota. In general, the heavy metal contents of the huge landfill investigated by these authors were higher than those observed here. This may be explained by the industrial importance of Barcelona.

Soil salinity in the landfills emerged as a factor that was more detrimental than soil heavy metal concentrations (Hernández et al., 1998b), although the landfills showed intensely varying concentrations of the different anions examined, as may be seen in Table 4, which is provided as an example. Soil EC was high in 9 of the landfills, NO_3 in 8 and SO_4 in six.

To assess the impact of pollutants from the main landfill of the city of Zagreb (Croatia) on the underlying soil, Ahel et al. (1998) determined a broad spectrum of inorganic and organic constituents in samples of solid waste, soil and aquifer sediments. The compounds identified in the landfill could be classified into the two main categories: markers of biological waste and of its microbial transformation (ammonia, dissolved organic carbon, short-chain aliphatic acids, phenols and derivatives of abiotic acid) and markers of anthropogenic waste (toxic metals, hydrocarbons chlorinated hydrocarbons, surfactant-derived compounds, phthalates and pharmaceutical chemicals). These authors noted that, besides the vertical infiltration of leachate from the solid waste, the hydrological groundwater regime also had a strong impact on contaminant distributions in soils beneath the landfill.

We also observed considerable differences in the organic pollutants appearing on our landfill slopes. The hydrocarbons analyzed often occurred at exceedingly high values. Some chlorinated insecticides (of the 13 studied) such as lindane also exist at harmful levels in the soils, according to the indicator values recommended for terrestrial organisms. In discharge areas, other organic pollutants detected were phenolic compounds, polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbons (PAHs). Slack et al. (2004) examined pollutants in leachates from household hazardous waste (HHW) in municipal landfills. Their results indicate that the risks associated with the disposal of HHW have not been fully elucidated. This review revealed that a broad range of xenobiotic compounds occurring in leachates could be linked to HHW. Mari et al. (2009) analysed the exposure to heavy metals and polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans (PCDD/Fs) of people living in the vicinity of a hazardous waste landfill (HWL) in Castellolí (Barcelona, Spain). The metals most often detected were again Zn and Cu, while Pb, Ni and Cd were lower and Cr higher than the values we recorded. Health risks were evaluated according to the distance from the sampling locations to the HWL (near and far-sites). Concentrations

Table 6
Differences in soil variables (Student's *t*-test) between landfill covers ($n = 59$) and reference ecosystems (92 pasture soils) in arkosic substrates.

Soil variable	Landfill covers		Reference ecosystems		Significance level
	X ± s.d.	Range	X ± s.d.	Range	
pH	7.1 ± 0.3	6.3–7.6	6.2 ± 0.5	5.1–7.1	99.9
OM	0.7 ± 0.4	0.2–1.6	3.5 ± 2.7	0.2–12.2	99.9
Fe	15291 ± 6455	5047–28453	9057 ± 4029	2315–21945	99.9
Mn	223 ± 113	46–552	112 ± 88	40–269	99.9
Zn	89 ± 157	13.8–960	32 ± 11	9.7–5.8	99.0
Cu	187 ± 741	2.5–3680	19.6 ± 32.1	0–63	
Pb	89.8 ± 313	0–797	8 ± 22.8	0–11.3	
Cd	1.9 ± 3.4	0–10	0.1 ± 1.6	0–6.1	90.0
Cr	4.6 ± 4.7	0–17	2.7 ± 2.8	0–6.0	
Ni	14.0 ± 9.2	0–32	7.4 ± 6.3	1.6–21	95.0
Co	1.9 ± 2.6	0–9	0.0 ± 0.0	0–1.9	99.0
Al	37404 ± 13696	15250–65790	24783 ± 11062	5700–54300	99.9
B	5.5 ± 11.8	0–50.0	2.6 ± 5.5	0–20.0	

Table 7
Mean and s.d. of electrical conductivity (EC) and soil anions in discharge areas of several landfills (L) located in different substrata versus soils from nearby reference ecosystems (R.E.).

Soil variable		Arganda del Rey		Alcalá de Henares		Pinto 1	
		L.	R.E.	L.	R.E.	L.	R.E
EC (μS/cm)	M	1430	530	3890	1200	11045	3940
	s.d.	156		735		6583	
Chlorides	M	33.5	5.0	22.0	5.0	76.5	18.0
(mg/100 g)	s.d.	7.8		2.8		10.6	
Sulphates	M	68.5	1.0	155.5	4.0	4544.0	412.0
(mg/100 g)	s.d.	3.5		3.5		5883.1	
Nitrates	M	4.2	2.0	3.9	0.5	31.3	19.8
(mg/100 g)	s.d.	0.5		0.9		19.1	

Table 8
Statistically significant differences recorded in 36 soil samples taken from landfills and 55 samples from a reference ecosystems in arkosic terrain.

Biological parameters / Plants	Landfill covers	Reference ecosystem soils	Significance level
Plant cover (%)	34.9 ± 17.1	60.6 ± 25.0	95
Average height of plant cover (cm)	14.9 ± 9.1	22.7 ± 10.2	
Plant diversity (No sp/m^2.)	15.5 ± 7.3	29.5 ± 11.4	95
Nematodes			
Total Density (No/100 cm^3)	45.6 ± 38.3	122.1 ± 50.7	99.9
Ectoparasites	10.1 ± 13.2	30.5 ± 15.7	99.9
Endoparasites	0.1 ± 0.4	0.9 ± 4.0	
Omnivores	10.6 ± 11.3	12.6 ± 10.2	
Bacterial feeders	71.3 ± 18.7	37.7 ± 17.5	99.9
Fungal feeders	8.0 ± 9.3	17.0 ± 13.3	95

of PCDD/Fs, as well as those of some metals, were found to be relatively higher in the HWL and Castellolí (the nearest village) than in samples collected far away, resulting in slightly increased exposure to those pollutants.

Open landfill dumping areas for municipal wastes in developing countries have received particular attention with regard to environmental pollution problems. Because of the uncontrolled burning of solid wastes, elevated contamination by several toxic chemicals including dioxins and related compounds in these dumping sites has been anticipated. Hung Minh et al. (2003) analyzed concentrations of PCDD/Fs and coplanar PCBs in soils from dumping sites in the Philippines, Cambodia, India, and Vietnam. Residue concentrations of PCDD/Fs and coplanar PCBs in dumping site soils were apparently greater than those in soils collected in agricultural or urban areas far from dumping sites, suggesting that dumping sites are potential sources of PCDD/Fs and related compounds. Observed PCDD/F concentrations in soils from dumping sites in the Philippines and Cambodia were comparable or higher than those reported for dioxin-contaminated locations in the world. Table 5 indicates the current values of organic variables recorded in our study. The contents of these pollutants were at times quite high. Values exceeding permissible levels according to Spanish legislation appear in bold.

Table 6 shows differences in soil variables between the arkosic landfills and their reference ecosystems. It may be observed that the differences recorded for most of these variables are highly significant. Heavy metal contents were higher in the landfill covers than the control soil samples. Table 7 shows differences in the electrical conductivity and soil anion levels in discharge areas of several landfills located in different substrates compared to soils from nearby reference ecosystems.

3.3. Impacts of landfill covers on the diversity of plants and nematodes

The topsoil layer of the landfills is inhabited by soil populations such as microorganisms or nematodes, and is also the main source of mineral nutrients for plant species growing in the soil (Hernández et al., 1998a; Urcelai et al., 2000; Pastor and Hernández, 2002). Our phytoecological and soil studies performed in the past years both on the capping soils and soils of the discharge zones of urban waste landfills have revealed certain features that may be of help when planning to ecologically restore this type of environmental setting. Table 8 shows the reduced diversity of plants and nematodes detected in the capping layer of the landfills, which is probably the result of adverse conditions in general, and toxicity due to heavy metals, high contents of salts or organic pollutants. This diversity is even lower than that reported for other highly degraded ecosystems of the area (Urcelai et al., 2000). Biederman et al. (2008) also investigated nematode community early development in ecological restoration.

Contrary to expectations, surface amendment treatments significantly increased bacterivorous, plant parasitic, omnivorous and predator nematode densities, but had no influence on fungi/root-tip feeding nematodes. In agreement with these authors, bacterial feeders were the predominant group detected at our landfills. Numbers of ectoparasites, endoparasites, fungal feeders and omnivores were significantly lower than in our reference ecosystems. Other observations in soil fauna were made by Eitminavièiûtë and Matusevièiûtë (2005) who summarized data on the domestic waste landfills of Vilnius (Lithuania). These authors focused on the influence of landfills on the soils of their environment. The structure of microarthropod complexes was monodominant, species composition was poor, and herbaceous plants intensively accumulated heavy metals. Even after fifteen years, the landfills remained polluted with high concentrations of Cd, Ni, Pb, and Zn.

Taking into account these findings together with those of Pastor et al. (1993a, b) and Hernández et al. (1998a, b), who also observed increased levels of salts in landfills, the difficulty in securing an environment of available nutrients for plants becomes clear, despite the appropriate pH of the soil (means of 7.1 were detected for landfills and 6.2 for control soil samples). Such high salt contents indicate a need for bioassays aimed at evaluating the suitability of native plant species as plant covers for landfills.

Table 9
Current pH, macroelement and boron contents (mg/l) of water samples obtained from the discharge areas of the landfills.

Landfills	pH	Ca	Mg	K	Na	P	S	Al	Fe	Mn	B
Colmenar Viejo	6.9	22.3	8.6	103.3	126.0	0.0	43.6	0.12	0.04	0.10	0.19
San Lorenzo	7.8	18.4	3.9	2.2	6.2	0.0	8.7	0.0	0.00	0.0	0.03
Móstoles	8.8	110.3	24.4	61.4	210.9	2.19	20.3	0.05	0.36	1.97	0.22
El Alamo	8.4	55.4	7.9	7.1	30.7	0.56	26.1	0.04	0.06	0.03	0.06
Getafe	8.1	530.0	244.4	4.1	71.3	0.0	567	0.06	0.06	0.08	0.18
Aranjuez	8.6	410.8	583.6	33.6	2736.5	0.0	2780	0.0	0.11	0.06	0.75

Table 10
Heavy metal (mg/l) contents of water samples collected from damp ground and streams at the foot of the landfills in early spring.

Landfills	Zn	Cu
Colmenar Viejo	0.06	n.d
San Lorenzo	n.d	n.d
Móstoles	0.034	0.011
El Alamo	n.d	0.025
Aranjuez	n.d	0.023

Pb, Cd, Cr, Ni, As: not detected.

3.4. Composition of waters in discharge areas

In a study in Denmark by Kjeldsen et al. (1998), an old municipal landfill emerged as a groundwater pollution source based on landfill history and leachate composition. Table 9 shows the current pH and composition of water obtained from damp ground and streams at the foot of the present landfills. Table 10 provides the heavy metal contents (mg/l) of these samples. Elevated values were recorded of pH, Na and S. High Ca and Mg contents were also detected in the waters close to the landfills of Getafe and Aranjuez.

Table 11 shows the electrical conductivity (μS/cm), chemical demand of oxygen (CDO) and anion contents (mg/l) of water samples collected from damp ground and streams in the discharge areas. Around 50% of the water samples collected showed CDO values greater than 150 mg/l. In contrast, nitrates and fluorides have undergone a considerable rise over the years. These trends differ according to the aquatic ecosystem affected by the landfills, although salinity also seems to be declining in the landfills on a granite substrate (Pastor et al., 1993c). Similar studies to ours reporting comparable findings include those by Mikac et al. (1998), who assessed groundwater contamination in the vicinity of a municipal solid waste landfill in Zagreb (Croatia), Paxéus (2000), who examined organic compounds in municipal landfill leachates, and Slack et al. (2004), who analyzed the hazardous components of household waste.

Table 12 shows the relationship between the organic compounds in the waters and their proximity to the landfills. Harmsen (1983) and Albaiges et al. (1986) identified organic indicators of groundwater pollution close to a biohazardous waste landfill. We observed a tendency towards higher carboxylic acid levels in some of our landfills, but more intense effects have been attributed to biohazardous waste in the landfills of Barcelona than those examined here. Albaiges et al. detected around double the concentration of phthalates, a group of colourless, odourless liquid chemical compounds mainly used as laminates, to that observed in our landfills. In Oman and Rosqvist's (1999) study of the transport fate of organic compounds in water flowing through landfills, the maximum concentration of diethyl phthalate (DEP) detected was 346 μl.

According to Jobling et al. (1995), a variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. Bauer and Herrman (1997) estimated the environmental impacts of phthalic acid esters leaching from household wastes. These authors recorded maximal concentrations of 2.6% di-(2-ethylhexil)phtalate and calculated this figure to translate to around 1 kg of phthalate esters per ton of dry waste and the leaching of some 1 g of phthalic acid esters per ton of dry waste. Staples et al. (1997) reviewed the global environmental fate of

Table 12
List of organic compounds detected in 2008 in water samples affected by the presence of a landfill.

Móstoles	El Álamo
Tetradecane	Undecane
4-tert-butyl benzoic acid	Octanoic acid
Dietiltoluamide	Tetradecane
Diethyl phthalate	Terbucarb
Heptadecane	Diethyl phthalate
Tetradecanoic acid	2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1, 3-propanodyl, propionate
Diisobutyl phthalate	**Diisobutyl phthalate**
Dibutyl phthalate	Butyl isobutyl phthalate
Hexadecanoic acid	Dibutyl phthalate
Oleic acid	Hexadecanoic acid
Octadecanoic acid	Pentadecane
Tricosane	Tricosane
Di-2-ethylhexyl adipate	Di 2-ethylhexyl adipate
Tetracosane	Tetracosane
Eicosane	Eicosane
di-2-ethylhexyl phthalate	Di 2-ethylhexyl phthalate
Squalene	

San Lorenzo	Colmenar Viejo
Octanoic acid	Butyric acid
3-hydroxy-2,2,4-trimethylpentyl isobutanoate	Undecane
Tetradecane	3-Hydroxy-2,2-dimethyl hexyl butyrate
Diethyl phthalate	Tetradecane
1,3-pentanediol, 2,2,4-trimethyl diisobutyrate	Diethyl phthalate
Tetradecanoic acid	1,3-Pentanediol, 2,2,4-trimethyl diisobutyrate
Diisobutyl phthalate	**Diisobutyl phthalate**
Butyl isobutyl phthalate	Pentadecanoic acid
Dibutyl phthalate	Dibutyl phthalate
Hexadecanoic acid	Hexadecanoic acid
Pentadecane	Octadecanoic acid
Tricosane	Pentadecane
Di 2-ethylhexyl adipate	Tricosane
Tetracosane	Bis 2-ethylhexyl adipate
Eicosane	Tetracosane
Di-2-ethylhexyl phthalate	Eicosane
Dotriacontane	Di-2-ethylhexyl phthalate
Squalene	Squalene

Aranjuez	
Undecane	Oleic acid
Octanoic acid	Octadecanoic acid
Tetradecane	Pentadecane
Diethyl phthalate	Tricosane
Tetradecanoic acid	2-etilhexil adipate
Diisobutyl phthalate	Tetracosane
Pentadecanoic acid	Eicosane
Butyl isobutyl phthalate	Bis 2-ethylhexyl phthalate
Dibutyl phthalate	Squalene
Hexadecanoic acid	

Most dangerous pollutant are represented in bold.

Table 11
Electrical conductivity (μS/cm), chemical demand of oxygen and anion contents (mg/l) of water samples collected from damp ground and streams close to the landfills.

Landfills	EC	CDO	F	Cl	NO_2	NO_3	PO_4	SO_4	CO_3	HCO_3
Colmenar Viejo	1281	261.0	0.12	269	n.d	67.7	n.d	130.9	0.0	0.0
San Lorenzo	265	168.0	0.17	4.46	n.d	5.2	n.d	26.2	0.0	48.8
Móstoles	2490	207.8	0.28	568.3	0.10	4.5	3.0	60.9	60.0	201.3
El Alamo	523	91.4	0.24	41.5	0.49	5.4	2.3	69.3	30.0	97.6
Aranjuez	13420	111.8	0.12	770	n.d	20.3	n.d	8341.0	48.0	207.4

phthalate esters determining that traces of these compounds are common in waters despite their relatively low solubility in water, high solubility in oils and low volatility. The most employed phthalate is the one used for PVC; others are used as solvents to elaborate perfumes, pesticides, enamels, adhesives, putties and paint pigments. The high quantities found in our water samples, despite their low solubility, is worthy of note, although there is much controversy regarding their effects on health. Several experiments have shown that the bioaccumulation of phthalate esters in the aquatic and terrestrial food chain is limited by biotransformations, which occur more frequently with increasing trophic level. Thus, they are usually considered to be low- or medium-level pollutants although high doses of some of them may cause abnormal hormone behaviour in rodents, as well as damage to the liver, kidneys, lungs and testicles. Fukuoka et al. (1997) suggested phthalic acid esters as a cause of testicular damage. In contrast, studies conducted in primates have not been able to confirm this theory. Diisobutyl phthalate (DIBP) is used as a plasticizer in coatings (e.g. in antislip coatings and epoxy repair mortars) and toxic effects of DIBP on reproduction and development have been described. Diisobutyl phthalate has also been described to have comparable anti-androgenic effects to di-n-butyl phthalate in foetal rat testis (Borch et al., 2006; Saillenfait et al., 2008).

It therefore seems evident that diisobutyl phthalate fulfils the criteria for its classification as a CMR (carcinogenic, mutagenic and reprotoxic) substance according to article 57a) of the REACH regulation.

The European Union, however, has not yet established any guidelines for the control of phthalic acid esters. We also detected large quantities of the resin 4-(1-pyrrolydinyl)-2(1H)-pyrimydinone, detergent components and the herbicide Terbucarb at our landfill sites.

4. Conclusions

Chemical analyses conducted on the soils and waters around landfills sealed 20 years ago continue to reveal the presence of heavy metals (Zn, Cu, Cr, Ni, Pb, Cd) in soils, and salts (sulphates, chlorides and nitrates) and high levels of organic pollutants in both soils and waters. The events and/or ever-changing uses given to the landfills after their initial sealing, together with their particular characteristics pose difficulties both for the revegetation and remediation of these soils that show great variation and heterogeneity in terms of the pollutants found, the density of nematodes (trophic groups) in the soil, and plant cover.

The existence of intense differences between the landfills and the surrounding reference ecosystems was identified in terms of the diversity of plants and nematodes, as well as differences in soil variables among the different landfills themselves.

Our findings reveal the real risks posed by these mixed industrial/household landfills in periurban areas and also illustrate how different concentrations of salts and ions can affect the biotic components of the surrounding ecosystems.

Acknowledgements

This study was funded by grants from the Spanish Ministry of Science and Innovation (Project CTM2008-04827) and Madrid's Regional Government (EIADES, Programme P2009/AMB-1478A).

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Journal of Environmental Management 95 (2012) S50–S54

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Ecotoxicological diagnosis of a sealed municipal landfill

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ARTICLE INFO

Article history:
Received 1 October 2009
Received in revised form
13 September 2010
Accepted 22 October 2010
Available online 13 November 2010

Keywords:
Soil pollution
Salinity
Heavy metals
Soil microbiology
Landfill vegetation

ABSTRACT

Assessing the environmental impact of a soil-topped landfill requires an accurate ecotoxicological diagnosis. This paper describes various diagnostic protocols for this purpose and their application to a real case: the urban solid waste (USW) municipal landfill of Getafe (Madrid, Spain). After their initial sealing with soil from the surroundings about 20 years ago, most USW landfills in the autonomous community of Madrid have continued to receive waste. This has hindered precise assessment of their impact on their environment and affected ecosystems. The procedure proposed here overcomes this problem by assessing the situation in edaphic, aquatic and ecological terms.

The present study focused on the most influential soil variables (viz. salinity due largely to the presence of anions, and heavy metals and organic compounds). These variables were also determined in surface waters of the wetland most strongly affected by leachates running down landfill slopes. Determinations included the characterization of plant communities and microbial biodiversity.

The study was supplemented with a bioassay under controlled conditions in pots containing soil contaminated with variable concentrations of Zn (as $ZnCl_2$) intended to assess ecochemical actions in a population of *Bromus rubens,* which grows profusely in the landfill.

1. Introduction

In previous work (Pastor and Hernández, 2007), we examined evidence obtained over the past 20 years of the difficulties involved in restoring ecosystems under the impact of urban solid waste (USW) in sealed landfills. Based on them, we concluded that an accurate ecotoxicological diagnosis cannot be solely based on the factors most closely related to the plant populations and communities at the sites under the impact of a landfill. This led us to develop an effective diagnostic protocol and check its effectiveness by application to a real case: the Getafe landfill (Madrid), a city located in the Spain central region. Although the diagnostic methodology used is consistent with theoretical foundations established in previous works (Ramade, 1995; Landis and Yu, 1999; Rapport et al., 2003), no similar ecotoxicological studies on closed solid waste landfills appear to have been conducted to date. Thus, we expect to show a conceptual and methodological framework which allows to validate a protocol for ecological diagnosis in these environments.

2. Materials and methods

Regarding the methodology, we have performed a revision and systematization of several works from our research group, related to sealed landfills in order establishes a protocol which allows its application in landfills. Below, we provide the methods and techniques used for a landfill in the region of Madrid.

2.1. Sampling and analyses

Soil samples were randomly collected from 8 zones in the landfill and associated dumps; all sampled zones previously exhibited soil spots suggesting alterations in the sealing edaphic material. Samples were collected with a hoe and a small shovel to obtain an average sample of topsoil (±2 kg) in each zone that was subsequently used for physico-chemical (Hernández and Pastor, 1989; Pastor and Hernández, 1989) and analyses including enzyme profiles (García et al., 2003). Total hydrocarbons were also determinated.

Plant specimens from the landfill and water samples from the main discharge zone for the wetland were also collected. The water samples were obtained at randomly distributed points across the wetland and stored in opaque glass bottles for determination of organic and inorganic compounds.

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0301-4797/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jenvman.2010.10.052

2.2. Assays under controlled conditions

The most heavily metal polluted soils in central Spain are located in areas occupied by old urban and industrial waste landfills or abandoned mine lands. Zinc is the most widely dispersed pollutant in them, where it can reach phytotoxic concentrations. In this work, we conducted a bioassay under controlled conditions involving the use of pots containing soils polluted with variable amounts of Zn in the form of $ZnCl_2$ in order to assess the eco-chemical action of a plant population of *Bromus rubens* L. growing profusely at the studied landfill site. The pot soil, a calcic luvisol, was kept under greenhouse conditions over a period of 18 weeks. The characteristics of this soil were similar to those of the landfill soil and included an alkaline pH (7.6) and the following contents: 0.8% OM; 0.063% total N; 14.2, 327, 8.7 and 1.4 mg K, Ca, Mg and Na, respectively, per 100 g soil; and 67 mg Zn/kg soil. Each pot was filled with 1 kg of previously sieved soil in triplicate for each Zn concentration used (100, 300 and 500 mg/kg/pot). A further 3 replicate plots were sown with 8 seeds from a plant specimen collected in the studied area. The seeds had previously been germinated in a growth chamber in the dark at 24 °C. The results of the treatments were compared by ANOVA, using LSD methodology as a post-hoc test to identify differences between means.

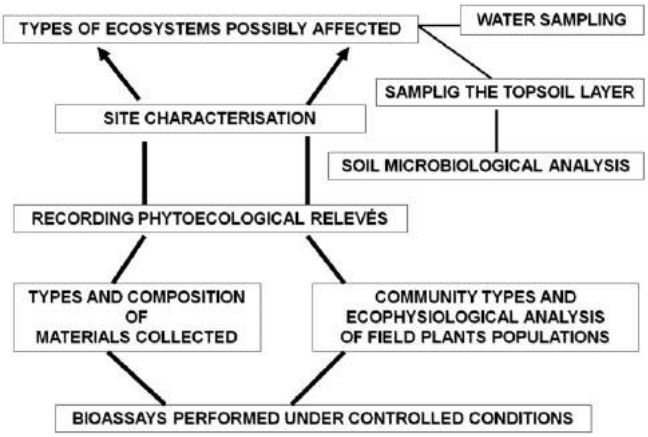

Fig. 2. Protocol of study.

3. Results and discussion

3.1. Conceptual framework: principal vectors of landfill pollution

We initially examined the vectors through which pollutants in a sealed landfill may flow or be transferred on a spatial and temporal scale (Fig. 1). We excluded the potential atmospheric impact of gaseous emissions and restricted the study to those vectors where the sealing edaphic material was the main component for ecotoxicological assessment of a given site. As far as the edaphic vector is concerned, the nature and depth of the sealing cover of a landfill is related to various soil physical properties hindering pollution (Hernández et al., 1998a) and often making landfill slopes unsuitable for development of the ecological succession in a landfill system. The loss and retention of chemical elements by the soil cover is related to pollution in the landfill system. The transfer of landfill pollutants to the autotrophic component causes remote ecosystems in the environment to be polluted.

One of the most immediate requirements was to identify the directions of underground water flows and their relationship to surface waters in the environment (Adarve et al., 1994). This factor is also associated to local or diffuse pollution of aquatic ecosystems,

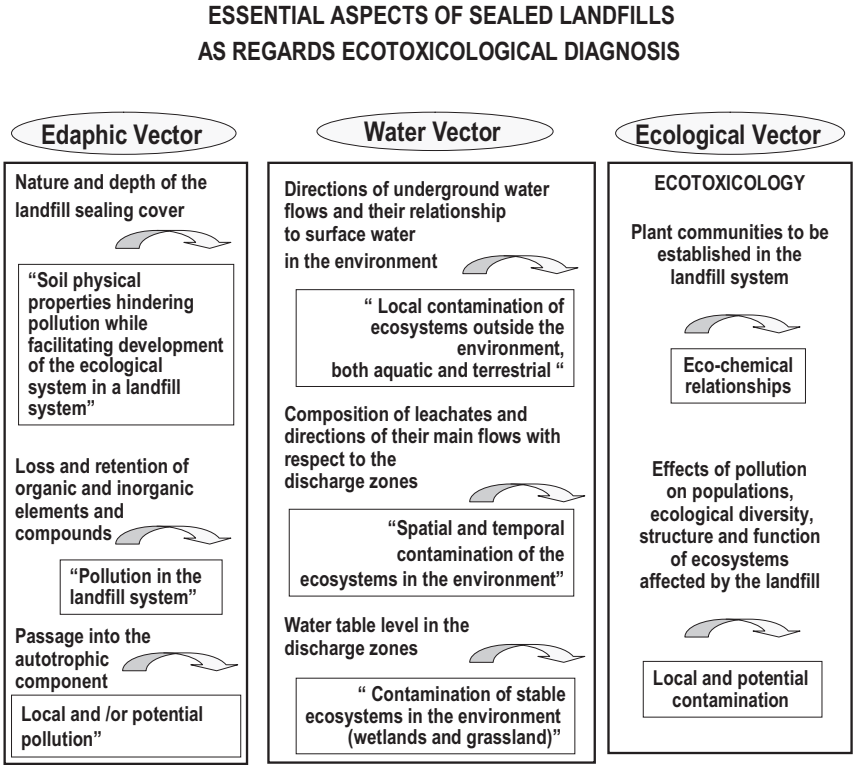

Fig. 1. (Essential aspect of sealed landfills as regards ecotoxicological diagnosis).

Table 1
pH, electrical conductivity (dS/m) and chemical composition (mg/l) of the wetland water in a very rainy spring.

No.	pH	F	Cl	$N0_3$	SO_4	Ca	K	Mg	Na	Fe	Cu	B	EC
1	7.6	n.d.	201	8.4	1683	509	583	143	120	0.124	0.020	0.38	0.322
2	8.1	3,8	177	28.5	2730	622	268	355	114	0.068	0.014	0.32	0.411
3	8.0	n.d.	90	32.8	2126	668	82	264	77	0.052	0.016	0.38	0.369

No detectable amounts of phosphates or nitrates were found.

surface water courses and terrestrial ecosystems, and also to the effect of pollution on animal populations with a potentially impact on the ecosystems structure. One other factor to be considered was the composition of leachates (Adarve et al., 1998) and the directions of their main flows in relation to the discharge zones; in fact, this component is associated to spatial and temporal pollution of ecosystems in the landfill environment. Water table levels in discharge zones play a role in the pollution of stable ecosystems in the environment (Hernández et al., 1998a; Pastor and Hernández, 2007). This justifies the need for an ecotoxicological study (Pastor et al., 1993a, b; Hernández et al., 1998b; Urcelai et al., 2000), one that should be conducted within the framework of risk analysis methodology.

We focused on the autotrophic component of the affected ecosystem for various reasons, namely: toxicity tests could be useful to examine the physiological and behavioural responses (mortality, injury, metabolic changes) of organisms, and also population (density, risk of extinction), and community-related variables (structure, diversity, biomass, nutrient flow changes). The accumulation of a heavy metal in the above-ground portion of a plant (phytoaccumulation) consumed by herbivores is also detrimental to health as it introduces the pollutant in the trophic network. Root systems may play a role in phytostabilizing heavy metals and preventing their passage into deeper soil layers. Finally, because fine materials in landfills and waste tip slopes may be easily eroded, vegetation can help prevent transfer of topsoil pollutants to other ecosystems (Hernández and Pastor, 2008).

3.2. Ecotoxicological diagnosis protocol

Wastes discharged at landfills first sealed in the late 1980s and subsequently reused for disposal of additional waste are of mixed nature (urban, industrial and inert) and have received no pretreatment. The sealing edaphic cover is never thicker than 40 cm. Tips are usually more 15 m tall and occasionally overlap by effect of the discharge of additional waste on previously sealed landfills.

In addition, many landfills have rather steep slopes (over 40% in some cases). Their characteristics influence not only plant colonization, but also the zones receiving runoff leachates. Even in the presence of a single tip, runoff exhibits a fan-shaped distribution pattern and affects biodiversity in the discharge area to a varying extent. Moreover, soil parameters usually vary widely in each area, even though the sealing soils often come from various substrates (Pastor and Hernández, 2002a).

Our recent assessment of the situation led us to conclude that the events and/or uses which have uninterruptedly followed the initial sealing of the landfills, and the characteristics of each landfill, have individual connotations that justify use of a special protocol (Fig. 2). In landfills capped with soils from their respective surroundings, the two processes involved in the ecological succession intermix. Thus, there can be a primary succession by effect of a new community starting in these recently arising systems; also, however, a secondary succession may arise through germination of the seed bank present in the soil used as cover. The two major points here are the need to arrest erosion and its effects (e.g. silting of water courses, eutrophication, and pollution of surface waters) and then ensure appropriate surveillance in order to avoid constant reuse of a sealed landfill for waste disposal, which can make any restoration efforts futile. In previous work, floristic relevés were recorded in twenty capped landfills lying on various types of substrates (granite and gneiss, arkoses, gypsum, limestone and marl) in central Spain.

Obvious differences in relation to diversity of plant species (all herbaceous) were observed between landfill soil covers and the corresponding reference ecosystems. An initial response to eco-chemical relationships was associated to the salinity of the landfills. These findings led us to examine the potential connection between salinity and pasture species in order to understand the ecological behaviour of the plants and apply this knowledge to our restoration strategies (Adarve et al., 1998; Hernández et al., 1998b; Pastor and Hernández, 2002a; 2002b). Since we examined behavioural patterns at both species and community level under control conditions, we also conducted supplemental tests under field conditions. Such tests revealed that the plants responded to some physical abiotic (slope gradient and orientation) and biotic factors (species growth habit in relation to horizontal soil cover, rooting type in shallow soils and seed production).

3.3. Application of the proposed protocol to the Getafe landfill (Madrid)

3.3.1. Description of the landfill and its discharge areas

After its initial sealing, in 1986 the landfill was in theory used to dump inert waste; in fact, discharges included industrial and organic waste. In 1993/94, it was reused to dump industrial waste (particularly steel slag). At present, it is used as a mixed landfill consisting of superimposed tips which have been expanded with two large dumps. The landfills contain a large number of rabbit dens. After the first sealing, the main discharge area was sown with

Table 2
Grain size distribution (%) of wetland soil at three randomly chosen points in late spring (once virtually all surface water had evaporated). Binding coefficients and pF values. SCCC = Soil Clay Compaction Coefficient; SIC = Silt Impermeabilization Coefficient.

Sand	Silt	Clay	SCCC	SIC	SCCC + SIC	Field capacity	Wilting point	Available water
35	62	3	0.03	0.61	0.64	28.3	17	11.3
36	57	7	0.07	0.55	0.62	44.2	25.7	18.5
40	57	3	0.03	0.55	0.58	31.9	17	14.9

Table 3
Comparison of soil parameters (means) between the landfill wetland soil and similar grassland in the environment not affected by the landfill.

Soil parameters	Landfills (USW)	Reference ecosystems
pH	7.3	7.2
Conductivity (μS/cm)	8065	2960
Chloride (mg/kg)	1775	70
Sulphate (mg/kg)	5210	3450
Nitrate (mg/kg)	91	25
Total N (%)	0.450	0.200

Table 4
pH, OM and N (%), available elements (mg/100 g), anions (mg/kg); and electrical conductivity (EC, dS/m) of the soils in the landfill tips.

Soil	pH	OM	N	P_2O_5	Ca	Mg	K	Na	F	Cl	NO_2	NO_3	PO_4	SO_4	EC
G-1	7.7	4.2	0.195	12	2414	184	21	94	8.6	7570	0.0	495	0.0	5918	0.822
G-2	7.7	2.0	0.110	16	915	20	30	5.4	2.9	199	2.3	29	0.0	3830	0.235
G-3	7.7	6.2	0.285	14	522	51	72	17	3.8	135	2.9	93	2.0	1938	0.149
G-4	7.8	7.8	0.365	22	603	58	58	8.5	1.6	77	1.5	0.0	0.0	3322	0.196
G-5	7.9	0.9	0.060	14	212	41	46	6.0	3.2	33	1.2	28	0.0	96	0.027
G-6	7.6	2.1	0.090	20	274	52	48	3.7	1.6	30	1.5	110	5.4	67	0.034
G-7	7.5	3.8	0.130	105	246	18	41	4.2	3.7	125	1.9	148	2.9	1647	0.149
G-8	7.8	3.8	0.100	7	216	29	29	5.7	2.9	43	1.0	34	0.0	1729	0.150

grain. The wetland is used for drinking by sheep herds grazing throughout the site and also by some bird populations. In addition, the area abounds with shells of a gastropod mollusc of the genus *Helicilla*. The site includes another area under a lesser impact that is sown with wheat.

3.3.2. Composition of the wetland water and soil slopes

The nature of the substrate (calcareous and gypsiferous marls) and the precipitation regime are the two factors most strongly influencing the composition of water in the studied wetland.

Table 1 shows the data for a highly rainy spring (2008). Another spring much drier on 2006 (unpublished data) the average chloride and sulphate concentrations were 770 and 8341 mg/l, respectively. No detectable amounts of phosphates or nitrites were found, and no appreciable levels of Zn, Cd, Pb or Ni in surface water were detected; by exception, Cu was found at 0.023 mg/l. However, as can be seen from Table 1, the metal contents of the water varied depending on the particular sampling location at the foot of the different tips. The properties of soil in a dry wetland are obviously dependent on its nearness to the water table.

Table 2 illustrates the capacity of the soil to retain moisture as measured via field capacity, wilting point and available water. In addition, it gives the cementation and impermeabilization ability of the soil associated to the clay and silt, respectively, it contains. Both factors are crucial with a view to assessing the potential impact of some chemical elements and compounds on underground water in the environment of a landfill. Soil salinity in the studied wetland was already high in the determinations performed twenty years ago (Table 3).

Tables 4 and 5 show other soil data, as the anions, of ecotoxicological interest. Chemical analyses (Tables 5 and 6) revealed the presence of heavy metals and non-agricultural organic compounds. Worth special note are the aliphatic and aromatic hydrocarbons detected in 4 of the 8 soil samples, which were present at very high levels in some. Also, some insecticides such as γ-HCH (lindane) (non published data) were found at detrimental levels for terrestrial organisms in soil. Their potential toxicity is closely related to their biodegradability. We conducted preliminary microbiological assays in order to evaluate the activity of enzymes involved in the C, N and P cycles (Table 7). The results obtained for the enzyme patterns revealed marked differences in enzyme activity between zones. The highest activities determined were those for acid phosphatase, alkaline phosphatase and β-glucosidase in sample G-4.

3.3.3. Plant communities

The studied wetland, overlying marl and gypsum materials where the presence of a water table or precipitation and the discharge of leachates released from the landfill tips and dumps have led to highly saline soils, now bears communities of *Juncus acutus* in addition to *Spergularia media*, *Sonchus crassifolius*, *Plantago maritima, Sphenopus divaricatus*, *Parapholis incurva*, *Galium parisiense* and *Minuartia hybrida*, all typical of saline soil lacking an upper organic horizon. In trough zones of the landfill, these communities are accompanied by pasture species such as clovers (*Trifolium scabrum, T. pratense*) in addition to *Medicago rigidula* and grasses (*Poa bulbosa* and *Bromus hordaceus*), which make them palatable to sheep and are in fact grazed despite their dubious quality a result of the polluted condition of the soil.

The tips are covered by a plant tapestry that differs between sunny and shady zones. Thus, the sunny zones are covered by an annual community that is virtually single-stratum and consists of short plants on the top layer of the landfill sealing soils; the community is composed largely of *Calendula arvensis* accompanied by *Bromus rubens*, *Centaurea melitensis*, *Malva parviflora, M. pusilla, Eruca sativa* and *Herniaria hirsuta*, among others. *Cardus bourgeanus*, *C. tenuiflorus* and *Sylibum marianum* form small spots in the bottom of the small gullies left by stream water from precipitations. These spots are easy to distinguish visually by their increased density and cover virtually all north-oriented tips; however, they are unpalatable to the ovine livestock.

The plant communities on the tips and dumps have evolved little in recent years, where they have been dominated by pioneer species with modest soil requirements. Obviously, these plant covers have helped stabilize the landfill sealing by forming thin humus layers; however, the methanogenic processes associated to the successive discharges have prevented germination and rooting of more demanding species. One other major factor for these covers is the high density of rabbits that feed on their roots. Because the landfill site is used for two primary purposes [viz. ovine grazing and gaming (rabbit)], a need exists to consider more frequent plant species which, by growing on

Table 5
Metal contents (mg/kg) of the soils on the landfill.

Soil	Fe	Mn	Zn	Cu	Pb	Ni	Cr
G-1	4775	205	238.1	18.4	56.9	4.6	2.9
G-2	18186	339	73.9	13.5	16.9	6.4	1.5
G-3	21604	306	527.7	36.5	117.0	8.6	6.6
G-4	21770	312	365.9	34.9	64.5	7.3	5.7
G-5	17963	328	61.1	9.5	18.4	5.1	2.3
G-6	23995	224	69.2	31.0	8.2	5.2	9.2
G-7	285480	1448	576.9	881.7	148.8	155.7	110.2
G-8	417320	1842	476.8	1260.0	139.4	212.7	152.7

Table 6
Total, aliphatic and aromatic hydrocarbon contents (mg/kg) of the soils in the Getafe landfill in the spring of 2006.

Site	Concentration		Aromatic/Aliphatic ratio	Total concentration
	Aromatic	Aliphatic		
Getafe 2	0.0	7.487	0.0	7.487
Getafe 4	0.0	5.129	0.0	5.129
Getafe 6	5.116	8.092	0.632	13.208
Getafe 8	0.0	854.414	0.0	854.414
Reference level				>50 mg/kg

Table 7
Enzyme activities as determined in a control soil and at four different points in the Getafe landfill.

Soil	Acid phosphatase	Alkaline phosphatase	β-Glucosidase	Invertase	Cellulase	ß-*N*-acetyl-glucosaminidase
Control	1.11	0.29	1.09	16.21	0.092	0.28
G-2	0.37	1.37	0.46	20.25	0.05	0.20
G-4	1.71	3.88	3.90	42.97	0.03	0.17
G-6	0.54	3.34	2.61	55.69	0.05	0.31
G-8	0.54	2.26	1.13	17.45	0.08	0.11

The phosphatase, β-glucosidase and β-*N*-acetyl-glucosaminidase activities are given in μmol *p*nitrophenol/gh, and the invertase and cellulase activities in μmol glucose/h.

Table 8
Average micronutrient levels (mg/kg) in the aerial portion of *Bromus rubens* plants as a function of the Zn content of the soil (mg/kg).

Soil Zn	Zn	Cu	Fe	Mn
Control	27.7 ± 2.6 a	7.7 ± 1.7 a	582.0 ± 207.4 a	288.3 ± 28.4 a
100	169.0 ± 20.6 b	9.3 ± 1.2 ab	420.7 ± 37.6 a	268.0 ± 68.2 a
300	330.0 ± 55.2 c	10.7 ± 0.9 b	1428.3 ± 1387.6 a	277.0 ± 30.0 a
500	506 ± 63.9 d	11.3 ± 0.5 b	627.0 ± 157.9 a	273.3 ± 36.2 a

Treatments sharing the same letter were not significantly different at $p = 0.05$.

polluted soils, can facilitate the passage of pollutants into other elements of the trophic network.

This entails not only analysing field material, but also conducting appropriate bioassays with variable concentrations of metals in the form of soluble salts applied to the soil and sowing various autochthonous species collected in the field. To this end, we chose *Bromus rubens*, which was one of the most abundant species among the grazed wetland communities and also on the tips and plateaux of the landfill and dumps.

3.4. Bioassay with Bromus rubens L.

Table 8 shows the Zn, Cu, Fe and Mn uptake by this species as obtained in the bioassay. The increase in soil Zn was found to have a favourable effect on Cu. Also, the increased Zn levels were correlated with decreased accumulation of Mn in plants such as corn. The accumulation of Zn at high levels in the above-ground portion of red brome can have toxic effects on grazing animals. Zinc tissue levels above 300 mg/kg are usually toxic (Seaker, 1991; Shuman et al., 2001). The Zn levels found in this species exceeded those previously reported for other Poaceae (Dudka et al., 1996).

Once should bear in mind that this species can grow in abandoned landfills and exhibit Zn levels similar to those found here; therefore, it may be accumulating similar of even greater amounts of Zn than those measured and eventually reach consumers through the trophic chain.

4. Conclusions

Assessing the environmental impact of a soil-topped landfill requires an accurate ecotoxicological diagnosis. For this purpose, this paper describes a diagnostic protocol and its application to a real case: the urban solid waste. The procedure proposed here overcomes this problem by assessing the situation in edaphic, aquatic and ecological terms.

Acknowledgements

To Spain's Ministry of Education and Science (Project CTM 2005-02165), Ministry of Innovation and Science (Project CTM2008-04827) and Madrid's Regional Government EIADES (Programme S-0505/AMB/0296).

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Journal of Environmental Management 95 (2012) S55–S60

Contents lists available at SciVerse ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

BTEX decomposition by ozone in gaseous phase

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ARTICLE INFO

Article history:
Received 30 September 2009
Received in revised form
27 June 2011
Accepted 13 September 2011
Available online 24 October 2011

Keywords:
Ozone
Volatile organic compounds
BTEX's gaseous phase ozonation

ABSTRACT

Environment management is turning its efforts to control the air pollution. Nowadays, gas phase contaminants coming from different sources are becoming into the main cause of serious human illness. Particularly, benzene, toluene, ethylbenzene and xylene (BTEX) are getting more and more attention from the scientific community due the high level of volatilization showed by these compounds and their toxicity. Decomposition of these compounds using different treatments is requiring lots of new strategies based on novel options. In the present work the use of ozone was proposed as possible alternative treatment in the gaseous phase of VOC's liberated from water by stripping. This study deals with the decomposition by ozone in gaseous phase of model mixtures of BTEX stripped from water. The experiments were realized in a tubular reactor with fixed length (1.5 m length and diameter of 2.5 cm). The experiments were conducted in two stages: in the first one, organics was ventilated by oxygen flow to liberate BTEX to the gaseous phase; second stage deals with the liberated BTEX decomposition by ozone in the tubular reactor. Ozonation efficiency was determined measuring the VOC's concentration at the output of the tubular reactor. This concentration was compared to the concentration obtained at the input of the reactor. The obtained results confirm the possibility to use of ozone for the VOC's decomposition in gaseous phase. Also, the dynamic relationship between degradation and liberation was studied and characterized.

1. Introduction

Air emissions coming from a variety of industries contain big amounts of VOC's and have been subject to increasingly stringent environmental regulations over last two decades (Park et al., 2008). For example, benzene, toluene, ethylbenzene, xylene (BTEX) and methyl-tert-butyl-ether (MTBE) are considered as predominant pollutants in areas near to large cities or industrial zones (EPA, 1992). Many VOC's are emitted to the environment from an extensive variety of sources including combustion products of wood and fuels, industrial production, adhesives preparation, use of degreasing agents and aerosols (Shih and Li, 2008). The presence of VOC's in atmosphere causes severe health problems (United States Solid waste and EPA 542-R-98-008, 1998). Even when not all VOC's pose a direct risk to human health, many of them can participate in photochemical smog cycle that is also a source of health danger. Besides, human exposition to VOC's occurs by ingestion (consuming contaminated water or food), inhalation or absorption through the skin, etc. (Sarafraz-Yazdi et al., 2009). The main problem with this kind of organic compounds is that their degradation products are known or suspected carcinogens. Following the previous ideas, effective treatments developments to remove these organics are necessary (Jia et al., 2008b).

A variety of air pollution control (APC) technologies, including adsorption, absorption, thermal oxidation, catalytic oxidation, and chemical scrubbing, are technically capable of recovering or destroying VOC's from industrial emissions (Beltrán et al., 2002). However, applications of these conventional APC technologies generally have disadvantages in treatment of dilute industrial VOC emissions. A number of methods have been developed to remove VOC's in the gaseous phase. Among them, the main methods are bio-filtration, incineration, the stripping, scrubbing and physical adsorption (Reza Iranpour et al., 2005; Jia et al., 2008a; Mascolo et al., 2008; Devinny et al., 1999; Saravanan and Rajamohan, 2009). All methods cited before have advantages and disadvantages that serve for adequate selection of method treatment of VOC's remediation. One common method of the VOC's emissions control is their adsorption in activated carbon (Shih and Li, 2008). This method has high removal efficiency and low cost, however, it requires disposal or a secondary treatment process to decompose the adsorbed VOC's (Ambrożek, 2008). Biological filtration (or bio-filtration) is a technology based on the biological oxidation of VOC's using microorganisms, but this method has been widely used when

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doi:10.1016/j.jenvman.2011.09.009

the VOC's concentration in air is relatively low (0.6–2.85 ppm). In addition, this method can also be limited by the biological reaction rate, that is, in the case of poorly biodegradable and/or toxic pollutants. Interestingly, some poorly biodegradable VOC's such as MTBE require a long start-up phase (months rather than days) before significant removal was observed (Reza Iranpour et al., 2005; Zhangli et al., 2007).

Additionally, there exists the problem of the treatment of ground and residual water as well as contaminated soil with high content of volatile organic compounds (VOC's), because of their stripping to atmosphere (Liberation of VOCs from the aqueous phase to the gaseous phase using the gas inflow as carrier) surrounding the contaminated water and soil. Indeed, some treatments have been proposed to recover the stripped contaminants with different technologies (Ambrożek, 2008; Budzianowski and Miller, 2008; Budzianowski and Koziol, 2005).

This stripping process can provoke air contamination in the case of incomplete decomposition of VOC's in the liquid and solid phases. Indeed, when the water is extracted from underground (to develop any possible on-site treatment), there is a possibility to lose VOC's (until 99.9% for low-weight volatile compounds) into the atmosphere without any previous treatment (Shah et al., 1995). These both aspects can serve as a justification to treat the dissolved VOC's in liquid phase and to treat the stripped compounds in gaseous phase. In the last case, the chemical oxidation is an alternative method to decompose hazardous contaminants to less toxic compounds (Beltrán et al., 1999). One of the important powerful oxidative agents is the ozone. Ozone treatment for contaminated water (Beltrán, 2004) and soil (Rivas, 2006; Rivas et al., 2009; Poznyak et al., 2007) has been deeply studied. Nevertheless, the ozone application on groundwater remediation still has similar problems about stripping. One possible option is the gasesous phase reaction between ozone and volatile organics (Razumovskii and Zaikov, 1984).

In the present study, the decomposition of the model VOC's (BTEX), dissolved in water and then stripped from it, by ozone in the gaseous phase was realized. The treatment was performed using a tubular reactor with fixed length (1.5 m length and diameter of 2.5 cm), at different gas flow and reagents concentration. The BTEX decomposition efficiency in ozonation was determined using the relationship of compounds concentration in the input and the output of tubular reactor that was measured by liquid chromatography (HPLC) (Bin and Roustand, 2000).

2. Materials and methods

2.1. Ozonation procedure

The ozone procedure was conducted in three sequential stages. In the *first stage*, the aqueous solution containing the dissolved VOC's was injected with oxygen. This procedure provokes the BTEX stripping from liquid phase enclosed in a continuous reactor (500 mL), which has a diffuser plate at its bottom for feeding the oxygen. Adsorption process was performed using an initial concentration of 200 mg L^{-1} for benzene, toluene and xylene while ethylbenzene was 150 mg L^{-1}. The treatment process was initialized with the stripping process that was performed with an input flow of 0.5 and 0.2 L min^{-1}. The volume of aqueous solution was 250 mL for all samples. The liberated BTEX were adsorbed in granular activated carbon (GAC) (1.0 g). The activated carbon was settled in a bed. This bed was prepared with 10 g of GAC in a small tube with 1 cm diameter. To determine the stripping dynamics, water samples were analyzed at different times (2, 5, 10, 15, 20, 30 until 60 min).

On the *second stage*, the stripped BTEX pass through a tubular reactor where the BTEX's ozonation is carried out in gaseous phase. The ozonation in a tubular reactor with fixed length (1.5 m length and diameter of 2.5 cm) was realized with the ozone concentration (30 and 15 mg L^{-1}) and the variation of gas flow (0.2 L min^{-1} and 0.5 L min^{-1}). The residual ozone, byproducts and final products of ozonation in GAC were adsorbed.

In the third stage, BTEX and products formed during ozonation were adsorbed into a bed of GAC located at the reactor output. The Fig. 1 shows the experimental set-up (Pedro et al., 2008), where the ozone generator "AZCO" with high-voltage was employed at the gas flow of 0.2–0.5 L min^{-1}. All experiments were carried at the ambient temperature. The measurements of ozone in gaseous phase at the reactor input was done with an ozone sensor model BMT 930, connected to a PC (using an acquisition data board NI-6024), to take the input concentration of ozone.

2.2. Analytical methods

Intermediates and final products obtained in ozonation in the gaseous phase were adsorbed on activated carbon. The solid samples of activated carbon were analyzed using the Soxlhet technique (Wang et al., 2006): the adsorbed compounds were extracted in

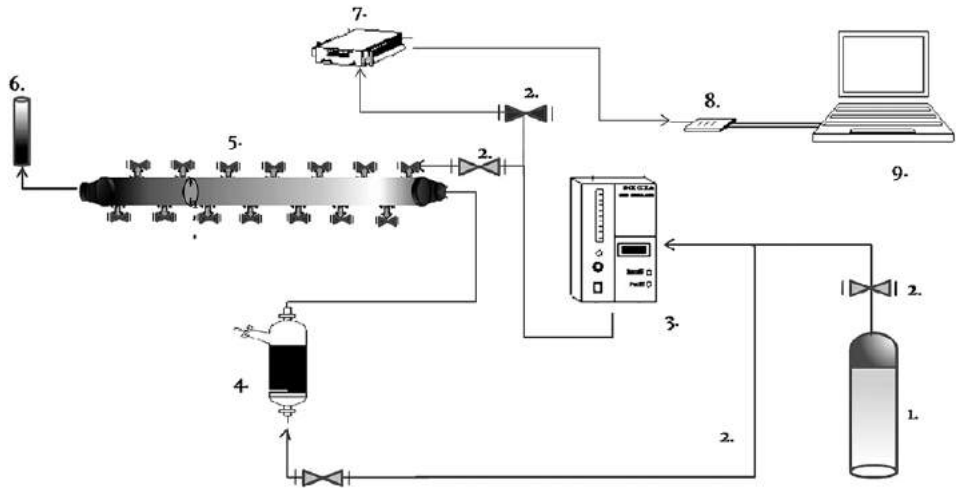

Fig. 1. Schematic diagram of the experimentation at laboratory scale: (1) oxygen tank, (2) valve step, (3) ozone generator, (4) reactor with model solution, (5) tubular gaseous phase reactor (6) activated carbon, (7) ozone analyzer, (8) data acquisition board, (9) PC.

methanol (30 mL per 0.5 g of GAC). Extracted samples were analyzed using the high performance liquid chromatography (HPCL). HPLC analysis was carried out by a liquid chromatograph Perkin–Elmer series 40 coupled with the UV/VIS detector and a chromatographic column Nova Pack C-18, 250 × 4.6 mm. BTEX analysis was performed using a mobile phase of water–acetonitrile (30.0:70.0) with the flow 0.5 mL min^{-1}. The injected sample volume was 30 μL and the used wavelength for benzene 264 nm, toluene 250 nm, ethylbenzene 262 nm and xylene 249 nm. Organic acids were determined using the same equipment but using 211 nm with the same sample volume. The mobile phase was not modified. Therefore, no acidification was needed to obtain the organic acids concentration.

3. Results and discussion

Results will be presented in two subsections: *First* one describes stripping of VOC's and their adsorption into activated carbon; *second* one describes the degradation of organics in the gaseous phase by ozone at different operating conditions to evaluate the BTEX decomposition degree.

3.1. BTEX stripping and adsorption in GAC

Fig. 2(a–e) displays the BTEX stripping and adsorption dynamics for benzene (Fig. 2b) at two gas flow. These adsorption dynamics are quiet similar to those obtained for toluene, ethylbenzene and xylene. So, as it can be observed, for the oxygen flow of 0.5 L min^{-1}, toluene and ethylbenzene were stripped before 10 min and for benzene and xylene were 15 and 30 min, respectively. The decrease of the oxygen flow to 0.2 L min^{-1} increases the stripping time for all hydrocarbons to 20–40 min. The stripping rate of the VOC depends on many factors related to the operating conditions, as well as the properties of the organics like vapor pressures and the water solubility. The mass transferring between both phases,

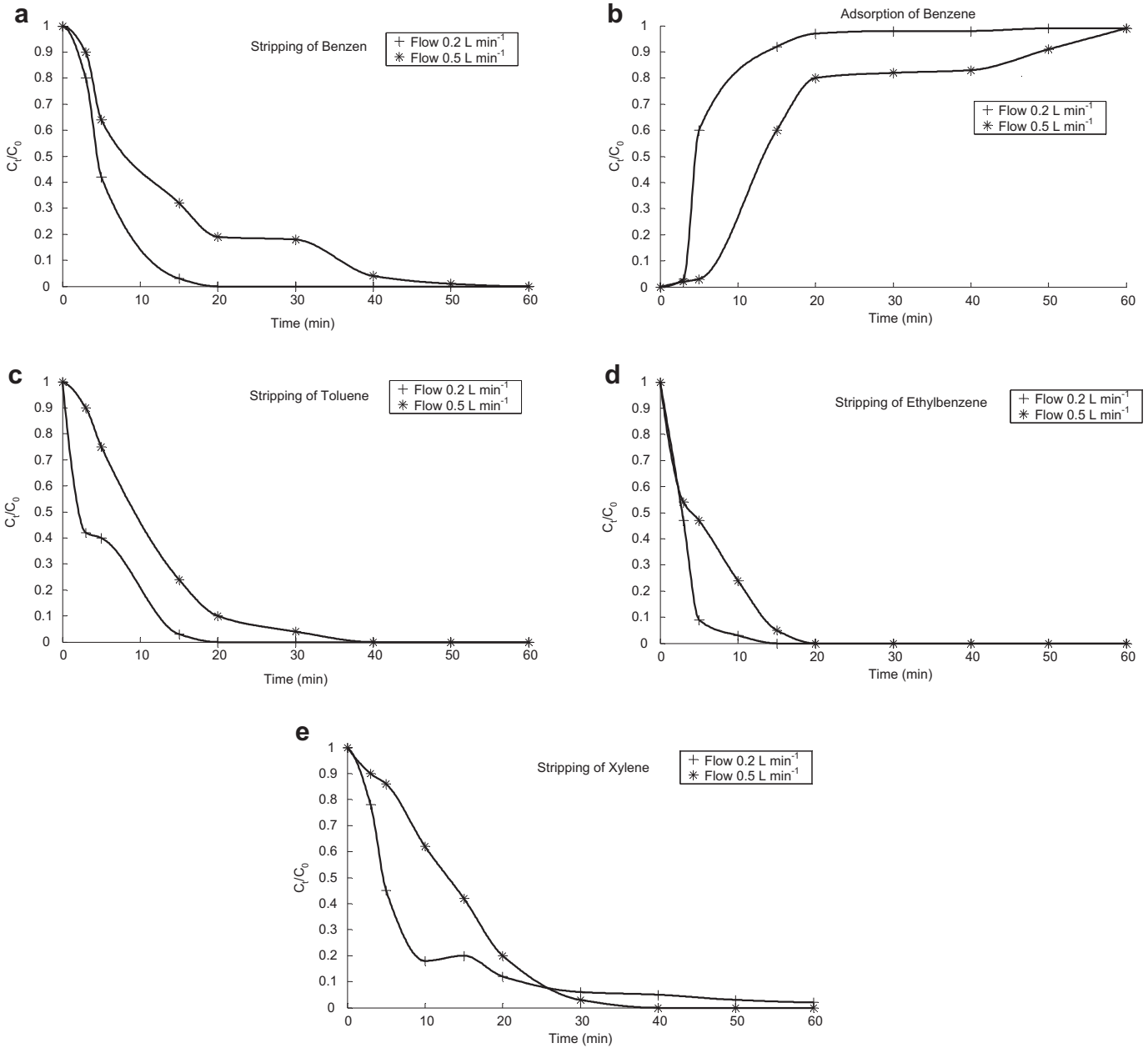

Fig. 2. Stripping dynamics of benzene (a), toluene (c), ethylbenzene (d) and xylene (e), and adsorption dynamics of benzene (b) at the gas flow of 0.2 and 0.5 L/min.

Table 1
Henry's Law coefficient, liberated constants of BTEX at two different flows (k, min^{-1}).

Compound	Vapor pressure, kPa at 20 °C	Henry's Law coefficient (atm m^3/mol)	k, min^{-1}	
			0.2 L min^{-1}	0.5 L min^{-1}
Benzene	10.0	5.49×10^{-3}	6.2×10^{-2}	8.7×10^{-2}
Toluene	2.9	6.44×10^{-3}	5.1×10^{-2}	8.8×10^{-2}
Ethybenzene	0.9	8.43×10^{-3}	5.7×10^{-2}	21.1×10^{-2}
Xylene	0.8		3.9×10^{-2}	5.4×10^{-2}

liquid and gaseous, may be characterized by the Henry's coefficient. In order to characterize the dependence of the BTEX stripping on their properties were calculated the rate constants of their liberalization for the two gas flows. In Table 1 these values were presented. As it can be observed, the values of the stripping constants calculated are not correlated with the values of the Henry's Law coefficient and the vapor pressure. In the base of this, we may conclude that the Henry's Law coefficient is a better predictor of the stripping behavior, which in turn coincides with the observations of other investigators (Deshusses and Johnson, 2000). In addition, the stripping time of VOC's is the same as the adsorption time.

3.2. Decomposition of BTEX

The suggested experimental scheme promotes a dilution of the ozone concentration and the stripped contaminant in the tubular reactor by oxygen. According to the obtained results, the BTEX degradation starts just after these organics were released along the reactor for all BTEX. However, as one can suppose, there is a remarkable difference between the reaction rates of the organics with ozone. So, xylene is decomposed faster (<2 min of ozonation) among the other BTEX. On the other hand, benzene cannot be decomposed completely under special reaction conditions (gas flow of 0.5 L min^{-1} and the initial ozone concentration of 15 mg L^{-1}). In the Fig. 3a–d the decomposition dynamics of studied BTEX under different operating conditions was presented. As it can be seen, benzene was decomposed under the gas flow of 0.2 L min^{-1} during 15 and 30 min, under 35 and 15 mg L^{-1}, respectively (Fig. 3a). In the case of toluene ozonation under the gas flow of 0.2 L min^{-1} and the ozone concentration of 35 mg L^{-1}, this is decomposed in 10 min. For all cases, ozone has an efficiency of 100% to decompose the toluene (Fig. 3b). Ethylbenzene was not completely decomposed under the tested operating condition (gas flow of 0.5 L min^{-1} and ozone concentration of 15 mg L^{-1}). There is a remaining of about 30%. Nevertheless, if the ozone concentration is increased (35 mg L^{-1}) and the gas flow is decreased (0.2 L min^{-1}), this organics is completely decomposed during 10 min. This fact is very important considering that among others BTEX, the ethylbenzene is stripped faster (during 10 and 15 min) (Fig. 3c).

The ratio Ct/Ci showed in Fig. 3 is different to that depicted in Fig. 2. This ratio was obtained using the same extraction method presented in the previous section. This relationship is valid considering that both concentrations were measured in the activated carbon. Initial concentration was obtained using a pure strpped process based on oxygen flow. The information presented in the figure corresponds to different experiments where the oxidation based on ozone was applied on renewed activated carbon samples.

Ozone was extremely effective to decompose xylene (Fig. 3d). If the ozone concentration is low (15 mg L^{-1}) and the higher gas flow was applied (0.5 L min^{-1}), the xylene is completely decomposed.

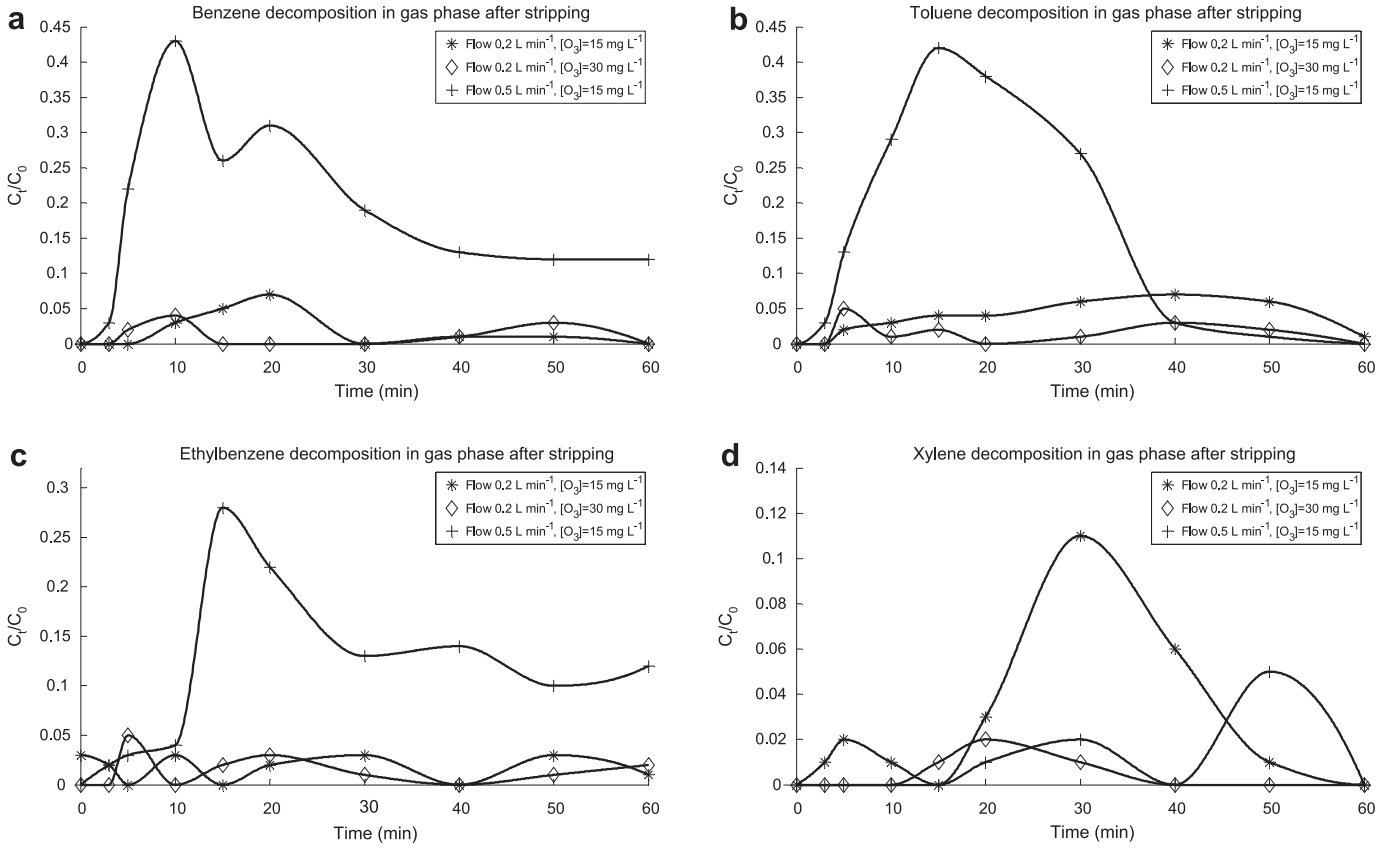

Fig. 3. Decomposition dynamics of: benzene (a), toluene (b), ethylbenzene (c) and xylene (d).

Table 2
Intermediates and final products formatted in the BTEX ozonation under different operating conditions (in the methanol extract/in the gaseous phase of the tubular reactor).

BTEX	Products	Concentration, mg L^{-1} extract in methanol/gaseous phase		
		0.5 L min^{-1} $[O_3]$ = 15 mg L^{-1}	0.2 L min^{-1} $[O_3]$ = 15 mg L^{-1}	0.2 L min^{-1} $[O_3]$ = 35 mg L^{-1}
Benzene	Benzoic acid	3.7/0.3	1.0/0.1	1.6/0.1
	Oxalic acid	4.0/0.3	6.4/0.5	9.0/0.7
	Formic acid	146.0/11.0	0.3/0.03	36.0/3.0
Toluene	Benzoic acid	1.0/0.1	ND	6.4/0.5
	Oxalic acid	97.0/8.0	12.0/0.9	ND
	Malonic acid	ND	28.0/2.0	ND
	Fumaric acid	ND	0.4/0.03	ND
	Formic acid	ND	1.9/0.2	ND
	Benzene	17.0/1.4	ND	5.0/0.4
Ethylbenzene	Benzoic acid	119.0/9.0	35.0/3.0	3.0/0.25
	Malonic acid	8.0/0.7	3.0/0.3	ND
Xylene	Oxalic acid	0.5/0.04	4.0/0.3	ND
	Malonic acid	188.0/15.0	44.0/3.5	19.0/1.5
	Formic acid	2.5/0.2	1.3/0.1	ND
	Benzene	ND	0.5/0.04	ND

ND = not detected.

This result corresponds with some results reported recently in studies describing the xylene ozonation (Kasprzyk-Hordern et al., 2005).

Concentrations showed in Figs. 2 and 3 are measured in the activated carbon. These values were obtained by the extraction method described in section 2. This procedure was designed to avoid the utilization of either on-line mass or liquid chromatography. Therefore, even when the contaminant concentration in the gas phase has been reduced to zero, the activated carbon still has a representative amount of such compounds (as it showed in the figure). This is an experimental restriction to describe the gas phase contaminant decomposition. However, results obtained by the method proposed in this paper seem to be representative of gas phase decomposition dynamics. Considering the activated carbon renovation after each experiment and the extraction procedure (that was developed just after each experiment was finished), one may claim that gas phase transient dynamics is close to that depicted in Fig. 2. On the other hand, the steady state concentration of each compound is no longer related to the actual gas phase concentration, because the adsorbed contaminant in the activated carbon cannot be eliminated by simple ozonation.

3.3. Identification of intermediates and final compounds formatted in the BTEX ozonation

In order to identify intermediates and final products of the BTEX decomposition in ozonation, the HPLC analysis at specific conditions (see *2.2*) was realized. In Table 2 are presented all products obtained in the BTEX ozonation with their concentration in the extract in methanol and in the gaseous phase in the tubular reactor. In the base of data presented in this table we may conclude that all intermediates and final products identified are presented in the gaseous phase as trace (<2.0 mg L^{-1}), except formic acid in the benzene decomposition, oxalic acid in the toluene decomposition, benzoic acid in the ethylbenzene decomposition and malonic acid in the xylene decomposition with concentrations in the range of 3.0–15 mg L^{-1}. In the comparison of all experimental conditions, the best results in the efficiency of the degradation of initials VOC's and intermediaries were obtained under a flow of 0.2 L min^{-1} and ozone concentration of 35 mg L^{-1}.

4. Conclusions

Experimental results show that the ozonation in the gaseous phase is effective for the reduction of BTEX's emission. The decomposition dynamics and the decomposition degree depended on the ozonation kinetics and the operating conditions that can be attributed to the chemical structure of studied VOC's. Particularly, under the experimental conditions, the best results about degradation efficiency of initials VOC's and intermediaries were obtained under the flow of 0.2 L min^{-1} and the ozone concentration of 35 mg L^{-1}. The main by-products obtained by the reaction between BTEX and ozone were identified and characterized. The relationship between the stripping and degradation processes is identified and explained by the relationship observed in the corresponding experiments. The study proposed in this paper uses the dynamic relationship between both these procedures.

Acknowledgments

The authors thank the Department of Graduate Study and Investigation of the National Polytechnic Institute of Mexico and the National Council of Science and Technology of Mexico - CONACyT (Project #49367) for the supporting of this investigation.

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Journal of Environmental Management 95 (2012) S61–S65

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Wastewater treatment by batch adsorption method onto micro-particles of dried *Withania frutescens* plant as a new adsorbent

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ARTICLE INFO

Article history:
Received 17 August 2009
Received in revised form
17 March 2011
Accepted 21 June 2011
Available online 30 July 2011

Keywords:
W. frutescens
Adsorption
Metallic elements
Phosphate
Nitrate
Wastewater treatment

ABSTRACT

A new adsorbent for removing metallic elements, nitrate and phosphate ions from municipal and industrial wastewaters has been investigated. This new adsorbent consists of micro-particles of dried *Withania frutescens* plant (<500 μm). Batch experiments were conducted to evaluate the removal of metallic elements and anions from raw wastewaters by *W. frutescens* particles. The results show that the micro-particles of *W. frutescens* plant presented a good adsorption of metallic elements, nitrate and phosphate ions from both real wastewaters. This adsorption increased with increasing of contact time. The percentage of metallic elements removal from industrial wastewater by *W. frutescens* plant was 98 ~ 99% for Pb(II), 92 ~ 93% for Cd(II), 91 ~ 92% for Cu(II) and 92 ~ 93% for Zn(II). The maximum adsorption capacity was dependent on the type of ions. The results also indicate that the values of chemical oxygen demand (COD) decrease after the contact with *W. frutescens* particles. Based on the results it can be concluded that the dried *W. frutescens* plant appears to be an economical and environmentally friendly material for wastewater treatment.

1. Introduction

Municipal and industrial wastewaters frequently contain heavy metals, nitrate and phosphate ions. Heavy metals pollution is spreading throughout the world because of the expansion of industrial activities. The industrial use of metals increases their concentrations in air, water and soil. The trace metals are widely spread in environment and may enter the food chain from the environment. It is well recognized that the presence of heavy metals in the environment can be detrimental to a variety of living species, including human. Unlike organic pollutants, metals are non-biodegradable and because of this the removal of heavy metal ions becomes essential. Also, nitrate and phosphates are commonly found in various raw wastewaters. They can cause serious water pollution and threaten the environment. It is therefore, essential to control and prevent their unsystematic discharge in the environment. For this reason, increased attention is being focussed on the development of technical know how for their removal from nitrate, phosphate and metal bearing effluents before being discharged into water bodies and natural streams.

A number of physico-chemical technologies such as chemical precipitation, coagulation and flocculation, ion exchange and membrane techniques are available for wastewater treatment. These methods often involve high capital and operational costs and may also be associated with the generation of secondary wastes, which present treatment problems. This had as result a need for innovative treatment technologies for anions and metallic elements removal. The adsorption is one of the techniques, which is comparatively more useful and economical at a low pollutant concentration. In recent years, considerable attention has been devoted to the study of different types of low cost materials as adsorbents such as tree bark, wood charcoal, saw dust, alum sludge, red mud, peanut hulls, peat, corncobs, cocoa shells and other waste materials for the adsorption of some toxic substances (Periasamy and Namasivayam, 1995; McKay and Porter, 1997; Reddad et al., 2002; Clave et al., 2004; Meunier et al., 2003; Kadirvelu and Namasivayam, 2001; Cengeloglu et al., 2002). The use of crushed and dried plants in the wastewaters treatment has been studied in recent years and the results of the laboratory investigations showed that dried plants are good adsorbents for the removal of nitrate, phosphate and heavy metals ions from wastewaters (Abdel-Halim et al., 2003; Benhima et al., 2008; Chiban et al., 2005, 2008). The plant selected to be used as an environmentally friendly adsorbent of wastewater treatment was *Withania frutescens* plant from the south–western part of Morocco. It is a shrub with little leathery leaves belonging to the Solanaceae family. The calyx of this plant increases after flowering and forms a small addition that completely hides reddish Bay

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doi:10.1016/j.jenvman.2011.06.044

(Ozenda, 1983). This plant is used against poisoning and skin diseases. It also has hepatoprotector (Montilia et al., 2006) and anti-inflammatory (Ahmad et al., 1990) properties.

For the present work the adsorptive properties of *W. frutescens* plant, which is a low cost adsorbent, have been evaluated for copper, cadmium, lead, zinc, nitrate and phosphate ions adsorption from two real wastewaters, the municipal and industrial ones. The objective of this study is to investigate the efficiency of a new process for wastewater treatment by batch adsorption method onto an abundantly available and a low cost adsorbent.

2. Materials and methods

2.1. Preparation of the natural adsorbent

W. frutescens plant was collected from the semi-arid zones of Morocco (Agadir zone). Then it was air-dried for 2–3 days and grinded. Afterward, *W. frutescens* powder was sieved using 500 μm sieves and used as such without any pre-treatment. The dried plants are used as adsorbents for wastewaters treatment and inorganic pollutants removal such as: cadmium, copper, lead, zinc, nitrate and phosphate, classified as major pollutants of domestic and industrial wastewaters (Benhima et al., 2008). A recent screening (Chiban et al., 2007) of chemical composition and surface characterization has shown that the major functional groups in *W. frutescens* plant are polar hydroxyl, aldehydic and carboxylic groups. These groups made *W. frutescens* plant to have great potential as an adsorbent for metal ions in aqueous solutions. These micro-particles of *W. frutescens* plant are also known to be non toxic (Montilia et al., 2006; Ahmad et al., 1990). Their selection was made in close relation to their relative abundance in the south–western Morocco zones where they are considered a worthless matter.

2.2. Collection and preparation of wastewater samples

The wastewaters used in this study were raw wastewaters collected from two Agadir zones, M'zar and Anza regions. Several raw wastewater samples were collected and stored in polyethylene bottles from two Agadir zones. M'zar and Anza wastewater samples are municipal and industrial wastewaters from Agadir zones. These samples were decanted and filtered on Whatman paper of 0.45 μm porosity. The pollutant charge of wastewater samples has been determined before using them for the batch experiments.

All the glassware and sample bottles which were used, were washed first with a detergent solution, rinsed with tap water, soaked with 1.0% subboiled HNO_3 for at least 12 h, and finally rinsed with Milli-Q water several times.

2.3. Batch adsorption studies

Batch adsorption experiments were carried out by batch process. 40 ml of wastewater solution with a given ions concentration, C_0, was mixed with 1 g of dried and grinded *W. frutescens* plant. The solutions put in contact with the plant matter were maintained at a constant temperature of 25 °C in a water bath thermostat, the mixture being vigorously stirred by means of a magnetic stirrer. The sampled solutions were then centrifuged at 8500 g for 15 min (Biofuge primo, Heraeus Instruments). The preliminary experiments had shown that ions adsorption losses to the walls of flask and during centrifugation process were negligible.

2.4. Apparent capacity measurements

The pollutant uptake was calculated by the simple method of concentration difference. The initial concentration, C_0 (mg/l) and ion concentrations at different contact time, C_t (mg/l) were determined and the uptake capacity (q_a, mg/g) and percentage removal (%) of pollutant were calculated as follows:

$$q_a = \frac{C_0 - C_t}{m} \times V$$

$$\%\text{Adsorption} = \frac{C_0 - C_t}{C_0} \times 100$$

where V (mg/l) is the volume of the solution and m (ml) is the mass of the adsorbent.

2.5. Instruments and apparatus

The instrument used for the determination of lead, cadmium, copper and zinc ions concentration was a Varian model 220FS atomic absorption spectrophotometer. The concentration of nitrate and phosphate ions was measured by Waters model capillary electrophoreses and HP model spectrophotometer, respectively. The pH values of the wastewater samples were measured by a Mettler-Toledo meter (MP120).

The specific surface of *W. frutescens* particles was determined by N_2/BET method (Micromeritics ASAP 2010) and found to be 3.8 m^2/g. The surface structure, morphology and the elements of *W. frutescens* particles were measured by scanning electron microscope coupled with energy dispersive X-ray analysis (SEM/EDX, LEICA-S-260) (IEM–Montpellier-France).

3. Results and discussion

3.1. W. frutescens adsorbent characterization

SEM images of *W. frutescens* micro-particles (Figure not shown) indicated the presence of grains in the structure. The morphology of this material can facilitate the adsorption of anions and metallic elements, due to the irregular surface of *W. frutescens* particles. Thus it makes possible the adsorption of anions and metallic elements in different parts of the material. So, based on the morphology, as well as on high amounts of amino acid and tannins (Chiban et al., 2007), can be concluded that this material presents an adequate morphological profile to retain heavy metal and anions. The qualitative EDX spectra for dried *W. frutescens* micro-particles (Fig. 1) indicated that Oxygen, Calcium, Carbon, Silicon,

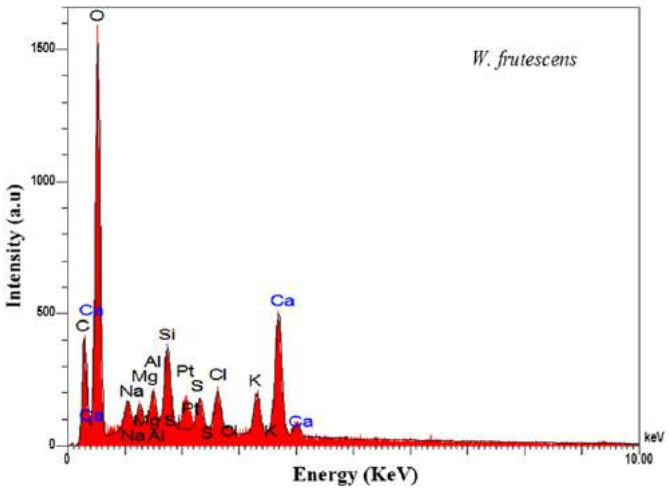

Fig. 1. EDX spectrum of *W. frutescens* particles.

Sulfur, Aluminum, Sodium, and Magnesium are the main constituents. These have been known as the main elements of *W. frutescens* plant.

3.2. Composition of wastewater samples

The characteristics of the raw wastewater samples selected in this study are presented in Table 1. The treatment of two different types of wastewaters in Agadir zone was studied. M'zar and Anza wastewater samples are municipal and industrial wastewater from Agadir zones (Morocco), respectively.

These results indicate that M'zar wastewater samples contain a low concentration of metallic elements but high concentration of NO_3^- and PO_4^{3-} ions. It also shows high values of Chemical Oxygen Demand (COD). The values obtained for nitrate and phosphate ions are superior comparing to the European norms. For Anza wastewater samples, we note that the wastewater samples contain higher concentrations of metallic elements such as Pb(II), Cu(II) and Zn(II) comparing to those of M'zar wastewater samples.

The adsorption studies of all pollutants from both real wastewaters on micro-particles of *W. frutescens* adsorbent are studied using the average concentration of these pollutants from several wastewater samples. The values of pH and temperature of M'zar wastewater samples are 7.6 and 24 °C respectively. The values of pH and temperature of Anza wastewater samples are 2.2 and 24 °C respectively.

3.3. Metallic elements adsorption from municipal and industrial wastewater samples

The M'zar wastewater samples contain a low concentration of metallic elements including Cu(II), Cd(II), Pb(II) and Zn(II) (Table 1). For this reason, it is not necessary to study the adsorption process at different contact times. It has been studied the removal of metallic elements by *W. frutescens* micro-particles after 3 h of contact time. The % removal of the metallic elements from M'zar wastewaters was noticed for all metal ions (Pb(II), Cd(II), Cu(II) and Zn(II)) at about 100%, using crushed plant as adsorbent.

The variations of the % removal of metallic elements ions by *W. frutescens* micro-particles from Anza wastewaters versus the contact time are presented in Fig. 2. These results show that the percentage of the removed metallic elements by 1 g of *W. frutescens* increased with the increasing of the contact time. For all heavy metal ions, adsorption was very fast and the equilibrium was reached within 60 min. Equilibrium adsorption efficiency for Pb(II) was achieved ~98–99% with an initial solution concentration of ~6.09 mg/l. About ~90% of the equilibrium Pb(II) uptake was adsorbed rapidly within first 15 min. This indicates a high

Table 1
Characteristics of municipal and industrial wastewaters.

	M'zar wastewater (mg/l)	Anza wastewater (mg/l)	ESDW (Directive 98/83/CE) (mg/l)
Pb^{2+}	0.40	6.093	0.050
Cd^{2+}	0	0.068	0.005
Cu^{2+}	0.75	2.13	1
Zn^{2+}	1.13	17.35	5
NO_3^-	123.5	2.97	50
PO_4^{3-}	98.5	81.58	0.05
COD	1274	1575	30
TSS	1023	1034	25
pH	7.6	2.2	6.5 < pH < 9.5
T (°C)	24	24	<25

All values are in mg/l except for pH and T, ESDW : European standard in drinking water (mg/l), COD : chemical oxygen demand, TSS : total suspended solids.

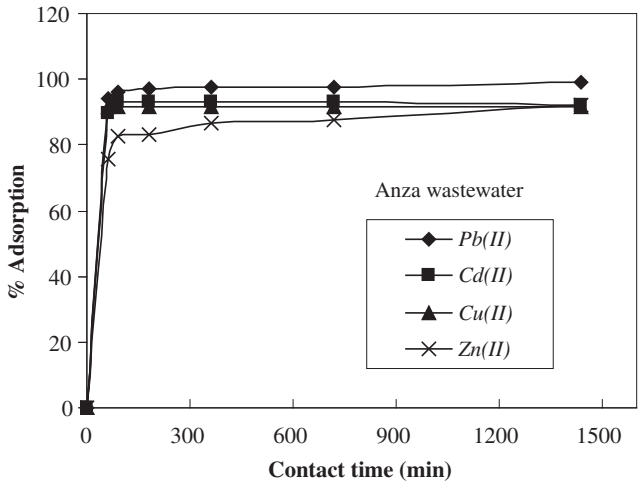

Fig. 2. Adsorption of metallic elements from Anza wastewater by 1 g of *W. frutescens* plant: $C_0(Cd) = 0.068$ mg/l, $C_0(Cu) = 2.13$ mg/l, $C_0(Pb) = 6.09$ mg/l, $C_0(Zn) = 17.35$ mg/l, m/V = 25 g/l, pH = 2.2 and $T = 25$ °C.

adsorption rate for metallic elements from real wastewaters. It is also noticed that : i) The adsorption quantities of Cu(II), Cd(II), Pb(II) and Zn(II) ions by *W. frutescens* particles are about ~78, ~2, ~241 and ~640 μg/g respectively and ii) the treated wastewater onto micro-particles of dried *W. frutescens* plant fulfills the requirements of World Health Organization (WHO, 2004). The results show that the percentage adsorption of metallic elements from wastewaters by *W. frutescens* plant was found to be ~99% for Pb(II), ~92% for Cd(II), ~91% for Cu(II) and ~92% for Zn(II). It is clear that the maximum %removal was depending on the type of the metal ions. Similar results were found for metallic elements removal onto *Carpobrotus edulis* plant (Chiban et al., 2008). These values are much lower comparing to those obtained for laboratory solution at various concentrations, i.e. the concentrations adsorbed by these inert materials of metallic elements from laboratory solution are 1.08 g/l for copper and 0.72 g/l for zinc (Chiban et al., 2006). In the studied conditions, the %adsorption of metal ions from Anza wastewater followed the order of Pb(II) > Cd(II) > Zn(II) > Cu(II). A similar trend has been noticed in the removal of divalent metal ions (Cu(II), Cd(II), Zn(II) and Pb(II)) by other plants (Benhima et al., 2008).

3.4. Nitrate and phosphate ions adsorption from real wastewaters

The variations of the % removal of nitrate and phosphate ions from selected municipal and industrial wastewaters, versus the contact time are plotted in Fig. 3 for *W. frutescens* plant as adsorbent.

For both nitrate and phosphate, we note that the % adsorption of *W. frutescens* particles increased with the increasing of the contact time. The equilibrium time was found to be 60, 240 min for nitrate and phosphate ions, respectively. It is also noticed a rapid kinetic adsorption with up to ~95% nitrate ions removal in the first 30 min. After the fast initial process, the adsorption continues at a slower rate, before reaching a constant level. The final nitrate ions concentrations ($C_f < 3$ mg/l) are lower than 25 mg/l, which is the European standard for drinking water (Directive 98/83/CE, 1998).

The adsorption process of phosphate ions by *W. frutescens* plant appeared to follow a process in two phases characterized by an initial fast retention step lasting at the maximum, less than 30 min, and corresponding to an uptake concentration of about ~48–~56% of the initial PO_4^{3-} concentration of Anza wastewater,

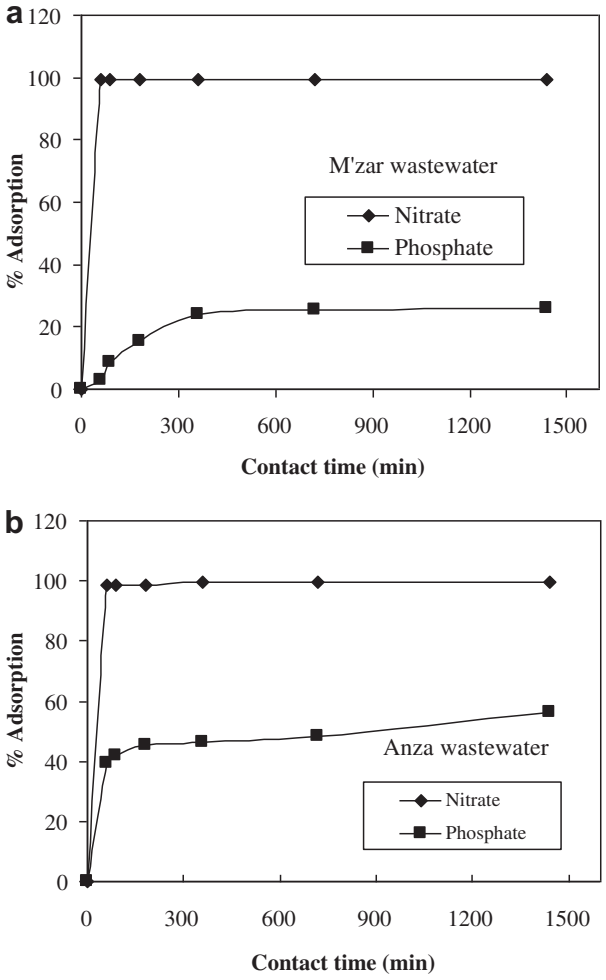

Fig. 3. Adsorption of NO_3^- and PO_4^{3-} ions by 1 g of *W. frutescens* plant from: (a): M'zar wastewater; $C_0(NO_3^-) = 123.5$ mg/l, $C_0(PO_4^{3-}) = 98.5$ mg/l, pH = 7.6 and $T = 25$ °C. (b): Anza wastewater; C_0 $(NO_3^-) = 2.97$ mg/l, C_0 $(PO_4^{3-}) = 81.58$ mg/l, pH = 2.2 and $T = 25$ °C.

followed by a much slower step lasting for hours and tending to a stable state. The equilibrium is attained in less than 5 h for M'zar wastewater and more than 5 h for Anza wastewater. This difference can be explained by the type of wastewater.

Under these experimental conditions, the maximum efficiency for nitrate and phosphate ions uptake by unit of weight of dried plant is higher than 4.91 mg/g. These values depend on the type of anions because the uptake of NO_3^- is higher than that for PO_4^{3-} ions. This indicates some specificity of the interactions between anions and active sites of *W. frutescens* plant responsible for the ions adsorption. These results are much lower in comparison with those obtained for the laboratory solutions. The concentrations retained by *W. frutescens* of nitrate and phosphate ions from aqueous solutions are 2.8 g/l (115 mg/g) for NO_3^- and 0.61 g/l (22 mg/g) for PO_4^{3-} ions (Chiban et al., 2006).

The results of adsorption onto micro-particles of *W. frutescens* plant with distilled water show that the negligible quantities of nitrate and phosphate ions are released by *W. frutescens* plant ($Q_{released}$ $(NO_3^-) << 0.01$ mg/g, after 24 h of contact time).

3.5. Chemical oxygen demand (COD)

The effect of the contact time under agitation on the supplement COD from two types of raw wastewaters (M'zar and Anza wastewaters) by *W. frutescens* particles is presented in Fig. 4. For Anza wastewater samples, the results show that COD supplement decreased with the increasing of the contact time. In the case of M'zar municipal wastewater, the results indicate that the release of the organic matter in the solution by the adsorbent obtained from *W. frutescens* increased with the increasing of the contact time. This difference can be explained by the variation of the pH of wastewater samples (pH(Anza) = 2.2, pH(M'zar) = 7.6). The tests of adsorption process with distilled water showed that the micro-particles of *W. frutescens* plant can release important amounts of organic matter in solution, which suggests the necessity of a pre-wash before using these micro-particles of dried *W. frutescens* plant.

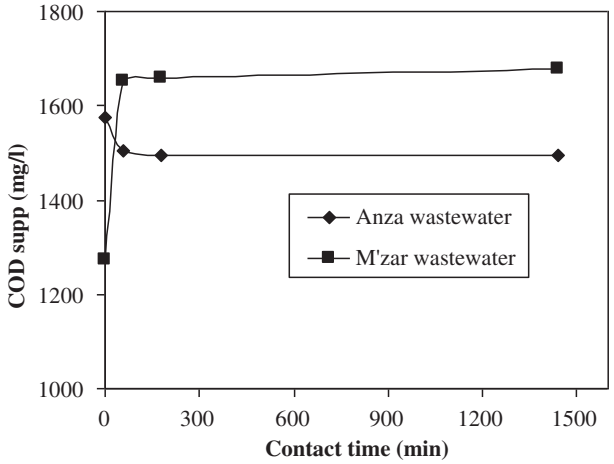

Fig. 4. Effect of contact time variation of the COD supplement by gram of *W. frutescens* plant: $T = 25$ °C, $m/V = 25$ g/l and natural pH.

4. Conclusion

In this study, tests were performed to evaluate the use of *W. frutescens* plant as an adsorbent for nitrate, phosphate and metallic elements. The results showed that the micro-particles of *W. frutescens* plant can be used as an adsorbent for the effective removal of Pb^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, NO_3^- and PO_4^{3-} ions from raw wastewater samples of Agadir zones (Morocco). It was found that zinc, lead, cadmium and copper were adsorbed by dried and crushed *W. frutescens* plant very rapidly (within the first 30 min), while equilibrium was reached in 60 min for metallic elements and nitrate and 240 min for phosphate ions. The % removal of metallic elements from Anza wastewater samples by *W. frutescens* plant was 98∼99% for Pb(II), 92∼93% for Cd(II), 91∼92% for Cu(II) and 92∼93% for Zn(II). The adsorption's percentage of metal ions from Anza wastewaters followed the order: Pb(II) > Cd(II) > Zn(II) > Cu(II).

The experimental results of this study can be used to design batch adsorption systems for the metallic elements, nitrate and phosphate removal. Such a batch system will be applicable to small industries which generate metallic elements-containing wastewaters.

Acknowledgments

This joint program was made possible thanks to the «comité Mixte inter Universitaire Franco- Marocain (A.I. N° 128/MA)». We gratefully acknowledge funding through European Membrane Institute for technical support and analytical screening of samples.

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Journal of Environmental Management 95 (2012) S66–S70

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Treatment of vinasse from tequila production using polyglutamic acid

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ARTICLE INFO

Article history:
Received 28 August 2009
Received in revised form
27 April 2011
Accepted 2 May 2011
Available online 19 May 2011

Keywords:
Vinasse
Poly-γ-glutamic acid (PGA)
Flocculating activity
Colour removal

ABSTRACT

Vinasse, the wastewater from ethanol distillation, is characterised by high levels of organic and inorganic matter, high exit process temperature (ca. 90 °C) and low pH (3.0–4.5). In this study, the treatment of tequila vinasse was achieved by a flocculation–coagulation process using poly-γ-glutamic acid (PGA). Results showed that the use of PGA (250–300 ppm) combined with sodium hypochlorite and sand filtration managed to remove about 70% of the turbidity and reduced chemical oxygen demand (COD) by 79.5% with the extra benefit of colour removal. PGA showed its best flocculating activity at pH 2.5–3.5 and a temperature of 30–55 °C. Such a treatment may be a solution for small tequila companies for which other solutions to deal with their vinasse may not be economically affordable.

1. Introduction

Vinasse is the wastewater produced from ethanol distillation (e.g. tequila). It is one of the most difficult waste products to dispose of, due to its low pH (3–4.5), high temperature, dark brown colour, high ash content and high biochemical oxygen demand (BOD) with values ranging from 35 to 50 g O_2/l (Nandy et al., 2002). Currently, two methods for disposal of vinasse are direct land discharge (Conde et al., 2009) and anaerobic digestion for methane production (Espinoza-Escalante et al., 2009). However, the former may result in severe damage to local agriculture (Conde et al., 2009).

In water treatment, flocculants are important in improving the efficiency of solid removal and reducing the processing time in solid–liquid separation for both water purification and wastewater treatment. At present, aluminium derived flocculants such as aluminium sulphate, poly-aluminium chloride (PAC) and synthetic polymers such as polyacrylamide (PAM) are widely used in coagulation treatment. However, aluminium is suspected to be related to Alzheimer's disease (Campbell et al., 2000) and the polyacrylamide monomer has been identified as a strong neurotoxin (Takahashia et al., 2005), two characteristics that could lead to their prohibition in the future. Thus, the search is on for alternative compounds without these drawbacks. Biodegradable flocculants could represent such an alternative to the presently used conventional flocculants. Reports are available on the production and application of bioflocculants such as polysaccharide from *Proteus mirabilis* (Zhang et al., 2010), and *Bacillus mucilaginosus* (Lian et al., 2008), polyamide from *Bacillus licheniformis* (Shih and Van, 2001), *Bacillus subtilis* DYU1 (Wu and Ye, 2007), and protein from *Bacillus* sp. DP-152 (Suh et al., 1997).

Among these bioflocculants, poly-γ-glutamic acid (PGA), a polyamide flocculant, is considered the best option because of its high yield, high flocculating activity and ability to flocculate a wide range of organic and inorganic compounds (Shih and Van, 2001). PGA is an anionic, naturally occurring, water-soluble homo-polyamide consisting of D- and L-glutamic acid monomers connected by amide linkages between α-amino and γ-carboxyl groups (Sung et al., 2005). It is biodegradable, edible, and non-toxic for humans and the environment (Shih and Van, 2001). The performance of polyglutamic acid-based bioflocculant PGA in processing kaolin suspension has been reported (Pan et al., 2009). It was observed that the efficiency of suspended solid removal was dose-dependent and that neither temperature nor pH were critical parameters affecting its flocculating activity. Practical applications of cross-

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0301-4797/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jenvman.2011.05.001

linked PGA flocculating activity in various polluted water samples have been reported. Successful treatment of samples high in BOD from rivers and lakes in Japan was accomplished using 20 μg/ml PGA. However, the addition of 2 μg/ml PAC was necessary to enhance the flocculating activity of PGA (Taniguchi et al., 2005). The treatment of vinasse from tequila using 200 mg/l PAM resulted in 95.1–100% removal of BOD. Similarly, the use of this synthetic polymer does not necessitate pH and temperature adjustment to ensure the best flocculating activity (Iñiguez-Cobarrubias and Peraza-Luna, 2007).

In this study, PGA was used initially for the treatment of vinasse from a tequila production facility to remove the suspended solids. The addition of an antioxidant such as NaClO was also tested in an attempt to remove the coloured compounds.

It is financially challenging for small industries to invest in new expensive technologies, therefore an effort to propose a simple method for the treatment of this wastewater was the main aim of this research. The principal objectives were to provide a method without the need of a pre-treatment and to process wastewater without modifying its characteristics (pH and temperature), using sand filters in place of a costly centrifuge machine.

2. Materials and methods

2.1. Vinasse characterisation

Raw vinasse was obtained directly from the storage tank of a tequila producing company and refrigerated until use. Both raw and treated vinasse were characterised by measuring COD, pH, turbidity and sedimentable solids (SS).

2.2. Jar testing apparatus

The optimum operating conditions for vinasse treatment were determined by the jar test procedure, as described in detail elsewhere (Satterfield, 2005). Briefly, samples supplemented with PGA from 0 to 300 ppm (in 50 ppm increments) were agitated at 120 rpm for 1 min, then reduced to 30 rpm for 30 min. Samples were allowed to settle and analysed for turbidity. PGA is commercially available as PGα21Ca™, a powder formulated with 14% (dry basis) poly-γ-glutamic acid and stabilised with minerals such as calcium sulphate. Throughout the paper, this is the formulation referred to. PGα21Ca™ was kindly provided by Poly-Glu de Mexico, S.A. de C.V. and Nippon Poly-Glu, Co. Ltd. Osaka, Japan. PGα21Ca™ was used directly in its powder form for the jar test.

2.3. Filtration using sand columns

The filtration tests were performed on tequila vinasse samples after treatment at 60 °C with 250 ppm PGA using the same reaction and flocculation time as in the jar test. The filtration column (0.71 L working volume, 30 cm height, 6 cm diameter) used for the separation of the flocks was packed under dry conditions with 596 g of fine (0.125–0.25 mm) and medium size (0.25–0.5 mm) sand river. The finer sand represented around 72% of the column volume. To ensure complete water saturation, the column was flushed with about 10 column volumes of distilled water before use.

2.4. Colour removal using sodium hypochlorite

Fifty ml of a 5.25% (w/v) solution of sodium hypochlorite was added to 950 ml filtrate from the sand column. This solution was maintained at 60 °C and agitated at 200 rpm to enhance the chemical reaction and at the end of the chemical treatment, the solution was percolated again through the sand column.

2.5. Assay of flocculating activity

Flocculating activity was based on the decrease in vinasse sample turbidity against a formazin calibration curve following a method described in the U.S. Geological Survey (Anderson, 2005). Samples (150 ml) were placed in beakers and supplemented with PGA at different concentrations and filtered as before. The turbidity, reported in nephelometric turbidity units (NTU), was measured by a UV–Vis Zeiss PM2K spectrophotometer at 420 nm and calculated according to the following equation:

$$\text{Flocculating activity } (\%) = [(B - A)/B] \times 100$$

where B = Initial turbidity (NTU), A = Final turbidity (NTU).

2.6. Effects of pH and temperature on flocculating activity

Raw vinasse samples were adjusted to different pH values (from 1 to 10 in one unit increments) and supplemented with 250 ppm PGA (the best dose ascertained from the jar test). The pH was adjusted with either 2 N H_2SO_4 or 2 N NaOH. Once the pH for the best flocculating activity was identified, another set of samples were prepared. PGA was added until 250 ppm was attained and the mixture was heated at different temperatures (30 °C, 45 °C, 60 °C and 75 °C).

2.7. Analysis

Vinasse COD before and after the various treatments was determined by the standard photometric method (APHA, 1998). Samples were digested using a thermoreactor (Model TR 300, Merck Pte. Ltd.) and analysed by a spectrophotometer (UV–VIS Hewlett Packard 8452, diode array). The volumetric sediment concentration of raw and treated vinasse were estimated after 24 h settling using Imhoff cones and reported as ml/l.

2.8. Statistical analysis

Data from two replicate experiments and three repetitions of the analysis for each sample were expressed as mean ± standard

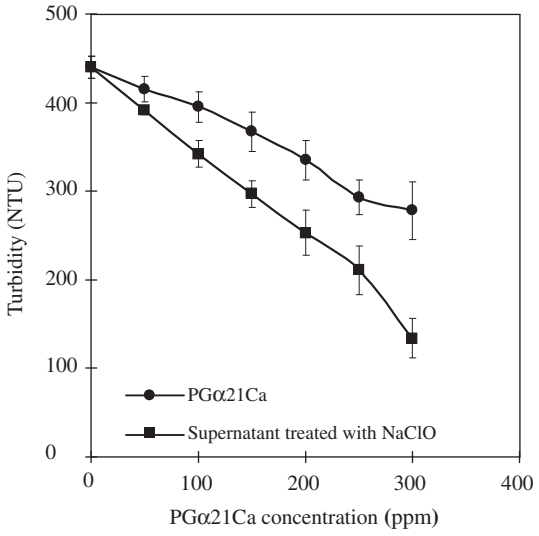

Fig. 1. Turbidity of the vinasses without and after treatment with PGα21ca™ at different concentrations followed by sand filtration (●) and additional chemical treatment with 5% (v/v) sodium hypochlorite followed by a second sand filtration (■).

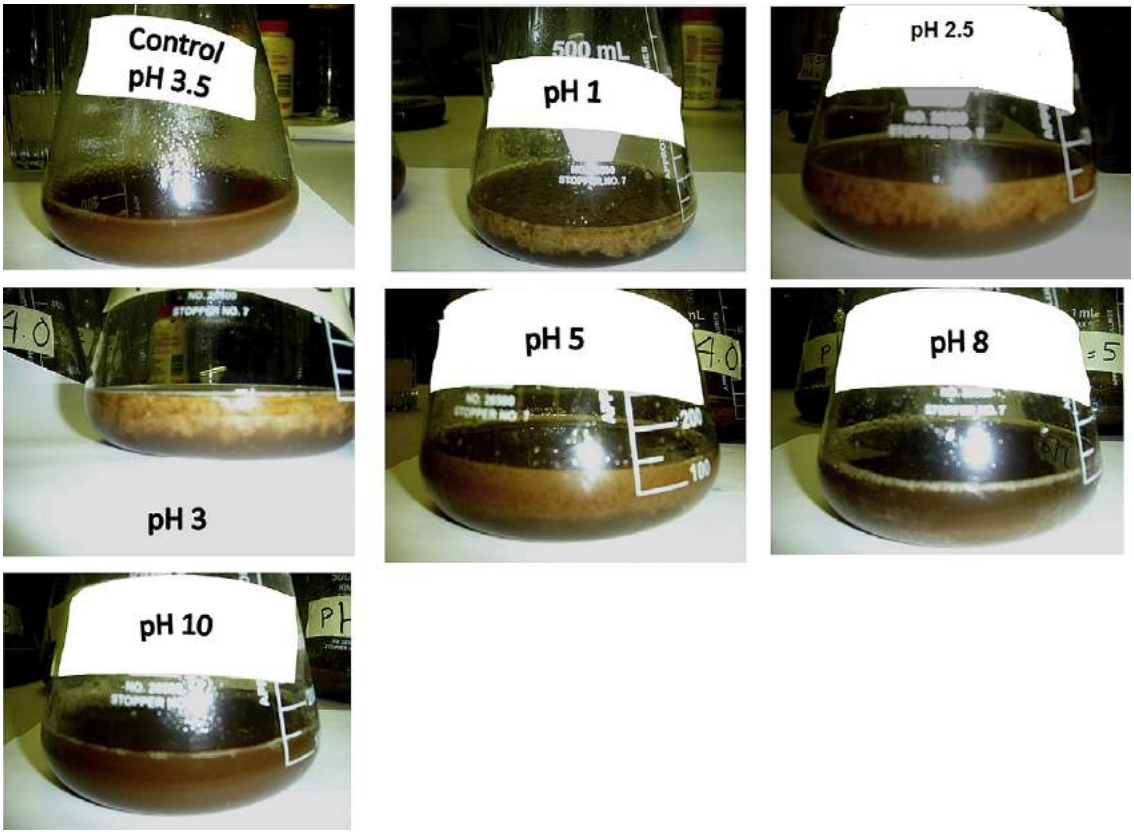

Fig. 2. Effect of pH on the flocculating activity of PGα21Ca.

deviation ($\bar{x} \pm SD$). A one-way analysis of variance (ANOVA) was used to compare the means of the studied treatment with *post hoc* Duncan multiple range tests at 5% for those results where a significant difference was indicated. Minitab version 12 statistical software was used.

3. Results and discussion

3.1. Effects of PGA concentration on flocculating activity

From the jar test, it was evident that flocculating activity was dose-dependent. PGA flocculated the suspended solids and the flocks were removed by a sand filter. This filtrate was analysed for turbidity and then was further treated with NaClO; the precipitated solids were removed again with a sand filter and the filtrate analysed for turbidity (Fig. 1). At this stage of the study, the treatments were performed without modification of the original vinasse pH (3.5).

Treatment with PGA, followed by sand filtration, revealed that the highest flocculating activity occurred between 250 and 300 ppm, and turbidity was reduced by 37% from 400 to 278 NTU (Fig. 1). The results obtained with 250 ppm were not significantly different from those obtained with 300 ppm ($p > 0.05$). Higher concentrations of PGA had no effect in decreasing turbidity. Further removal of suspended impurities remaining in the clarified vinasse (already treated by PGA and sand filtration) with a new PGA addition was not observed. These suspended impurities that still caused appreciable turbidity could be substantially removed by means of a chemical treatment with NaClO. The combined PGA − sand filtration−NaClO − sand filtration treatment finally resulted in 70% turbidity removal (Fig. 1).

3.2. Effects of pH and temperature on flocculating activity

It has been reported that factors such as temperature, pH, polymer concentration, and ionic strength affect both the orientation of PGA functional groups and the charge of the molecule and, as a consequence, its overall conformation and local structure (Inbaraj et al., 2009). Since these charges may have potential consequences for PGA flocculation capacity, it was necessary to study flocculation at different pH values and temperatures in order to define the conditions where an optimal particle aggregation can be achieved.

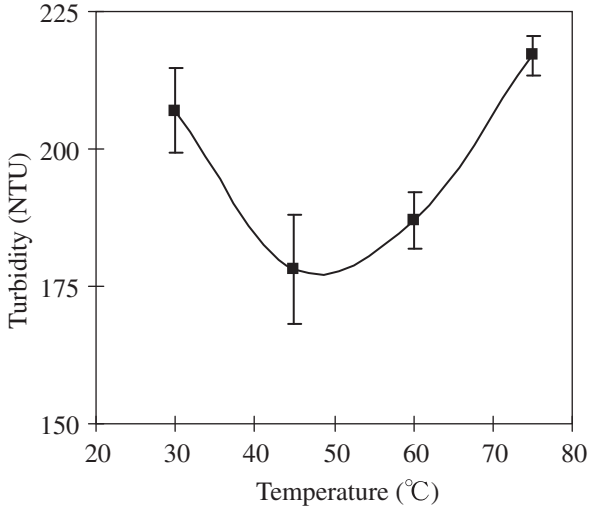

Fig. 3. Effect of temperature on the flocculating activity of PGα21Ca.

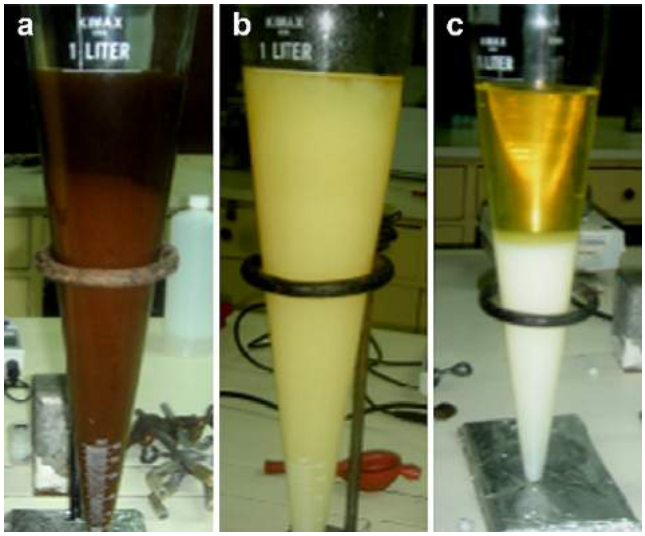

Fig. 4. Colour removal by chemical treatment a) control, b) treatment with 5.25% (w/v) of a sodium hypochlorite solution (NaClO), and c) sedimentable solids after 24 h in static conditions.

In terms of pH, acidic conditions promoted the formation of fast settling macro-flocks while alkaline conditions favoured the formation of much smaller ones (Fig. 2). An optimum flocculation was achieved at pH 2.5, which is very close to the natural pH of the tequila vinasses (3.5). Flocculation efficiency at these two pH values was comparable. PGA flocculation efficiency was directly proportional to temperature within the range 30–55 °C. At both lower and higher temperatures, however, this efficiency was affected (Fig. 3). The effect of temperature on flocculation has not been the subject of many studies (Mohtadi and Rao, 1973; Fitzpatrick et al., 2004; Pan et al., 2009). In particular, Pan et al. (2009) found an optimum range (50–60 °C) for flocculation when PGA was added to kaolin suspensions. All this indicates that too high or too low a temperature is unfavorable for flocculation (Fig. 3), a disadvantage for vinasse treatment, since the exit temperature of the tequila distillation process is around 80–90 °C. However, during transport and storage of the vinasse in tanks, the temperature decreases to around 50–70 °C which allows treatment with PGA without compromising flocculating activity.

Pan et al. (2009) stated a physico-chemical explanation for the increase of flocculation with temperature: (a) "suspended particle moves faster and collision frequency is greater at higher temperatures, thus contributing to the increase in reaction rate". Li and Logan (1997) stated "there is a low capture of particles due to low flow through macropores formed between large clusters within the aggregates".

Therefore at higher temperatures (upper limit of range), although the reaction speeds up, small flocks are formed with a stronger hydrating tendency, making separation by precipitation more difficult.

At temperatures at or below the lower limit, the reaction slows down and the increase in water shear in the flocculant yields small flocks which also make it difficult to separate by precipitation (Pan et al., 2009).

This confirms the effect of temperature on flocculation performance, as mentioned above. In this study, it was also obvious that the samples had properties which enhanced PGA flocculating activity.

3.3. Removal of colour from vinasse

Vinasse colour notably decreased with the addition of sodium hypochlorite. This oxidant promotes precipitation of additional organic material that can be removed from the bulk liquid by sedimentation. Fig. 4 shows the difference between the colour of the original sample and the final colour after chemical treatment. A total of 150 ml solids could be recovered following the treatment after 24 h settling (Table 1). The efficiency of this process can be improved, depending on the nature of the vinasse, which may vary between each process, and even from batch to batch. These variations are influenced by the types of raw materials used or by the final use of the ethanol produced (industrial grade, biofuels, and beverages).

The purpose of colour removal was to eliminate additional particles and colloidal materials from the vinasse. Highly sedimentable organic matter such as cellulosic materials could easily be removed by sedimentation. However, the main problem was the presence of less dense suspended particulate matters and colloidal materials. The former may be removed by sedimentation, but only with extended residence times. This may not be feasible in factories where the daily generation of vinasse is higher than 100 m^3. Colloidal particles, on the other hand, consist mainly of coloured compounds of the melanoidin group formed during the cooking of raw materials or during broth sterilisation for the fermentation process (Iñiguez-Cobarrubias and Peraza-Luna, 2007). Although the concentration of coloured compounds may be low, it is still necessary to consider the treated vinasse from an environmental point of view, since it does not satisfy the minimum requirements outlined in environmental regulations.

In vinasse treatment, the most difficult task is the removal of these coloured compounds. Pan et al. (2009) reported that PGA does not remove coloured compounds, but, as shown in the present study, with the use of NaClO it was possible to eliminate these compounds to some extent.

The processes of coagulation/flocculation have been used intensely for colour and turbidity removal, as well as for lowering COD of different industrial effluents. In the case of vinasse from the tequila industry, it can be observed that, with different PGA doses and at a constant pH, turbidity and COD decreased by 70% and 79.5% respectively (Table 1) in a combined (coagulation/flocculation)/sand filtration/NaClO/sand filtration treatment. Turbidity removal by a coagulation–flocculation treatment reached approximately 34% (Fig. 1). This relationship between flocculant concentration and its flocculating activity has been reported before (Wu

Table 1
Summary of COD, pH, turbidity and sedimentable solids (SS) parameters obtained after a combined (coagulation/flocculation)/sand filtration/NaClO/sand filtration treatment using different PGA concentration for the coagulation/flocculation step.

PGα21Ca (mg L^{-1})	COD (mg L^{-1})[a]	Removal (%)[a]	Turbidity (NTU)[a]	Removal (%)[a]	Sedimentable solids (mL)	pH
0	40,000 ± 3850		440 ± 40		420	3.5
100	27,250 ± 2580	31.9 ± 6.45	342 ± 18	22 ± 4.1	310	5.6
200	12,700 ± 1790	68.3 ± 6.6	253 ± 23	43 ± 5.2	250	5.6
300	8200 ± 920	79.5 ± 11.2	134 ± 33	70 ± 7.5	150	5.6

The original vinasse had a pH of 3.5 and the final pH was 5.6 after the addition of the basic NaClO solution (pH 10–11).
[a] Data are mean values ± SD of 3 determinations.

and Ye, 2007; Yokoi et al., 1995). The results obtained showed that PGA has a remarkable effect on turbidity removal.

4. Conclusions

The use of PGA is an appropriate alternative as a harmless biodegradable biopolymer flocculant for the removal of suspended solids as for instance for vinasse produced from tequila distilleries. The treatment of vinasse using PGA is attractive, since this can be accomplished without the necessity of pre-treatment processes and the low pH of the vinasse actually enhanced its flocculating activity. However, use of PGA at temperatures higher than 55 °C is not recommended due to the reduction in flocculating efficiency. Removal of coloured compounds was achieved using a mild chemical treatment using NaClO which concomitantly enhances COD reduction of the vinasse.

At first sight, this work could be interpreted as the transfer of a pollution problem from a liquid (vinasse) to a solid form (sludge from the flocculation process). However, the chemical composition of vinasse is known to fertilize, therefore the sludge from this treatment should maintain this property. One example of this application can be consulted in Irizarri-Navalpotro (2009) who presented a patent application to the United States Patent and Trademark Office for the use of products derived from vinasse as fertilizer.

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Journal of Environmental Management 95 (2012) S71–S76

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Phytoplankton as bioindicator for waste stabilization ponds

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ARTICLE INFO

Article history:
Received 30 September 2009
Received in revised form
22 June 2011
Accepted 12 July 2011
Available online 6 August 2011

Keywords:
Phytoplankton
Waste stabilization pond
Operation & maintenance
Location
Bioindicators

ABSTRACT

Waste stabilization ponds are an appropriate technology for domestic onsite wastewater treatment. It is a low-cost technology, requires low maintenance, is highly efficient, mostly natural and remarkablably sustainable. In facultative ponds, the existence of an algal population is very important for the stability of the symbiotic relation with aerobic bacteria. The aim of this work is to determine the pattern of microalgae in the facultative and maturation ponds to obtain information for the operation and maintenance work. The important parameters for phytoplankton measured in this study are the organic load, temperature, light penetration, dissolved oxygen and nutrients. Methodology consists in: analysis of main water quality parameters, plankton taxonomic determination and abundance calculation related with the maintenance operations. Results show that cyanobacteria are present in under-loaded conditions and chlorophyceae are present when the pond is overloaded. Using this methodology over time we can obtain a year round pattern to use the phytoplankton as a bioindicator of the pond's conditions. Our conclusion is that the phytoplankton determination and density can be used to know the pond's performance and help the operation and maintenance tasks.

1. Introduction

Sustainable water management and integrated water resources are a priority for science and research. To avoid export of the problem over time or space, the solution should be based on a long and global view (Balkema et al., 2002) and sometimes can found solutions on the decentralized systems (Tchobanoglous, 1996). Besides the savings on transport and treatment costs, the waste is treated in the same spot preventing externalization to further zones and using it as a resource (Crites and Tchobanoglous, 1998). Natural treatment systems uses natural processes to achieve the goal of recycling (Tchobanoglous, 1996) without depending exclusively of external energy for their performance (Reed et al., 1995). On limnology science it is considered a stressed ecosystem acting as an open system constantly fed with organic matter. As a consequence succession is disrupted and the ecosystem remains in a primitive state, mainly with phytoplankton (Margalef, 1983).

Waste stabilization ponds (WSP) are a satisfactory solution for small communities (Racault et al., 1995), (Hosetti, 1995). They consist of a set of connected basins in which biological processes break down the organic matter at a natural rate thanks to forces of nature such as temperature, wind, sunlight and the biological interaction of microorganisms (Mara, 2009). WSP are considered environmentally sustainable given their low energy consumption (Muga and Mihelcic, 2008), the associated carbon dioxide emissions reduction (Shilton et al., 2008) and because they return nutrients to the surrounding environment (Muga and Mihelcic, 2008). WSP generates important savings on operation and maintenance costs (Tsagarakis et al., 2003).

Wastewater treatment plants, either pilot or full-scale in campus areas, to be used as a teaching and research tool, are located on some university campuses in Spain. There are two public universities treating some wastewater in a decentralized plant onsite: the Espinardo campus in Murcia and La Laguna campus in Gran Canaria. (1) The plant in Murcia was launched in 1980 and consists on a deep pond with a daily rate of 102 m^3/day. Researches emerging from it reveal the hydrodynamic behaviour, performance models (Torres et al., 1997). (2) The plant in Gran Canaria was built in 1995, combining constructed wetlands and maturation ponds. Research has shown optimal BOD removal on the gravel filter and better nitrogen elimination on the pond although its best results are attributed to the combination between microorganisms from both environments (Herrera et al., 2009).

The University of the Balearic Islands gave its support to decentralized wastewater treatment in 2001 by deciding to build a WSP on the campus, to be used as a primary wastewater treatment system. We present the first results on the pond's performance, focused specially on the phytoplankton role in the aeration process in relation with the operation and maintenance tasks.

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doi:10.1016/j.jenvman.2011.07.008

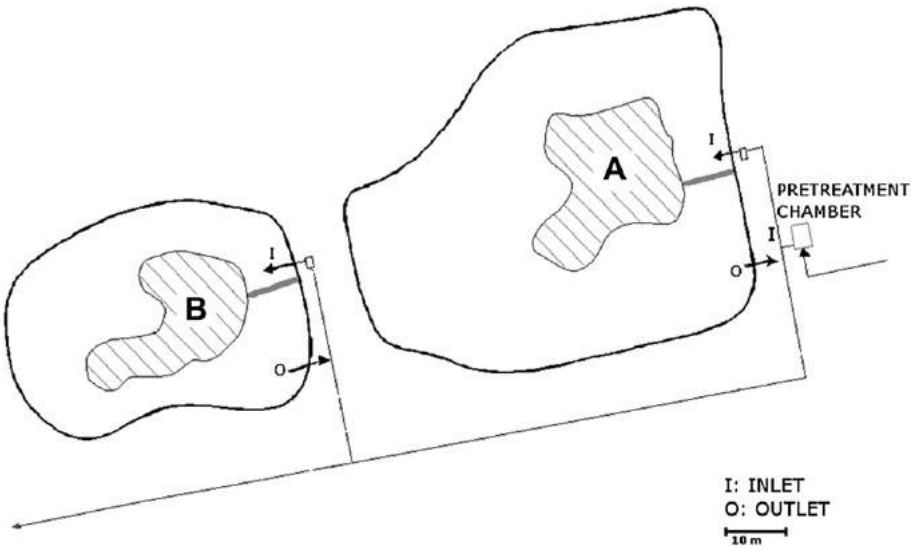

Fig. 1. Pond layout showing the hydraulic circulation and pond nomenclature.

Natural treatment systems are often considered to function by themselves. This is a common misconception, but many authors (EPA, 1992), (García et al., 2000), (Crites and Tchobanoglous, 1998), (USEPA, 2002) and associate the failure of small-scale systems to lack of proper operation and maintenance (O&M) procedures.

2. Materials and methods

2.1. Case study

The University of the Balearic Islands' campus is located 7.5 km north-west of Palma city. The climate is typically Mediterranean, with an annual rainfall of 529.5 mm and an average temperature of 17.6 °C. The campus was constructed in 1980 and has an extension of about 100 Ha, with a mixture of urbanization and rural landscape of non irrigated tree crops. After a severe drought, the board decided to build an onsite wastewater treatment plant to reclaim water to be used for landscape watering and to indirectly contribute to the groundwater recharge.

The wastewater treatment plant is a full-scale experimental stabilization pond; it was constructed in 2002 following Wolverton (1979) and was designed following the theories of Oswald and Gotaas (Middlebrooks et al., 1982), based on the relation established between solar radiation and organic load. The design equation is:

$$A = \frac{N \cdot C}{L_S}$$

Where A is the pond area (Ha); N number of inhabitants; C the organic load (kg BOD/hab·day) and L_s is a constant = $\text{Radiation}_{\text{min}}$ * 1,12/1,5. The plant consists of primary sedimentation and greases separation, influent screening (2 mm), a first facultative pond, volume = 2.278 m^3 (A), and a second maturation pond, volume = 916 m^3 (B) (See Fig. 1), designed for pathogen elimination through ultraviolet radiation (Brissaud et al., 2000). It has a round shape with an island in the middle acting as a baffle between the inlet and outlet. Rounded shapes give better results in hydraulic circulation (Alamancos et al., 1999) and buffered geometries are preferred (Pearson et al., 1995). The capacity of the whole plant is enough to treat 34% of the total wastewater production of the university, corresponding to 18.250 m^3 per year (50 m^3/day), the same as 225 equivalent inhabitants. The daily organic load corresponds to 160 kg BOD/Ha·day. The total water surface is 2.686 m^2, the average depth is 1.27 m for the first and 1.03 m for the second, and the maximum depth is 2.5 m. The hydraulic retention time resulting is 48 days for the first pond and 21 days for the second.

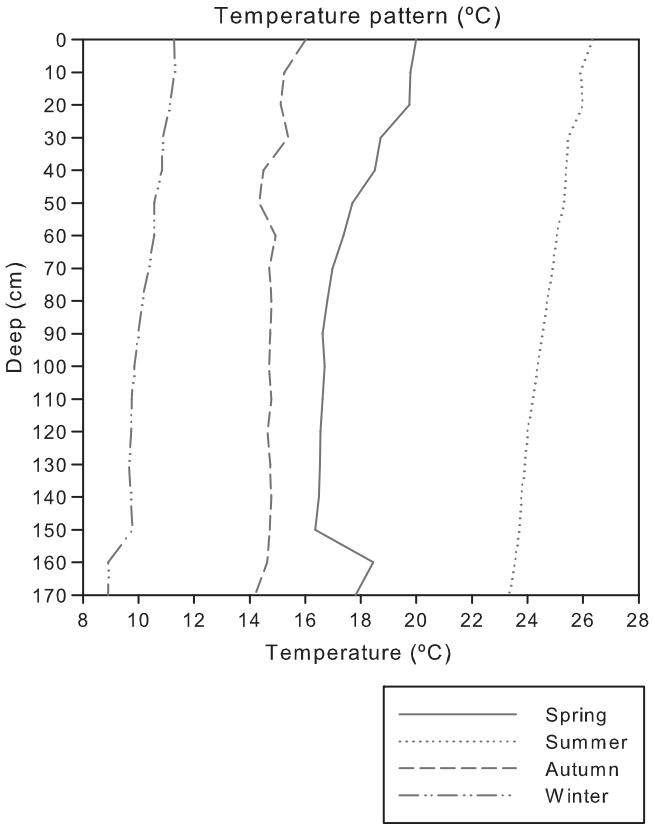

Fig. 2. Annual temperature pattern.

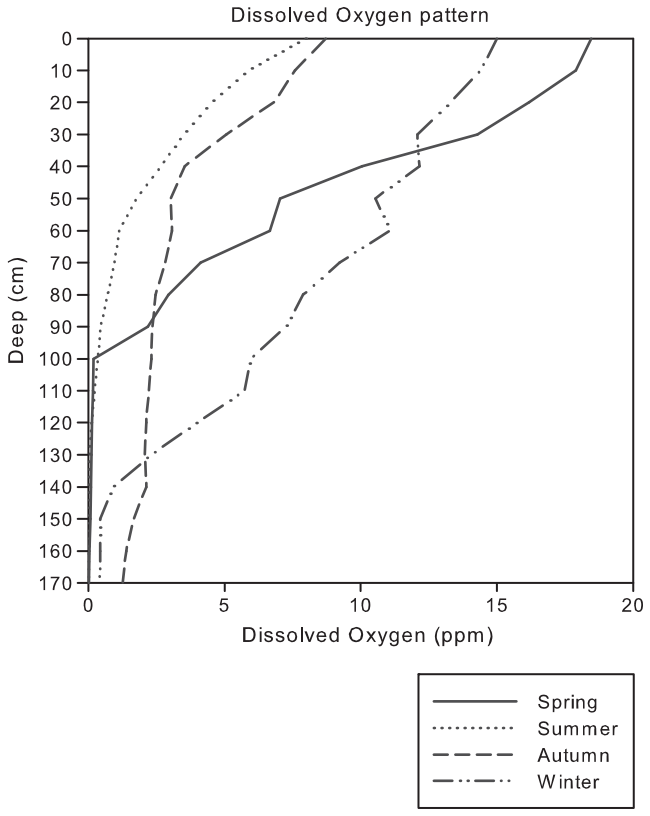

Fig. 3. Dissolved oxygen pattern.

2.2. Samples and analysis

Inlet flows were measured using an ultrasonic flow meter after the sedimentation tank. A monitoring program was carried out from August 2006 to May 2008. Raw wastewater (after the sedimentation tank), and input and output samples were analysed monthly according to Standard Methods (APHA, 1992) and included: pH, total suspended solids (TSS), total and dissolved chemical oxygen demand (COD), total and dissolved biological oxygen demand (BOD_5), total nitrogen (TN), total phosphorus (TP), faecal coliforms (FC) and chlorophyll *a* (Cha). Parameters as temperature (T), dissolved oxygen (DO) and electrical conductivity (EC) were measured weekly *in situ* at 11 am every 10 cm on the water column on pond A using a WTW probe (Oxi 1971 and Cond 1971). Water samples (100 ml) for the determination of phytoplankton and zooplankton composition biomass were collected weekly at two points of each pond. Surface water samples were collected at 1 m away from the shore. Fresh samples were used for identification and for plankton enumeration they were fixed with Lugol's iodine solution. Zoo- and phytoplankton identification was carried out using and optical microscopy (OLYMPUS BX 60). Determination of phytoplankton abundance was performed using the Utermöhl technique (Utermöhl, 1958). Enumeration of phytoplankton taxa was carried out in sedimentation chambers using the optical inverted microscope (ZEISS AXIOVERT 100). Spearman's rank correlations analysis was performed to determine the relationships between study variables (e.g. nutrients, organic matter and algal cell density). All statistical analyses were performed with JMP version 7.0.1 software (SAS Institute Inc., Cary, NC, USA).

2.3. Operation and maintenance

Daily inspection has been carried out every working day at 7:30 h, requiring 45 min of work, and recorded in a routine form with the observations. The maintenance programme has been recorded from July 2007 to October 2009. The data collected is stored in a database and percentages have been used to determine patterns on operator observations.

3. Results and discussion

3.1. Water quality

Typical seasonal fluctuations in temperature are observed in Fig. 2 ranging from 9 °C in the winter to 26 °C during the summer and a temperature gradient of decreasing depth in each one. The average temperature difference in the first meter reaches 3.3 °C during the spring. Diurnal stratification is represented in Fig. 3, where surface temperature is increased 1.4 °C and a thermocline appears with a difference of 0,8 °C between the layers of 20 and 30 cm. A thermal gradient of 0.6°C/m proved satisfactory as a limit value for identification of the occurrence of thermal stratification for tropical climatic regions (Kellner and Pires, 2002). Higher dissolved oxygen (DO) concentrations visualized on Fig. 3, corresponding to pond A, were associated with algal bloom development during the spring, and the minimum during the summer is because the DO decreases with high temperature. DO values from 100 cm deep are associated with wind episodes during the winter that can affect the whole water mass (Alamancos et al., 1999). In Table 1 we display the main average water quality

Table 1
Pond performance as an average of two years' results. Inlet and Outlet points of each pond are indicated on Fig. 1.

Parameter	Units	Inlet	Pond A		Pond B		Reduction %
			Inlet	Outlet	Inlet	Outlet	
pH		7.16	8.51	8.75	9.03	9.2	
Turbidity	NTU	154.75	45.17	33.9	50.5	51.97	66.42
SS	mg/l	199.31	66.26	54.67	69.86	66.67	66.55
BOD5	mg/l	209.89	66.33	57.56	26.56	23.89	88.62
BOD5 filtered	mg/l	90.67	43.33	17	17.7	7.97	91.21
COD	mg/l	1672.41	329.85	199.19	343.7	375.05	77.57
COD filtered	mg/l	311.33	117.62	109.57	79.01	62.49	79.93
T Nitrogen	mg/l	131.49	30.15	29.8	18.19	17.69	86.55
Nitrate	mg/l	0.39	1.36	1.59	2.1	2.4	
Nitrite	mg/l	0.02	0.1	0.11	0.02	0.01	31.25
Amonium	mg/l	36.3	5.2	5.07	0.19	0.27	99.26
T Phosphorous	mg/l	11.16	4.85	4.88	3.23	3.01	97.31
Chlorophyl a	mg/l	0	31.3	37.4	38.8	43.8	–
Faecal coliforms	UFC/100 ml	$3 \cdot 10^5$	$2 \cdot 10^3$	$1 \cdot 10^3$	$5 \cdot 10^{-1}$	$4 \cdot 10^{-1}$	99.99

parameters with the reduction ratios on percentage. The concentrations of main nutrients, analyzed as total nitrogen and phosphorous, are highly reduced due to the biomass consumption. The pH values are related with high Chlorophyll *a* values. Turbidity and suspended solids are decreased by the biomass effect, from the inlet to the A pond, and the same paremeter is slightly increased in the B pond compared to A, because there are more algae density on the second pond. Faecal coliforms are dramatically reduced,

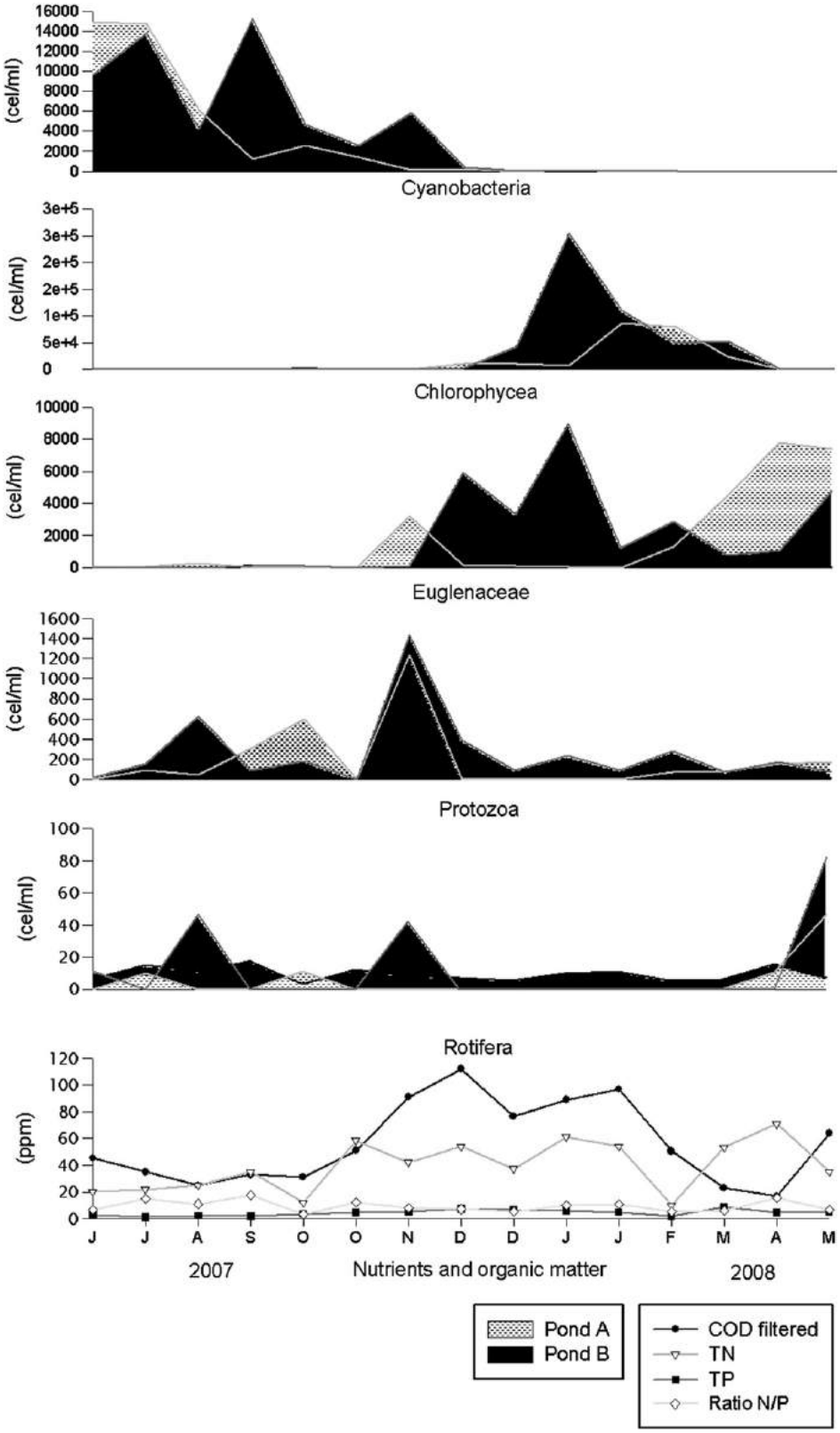

Fig. 4. Plankton succession in taxonomic orders compared with nutrients and organic load for the period between July 2007 and May 2008. Big differences between scales are caused by the ecological characteristics of the groups.

especially on the second pond due to the high pH, solar radiation (Curtis et al., 1992) and long hydraulic retention time.

3.2. Plankton and nutrients

Although there is a succession of dominant algal species during the year, generally only one or two species will be dominant at any one time in the facultative pond. The most commonly recorded genera are: *Chlorella, Scenedesmus, Chlamydomonas, Micractinium, Euglena, Ankistrodesmus, Oscillatoria,* and *Microcystis.* The microorganisms observed are common on literature concerning WSP (Mara and Pearson, 1998). The dominant algal species is determined by the organic loading, with those algae able to tolerate anaerobic conditions being recorded in ponds receiving heavy organic loads, e.g. *Chlamydomonas* spp. and *Euglena* spp. The main function of algae is as phtototrophs, producing oxygen to maintain the aerobic condition of the pond. A supplementary role, but a very important one, is the removal of plant nutrients such as nitrogen and phosphorus. Nutrients are also precipitated out of solution as a consequence of the pH change brought about by photosynthesis, which reduces the concentration of carbon dioxide in the water. Above pH 8, phosphates are precipitated out as calcium phosphate, and at higher pH values nitrogen can be lost as ammonia. However, above pH 9, the conditions are no longer optimal for normal aerobic and facultative bacterial activity. Maximum dissolved oxygen concentrations reach a peak in mid afternoon, falling to a minimum during the night as photosynthesis ceases but respiration continues. During periods of rapid photosynthesis, algal demand for carbon dioxide exceeds that produced by bacterial respiration. At this point, carbonate and bicarbonate ions dissociate to produce carbon dioxide, which is used by the algae, and hydroxyl ions, which accumulate, raising the pH even further to 10 and above. Once photosynthesis declines, free carbon dioxide accumulates and the pH returns to normal parameters. This is a major mechanism for the destruction of faecal bacteria in ponds.

Variation in phytoplankton composition is closely linked to changes in the physico-chemical properties of the pond water. Sometimes, when dominated by green algae, the pond exhibited a dark green appearance, which is indicative of a healthy algal population (Mara and Pearson, 1998). Instead, a surface scum characterized the pond water when the cyanobacteria were dominant (Kotut et al., 2010).Fig. 4 represents the plankton succession related to two important factors: nutrients and organic load. Cyanobacteria have a bloom when organic load is low (20 m^3/day). Main genus determined on this division are *Spirulina* sp., *Microcystis aeruginosa*, and rarely *Anabaena flos-aquae* developed in a quasi monospecific environment. The cyanobacteria, both *Microcystis* and *Anabaena*, are well known for their ability to produce potent toxins causing animal deaths (Kotut et al., 2010). When the N/P relation is below 1/16 cyanobacteria are sensitive to fix atmospheric nitrogen, becoming more competitive vis-à-vis other groups (Margalef, 1983). From November 2007 onwards, the organic load increased by a higher daily flow (60 m^3/day); under these conditions Euglenophyta prevail and Chlorophyta are more competitive. The zooplankton is represented mostly by Protozoa, and secondly by rotifers. Presence in the A pond indicates a complexity on the food web characteristic of complex aquatic ecosystems. This group is represented mostly by *Brachionus* sp. and *Filinia* sp. On the later episode (Spring 2008) population is dominated by the Euglenophyta, because they are mixotroph and can alternate the carbon source, so when we increment the in-flow they are more competitive. This group is represented by diverse species of *Euglena* and *Phacus,* included *Euglena sanguinea*. Euglena have the advantage to keep a double ecological niche (Reynolds, 2006), and they can feed from primary production or from particulate organic matter present on water. Protozoa appear in a moment of change between Cyanobacteria and Euglenophyta. Protozoa group is represented by *Amoebae*, heliozoans, flagellate and ciliates. A common pattern on the ponds is that because they are on serial the bloom starts on A pond and continues in the B pond. Chlorophyta algae is present mostly with: *Chlamydomonas* sp., *Oocystis* sp., *Pandorina morum*, *Eudorina elegans* and *Scenedesmus* spp. The bloom of *Chlamydomonas* on January of 2008 produced the high peak on the Fig. 4, with values of five orders of magnitude. In Table 2 we show the statistical analysis made with Sperman's correlation, where Euglenophyta and Cyanobacteria are negatively correlated, the same as rotifers and Chlorophyta. In relation to nutrients, Cyanobacteria are negatively correlated with phosphorus and nitrogen. In summary a relation can be established between water quality and phytoplankton development. Fluctuations of nutrients and organic load have an effect on the unicellular density of the ponds.

Table 2
Sperman's correlation between main algal groups (Chlorophyta, Cyanobacteria, Euglenophyta); zooplankton (Protozoa and Rotifers) and water parameters (COD filtered, Phosphorus and relation Nitrogen Phosphorus).

Variable	by Variable	Spearman ρ	Prob>|ρ|
Chlorophyta	Cyanobacteria	−0.4	0.03
Euglenophyta	Cyanobacteria	−0.62	0
Euglenophyta	Chlorophyta	0.28	0.14
Protozoa	Cyanobacteria	0.11	0.56
Protozoa	Chlorophyta	−0.18	0.33
Protozoa	Euglenophyta	0.33	0.07
Rotifers	Cyanobacteria	0.04	0.85
Rotifers	Chlorophyta	−0.57	0
Rotifers	Euglenophyta	0.04	0.82
Rotifers	Protozoa	0.31	0.1
COD filtered	Cyanobacteria	−0.29	0.13
COD filtered	Chlorophyta	0.32	0.09
COD filtered	Euglenophyta	−0.12	0.54
COD filtered	Protozoa	−0.32	0.09
COD filtered	Rotifers	−0.42	0.02
Phosphorus	Cyanobacteria	−0.59	0
Phosphorus	Chlorophyta	0.35	0.07
Phosphorus	Euglenophyta	0.37	0.06
Phosphorus	Protozoa	−0.32	0.1
Phosphorus	Rotifers	−0.15	0.45
Phosphorus	COD filtered	0.35	0.07
Relation N/P	Cyanobacteria	0.11	0.6
Relation N/P	Chlorophyta	−0.18	0.38
Relation N/P	Euglenophyta	−0.08	0.7
Relation N/P	Protozoa	0	0.98
Relation N/P	Rotifers	−0.08	0.68
Relation N/P	COD filtered	0	0.98

3.3. Operation and maintenance

The register data ($n = 273$) were stored and treated on a database software. One of the variables is to identify the presence of floating elements on the ponds. On 63% of the days there were algae accumulations on pond A and on 54% of the days on the pond B. The presence of superficial foam is also related with the oxygen production of phytoplankton and was identified in 33% of the registers on pond A and in 24% on pond B. During the cyanobacteria bloom two wild ducks were found dead on the pond and recorded on the daily register. This is another important point: the plankton species determine the maintenance tasks and the security of the public facility.

4. Conclusions

Onsite wastewater treatment technology can be a reality as a university campus facility using natural systems. The WSP have

demonstrated effectiveness at nutrients and organic matter removal and especially on faecal coliforms elimination. The results on the effluent water quality show this water to be suitable for landscape watering. Identifying the phytoplankton group's presence and relating it to the water quality parameters can help to understand the ecological dynamics of the system. The operation and maintenance tasks are necessary, and floating algae accumulation and foams have been found to be a common problem. The natural plankton succession is related to changes in nutrients and organic load. The knowledge of plankton fluctuation and response can help the pond's management and maintenance operations through the anticipation of certain harmful phases, as the dominance of cyanobacteria.

Acknowledgements

We wish to thank the willingness of the UIB to try using natural treatments, the IBASAN and CAM for funding the construction, the CAM for funding my studies, and Miquel Truyols for the Operation and Maintenance tasks.

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Journal of Environmental Management 95 (2012) S77–S82

Contents lists available at ScienceDirect

Journal of Environmental Management

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Experimental design for the optimization of copper biosorption from aqueous solution by *Aspergillus terreus*

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ARTICLE INFO

Article history:
Received 30 September 2009
Received in revised form
26 November 2010
Accepted 4 January 2011
Available online 3 February 2011

Keywords:
Copper
Biosorption
Aspergillus terreus
Optimization
Response surface methodology

ABSTRACT

An experimental design methodology was applied to study the effects of temperature, pH, biomass dose, and stirring speed on copper removal from aqueous solutions by *Aspergillus terreus* in a biosorption batch system. To identify the effects of the main factors and their interactions on copper removal efficiency and to optimize the process, a full 2^4 factorial design with central points was performed. Four factors were studied at two levels, including stirring speed (50–150 min^{-1}), temperature (30–50 °C), pH (4–6) and biosorbent dose (0.01–0.175 g). The main factors observed were pH and biomass dose, along with the interactions between pH and biomass, and stirring speed. The optimal operational conditions were obtained using a response surface methodology. The adequacy of the proposed model at 99% confidence level was confirmed by its high adjusted linear coefficient of determination ($R^2_{Adj} = 0.9452$). The best conditions for copper biosorption in the present study were: pH 6, biosorbent dose of 0.175 g, stirring speed of 50 min^{-1} and temperature of 50 °C. Under these conditions, the maximum predicted copper removal efficiency was 68.52% (adsorption capacity of 15.24 mg/g). The difference between the experimental and predicted copper removal efficiency at the optimal conditions was 4.8%, which implies that the model represented very well the experimental data.

1. Introduction

Electroplating, textile, storage batteries, ceramic, glass and metal-processing industries discharge large amounts of heavy metals into the environment. These pollutants are highly toxic, non-biodegradable and could accumulate in living organisms. Although copper is essential to living organism at trace levels, high concentrations can cause several physiological and health problems or even death. Several treatment processes have been reported to remove copper from wastewater. These processes include precipitation, electrochemical treatment, membrane filtration, ion exchange and adsorption (Han et al., 2006; Ng et al., 2008; Reyes et al., 2006). Biosorption of heavy metals by microorganisms could be an effective and eco-friendly treatment method for metal removal from aqueous solutions. The use of dead biomass is preferred because it does not require nutrients, it is not affected by toxic pollutants, and may be regenerated and reused in a number of adsorption–desorption cycles (Kumara et al., 2008; Wang et al., 2004). Fungal biomass is useful in biosorption processes because of its high ability to bind heavy metals and high availability from industrial residues (Mullen et al., 1992; Amini et al., 2009; Pakshirajan and Swaminathan, 2009; Preetha and Viruthagiri, 2007).

Aspergillus terreus, by-product of the fermentative process of lipase production, has been tested as a biosorbent for copper removal (Gulati et al., 2002); however, the optimal conditions for adsorption processes have not yet been obtained. The experimental design is a helpful tool to identify significant variables that affect the process and to determine optimal conditions in several processes with minimal experimental runs (Amini et al., 2009; Cruz-González et al., 2010; Freitas et al., 2009; Gulati et al., 1999; Yus Azila et al., 2008).

This research examined *A. terreus* (strain ATCC-20516) as a biosorbent for copper removal from aqueous solutions. In addition, factors with significant effects on cooper removal efficiency were studied to obtain the optimal conditions.

2. Materials and methods

2.1. Preparation of biosorbent

A. terreus strain ATCC-20516 was the microorganism used in this study. The fungi were cultivated in a sterile medium containing

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doi:10.1016/j.jenvman.2011.01.004

20 g dextrose/L, 5 g yeast extract/L, 5 g soya flour/L, 5 g NaCl/L and 5 g K_2HPO_4/L. Culture medium was adjusted to pH 7 using HCl (6 N). After four days of incubation at 28 °C and 150 min^{-1}, the living microorganism was recovered by centrifugation, washed several times with NaCl solution (0.96% w/v) and dried at 70 °C for 4 h. Dried biomass was milled and stored at 10 °C previous to the adsorption experiments.

2.2. Batch biosorption experiments

Copper solution was prepared by dissolving copper nitrate (analytical reagent grade) in bi-distilled water to obtain a copper concentration of 50 mg/L. Batch adsorption experiments were carried out in 250-mL Erlenmeyer flasks containing 100 mL of copper solution (50 mg/L), and different biomass quantity, solution pH and temperature. Aliquots were withdrawn at the beginning of the experiment and after 3 h once the equilibrium was reached. These experiments were carried out in triplicate. Collected aliquots at the equilibrium were centrifuged at 3000 min^{-1} for 10 min, and the copper concentration of the supernatant (Table 2) was determined by an atomic absorption spectrophotometer (GBC Scientific model 932AA). Copper concentration deviation was less than 3%. Copper removal efficiency was estimated according to the following equation:

$$\%R = \frac{(C_0 - C_e)}{C_0} \times 100 \quad (1)$$

where $\%R$ is the copper removal efficiency by *A. terreus*, and C_0 and C_e are the initial and equilibrium concentrations of copper in mg/L, respectively.

2.3. Experimental design for optimization

2.3.1. Full factorial design with central points

A 2^4 factorial design with central points was used to study the effects of the independent process variables (factors) and their interactions on copper removal efficiency. The selected independent factors were stirring speed, temperature, pH and biosorbent dose. The ranges and the levels of the variables (coded as −1 or low, and +1 or high) studied in this research are shown in Table 1: pH (4, 6), biomass dose (0.01, 0.175 g), stirring speed (50, 150 min^{-1}) and temperature (30, 50 °C).

To detect curvature in the response surface, replicates at a central point were added to the full 2^4 factorial design (Myers et al., 2009). This factorial design with central point consisted of 55 experiments including 48 factorial points, and seven central points (coded as 0).

The regression model was built to determine whether the response surface would be a plane or a curved surface in the range of the factors studied. The regression model represented by Eq. (2) includes the factors and their interactions.

$$Y = \beta_0 + \sum_{i=1}^{l} \beta_i x_i + \sum_{i=1}^{l} < \sum_{j=1}^{l} \beta_{ij} x_i x_j + \sum_{i=1}^{l} < \sum_{j=1}^{l} < \sum_{h=1}^{l} \beta_{ijk} x_i x_j x_k + \varepsilon \quad (2)$$

Table 1
Factors and levels used in the 2^4 factorial design with central points.

Independent variable	Factor	Coded levels		
		−1	0	+1
		Actual levels		
Stirring speed (rpm)	A (x_1)	50	100	150
Temperature (°C)	B (x_2)	30	40	50
pH	C (x_3)	4	5	6
Biosorbent dose (g)	D (x_4)	0.01	0.0925	0.175

where Y is dependent variable or response, β_0 is a constant coefficient, β_i, β_{ij}, β_{ijk} are the coefficients for the linear, double, and triple interaction effects, respectively; x_i, x_j, x_k are the independent variables or factors, and ε is the random error.

A measure of the variability of the linear model was explained by the adjusted linear coefficient of determination (R^2_{Adj}) in Eq. (3).

$$R^2_{Adj} = 1 - \frac{SS_{Residual}/DF_{Residual}}{(SS_{Model} + SS_{Residual})/(DF_{Model} + DF_{Residual})} \quad (3)$$

where SS is the sum of squares and DF is the freedom degrees.

Data analysis and response surface graphics were performed using Design Expert (version 6.0.1, Stat-Ease, Inc., USA). Analysis of variance (ANOVA) was used to determine if the regression model is able to represent the experimental data; additionally, this was verified by the adequate precision ratio (Eq. (4)) (Box et al., 1978; Myers et al., 2009).

$$\text{Adequate precision} = \frac{\text{Max}\left(\widehat{Y}\right) - \text{Min}\left(\widehat{Y}\right)}{\sqrt{\overline{V}\left(\widehat{Y}\right)}} = \frac{\text{Max}\left(\widehat{Y}\right) - \text{Min}\left(\widehat{Y}\right)}{\sqrt{\frac{p\sigma^2}{n}}} \quad (4)$$

where $\overline{V}(\widehat{Y})$ is the average variance of the predicted values, p is the number of model parameters (including β_0), σ^2 is the residual mean square, and n is the number of experiments.

The selected regression model was used to predict copper removal efficiency by *A. terreus* and to build a response surface to explore the design space. The maximum amount of copper biosorption was observed on the three-dimensional response, the contour, and the cube plot.

3. Results and discussion

3.1. Model for copper biosorption process

The experimental design matrix derived from factorial design 2^4, and the predicted and observed responses (copper removal efficiency and adsorption capacity) are shown in Table 2. The average experimental data of copper removal efficiency and adsorption capacity ranged from 6% to 72%, and from 7.86 to 268.67 mg/g, respectively. A linear model was built to estimate the response as a function of stirring speed, temperature, pH and biosorbent dose.

The analysis of variance (ANOVA) is shown in Table 3, and the results show that there is no inflection point on the response surface because the curvature is non-significant (Prob > 0.37). Therefore, the linear model is appropriate to represent the design space, this was confirmed by other tests included in the ANOVA analysis that are described as follows. The linear regression model was highly significant at 99% confidence level (Prob > 0.0001). The adjusted determination coefficient (R^2_{Adj} = 0.9452, a value higher than 0.70 is desirable) was very high, which means that the model represented 94.52% of response total variation, and only 5.48% of total variations was due to random error. Finally, the adjusted linear model obtained in this study showed an adequate precision ratio of 33.12, which indicates an adequate signal-to-noise ratio because a value higher than four is desirable. In that case, the copper removal efficiency model can be used to predict, build and explore the design surface.

The normal probability plot (Fig. 1) showed the magnitude of negative or positive effect of each individual variable, and their interactions on copper removal efficiency. A positive value of effect means an increase of copper removal efficiency when the factor level increases; on the other hand, a negative value of effect means

Table 2
Experimental design and results for percentage of metal adsorbed from aqueous solution.

Trial	Coded values				Copper removal efficiency (%)					$\bar{q}$ (mg/g)
	A	*B*	*C*	*D*	Runs			Average	Predicted	
					1	2	3			
1	−1	−1	−1	−1	11.31	10.92	14.58	12.27	14.58	57.37
2	+1	−1	−1	−1	7.68	7.07	3.34	6.03	6.81	28.21
3	−1	+1	−1	−1	9.66	17.28	9.95	12.30	11.25	57.51
4	+1	+1	−1	−1	5.99	6.98	3.61	5.53	3.49	25.84
5	−1	−1	+1	−1	37.27	32.21	48.45	39.31	40.02	183.84
6	+1	−1	+1	−1	30.44	45.43	34.79	36.89	39.27	172.50
7	−1	+1	+1	−1	55.80	59.89	56.67	57.45	55.48	268.67
8	+1	+1	+1	−1	52.95	57.29	57.30	55.85	54.73	261.16
9	−1	−1	−1	+1	34.31	38.90	38.02	37.07	36.50	9.91
10	+1	−1	−1	+1	28.86	28.60	30.79	29.42	26.90	7.86
11	−1	+1	−1	+1	40.20	40.58	40.15	40.31	42.14	10.77
12	+1	+1	−1	+1	31.62	31.35	30.91	31.30	32.55	8.36
13	−1	−1	+1	+1	59.58	59.78	60.04	59.80	62.02	15.98
14	+1	−1	+1	+1	50.47	51.53	50.14	50.71	45.41	13.55
15	−1	+1	+1	+1	67.35	77.61	71.04	72.00	68.52	15.24
16	+1	+1	+1	+1	47.25	47.38	41.37	45.33	51.90	12.11
17	0	0	0	0	38.34	36.23	31.59	35.38	36.97	17.89
					36.01	34.14	38.83			
					32.53					

$\bar{q}$: average experimental adsorption capacity.

a decrease of copper removal efficiency when the factor level increases. The factors located to the right of the central line (Fig. 1) were significant at a 1% significance level and have a positive effect (*C*, *D*, *B* and *BC*) on copper efficiency removal. It is also observed that copper efficiency removal increases with increasing biomass dose: an increase of functional groups on the biosorbent surface could explain this behavior. A similar effect was observed when the solution pH was increased and could be explained based on the biosorbent surface charge which become more negative indicating a rise of electrostatic attraction between the biosorbent functional groups and copper species. On the other hand, the effect of temperature (*B*) suggest an endothermic process, which means that an increase of temperature increases the equilibrium constant value. In the adsorption process, it is always necessary to study the effect of temperature to determine if the process will be favored at low or high temperature values. Different authors have reported both positive and negative effects for temperature (Preetha and Viruthagiri, 2007; Veit et al., 2005).

The factors to the left of the central line are also significant but have a negative effect (*A*, *CD*, *BCD*, *AD* and *ACD*) on copper removal efficiency. In addition, the factors located on the central line, that crosses the zero value at the abscissa, were non-significant (*BD*, *ABD*, *AB*, *ABCD*, *ABC* and *AC*) at a 1% significance level. Taking into account these main factors, the model coefficients were estimated to build the linear regression model and to predict copper removal efficiency. This model, in terms of coded factors, is shown in Eq. (5):

$$\hat{Y} = 36.97 - 4.34A + 3.03B + 15.20C + 8.77D - 2.21AD + 2.46BC - 3.98CD - 1.75ACD - 2.24BCD \quad (5)$$

Table 3
Analysis of variance for factorial design with central points.

Variation source	Sum of square	Degree of freedom	Mean square	*F*-value	Prob.
Model	17983.95	8	2247.99	153.84	<0.0001
Curvature	15.49	1	15.49	1.06	0.3087
Residual	657.58	45	14.61		
Lack of fit	176.59	7	25.23	1.99	0.0817
Pure error	480.99	38	12.66		
Total	18,657.02	54			

The linear regression model (Eq. (5)) built from the analysis of variance was used to estimate copper removal efficiency and the values of effects. The results showed that pH (Prob < 0.0001) has the greatest effect on copper removal efficiency, followed by biosorbent dose (Prob < 0.0001), stirring speed (Prob < 0.0001) and the interaction between pH and biosorbent dose (Prob < 0.0001). Yus Azila et al. (2008) observed a similar effect of this interaction on lead biosorption using *Pycnoporus sanguineus*. The magnitude of the effect of the triple interaction between stirring speed, pH and biomass dose (Prob < 0.0082), the double interaction between stirring speed and biomass dose (Prob < 0.0011), the triple interaction between temperature, pH and biosorbent dose (Prob < 0.001), and the double interaction between temperature and pH (Prob < 0.0004) are less significant for copper removal efficiency.

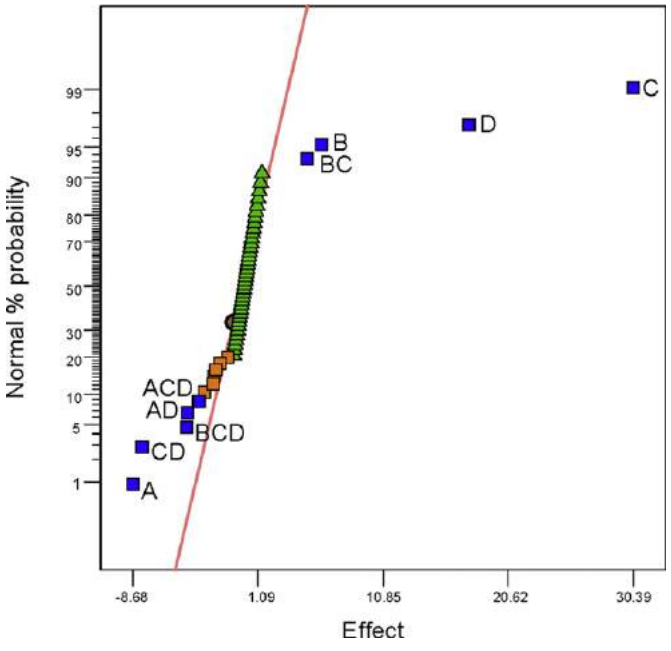

Fig. 1. Normal probability plot of effects at a 99% confidence level.

The obtained model can be used to represent copper removal efficiency as a function of the process factors (pH, temperature, biomass dose, stirring speed, and their interactions). Although the obtained model is a statistical model, the response variable (removal efficiency) can be adequately predicted by the model (including main factors and their interactions). However, this model does not physically explain the biosorption phenomena.

3.2. Optimal conditions of copper biosorption process

The hierarchical linear model (Eq. (5)), obtained in this study, was used to represent the response surface. The optimization of copper biosorption was carried out using the cube plot, the response surface plot, and the contour plot of the response.

To evaluate the effect of stirring speed on copper removal efficiency, Fig. 2 was built from the regression model by varying the stirring speed and biomass dose from low to high at middle point of pH, and temperature. It was observed that copper removal efficiency decreases, at high biomass dose, when the stirring speed increases from 50 to 150 min^{-1}. This behavior could be explained by the mechanical biomass damage caused by shear stress. Therefore, the level of stirring speed was selected as 50 min^{-1} for the following analysis steps because copper removal efficiency was better at this level than at 150 min^{-1}.

Fig. 3 shows the cubic representation of the triple interaction between temperature, pH and biosorbent dose at a low stirring speed level (50 min^{-1}). The results showed that a rise of temperature at high pH level and low biosorbent dose level caused an increase of copper removal efficiency from 40.02 to 55.48%. Copper removal efficiency greatly increased from 42.14 to 68.52% when the pH level was high along with high levels of temperature and biosorbent dose. On the other hand, copper removal efficiency increased from 55.48 to 68.52% when increasing biosorbent dose from 0.01 to 0.175 g (at high levels of both temperature and pH). Other microorganisms have shown different behavior (Amini et al., 2008; Basha et al., 2009) due to the loss of active sites by biomass agglomeration. Although, in this study the agglomeration phenomenon was experimentally observed, the amount of available sites at high biomass dose were enough to adsorb copper as the biomass amount was 17 times higher than at low biomass dose.

As shown by its overall effect and strong interaction with other factors, pH was the most important factor affecting copper biosorption (Fig. 1). Gulati et al. (2002) previously observed the importance of the effect of pH on copper biosorption by *A. terreus*; however, the interaction between pH and biomass dose was not studied. Copper biosorption strongly increased to 68.52% when the pH level increased, and biosorbent dose increased as shown in Fig. 4a and b; in addition, stirring speed was low, and temperature was high. The effect of pH can also be explained in terms of point of zero charge ($pH_{PZC} = 5$, data not shown) of the biosorbent; at this pH value, the number of positive and negative charges is equal, and thus the total surface charge becomes neutral. Furthermore, ion copper adsorption is favorable at pH values higher than pH_{PZC} due to the increased electrostatic attraction force between *A. terreus* and copper, because the augmentation of pH causes the biosorbent surface to become more negative. On the other hand, the decrease of copper removal efficiency with decreasing pH can be explained by the competition between copper ions and hydrogen ions for the active sites of *A. terreus*. Although, at pH > 6 the biosorbent surface charge become more negative and, therefore, copper removal efficiency may be increased, it is not recommended to rise the solution pH higher than 6 because copper can be precipitated instead of being adsorbed on the biosorbent.

Based on these results, the optimal conditions for copper biosorption from aqueous solution using *A. terreus* as a biosorbent are a pH of 6.0, a biosorbent dose of 0.175 g, a stirring speed of 50 min^{-1}, and a temperature of 50 °C. The maximum quantity of adsorbed copper predicted by the model was 68.52%, as can be seen in the response surface and contour plots (Fig. 4).

The final copper concentration at the optimal tested conditions was 14 mg/L (average copper removal efficiency of 72%) and, at this final concentration, the treated effluent can be discharged onto public sewer according to the recommended discharge concentration in Mexico (NOM-002-SEMARNAT-1996: daily average of 15 mg/L).

In copper plating processes, the operation temperature varied from 20 to 75 °C by using copper cyanide or copper sulfate. These operational plating conditions could allow treating the exhausted water bath at this temperature range (United States Patent US4933051). If the wastewater is treated by biosorption processes

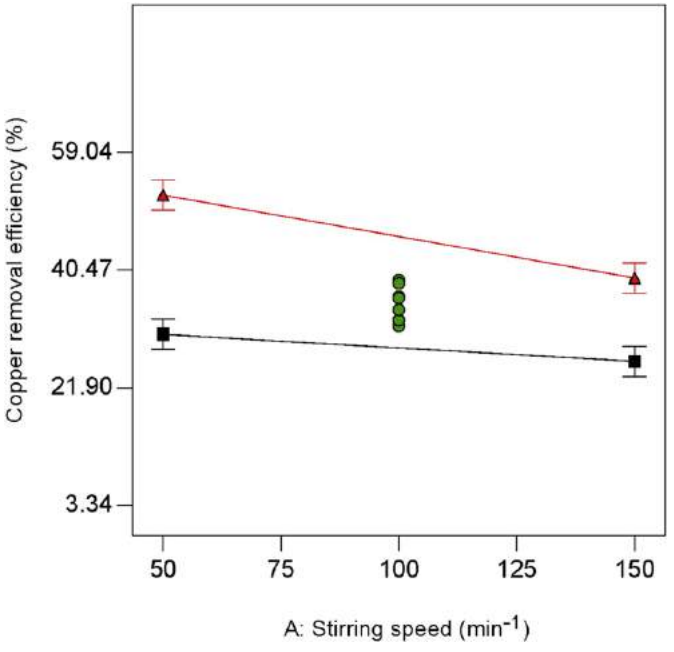

Fig. 2. Factor plot of stirring speed and biomass dose effect, with the other factors remaining constant (40 °C, and pH 5). Biomass dose: □ 0.01 g, Δ 0.175 g.

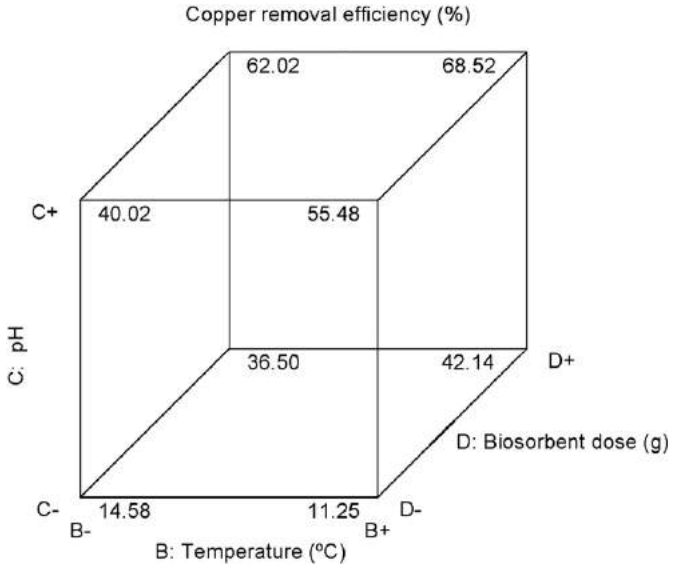

Fig. 3. Cube graph of the triple interaction between temperature, pH and biosorbent dose: stirring speed 50 min^{-1}.

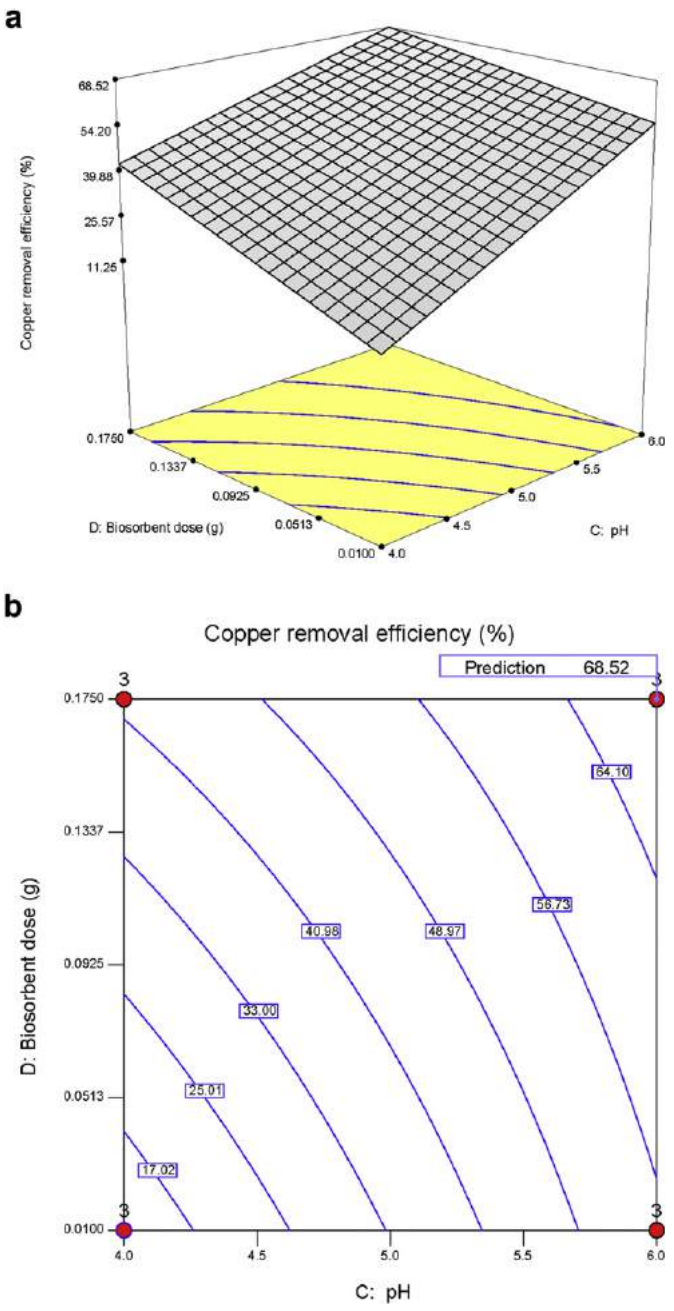

Fig. 4. Optimum conditions of the process: stirring speed 50 min^{-1} and 50 °C; pH level of 6 and 0.175 g of biomass: (a) response surface plot (b) contour plot.

by using *A. terreus* biomass at 30 °C (pH 6 and 0.175 g biomass) copper removal efficiency achieved 62% whereas at 50 °C removal efficiency increased up to 72%.

The factorial design applied in this research was a valuable tool to optimize the copper adsorption process using *A. terreus*. At the optimal conditions, adsorption capacity was 268.67 mg/g; this value is 1.7 times higher than the reported value by Gulati et al. (2002). These dissimilar results may be attributed to the solution pH: at pH around 6 the copper removal could be associated to an adsorption–microprecipitation coupled process. Other types of fungi have shown an adsorption capacity of 1.52–89 mg/g in copper biosorption process at different operational conditions; therefore, a direct comparison between these different results is not appropriate (Veit et al., 2005; Gopal et al., 2002; Gabriel et al., 2001; Kapoor et al., 1999).

Surface response methodology allows obtaining the optimal conditions for treating industrial wastewaters but other pollutants may reduce the copper removal efficiency reported in this research, where synthetic aqueous solution was used for adsorption experiments. These pollutant species may compete for functional groups of *A. terreus* biomass. This effect requires to be explored experimentally to assess the final copper removal efficiency.

The optimal conditions obtained in this research can be used to carry out column adsorption studies to treat wastewaters containing copper in solution.

4. Conclusion

This study demonstrates the usefulness of a factorial experimental design to model copper biosorption from aqueous solutions by *A. terreus* biomass. The most significant factors affecting copper removal efficiency were: pH, biosorbent dose, and the double interaction between pH and biosorbent dose. The selected model was adequate to represent the response surface and to obtain the optimal conditions for copper biosorption by *A. terreus* from aqueous solution; these were pH 6.0, 0.175 g of biomass, 50 min^{-1} and 50 °C. At these conditions, the predicted copper removal efficiency was 68.5% that is similar to the experimental copper removal efficiency (72%).

Acknowledgments

The authors gratefully acknowledge Facultad de Ciencias Químicas de la Universidad Autónoma de Nuevo León and, Secretaría de Educación Pública (SEP), Mexico for financial support (PROMEP/103.5/04/1371). In addition, the authors acknowledge to Professor Jonh Rosazza for your valuable contribution and Dr. R.B. García-Reyes for critical review.

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Journal of Environmental Management 95 (2012) S83–S87

Contents lists available at ScienceDirect

Journal of Environmental Management

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Chlorophenol degradation in soil columns inoculated with *Anthracophyllum discolor* immobilized on wheat grains

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ARTICLE INFO

Article history:
Received 22 September 2009
Received in revised form
3 September 2010
Accepted 26 September 2010
Available online 23 October 2010

Keywords:
Anthracopyllum discolor
Chlorophenols
Soil columns
Biodegradation

ABSTRACT

The white-rot fungus *Anthracophyllum discolor* immobilized on wheat grains was evaluated for chlorophenol (2,4-dichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol) degradation in allophanic soil columns activated by acidification. Columns without inoculation were used as the control to evaluate the adsorption capacity of the soil columns. The chlorophenols were removed efficiently in soil columns by both adsorption and degradation processes. In inoculated soil columns, 2,4-dichlorophenol was highly degraded and this degradation is associated with a high production of manganese peroxidase. 2,4,6-trichlorophenol was degraded to a lesser extent compared with 2,4-dichlorophenol. Pentachlorophenol was first removed by adsorption and then through degradation by the fungus. Manganese peroxidase activity was lowest when the column was fed with pentachlorophenol and highest when the column was fed with 2,4-dichlorophenol. Laccase was also produced by the fungus but to a lesser degree.

1. Introduction

The use of soil or natural adsorbents for the removal of organic compounds from contaminated wastewater is considered a beneficial method, as in this system the contaminants can be removed by both adsorption and degradation processes (Kookana and Rogers, 1995; Lin and Juang, 2009; Uddin et al., 2009). Taking adsorption processes into account, fixed-bed columns packed with allophanic soil (Andisol) have proven to be effective in the removal of chlorophenols present in kraft mill wastewater (Navia et al., 2003, 2005; Diez et al., 2005). However, no information is available regarding the biodegradation of chlorophenols by immobilized white-rot fungi in a fixed-bed column packed with allophonic soil. Chilean Andisol is an effective support for this system due to its high organic matter content with a great affinity for pollutants due to the presence of humic and fulvic acids and a reactive clay fraction (allophane) that contains Al and Fe hydroxide groups and a high specific surface area (Mora et al., 1994; Diez et al., 2005). In addition, pH plays an important role in the adsorption of compounds with acidic functional groups due to their neutral and ionic forms (Kookana and Rogers, 1995; Diez et al., 2005; Cea et al., 2007). In a pH-dependent variable surface charge soil, Diez et al. (1999) demonstrated that phenolic compounds adsorption increased as the pH decreased, as a result of electrostatic repulsion between the compounds and the resulting negative surface charge.

White-rot fungi are microorganisms with a well known capacity for degrading a wide range of organic compounds, attributed to their extracellular enzymatic system conformed by lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac) involved in the degradation of lignin compounds. *Phanerochaete chrysosporium* and *Trametes versicolor* have been the most widely used fungi for chlorophenol degradation. However, several studies have been performed to evaluate new fungal strains with a high ability for degrading recalcitrant organic compounds (Levin et al., 2004; Tortella et al., 2008), new technological processes such as degradation of pentachlorophenol in soil slurry cultures by *Bjerkandera adusta* and *Anthracopyllum discolor* (Rubilar et al., 2007) and biodegradation of 2,4-dichlorophenol in columns packed with immobilized *P. chrysosporium* (Wu and Yu, 2008). Tortella et al. (2008) characterized several Chilean native wood-rotting fungi, and showed that the selected strains presented high lignin peroxidase (LiP) and manganese peroxidase (MnP) activity. Their ability to degrade 2,4-dichlorophenol (2,4-DCP), 2,4,6-trichlorophenol (2,4,6-TCP) and PCP especially by the fungus *A. discolor* (Sp4) was also shown.

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0301-4797/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jenvman.2010.09.024

Several aspects of white-rot fungi degradation properties have been reviewed recently (Tortella et al., 2005; Gianfreda and Rao, 2008; Rubilar et al., 2008). The application of white-rot fungi in a fixed-bed column has not been investigated in any depth (Wu and Yu, 2008), in spite of the fact that they can degrade some complex substances which are beyond the metabolic abilities of bacteria. The main purpose of this study was to evaluate chlorophenol degradation in columns packed with allophanic soil and inoculated with the white-rot fungus *A. discolor* immobilized on wheat grains.

2. Materials and methods

2.1. Column preparation

Glass columns (35 cm long with 5 cm internal diameter) packed with a mixture of quartz sand and allophanic soil (1:1) were tested for 2,4-DCP, 2,4,6-TCP and PCP adsorption. The allophanic soil used was an Andisol, belonging to the Temuco Series, located in southern Chile. The soil sample was taken from 0 to 20 cm depth, air dried at room temperature and sieved through a 2 mm mesh. The allophanic soil used has a pH(w) of 5.9 and 14.6% organic matter (Cea et al., 2007). The quartz sand was washed with distilled water, dried at 100 °C and stored in a vacuum desiccator prior to use. Columns were packed with 300 g of total mass (bulk density (ρ) was 1.26 g/mL) and were pre-conditioned to pH 4.5 by eluting the columns with H_2SO_4 0.1 M so as to activate the soil surface (Diez et al., 1999). The columns were covered with aluminium foil to avoid oxidation processes.

2.2. Fungus immobilization

A white-rot fungus *A. discolor*, isolated from decayed wood in the rain forest of southern Chile (Tortella et al., 2008) was used in this study. The fungus was transferred from slant tubes (maintained at 4 °C) to glucose malt extract agar plates (15 g/L agar, 3.5 g/L malt extract, and 10 g/L glucose) and kept at 30 °C for 5–7 days before its use as inoculum. To immobilize the fungus, 30 g of wheat grains were put in a flask of 250 mL and moistened with 30 mL of distilled water and sterilized at 121 °C for 15 min. Then, the flask was inoculated with 5 agar disks (6 mm in diameter) of active mycelia of *A. discolor* from 5-day-old cultures on LBM medium (Tortella et al., 2008) and put in darkness at 25 °C for 6 days approximately (or until the mycelia covered completely the wheat grains). To evaluate the degree of immobilization, samples were analyzed using scanning electron microscope JEOL JSM-6380LV.

2.3. Columns operation

Columns were inoculated with white-rot fungus *A. discolor* previously immobilized on wheat grains. The colonized wheat grains were placed on the upper part of each column (5 cm). The columns were operated in continuous systems and were fed with the respective chlorophenol (100 mg/L) at a flow rate of 1.5 mL/min, at room temperature for approximately 29 days (until saturation point C/Co >0.7). Samples were taken from the effluent of the columns throughout the time and analyzed for phenols (2,4-DCP, 2,4,6-TCP, PCP), manganese peroxidase (MnP) and laccase (Lac) enzymes. All the experiments were carried out in duplicate and the average values were used for further calculations. Columns without inoculation with *A. discolor* were used as the control to evaluate the adsorption capacity of the soil columns. The evaluation of the column performance was conducted by plotting chlorophenol concentration in effluent to chlorophenol concentration in influent (C/Co) as a function of flow time (min).

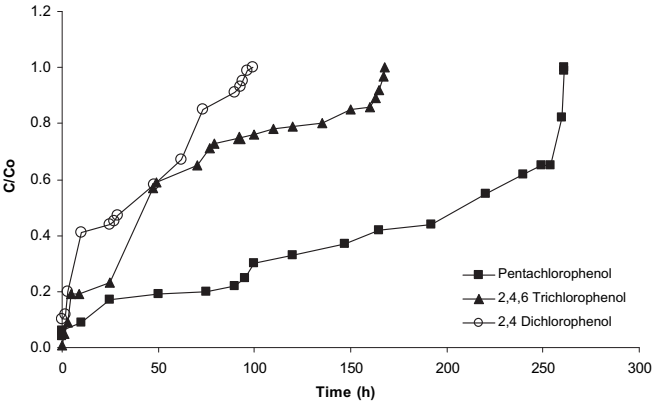

Fig. 1. 2,4-DCP, 2,4,6-TCP and PCP adsorption breakthrough curves in soil columns (pH 4.5) without inoculation with *A. discolor*.

2.4. Chlorophenol analyses

2,4-DCP, 2,4,6-TCP and PCP analyses were performed using HLPC, with a Merck Hitachi L-7100 pump, a Rheodyne 7725 injector with 20 μL loop diode array detector. The detection was set up at 215 nm and the column was a reverse phase (Lichrosphere 60RP select B, 5 μm, 4 mm diameter × 250 mm long). The mobile phase consisted of acetonitrile/phosphoric acid 1% (1:1 v/v) delivered at a flow rate of 1 mL/min, at room temperature (22 ± 1 °C). The sample was filtered (0.2 μm) before injection into the chromatograph.

2.5. Determination of enzyme activity

Laccase (Lac) was assayed as peroxide-independent degradation of 2,6- dimethoxyphenol (2,6-DMP) at pH 4.5 at 468 nm. The mixture contained 200 μL (250 mM, pH 4.5) of sodium malonate, 50 μL (20 mM) of 2,6-DMP, and 600 μL of supernatant (10 min at 5000 rpm) in a total volume of 1 mL. One laccase activity unit (U) was defined as the quantity of enzyme that produced 1 μmol of oxidized product per minute. Manganese peroxidase (MnP) activity was measured from the supernatant of a previously centrifuged sample (10 min at 5000 rpm). MnP activity was determined by monitoring the oxidation of 2,6-dimethoxyphenol (2,6-DMP) spectrophotometrically at 30 °C (Cecil CE 7200, UK). The reaction mixture (1 mL) contained 200 μL (250 mM, pH 4.5) of sodium malonate, 50 μL (20 mM) of 2,6-DMP, 50 μL (20 mM) of $MnSO4 \cdot H_2O$, and 600 μL of supernatant (10 min at 5000 rpm). The reaction was initiated by adding 100 μL (4 mM) of H_2O_2. The absorbance of the colored product was measured at 468 nm and corrected for Lac activity (Wariishi et al., 1992). One MnP activity unit (U) was defined as the amount of enzyme transforming 1 μmol of 2,6-DMP per minute.

3. Results and discussion

3.1. Chlorophenol breakthrough curves without inoculation

Fig. 1 shows chlorophenol (2,4-DCP, 2,4,6-TCP and PCP) adsorption breakthrough curves in soil columns (pH 4.5) without *A. discolor* inoculation. The soil column fed with 2,4-DCP solution was rapidly saturated (90 h), showing a lower adsorption capacity for this contaminant; by contrast, the columns fed with 2,4,6-TCP and PCP were saturated after 160 and 250 h, respectively. In Fig. 1, it is clearly stated that the total area under the PCP breakthrough curve is much higher than those of 2,4-DCP and 2,4,6-TCP, suggesting a higher affinity of PCP to allophonic soil under these experimental

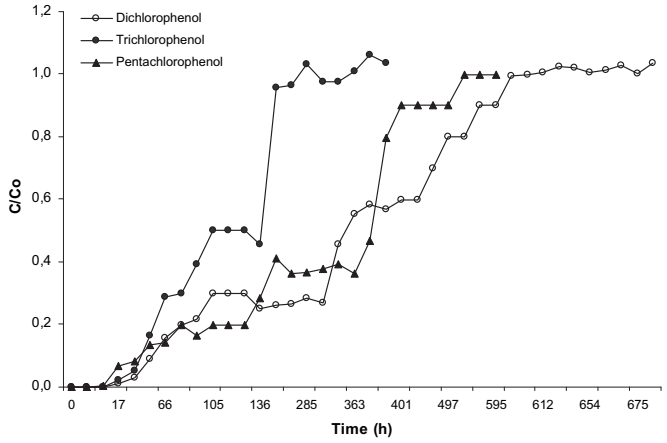

Fig. 2. 2,4-DCP, 2,4,6-TCP and PCP adsorption breakthrough curves in soil columns (pH 4.5) inoculated with *A. discolor*.

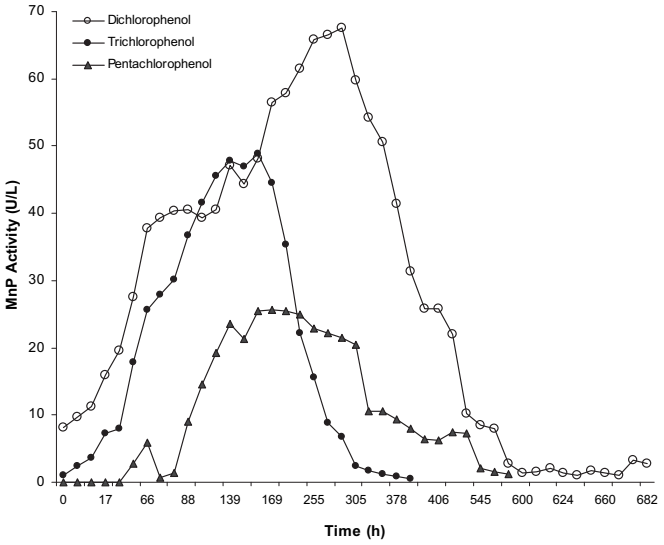

Fig. 3. Manganese peroxidase activity in soil columns (pH 4.5) inoculated with *A. discolor*.

conditions. Indeed, under the experimental pH condition (4.5), PCP should be present at about 50% in anionic form, and likely adsorbed completely (Diez et al., 1999). This is not the case for 2,4-DCP and 2,4,6-TCP pollutants, which are present in their non-ionic form at pH 4.5, leaching through the column and decreasing their adsorption. Previous studies have demonstrated that the presence of MnP and laccase enzyme activity in columns packed with allophonic soil without inoculation is not significant (Diez et al., 2006), suggesting that the removal of chlorophenols under these conditions may be associated mainly with adsorption processes.

PCP presents a high affinity with soil organic matter (14.6% in the allophonic soil used in this study), associated with its log K_{ow} value of 5.01 compared to the 2,4-DCP K_{ow} value of 3.08. The pKa values of PCP, 2,4,6-TCP and 2,4-DCP are 4.75, 6.15 and 7.85, respectively, with their adsorption being strongly affected by soil pH in allophanic soil (Diez et al., 1999; Cea et al., 2007).

Chlorophenol adsorption capacity of allophanic soil in batch processes has been studied under different operational and environmental conditions (Diez et al., 1999, 2005; Cea et al., 2005; Cea et al., 2007), and it has been demonstrated that its use is technically feasible with a high removal efficiency. Cea et al. (2007) described the adsorption capacity of this allophanic soil in three depths for 2,4-DCP and PCP, showing that PCP adsorption was higher than that observed for 2,4-DCP, and it decreased as organic matter fell with soil depth. The multiple regression analysis between *K*d and various soil properties showed that soil organic carbon content is a strong indicator of chlorophenol adsorption. In addition to organic carbon, pH is an important parameter controlling adsorption behavior (Cea et al., 2007). Columns assays using this allophanic soil for chlorophenol removal from contaminated wastewater have also been reported (Navia et al., 2003, 2005, 2006), and the operational conditions, the irrigation model, and the kinetic parameters have been established, leading to the conclusion that the adsorption rates are comparable to other adsorption systems and adsorbent materials.

3.2. Chlorophenol breakthrough curves with inoculation

Fig. 2 shows chlorophenol (2,4-DCP, 2,4,6-TCP and PCP) breakthrough curves in soil columns (pH 4.5) inoculated with the fungus *A. discolor* immobilized on wheat grains. The adsorption of the chlorophenols into the *A. discolor* mycelium was about 2%; therefore, it was not considered in the evaluation. In general, it can be observed that the operational time of the inoculated columns increased compared to the non-inoculated columns, showing the following trend: 2,4-DCP>PCP>2,4,6-TCP. The 2,4-DCP breakthrough curve shows high degradation of the contaminant compared with the 2,4-DCP breakthrough curve in the soil column without inoculation (Fig. 1). When using the inoculated column, 2,4-DCP degradation was constant between 100 and 320 h with C/Co of approximately 0.2 in this period of time. Then, the C/Co ratio increased until complete saturation of the column after 600 h of operation. The high 2,4-DCP removal was associated with degradation processes and with the high production of the ligninolytic enzyme manganese peroxidase (MnP) produced by fungus *A. discolor* (Fig. 3). MnP activity increased during the operation of the column, with the maximum value of 70 U/L being attained after 280 h.

The 2,4,6-TCP breakthrough curve in the column inoculated with the fungus *A. discolor* shows degradation of the contaminant (Fig. 3), but to a lesser extent than 2,4-DCP. The removal of 2,4,6-TCP was higher compared with the 2,4,6-TCP retention in the soil column without inoculation (Fig. 1). 2,4,6-TCP degradation was almost constant between 80 and 185 h with C/Co of approximately 0.4 in this period. Then, the C/Co ratio increased until complete saturation of the column after 270 h of operation. 2,4,6-TCP degradation using the inoculated column was associated with the production of the ligninolytic enzyme manganese peroxidase (MnP) produced by the fungus *A. discolor*. MnP activity increased during the operation of the column, with the highest value of 50 U/L being attained after 150 h.

The breakthrough curves of PCP in both columns (with and without inoculation) were similar until 150 h of operation (Figs. 1 and 2), indicating that the adsorption process for PCP removal is predominant in this period. After 150 h, PCP was degraded in the inoculated column, and the degradation remained constant between 150 and 350 h (C/Co of 0.4). The total saturation of this column was obtained after 420 h of operation. The degradation of PCP was associated with MnP production by the fungus during the column operation (Fig. 3). MnP activity increased during the operation of the column, with the maximum value of 30 U/L being attained after 300 h.

The enzyme activity of manganese peroxidase (MnP) and laccase during the operation of the inoculated columns are shown in Figs. 3 and 4. In general, it can be observed that MnP activity was

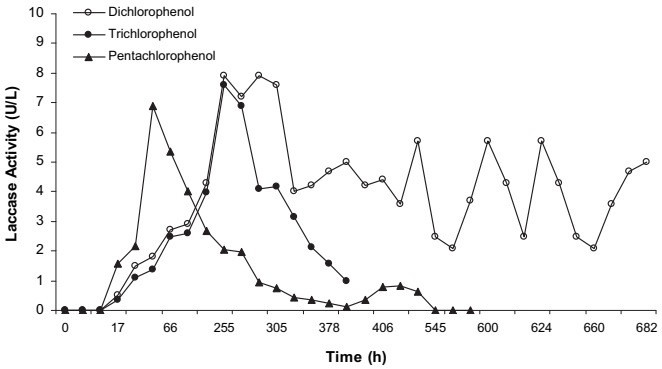

Fig. 4. Laccase activity in soil column (pH 4.5) inoculated with *A. discolor*.

higher (up to 70 U/L) when the column was fed with 2,4-DCP and lower (up to 30 U L^{-1}) when the column was fed with PCP. However, the highest MnP activity was obtained during the constant degradation period for the three chlorophenols. Laccase was also produced by the fungus but to a lesser extent, reaching less than 10 U/L values for the three chlorophenols.

The degradation capacity of white-rot fungi has been attributed to enzyme ligninolytic activity (Gianfreda and Rao, 2008). In our work, the highest MnP activity was obtained in the column fed with 2,4-DCP and was lower when the column was fed with PCP. This addition inhibited MnP production by *A. discolor* in the soil column; however, its degradation was not affected (Fig. 2). These results agree with the results obtained by Rubilar et al. (2007). The authors reported that the PCP degradation capacity of *A. discolor* was not affected when MnP activity decreased in a soil slurry culture, and that MnP activity was negatively affected (up to 75% reduction) when initial PCP concentrations were increased from 100 to 250 mg/kg of soil. The degradation of these compounds may also be attributed to the action of other cellular and extracellular fungal enzymes, such as phenoloxidases and cellobiose dehydrogenases, which may participate concomitantly with the ligninolytic enzymes in degradation processes (Montiel et al., 2004; Tortella et al., 2008).

Table 1 shows column data and parameters obtained for chlorophenols in the soil column with and without *A. discolor* inoculation. The equilibrium sorption capacity (q_{eq}) of 2,4-DCP was ten times higher when inoculated columns were used. For 2,4,6-TCP and PCP, the q_{eq} values were approximately two times higher when inoculated columns were used, while a similar situation was observed with respect to saturation time (t_p). The total removal efficiency (R) was almost two times higher for 2,4-DCP and 2,4,6-TCP when inoculated columns were used; however, it was found to be slightly lower in the case of PCP. Although there are no similar studies to this one in the literature for performing a proper comparison, we can show that Wu and Yu (2008) studied the biosorption of 2,4-DCP from aqueous solutions by immobilized *P. chrysosporium* biomass in a fixed-bed column. The authors found values of q_{eq} between 5.2 and 12.2 mg/g and, total removal capacity between 22.3 and 72.6 (%) depending on the flow rate (1.0–3.0 mL/min), influent concentration (20.9–80 mg/L) and bed depth (15–26 cm) used in their study.

Table 1
Column data and parameters obtained for chlorophenols (100 mg/L initial concentration) in the soil columns non-inoculated and inoculated with *A. discolor* (1.5 mL/min flow rate).

Column	Chlorophenol	q_{eq} (mg/g)	t_p (h)	R (%)
Non inoculated	2,4-DCP	1.69	90	30.3
	2,4,6-TCP	3.23	160	32.1
	PCP	9.46	250	61.2
Inoculated	2,4-DCP	20.25	600	56.1
	2,4,6-TCP	8.09	270	52.1
	PCP	16.5	420	46.2

q_{eq} = equilibrium sorption capacity.
t_p = saturation time.
R = total removal efficiency.

The use of *A. discolor* immobilized on wheat grains promoted chlorophenol degradation in the allophanic soil columns, coupled with ligninolytic enzyme production. The growth and colonization of wheat grains by *A. discolor* completely covered the lignocellulosic support after 7 days of incubation. The effect caused by wheat grains on fungus growth is probably due to their high content of carbohydrates and starch, which are not present in the other supports, and it provides a major source of energy for the fungus.

4. Conclusions

Chlorophenols were removed using allophanic soil columns with and without *A. discolor* inoculation by both adsorption and degradation processes. Chlorophenol degradation increased in allophanic soil columns inoculated with the fungus *A. discolor* immobilized on wheat grains, increasing the capacity of allophanic soil columns to adsorb and eliminate these contaminants. Equilibrium sorption capacity and retention time increased when inoculated soil columns were used. The higher degradation capacity of the inoculated soil columns was associated with the presence of the ligninolytic enzymes manganese peroxidase and laccase.

Acknowledgements

Research supported by FONDECYT 1050614 and DIUFRO GAP 2009–2010 projects.

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Journal of Environmental Management 95 (2012) S88–S92

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Aerobic granular SBR systems applied to the treatment of industrial effluents

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ARTICLE INFO

Article history:
Received 19 August 2009
Received in revised form
25 January 2011
Accepted 15 March 2011
Available online 7 April 2011

Keywords:
Granulation
Nitrogen removal
Organic matter
Sequential batch reactor

ABSTRACT

Four lab scale sequencing batch reactors (SBRs) were operated to remove organic matter and nitrogen from four different industrial wastewaters. The biomass grew in the reactors in the form of aerobic granules characterized by good settling properties. The high biomass concentrations achieved inside the reactors allowed reducing the solids concentration in the effluent down to 0.2 g VSS L^{-1}. The organic loading rates (OLR) applied to reactors ranged between 0.7 and 5.0 g COD L^{-1} d^{-1} with removal efficiencies of 60–95%. The nitrogen loading rates (NLR) applied varied between 0.15 and 0.65 g NH_4^+-N L^{-1} d^{-1} with variable removal efficiencies in the four systems (between 15% and 76%).

1. Introduction

The biological wastewater treatment is normally accomplished in the WWTPs (wastewater treatment plants) in conventional activated sludge systems, which generally require large surface areas for implantation and the presence of biomass separation units due to the poor settling properties of the sludge. Systems based on aerobic granular biomass are an alternative because the footprint of this technology is only 25% compared to that of the conventional activated sludge one (de Bruin et al., 2004). This is due to the fact that the reactor design and the properties of the biomass make unnecessary the construction of secondary settlers. Large organic and nitrogen loads can be treated in these systems. Furthermore simultaneous carbon, nitrogen and phosphorus removal is feasible (de Kreuk et al., 2005).

A number of factors can affect the granulation process (Liu, 2006), being one of them the type of substrate. Up to date, the results from several research works seem to indicate that the formation of aerobic granules is possible treating different substrates but evidence shows that the microbial structure and diversity of mature granules are closely related to them (Liu et al., 2003; Tay et al., 2001). The development of aerobic granular biomass has been studied treating different synthetic mediums using as carbon source ethanol (Beun et al., 1999), acetate (Chen et al., 2008), glucose (Tay et al., 2001) and phenol (Chiu et al., 2005). Furthermore the application of this technology to treat industrial wastewaters (Arrojo et al., 2004; Cassidy and Belia, 2005; Inizan et al., 2005; Schwarzenbeck et al., 2004; Schwarzenbeck and Wilderer, 2005; Wang et al., 2007) indicates that it is possible to grow aerobic granules with complex substrates. This is of interest for the industries that normally have limitations in the surface for the WWTP installation.

The main objective of this work was to study the feasibility of the development of aerobic granular systems to treat four different industrial effluents. Results will be compared in terms of physical properties of obtained granular biomass and of carbon and nitrogen removal efficiencies.

2. Materials and methods

2.1. Experimental set-up

Four SBRs, each one with a total volume of 2.5 L and a working volume of 1.5 L, were used. Dimensions of the units were: height of 465 mm and inner diameter of 85 mm. The height to the diameter ratio (H/D) being 5.5. The maximum level of the liquid was 264 mm,

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doi:10.1016/j.jenvman.2011.03.019

and the minimum level of 132 mm after effluent withdrawal. Oxygen was supplied to the reactors by using spargers to promote the formation of small air bubbles (Fig. 1). A set of two peristaltic pumps was used to feed (on top of the reactor) and to discharge the effluent (at medium height in the column reactor), respectively in each reactor. The exchange volume was fixed at 50%. A programmable logic controller (PLC) Siemens model S7-224CPU controlled the actuations of the pumps and valves and the length of every operational period in the SBRs.

2.2. Operational conditions

Reactors were operated at room temperature (15–20 °C) and at oxygen concentrations between 4 and 8 mg O_2 L^{-1}. The cycle of operation was of 3 h and distributed as follows according to Arrojo et al. (2004): 3 min of feeding, 171 min of aeration, 1 min of settling and 5 min of effluent withdrawal.

The reactors were fed with four different industrial effluents produced in: a laboratory for analysis of dairy products characterized by having a high concentration of suspended solids (R1), a fish canning industry with 30 g NaCl L^{-1} (R2), a plant processing marine products with a previous physical-chemical treatment (R3) and a pig farm characterized by its high organic matter and nitrogen content (R4). The composition of the feeding media used in each reactor is shown in Table 1. All the reactors were operated during 200 days except for R4 which was operated during 100 days.

Each reactor was inoculated with flocculent activated sludge of different origin: R1 and R3 inocula were collected from the WWTPs operated in the laboratory of analysis of dairy products (SVI = 200 mL(g VSS)$^{-1}$) and in the plant processing marine products (SVI = 125 mL(g VSS)$^{-1}$), respectively; R2 and R4 inocula were collected from two urban WWTPs with SVI values of 100 and 115 mL(g VSS)$^{-1}$, respectively.

2.3. Analytical methods

The ammonia, nitrate, nitrite, total suspended solids (TSS), volatile suspended solids (VSS) and sludge volumetric index (SVI) were determined according to the Standard Methods (APHA-AWWA-WPCF, 1998). Concentrations of total carbon (TC), total organic carbon (TOC) and inorganic carbon (IC) were measured with a Shimadzu analyser (TOC-500). Chemical oxygen demand (COD) was determined by a semi-micro method (Soto et al., 1989); total COD (COD_T) was measured directly in the sample and the soluble COD (COD_S) from the sample filtered through 0.45 μm pore size filters. The morphology and size distribution of the granules were measured regularly by using an Image Analysis procedure (Tijhuis et al., 1994) with a stereomicroscope (Stemi 2000-C, Zeiss). Biomass density, in terms of g VSS per litre of granules, was determined with dextran blue, which is not absorbed by the biomass (Jiménez et al., 1988) and following the methodology proposed by Beun et al. (1999).

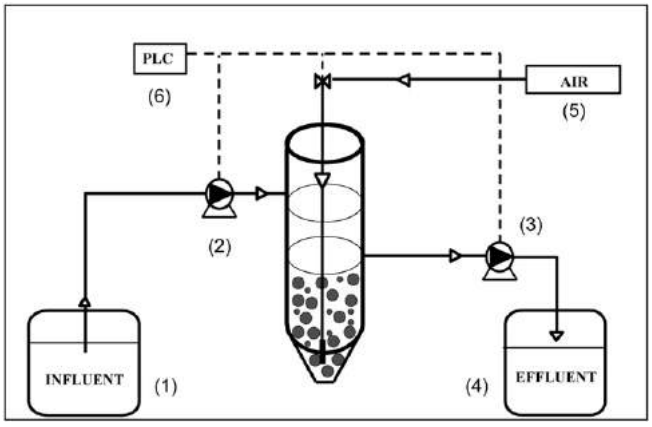

Fig. 1. Experimental set-up: (1) feeding tank; (2) feeding pump; (3) effluent pump; (4) effluent tank; (5) air; (6) PLC.

Table 1
Composition of the feeding media of each SBR.

Parameter	R1	R2	R3	R4
COD_T (mg O_2 L^{-1})	400–1750	350–530	500–1500	400–1550
COD_S (mg O_2 L^{-1})	200–1000	300–470	400–1300	375–1250
NH_4^+ (mg NH_4^+-N L^{-1})	25–185	40–70	50–150	70–220
TSS (mg TSS L^{-1})	100–900	30–200	30–150	70–520
VSS (mg VSS L^{-1})	50–700	20–70	20–100	60–200
pH	–	6.6–7.5	6.0–7.4	7.2–7.9

3. Results and discussion

3.1. Granule formation and properties

During the first days of operation an almost complete biomass washout occurred in the four reactors. This was a result of the operational strategy of the systems, in which a very short settling and a fast effluent withdrawal period were applied. Then the process of granules formation coincided apparently with that proposed by Beun et al. (1999).

In the four reactors the time needed for the granules development was different. In R1, three weeks after the start up, the formation of aggregates with 2.3 mm was observed. Mature granules of 3.5 mm of average diameter were measured on day 60. Granules were formed on day 45 in R2 characterized by small fibrous structures on their surfaces which gradually disappeared. Mature granules with an average diameter of 2.2 mm were only observed after day 75. In R3 on day 35 filamentous aggregates were observed with an average diameter of 2.97 mm that were grown and on day 91 had a diameter of 5.41 mm. Then these aggregates fell apart due to lysis and on day 144 mature granules were observed with 2.9 mm. In R4 the first aggregates were observed on day 11 with a diameter of 1.9 mm, but mature granules were obtained on day 50 with a diameter of 3.7 mm. These values are higher than those obtained by Cassidy and Belia (2005), who operated an aerobic granular sludge reactor treating an abattoir wastewater and obtained granules with a mean diameter of 1.7 mm during steady state operation (day 16 to day 76).

Once stable granules are formed (Fig. 2) larger biomass concentrations can be maintained inside the reactors. The physical properties of granules were very good in four cases, with density values between 15 and 60 g VSS($L_{granule}$)$^{-1}$ and SVI values between 30 and 60 mL(g VSS)$^{-1}$ (Table 2). Other authors obtained similar results of SVI (30–50 mL(g VSS)$^{-1}$) treating industrial wastewater (Schwarzenbeck et al., 2004; Schwarzenbeck and Wilderer, 2005; Wang et al., 2007). Taking into account that similar operational conditions were imposed in all cases the differences in the obtained values in the four reactors can be attributed to the kind of treated wastewater.

3.2. Solids concentrations and carbon and nitrogen removal

The settling time shortage during the start-up period temporarily increased the effluent solids concentration due to the floc-forming biomass washout. However, once the granular biomass was formed, the solids concentrations in the reactors increased

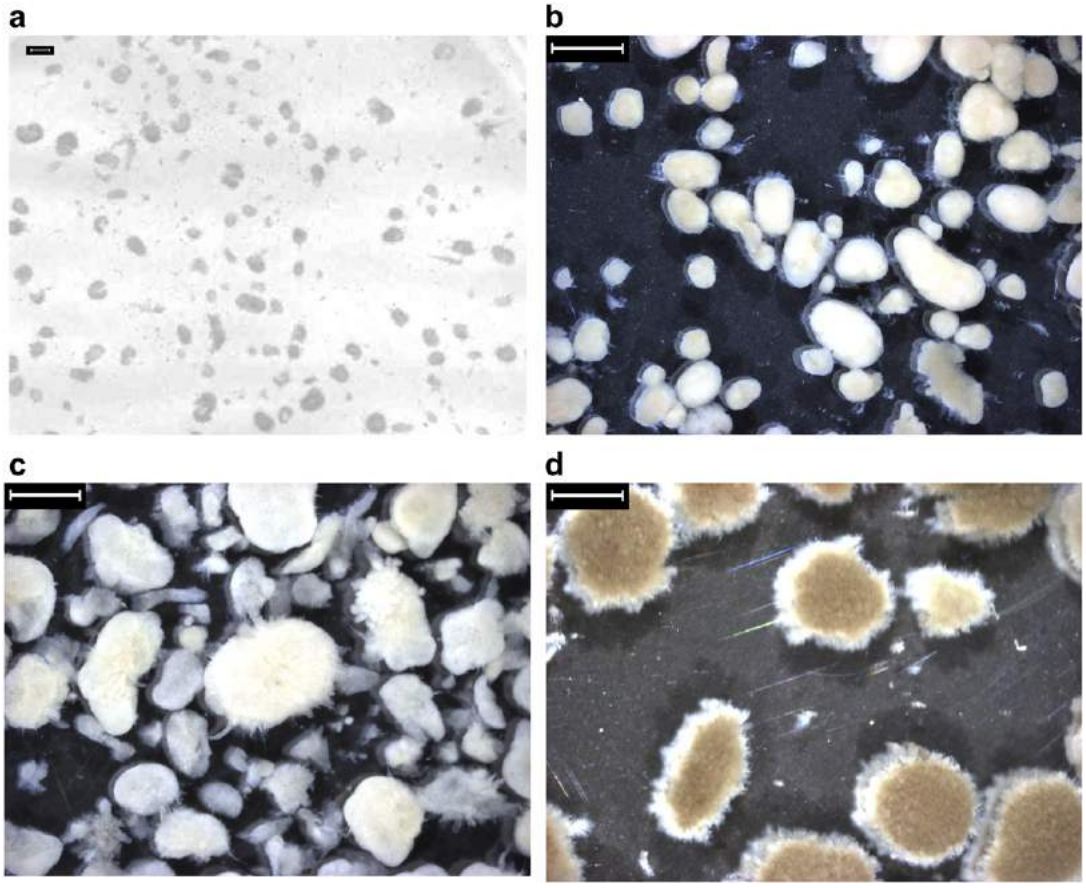

Fig. 2. Pictures of the aerobic granules in R1 day 72 (a), in R2 day 79 (b), in R3 day 144 (c) and in R4 day 51 (d). The size bar is 2 mm.

because aerobic granular systems promote better biomass retention, and the amount of solids in the effluent decreased to less than 0.2 g VSS L^{-1}, except in R4 where they were between 0.2 and 0.4 g VSS L^{-1} (Fig. 3a–d). The increase of solids in the effluent up to these values was caused by natural purge of biomass from the reactor, because the granules reached the level of the withdrawal port due to the biomass accumulation inside the reactor. In R2, R3 and R4 solids concentration inside reactor was larger than 10 g VSS L^{-1}, whereas in R1 the maximum reached value was of 5 g VSS L^{-1}. The solids concentrations followed a similar trend as the organic loading rates fed, as it can be observed comparing the figures a–e, b–f, c–g and d–h (Fig. 3), and the type of wastewater.

A comparison of the results obtained with these reactors, corresponding to stages with similar biomass concentrations inside each unit (5–6 g VSS L^{-1}), is resumed in Table 3. All the reactors achieved organic matter removal percentages between 80% and 93%. However the nitrogen removal efficiencies were more variable. Reactors fed with wastewaters from the laboratory of dairy products (R1) and piggery slurries (R4) treated higher nitrogen loads and achieved the best values in terms of nitrogen removal of 76% and 68%, respectively. In the case of R2 and R3 comparable values were obtained due to the similarity of the treated wastewater and, although the ammonia oxidation efficiency was 87% and 51%, respectively, the overall nitrogen removal was only 15% in both reactors. In the case of R2 this low value of the overall nitrogen removal was due to the lack of organic matter to accomplish the complete denitrification. In the case of R3 the limiting step was the low nitrifying capacity of the reactor which involved the accumulation of ammonia in the effluent and reduced the overall nitrogen removal by denitrification.

Table 2
Physical properties of granules in four SBR.

Reactor	Day of operation	Density (g VSS$(L_{granule})^{-1}$)	SVI (mL(g VSS)$^{-1}$)
R1	60	15	60
R2	90	60	30
R3	170	60	35
R4	100	44	37

The organic matter removal efficiency, expressed in terms of COD_S removal, was between 60% and 95% (Fig. 3e–h) and in accordance with other authors treating synthetic wastewater (Chen et al., 2008; Tay et al., 2002) and industrial wastewater (Cassidy and Belia, 2005; Inizan et al., 2005; Schwarzenbeck et al., 2004; Schwarzenbeck and Wilderer, 2005; Wang et al., 2007). The maximum OLR treated in each reactor with stable granular biomass was: 4 g COD L^{-1} d^{-1} in R1, 2 g COD L^{-1} d^{-1} in R2, 3 g COD L^{-1} d^{-1} in R3 and 5 g COD L^{-1} d^{-1} in R4. The loading rates treated in R1 and R4 were higher than those treated by Wang et al. (2007) using brewery wastewater (3 g COD L^{-1} d^{-1}), while the loading rates of R2 and R4 were similar to Cassidy and Belia (2005) treating abattoir wastewater (2.6 g COD L^{-1} d^{-1}).

Concentrations of the carbon and nitrogen compounds were periodically tracked in the liquid phase during selected operational cycles for each reactor in order to establish their profiles. As an example the profiles for R3 on the operation day 183 are shown in Fig. 4 (the trends in R1, R2 and R4 were similar, data not shown). The dissolved oxygen (DO) concentrations were in the first minutes

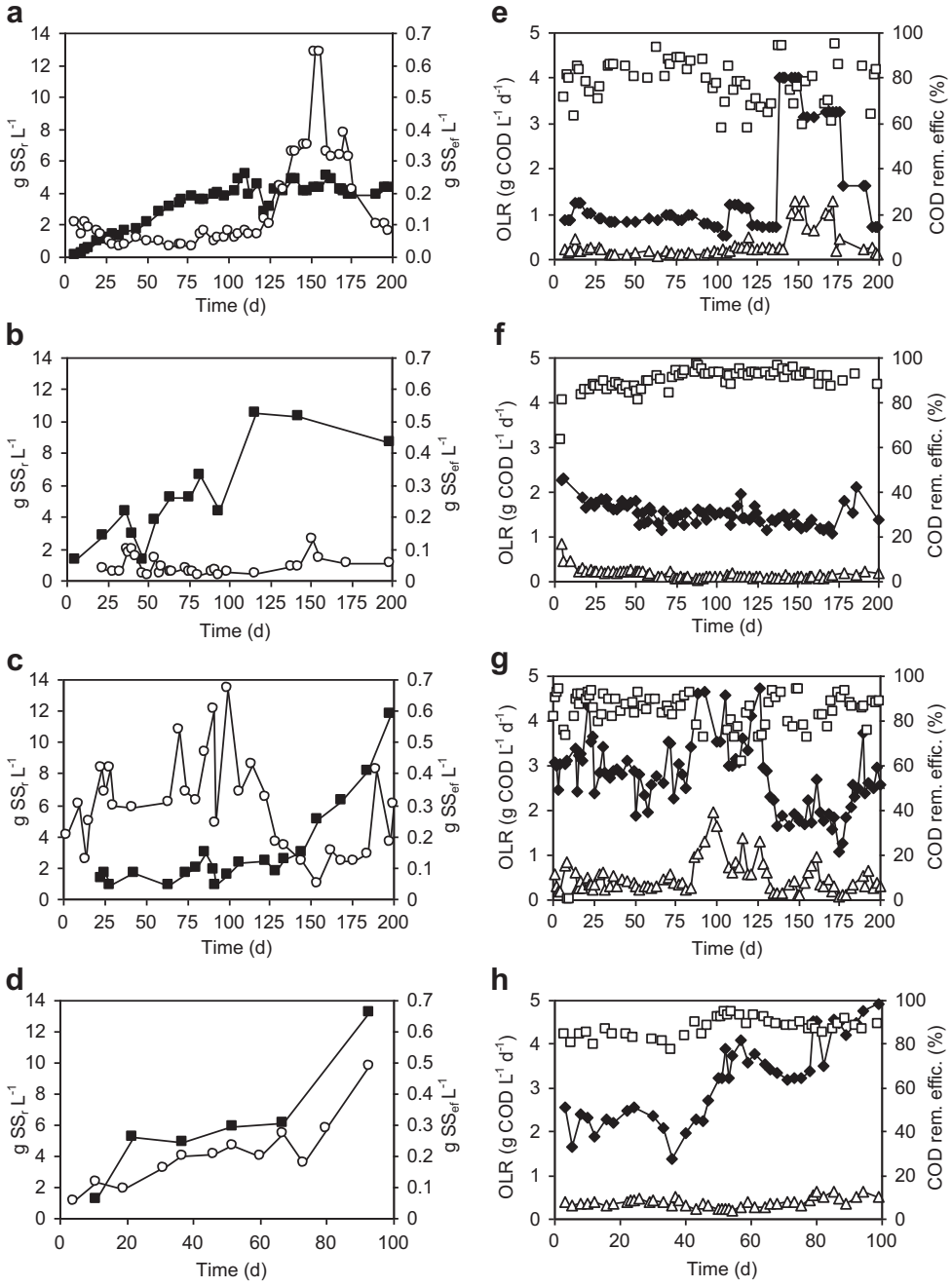

Fig. 3. Solids concentrations (VSS) in the reactor (■) and in the effluent (○) in R1 (a), R2 (b), R3 (c) and R4 (d). Organic loading rate in the influent (◆) and in the effluent (△), COD removal efficiency (□) in R1 (e), R2 (f), R3 (g) and R4 (h).

Table 3
Operational conditions in the four reactors when the biomass concentration was of 5–6 g $VSS_r\ L^{-1}$.

Reactor	R1	R2	R3	R4
Day operation	160	80	154	67
C/N ratio	5	8	8	6
g $VSS_{ef}\ L^{-1}$	0.30	0.02	0.05	0.27
OLR (g COD $L^{-1}\ d^{-1}$)	3.14	1.27	1.67	3.43
NLR (g NH_4^+-N $L^{-1}\ d^{-1}$)	0.60	0.15	0.22	0.62
% Organic matter removal	80	93	80	90
% N removal	76	15	15	68
% NH_4^+-N oxidized to NO_2^--N_{ef}	0	70	28	1
% NH_4^+-N oxidized to NO_3^--N_{ef}	4	2	8	28

(feast period) almost constant at values between 4 and 6 mg $O_2\ L^{-1}$, and during time left (famine period) near the saturation value (8 mg $O_2\ L^{-1}$). The biodegradable organic matter was almost fully removed at the beginning of the cycle (Fig. 4a) and the time of feast period for each reactor was approximately: 10 min R1, 20 min R2, 40 min R3 and 30 min R4 (data not shown). The amount of organic matter measured at the end of the cycle could be attributed to the fraction of slowly or non biodegradable substrate contained in the wastewater. Ammonia was oxidized to nitrite, and nitrite to nitrate, during the aerobic period immediately after the disappearance of biodegradable organic matter from the liquid phase. Nitrite and nitrate accumulated at the end of the cycle were consumed via denitrification during the first minutes of the next cycle while biodegradable organic compounds might be partly aerobically

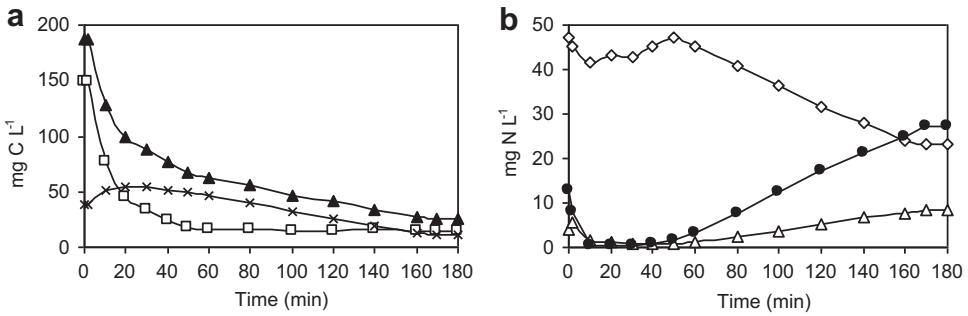

Fig. 4. (a) TC (▲), IC (×) and TOC (□) and (b) NH_4^+-N (◇), NO_2^--N (●) and NO_3^--N (△) concentrations for R3 during a cycle on day 183.

oxidized, partly used as electron donor for denitrification and partly stored in the biomass.

4. Conclusions

From the obtained results it can be concluded that production and operation of granular biomass is possible with different industrial effluents containing readily biodegradable organic matter.

In the four reactors granules with good settling properties were obtained (SVI around 60 mL(g VSS)$^{-1}$). In R2 and R3 the granular biomass reached similar values for SVI (30–35 mL(g VSS)$^{-1}$) and density (60 g VSS($L_{granule}$)$^{-1}$) since in both cases the treated wastewater was obtained from the seafood industry. These characteristics of biomass provided low solids concentrations in the effluent and, therefore, large biomass concentrations could be retained in the reactors.

In all the reactors combined removal of organic matter and nitrogen was achieved. Organic matter removal percentages of 60–95% were obtained with the four types of industrial wastewater, whereas the removal of nitrogen compounds was between 68% and 76% in R1 and R4 and of 15% in R2 and R3.

Acknowledgements

This work was funded by the Spanish Government (BIOGRAMEM project CTQ2005-04935/PPQ and NOVEDAR_Consolider CSD2007-00055) and the Xunta de Galicia (project coordinated by Espina y Delfin S.L. PGIDIT06TAM004). Authors want to thank Mar Orge, Mónica Dosil and Miriam Vieites for their support in the analytical techniques.

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Journal of Environmental Management 95 (2012) S93–S98

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Aerobic biodegradation of sludge with high hydrocarbon content generated by a Mexican natural gas processing facility

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ARTICLE INFO

Article history:
Received 30 September 2009
Received in revised form
22 March 2011
Accepted 28 April 2011
Available online 19 May 2011

Keywords:
Oil sludge
Biodegradation
Hydrocarbons
Biological treatment

ABSTRACT

The biodegradation of oil sludge from Mexican sour gas and petrochemical facilities contaminated with a high content of hydrocarbons, 334.7 ± 7.0 g kg^{-1} dry matter (dm), was evaluated. Studies in microcosm systems were carried out in order to determine the capacity of the native microbiota in the sludge to reduce hydrocarbon levels under aerobic conditions. Different carbon/nitrogen/phosphorous (C/N/P) nutrient ratios were tested. The systems were incubated at 30 °C and shaken at 100 rpm. Hydrocarbon removals from 32 to 51% were achieved in the assays after 30 days of incubation. The best assay had C/N/P ratio of 100/1.74/0.5. The results of the Microtox® and Ames tests indicated that the original sludge was highly toxic and mutagenic, whereas the best assay gave a final product that did not show toxicity or mutagenicity.

1. Introduction

A considerable quantity of oil sludge is generated during the processing chain in the petroleum industry, including exploration processes, crude oil production, transportation, storage, and refining (Xu et al., 2009). It has been estimated that for every 500 tons of crude oil processed, one ton of oil sludge waste is generated (van Oudenhoven et al., 1995). Oil sludge is a complex chemical mixture with dissimilar physicochemical properties, showing a wide range of toxicity levels.

Environmental regulations from various countries in Europe, Asia and America, including Mexico, consider oil sludge as a hazardous waste (Machín-Ramírez et al., 2008; Mater et al., 2006; NOM-052-SEMARNAT, 2005; Xu et al., 2009). The safe disposal of oil sludge is one of the main problems encountered by the oil industry.

Mexico is one of the major crude oil producing countries (Gallegos-Martínez et al., 2000). The Mexican oil industry generates an annual average of 31,000 t of oil sludge, mostly from refining and petrochemical processing (PEMEX, 2007).

Current technologies for oil contaminated soil are used to remediate oil sludge. These techniques include ultrasound, solidification, pyrolysis, photocatalysis, incineration, chemical treatments, heat cleaning, and extraction (Castañeda et al., 2001; Liu et al., 2009; Mater et al., 2006; da Rocha et al., 2010; Xu et al., 2009). Most of them, however, are considered costly or ineffective due to the high hydrocarbon concentration and waste complexity. Biological processes can offer a combination of low cost and efficiency (Semple et al., 2001). An assessment of their feasibility is required based on a determination of the extent to which they can biodegrade the contaminated sludge.

The nutrients needed for microbial activity must be supplied in a suitable proportion to achieve a balance related to the amount of carbon in the waste to be treated. Consequently, relevance should be given to a characterization of the residue (sludge, soil, sediment) and an assessment of the bioavailability of the nutrients. Furthermore, the most adequate C/N and C/P ratios for the degradation of these pollutants must be determined (Rojas-Avelizapa et al., 2007, 2006).

Some research has reported on the biological removal of total petroleum hydrocarbons (TPH) contained in sludge from the oil industry (Admon et al., 2001; Kuyukina et al., 2003). However, few studies have addressed the biological hydrocarbon removal from oil sludge or contaminated soils with concentrations above 100 g TPH kg^{-1}. Marín et al. (2006) described the utilization of a biopile system for the remediation of oil sludge with concentrations of up to 300 g TPH kg^{-1}, where an efficiency of 60% was achieved after a 3-month treatment period.

The difficulty in treating oil sludge lies in the complicated structure of the component blend, as well as its concentration, implying that each type of sludge has a specific problem that must be evaluated in order to determine treatment feasibility. For

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0301-4797/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jenvman.2011.04.014

example, the hydrocarbon and metal contents in sludge are variable and can affect the performance of biological systems (Sandrin and Maier, 2003). Moreover, changes in sludge toxicity caused by biological activity have hardly been evaluated (Mazlova and Meshcheryakov, 1999).

The aim of this research was to evaluate the biostimulation of native microbiota at different nutrient ratios for the potential treatment of oil sludge with a high content of hydrocarbons. Changes in organic extract toxicity caused by biological activity in the sludge were also examined.

2. Materials and methods

2.1. Sludge sample

Oil sludge was obtained from a natural gas processing facility located in Tabasco, Mexico. The residues of the facility are deposited in an uncovered lagoon where solids, the aqueous phase, and most of the oil are separated by gravity. Hydrocarbons floating on the surface of the lagoon are removed and placed in a storage tank, and the aqueous phase is pumped to a treatment plant prior to discharge outside the industrial facility. The untreated sediment remains in the lagoon and was the source of the oil sludge used in this work. Samples were collected in plastic containers and were transported and stored at 4 °C until use.

2.2. Biodegradability assays

The aerobic biodegradation assays were performed in 125 ml serum bottles with 15 g of sludge. Six biodegradability microcosm assays, as described in Table 1, were prepared in order to evaluate the effect of different C/N/P ratios. The bottles identified as MA, MB, MC and MD were supplemented with external nitrogen (NH_4Cl) and phosphorus (K_2HPO_4) in order to biostimulate the native microbiota, whereas those identified as MF and MG received only phosphorus. The addition of nutrients did not cause a considerable dilution of the sludge. No nutrients were added to the control assay (C), which was sterilized by autoclaving. The C/N/P ratio for this assay corresponds to the original N and P contents of the sludge. The bottles were sealed with acrylonitrile rubber stoppers and aluminium crimps, incubated at 30 °C and shaken at 100 rpm in an orbital shaker for a 30-day period. The microcosms were opened under sterile conditions once a day for 30 min to maintain the oxygen supply. Sampling took place at the onset (0 day) and at 15 and 30 days. Six bottles were prepared for each assay and two bottles were analysed on each sampling occasion. The total contents of the bottles were analysed for each assay. The moisture content, pH level, heterotrophic (HB) and hydrocarbon-degrading bacteria (HDB) and TPH contents were quantified.

2.3. Analytical methods

Enumeration of HB and HDB was performed by the plate-count method (Alef and Nannipieri, 1995) in selective media, according to Fernández et al. (2006). The entire sample from a bottle was manually mixed and 1 g was taken for microbial analyses. Nutrient agar was used for HB, and mineral medium with noble agar for HDB. The plates were incubated at 30 °C and counted on day 3 for HB and on day 5 for HDB.

In order to determine TPH contents, sludge samples (1 g dm) were taken from each bottle and extracted with dichloromethane by following a modified shaking/centrifugation method (Arce et al., 2004). The organic extracts were asphaltenes free and were precipitated by hexane. Concentrated samples (1 μl) were analysed by FID-gas chromatography (Agilent Technologies, model 6890) under the conditions described by Rojas-Avelizapa et al. (2006), but increasing the time in the last step to 25 min. Helium was used as the carrier gas at a flow rate of 1.4 ml min^{-1}. Injector and detector temperatures were set at 250 °C. The range of C-atom hydrocarbons present in the sludge was determined by the boiling point distribution using the ASTM D7169-05 method. The analysis of hydrocarbon fractions was achieved using the SARA method (ASTM D2007-03).

Total organic carbon was quantified using an IR Shimadzu (Rojas-Avelizapa et al., 2007), total nitrogen by the Kjeldahl method, total sulphur according to the ASTM D4294-10 method, phosphorus by the Bray–Kurtz method (Bray and Kurtz, 1945; Sims, 2000) and total metal contents were analysed by inductively coupled plasma–atomic emission spectrometry. The sludge samples required acid digestion prior to analysis using the EPA-6010C technique. The pH was measured in a suspension of 1 g sludge in 9 ml distilled water, using an Orion pH meter. Moisture levels were measured by the gravimetric method. All analyses were carried out in duplicate.

2.4. Toxicological evaluation

2.4.1. Preparation of organic extracts

A sample from the organic extracts obtained to determine the TPH content was taken for toxicological evaluation. Dichloromethane was removed from the organic extracts with N_2 gas (purity 99.9%). The pH was adjusted with 0.1 N NaOH to the levels required for the Microtox® and Ames assays. All organic extracts were stored in amber vials with Teflon-lined screw caps at 4 °C in the dark. The Microtox® and Ames tests were carried out by diluting extracts in 1% v/v dimethylsulphoxide (DMSO). The Microtox® bacterial reagent, the reconstitution solution, the diluents and osmotic adjusting solution were purchased from Azur Environmental.

2.4.2. Acute toxicity assays

The Microtox® assay is based on the use of the bioluminescent marine bacterium *Vibrio fischeri*. The light emitted by these bacteria is reduced upon exposure of the test organisms to an organic extract sample, and this reduction is directly related to the relative toxicity of hydrocarbons. The effective concentration for 50% inhibition (EC_{50}) of luminescence after 5 and 15 min incubation was calculated using Microtox® data software (Azur Environmental, 1998; Microbics Corporation, 1992). Phenol (Sigma–Aldrich) was used in a 100 mg ml^{-1} solution as a reference substance in order to monitor the quality of all bioassays.

2.4.3. Mutagenicity assays

The Ames test (*Salmonella*-his reversion test) for evaluating mutagenicity was performed following the Maron and Ames (1983) methods. This assay employed two histidine auxotroph mutant strains of *Salmonella typhimurium*: the TA98 strain detects frameshift mutations while the TA100 strain detects base pair substitutions. The tests were performed with and without S9 activation

Table 1
Codes used for the different assays with the respective C/N/P ratios tested.

Assays	C/N/P ratio	C/N ratio	C/P ratio
MA	100/6.9/0.75	14.49	133.3
MB	100/6.9/0.5	14.49	200.0
MC	100/3.0/0.75	33.33	133.3
MD	100/3.0/0.5	33.33	200.0
MF	100/1.74/0.75	57.47	133.3
MG	100/1.74/0.5	57.47	200.0
Control	100/1.74/0.21	57.47	476.2

C, N, P: Carbon, Nitrogen and Phosphorous.

(mouse liver microsomal suspension). Positive controls were developed using N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) and 2-aminoanthracene (2-AA).

2.5. Data analysis

The effects of the C/N and C/P ratios were statistically analysed by a variance analysis (ANOVA) and response surface methodology using STATISTICA V. 6 software. The hydrocarbon degradation results were also statistically analysed using Fisher's least significant difference (LSD) method to compare the means (Montgomery, 2001).

3. Results and discussion

3.1. Characterization of the sludge

Table 2 shows the results of the physicochemical characterization of the sludge. A high initial TPH concentration and high concentrations of iron, zinc, nickel, cadmium and sulphur were observed. The phosphorus content in the sludge was low compared to the optimal levels reported in the literature for bioremediation (Rojas-Avelizapa et al., 2007); hence phosphorus was added as phosphates to the assays. Although nitrogen was detected in the original sludge, uncertainty emerged regarding its bioavailability; therefore, NH_4 was added in some treatments.

Chromatographic analysis of the original sludge oil extract evidenced a complex mixture of different hydrocarbons; most of them were heavy compounds. The range of C-atom hydrocarbons contained in the oil sludge was from C_6 to C_{73}; the range from C_{23} to C_{36} was dominant (Fig. 1). SARA analysis revealed that the sludge contained 47.0% aromatic compounds, 45.73% saturated compounds, 5.47% resin (polar compounds) and 1.77% asphaltenes. The sludge also presented high metal and sulphur contents (Table 2). Some biodegradation studies with oil sludge have reported high concentrations of hydrocarbons (Marín et al., 2006; Ouyang et al., 2005); however, neither metal concentrations nor sulphur contents were reported, which are two elements known to negatively impact the degradation of hydrocarbons (Benka-Coker and Ekundayo, 1998; Sandrin and Maier, 2003).

3.2. pH and moisture evaluation

All oil sludge samples amended with different N and P amounts had initial pH values between 7.5 and 8, which decreased to 5.3–4 after 30 days of incubation. In contrast, the pH of the control remained unchanged. This decrease in pH might be attributed to the presence of microorganisms in the sludge capable of producing acids. In the original sludge the total sulphur content was 2.68% w/w (26.8 g kg^{-1}) and the sulphate concentration was 5.08 g kg^{-1}. After 30 days of incubation the sulphate concentration increased up to 64.8 g kg^{-1} (data not shown), suggesting the production of sulphuric acid. It is well known that some acidophilic microorganisms such as *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* can catalyse the oxidation of sulphide and elemental sulphur to sulphuric acid (Chazal and Lens, 2000; Kuenen et al., 1992).

Table 2
Characterization of the oil sludge before treatment.

Parameter	Concentration
Total petroleum hydrocarbons TPH (g kg^{-1} dm)	334.7 (±7.0)
Total organic carbon (g kg^{-1} dm)	234.3 (±3.1)
Total sulphur (% weight)	2.68 (±0.4)
Total nitrogen (g kg^{-1} dm)	4.08 (±0.17)
Total phosphorous (g kg^{-1} dm)	0.5 (±0.01)
Total iron (g kg^{-1} dm)	60.2 (±1.7)
Total chrome (g kg^{-1} dm)	0.2 (±0.01)
Total zinc (g kg^{-1} dm)	8.2 (±1.1)
Total nickel (g kg^{-1} dm)	0.1 (±0.03)
pH	7.82
Moisture (%)	73.9 (±0.9)

dm: dry matter.

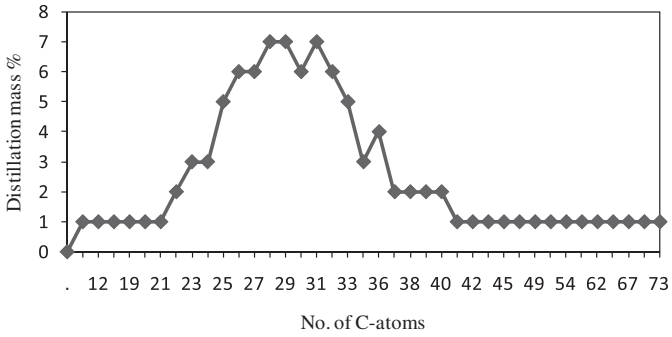

Fig. 1. Distribution of C-atoms of the hydrocarbon compounds in the oil sludge.

The pH level can also be influenced by the nitrogen source during hydrocarbon biodegradation; Wrenn et al. (1994) found that the pH level could dramatically decrease in cultures containing NH_4Cl but not in those supplied with KNO_3. Extreme pH values can adversely affect the activity of HDB populations and may explain why the hydrocarbon removal in our case was limited to 50–60%. Several reports recommended controlling pH values between 6 and 8 in order to maintain microbial hydrocarbon-degrading activity, a pH range that was found to be optimal for hydrocarbon degradation (Cunningham and Philip, 2000). Nevertheless, in our assays, a drastic decrease in the pH level was unexpected and was only seen at the end of the study before pH control could be implemented.

All systems had moisture contents of 75–77% and no significant modifications were noticed. These moisture values are in agreement with the 40–85% range suggested by von Fahnestock et al. (1998) for biological remediation systems.

3.3. Microorganisms

The microbial count is an indication of microorganism viability and the biodegradation potential in a contaminated system (Mishra et al., 2001). The native populations of HB and HDB in the oil sludge were (at the beginning) 7.1×10^5 and 7.08×10^4 CFU g^{-1} dm, respectively. After 15 days of treatment, all systems showed an increase from two to three orders of magnitude in the microbial count of both bacterial groups (data not shown), probably as a result of the nutrient supply and aeration.

After 30 days of treatment, all systems displayed a reduction in the microorganisms from one to three orders of magnitude compared to the number of microorganisms recorded at 15 days. This is most likely due to the drop in pH to 5.5 or below observed after 30 days in most of the microcosms.

3.4. Hydrocarbon removal

The most relevant response parameter in biodegradability tests is hydrocarbon removal (% TPH). Fig. 2 shows the percentage hydrocarbon removal from the different assays after 30 days of treatment. Control (C) exhibited a very low hydrocarbon removal (6.9%) and the assay did not produce CO_2. This difference was attributed to abiotic causes, likewise the loss of volatile compounds during the aeration period or the extraction process.

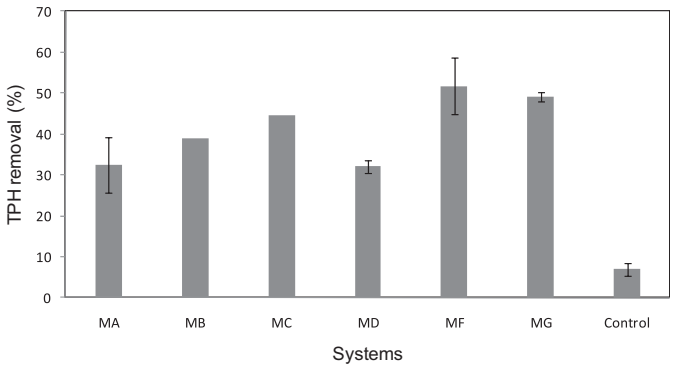

Fig. 2. Removal of the hydrocarbons present in the sludge and the control after 30 days of incubation.

The TPH contents in the oil sludge for all assays decreased between 32 and 51.7%. Assays MF and MG showed the highest TPH removal. The aeration, stirring and nutrient addition to the systems were adequate as they stimulated microbial activity.

The degradation rates (3.6–5.7 g TPH kg^{-1} dm d^{-1}) obtained in this study were equivalent or higher than data estimated by other authors (Admon et al., 2001; Lazar et al., 1999; Rojas-Avelizapa et al., 2007) who also utilized high hydrocarbon concentrations in their systems. However, other pollutants, such as metals and sulphur, were not reported in these cases. Marín et al. (2006) found that the treatment of a refinery oil sludge containing 280 g TPH kg^{-1} resulted in a hydrocarbon removal of 60% after 3 months. In this work, hydrocarbon biodegradation was performed in 30 days and TPH contents decreased up to 51%, even in the presence of a high metal concentration. Some authors found that Ni inhibited the biodegradation of organic compounds at concentrations ranging from 5.1 to 20 mg l^{-1}. The same effect was observed for Zn concentrations between 0.43 and 10 mg l^{-1} (Benka-Coker and Ekundayo, 1998; Sandrin and Maier, 2003). Nickel and zinc concentrations (Table 1) in our work exceeded the inhibitory values mentioned above.

The previous results suggest that hydrocarbon removal can probably be increased by controlling the pH of the assays, by adding other nutrients or bulking agents or by increasing the oxygen available and the treatment time.

Fig. 3 shows the hydrocarbon chromatographic profile for the MG assay at 0, 15 and 30 days. The decrease in TPH contents could mainly be attributed to the removal of light and medium weight hydrocarbon fractions.

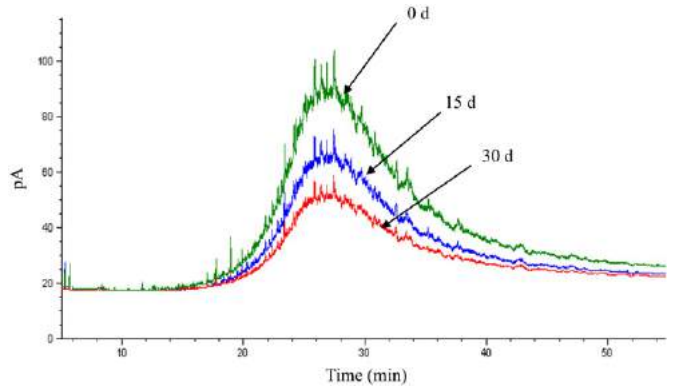

Fig. 3. Chromatogram of TPH for the biostimulation treatment of MG after 0, 15 and 30 days of the experiment.

Statistical analysis was performed in order to determine the major differences between the six assays and the control by using Fisher's least significant difference (LSD) test with a significance level of $\alpha = 0.05$ (Montgomery, 2001). The outstanding assays were MF and MG, which showed a significant difference when compared to the other treatments, but not between themselves. The least significant differences, calculated as removal percentages, were higher than the $LSD_{theoretical}$ (9.9).

Eq. (1) was obtained from the regression analysis:

$$\text{HC removal}(\%) = 25.0058 + 0.0987x + 0.1947y - 0.0001x^2 - 0.0026xy + 0.0081y^2 \quad (1)$$

where:

x : C/P ratio
y : C/N ratio

The analysis of variance (ANOVA) revealed that the main effect was given by the C/N ratio with a significant $p = 0.0125$ and $\alpha = 0.05$.

The response surface of hydrocarbon removal was plotted as a function of the C/N and C/P ratios (Fig. 4). The highest theoretical hydrocarbon removal (58%) was reached for a C/N/P ratio of 100/1.66/0.83, which was close to those obtained for the MF and MG assays after 30 days. This implies that the nitrogen in the original sludge was of a suitable amount and was available for hydrocarbon removal. In contrast, the analysis indicated that the best result was obtained from the lowest C/P ratio; therefore, the addition of phosphorus was necessary for treating this kind of oil sludge.

3.5. Toxicological evaluation

The assay with the highest hydrocarbon removal rate (MG) and the controls were subjected to a toxicological evaluation. In order to determine the level of toxicity, the results from the assays were

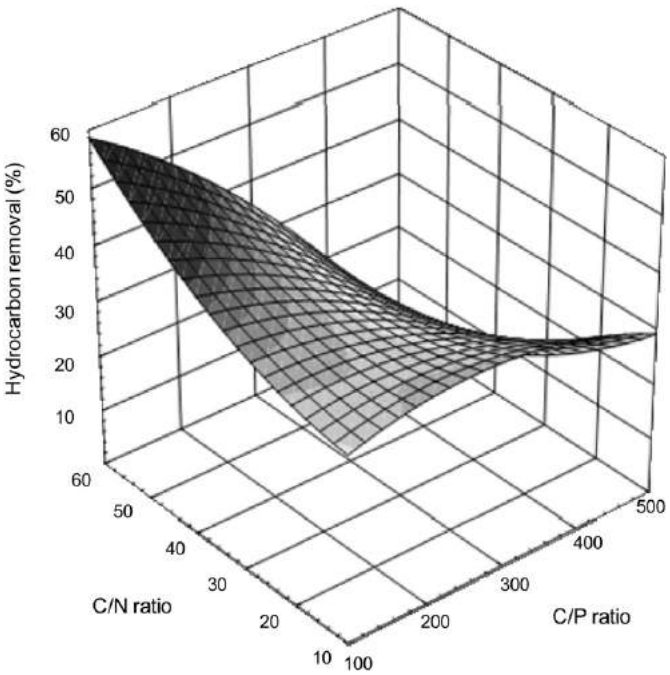

Fig. 4. Response surface plot of hydrocarbon removal as a function of C/N and C/P ratios after 30 days of incubation.

Table 3
Toxicity of the organic extracts of the oil sludge after 30 days of treatment.

Organic extract	Microtox® EC_{50} Toxic effect (%)		Mutagenic potential[a]	
			TA98	TA100
	5 min	15 min	S9	S9
MG	4.385	2.1	Negative	Negative
Control	0.0202	0.0203	Positive	Negative

[a] Strains TA98 and TA100 of *Salmonella typhimurium* that have mutations in genes involved in histidine synthesis.

referred to effect categories: according to Brower et al. (1990), EC_{50} values higher than 2% denote non-toxic effects, 1–2% uncertain toxicity and 0.75–0.99% lower toxicity. Additionally, values from 0.5 to 0.74% are considered as low toxicity, 0.25–0.49% toxic and 0.0–0.24% very toxic (Bennett and Cubbage, 1992).

The control assay showed EC_{50} values of 0.0202% at 5 min and 0.0203% at 15 min; thus the sludge was very toxic at the start of this study in all systems. After 30 days, the EC_{50} values of the MG assay were 4.38 and 2.10% (5 and 15 min, respectively); thus the oil sludge became non-toxic (Table 3).

The control sludge disclosed a mutagenic potential with the *S. typhimurium* TA98 strain at a concentration of 120 mg TPH l^{-1} with metabolic activation (S9) at the beginning of the experiments and after 30 days of incubation. The MG assay showed mutagenic potential with the TA98 strain at time zero. However, no mutagenic effects were detected with TA98 and TA100 at day 30. It is important to emphasize that the *S. typhimurium* TA98 strain has been used to detect mutagenic agents such as aromatic compounds (Maron and Ames, 1983). The findings of this work suggest that mutagenic compounds present in the sludge could be transformed to non-mutagenic forms along the biodegradation process. Furthermore, biodegradation studies should be complemented with toxicity and mutagenic tests to make sure that the biodegradation products are less toxic to the environment than the original sludge.

4. Conclusions

The results obtained showed that the native microorganisms present in the oily sludge can be used to treat this type of waste with high heavy hydrocarbon and metal contents. An adequate balance of nutrients and oxygen favourably affects hydrocarbon mineralization. A TPH removal of 173 g kg^{-1} dm was achieved and the degradation rates reached 3.6–5.7 g TPH kg^{-1} dm d^{-1}. The toxicological analysis showed that the biological treatment generated end products that had no toxic or mutagenic effects.

Acknowledgements

This research was supported by the IMP-D.00411 Project “Alternativa potencial para tratamiento biológico y disposición final de lodos de instalaciones PGPB”.

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Journal of Environmental Management 95 (2012) S99–S103

Contents lists available at ScienceDirect

Journal of Environmental Management

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Conversion of phenols during anaerobic digestion of organic solid waste – A review of important microorganisms and impact of temperature

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ARTICLE INFO

Article history:
Received 25 September 2009
Received in revised form
10 August 2010
Accepted 7 October 2010
Available online 3 November 2010

Keywords:
Anaerobic degradation
Digestate
Microbial community
Phenol
Potential ammonia oxidation assay
Soil
Temperature

ABSTRACT

During anaerobic digestion of organic waste, both energy-rich biogas and a nutrient-rich digestate are produced. The digestate can be used as a fertiliser in agricultural soils if the levels of hazardous compounds and pathogens are low. This article reviews the main findings about phenols in anaerobic digestion processes degrading organic solid wastes, and examines the effect of process temperature on the anaerobic degradation of phenols, the microbial community and the quality of the digestate. The degradation efficiency of a number of different phenols has been shown to be correlated to the process temperature. Higher degradation efficiency is observed at mesophilic process temperature than at thermophilic temperature. Possible explanations for this variation in the degradation of phenols include differences in diversity, particularly of the phenol-degrading bacteria, and/or the presence of temperature-sensitive enzymes. Chemical analysis of digestate from bioreactors operating at thermophilic temperature detected a higher content of phenols compared to mesophilic bioreactors, verifying the degradation results. Digestate with the highest phenol content has the greatest negative impact on soil microbial activity.

1. Introduction

Anaerobic digestion of different organic solid wastes results in a digestate rich in plant nutrients (N, P, K and Mg) that is suitable for use as a fertiliser in agricultural soil. Digestate has been shown to have positive effects on the soil quality by improving the soil structure, increasing the water-holding capacity and stimulating the microbial activity (Debosz et al., 2002; Marinari et al., 2000; Arthurson, 2009). Furthermore, fertilisation with digestate is reported to give higher crop yields and better grain quality in comparison with unfertilised soil, and equivalent effects after the application of artificial fertiliser (Arthurson, 2009). To maintain the long-term fertility of the soil and to avoid risks during the production of food and feed, it is important that the digestate contains adequate nutrient levels, however no pathogens or hazardous compounds such as various organic pollutants. Organic contaminants, if not sufficiently degraded in the anaerobic digestion process, can result in a digestate with a lower value as a fertiliser.

Organic material used for biogas production, such as organic industrial wastes, animal manure and organic household wastes, may contain a variety of organic pollutants. Compounds shown to be present in such materials and in digestate include dioxin-like compounds (Brändli et al., 2007c; Engwall and Schnürer, 2002; Olsman et al., 2002, 2007), polyaromatic hydrocarbons (PAH) (Angelidaki et al., 2000; Brändli et al., 2007a, 2007b), polychlorinated biphenyls (PCBs) and pesticides (Brändli et al., 2007a, 2007b; Nilsson, 2000), chlorinated paraffins (Brändli et al., 2007c; Nilsson et al., 2001), phthalates (Angelidaki et al., 2000; Brändli et al., 2007c; Hartmann and Ahring, 2003; Nilsson et al., 2000) and phenolic compounds (Angelidaki et al., 2000; Levén et al., 2006; Levén and Schnürer, 2005; Wu et al., 1999). The presence of these compounds can negatively affect the microorganisms in the anaerobic digestion process and also result in digestate not suitable for use as a fertiliser in agricultural soils. This paper reviews the main findings about phenols in anaerobic digestion processes degrading organic solid wastes in terms of: (i) the effect of temperature on phenol degradation capacity and pathways; (ii) the effect of temperature on anaerobic microbial community diversity and its correlation to phenol degradation efficiency; and (iii) the effect of phenol content in the digestate on soil microbial activity.

2. Sources of phenols in digestate

Phenols are widespread compounds and occur for example in different industrial wastewaters (Khardenavis et al., 2008; Veeresh

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doi:10.1016/j.jenvman.2010.10.021

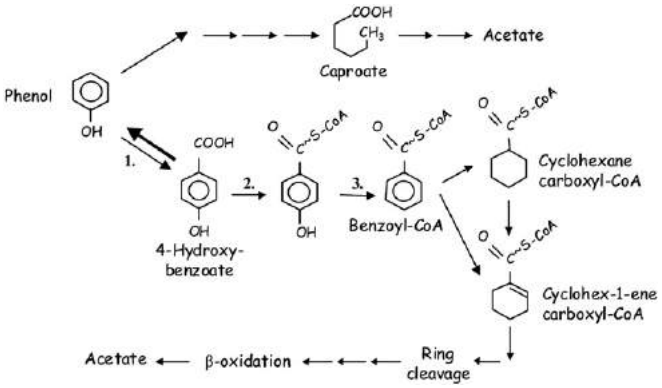

Fig. 1. Two alternative degradation pathways for phenol under methanogenic conditions, either via caproate or via 4-hydroxybenzoate and the benzoyl-CoA pathway (Elshahed et al., 2001; Fang et al., 2006; Li et al., 2000). Enzymes involved in transformation of phenol; 1. phenol carboxylase/4-hydroxybenzoate decarboxylase; 2. 4-hydroxybenzoate-CoA ligase; 3. 4-hydroxybenzoate-CoA reductase.

et al., 2005) and sewage sludges (Angelidaki et al., 2000). They have also been found in digestate from large-scale and laboratory-scale bioreactors anaerobically treating different types of organic solid wastes, mainly slaughter house waste, animal manure and food wastes (Levén et al., 2006; Levén and Schnürer, 2005). The occurrence of phenols in anaerobic digestate can be due to the biodegradation of different xenobiotic compounds, such as pesticides, but also due to biodegradation of naturally occurring aromatic amino acids and aromatic polymers, e.g. humic acids, lignins and tannins, in plant materials (van Schie and Young, 1998). Swine manure has also been identified as one likely source of phenols, as it is known to contain phenolic compounds (Wu et al., 1999) and the phenol content in digestate has been shown to be higher with a higher input of swine manure (Levén et al., 2006). In the future, a major source of substrate for biogas production is likely to be second generation substrates such as straw or energy forest. To increase the biogas production from these materials, different pre-treatment techniques are often needed. However, such pre-treatment can result in the production of e.g. a variety of phenols (Chen et al., 2006; Klinke et al., 2004). These compounds can affect downstream microbial processes and, if they are not degraded, the quality of the digestate. Negative effects of phenols on microbial activities have been reported for several different microorganisms (Dyreborg and Arvin, 1995; Hernandez and Edyvean, 2008; Levén et al., 2006; Olguin-Lora et al., 2003; Varel and Miller, 2001), illustrating the toxicity of these compounds.

3. Anaerobic degradation of phenol and the effect of temperature

Under methanogenic conditions, the mineralisation of phenols can proceed through different pathways and requires a consortium of various microorganisms. As an example, two possible pathways have so far been reported for phenol; either via 4-hydroxybenzoate into the benzoyl-CoA pathway or via caproate to acetate (Fig. 1). The degradation of phenol to benzoate is well documented and has been demonstrated for several methanogenic consortia (Béchard et al., 1990; Chen et al., 2008; Fang et al., 2004; Karlsson et al., 1999; Knoll and Winter, 1989; Kobayashi et al., 1989; Levén et al., 2006; Levén and Schnürer, 2005, 2010; Sharak Genthner et al., 1991) and isolates of bacteria (Juteau et al., 2005; Qiu et al., 2008). However, although caproate has been identified as an intermediate, neither the bacteria responsible for the production nor the degradation pathway are currently known (Fang et al., 2006). Previously described methanogenic consortia and isolated bacteria degrading phenol are listed in Tables 1 and 2.

Anaerobic degradation of phenol has been reported to occur at both mesophilic (37 °C) and thermophilic temperatures (55 °C) (Chen et al., 2008; Fang et al., 2006; Karlsson et al., 1999; Levén et al., 2006; Levén and Schnürer, 2005). However, the majority of the known phenol-degrading consortia and the isolated bacteria are mesophilic (Béchard et al., 1990; Juteau et al., 2005; Karlsson et al., 1999; Knoll and Winter, 1989; Kobayashi et al., 1989; Levén and Schnürer, 2010; Qiu et al., 2008; Sharak Genthner et al., 1991). Investigations of phenol degradation have shown effects of temperature on both the degradation efficiency and the degradation pathway. Comparisons of degradation at mesophilic and thermophilic temperature have revealed a strong influence of temperature, with more efficient phenol degradation in anaerobic organic waste digestion processes at the mesophilic temperature (Levén et al., 2006; Levén and Schnürer, 2005). These degradation studies have shown that no or very slow degradation, often with a long lag phase, occurs for both phenol and *p*-cresol at the thermophilic temperature, while both compounds are quickly converted to methane at the lower temperature. Furthermore, lowering of temperature from a thermophilic to a mesophilic temperature triggers the degradation of both phenols and enhances the degradation rate by the microbial consortia. A more detailed study of the importance of temperature has revealed that phenol degradation rate is stimulated when the temperature is lowered below 50 °C (Levén and Schnürer, 2005, 2010). Chemical analysis of digestate from different bioreactors anaerobically treating organic solid wastes has confirmed this difference in degradation of phenols, with a lower content of phenols after digestion at mesophilic temperature (Levén et al., 2006; Levén and Schnürer, 2005).

Temperature has previously been shown to have an impact also on the degradation pathway of phenol in different methanogenic systems (Fang et al., 2006; Karlsson et al., 1999; Levén and Schnürer, 2005). This might be a possible explanation for the difference in degradation efficiency of a number of different phenols observed during anaerobic digestion of solid organic waste (Levén and Schnürer, 2005). At mesophilic temperature, benzoic acid is commonly seen as an intermediate during degradation of phenol,

Table 1
Important populations in seven different phenol-degrading methanogenic consortia. UASB = Upflow anaerobic sludge blanket reactor, WWTP = wastewater treatment plant, CSTR = continuous stirred tank reactor, OMSW = organic municipal solid waste, GAC-AFB = granular activated carbon-anaerobic fluidized bed. 4-OHBa = 4-hydroxybenzoate.

Important populations	Origin	Temperature[a] (°C)	Substrate[b]	References
Desulfotomaculum subcluster Ih	UASB treating phenol-containing wastewater	26	Phenol	Zhang et al., 2005
Clostridia, *Syntrophorhabdaceae*	UASB treating phenol-containing wastewater	55	Phenol	Fang et al., 2006
Syntrophorhabdaceae	WWTP[c]	37	Phenol, benzoate, terephthalate	Chen et al., 2008
Desulfotomaculum subcluster Ih	WWTP[c]	55	Phenol, benzoate, terephthalate	Chen et al., 2008
Syntrophorhabdaceae Syntrophus	Full-scale GAC-AFB treating phenolic wastewater	35	Phenolic wastewater	Chen et al., 2009
Syntrophorhabdaceae	Mesophilic CSTR treating OMSW[c]	37	Phenol, 4-OHBa, benzoate	Levén and Schnürer, 2010
Desulfotomaculum subcluster Ih	Thermophilic CSTR treating OMSW[c]	37	Phenol, 4-OHBa	Levén and Schnürer, 2010

[a] Process temperature.
[b] Substrate used for enrichment of methanogenic consortia.
[c] Enrichment cultures.

Table 2
Phenol-degrading bacteria isolated from methanogenic environments.

Isolate	Origin	T opt[a] (°C)	Substrate[b]	References
Sedimentibacter hydroxybenzoicum	Freshwater sediment sample	33–34	Phenol, catechol, 4-OHBa	Zhang et al., 1994
Cryptanaerobacter phenolicus	Culture mix of swamp water, sewage sludge, swine waste, soil	30–37	Phenol, 4-OHBa	Juteau et al., 2005
Syntrophorhabdus aromaticivorans	Terephthalate manufacturing wastewater treatment plant	35–37	Phenol, p-cresol, benzoate, 4-OHBa, isophthalate	Qiu et al., 2008

[a] Temperature optimum for growth.
[b] Substrate utilized by the bacterium.

whereas it has not been detected at 55 °C. (Fang et al., 2006; Karlsson et al., 1999; Levén et al., 2006). This indicates the simultaneous turnover of benzoate or an alternative pathway. Fang et al. (2006) suggested that phenol is transformed via caproate instead of benzoate at thermophilic temperature.

4. Effect of temperature on microorganisms and enzymes

The previously shown temperature effect on the degradation capacity with a higher degradation capacity at the lower temperature likely has a biological explanation (Levén et al., 2006; Levén and Schnürer, 2005). Since phenol degradation is dependent on a consortium of microorganisms and sometimes also on unknown growth factors produced by some microorganisms, differences in the general microbial diversity (Levén et al., 2007) can be one reason for the difference in the degradation capacity (Levén and Schnürer, 2005). Only a few studies have isolated the impact of temperature on the microbial community by using two bioreactors that use the same substrate and similar performance, except for the temperature (Hernon et al., 2006; Levén et al., 2007; Pender et al., 2004; Sekiguchi et al., 1998). In all the studied anaerobic bioreactors, the microbial diversity of both *Bacteria* and *Archaea* is reported to be comparably lower at thermophilic temperature than at mesophilic temperature (Hernon et al., 2006; Karakashev et al., 2005; Levén et al., 2007; Pender et al., 2004; Sekiguchi et al., 1998). Furthermore, the phlyogenetic distribution of the microbial populations has clearly been shown to be affected by temperature (Ariesyady et al., 2007; Chouari et al., 2005; Dollhopf et al., 2001; Fang et al., 2006, 2004; Hernon et al., 2006; Levén et al., 2007; Sekiguchi et al., 1998; Weiss et al., 2008).

Temperature not only influences the community structure in general, but also the methanogenic consortia degrading phenol. A great difference in the microbial community structure has been observed between methanogenic consortia degrading phenol at different temperature (Chen et al., 2008). In the study of Chen et al. (2008), one conceivable phenol degrader belonging to the *Syntrophorhabdaceae* and one to the subcluster Ih within *Desulfotomaculum* was found at mesophilic and thermophilic temperature, respectively (Table 1). Similar results are shown by Levén and Schnürer (2010). These clusters have been shown to be two important groups of bacteria capable of degrading aromatic compounds, in particular phenols and phthalates, under methanogenic conditions (Chen et al., 2009, 2008; Fang et al., 2006; Fang et al., 2004; Levén and Schnürer, 2010; Qiu et al., 2004; Zhang et al., 2005).

Yet another possible explanation for the differences in the degradation capacity could be that some enzyme(s) involved in the degradation of phenol to benzoate are temperature-sensitive. Temperature has previously been shown to affect enzyme activities and the degradation of different aromatic compounds such as chlorophenols and PCBs (Kohring et al., 1989; Wu et al., 1996). The impact of temperature on enzyme activity is difficult to evaluate for phenol degradation, since one of the possible temperature-sensitive enzymes (phenol carboxylase/4-hydroxybenzoate decarboxylase) in phenol degradation has a strong reversible activity and thus it is difficult to measure (Li et al., 2000). However, results from NMR (nuclear magnetic resonance) analyses performed with washed, dense cell cultures supported the hypothesis that the inability of the thermophilic community to degrade phenol above 48 °C was due to temperature inactivation of one of the initial enzymes in the degradation pathway (Levén and Schnürer, 2005).

5. Effect of phenols on methanogens and soil microbial functions

Phenols that are introduced or produced in the anaerobic digestion process can cause problems due to their inhibition of acetate-utilising methanogens (Hernandez and Edyvean, 2008; Olguin-Lora et al., 2003). In a digestate from a Swedish biogas plant treating a high proportion of swine manure, the phenol concentration (274 mg/kg digestate) was found to be in the same range as the minimal inhibitory concentration for these methanogens (Levén et al., 2006).

In addition to negative effects in the anaerobic digestion process, the addition of hazardous compounds to the soil by the application of digestate as fertiliser can also pose a serious threat to microbial functions and subsequently the productivity and sustainable use of the soil (Pell and Torstensson, 2002). One way to investigate the effects of such toxic compounds on the microbial transformation of carbon and nitrogen in soil is to use different microbial assays as stress indicators (Pell and Torstensson, 2002). One such assay is the PAO (potential ammonia oxidation) assay, which has frequently been used for studying the effects of different pollutants applied to the soil, as well as different agronomic treatments (Chang et al., 2001; Hastings et al., 1997; Nyberg et al., 2004; Pell et al., 1998; Petersen et al., 2003).

One difficulty in investigating the effects of digestate application to the soil is that it causes a general stimulation of soil microorganisms due to the presence of inorganic nutrients and organic matter (Petersen et al., 2003). This stimulation might hide the underlying effects of pollutants on more specific microbial groups (Nyberg et al., 2004). However, by extracting the organic fraction of the digestate and then using the extract in soil microbial tests, it is possible to isolate the effects of the pollutant (Levén et al., 2006; Nyberg et al., 2004). Using this approach, organic extracts of digestate from large-scale bioreactors, as well as from swine manure, have been shown to inhibit the activity of ammonia oxidising bacteria (AOB) in soil, indicating the presence of toxic organic substances (Levén et al., 2006; Nyberg et al., 2006, 2004). There is a clear positive relationship between the degree of inhibitory effects on AOB activity and the content of phenols in the digestate (Levén et al., 2006). This correlation is further supported by the fact that the addition of pure phenols to the soil causes similar inhibitory effects (Levén et al., 2006). For some digestate samples the amount of phenols in the soil after application of digestate are on the same level as the guideline value (1.5–5 $\mu g\ g^{-1}$ dry weight of soil) set by the Swedish Environmental Protection Agency for phenol and cresol in contaminated soil (http://www.naturvardsverket.se, 2010-06-28).

This value should not be exceeded due to the potential risk for human health and/or the environment. Therefore, to avoid inhibitory effects in the anaerobic digestion process and in the soil, it is important that phenolic compounds are efficiently removed in the anaerobic degradation process. Alternatively, the levels of phenols can be controlled by regulation of the combination of substrates that are treated in the biogas process.

6. Summary

The process temperature in anaerobic digestion processes degrading organic solid waste has been shown to have a strong impact on the degradation of a number of phenols, with higher degradation efficiency at mesophilic temperatures than at thermophilic. As a consequence, digestate from thermophilic processes generally contains comparatively higher levels of phenols, if phenols are present in the anaerobic digestion process. Digestate with high phenol content cause inhibition of AOB activity in soil, implying risks for environmental disturbances. One possible explanation for the limited degradation of phenols at the higher process temperature is the presence of different phenol-degrading consortia at different temperatures. The lower microbial diversity in processes operating at the thermophilic temperature may also cause differences in the degradation capacity. Another possibility is that the activity of enzyme(s) involved in anaerobic phenol degradation is strongly regulated by temperature. Thus, it is important to consider the process temperature when anaerobically treating phenol-rich material. For such materials, mesophilic process temperatures are recommended.

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Journal of Environmental Management 95 (2012) S104–S109

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Influence of organic matter transformations on the bioavailability of heavy metals in a sludge based compost

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ARTICLE INFO

Article history:
Received 15 September 2009
Received in revised form
4 April 2011
Accepted 27 April 2011
Available online 13 May 2011

Keywords:
Sewage sludge
Compost
Humic substances
Humin
Heavy metals
Bioavailability

ABSTRACT

The agricultural use of anaerobically digested sewage sludge (ADSS) as stable, mature compost implies knowing its total content in heavy metals and their bioavailability. This depends not only on the initial characteristics of the composted substrates but also on the organic matter transformations during composting which may influence the chemical form of the metals and their bioavailability.

The objective of this work was to examine the relationships between the changes in the organic matter content and humus fractions, and the bioavailability of heavy metals.

A detailed sampling at 0, 14, 84, and 140 days of the composting process was performed to measure C contents in humic acids (HAs), fulvic acids, (FAs) and humin, the total content of Zn, Pb, Cu, Ni, and Cd, and also their distribution into mobile and mobilisable (MB), and low bioavailability (LB) forms.

Significant changes of C contents in HA, FA, and Humin, and in the FA/HA, HA/Humin and C_{humus}/TOC ratios were observed during composting. The MB and LB fractions of each metal also varied significantly during composting. The MB fraction increased for Zn, Cu, Ni, and Cd, and the LB fraction increased for Pb. Stepwise linear regressions and quadratic curve estimation conducted on the MB and LB fractions of each metal as dependent on the measured organic variables suggested that Zn bioavailability was mainly associated to percentage of C in FAs. Bioavailability of Cu, Ni and Cd during composting was associated to humin and HAs. Pb concentration increased in the LB form, and its variations followed a quadratic function with the C_{humus}/TOC ratio. Our results suggest that the composting process renders the metals in more available forms. The main forms of metal binding in the sludge and their availability in the final compost may be better described when metal fractionation obtained in sequential extraction and humus fractionation during composting are considered together.

1. Introduction

The agricultural use of anaerobically digested sewage sludge as an organic amendment to improve soil fertility is becoming increasingly important. The management of the raw sludge involves many problems such as pathogens, plant seeds, odors, and a pasty structure with high water content. This later feature makes it hard to store and may lead to immobilization and volatilization of plant nutrients (Cambardella et al., 2003). One of the possibilities to convert sewage sludge into a marketable organic amendment or fertilizer is to co-compost it with different bulking agents, such as wood chips, thus obtaining a humus-like material that is easy to store (Gallardo et al., 2007). The addition of such a bulking agent for composting may optimize substrate properties such as air space, moisture content, C/N ratio, particle density, pH and mechanical structure, affecting positively the decomposition rate. In this sense, ligno-cellulosic by-products such as wood chips and sawdust are commonly used as bulking agents (Maboeta and van Rensburg, 2003; Pasda et al., 2005; Neves et al., 2009). In the case of anaerobically digested sewage sludges with high contents of nitrogen, heavy metals, and other toxic or phytotoxic substances, bulking

Abbreviations: C_{HA}, Carbon in humic acids; C_{FA}, Carbon in fulvic acids; C_{Humin}, C in hydrolisable humin; C_{humus}, (sum of C contents in FAs, HAs and hydrolyzable humin); TOC, Total organic carbon; X_{MB}, mobile and mobilisable metal form; X_{LB}, low bioavailability metal form.

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doi:10.1016/j.jenvman.2011.04.015

agents like sawdust are recommended because of the dilution effect (Banegas et al., 2007).

Composting of organic wastes has been defined as a well-established method, which leads to a stabilized product rich in humic substances that resemble native soil humic substances (De Bertoldi et al., 1996). In addition, the agricultural use of stable, mature sewage sludge-based compost means knowing its content (Cec, 1986; Royal Decree, 1990), and the biogeochemical forms of the heavy metals present (Hsu and Lo, 2001).

Most of the studies on the speciation of heavy metals have been carried out in raw or composted sludges-amended soils (Petruzzelli et al., 1994; Kunito et al., 2001; Maboeta and van Rensburg, 2003; Hanc et al., 2009). Only a few were dedicated specifically to study the bioavailability of the heavy metals during composting of sewage sludges (Amir et al., 2005; Liu et al., 2007; Tandy et al., 2009). Studies relating changes in bioavailability of heavy metals with changes in humus fractions during composting are scarce (Amir et al., 2005; Liu et al., 2007).

The speciation of each metal in the sewage sludge-based compost may depend not only on its initial chemical state in the sewage sludge which also depends on their nature and processing (Fuentes et al., 2004; Walter et al., 2006), but also on the organic matter transformations during composting. These could influence the metal distribution through metal interaction with the newly formed humic substances (Petruzzelli et al., 1994; Amir et al., 2005; Liu et al., 2007).

The objective of this work was to examine the relationships between the changes in the organic matter content and humus fractions, and the bioavailability of heavy metals. This was tested in a 70:30 (on wet basis) mixture of ADSS and wood chips with an initial C/N ratio of 30.4, during its aerobic batch composting at 30 °C of external temperature in an open type lab-scale reactor without lixiviation.

2. Materials and methods

2.1. Composting

The raw material used in the composting process was a mixture of sewage sludge and wood chips as bulking agent, in the ratio 70:30 (on a wet basis). Sewage sludge was an anaerobically digested dewatered cake of sludge (FACSA Sewage Treatment Plant in Castellón, Spain). The characteristics of the raw sludge were 94.3% moisture content, pH8.51, EC 1.51 dS m^{-1}; 42.2% TOC; 6.37% total N; C/N 6.62; and total Zn, Pb, Cu, Ni, and Cd contents of 1660, 310, 256, 16.0, and 1.95 mg kg^{-1}, respectively (all results expressed in dry basis). The C/N ratio of the wood chips was 64.5, its moisture content was 8.5%, and its total N content was 0.83% (Gallardo et al., 2007). Normally, bulking agents have high C/N ratios, which can compensate for the low values of the sewage sludge because of the dilution effect (Banegas et al., 2007; Neves et al., 2009).

The pilot-scale composting experiments were carried out in five 65 L capacity open type lab-scale reactor without drainage of lixiviates. Aeration was controlled daily, moisture every five days, and mixture turned every 15 days. Composting was monitored for 140 days, when oxygen consumption finished (García et al., 1992). According to temperature measurements (Gallardo et al., 2007) composting developed in a first very active phase with high oxygen consumption until day 20; a second phase in which the activity dropped to a medium level until day 90; and a third phase with low activity, which lasted until day 140.

2.2. Physico-chemical analysis

To obtain representative samples for the physico-chemical analysis of the sludge based compost during the time of composting, good homogenization was ensured, and five aliquots of about 80 g (on dry basis) were taken and mixed at every sampling date. Three replicates of each composite sample were analysed at 0, 14, 84, and 140 days of composting. The time intervals were determined according to the changes of composting temperature and oxygen consumption (Gallardo et al., 2007). To determine their main physico-chemical properties we followed standard methods (MAFF, 1996): organic carbon by partial oxidation with potassium dichromate, total nitrogen by the Kjeldahl method, and pH and electrical conductivity (EC), respectively, in a 1/2.5 and a 1/5 sample/water ratios. The total concentrations of metals were determined through inductively coupled plasma-ICP (EPA, 1990) using a Perkin Elmer ICP/-5500 after the microwave digestion of the samples with HNO_3:$HClO_4$ (Polkowska-Motrenko et al., 2000).

Compost samples were extracted with 0.1 M NaP_2O_7 (pH 9.8) at room temperature using a sample/extractant ratio of 1/10. Each extraction was repeated 3 times. For each extraction step, the mixture was shaken for 3 h, centrifuged at 15,000 g for 15 min and the supernatant was filtered through a Whatman 31 filter paper. The combined alkaline extracts (soluble humic substances) were then acidified with concentrated H_2SO_4 to pH 1, left standing for 24 h in a refrigerator to allow the complete precipitation of HAs, and then centrifuged at 15,000 g for 30 min to separate the supernatant FAs fraction. Since the alkali-insoluble humin fraction may contain humic-like substances (i.e. proteinaceous compounds linked to decomposed ligno-cellulosic materials), we determined the hydrolyzed humin (Zaccheo et al., 2002).The hydrolyzed humin was obtained after acidification of the sample retained in the filter paper with concentrated 6 N HCl for 10 h, filtration and washing with deionized water. This fraction was considered as forming part of the humus in the compost. The total alkali extractable (soluble humic substances), the FAs, and the hydrolyzed humin were analyzed for C. The C in HAs was obtained by the difference between C in the total alkali extractable and C in FAs (MAFF, 1996). C_{humus} was obtained as the sum of C contents in FAs, HAs and hydrolysable humin.

Heavy metal fractionation for Zn, Cu, Pb, Ni, and Cd was determined according to Sposito's procedure (Amir et al., 2005). In each of the three replicates taken from the composite samples, a series of reagents were sequentially applied with a compost/extractant ratio of about 1/4. The sequence of reagents application to collect the medium -bioavailable fraction MB (mobile and mobilisable) was: H_2O (shaking during 2 h at 20 °C, three times); KNO_3 0.5 M (shaking during 16 h at 20 °C); NaOH 0.5 M (shaking during 16 h at 20 °C), and EDTA 0.05 M (shaking during 16 h at 20 °C). Finally, to collect the low-bioavailable fraction LB (bound to sulphides; hardly mobilisable) the samples were treated with HNO_3 4 M (shaking during 16 h at 80 °C). Metal concentration was measured after each step treatment, and referred to dry weight. All filtered supernatants were analyzed by ICP (EPA, 1990).

The levels of bioavailability considered in this work are:

1) Medium, MB (mobile and mobilisable fractions):

$$MB = X - H_2O + X - KNO_3 + X - NaOH + X - EDTA$$

2) Low, LB (Sulphides. Hardly mobilisable fraction):

$$LB = (X - HNO_3)$$

2.3. Statistical analysis

Statistical analyses were performed with the SPSS v.17.0 statistical software. A one-way ANOVA was used to detect the significant

effect of time of composting on different compost parameters. The Tukey's *t*-test was used for mean comparison and significant differences at 95% level on data obtained at the different composting times. To describe more clearly the metal and humus fraction variations through time, also linear and curvilinear adjustments were performed. In order to ascertain the best-fit model between variations in the metal fractions during composting and changes in the organic fractions, stepwise linear regressions and quadratic curve estimations were performed on the MB and LB fractions of each metal as dependent variables. The independent organic variables were C_{FA}, C_{HA}, C_{Humin}, and C_{FA}/C_{HA}, C_{HA}/C_{Humin}, and C_{humus}/TOC ratios.

3. Results and discussion

3.1. Compost properties

The main physico-chemical properties of the composted sludge at different times of the process, the ANOVA and the Tukey's *t*-test results are presented in Table 1.

Because of the high moisture content of the raw sludge, the ratio of sewage sludge and wood chips on a dry weight basis was 15:85. As shown by Pasda et al. (2005) this product is not easy to decompose because its high lignin/tannins ratio. This fact likely provoked that temperature in the reactors during the composting process was always below 35 °C. No significant changes were detected for pH, EC and total N. The high value of pH in the raw sewage sludge may compensate for the decrease of this parameter during composting (Amir et al., 2005; Liu et al., 2007). The pH during composting was in the optimal range for the development of bacteria and fungi (Zorpas et al., 2003).

Total organic C content (TOC) decreased significantly during composting (Table 1), which is consistent with the decomposition of the organic matter through microbe respiration in the form of CO_2 and even through mineralization. The overall decomposition observed in this work (37%) contrasts with the 60% observed by Jouraiphy et al. (2005) during 135 days of composting of a mixture of sewage sludge and green plant waste, and the 19.6% of Amir et al. (2005) during 180 days with straw as bulking agent. At difference with other authors (Soumaré et al., 2002), the organic matter decomposition during composting did not cause an increase in total N during the process. Although N variations were not significant, the trend was to decrease. In agreement with the results by García et al. (1995), it is interpreted because heavy metal concentration of the sewage sludge may have affected to certain extent the mineralization rate of N in our sewage sludge based compost.

The C/N ratio significantly decreased from 30.4 in the initial mixture to 21.6 at 140 days. This relatively high C/N ratio at the end of composting indicates that organic matter has not achieved an adequate stabilization (De Bertoldi et al., 1996), likely due to the quality of the bulking agent (Pasda et al., 2005).

Since metal loss by leaching did not occur in our experiment, we observed a continuous increase of total heavy metal concentration in the compost due to the weight loss during composting, the release of carbon dioxide and water, and the mineralization process as shown by Lazzari et al. (2000). Although the trend was to increase, no significant differences with time were detected for total Zn. The increase of the total metal concentration during composting was significant for other metals which had high concentration in the sludge (Pb and Cu), but also for metals with low concentrations (Ni, Cd). The total heavy metal concentrations in the obtained compost were below the maximum permitted in Spain for application of sewage sludge in soils (Royal Decree, 1990).

3.2. Heavy metals bioavailability

The sum of the amounts extracted by sequential extraction (MB and LB fractions, Table 2) for the most abundant metals (Zn, Pb, Cu, Ni), and also for Cd, was, respectively, almost four or two times lower than the total amount of metal. This result indicates that most metals are mainly bound to residual forms. Our results agree with those by Amir et al. (2005), who found recoveries of 20–30% using this sequential extraction procedure. The fact that the residual fraction is so abundant indicates that an important proportion of metal is probably occluded in minerals present in the sludge as has been referred by Wong et al. (2001) for some metals such as Pb.

For all metals except Pb, the concentration of the MB forms is higher than that in the LB forms (Table 2). This suggests that composting enhances the availability of most of metals. The MB and LB fractions of Zn vary in a quadratic function with time. The amount of Zn_{MB} increases to a maximum at day 84 and decreases thereafter to concentrations that are similar to those at day 14. Zn_{LB} follows the reverse trend (Table 2). It decreases to a minimum at day 84, and increases at day 140 to concentrations that are similar to those at the beginning of composting. This result suggests that important changes in the Zn speciation occur in the final period.

For Cu, Ni and Cd, the concentrations of the MB fraction follow a linear significant increase with time of composting. Cu_{LB} also linearly decreases with time although with the b parameter (absolute value) lower than the corresponding parameter of the Cu_{MB} model. This result indicates that the increase in the MB fraction of Cu occurs at expenses of both the LB fraction and the residual fraction. The changes in the LB fractions of Ni and Cd during composting follow a curvilinear trend, reaching a maximum at day 84 and decreasing thereafter, especially Ni. The decrease of the Ni_{LB} in the last period of composting suggests that some moieties of the Ni_{LB} become more available and increase the Ni_{MB} fraction, whereas some other could join the residual fraction. For Cd, the decrease in the LB fraction is lower than the corresponding increase of the MB fraction, and suggests that some Cd_{MB} forms also at expenses of residual Cd. The MB fraction of Pb remains constant

Table 1
Physico-chemical properties ($n = 3$) of the sludge based compost at different days of composting. All results expressed in dry basis.

Time[a]	Moisture (%)	pH	EC (dSm-1)	TOC (%)	Total N (%)	C/N	Total Zn	Total Pb	Total Cu	Total Ni	Total Cd
							mg kg $^{-1}$				
0	71.8 a	7.07 a	1.06 a	50.0 a	1.64 a	30.4 a	259.8 a	45.3 a	37.7 a	2.24 a	0.29 a
14	71.0 a	7.03 a	1.14 a	45.2 b	1.54 a	29.3 ab	262.1 a	49.5 ab	41.3 a	2.38 a	0.33 ab
84	69.0 b	7.01 a	1.16 a	37.4 c	1.51 a	24.8 ab	267.1 a	53.7 ab	43.1 ab	2.69 b	0.40 ab
140	68.8 b	7.01 a	1.12 a	31.3 d	1.45 a	21.6 b	278.2 a	57.4 b	49.5 b	2.76 b	0.45 b
ANOVA											
F	15.942	1.848	0.243	1960.23	1.896	14.340	2.924	14.141	12.446	19.310	12.365
p	0.001	0.217	0.864	0.000	0.209	0.001	0.100	0.001	0.002	0.001	0.002

[a] Days of composting. Mean value followed by different letters is statistically different (Tukey's *t*-test, $p < 0.05$).

Table 2
Evolution of heavy metals in medium bioavailable forms (MB) and in low bioavailable forms (LB), and evolution of humic (FA, HA), humic-like substances (hydrolysable humin), and their ratios during composting. All metal concentrations are expressed in mg kg^{-1} dry matter ($n = 3$).

Dependent Variable	Means and Tukey's *t*-test Time of composting (days)				ANOVA curvefit		Best-fit Model parameters			
	0	14	84	140	F	P	a	b	c	R^2
Zn_{MB}	39.4 a	46.2 b	53.6 c	46.1 b	80.533	<0.001	40.41	0.347	−0.002	0.947
Zn_{LB}	25.6 a	19.4 b	13.2 c	23.4 a	94.132	<0.001	24.77	−0.343	0.002	0.954
Cu_{MB}	5.09 a	6.15 b	7.00 c	8.92 d	153.23	<0.001	5.349	0.024		0.939
Cu_{LB}	4.34 a	4.18 a	3.78 b	3.45 c	160.85	<0.001	4.300	−0.006		0.941
Pb_{MB}	5.27 a	5.42 a	5.43 a	5.25 a		0.7540				
Pb_{LB}	6.06 a	6.96 b	7.99 c	8.49 d	87.080	<0.001	6.263	0.031	−0.0001	0.951
Ni_{MB}	0.27 a	0.24 ab	0.29 ab	0.46 b	34.852	<0.001	0.234	0.001		0.777
Ni_{LB}	0.31 a	0.36 ab	0.38 ab	0.14 b	16.597	0.0010	0.309	0.001	−0.00004	0.787
Cd_{MB}	0.06 a	0.07 b	0.09 c	0.13 d	222.03	<0.001	0.065	0.0004		0.957
Cd_{LB}	0.08 a	0.09 b	0.11 c	0.10 d	882.99	<0.001	0.081	0.0001	−0.000004	0.995
C_{FA}	5.80 a	6.72 b	8.80 c	9.07 c	64.860	<0.001	6.217	0.023	−0.0002	0.866
C_{HA}	2.11 a	2.24 a	4.82 b	5.87 c	354.86	<0.001	2.057	0.029		0.973
C_{Humin}	7.30 a	8.34 a	11.4 b	13.0 c	100.51	<0.001	7.616	0.040		0.941
C_{FA}/C_{HA}	2.75 a	3.02 a	1.82 b	1.54 c	84.349	<0.001	2.904	−0.010		0.894
C_{HA}/C_{Humin}	0.29 a	0.27 a	0.42 b	0.45 c	104.43	<0.001	0.277	0.001		0.913
C_{humus}/TOC	0.30 a	0.38 b	0.67 c	0.89 d	1383.5	<0.001	0.315	0.004		0.993

Metal fraction (mg kg^{-1} dry basis): MB = Mobile + Mobilisable (Bioavailable), LB = low bioavailability. FA = % C Fulvic acids; HA = % C Humic acids; TOC = % Total organic carbon. Means in a row followed by the same letter are not significantly different at $\alpha = 0.05$ according to the Tukey's *t*-test. P= P values of the F test in ANOVA curvefit for linear and quadratic models. Model parameters: a = constant; b = coefficient of x in linear and quadratic models; c = coefficient of x^2 in the quadratic model. Independent variable = Time of composting (days).

during composting, but the curvilinear trend of increase observed for Pb_{LB} indicates that this form likely increases at expenses of the residual forms during composting and reaches a maximum at the end of the process. Although the quadratic fit may indicate a further decrease, this cannot be tested because of the lack of data beyond 140 days. Overall, the results for Pb indicate that this metal accumulates in the very stable organic fractions and unavailable mineral forms.

Increased availability for Zn and Cu through composting is in agreement with results obtained by several authors (Wong et al., 2001; Amir et al., 2005). The observed increase of Cd availability is in agreement with results by McGrath and Cegarra (1992), who found high extractable Cd levels in sludge-amended soils. For composted sludges Walter et al. (2006) found increased mobility for Zn, Cu and Cd during composting. Richards et al. (1997) found reductions in Pb mobility and an increase in Cd and Cu mobility because of the composting process.

3.3. Humic substances

The changes in C in humic (FAs, HAs) and humic-like substances (hydrolyzed humin) and their ratios during composting are shown at the bottom of Table 2. All of them changed significantly with time of composting indicating that transformations of the organic matter and humification have occurred. C in FAs increased mainly during the initial phases of composting. The best model describing the FAs changes is quadratic and reflects that stop increase beyond day 84 of composting. C in FAs was higher than that in HAs thus indicating that among the soluble humic substances the most abundant are those of low molecular weight. Some of them may have polymerized in the last phases of composting likely forming more condensed structures such as HAs thus explaining some of the increase of C in HAs and also the linear decrease of the C_{FA}/C_{HA} ratio (Table 2).

C in HAs increased linearly during composting. Its rate of increase was similar or even higher than that of C in FAs, as deduced from the b parameters of models. The linear rate of increase observed for C in hydrolyzed humin is higher than that of C in HAs as deduced by comparison of the corresponding b parameters. This suggests that both FAs and HAs of the sludge may polymerize in the form of humin. The abundance of aliphatic compounds in sewage sludges may have a negative effect on the formation of the condensed structures typical of the true HAs (García et al., 1989). Likely, the dilution of the sludge with the bulking agent in our compost has lowered this negative effect and even facilitated HAs and likely humin formation through polymerization of FAs with some ligno-cellulosic derivates coming from the partial degradation of the wood chips. The slope of the linear model describing the changes of the HA/Humin ratio (Table 2) is an order magnitude lower (absolute value) than that of FA/HA ratio. This result may indicate that the transformation of FAs into HAs is higher than that of HAs into humin, but also that some compounds in humin may transform into HAs.

Finally, the progressive increase of the C_{humus}/TOC ratio indicates that the proportion of humified organic matter (sum of FAs, HAs, and hydrolyzed humin) increases linearly through composting.

3.4. Metal fraction-humic substances relationships

Table 3 summarizes the best-fit models (highest R^2) containing the humus fractions in the compost that are most related to the

Table 3
Best-fit models for major metal fractions varying in the sewage sludge compost and C in humus fractions during composting.

Dependent variable	Model	Best-fit Model parameters			
		Coefficient	SE	*p*-value	R^2
Zn_{MB}	Constant	8.271	2.897	0.019	0.975
	C_{FA}	14.919	1.412	<0.001	
	C_{Humin}	−7.515	0.848	<0.001	
Cu_{MB}	Constant	3.538	0.304	<0.001	0.965
	C_{humus}/TOC	5.785	0.499	<0.001	
Pb_{LB}	Constant	3.578	0.533	<0.001	0.982
	C_{humus}/TOC	10.330	2.051	0.011	
	$(C_{humus}/TOC)^2$	−5.418	1.710	<0.001	
Ni_{MB}	Constant	0.588	0.031	<0.001	0.990
	C_{humus}/TOC	0.908	0.041	<0.001	
	C_{FA}	−1.030	0.007	<0.001	
Cd_{MB}	Constant	0.033	0.004	<0.001	0.951
	C_{humus}/TOC	0.101	0.007	<0.001	
C_{humus}/TOC	Constant	−0.084	0.104	0.440	0.990
	C_{Humin}	0.090	0.030	0.018	
	C_{HA}	0.073	0.029	0.036	
	C_{FA}	−0.069	0.032	0.065	

changes in the main metal forms during composting. FAs and humin explained the Zn_{MB} fraction. The stepwise regression procedure selected C_{humus}/TOC ratio as the independent variable predicting metal fractions of the rest of metals. The C_{humus}/TOC ratio was also dependent on the three humus fractions considered in this work. The best model explaining the variations of the C_{humus}/TOC is shown at the bottom of the table. C_{humus}/TOC ratio increases when C in hydrolyzed humin and HAs increase, and when C in FAs decreases.

Overall, results in Table 3 suggest that the bioavailability of metals clearly depend on the organic compounds present and formed during composting, which may increase or restrict it.

Regressions indicate that Zn availability is positively associated with the FA fraction and negatively with humin. This result agrees with those by Moreno et al. (1996) who questioned the capability of Zn to form complexes with organic compounds. Alloway and Jackson (1991) found Zn associated to organic matter of low molecular weight. The negative dependence with humin may be indicating a decrease in Zn bioavailability at the end of the composting process due to a relative decrease of FAs amount in much more stable forms such as humin.

The C_{humus}/TOC ratio explains both Cu_{MB} and Cd_{MB} fractions. Cu_{MB} increases at expenses of the LB fraction (Table 2), and this increase is explained by the increase of C in humin and HAs at expenses of transformations of the sulphide forms. It follows that more than a half of Cu_{MB} must be attached to alkali-insoluble EDTA extractable organic forms such as humin, and the rest bound to alkali soluble HAs. This explanation also follows for Cd, although, as deduced from data (Table 2), Cd_{MB} increases at expenses of the residual form of this metal.

Ni_{MB} increases when C_{humus}/TOC ratio increases and C in FAs decreases because some FAs polymerize into HAs and humin. These results suggest that Ni_{MB} follows the same trend as Cu and Cd, and the reverse trend as Zn_{MB}.

As indicated in Table 1, the order of abundance of Cu, Ni, and Cd in the sludge is Cu > Ni > Cd. However, comparing the amount of metal in the MB fraction at the beginning and at the end of composting, their relative availability increase in the order $Cd_{MB} > Ni_{MB} = Cu_{MB}$. This suggests that the main factor explaining their bioavailability during composting was not the initial metal concentration but the stability of complexes with humic-like substances and HAs, which likely increase in the order Cu > Ni > Cd. Other authors (Canet et al., 1997) have also attested to the high stability of Cu-organic matter complexes. Soler Rovira et al. (2010) found that the complexing capacity of Cu (II) increased as the humification degree increased. Our results suggest that it may occur also for Ni and Cd.

Finally, the model for Pb differs from the rest of metals. Since the MB fraction did not change through composting (Table 2), the increase of the Pb_{LB} fraction in a quadratic model with the C_{humus}/TOC ratio may indicate the amount of residual Pb changing to LB forms. The quadratic fit would open the question to a further decrease of Pb_{LB} forms (decreasing branch of the curve) depending on the compost maturation.

4. Conclusions

The 140 days composting process of a mixture of sewage sludge and wood chips (C/N ratio of 30.4), resulted in a product with a relatively high C/N ratio of 21.6, a relatively low stabilization of the organic matter if considered the dominance of FAs over HAs, and total heavy metal concentrations below the maximum permitted for land application. With exception of Pb, the relative bioavailability of metals increased with composting. Zn bioavailability was mainly associated to percentage C in FAs. Bioavailability of Cu, Ni and Cd during composting was associated to percentage C in humin and HAs. Pb concentration increased in unavailable forms, and followed a quadratic function of the C_{humus}/TOC ratio.

Our results suggest that the composting process renders the metal in more available forms. The main forms of metal binding in the sludge and their availability in the final compost may be better described when metal fractionation obtained in sequential extraction and humus fractionation during composting are considered together.

Acknowledgments

This work was part of the Generalitat Valenciana projects IIARCO2004-A-196, IIARCO2004-A-212, and IIARCO/2004/213, and the national project 135/2004/3 (Ministerio de Medio Ambiente). We thank FACSA Sewage Treatment Plant (Castellón, Spain), and University Jaume I of Castellón (Spain) for providing materials and technical assistance. We thank the two anonymous reviewers for their useful comments on the manuscript.

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Journal of Environmental Management 95 (2012) S110–S115

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Assessing fly ash treatment: Remediation and stabilization of heavy metals

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ARTICLE INFO

Article history:
Received 20 August 2009
Received in revised form
25 October 2010
Accepted 4 November 2010
Available online 17 December 2010

Keywords:
Fly ash
Electrodialytic process
Mortar stabilization
Leachability tests

ABSTRACT

Fly ashes from Municipal Solid Waste (MSW), straw (ST) and co-combustion of wood (CW) are here analyzed with the intent of reusing them. Two techniques are assessed, a remediation technique and a solidification/stabilization one. The removal of heavy metals from fly ashes through the electrodialytic process (EDR) has been tried out before. The goal of removing heavy metals has always been the reuse of fly ash, for instance in agricultural fields (BEK). The best removal rates are here summarized and some new results have been added. MSW fly ashes are still too hazardous after treatment to even consider application to the soil. ST ash is the only residue that gets concentrations low enough to be reused, but its fertilizing value might be questioned. An alternative reuse for the three ashes is here preliminary tested, the combination of fly ash with mortar. Fly ashes have been substituted by cement fraction or aggregate fraction. Surprisingly, better compressive strengths were obtained by replacing the aggregate fraction. CW ashes presented promising results for the substitution of aggregate in mortar and possibly in concrete.

1. Introduction

Fly ash is a fine grained waste/material, with high specific surface area (e.g. Yang and Yang, 1998; Ferreira et al., 2003) – 2 to 6 $m^2\ g^{-1}$ – where the attributed hazardousness may be directly dependent on the fuel that is combusted (Lima et al., 2008a). Heavy metals are the most recurrent contaminants preventing fly ash reuse and may be found in problematic concentrations, depending on the type of fly ash. For instance, MSW fly ash is considered hazardous (172/2007/EC; 2000/532/EC). As for fly ash resulting from the combustion of biomass (e.g. wood and straw), regulations have been made for its reuse in agricultural fields, e.g. Denmark (BEK39, 2002). But frequently this type of fly ash is very prone to leach metals and chlorides, mainly due to its content in soluble salts and particles high specific surface area (Pedersen, 2002; Lima et al., 2008b).

In the evaluation of fly ashes, it is relevant to distinguish the source, i.e. the combusted waste. MSW fly ash management normally includes stabilization prior to direct disposal in landfill (Tchobanoglous et al., 1993; Levy and Cabeças, 2006). Stabilization and remediation techniques have been widely studied for reusing purposes, since fly ashes possess valuable characteristics for further applications (Ferreira et al., 2003).

The electrodialytic process (EDR) is a remediation technique used for the removal of heavy metals and chlorides from fly ashes (Pedersen et al., 2005; Ottosen et al., 2006; Lima et al., 2009). Authors have been circulating their achievements in all ranges of ashes, since MSW (Pedersen et al., 2003, 2005) to bioashes (Lima et al., 2009), namely straw ashes (Hansen et al., 2004; Lima et al., 2009). Together with heavy metals, alkalis are also removed, which may decrease the fertilizing value of the remaining solid residue for agricultural applications (Lima et al., 2008b).

A possibility for fly ash management could then be a solidification/stabilization (S/S) technique, e.g. concrete. Since concrete production is one of the top ten material categories with the highest environmental impacts (European Environment Agency, 2007), there is a growing need to minimize its environmental costs. Green or mixed concrete with different wastes has been the subject of several studies (Bishop et al., 1992; Cenni et al., 2001; Shih et al., 2005), including MSW fly ash (Rémond et al., 2002). Fly ashes from biomass combustion may also have potential to be reused in concrete. Ganesan et al. (2007) have already studied straw ashes in concrete. But regarding the introduction of waste materials in concrete, leachability of heavy metals should be addressed prior to its use in civil constructions. Usually leachability studies are connected to landfill disposal and only some literature is found regarding heavy metal leachability (Schmid et al., 2000). This

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doi:10.1016/j.jenvman.2010.11.009

work is an attempt to alert the need of leachability tests when applying waste materials to mortars and concrete.

2. Material and methods

2.1. Fly ashes

The following fly ashes were used in this study:

PT. Portuguese MSW Incinerator, ValorSul
ST. Danish bioash from straw combustion, Avedøre Unit 2
CW. Danish ash from co-combustion of wood, fuel oil and natural gas (in the proportion of 11/1.5/4), Avedøre Unit 2

The studied fly ashes were chemically analyzed. Total concentration of Cd, Cr, Cu, Ni and Pb in the fly ash was microwave assisted in a pressurised digestion with concentrated HNO_3. This scheme was used according to the modified Danish standard DS 259 (BEK39, 2002). The liquid-to-solid (L/S) ratio used in the digestion was 20 and after digestion the samples were vacuum filtered through 0.45 μm filter. Series of three replicates were carried out and metals concentration analyzed in Atomic Absorption Spectrophometer (AAS).

Ash dissolution, the mass of solid that is dissolved, was performed using distilled water in an L/S of 4 (100 g of fly ash in 400 ml of distilled water). The samples were filtered after 24 h of contact in 0.45 μm filter and weighted. pH_{H2O} was measured in an L/S of 5 using a combined Radiometer pH electrode (after 1 h of contact). Carbonate content was determined by a Scheibler apparatus, where a standard curve was first performed using different $CaCO_3$ contents combined with HCl. The fly ashes were mixed with HCl and $CaCO_3$ was determined. Chloride content was measured in H_2O extractions (L/S ratio of 2.5) by a Dionex ion chromatograph DX120. X-ray diffraction (XRD) analysis were performed by a Philips PW 1050/25 (vertical Goniometre) with automatic divergence slit, 0.2° receiving slit and 1.0° scatter slit at a spectrum of 2θ from 5 to 65°. All methods have been previously described and used by e.g. Pedersen (2002).

2.2. Electrodialytic experiments

For the remediation of CW fly ashes, an electrodialytic cell was used (see e.g. Lima et al., 2009). Two experiments were carried out for 10 and 14 days. The design and characteristics of this cell can be found elsewhere (e.g. Lima et al., 2009). Before the EDR period started, washing of the ash was carried out. The procedure was similar to ash dissolution, but instead of filtering the suspension, a decantation was used to grossly separate the solid from the liquid. The remaining suspension was filled up to L/S of 4 using distilled water.

Anion-exchange membrane 204 SZRA B02249C and cation-exchange membrane CR67HUYN12116B from Ionics were used. Platinum coated electrodes from Permascand were used as working electrodes and a power supply (Hewlett Packard E3612A) maintained a constant current of 40 mA, corresponding to a current density of about 0.8 mA cm^{-2}. As anolyte and catholyte 500 ml of 0.01 M $NaNO_3$ was used, with pH adjusted to 2 with HNO_3. Both anolyte and catholyte pH was daily adjusted to 2 with HNO_3 during the experiments.

At the end of the experiments the suspension in the central compartment was filtered at normal pressure. Ash digestion with acid was preceded, according to the described in Section 2.1. Concentrations of Cd, Cr, Cu, Ni and Pb were determined by AAS.

2.3. Mortar bars

Each fly ash was blended with cement, water and standard quartz sand in different proportions and in substitution of the two solid fractions: cement and aggregate. The substitution percentage was based on dry weight. ST was used in 5%, 12.5% and 25% as substitute of cement and aggregate; CW ash in 5%, 12.5% and 25% in substitution of cement and in 5%, 10%, 12.5%, 15% and 25% in substitution of aggregate; and finally MSW ash was used in 5% and 10% of the aggregate fraction. The type of cement used was alkali cement from Merter. Fine sand was used as aggregate, with diameter (φ) size inferior to 2 mm. Water to cement (W/C) ratio was kept constant at 0.5 in all experiments. Mortar bars cylinders were then prepared with dimensions 6 cm φ by 12 cm high. After a curing period of 28 days, the compressive strength was tested in a Werk Nürnberg device. Three replicates were carried out. Mortar bars were then crushed and tested for heavy metals leachability. The test was performed according to NEN 7371:2004 and Ni, Pb, Cr, Cu and Cd were determined through AAS quantifications.

3. Results and discussion

3.1. Fly ash characterization and electrodialytic remediation

Table 1 summarizes the main characteristics of MSW, ST and CW fly ashes. The EDR has previously been tested in MSW and ST ashes, and a resume of the best-achieved removal rates is presented in Table 2. In addition, two new experiments were carried out with CW ashes and results are also presented in Table 2.

Table 1
Characteristics of the analyzed fly ashes (adapted from Lima et al., 2008a).

Parameter	MSW	ST	CW
Fuel	MSW	Straw	Wood and oil
Flue gas Temperature (°C)	420	580	580
pH	11.9 ± 0.0	5.9 ± 0.0	12.0 ± 0.2
Ash Dissolution (%)[a]	23.22	60.96	12.69
Total Ca Content (%)	22.2	0.76–0.86[a]	8.7
Cl Content (%)[a]	9.81	21.14	0.34
Carbonate Content (%)	8.49 ± 0.7	11.85 ± 0.0	13.21 ± 0.0
Mineral Species found in fly ash	Al_2O_3, $CaSO_4$, $CaCO_3$, Fe_2O_3, KCl, NaCl, SiO_2	Fe_2O_3, KCl, K_2SO_4, $MgCO_3$	$CaSO_4$, Fe_2O_3, $MgCO_3$, Mg_2SiO_4, SiO_2
Cd (mg kg^{-1})	83.4 ± 0.8	11.3 ± 0.0	22.4 ± 0.0
Cr (mg kg^{-1})	185 ± 6.0	13.5 ± 0.4	185.0 ± 0.0
Cu (mg kg^{-1})	586 ± 8	80.5 ± 1.1	232.9 ± 0.0
Ni (mg kg^{-1})	61 ± 1	n.d.	621 ± 0.0
Pb (mg kg^{-1})	2462 ± 71	17.3 ± 0.9	1224 ± 0.0

[a] ash dissolved after 24 h contact with water in a proportion of 1–4 solid to liquid.

Table 2
Maximum electrodialytic removal rates from ST, MSW ashes. New results are presented CW fly ashes electrodialytic treatment.

Removal Rate	MSW	ST	CW
Cd (%)	70[e]	40[a]; 97[b]	18
Cr (%)	40[f]	13[g]	n.r.d.
Pb (%)	41[d]	66[c]	19
Ni (%)	–	78[c]	17
Cu (%)	90[d]	29[g]	n.r.d.
Cl (%)	98[d]	–	–

[a] Lima et al., 2008b – Large scale experiment.
[b] Hansen et al., 2004.
[c] Ottosen et al., 2007.
[d] Ottosen et al., 2006.
[e] Pedersen et al., 2005.
[f] Pedersen et al., 2003.
[g] Lima et al., 2009 n.r.d. – no removal detected.

ST ash exhibits high solubility due to its KCl and K_2SO_4 content, where pH is neutral (Hansen et al., 2001; Lima et al., 2008a). The electrodialytic removal of heavy metals, namely Cd, from straw ashes has been quite successful – see Table 2 (Hansen et al., 2004; Ottosen et al., 2007, 2006; Pedersen et al., 2003). Inclusively, the scale-up of the process has been tried out for the remediation of straw ash, and achieved a 40% removal of Cd (Lima et al., 2008b). In this study, the final concentration of Cd in ST ashes was 6.8 mg Cd/kg, very close to the target of 5 mg Cd/kg established by Danish regulations (BEK39, 2002). This result was quite promising for the scale-up of EDR, with further technical adjustments, and (re) application of ST ashes in agricultural soils.

CW ash is a low chloride content (0.34%) material with a high pH (12) and low ash dissolution when in contact with water (12.69%) (Table 1). Among bioashes, CW ashes presented high heavy metal content. The electrodialytic treatment of such ashes proceeded in a very simple manner, in an acidic environment, with no assisting agent (Lima et al., 2009). Some new results are presented here and it ranged no observed removal of heavy metals to a figure of 18% (Table 2). Initial washing of fly ashes presented no detectable heavy metals release.

MSW is the most hazardous of all studied fly ashes. The electrodialytic treatment of MSW fly ashes has been tried by several authors, as seen in Table 2. The literature is considerable regarding the EDR treatment of MSW ashes. The removal rate of 30% of Pb was the best result achieved by Ferreira et al. (2005). Pedersen et al. (2005) have achieved 86% Cd, 20% Pb, 62% Zn, 81% Cu and 44% Cr removal of such metals. In all these studies, assisting agents were used such as a solution of Na-gluconate and ammonium citrate and ammonia, respectively. Further on, Ottosen et al. (2006) have removed 90% of Cu and 41% of Pb without assisting agent, but the dissolution of the ash was very high. Hence, either the potential value of the ashes is decreased by the addition of assisting agent, or the solid fraction is dissolved.

Summing up:

i. The idea of assisting agent addition is very positive since it enhances removal rates and decreases remediation times, depending on the used agent. However, the fly ashes get impregnated with agents and might prevent its further valorisation, e.g. ammonium citrate is hazardous for concrete;
ii. EDR favours fly ash pH decrease and dissolution. The natural pH of the fly ashes is rather high (around 12) and an EDR remediation imposes acidity to the system, which is mainly the driving force for metals mobility. With fly ashes dissolution, some characteristics are lost such as the high pH, or the depletion of Ca and OH;
iii. EDR removal rates are based on the total concentration of the metals in the fly ash. Some of the heavy metals may be found in the least soluble fraction of the ashes, and therefore immobilized. Removal efficiencies should better translate heavy metals leachability rather than its total content.

In addition to EDR difficulties, standardization of fly ashes has been pointed out as an obstacle for its reuse. In fact, it may be unfeasible since characteristics of MSW ash float deeply according to the combusted debris (Wiles, 1996; Ferreira et al., 2005; Lima et al., 2008a). However, MSW ash regularly presents a high pH (Wiles, 1996) and the presence of minerals such as Al_2O_3, $CaSO_4$, $CaCO_3$, Fe_2O_3, and SiO_2 are good indications for possible reuse, if a remediation technique does not remove such minerals. With this in mind, raw application of MSW fly ash has been tried out in mortar for leachability of metals, since there are studies presenting a good ground for it (Rémond et al., 2002).

3.2. S/S technique

MSW, ST and CW ashes are here added to mortar and tested for resulting compressive strength and Ni, Pb, Cr, Cu and Cd leachability according to NEN 7371:2004. The fly ashes had no pretreatment.

The reference mortar presented an average compressive strength of 40 MPa (Figs. 1–3). This is in range of mortar and cement pastes compressive strength, 35 MPa for a W/C ratio of 0.5

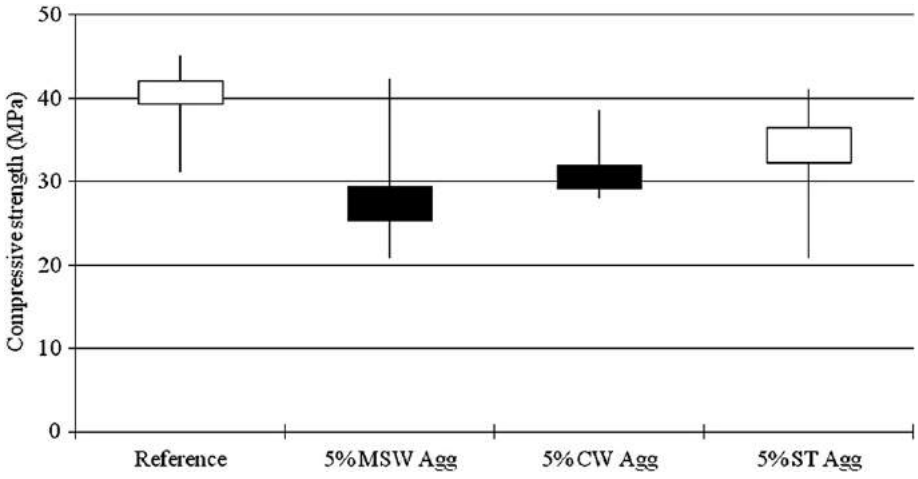

Fig. 1. Resulting compressive strength of samples with 5% fly ash (ST, MSW and CW) substitution by the aggregate fraction.

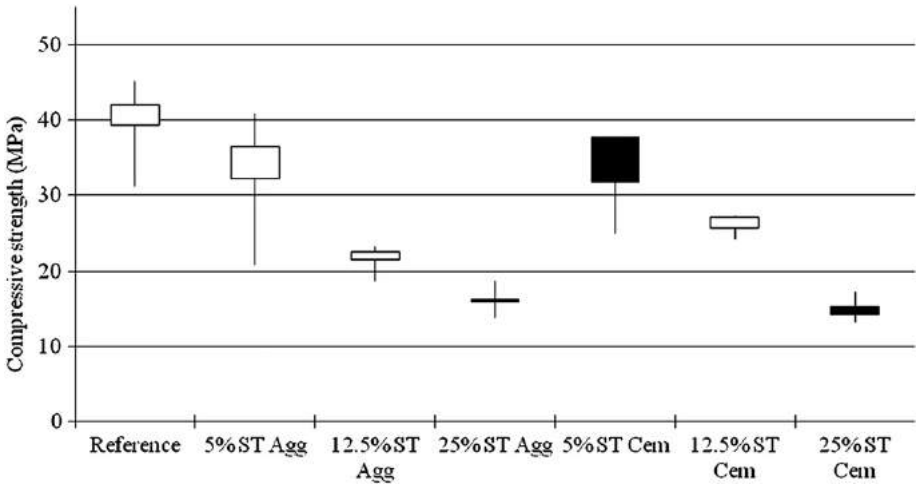

Fig. 2. Resulting compressive strength on a reference mortar and samples with substitution of either aggregate or cement by ST ash.

(Neville, 1995). All the mixed mortar presented strengths inferior to the reference sample (Figs. 1–3). The least consistent results were from both ST and MSW ashes. A high standard deviation at low substitution percentages was observed for both, and a consistent decrease of strength was observed with increasing substitution percentage (Fig. 1).

Since ST ash presents high alkali/chloride and low SiO_2 content (Lima et al., 2008a), some problems may have occurred in the development of mortar strength. For instance, the excess of alkali in concrete enhances cracking incidence (Taylor, 1990) and the incorporation of soluble Cl may provoke steel corrosion (Chen and Liew, 2003). According to Cenni et al. (2001) high Cl content accelerates hardening at the same time that free Cl in induces steel corrosion. Since the main mineral found in ST ash was the highly soluble KCl (Lima et al., 2008a), it is likely that it would become threshold Cl^- in concrete systems.

CW ash is a fine and heavy ash, with high pH, high OH^- and low Cl^- content (Table 1), resembling the appearance of clayey soil. Combined with $CaSO_4$, Fe_2O_3, $MgCO_3$, Mg_2SiO_4, SiO_2 as constituents' minerals and total calcium content of 8.7% Ca (Table 1), CW ash presents promising basis for mortar incorporation. Fig. 3 shows promising compressive strength results, where fly ash substitution of the aggregate fraction shows higher values. In particular, 12.5% and 15% CW ash in substitution of aggregate (Fig. 3).

MSW ash is a high pH material with moderate ash dissolution (20%), low Cl content (<10%) and high OH^-, which makes this waste a possible pozzolanic material for concrete production (Table 1). Extensive literature can be found on the application of MSW ashes into concrete production (*e.g.* Bishop et al., 1992; Cenni et al., 2001; Rémond et al., 2002; Juric et al., 2006). In this study, the presented results serve only as a basis of comparison. The handling of such a hazardous material should always be addressed regarding the precautionary principle (European Commission, 2000; Tukker, 2002). No precise conclusion might be drawn from the present compressive strength test (Fig. 2), however Rémond et al. (2002) obtained much higher strengths, around the order of the 60 MPa. This poor result substantiates the high heterogeneity of MSW ashes.

Overall, the mortars with the aggregate fraction substituted by fly ashes presented higher compressive strengths.

3.2.1. Leachability tests

Heavy metals are considered foreign cations in a cement bed-matrix and are found in considerable concentrations on ash materials (Table 1). But when higher than 0.1%, heavy metals seem to inhibit the setting of the cement, probably due to the formation of protective layers in the cement grains (Taylor, 1990). Whereas no consistency is found on heavy metal effect in concrete, there is certainly an environmental issue regarding their leachability/availability.

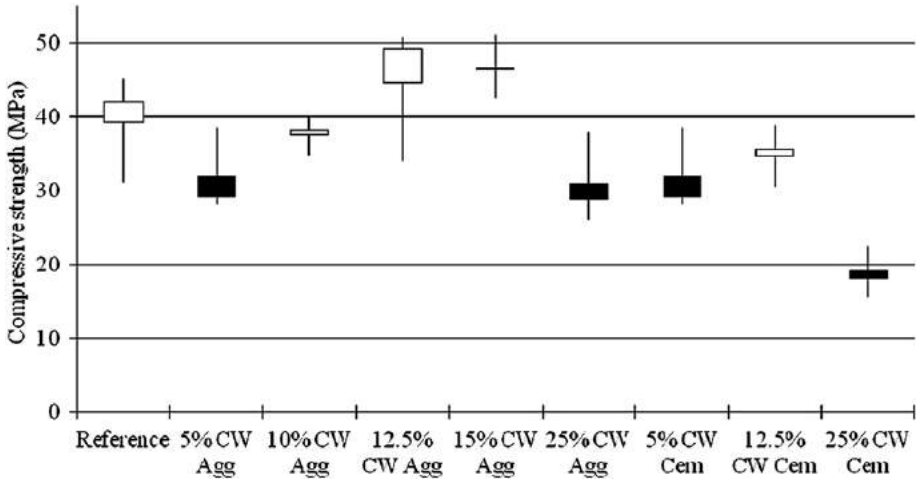

Fig. 3. Resulting compressive strength on mortar samples with CW fly ash as a substitute of aggregate and cement fraction.

Table 3
EEA landfill criteria for metals leachability; Experimental data obtained by NEN 7371:2004 on mortars with aggregate substitution.

	EEA landfill criteria (mg l^{-1})	Experimental Data – NEN 7371:2004 (mg l^{-1})					
	Class I	Ref.	5% MSW Agg	5% ST Agg	5% CW Agg	12.5% CW Agg	25% CW Agg
Cd	≤0.1	0.04	0.07	0.04	*0.09*	0.13	0.07
Cr	≤0.5	0.07	0.10	0.06	0.10	0.09	0.08
Pb	≤0.5	0.03	0.02	0.02	0.03	0.03	0.03
Ni	≤0.5	0.02	0.04	0.03	*0.05*	0.05	0.02
Cu	≤2	0.015	*0.020*	0.016	0.019	0.022	0.016
pH	5.5–12	12.57	12.60	12.57	12.61	12.60	12.60

Concrete's high pH is believed to immobilize heavy metals. But due to fly ash high specific surface, some of the heavy metals are placed there and prone to leach (Pedersen, 2002). Heavy metals such as Ni, Pb, Cu and Cd are susceptible of leaching from MSW fly ashes (Wiles, 1996) and there is no legal regulation limiting the leachability of heavy metals from regular concrete. Table 3 presents the European Environment Agency (EEA) criteria for landfill deposition of waste and leachability values of the mortars after NEN 7371:2004. This gives an overview of the increase on metal leachability from the blended mortar.

The reference mortar presented the lowest leachability of all the studied heavy metals, with exception of Cr. Observing Table 3 for the 5% substitution of aggregate with all ST, CW and MSW ashes, no conclusion could truly be drawn about their hazardousness. 5% ST Agg sample presented the lowest leachability of the three; 5% MSW ash was leaching the most of Cr and Cu; and 5% CW of Ni and Cd. According to Shih et al. (2005) Ni, Cr and Cu are almost totally trapped/incorporated into clinker, not presenting a threat to leach. But heavy metals speciation may play an important role on their leachability. Ni, Pb and Cu seem to leach more easily from CW and MSW blended mortar, meaning that extra caution should be given to these metals in future research. Only a small percentage of MSW ash was included in the mortars and consequently low concentrations of heavy metals were observed in the leachates. However, Gao et al. (2008) found out that by including complexing agents to the concrete mixture, MSW fly ash leachability is highly reduced.

Regarding CW ash, its initial total content of Ni was 10 times higher than the one in MSW ash (Table 1), but the leachability did not present the same relation. Indeed, Yu et al. (2005) concluded that metals' leachability depends on the kind of solidified fly ashes but not on their initial heavy metal content. In addition, Cr and the more concerning Cr (VI) did not increase leachability due to fly ash input. It is actually supposed that Cr (VI) soluble species are effectively immobilized by the cement base material and can be found adsorbed or precipitated with silicates or calcium compounds, as $Ca_2CrO_5 \cdot 3H_2O$ or in C–S–H gel, where SiO_4^{4-} is substituted by CrO_4^{2-} (Omotoso et al., 1998; Yu et al., 2005). Furthermore, dissolved Cr and Cr (VI) is not a constituent of concern with respect to surface pavement materials alone (Kayhanian et al., 2009).

CW ash was further investigated since the results from the compressive strength test were quite promising (Fig. 3) and Table 3 shows the leachability results. The sample with 12.5% CW Agg, with the most promising compressive strength (Fig. 3), was the one leaching the most, especially Ni. Compressive strength and leachability of metals may be somehow interconnected. It may be that the less heavy metal is absorbed into the matrix, the more strength the mortar develops. Hypothetically, a reduced sorption of foreign cations by the silicate matrix may enhance strength and induce leachability of the respective cation (e.g. Ni). This is certainly a mechanism that deserves some further research for better understanding of heavy metal sorption in a mortar matrix and its environmental implications.

4. Conclusions

Summarizing, three fly ashes were here compared regarding efficiency for EDR remediation and mortar application. While ST ashes proved to be quite successful removal of metal during EDR and potential to be reused in agricultural land, but CW ash did not. MSW fly ash has been deeply investigated and high removal rates obtained. However, the resulting final product may have reduced reuse value.

A solidification/stabilization technique was then applied where untreated fly ashes were directly applied to mortar. Overall, the mortars with the aggregate fraction substituted by fly ashes presented higher compressive strengths. We show here that addition of only 5% fly ash affects the leaching of heavy metals from mortar. The leachability values are orders of magnitude lower than the EEA landfill criteria for the deposition of waste. MSW ashes deserve further investigation, since only a small substitution percentage was studied. ST ashes poor results might be explained by their high salt content. CW ash presented consistent compressive strength results, but the high Ni content should be addressed.

Overall, the integration of waste in the synthesis of new "green" materials should always consider the precautionary principle, where environmental and health related tests should consistently be carried out.

Acknowledgments

The authors would like to acknowledge CIRIUS for A. T. Lima scientific interchange to the Technical University of Denmark. The research work described in this paper was partly funded by the Utrecht University award for KAUST Center-in-Development SOWACOR, Award No. KUK-C1-017-12.

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Journal of Environmental Management 95 (2012) S116–S121

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Enhancement of cometabolic biodegradation of 4-chlorophenol induced with phenol and glucose as carbon sources by *Comamonas testosteroni*

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ARTICLE INFO

Article history:
Received 3 September 2009
Received in revised form
22 July 2010
Accepted 3 September 2010
Available online 22 October 2010

Keywords:
4-Chlorophenol
Comamonas testosteroni
Cometabolism
Glucose
Phenol

ABSTRACT

The biological degradation of phenol and 4-chlorophenol (4-CP) by *Comamonas testosteroni* CECT 326T has been studied. Phenol and 4-CP were treated alone as a sole carbon and energy source, but only phenol was completely degraded by *C. testosteroni*. Since the presence of cosubstrates can enhance the toxic compounds removal by pure cultures, phenol and glucose were added as growth substrates for cometabolic transformation of 4-CP. High efficiencies were obtained in all the experiments carried out in presence of both cosubstrates. In spite of the fact that the addition of glucose reduced the lag phase of 4-CP removal, lower phenol concentrations were required to obtain the same degradation efficiencies. The cometabolic transformation of 4-CP was closely related with the extent of phenol removal. The values of the 4-CP/biomass concentration ratio (S/X) obtained for discriminating between complete ($S/X \leq 0.11$) and partial 4-CP ($S/X \geq 0.31$) transformation showed a narrower range than that reported in the literature. The extent of the cometabolic 4-CP transformation in the presence of phenol could be further enhanced by using glucose as an additional carbon and energy source. However, no significant influence of glucose concentration on 4-CP removal was observed over the concentration range studied.

1. Introduction

Nowadays environmental contamination by toxic xenobiotic compounds is a serious worldwide problem. Among these toxic pollutants, phenol and chlorophenols have gained relevance due to their presence in the environment and biota because of the widespread use in many industrial processes such as the production of resins, nylon, plastics, antioxidants, lubricant additives, wood protectors, bleached pulp, pesticides, textile, dyes, explosives, disinfectants or biocides. The yearly industrial production of chlorophenols was estimated at 200,000 tons in 1989 (WHO, 1989; Field and Sierra-Alvarez, 2007). In 1999, 56,000 tons of waste phenol and 1900 tons of waste chlorophenols were generated by industries in the United States (Tarighian et al., 2003). Wastewaters from the industrial activities are characterized by variable concentrations of phenolic compounds (500–4000 mg/L) (Dojlido and Best, 1993; Park and Keane, 2003). Concentrations of monochlorophenols have been reported to range from non-detectable to 20 mg/L (WHO 1989). Some of the characteristics of chlorophenols are their acute toxicity and poor biodegradability (Armenante et al., 1999). Therefore, the treatment of the wastewaters, containing variable concentrations of those pollutants, has generated a great interest in the last years.

Traditionally, those effluents have been treated by physical or chemical methods. Activated carbon adsorption or air stripping simply transfer the chlorinated organics from water into another medium (Prübe et al., 2008). Oxidation processes such as wet air oxidation, Fenton, or photochemical processes show several drawbacks like relatively high temperatures and/or pressures, large amounts of reagents and complex equipment, respectively (Santos et al., 2002; Pera-Titus et al., 2004). Catalytic hydrodechlorination shows a high efficiency for the removal of chlorophenols but so far it is still in an early stage (Diaz et al., 2008). Generally, chemical processes are much more energy-intensive than biological treatments due to severe reaction conditions, more expensive for higher contaminants loadings and might yield byproducts with similar or even higher toxicity than those of the starting pollutants (Prübe et al., 2008). In some cases these processes can be coupled to a biological treatment once the toxicity of the wastewater has been reduced by some previous treatment (Felis et al., 1999).

Novel biological processes based on aerobic, anaerobic and combined anaerobic–aerobic schemes have been postulated as emerging technologies for the degradation of halogenated organic compounds since they have the potential of mineralizing toxic compounds at relatively low cost. Several studies are available on the biological treatment of chlorinated phenols in aqueous effluents.

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doi:10.1016/j.jenvman.2010.09.030

Despite their recalcitrant nature, different microorganisms such as yeast (Hofrichter et al., 1994; Polnisch et al., 1992), bacteria, fungi and algae (Field and Sierra-Alvarez, 2007; Kim et al., 2002; Wu et al., 2004) have been used for biological degradation of phenolic and chlorophenolic compounds. In the present study, a pure culture of *Comamonas testosteroni* has been tested for the biological degradation of 4-chlorophenol (4-CP), which was selected as target toxic pollutant. *C. testosteroni* can be isolated from soil and wastewater by using phenolic and chlorophenolic compounds as a sole carbon and energy source (Chen et al., 2003; Hollender et al., 1994).

It is known that biodegradation of industrial wastewaters can be improved by using microorganisms previously adapted to the specific toxic compounds (González et al., 2001). Cometabolic removal of toxicants is also a well-established method to enhance their biodegradation (Sahinkaya and Dilek, 2006). Phenol has been claimed as a good growth substrate in the biodegradation of chlorophenolic compounds because of its similar chemical structure and lower toxicity. Although glucose has been a widely used conventional carbon source in biotransformation studies, it has never been used in the cometabolic transformation of 4-CP by *C. testosteroni*.

Previous works have studied the removal of phenolic and chlorophenolic compounds by *C. testosteroni* at temperatures between 25 and 32 °C (Hollender et al., 1994, 1997; Kim et al., 2002; Yap et al., 1999). However, lower temperatures can be commonly found in wastewater treatment plants. Therefore, the study of the influence of temperature on the degradation of phenolic compounds by *C. testosteroni* could elucidate if this species could be considered as a promising specialist degrader bacteria for the bioaugmentation of activated sludge systems.

A major part of the research on diauxic phenomena is concerned with cell growth on binary mixtures with sugars. Diauxic growth implies the inhibition of the consumption of one growth substrate by the presence of another, which requires a larger acclimation period for the utilization of the second substrate. In contrast to diauxic growth patterns, concurrent utilization of multiple substrates in natural ecosystems and in wastewater treatment systems is commonly observed. In general, the use of mixed substrates is desirable because of the enhancement of the removal rates and degradation efficiencies. In addition, the complete or partial biodegradation of 4-CP in presence of cosubstrates would depend on the 4-CP/biomass (S/X) concentration ratio. That dependence has been studied in the literature for *Pseudomonas putida* (Saez and Rittmann, 1993) but there is a lack of information in that respect relative to *C. testosteroni*. Although an enhancement of both the rate and extent of cometabolic 4-CP transformation by other microorganisms has been reported, the 4-CP removal by *C. testosteroni* in ternary systems has not been previously studied. The presence of a conventional carbon source mitigated the toxic effects of 4-CP while phenol induced the production of the enzymes needed for its cometabolic transformation by *P. putida* (Wang and Loh, 2000).

The aim of this work is to study the enhancement of 4-CP biodegradability by *C. testosteroni* in presence of cosubstrates. In the present work glucose has been used as a conventional carbon source, either as a sole growth substrate or in combination with phenol for the 4-CP degradation by *C. testosteroni*. Total Organic Carbon (TOC) removal efficiencies obtained by using both phenol and glucose in the transformation of 4-CP are compared.

2. Materials and methods

2.1. Microorganism and growth conditions

C. testosteroni strain CECT 326T used in this study was obtained from the Spanish Type Culture Collection (Coleccion Española de Cultivos Tipo CECT, Valencia). The microorganism was maintained in frozen stock in microtubes at −40 °C in a nutrient medium with 15% (v/v) of glycerol. *C. testosteroni* was transferred to a nutrient medium containing 1 g beef extract, 2 g yeast extract, 5 g peptone and 5 g NaCl per liter of deionised water. The cell suspension resulting from the late exponential growth phase was subcultured in a mineral salts medium (Farrell and Quilty, 1999) with phenol (25 mg/L) as a sole carbon source and grown at 30 °C for 10–12 h in a thermostated orbital shaker (SW2L, Julabo). Agitation was maintained at an equivalent of 120 rpm. The resulting culture was inoculated at 2% (v/v) into conical flasks with a working volume of 150 ml containing mineral salts medium with either phenol or 4-CP as sole carbon sources or 4-CP/phenol, 4-CP/glucose and ternary mixtures. The aerobic batch cultures of *C. testosteroni* were carried out at 30 °C, 120 rpm and pH 7.2. Different concentrations of phenol (80–240 mg/L) and 4-CP (15 and 30 mg/L) were tested when they were treated alone. Studies of cometabolism were carried out at two 4-CP concentrations (20 and 40 mg/L) over a wide range of phenol (40–180 mg/L) and glucose (10–250 mg/L) concentrations. The results reported were the average values from duplicate runs. In all the cases, the standard errors were lower than 10%.

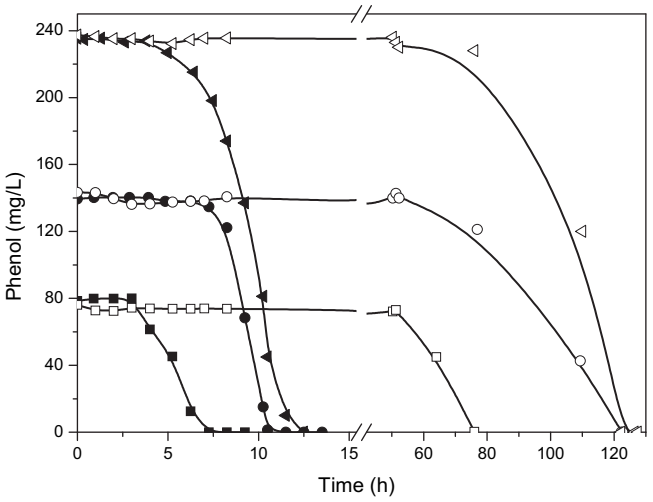

Fig. 1. Time-evolution of phenol concentration at 15 (open symbol) and 30 °C (filled symbol). Initial phenol concentration: (■, □) 80 mg/L, (●, ○) 150 mg/L and (►, ◁) 240 mg/L.

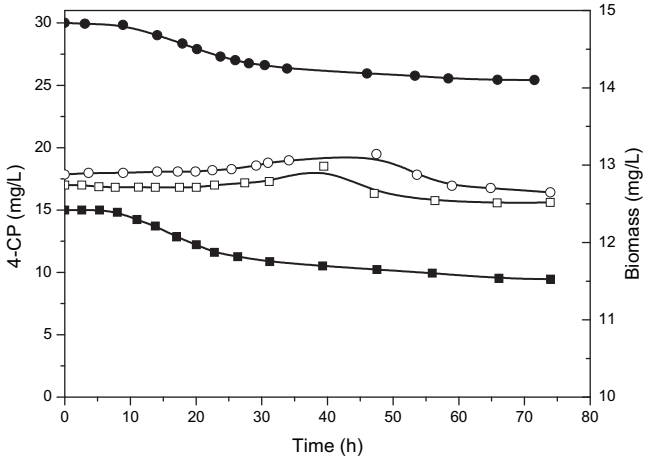

Fig. 2. Time-evolution of 4-CP (filled symbol) and biomass (open symbol) during the growth of *C. testosteroni* with 4-CP as a sole carbon source. Initial 4-CP concentration: (■, □) 15 mg/L and (●, ○) 30 mg/L.

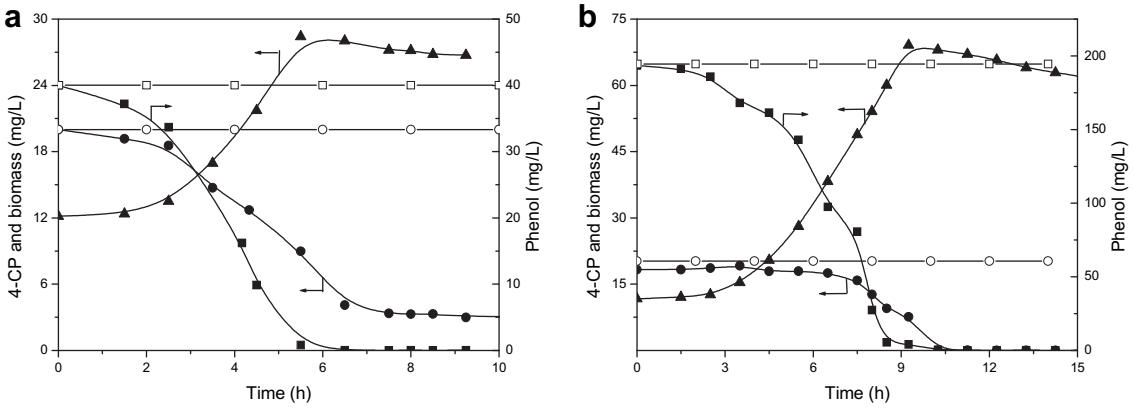

Fig. 3. Time-evolution of phenol (■), 4-CP (●) and biomass (▲) concentration working with mixtures of 4-CP (20 mg/L) and phenol: (a) 40 mg/L and (b) 200 mg/L. Abiotic blank experiments: phenol (□) and 4-CP (○).

Samples were periodically taken for biomass and substrate concentration measurements. Samples were centrifuged (Orto Alresa, mod. Digicen, Madrid, Spain) at 4300×*g* for 10 min at room temperature. The supernatant fraction was then filtered (pore size 0.22 μm; Whatman) and stored at −40 °C for subsequent analyses.

2.2. Analytical methods

Biomass concentration was determined by optical density measurements (Cary 50 conc, Varian) at 600 nm which were converted to cell dry weight using a previously obtained calibration curve. Aromatic compounds were analysed by HPLC/UV (Prostar, Varian) using a C_{18} column as stationary phase (Microsorb MW-100-5) and a mixture of acetonitrile and H_2O (40:60, vol.) as mobile phase. The flow rate was maintained at 1.0 ml/min and a wavelength of 280 nm was used. TOC was measured by an OI Analytical Model 1010 TOC apparatus.

Contribution of abiotic processes such as adsorption, volatilisation and photodegradation was measured. The adsorption assays were carried out at biomass concentrations and operating conditions comparable to those occurring during the biodegradation runs. Adsorption experiments were performed by using bacteria grown in different media (phenol, glucose and mixtures of 4-CP and both cosubstrates). Adsorption of phenol and 4-CP was determined on biomass samples after extraction with Soxhelt following the US-EPA method 8041. Volatilisation and photodegradation tests were performed under identical operating conditions to those employed in the biodegradation experiments but in the absence of biomass. In order to avoid photodegradation and photosynthesis the flasks were protected from light.

3. Results and discussion

3.1. Phenol degradation

The time-evolution of phenol concentration at 15 and 30 °C and different starting concentrations (80, 150 and 240 mg/L) when phenol was used alone is shown in Fig. 1. Although phenol exhaustion was achieved at both temperatures, a great influence of temperature on phenol removal rate can be observed. Thus, the values for degradation rates of phenol at 30 °C (20–36 mg/L h) are nearly ten times of those obtained at 15 °C (2–3.3 mg/L h) over the range of initial phenol concentrations studied. An increase of the temperature leads to a dramatic reduction of the lag time and to a much sharper decay of phenol. Therefore, in the following all the assays were carried out at 30 °C.

No significant influence of initial phenol concentration on the length of the lag phase was observed. However, phenol concentrations higher than those shown in Fig. 1 led to an increase of the length of this phase (data not shown). This phenomenon is related with the toxic action of phenol which affects the integrity of the cytoplasmic membrane (Keweloh et al., 1990; Heipieper et al., 1991).

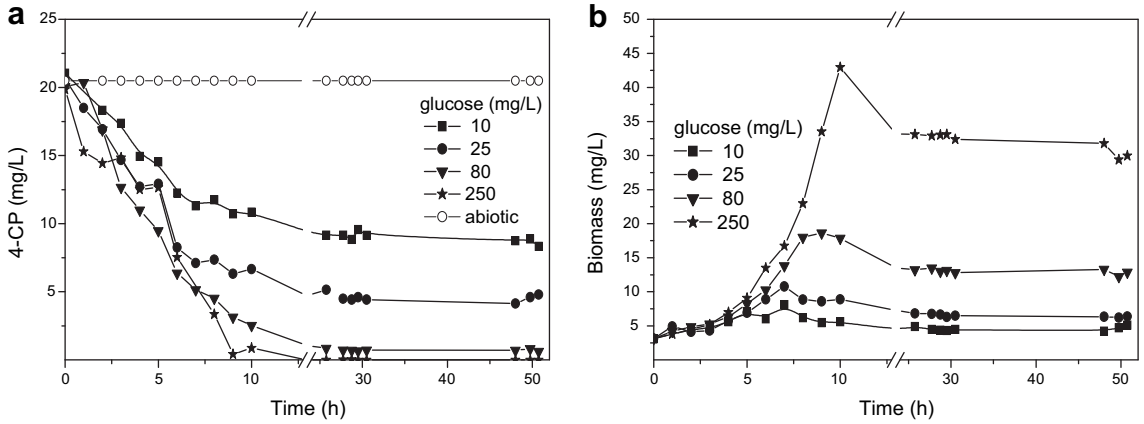

Fig. 4. Time-evolution of 4-CP (a) and biomass (b) concentration during the cometabolic removal of 4-CP (20 mg/L) with glucose as cosubstrate. Abiotic blank experiments (○).

3.2. 4-CP degradation

The degradation of 4-CP alone was studied at two different initial concentrations (15 and 30 mg/L). The time-evolution of 4-CP and biomass concentrations are shown in Fig. 2. As can be seen, low 4-CP transformation rates and conversions were achieved (36.9 and 15.3% for 15 and 30 mg 4-CP/L, respectively). This incomplete transformation was due to the toxicity of 4-CP on cell growth and consequently degradation ability. Similar results were found by Loh and Wang (1998). As the total amount of 4-CP removed is similar at both concentrations, the maximum biomass concentration reached was nearly the same. However, the higher toxic effect at 30 mg/L led to higher biomass decay during the final stage when the 4-CP concentration is stabilised.

3.3. Effect of phenol on 4-CP degradation

Fig. 3 shows the evolution of phenol, 4-CP and biomass concentrations along the degradation process. The results from the abiotic tests showed negligible effects of either stripping or adsorption, so any decrease of the target compounds concentration can be attributed exclusively to biological degradation. In all the experiments, shorter lag times in comparison with those observed when using phenol or 4-CP alone were found. Phenol, which was used as growth substrate, was transformed more rapidly than 4-CP over all the range of phenol concentrations tested. It was found that 4-CP was transformed rapidly only after phenol was almost fully depleted. Similar observations have been reported by Loh and Wang (1998) for phenol and 4-CP transformation by *P. putida*. Once phenol was exhausted, biomass started decaying. These results indicate that 4-CP cannot support cell growth, even in the presence of phenol.

In the cometabolic transformation of 4-CP, phenol is an excellent primary substrate since it not only easily induces the monooxygenase required for 4-CP transformation, but the phenol oxidation can also efficiently regenerates the consumed NADH (Bali and Sengül, 2002). Moreover, the addition of phenol greatly accelerated the degradation of 4-CP due to the increase of biomass production as reported by Bae et al. (1997).

The complete or partial biodegradation of 4-CP can be determined from the 4-CP/biomass (S/X) concentration ratio at the point where no further phenol removal is observed. In this work it was established that partial-removal was found for $S/X \geq 0.31$ (Fig. 3a), while complete 4-CP removal was achieved at $S/X \leq 0.11$ (Fig. 3b). Saez and Rittmann (1993) discriminated between complete ($S/X \leq 0.21$) and partial 4-CP ($S/X \geq 0.38$) removal by *P. putida*.

3.4. Effect of glucose on 4-CP degradation

In order to study the feasibility of using glucose as a growth substrate for 4-CP cometabolization, runs at two different 4-CP concentrations (20 and 40 mg/L) were carried out with glucose as the only added growth substrate over a wide range of initial concentrations (10–250 mg/L). Negligible adsorption of 4-CP onto biomass was found as can be seen in Fig. 4a. No lag phase in 4-CP biodegradation took place in any case, whereas complete removal of 4-CP was only achieved at the highest concentration of glucose tested. Although 4-CP retards the cell growth, its toxicity can be greatly attenuated by adding a primary substrate (Wang and Loh, 1999). Although the steep branch of the 4-CP decay curves is time-coincident with the growing region of the biomass curves (Fig. 4b), the biomass growth must be attributed to glucose consumption since *C. testosteroni* growth on 4-CP is limited as shown previously.

Fig. 5 compares both substrates, phenol and glucose, when used as the sole primary carbon and energy sources in the cometabolism of 4-CP. As can be seen, although the lag period of 4-CP removal

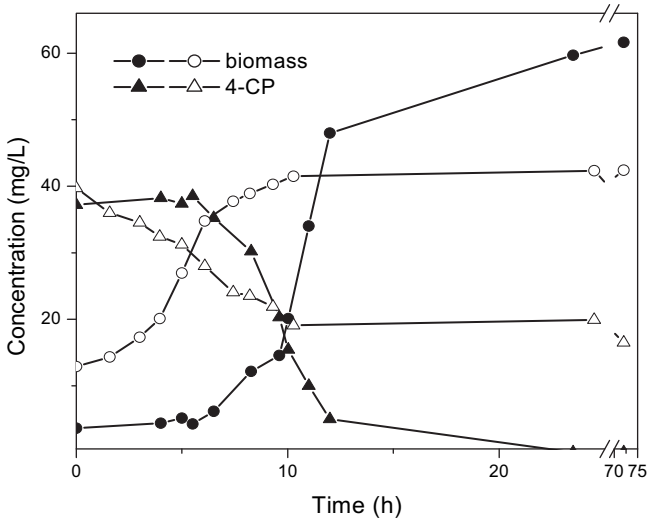

Fig. 5. Evolution of 4-CP and biomass concentration upon cometabolism of 4-CP (40 mg/L) with primary substrate (200 mg/L): phenol (filled symbol), glucose (open symbol).

disappears when using glucose (Tarighian et al., 2003) the presence of phenol leads to a higher removal rate once biodegradation starts. Complete 4-CP transformation was obtained using 200 mg/L of phenol, whereas only 58.4% was removed by adding the same glucose concentration.

Similarly, TOC removal efficiencies obtained with phenol as growth substrate were higher than those with glucose (Fig. 6). A comparison of the TOC removal efficiencies during 4-CP cometabolism showed no effect of the cosubstrate concentration on the final TOC values for concentrations higher than 80 and 250 mg/L of phenol and glucose, respectively. When phenol was added at concentrations higher than 80 mg/L, a residual TOC fraction of 20% was detected after the complete phenol depletion in all the experiments, regardless of the initial phenol concentration. This fact indicates the presence of unidentified refractory species derived from 4-CP biodegradation. Studies about the accumulation of chemical oxygen demand (COD) during phenol/4-CP degradation by *Acinetobacter* species have also indicated the presence of

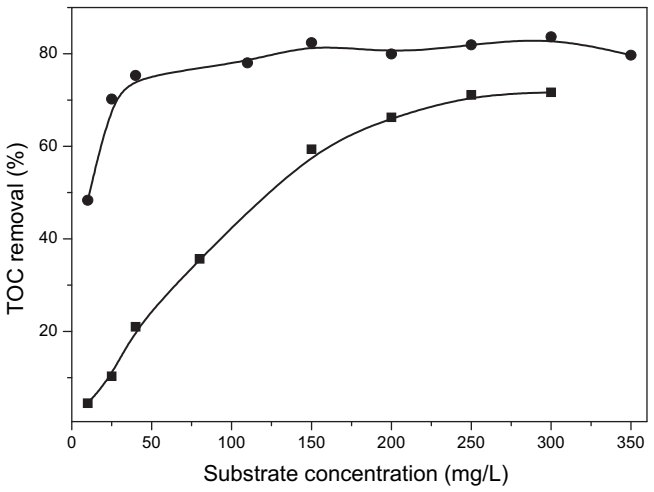

Fig. 6. TOC removal in cometabolism of 4-CP (20 mg/L) using glucose (■) and phenol (●) as the carbon substrates.

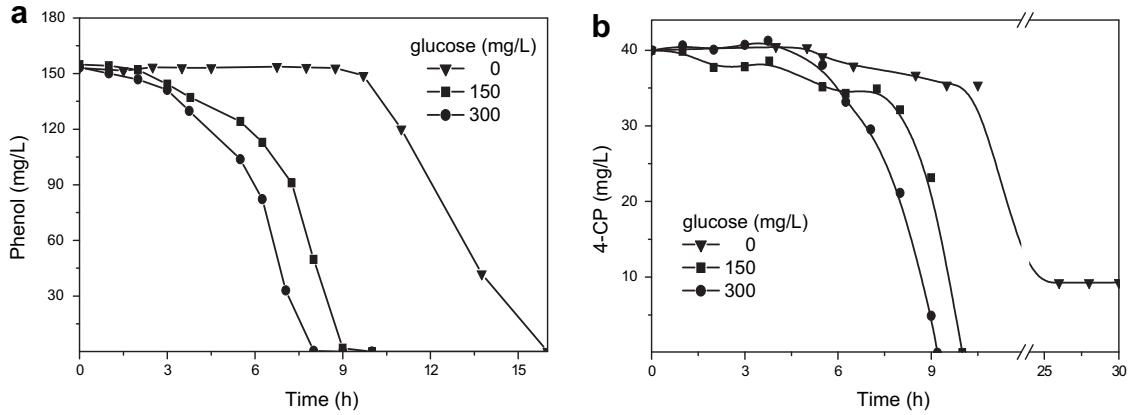

Fig. 7. Time-evolution of phenol (a) and 4-CP (b) treating mixtures of 4-CP (40 mg/L) and phenol (150 mg/L) and glucose (150 and 300 mg/L).

excreted products resulted from 4-CP transformation (Kim and Hao, 1999).

3.5. Effect of phenol and glucose on 4-CP degradation

Fig. 7 shows the degradation of phenol and 4-CP by *C. testosteroni* when glucose was added. As can be seen, the cometabolic transformation of 4-CP in the presence of phenol can be enhanced by adding a conventional carbon source like glucose. Although the use of sugars as a primary substrate does not require oxygenases for metabolism, it can support cometabolism of 4-CP through the generation of NADH (Wang and Loh, 1999). The addition of glucose reduced significantly the lag time observed for both phenol and 4-CP removal when phenol was used as a sole primary substrate. In these conditions the toxicity and inhibition of 4-CP can be attenuated and both cell growth and degradation rates can be significantly enhanced (Loh and Wang, 1998). No inhibition of degradation of either phenol or 4-CP by the presence of glucose was found. Increasing the glucose concentration from 150 to 300 mg/L did not reduce significantly the time required for complete removal of phenol and 4-CP.

4. Conclusions

C. testosteroni is capable of degrading phenol as a sole carbon and energy source within the range of concentrations tested (80–240 mg/L). However, results showed that the 4-CP transformation capacity was clearly deficient. Phenol and glucose acting as a primary growth substrate enhance 4-CP biodegradation. Nevertheless, higher 4-CP removal efficiencies can be obtained in presence of phenol than with glucose at the same initial concentrations. Whilst partial-removal was found for $S/X \geq 0.31$, complete 4-CP transformation was achieved at $S/X \leq 0.11$ when using phenol as cosubstrate. The simultaneous addition of phenol and glucose greatly reduces the minimum time required for complete 4-CP biodegradation. The complete removal of 4-CP and phenol is important for bioremediation purposes since both compounds are frequently found together in hazardous wastes.

Acknowledgements

This work was supported by Comunidad de Madrid-Universidad Autónoma de Madrid through the project CCG06-UAM/AMB-0048 and by Spanish MCI through the project CTM2007-60959. Monsalvo V. M. wishes to thank the MCI and FSE for a research grant.

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Journal of Environmental Management 95 (2012) S122–S127

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Biogasification of biowaste and sewage sludge – Measurement of biogas quality

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ARTICLE INFO

Article history:
Received 30 September 2009
Received in revised form
16 May 2010
Accepted 4 January 2011
Available online 5 February 2011

Keywords:
Biogas
Trace gas compound
Biogasification
Anaerobic digestion
Biogas measurement

ABSTRACT

Biogas quality, the presence of some trace components (siloxanes, sulfur compounds, volatile organic compounds, VOCs) in biogas, is in a decisive role when determining the biogas utilization and the purification requirements and equipments. In the present work, the effects of process changes related to reactor loading variations on the concentrations of selected trace compounds in biogas were studied. Source separated biowaste and sewage sludge were co-digested in a mesophilic pilot reactor (200 L) for four months during which the organic load was stepwise increased. The results showed that the process worked steadily up to the load of 8 kgVS $m^{-3}d^{-1}$. Also the community composition of methanogenic archae stayed largely unaffected by the load increase, and was at all stages typical for a mesophilic biogasification process. Gaseous concentrations of siloxanes, hydrogen sulfide and most VOCs remained at a constant low level, showing no sensitivity to variations in the load and related process changes. However, the total siloxane concentration in the biogas was dependent on feed quality, and the detected concentrations require removal prior to use in turbines or fuel cells. Otherwise, after the removal of siloxanes, the biogas studied in this work is well applicable in various electricity production options, like in gas engines, turbines, microturbines and fuel cells.

1. Introduction

Biogasification, i.e. anaerobic digestion, is a well known sustainable option for the management of organic solid wastes and sludges. The produced biogas is a valuable biofuel for the replacement of fossil fuels in various technical applications (e.g. heating, electricity, transport fuel), which in turn determine its quality requirements. Elevated concentrations of certain trace components, such as sulfur compounds (sulfides, disulphides, thiols), siloxanes (organic silicon compounds), halogenated compounds and ammonia, can be harmful in many biogas utilization applications (Persson et al., 2006; Arnold, 2009; Trogisch et al., 2005). Data have been reported providing information about the presence and concentration levels of the trace compounds in biogas in landfills or biogasification plants (e.g. Persson et al., 2006; Rasi et al., 2006; Urban et al., 2008; Arnold and Kajolinna, 2008). In the earlier studies, large variation in the trace gas concentrations has been detected and the composition of waste/feed has been found to be in a decisive role but also the process conditions may have an effect on the concentrations.

The measurement of trace compounds in biogas has typically been done by gas sampling and subsequent analysis in a laboratory. The method is laborious and time-consuming. In this work, on-line measurements were conducted to continuously follow up the concentrations of the main biogas trace compounds formed during co-digestion of municipal biowaste and sewage sludge. The aim of the study was to identify and quantify the major biogas trace compounds and to study the effects of variations in organic loading and related process changes on their concentrations. A sudden and significant increase in organic loading of a biogas reactor is a risk for process disturbances. Therefore, the load-increasing points of the process were chosen as main focus of the conducted analyses of the physical, chemical and microbiological process parameters.

2. Materials and methods

2.1. Anaerobic reactor and test period

An anaerobic reactor (200 L; operating volume of 150 L) was operated semi-continuously (fed once per day) for four and half months with a mixture of biowaste and sewage sludge (30 and 70% of total wet weight, respectively). The reactor was mixed (ca. 160 rpm) for 30 min after every 2 h. The reactor temperature was in a mesophilic range, 35–37 °C. The organic loading rate (OLR) was increased

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doi:10.1016/j.jenvman.2011.01.003

Table 1
Loading data of the pilot reactor during the test period of 9.10.2007–20.2.2008.

Date	Organic load kgVS $m^{-3}d^{-1}$	TS content of feed mixture %	Retention time days
9.10–12.10.2007	1	7.5	58
15.10–4.11.2007	1.5	6.2–8.0	30–44
5.11–13.11.2007	2	7.8–8.0	29–32
14.11.07–9.1.2008	3	7.4–8.4	18–24
10.1–27.1.2008	5	10.0–13.1	15–17
28.1–12.2.2008	8	9.1–13.0	9–10
13.2–20.2.2008	10	9	8

stepwise from 1 to 10 kgVS $m^{-3}d^{-1}$ (kg volatile solids per m^3 reactor volume and day). At the same time, HRT (hydraulic retention time) was decreased stepwise from 58 days to 8 days (Table 1).

The physico-chemical state of the process (pH, T, alkalinity, volatile fatty acids, ammonia, biogas production and its CH_4-content) was monitored throughout the test period, whereas the detailed composition of the biogas, as well as community structure of methanogenic archaea, were analyzed in transient reactor conditions, i.e. at the load-increasing points: from 2 to 3 kgVS $m^{-3}d^{-1}$; from 3 to 5 kgVS $m^{-3}d^{-1}$; from 5 to 8 kgVS $m^{-3}d^{-1}$ and at the end of the test period at the load of 10 kgVS $m^{-3}d^{-1}$.

2.2. Feed mixture

The source separated biowaste was used as a feed at a full-scale biogasification plant where it was finely minced, homogenized and hygienized (70 °C) before mixing with other wastes and feeding to a reactor. Five different batches (deliveries) of that biowaste were used during the test period. The biowaste, divided into doses, was stored frozen. TS (total solids) and VS (volatile solids) content of biowaste varied between 22.1–32.2% and 19.2–26.2%, respectively. Total nitrogen and carbon contents were around 2.6–3.2% and 41–49% of dry solids, respectively.

The sewage sludge originated from a local municipal biological-chemical wastewater treatment plant. Eight different batches (deliveries) of that sludge were used during the test period. The sludge was stored in a cold room (4 °C). TS and VS content of sludge varied between 3.4–5.9 and 2.2–3.6%, respectively. Total nitrogen and carbon contents were around 3.3–4.1% and 32% of dry solids, respectively.

The feed mixture was made by diluting biowaste and sludge with water to a selected TS content of the mixture: at first the TS content was ca. 8%; at the load of 5 kgVS $m^{-3}d^{-1}$ it was increased to 10% and finally it was ca. 13% (i.e. no dilution with water). The TS content was increased in order to keep the HRT long enough. On the other hand, the feeding pump limited the highest value of TS to around 13%. In the feed mixture, the ratio of biowaste and sludge based on wet weight was kept constant: 30% biowaste and 70% sludge. As there was variation in their TS and VS contents, the ratio based on TS or VS in the feed mixture varied slightly, but around 70–80% of the total VS amount of the mixture originated from the biowaste.

2.3. Gas analyses

The total biogas volume (KIMMON SK35 gas meter) and its CH_4-content (Simrad GD10 IR gas detector) were followed up on-line throughout the test period. The gas meter was read once a day and CH_4-data were recorded automatically every 15 min. Furthermore, the main biogas trace compounds, such as siloxanes, sulfur compounds (H_2S, DMS, MeSH, ethylthiol), volatile organic compounds (VOCs), ammonia and nitrous oxide (N_2O) were determined during selected transient biogasification reactor conditions, i.e. at the load-increasing points: from 2 to 3 kgVS $m^{-3}d^{-1}$; from 3 to 5 kgVS $m^{-3}d^{-1}$; from 5 to 8 kgVS $m^{-3}d^{-1}$ and at the end of the test period at the OLR of 10 kgVS $m^{-3}d^{-1}$ (Fig. 1A). Siloxanes, sulfur compounds and VOCs were measured on-line with gas chromatography (Voyager Perkin Elmer). The main gas components, CH_4 and CO_2, as well as ammonia and nitrous oxide were determined by FT-IR-analysis (Gasmet, Temet Instruments) (Arnold and Kajolinna, 2008). These on-line measuring periods lasted from 3 to 14 days, starting at least a day before the load-increasing step.

2.4. Molecular microbiological analyses

Total DNA was extracted from 0.25 ml of the reactor's digested sludge at the load-increasing steps from 2 to 8 kgVS $m^{-3}d^{-1}$ using a FastDNA SPIN kit for soil (Bio 101, Inc., La Jolla, CA, USA) according to the manufacturer's instructions. PCR-primer set of *mcrA*-F and *mcrA*-R by Luton et al. (2002) was used for methanogen-specific amplification. Methyl coenzyme M reductase is a key enzyme for the terminal step in methanogenesis, and the *mcrA* gene is present in all methanogens, which makes it a unique molecular marker for methanogenic Archaea (Friedrich, 2005). Denaturing gradient gel electrophoresis (DGGE) and direct clone library analyses of the methanogens in the sludge were performed on the PCR-products obtained with *mcrA*-primers. The PCR-DGGE products (with GC-clamp) were separated by a denaturing gradient from 40 to 60% (100% denaturant contains 7 M urea and 40% formamide) in polyacrylamide gels containing 6% (w/v) acrylamide-bisacrylamide. The gels were run at 250 V for 6 h at 60 °C in the DcodeTM system universal mutation detection system (Bio Rad, Hercules, USA). After electrophoresis, single bands from the gels were cut out, and reamplified with *mcrA*-primers (without GC-clamp). The *mcrA* clones and the PCR-products from the DGGE bands were subjected to sequencing using Big Dye terminators with API genetic analyzer 373 (Applied Biosystems, USA). Phylogenetic analysis of the recovered *mcrA* sequences were done as described by Kurola et al. (2005).

3. Results and discussion

3.1. Physical, chemical and microbiological process behavior

The biogasification process responded to loading increases very steadily up to the load of 8 kgVS $m^{-3}d^{-1}$. The biogas production followed closely the increase in loading and, correspondingly, the biogas production rate increased linearly with increasing VS load until 8 kgVS $m^{-3}d^{-1}$ was reached (Fig. 1). In steady state conditions, the biogas production was around 680–700 L $kg^{-1}VS^{-1}$ and CH_4-content 62–65%. TS and VS reductions were 61–64% and 72–75%, respectively. The biogas yield was high, around 90–95% of the maximum biogas potential, which had been determined by separate lab-scale batch tests for each feed fraction (data not shown). This indicates that the process operated very effectively up to exceptional high loads of over 5 kgVS $m^{-3}d^{-1}$. The stability of the process up to the load of 8 kgVS $m^{-3}d^{-1}$ was also shown by other process parameters: pH was between 7.3 and 7.6; alkalinity between 6000 and 6700 mg $CaCO_3$ L^{-1}; COD_{sol} between 1 and 7 g L^{-1}, and ammonia nitrogen around 1.3 g L^{-1}. At load-increasing points (3 → 5 kgVS $m^{-3}d^{-1}$ and 5 → 8 kgVS $m^{-3}d^{-1}$) a three to five fold increase in the concentrations of individual volatile fatty acids (VFAs, acetate, propionate) was noticed. The concentrations normalized in one or two days. Thus, according to all above mentioned traditional physico-chemical analyses of the process, individual VFAs showed the only clear indication of a typical short-time process disturbance due to a sudden increase in organic load.

The community composition of methanogenic archaea was analyzed at load-increasing steps from 2 to 8 kgVS $m^{-3}d^{-1}$ with

PCR-DGGE, and the methanogens in the process were identified with direct cloning and sequencing, or sequencing the individual DGGE bands. The phylogenetic analyses of retrieved methanogenic *mcrA* gene sequences (Fig. 2) showed that the major methanogenic population in this digestion process consisted of *Methanosarcina* sp. and other members of *Methanosarcinales*. Two clones of *Methanomicrobiales* were also detected. The results indicate that the diversity of the methanogenic community in the process was low, dominated by *Metanosarcina* sp. at all loading steps, and the increased loading did not affect the general community composition of methanogens. The results suggest moreover that the primary metabolic pathway for methane production in the process was acetoclastic. In previous studies (Garcia, 1990; Garcia et al., 2000; Lozano et al., 2009), the ability of *Methanosarcina* sp. to use different substrates (including H_2 and formate) is suggested to explain its high growth rate and predominance in acetoclastic digestion processes.

3.2. Trace compounds in biogas

Siloxane analyses included six different siloxanes, L2, L3, L4, D3, D4 and D5. The letters L and D refer to linear and cyclic organic Si- compounds, respectively, and the subsequent number refers to the number of Si-atoms in the compound. The above mentioned siloxanes are the most common ones found in landfill gas and biogas (Hagmann et al., 1999; Schweigkofler and Niessner, 1999; Wheless and Pierce, 2004; Pierce, 2005). In our biogas measurements, D5 (decamethylcyclopentasiloxane) was found to be the dominant siloxane representing around 70–85% of the total amount of the siloxanes measured in this digestion process. The result is consistent with the earlier findings of the most abundant siloxane components being D5 and D4 by Vesterager and Matthiesen (2004) The total siloxane concentration varied between 0.2 and 0.7 ppm (Fig. 3). The low biogas siloxane concentrations (0.5–4 ppm) have been reported to be typical in municipal sewage sludge digesters in less industrialized regions (Rossol and Schmelz, 2005). Siloxanes are known to end up in sewage sludge or biowaste e.g. from hygienic and cosmetic products or industrial processes, and as semi-volatile compounds they are released into the gas phase during biogasification. In our measurements, the concentration of siloxanes in biogas was not sensitive to variations in the load and related process changes. On the contrary, a clear change in the siloxane concentration was found when shifting to a new feed batch (Fig. 3), indicating that the level of siloxane in the biogas was dependent on feed batches.

Very diverse values for hydrogen sulfide (H_2S) concentrations in biogas have been reported e.g. by Urban et al. (2008): 10–30 000 mg m^{-3}, depending on feed material and process conditions. In our work, the content of H_2S remained < 10 ppm in all measurements. One reason for the low value may be the presence of ferro sulfate ($FeSO_4$) in the sewage sludge used as feed. Ferrous salts are known to precipitate sulfide in anaerobic conditions (Urban et al., 2008). Other sulfur compounds identified in the biogas were dimethyl sulfide (DMS), methyl mercaptan (MeSH) and ethylthiol. Their concentrations were <0.2 ppm in all measurements. The

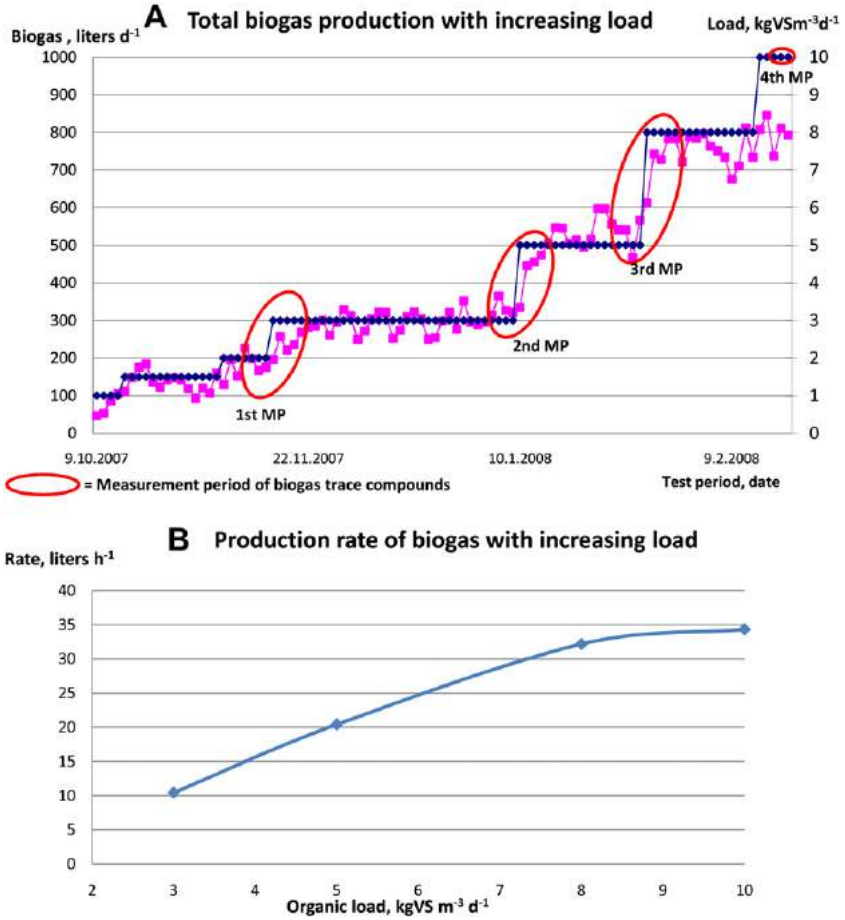

Fig. 1. The increase in organic loading (kgVS $m^{-3}d^{-1}$) and related biogas formation (l d^{-1}) during the test period of ca. 4 months. The four measurement periods of biogas trace compounds were performed at the three load-increasing points and at the final overloading situation. (Panel A) Production rate of biogas (l h^{-1}) as a function of organic loading (kgVS $m^{-3}d^{-1}$). (Panel B).

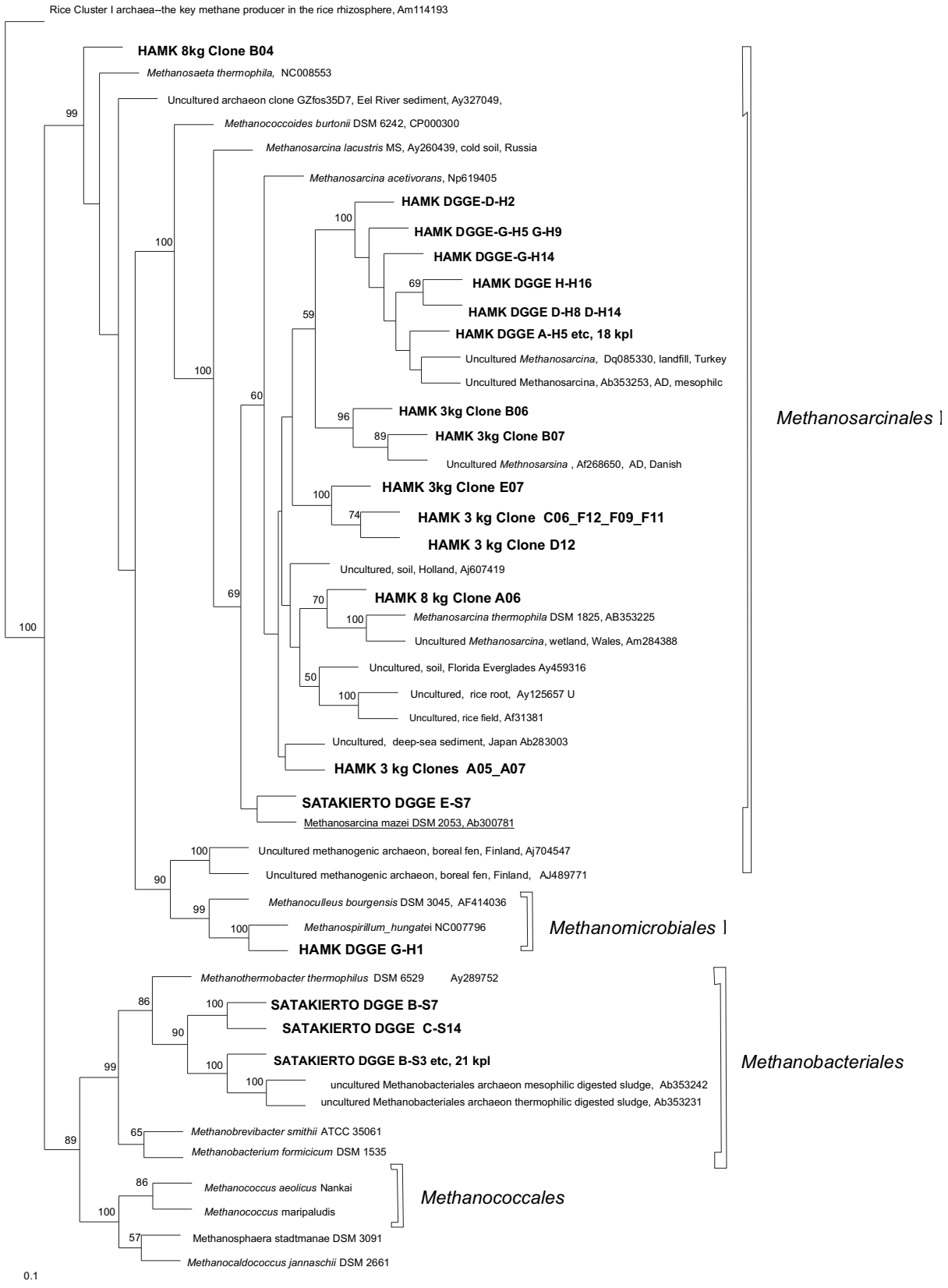

Fig. 2. Phylogenetic relationship between the clones and the DGGE separated PCR-products amplified using *mcrA*-F and *mcrA*-R primer sets (Luton et al., 2002) and recorded methanogenic archaea. The sequences recovered from the pilot reactor are designated with HAMK (3 kg and 8 kg = reactor load of 3 and 8 kgVS $m^{-3}d^{-1}$). Numbers at nodes represent bootstrap values (100 replicates).

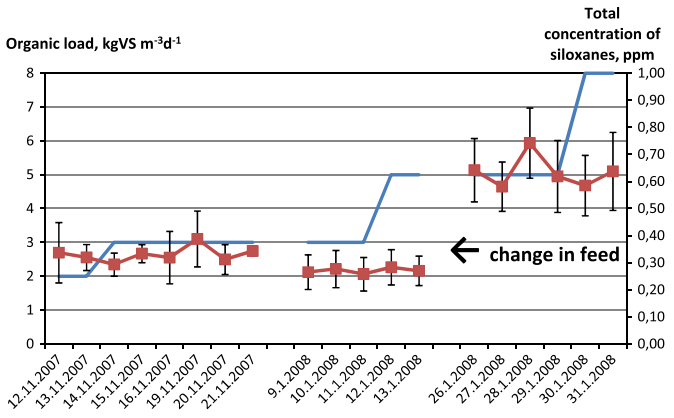

Fig. 3. The effect of load increase and change in feed on the total concentration of siloxanes in biogas.

ammonia concentration remained below the detection limit of 5 ppm in all measurements. Instead, nitrous oxide (N_2O) was found in a few occasions. The reason for this was probable penetration of air into the reactor during the feedings resulting in a temporary short-time formation of N_2O. When the penetration of air during the feedings was successfully prevented, no formation of N_2O was detected anymore.

The major part of all VOCs detected, such as toluene, ethyl benzene, nonane, octane, o-xylene, m-xylene, remained at a very low level, < 1 ppm, during all measurements. Slightly higher concentrations of limonene, p-xylene and α-pinene about 200, 1–2 and 1–8 ppm, respectively, were detected at all loading rates. Interestingly, the ethanol concentration was found to vary significantly and to increase after the load-increasing points (Fig. 4, Panels A and B), indicating that formation of ethanol was related to process changes. Fermentative acidogenic bacteria (i.e., acid-forming bacteria), which is one of the main groups implicated in anaerobic digestion, convert sugars, amino acids, and fatty acids to organic acids and also alcohols and ketones. The increased formation of ethanol can thus be analogous to microbiological processes resulting in increased VFA-concentrations in the liquid phase, which is a recognized indicator of process instability.

Table 2
Biogas quality criteria in electricity production (EPRI, 2006). Content calculated as ppm in methane.

	Reciprocating engine	Turbine	Microturbine	Fuel cell (MCFC)	Stirling engine
Sulfur, ppm	545–1742	< 10,000	25–70,000	< 10[a]	280
Total silicon, ppm	9–44	0.087	< 0.01	< 1[a]	0.42 (D_4)
Halogens, ppm	60–491 (Cl)	1500	200	< 0.1[a]	232 (as HCl)

[a] as ppm in biogas.

4. Conclusions

The mesophilic co-digestion of biowaste and sewage sludge resulted in good quality biogas. Trace compounds potentially impeding the energy utilization of biogas, such as hydrogen sulfide, ammonia and certain VOCs, were found to remain at very low levels showing no sensitivity to process changes. The formation of siloxanes was also independent of process changes, depending on the

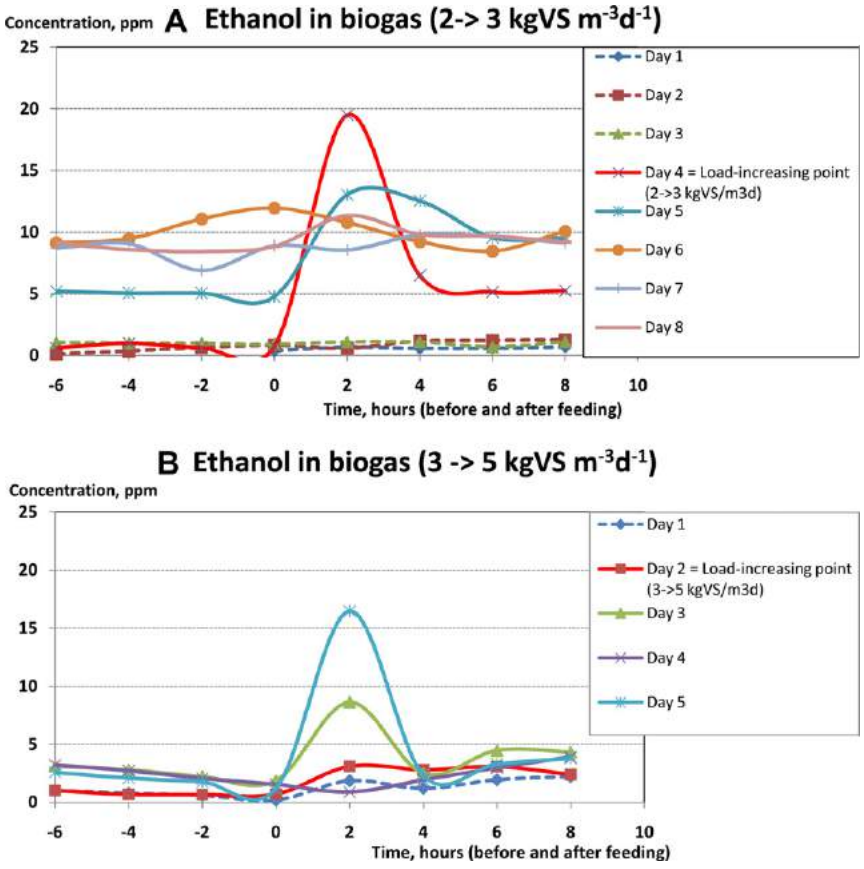

Fig. 4. Formation of ethanol in biogas after load-increasing points of 2–3 kgVS $m^{-3}d^{-1}$ (Panel A) and from 3 to 5 kgVS $m^{-3}d^{-1}$ (Panel B).

feed. However, the detected concentration of siloxanes (mostly D5) is critical if the biogas is utilized in turbines or fuel cells and might require removal prior to use. Concerning electricity generation in gas engines, all measured trace compounds were again below the recommended levels (see Table 2). The physical, chemical and microbiological parameters indicated that the co-digestion process was well under control at all load-increasing steps (from 2 to 8 kgVS $m^{-3}d^{-1}$), and increase in loading caused no clear changes in the concentrations of trace compounds in biogas, except that of ethanol. Changes in ethanol concentration after the feedings close to load-increasing points were significant thus probably having potential for process control purposes.

Acknowledgments

The financial supports from the Finnish National Technology Agency (Tekes) and the companies Preseco Oy, Kiertokapula Oy, Ekokem Oy and Doranova Oy are greatly acknowledged. Tuula Kajolinna from VTT is acknowledged for her excellent work on gas phase analyses, and the project workers from HAMK on their help in everyday reactor-keeping and analysis work: Markus Kaarnakoski, Sebastian Antonzcyk, Laura Soto Vázquez, Piia Lindfors and Laura Kannisto.

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Journal of Environmental Management 95 (2012) S128–S133

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Kinetics of forced aerated biodegradation of digested sewage sludge-reed mixtures at different temperatures

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ARTICLE INFO

Article history:
Received 30 August 2009
Received in revised form
4 May 2011
Accepted 11 May 2011
Available online 1 June 2011

Keywords:
Sludge biodegradation
Kinetics
Temperature influence
Easily biodegradable
Slowly biodegradable

ABSTRACT

This work presents a kinetic analysis of the aerobic biodegradation of anaerobically digested sewage sludge and dried reed mixtures at different temperatures. Batch experiments were conducted in laboratory-scale reactors with temperature (T) control and forced aeration of the solid mixture. The biowaste mixture was treated at four different temperatures: 25, 40, 50 and 60 °C, with moisture controlled and samples taken weekly for carbon (C) and volatile solids (VS) measurements. The duration of experiments was either 90 d (at 25 °C) or 60 d (at 40, 50 and 60 °C). Two different kinetic models were used to fit the carbon mineralisation curves: the 2C model, which considers two organic fractions (biodegradable and non-biodegradable) and the 3C model, which considers three fractions (easily biodegradable, slowly biodegradable and non-biodegradable). In both cases, the kinetic rate constants were calculated by mathematical fitting and were compared with previously reported values. The temperature influence on the rate constants was studied for both models using a T-dependent equation. The calculated kinetic rate constants were in agreement with previously published values, and good fitting of the experimental data was obtained with both models. Similar rate constant values were obtained for mineralisation of the biodegradable fraction (2C model) and the easily biodegradable fraction (3C model). The rate constants for the slowly biodegradable fraction (3C model) were much lower. A good correlation between rate constants and T was observed. Different optimum temperature values were obtained for each rate constant depending on which carbon fraction was degraded. The T-dependent rate constant values obtained could be used for modelling the C mineralisation of real variable-temperature composting processes.

1. Introduction

An understanding of the kinetics of aerobic organic waste biodegradation is very important for designing a composting facility. Obtaining kinetic data is one of the first steps necessary when attempting to model the complex composting process. Some authors have emphasized the importance of devoting efforts to study the modelling of composting or obtaining new kinetic data from different types of wastes or under different working conditions (De Guardia et al., 2010; Mason, 2009). The kinetic study of a solid waste biodegradation process can be operated through the use of inductive or deductive models. Hamelers (2004) and Mason (2006) reported that inductive models are most commonly used. They also indicated a group of important factors which should be considered in a model (e.g., temperature, aeration, moisture, and porosity) because they clearly influence biodegradation rates.

Researchers typically use oxygen consumption or CO_2 production experimental data for kinetic studies (Mohajer et al., 2010; Nakasaki and Ohtaki, 2002; Tremier et al., 2005). Alternatively, carbon (C), organic matter (OM) or volatile solids (VS) removal data have been used (Bari et al., 2000; Kulcu and Yaldiz, 2004; Paredes et al., 2002). These experimental data usually are obtained in lab-scale controlled experiments or full-scale composting processes using a wide range of wastes that combine a nitrogenous or animal origin material with a carbonaceous or vegetal material as a bulking agent.

First-order models are the most commonly used, followed by zero-order and Monod-type models. First-order kinetic models

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doi:10.1016/j.jenvman.2011.05.014

have been proposed by Keener and Marugg (1992) and are widely used to predict substrate degradation in composting processes:

$$\frac{\mathrm{d}\,m}{\mathrm{d}\,t} = -k\cdot(m - m_e) \quad [1]$$

$$k(x_1, x_2, \ldots, x_n) = k_{\mathrm{opt}}\cdot f_1(x_1)\cdot f_2(x_2)\ldots\cdot f_n(x_n) \quad [2]$$

where m (kg) is the mass amount measured at any time, k (h^{-1}) is the degradation rate constant, x_i are the main environmental factors affecting the reaction rate in composting processes, t (h) is the composting time, m_e is the residual mass remaining after an infinite time of composting, k_{opt} is the rate constant in optimal conditions, n is the number of factors that are taken into account and f_i is the function defining the dependence of the reaction rate constant on the factor i studied. Several authors have also obtained good correlations using first-order kinetic models when fitting experimental data (Baptista et al., 2010; Bari et al., 2000; Komilis, 2006; Kulcu and Yaldiz, 2004; Nakasaki and Ohtaki, 2002; Paredes et al., 2002).

The type of organic compound determines its degradation rate. Wastes used in composting cannot be characterized as pure organic compounds, and their composition is usually indicated by carbonaceous fractions with different biodegradation rates. Models taking into account different carbon fractions according to their respective biodegradability have been previously proposed. These models have considered two (Nakasaki and Ohtaki, 2002) or three biodegradable fractions (De Guardia et al., 2008; Komilis, 2006; Mohajer et al., 2010; Tremier et al., 2005).

Temperature (T) is one of the most important environmental factors affecting the composting process. The Arrhenius equation is commonly applied to predict the temperature dependence of the reaction rate constant. The integrated equation can be expressed as follows:

$$k_2 = k_1\cdot e^{(\theta\cdot(T_2 - T_1))} \quad [3]$$

where k_2 is the rate constant obtained at a temperature T_2, k_1 is the rate constant obtained at a temperature T_1 and θ is assumed to be constant in the typical temperature range (20–80 °C) of composting processes (Haug, 1993).

There have been few studies showing the kinetics of aerobic biodegradation of anaerobically digested sewage sludge (SS) and dried reed (R) solid mixtures at different temperatures, and this work tries to offer some new information on this topic. Two different first-order kinetic models (2C and 3C) were used to fit the carbon mineralisation curves. The dependence of the rate constants on the temperature was modelled using a modification of equation (3).

2. Materials and methods

2.1. Materials

Anaerobically digested sewage sludge from a conventional domestic wastewater treatment plant was used. Reed (*Phragmites australis*) harvested from a wetland in central Spain was milled and used as a co-substrate and bulking agent. Table 1 shows the characterisation of the digested sludge, the bulking agent and the sludge/reed waste mixture used in the experiments. The bulking agent was used to increase the sludge porosity. Reed is a frequently used material for this common practice in sludge composting technology (Manios et al., 2003).

2.2. Experimental system

Lab-scale batch reactors were used for the aerobic biodegradation experiments at constant temperatures. Each reactor consisted of a 2-L glass container with an external cooling jacket through which water flowed from a temperature-controlled water bath. Forced aeration was continuously supplied by an air blower to provide an excess oxygen level. Inlet air was humidified and heated to the desired experimental temperature before entering the reactor, and outlet air was collected periodically in *Tedlar*® plastic bags to measure oxygen concentration. The biowaste inside the reactor was supported on a porous plate (Fig. 1).

Table 1
Initial characterisation of the materials and the waste mixture (d.w.: dry weight).

Parameter	Digested sewage sludge (Fernández et al., 2010)		Reed (bulking agent)		Waste mixture (sludge and bulking agent)	
	Mean value	SD ($n = 10$)	Mean value	SD ($n = 3$)	Mean value	SD ($n = 3$)
Moisture (%)	82.1	4.10	24.7	2.6	63.0	1.5
C_{tot} (%) (d.w.)	27.2	2.98	47.9	8.8	42.2	6.8
N_{tot} (%) (d.w.)	3.32	0.30	0.62	0.19	1.39	0.42
VS (%) (d.w.)	93.6	6.27	68.8	3.5	75.9	6.3
P_{tot} (%) (d.w.)	33.4	3.23	0.3	0.12	1.24	0.27
pH	8.5	0.17	7.7	0.11	8.0	0.09

SD: standard deviation.

2.3. Experimental procedure

Four different experiments were performed at constant temperatures of 25, 40, 50 and 60 °C. The biowaste amount used in each experiment was 1 kg of a mixture of SS and R in a ratio of 2:1 (wet weight) and 2:5 (dry weight) to obtain a C/N ratio of 30 and a porosity of approximately 0.3. Porosity was calculated as reported by Fernández et al. (2010). The biowaste was introduced into the reactor and air was supplied. Each constant-T experiment was performed in triplicate. Processing time was 90 days for the 25 °C experiment and 60 days for the remaining experiments because it was expected that a higher T would result in faster completion. Temperature was maintained, although it dropped for a short time (less than 1 h) after sampling, particularly for experiments at 50 and 60 °C. Moisture content was kept in the range of 50–55%. For this purpose, leachate was collected at the bottom of the reactor (with deionised water if necessary) and added to the biowaste when the moisture content fell below 50%. To avoid oxygen diffusion limitations, a continuous air flow of 3 L min^{-1} was supplied. Each reactor was opened periodically throughout the experiment and the entire contents were removed and mixed thoroughly before taking a representative 50-g sample. The remaining reaction mass was returned to the reactor. The biowaste was mixed but never subjected to compaction within the reactor, to ensure a relatively constant density and porosity during the experiment. Fresh samples were used for pH measurements in a 1:10 w/w water extract. Moisture content in the solid samples was calculated by drying at 105 °C for 24 h, and volatile solids (VS) content was determined by measuring weight loss on ignition at 550 °C for 2.5 h. About 15 g of each sample was dried and milled to a particle size smaller than 1 mm in an IKA®-A-11 basic mill and was then used to measure the total carbon concentration (C) by dry combustion, followed by infrared detection of CO_2 (TOC Shimadzu 5050A).

3. Results and discussion

3.1. Evolution of the main variables

Several variables were controlled (T, moisture) or measured (oxygen in exhaust air, pH) throughout the process. Moisture was

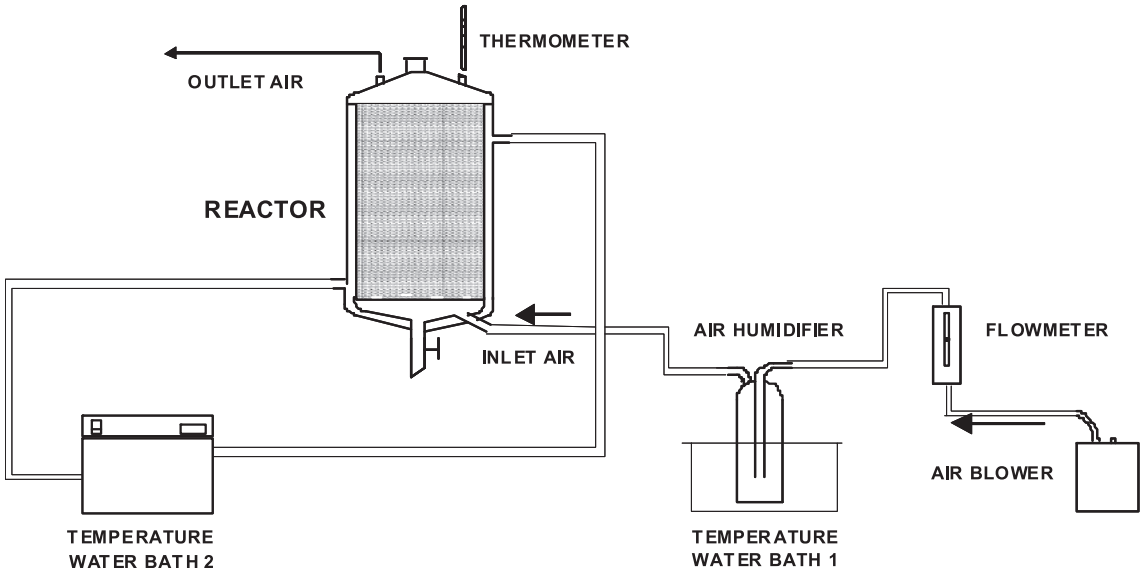

Fig. 1. Scheme of the lab-scale experimental system.

kept in a similar range during each experiment, with the exception of the experiment performed at 25 °C, where initial levels were over 65% for the first 20 days. Air was supplied in excess so that oxygen concentration was always over 15%, which assured aerobic conditions. Higher initial oxygen consumption at 60 °C during the first 25 days was observed due to the higher microbial activity. No initial optimisation was necessary for the pH level. Moreover, during the composting process, pH naturally auto-regulated and did not suffer sudden variations. Fig. 2 shows the volatile solids (VS) evolution in the experiments. Organic matter and VS concentrations related parameters of biowaste, and either can be used to monitor the biowaste degradation. VS measured in the experiments dropped throughout the process and to a greater extent at higher temperatures. However, the quantification of the degradation rates was performed using the C concentration data.

3.2. Kinetics of carbon degradation

A first-order kinetic model was selected to obtain the C degradation rate. Carbon concentrations were expressed in relation to the initial dry mass (DM_0). Two different models were used in this work. A "2C" model only considered two main carbon fractions present in the biowaste mixture, a biodegradable one and a residual non-biodegradable carbon fraction. Equations (4) and (5) show the differential and integrated expressions of the mathematical model, respectively.

$$\left(\frac{-\mathrm{d}\,C}{\mathrm{d}\,t}\right) = k\cdot(C - C_{nb}) \quad [4]$$

$$C = \left[(C_0 - C_{\mathrm{nb}})\cdot e^{-k\cdot t} + C_{\mathrm{nb}}\right] \quad [5]$$

where C is total carbon concentration at any time (kg C kg DM_0^{-1}), C_0 is the initial carbon concentration (kg C kg M_0^{-1}), C_{nb} is the residual non-biodegradable carbon concentration (kg C kg M_0^{-1}), t is the reaction time (d),and k is the carbon degradation rate constant (d^{-1}).

The "3C" model considered three different carbon fractions: a readily biodegradable carbon fraction (C_{rb}), a slowly biodegradable carbon fraction, (C_{sb}) and a non-biodegradable carbon fraction (C_{nb}). The expression of the model (Equations (6) and (7)) takes into account different first-order decay for each biodegradable fraction:

$$\left(\frac{-\mathrm{d}\,C}{\mathrm{d}\,t}\right) = \left(\frac{-\mathrm{d}\,C_{\mathrm{rb}}}{\mathrm{d}\,t}\right) + \left(\frac{-\mathrm{d}\,C_{\mathrm{sb}}}{\mathrm{d}\,t}\right) \quad [6]$$

$$C = \left[C_{\mathrm{rb0}}\cdot e^{-k_{\mathrm{rb}}\cdot t} + C_{\mathrm{sb0}}\cdot e^{-k_{\mathrm{sb}}\cdot t} + C_{\mathrm{nb}}\right] \quad [7]$$

where C_{rb0} is the initial readily biodegradable carbon concentration (kg C kg DM_o^{-1}), k_{rb} is the carbon degradation rate constant for C_{rb} (d^{-1}), C_{sb0} is the initial slowly biodegradable carbon concentration (kg C kg DM_0^{-1}), k_{sb} is the carbon degradation rate constant for C_{sb} (d^{-1}), C_{nb} is the non-biodegradable carbon concentration (kg C kg M_0^{-1}) and t is the reaction time.

The readily biodegradable carbon fraction is usually associated with sugars, lipids and starches, whereas the slowly biodegradable carbon fraction is related to lignocellulosic carbon (Kaiser, 1996; Tuomela et al., 2000). In this work, the initial percentage of slowly biodegradable carbon was estimated from the lignocellulosic content of the initial mass. Since digested sewage sludge and reed

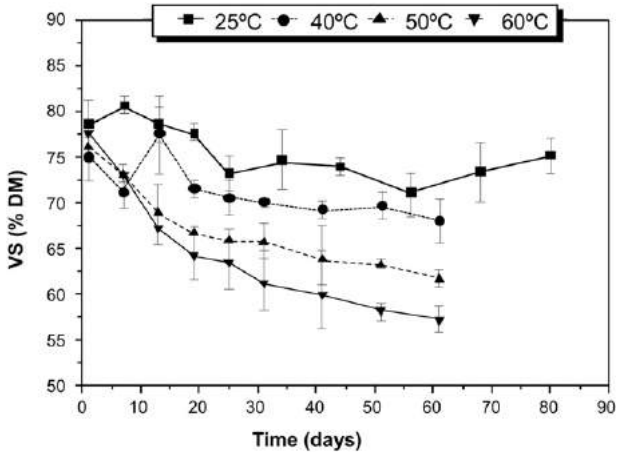

Fig. 2. Volatile solids evolution during the experiments (DM: dry matter).

are very common and well-known materials, default C_{sb0} values from the literature were used for this work. Lignin content (31.8%) and cellulose-hemicellulose content (30.1%) for SS were obtained from data reported by Guoxue et al. (2001) and lignin (10.9%), cellulose (38.4%) and hemicellulose (29.7%) contents for Reed were obtained from data reported by Alburquerque et al. (2004) and Boateng et al. (2006). Concentrations were measured on a dry weight basis. These values were used to calculate the initial lignocellulosic carbon concentration in the SS/R mixtures and a value of 61.0% was obtained. The initial percentage of readily biodegradable carbon in the mixture was obtained from pilot-plant composting experiments performed with the same biowaste mixture under optimal conditions (data not shown) with a value of 37.9%. These values are very similar to those reported by Bernal et al. (1998) for different biowaste mixtures. The authors obtained average values of 35% and 65% (dry weight basis) by fitting carbon-concentration data to a combined first/zero-order equation for the readily biodegradable and slowly biodegradable fractions, respectively.

Carbon concentration data were fitted to both models. Fig. 3 shows the experimental carbon concentration (mean values), the model-adjusted values and the non-linear correlation coefficients for each temperature. Vertical bars represent the standard deviation of three measurements. The values of the rate constants (k) and the C_{nb} concentrations were obtained from the mathematical fitting to the 2C model (Table 2); likewise, the values of the rate constants (k_{rb} and k_{sb}) were obtained from the mathematical fitting to the 3C model, (Table 3). The remaining values shown in Tables 2 and 3 were obtained by mass balance as previously explained.

Both models gave good predictions for the carbon degradation process with similar correlation coefficients. The simulation profiles in Fig. 3 were quite similar, which raises the question of where the real differences between the two models occur. To observe differences, theoretical simulation profiles were calculated for a higher experimental period of 300 d (figures not shown). Based on the C profiles, it was concluded that the 2C model, which was formulated with fewer theoretical presumptions, is only valid for the given experimental time frame. The C_{nb} values obtained by the model were quite high, while the biowaste seemed able to undergo further degradation, based on the experimental data trends.

Table 2
Values for C_0, k and C_{nb} obtained after fitting to the 2C model.

T (°C)	C_0 (kg C kg DM_0^{-1})		C_{nb} (kg C kg DM_0^{-1})	k (d^{-1})
	Mean value	SD ($n = 3$)		
25	0.445	0.02	0.210	0.040
40	0.425	0.08	0.190	0.049
50	0.443	0.08	0.208	0.070
60	0.435	0.05	0.190	0.085

SD: Standard deviation.

In contrast, the 3C model, which starts from a higher level of *a priori* knowledge of the biowaste composition, takes into account the subsequent degradation that will take place in the biowaste at a low rate. Thus, the main simulation differences between the two models would be detected at degradation times greater than 60 d. For instance, reaction times of approximately 100 d offered significant differences at 40, 50 and 60 °C. The difference between the two models was previously noted for higher temperatures.

According to the 3C simulation results, a readily biodegradable C fraction (approximately 31%) was always removed during the first 20–25 days (a little faster at 60 °C), whereas a low degradation rate was observed after this time.

Several authors have obtained similar rate values at constant temperature for different biowaste mixtures. Considering two-carbon fractions in the biowaste, Bari et al. (2000) obtained constant values of 0.008 d^{-1} at 25 °C and 0.04 d^{-1} at 50 °C for food waste, paper mill and sawdust mixtures. Komilis and Ham (2000) obtained values in the range of 0.08–0.14 d^{-1} for different biowastes at 55 °C; and Paredes et al. (2002) reported a value of 0.018 d^{-1} for sludge/cotton mixtures.

Some previous studies have considered three-carbon fractions in the biowaste. Haug (1993) and Nakasaki et al. (1998) reported rate constant values from 0.015 to 1.90 d^{-1} for the readily biodegradable carbon fraction and from 0.004 to 0.11 d^{-1} for the slowly biodegradable carbon fraction. Komilis (2006) reported values of 0.06–0.1 d^{-1} (readily hydrolysable carbon fraction) and 0.005–0.06 d^{-1} (moderately hydrolysable carbon fraction) when composting municipal solid waste and yard waste components. The 3C model has been

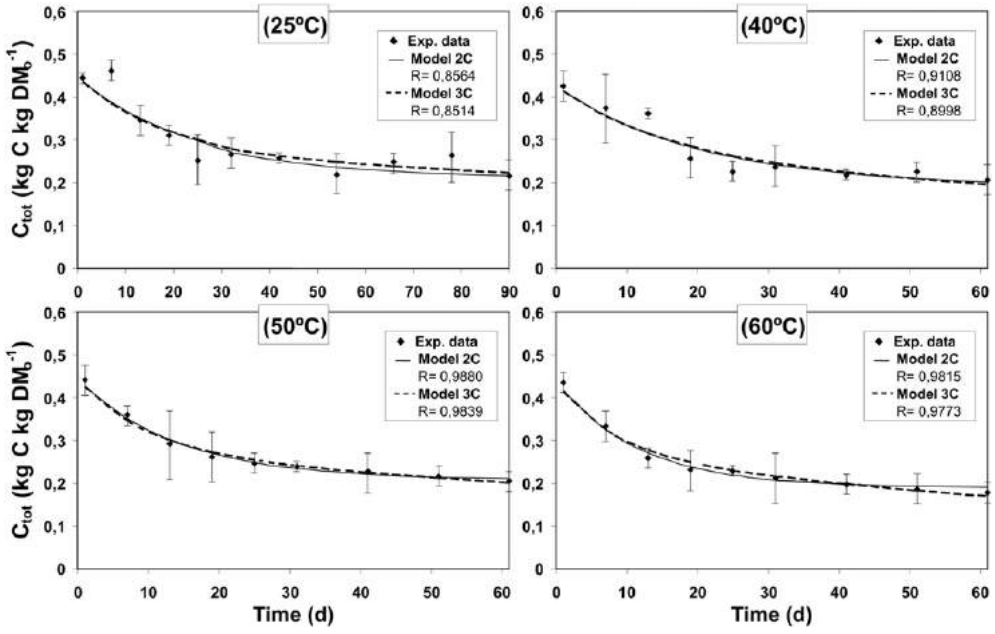

Fig. 3. Carbon degradation profiles in the experiments. Symbols represent experimental data and solid/dashed lines represent the model predictions.

Table 3
Values for C_{rb0}, C_{sb0}, C_{nb}, k_{rb} and k_{sb} obtained after fitting to 3C model.

T (°C)	C_{rb0} (kg C kg DM_0^{-1})	C_{sb0} (kg C kg DM_0^{-1})	C_{nb} (kg C kg DM_0^{-1})	k_{rb} (d^{-1})	k_{sb} (d^{-1})
25	0.169	0.272	0.004	0.057	0.0025
40	0.161	0.259	0.005	0.067	0.0052
50	0.168	0.270	0.005	0.103	0.0052
60	0.165	0.266	0.004	0.125	0.0079

extensively studied by De Guardia et al. (2008), Mohajer et al. (2010) and Tremier et al. (2005) using different types of wastes.

3.3. Temperature effect on the carbon degradation rate

To study the effect of temperature on the reaction rate, the rate constants were adjusted by a modification of Equation (3).

$$k(T) = k_{20} \cdot \theta_1^{(T-20)} - k_{60} \cdot \theta_2^{(60-T)} \quad [8]$$

where k_{20} is the rate constant at 20 °C (obtained in this work after extrapolation from experimental data) and k_{60} is the rate constant at 60 °C. Values of θ_1 and θ_2 were estimated for both models using mathematical fitting. The θ values obtained are presented in Tables 4 and 5, along with the R coefficients for the model predictions. The theoretical model predicts an increase in k until a maximum degradation rate is reached (at temperature $T_{k,max}$) followed by a decrease to zero (at temperature $T_{k=0}$, the temperature corresponding to thermal inactivation). This shape of the $k(T)$ curve has been previously reported for readily and slowly biodegradable carbon fractions (De Guardia et al., 2008; Tremier et al., 2005). However, in the present work no k experimental data were obtained in the short interval between $T_{k,max}$ and $T_{k=0}$, which would confirm the model predictions. Tables 4 and 5 include $T_{k,max}$ and $T_{k=0}$ for both 2C and 3C models.

The shapes of the theoretical $k(T)$ curves represent the behaviour of the rate constant and thus, the microbial activity with respect to the temperature. An initial exponential increase was observed when temperature was increased, but there was an optimum value after which the effect of temperature inactivation of the micro-organisms began to dominate (Mason, 2006).

As indicated previously, authors have obtained similar curves for the temperature dependence of the degradation rate in composting. The main differences can be found in the temperature for the maximum degradation rate. Haug (1993) gave values for optimal temperature around 80 °C, while in the present study, the optimal temperature predicted by the models was in the range of 65–70 °C, indicating a higher influence of thermal inactivation than predicted by Haug. De Guardia et al. (2008) reported $T_{k,max}$ values of 50–65 °C and 55–65 °C for k_{rb} and k_{sb}, respectively and inactivation temperatures higher than 70 °C for k_{sb}. Tremier et al. (2005) also predicted a similar thermal behaviour for biomass growth rate and hydrolysis rate.

Notably, optimal and total thermal inactivation temperatures for k_{sb} were lower for the 3C model than those obtained with the 2C model for k_{rb}. This observation could be related to the type of micro-organisms that are predominantly involved in degrading each fraction. Bacteria thrive at higher temperatures and are more likely to degrade sugars, proteins and starch from the readily biodegradable carbon fraction, while fungi, with lower optimal temperatures, are the main group of micro-organisms involved in lignocellulosic materials degradation (Kakezawa et al., 1990). A microbiological analysis performed by Tuomela et al. (2000) in the thermophilic phase of a composting process found that the main group of micro-organisms detected was bacteria, while in the mesophilic phase, growth of actinomycetes and fungi was higher. Values of θ were similar for both models and comparable to those obtained by Haug (1993).

Table 4
Parameters and results obtained from fitting 2C model rate constants to Equation (8).

k_{20} (d^{-1})	k_{60} (d^{-1})	θ_1	θ_2	$T_{k,max}$ (°C)	$T_{k=0}$ (°C)	R
0.035	0.085	1.040	0.938	68	88	0.956

Table 5
Parameters and results obtained from fitting 3C model rate constants to Equation (8).

Readily biodegradable (rb) fraction		Slowly biodegradable (sb) fraction	
k_{rb20} (d^{-1})	0.050	k_{sb20} (d^{-1})	0.0020
k_{rb60} (d^{-1})	0.124	k_{sb60} (d^{-1})	0.0079
θ_{rb1}	1.041	θ_{sb1}	1.053
θ_{rb2}	0.934	θ_{sb2}	0.915
$T_{k,max}$ (°C)	70	$T_{k,max}$ (°C)	65
$T_{k=0}$ (°C)	90	$T_{k=0}$ (°C)	78
R	0.933	R	0.878

4. Conclusions

Two kinetic models to estimate the rate constants were tested: the 2C model, which considers two different carbon fractions, and the 3C model, which considers three different carbon fractions; both gave good correlations. On one hand, the 2C model required less *a priori* knowledge about the biowaste while for the 3C model, the different carbon fractions present in the biowaste must be set before fitting experimental data. This initial effort in setting carbon fractions resulted in a better physical sense and a longer-term validity for the 3C model. Temperature effects on the degradation rate constants were studied. In all cases, an exponential increase of the reaction rate with temperature was followed by a more rapid decrease caused by thermal inactivation. The maximum reaction rates were observed in the range of 65–70 °C. These temperatures were lower for the slowly biodegradable carbon fraction in the 3C model which could be related to the optimal growth temperature of the micro-organisms involved in the degradation of lignocellulosic carbon.

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Journal of Environmental Management 95 (2012) S134–S138

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Respiration indices and stability measurements of compost through electrolytic respirometry

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ARTICLE INFO

Article history:
Received 26 August 2009
Received in revised form
24 September 2010
Accepted 23 October 2010
Available online 12 November 2010

Keywords:
Sludge composting
Stability
Sapromat
Electrolytic respirometry
Respiration index
Rotary drum reactor

ABSTRACT

An experimental technique for compost stability measurements based on Sapromat electrolytic respirometry was optimised and subsequently applied to a sludge composting process. Anaerobically digested sewage sludge mixed with reed was composted during 90 days in a pilot-scale rotary drum with forced aeration. Periodic solid samples were taken, and a previously optimised respirometric procedure was applied in order to measure the oxygen consumption. The respirometric experiments were made directly with a few grams of solid samples, optimum moisture and 37 °C over a period of 96 h. The results obtained showed how the respiration activity of the sludge decreased during the composting experiment under the specific operating conditions. The specific oxygen uptake rate (SOUR) instant values from the oxygen consumption curves were obtained, and two commonly used respirometric indexes (RI_{24} and AT_4) were calculated for all samples. Both RI_{24} (a mean of the SOUR values during the 24 h maximum activity period) and AT_4 (total oxygen consumption after 4 days) were the recommended parameters for the estimation of compost stability by the European Union in the second draft of the Working Document on the Biological Treatment of Biowaste in 2001. Both indexes exponentially decreased with the composting time, and a good linear correlation between them was observed. Final values of RI_{24} and AT_4 after 90 days were 600 mg O_2 $kgVS^{-1}$ h^{-1} and 26 mg O_2 gTS^{-1}, respectively. We also considered if this technique could be classified as a Dynamic or Static method, the two primary respirometric techniques for measuring compost stability. Supposing that the proposed procedure is considered a dynamic method (no limitations on the amount of oxygen supply), the final RI_{24} obtained was compared with the dynamic respiration index (DRI) proposed by the EU (1000 mg O_2 $kgVS^{-1}$ h^{-1}). Our result indicated that stable compost was obtained after 90 d. However, if a static limit was considered (AT_4 lower than 10 mg O_2 gTS^{-1} as proposed by the EU), our result would indicate that more residence composting time would be needed. Taking into account these results, the advantages and disadvantages and the validity of the proposed method are discussed.

1. Introduction

Composting is a natural process by which a biowaste is oxidised by microorganisms using atmospheric oxygen under controlled conditions. The final compost is a valuable product for agriculture and should be a stabilised material from an environmental point of view. Maturity and stability are two important compost characteristics. While maturity relates primarily to the agronomical characteristics, stability can be defined as the extent to which readily biodegradable material has decomposed (Barrena et al., 2006). An unstable biowaste contains high amounts of biodegradable organic material that cause pollution in the environment. Good compost should be free of such material and considered stable.

Different methods have been proposed to measure compost stability, and many scientific studies are available (Cossu and Raga, 2008; Barrena et al., 2009; Wagland et al., 2009). Respirometric techniques are considered one of the most adequate methods for measuring stability. These techniques are based on either oxygen consumption or CO_2 production measurements by an unstable biowaste; however, O_2 consumption methods are more generally recommended in the literature. The Respiration Index of a biowaste (RI) is defined as the rate of oxygen uptake and can be measured by different kinds of respirometric techniques. These techniques usually include oxygen uptake by carbon and also by nitrogen degradation.

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0301-4797/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jenvman.2010.10.053

There are several methods proposed that have been compared and widely described by Lasaridi and Stentiford (1998), Adani et al. (2003) and Barrena et al. (2006), among others.

Electrolytic respirometry is a technique based on the manometric Warburg respirometer (Ros, 1993). This technique has been usually named as the Sapromat® system and it has been widely used to measure oxygen consumption by organic substances with different objectives: wastewater biodegradability, toxicity and inhibition tests, modelling and kinetics of biodegradation, anaerobic treatability, respiration of polluted soils, compost respiration. It has been previously used by the authors of this work for biological wastewater treatment studies (Cañizares et al., 2000; De Lucas et al., 2005; De Lucas et al., 2007). It was supposed that the application of the electrolytic system, with a solid-phase respirometry, it would be an interesting tool to monitor the respiration evolution of a sludge composting process.

Thus, the aim of this work is to study the application of electrolytic respirometry for the monitoring of a sludge composting process by measuring respiration indexes with respect to composting time, including stability measurements of the final compost obtained. First, the operating conditions were optimised, and then, the sludge-reed composting process was monitored. Finally, this technique was classified into one of the different groups of respirometric methods, and thus, the corresponding proposed RI threshold values were used to evaluate if the produced compost was stable. However, it is not the objective of this work to prove if this technique is better than the others. It would need a large experimental work. Electrolytic Respirometry using solid-state samples can offer advantages and disadvantages which will be explained at the Discussion section.

2. Materials and methods

2.1. Respirometer

A Sapromat electrolytic respirometer (Bioscience BI-1000, Bethlehem, PA, USA), previously described by Cañizares et al. (2000), was used. The respirometer is a closed system that contains several test vessels with electrolytic cells (Fig. 1).

The cells act as manometers and oxygen generation systems. The reaction temperature can be controlled by a water bath. The CO_2 produced by respiration is absorbed on traps containing a concentrated KOH solution. The electrolytic release of oxygen from a sulphuric acid solution begins when the manometer detects a pressure drop. When the initial pressure is re-established, the electrolysis is stopped. The amount of electricity consumed for electrolysis is proportional to the amount of oxygen consumed during microbial degradation. By coupling the measuring unit with a computer, a continuous recording of the oxygen consumption is obtained.

2.2. Materials and experimental procedure

A composting process was developed in a pilot-scale closed rotary drum with forced aeration (Fig. 2). The reactor contained biowaste and worked as a fixed bed that was eventually turned using the rotary system. A humidified and heated atmospheric air inlet was placed at the bottom. Leachates were re-introduced in the reactor, which helped to maintain the moisture.

The biowaste was a mixture of anaerobically digested sewage sludge and reed in a ratio of 2:1 (wet weight) and 2:5 (dry weight), and the characteristics of the mixture are indicated in Table 1. The sewage sludge was taken from a conventional domestic wastewater treatment plant. Reed (*Phragmites Australis*) harvested from a wetland in central Spain was milled and used as a co-substrate and amendment.

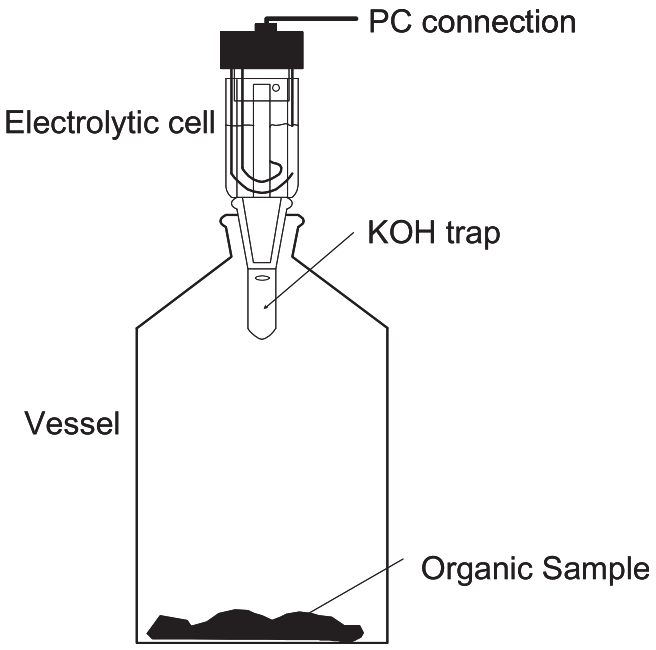

Fig. 1. Reaction vessel of an electrolytic respirometer.

The sludge/reed mixture (90 kg, wet weight) was composted using the following conditions: excess oxygen (always more than 18% in exhaust air) and an air flow of 0.5–1 l min^{-1} kg^{-1} SV_o, with moisture between 40 and 60%. Total residence time (fermentation and maturation periods) was 90 days. Representative samples of 1 kg were taken (by triplicate) at days 1, 13, 33, 53 and 90.

A small portion of each sample (sub-sample) was introduced into a vessel of the respirometer. The vessel with the electrolytic cell was closed, and the oxygen generation system was connected. Then, the respirometer recorded the oxygen consumption of the solid sample. The following conditions were used in the respirometric tests: 30 g of sub-sample, 37 °C, moisture between 40 and 60% (that is, the same moisture maintained in the composting experiment), particles size less than 10 mm and residence time of 96 h (4 days).

Also, the composting trial was monitored. Another portion of each solid sample was mixed and a 250 g aliquot was used for analysis, while the rest of the sample was discarded and returned to the reactor. The aliquot was homogenised before analyses were performed. The parameters measured were the following: total C (%), total N (%), Volatile Solids (VS, %), NH_4^+/NO_3^- ratio, and the percentage of humic acid-like carbon ($P_{HA}=(C_{HA}/C_{EX})\times100$) and the

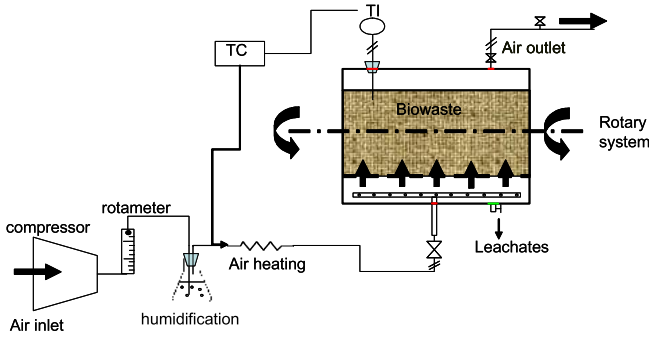

Fig. 2. Scheme of the pilot-scale composting experimental system.

Table 1
Initial characterisation of the waste mixture (d.w.: dry weight).

Parameter	Value	Parameter	Value
Moisture (%)	63.0	VS (%) (d.w.)	75.9
C_{tot} (%) (d.w.)	42.2	P_{tot} (%) (d.w.)	1.24
N_{tot} (%) (d.w.)	1.39	pH	8.0

polymerisation rate (C_{HA}/C_{FA}) at the end of the experiment. All analyses were done in triplicate, following the same procedure as previously reported by Fernández et al. (2010).

3. Results and discussion

3.1. Experimental conditions selected

The respirometric tests were made using the operating conditions indicated at the end of section 2.2. These conditions were selected according to the recommendations reported in the literature (Adani et al., 2003; Barrena et al., 2006; Iannotti et al., 1993; Liang et al., 2003). Temperature (37 °) and residence time (4 days) were commonly recommended values.

Also, some previous experiments helped us select the amount of sample and particle size. Big samples (100 g approximately) were previously used. Such high amount of sample caused difficulties to observe changes in the oxygen uptake curves. Sometimes more than 500 h were needed to observe a decrease in the respiration rate, and the electrolytic system was running (generating oxygen) at the maximum power without stopping, causing some experimental problems. Regarding the particle size, previously to the respirometric tests, samples were passed through sieves with different holes sizes. The humidity caused particles smaller than 10 mm to clump together again, which is one of the disadvantages that the biowaste remains static.

Moisture was easily controlled (and remained nearly constant in the closed system) and was one of the factors that determined the use of solid samples instead of water suspensions.

3.2. Results

Fig. 3 shows the oxygen uptake curve and the instantaneous Specific Oxygen Uptake Rate (SOUR), also called the respiration index (RI), obtained from one sample at composting day one. Each respirometric test was done by triplicate, and so we obtained three curves each time for five different composting times (days 1, 13, 33, 53 and 90). The discussion based on Fig. 3 also extends to other data that are not shown.

From the observations of all respirometric experimental results, it was detected that the total accumulated oxygen consumption after 96 h always decreased with the time of composting. The oxygen uptake curves did not show the classical asymptotic profile (typical in BOD_5 wastewater analysis), with the exception of some samples at the end of the composting process. These curves indicated that a high amount of biodegradable material was contained in the samples. Longer respiration times or smaller samples would be needed to observe the asymptotic profile, but smaller samples were considered not to be representative. The RI evolution indicated a slow decrease from the higher values during the first 25–50 respiration hours, to the lower values at the end of the test. In addition, important RI changes were suddenly detected during short periods (for example, Fig. 3 at times 40 or 88 h), although they did not change the global decreasing RI trend observed and so, they should not be considered as important results. These changes, with respect to the general RI evolution, were supposed to be related to possible physical changes during the solid biowaste degradation that could have resulted in the ability of oxygen to access new zones in the waste with raw organic material. The reason of this behaviour would be the lack of homogenous conditions in a solid sample, compared to respirometric tests using wastewater or water-suspended compost samples.

3.3. RI_{24} and AT_4 calculations

Two commonly used respirometric indexes, RI_{24} and AT_4, were calculated from the respirometric tests. Both RI_{24} (a mean of the SOUR values during the 24 h maximum activity period) and AT_4 (total oxygen consumption after 4 days) were the recommended parameters for the estimation of compost stability by the European Union in the second draft of the Working Document on the Biological Treatment of Biowaste (European Union, 2001). Fig. 4 shows (a) the mean values of RI_{24} and AT_4 and (b) the evolution of RI_{24} compared with the temperature during the composting process. It is interesting to see how the sludge respiration activity decreased rapidly in first 20 days using this rotary drum reactor. Final values after 90 days were 600 mg O_2 $kgVS^{-1}$ h^{-1} and 26 mg O_2 gTS^{-1}, respectively. No lag phase was observed in any oxygen consumption profiles, thus it was not considered during AT_4 calculations.

Fig. 4 shows that both RI_{24} and AT_4 strongly decreased during the first period (approximately 30 d) corresponding to the maximum biological activity period as indicated in the *T* profile. Fig. 5 shows the linear relationship ($p < 0.05$) between the mean values of RI_{24} and AT_4. Data obtained at day 13 in the composting trial, has not been included because is very far from the overall

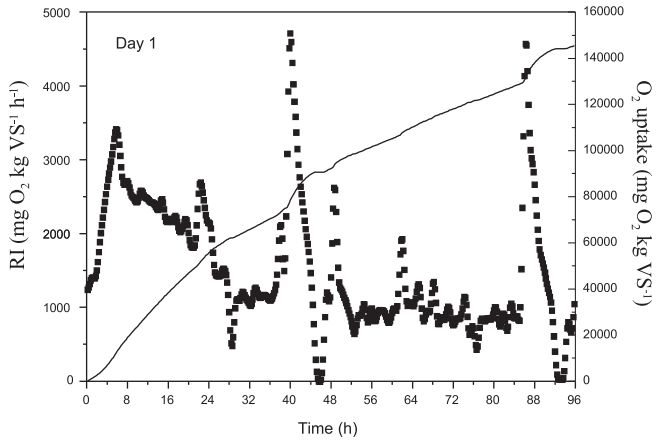

Fig. 3. O_2 consumption (line) and RI (points) in the 96 d-respirometric test made using one of the samples taken from the composting process on day one.

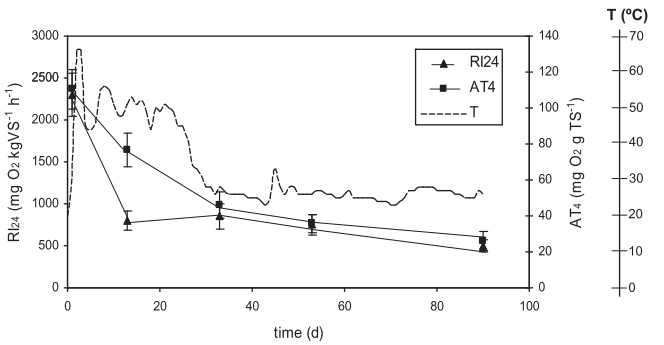

Fig. 4. RI_{24} and AT_4 mean values, and temperature evolution during the composting process.

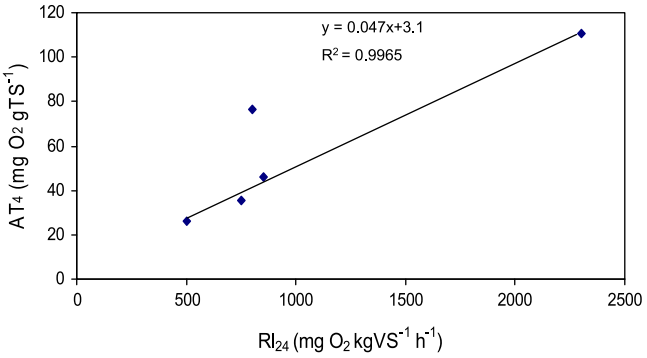

Fig. 5. Linear correlation between mean values of RI_{24} and AT_4, (data at day 13 has not been included in the correlation).

trend. The good correlation ($r^2 = 0.9965$) indicates that the same information and results could be obtained by using both indexes, and thus, either of them would be adequate. A wide work published by Ponsá et al. (2010a) studied linear correlation between RI_{24} and AT_4 in 58 samples of different organic solid wastes. Their results have been compared with the one obtained in the present work (Table 2). They reported r^2 values between 0.2920 and 0.9142 (average value $r^2 = 0.8698$) and AT_4/RI_{24} slopes between 51.17 and 101.24 h (average value 65.81 h). The AT_4/RI_{24} value obtained in this work is similar to the one reported for anaerobic digested sludge (Table 2) and the one reported by Adani et al. (2003). Also, Ponsá et al. (2010b) obtained $r^2 = 0.93$ in a correlation between cumulative and dynamic respiration indices. The advantage of RI_{24} is that only 24 h are needed (usually the first 24 h), while AT_4 has the advantage that no calculations are needed and the final AT_4 can be directly obtained from the test.

3.4. Comparison of results with the established limits of compost stability

Different limits have been established for the use of respiration indices as a biological stability parameter. Barrena et al. (2006) reported a complete review, and two of them have been selected in this work. First, the Static Respiration Index (SRI) proposed by the European Union (2001) is an AT_4 lower than 10 mg O_2 gTS^{-1}. The second index, known as the Dynamic Respiration Index (DRI), is an RI_{24} lower than 1000 mg O_2 $kgVS^{-1}$ h^{-1}.

The question now becomes, how can we classify the electrolytic method described in this work? The main methods used are the following: static-liquid methods, which use small compost samples suspended in water with oxygen consumption measured through dissolved oxygen probes (Chica et al., 2003; Lasaridi and Stentiford, 1998); static-solid methods, which use solid samples in closed vessels and oxygen consumption is measured by the decrease in the atmospheric oxygen partial pressure; and finally, dynamic-solid methods where an air flow passes through the solid sample and the oxygen consumption is measured as the difference between the inlet and outlet gas phase oxygen concentrations. Adani et al. (2003) reported a comparison between these three methods.

The electrolytic method described in this work has a mixture of characteristics when compared with the three methods indicated above. The main advantages of the method described here are that the electrolytic system ensures no oxygen supply limitations (as in dynamic methods); it is easy to control experimental conditions, temperature and moisture, because it is a closed system; also, a solid-state procedure can simulate the real respiration behaviour of a compost sample in the open environment, while liquid-suspended methods don't do it.

On the contrary, the main disadvantages are that the static procedure causes oxygen transfer limitations which could underestimate oxygen uptake values (as in static methods) or even produce anaerobic conditions in the inner parts of the solid sample; and also, that solid samples homogenization is difficult compared with liquid-suspended methods. According to the description of the respirometric methods, we would classify the technique as static.

Taking into account the above discussion, the AT_4 value obtained (26 mg O_2 gTS^{-1}) should be compared with the limit (10 mg O_2 gTS^{-1}), indicating that the compost was still unstable and more than 90 d composting is necessary. If we compare the RI_{24} value (600 mg O_2 $kgVS^{-1}$ h^{-1}) with the proposed limit (1000 mg O_2 $kgVS^{-1}$ h^{-1}), the opposite conclusion is derived.

The additional results obtained at the end of the composting trial (Fernández et al., 2010) could help to solve these contradictory results regarding the stability level obtained. Compost characteristics after 90 d were the following: $C/N = 19.5$, VS = 54% (dry basis), $NH_4^+/NO_3^- = 0.55$, $P_{HA} = 51\%$ and $C_{HA}/C_{FA} = 0.95$. It is possible that $C/N = 19.5$ is still not a low enough value to ensure stability. A C_{HA}/C_{FA} value lower than 1.0 indicated also low maturity level (Chefetz et al., 1996), and very low increases of C_{HA}/C_{FA} and P_{HA} values were measured during the experiments. Regarding the NH_4^+/NO_3^- ratio value, it has been reported that this ratio should be lower than 0.16 (Bernal et al., 1998). Thus, according to this additional information, it has been supposed that stability was not reached and a short additional reaction period would finalise the maturation. So, the information obtained from RI_{24} would not be adequate, and it is supposed that the RI_{24} values could be underestimated because of oxygen transfer limitations, especially during the thermophilic stage with higher biological activity (day 13, as can be observed in Fig. 5).

Adani et al. (2003) compared the different respirometric methods, and based on this work, Barrena et al. (2006) proposed equivalences amongst different stability limits for the most commonly used respiration indexes and calculated a new limit for AT_4 (45.39 mgO_2 gVS^{-1}) when using Sapromat® respirometers, a kind of respirometer similar to the electrolytic respirometer. This limit would suggest that our compost reached stability after 90 d. Thus, some confusion remains and there is no general agreement on the interpretation of the biological stability of a material.

Table 2
Linear correlation between AT_4 and RI_{24} and comparison with previously reported values (Ponsá et al., 2010a).

Waste	Slope AT_4/RI_{24}	R^2
ADS/reed (this work)	47.00 kg_{VS} h kg_{TS}^{-1}	0.9965
ADS	51.17 h	0.2920
OF and MBT-OF	71.81 h	0.9063
RS	101.24 h	0.9142
MBT-MSW	80.08 h	0.9017

ADS: Anaerobic Digested Sludge; OF: Organic Fraction of MSW; MBT: Mechanical-Biological Treatment; RS: Raw Sludge; MSW: Municipal Solid Waste.

4. Conclusions

Electrolytic respirometry was successfully used as a tool to measure the sludge respiration evolution during sludge composting and the final compost stability. Two different respiration indexes, RI_{24} and AT_4, were calculated and similar information could be extracted from both. The electrolytic method had a mixture of characteristics when compared with either the static or dynamic methods; however, the electrolytic technique was eventually classified as static in this work. The main advantage is that there are no limitations on oxygen supply, while the main

disadvantage is a limitation on the oxygen transfer rate. Depending on the limit selected for comparison, the compost obtained in this work could be classified as either stable or unstable, suggesting that more work is needed to establish agreement among methods and establish limits for biological stability.

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Journal of Environmental Management 95 (2012) S139–S143

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Comparison between ozone and ultrasound disintegration on sludge anaerobic digestion

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ARTICLE INFO

Article history:
Received 30 September 2009
Received in revised form
26 May 2010
Accepted 18 July 2010
Available online 16 August 2010

Keywords:
Ultrasound
Ozone
Sewage sludge
Anaerobic digestion
Dewaterability

ABSTRACT

This paper deals with the comparison of ultrasound (mechanical) and ozone (chemical) pre-treatment on the performances of excess sludge semi-continuous digestion. Sludge solubilisation has been investigated by varying specific energy input. For each pre-treatment, long anaerobic digestion tests were carried out by two parallel digesters: one reactor, as control unit, was fed with untreated waste activated sludge, and the other one was fed with disintegrated sludge. To evaluate and compare the efficacy of both pre-treatments, the specific energy was maintained approximately the same. The digestion tests were carried out to investigate the feasibility of anaerobic digestion performance (total biogas production, volatile solids removal, sludge dewaterability) and to assess the heat balance. Results obtained from the digestion of sonicated sludge at 4% disintegration degree (~2500 kJ/kg TS) showed that the ultrasound pre-treatment may be effective both in increasing VS destruction (+19%) and cumulative biogas production (+26%). On the contrary, the digestion test with ozonized sludge (ozone dose of 0.05 g O_3/g TS corresponding to ~2000 kJ/kg TS) did not indicate a significant improvement on the digestion performances. By doubling the ozone dose an improvement in the organics removal and cumulative biogas production was observed. Relevant differences in terms of colloidal charge and filterability were discussed.

1. Introduction

Sludge management in an economically and environmentally acceptable manner is one of the critical issues facing modern society, due to the very fast increase in sludge production, as a result of the implementation of the Directive 1991/271. The challenge in the coming years will be both to sustain agricultural use of good quality sludge, and to reduce as much as possible disposal of polluted sludge especially in landfill. Upgrading of activated sludge treatment might be required in order to increase stabilization minimizing digestion residence time and maximizing biogas production. Waste activated sludge is very difficult to digest due to the rate-limiting cell lysis (Lafitte-Trouque and Forster, 2002). In the last years a renewed interest for the anaerobic digestion of sludge raised from the possibility of a significant gain in solids degradation and energy recovery by applying an appropriate sludge pre-treatment that leads to the breakage of flocs, cell walls, and bacteria membranes enhancing the hydrolysis of sludge volatile solids (Carballa et al., 2007; Khanal et al., 2007; Müller et al., 2003).

This paper deals with the comparison of ultrasound (mechanical) and ozonation (chemical) pre-treatments. Ultrasonic pre-treatment leads to cavitation bubbles formation in the liquid phase. These bubbles grow and then violently collapse causing high shearing forces in the surrounding liquid phase and formation of radicals. Ozone, on the contrary, is used for water and wastewater treatment due to its strong oxidative properties. During sludge ozonation, because of the complex composition of sludge, ozone decomposes itself into radicals and reacts with the whole matter: soluble and particulate fractions, organic or mineral fractions (Böhler and Siegrist, 2004). If activated sludge is exposed to low-dose ozone at less than 0.02 g O_3/g TS, ozone firstly destroys the floc, leading to the disruption of the compact aggregates (Chu et al., 2009). Moreover, Zhang et al. (2009) found that, by applying ozone dose at 0.05 g O_3/TS, the soluble COD increased linear with the ozonation time during the first 105 min and then stagnated, probably because of the balance of sludge lysis and mineralization, showing that too long time was unnecessary. Extra ozone dose or long treatment time resulted in mineralization of dissolved organics and should be avoided.

As regards the effect of pretreatments on the filterability, high-energy ultrasonic treatment can disrupt flocs and increase the number of fine particles and bound water. Therefore, only low

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0301-4797/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jenvman.2010.07.030

energy ultrasound treatment is generally used to improve sludge dewaterability (Huan et al., 2009). Moreover several researchers reported that sludge filterability quantified by capillary suction time (CST) was deteriorated by ozone treatment (Muller et al., 1998; Scheminski et al., 2000; Weemaes et al., 2000). In particular a pilot scale ozone treatment test showed that filterability deteriorated up to an ozone dose of 0.2 g O3/g DS and then improved tremendously by higher ozone dose (Park et al., 2010).

Only few studies (Braguglia et al., 2009; Feng et al., 2009; Huan et al., 2009; Wang et al., 2006) report the effect of ultrasound and ozone on the dewaterability during sludge anaerobic digestion, and consequently more research is needed.

The performance of the various methods can be compared by the specific energy, which is defined as the amount of energy stressing a certain amount of sludge. Previous works in the laboratory (Braguglia et al., 2008, 2009; Tomei et al., 2008) were addressed to determine optimum conditions of ultrasounds pretreatment and anaerobic digestion. In this work we applied a specific energy range of ~2500–5000 kJ/kg TS that simulated the energy values generally adopted for full scale applications of ultrasound apparatus.

The corresponding ozone doses were in the range 0.05–0.07 g O_3/g TS, much lower with respect to the limit value of 0.15 g O_3/g TS suggested by Bougrier et al. (2006) for mineralization. For each pre-treatment, semi-continuous digestion tests were carried out to investigate the feasibility on anaerobic digestion performance (total biogas production, volatile solids removal, sludge dewaterability) and to assess the heat balance.

2. Materials and methods

2.1. Sludge

Waste activated sludge (WAS) was sampled from the municipal "Roma-Nord" wastewater treatment plant, one of the four wastewater treatment plants serving the city of Rome with an organic load of about 700.000 p.e. The plant includes screening, primary clarification and secondary treatment by activated sludge with a quite high sludge age (20 d). The activated sludge was sampled from the recycle stream before thickener, and the anaerobic inoculum from the full scale digester of the plant fed with primary and secondary sludge.

2.2. Matter composition

Total and volatile solids (TS and VS) were determined according to the standard methods (APHA et al., 1998).

To analyse the soluble phase, the particulate sludge matter was removed by centrifugation (10 min at 5000 rpm) and resulting centrate was filtered through 0.45 μm pore size membrane filters. Soluble COD, measured in duplicates, was determined by photometric determination of chromate consumption by the organic compounds, subsequent to digestion in concentrated sulphuric acid solution for 2 h at 148 °C by means of COD Cell Test by Spectroquant Merck (EPA method 410.4). Sludge filterability was estimated using a capillary suction apparatus supplied by Triton Electronics Ltd., England. A stainless-steel tube with an inner radius of 0.535 cm and filter paper Whatman No. 17 was used. Each sludge was analysed 5 times and the results were averaged (standard deviation within 10%), before being standardised to the TS concentration as detailed in Standard Methods (APHA et al., 1998). The charge density determinations were performed by a Particle Charge Detector PCD02 (Mütek GmbH, Herrsching). The PCD (Particle Charge Detector) operates on the principle of the so-called "streaming current detector". Since the SC (streaming current) is proportional to the electric charge of the colloids, it may provide an indication of charge-related particle destabilization in a manner similar to zeta potential (Abu-Orf and Dentel, 1997). To measure quantitatively the overall charges in a system polyelectrolyte titration was used. A titrant (organic polylelectrolyte) of opposite charge was added to the sample until the latter reaches the point of zero charge. The original charge amount is calculated from the titrant consumption. The anionic standard polyelectrolyte used was 0.001 N sodium polyethylene sulphate solution (Na-PES), the cationic standard was a 0.001 N polydiallyldimethylammoniumchloride solution (Poly_-Dadmac). Sludge samples were centrifuged at 5000 rpm for 10 min, the centrate was then filtered through a 1.2 μm filter and titrated in the PCD to determine the quantity of charge related to the colloidal particles, performing two replicates per sample.

2.3. Pretreatment procedure

The disintegration by ultrasound was performed with an ultrasonic processor UP400S (dr. Hielscher, Germany) operating at 255 W and 24 kHz. The sonotrode has a diameter of 22 mm making the device suitable for sample volumes of 500 mL. Sonication energy was 0.7 kWh kg^{-1} dry solid on 500 mL of waste activated sludge placed in 1 L beaker with the probe allocated at 3 cm above the beaker bottom.

The ozonation process was performed with the ozone generator BMT802M, supplied by Air Liquide Italia. The sludge (sample volume 0.4 L) was transferred to 1 L contact chamber, where it was maintained for a prefixed duration under the ozonating flux. The ozone transfer efficiency was computed by in and off-gas measurements using an ozone detector. The transfer efficiency was always over 90%.

The degree of sludge disintegration (DD_{cod}) was calculated as the ratio of the soluble COD increase by sonication to the maximum possible soluble COD increase (Braguglia et al., 2006).

The degree of disintegration was dependent on the specific energy supplied (E_{spec}), which can be calculated by equation (1) considering that P is the power, t the treatment time, V the sludge treated volume, and TS the sludge total solid content:

$$E_{spec} = \frac{P \times t_t}{V \times TS} \quad (1)$$

2.4. Digester system

Digestion of sludge was carried out using two anaerobic digesters operated in semi-continuous mode under the same conditions at the same HRT of 10d. One reactor, as control unit, was fed with untreated WAS, and the second one with the same sludge but after sonication (test #1) or ozonation (test #2 and test #3). Both jacketed reactors (7 L) were completely mixed and maintained at the constant temperature of 37 °C. Table 1 lists the operating conditions of the anaerobic digestion tests. Data were collected in steady state conditions, i.e. after reaching constant specific biogas production. In test #1 (with ultrasound) and in test # 2 (with ozone) sludge was pre-treated maintaining the energy value constant (~2000–2500 kJ/kg TS). Test #3 was carried out with ozone by doubling the specific energy.

Table 1
Operating conditions of the anaerobic tests at steady state conditions.

Test #	1(US)	2(Oz)	3(Oz)
Test duration (d)	63	74	49
HRT (d)	10	10	10
Sonication energy (kJ kg^{-1} TS)	~2500	~2000	~4500
Ozone dose (g O3/g TS)	–	0.05	0.07
Disintegration degree (%)	~4.0	~2.4	~6

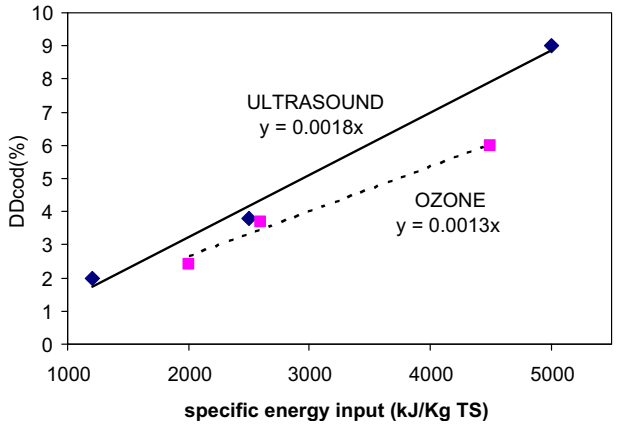

Fig. 1. Effect of the specific energy on the sludge disintegration degree.

The produced biogas was collected by water displacement in a biogas collection unit. The gas meter consists of a volumetric cell for gas–liquid displacement, a sensor device for liquid level detection, and an electronic control circuit for data processing and display.

A Gas sensor (Detcon Model IR-700-CH) was used to estimate the methane content. The sensor was connected directly to the biogas collection system, allowing easy and continuous measurements.

3. Results and discussion

3.1. COD solubilisation

The main objective of these pretreatments was to improve the bioavailability of sludge particulate material. An increase of the specific energy led to an increased release of dissolved organic components (i.e. of soluble COD). Fig. 1 presents the degree of sludge disintegration *vs* the specific energy input, defined as the amount of energy that is related to the sludge dry solids content. Ultrasounds were more efficient in sludge disintegration with respect to ozone (the slope of the line was 0.0018 *vs* 0.0013), nevertheless both mechanical and chemical disintegration depended linearly from the specific energy input applied, according with Tiehm et al. (2001).

For each experiment, while the energy input increased, the total solids and the volatile solids concentration did not change, suggesting that in these conditions pretreatments did not induce evaporation and mineralization effects.

3.2. Effect of pre-treatment on VS and COD removal

The WAS sampled from the WWTP and used for the tests was characterized by a quite low VS content due to its high sludge age. It must be pointed out that these semi-continuous anaerobic tests lasted very long so the results could be affected by the variability of

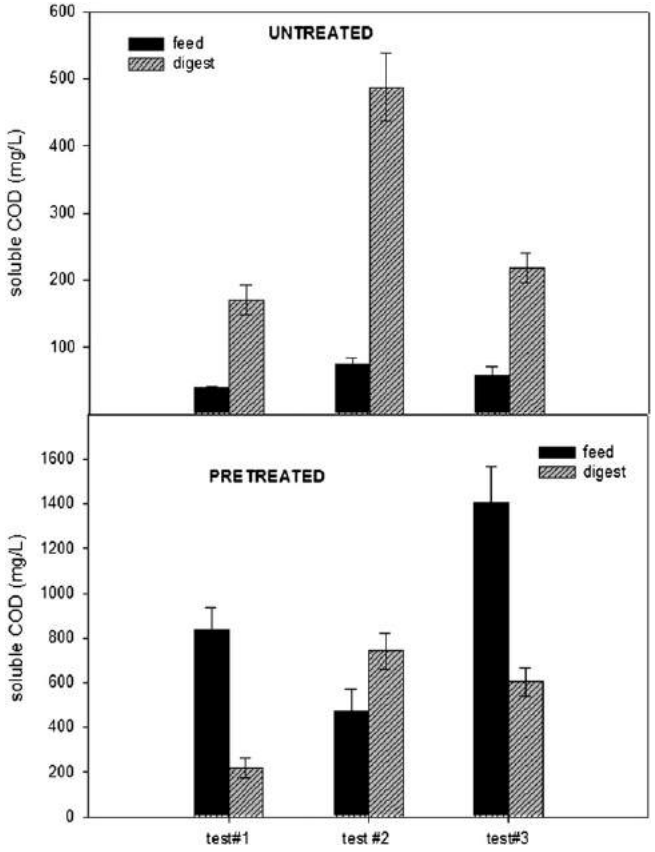

Fig. 2. Soluble COD at steady state conditions of the digesters for untreated and pretreated sludge.

the feed during the experimental campaign. With ultrasound pretreatment, VS removal increased in test #1 from 32 to 38%, and the improvement was statistically significant, considering the standard error reported in Table 2. In fact, the calculated *t*-statistic for comparing these two values was $t = 2.14$ which reflects that the null hypothesis, that the means of the two groups are the same, should be rejected at the $\alpha = 0.05$ level.

As reported also in Tomei et al. (2008) ultrasound pre-treatment accelerated considerably the reaction rate (k values are doubled) and the gain in VS removal increased by decreasing the OLR (Braguglia et al., 2008). On the contrary, by pretreating the sludge with ozone at the same energy input (test #2) the improvement was negligible. By doubling the ozone energy the VS removal increased from 27 to 34%.

By comparing the trend of the soluble COD at steady state conditions (Fig. 2), in the digestion tests of untreated sludge the solubilised organic matter increased markedly, probably due to the higher rate of hydrolysis with respect to anaerobic conversion rate. On the contrary in the parallel tests with sonicated sludge in test #1 and ozonized sludge in test #3 the quite high initial soluble COD

Table 2
Results of the anaerobic semi-continuous tests at steady state conditions.

Test #	1		2		3	
	untreated	sonicated	untreated	ozonized	untreated	ozonized
VS removal (%)	32 ± 1.6	38 ± 2.3	27 ± 1.4	29 ± 1.0	27 ± 1.7	34 ± 2.4
Cumulative biogas production (NL)	137	172	139	131	108	126
Specific biogas production ($Nm^3\ kg^{-1}$ VS destroyed)	0.66	0.84	0.80	0.75	0.75	0.70

due to pre-treatment disintegration was progressively removed. The COD removal in the digester was 74 and 57% for test #1 and test #3, respectively. In test #2 no removal of the soluble organic matter in the digester was observed, and the soluble COD in the digester increased indicating that the biological hydrolysis step is, in any case, predominant because of the scarce effect of the ozone pre-treatment. So, by comparing the results of test #1 and test #2 where the sludge was pre-treated with the same specific energy it was evident that the disintegration obtained with the ultrasound was more effective with respect to the ozone as regard organics removal, and the effluent of the digester fed with ozonized sludge presented a significant high organic content requiring an "additional" effluent post treatment.

3.3. Effect of pre-treatment on sludge dewaterability

Disintegration pretreatments alone or combined with the anaerobic digestion cause many changes on the physical characteristics of sludge (floc structure, bound water, etc) affecting generally dewaterability and filterability of sludge. Several analyses were carried out to evaluate the filterability and the colloidal charge of sludge, before and after the anaerobic digestion of untreated and pre-treated sludge (Table 3 and Fig. 3). The floc disintegration by ultrasound at $E_{spec} = 2500$ kJ/kg TS caused a significant worsening of sludge filterability (CST increased from 1 to 15 s L/gTS) due to the increase of fine particles and bound water, whereas in the case of the ozone pre-treatment (E_{spec} = 2000–4500 kJ/kg TS) only a slight increase of the CST value was observed. Although the specific colloidal charge increased noticeably for both ultrasound and ozone disintegration, the effect on the filterability was different. The colloidal particles produced from the ozone pre-treatment presented probably high polarity and surface characteristics that did not impair significantly the filterability, whereas sonication generated fines that probably blind the sludge cake and filter medium during filtration.

The untreated digested sludge was always harder to filter (CST increases after digestion) because of the high release of colloidal particles in solution due to the hydrolysis step. In fact, the biological hydrolysis of the untreated sludge caused a large release of dispersed charged fines and biocolloids and consequently the increase of the colloidal charge of the particles (Fig. 3), as the soluble COD. In fact, the degradation of sludge solids during the anaerobic digestion process generated changes in the physical–chemical properties of the floc, releasing its intracellular components, changing its morphology and increasing "true" colloidal content (Braguglia et al., 2009).

On the contrary, the digestion of the pre-hydrolyzed sludge in test #1 (with ultrasound) and #3 (with ozone) caused a significant removal of colloidal particles during digestion (Fig. 3). In the case of ozonized sludge at 2000 kJ/kg TS (test #2), no removal of the colloidal charge was observed, as noticed for soluble COD, too.

The digestion of sonicated sludge, by the consumption of the released organic matter, attenuated the negative effects of pre-treatment and the filterability ameliorated during the digestion process (CST value decreases from 375 to 209 s). On the contrary the removal of colloidal charge during the digestion of the ozonized sludge (test #3) did not imply a gain in the filterability.

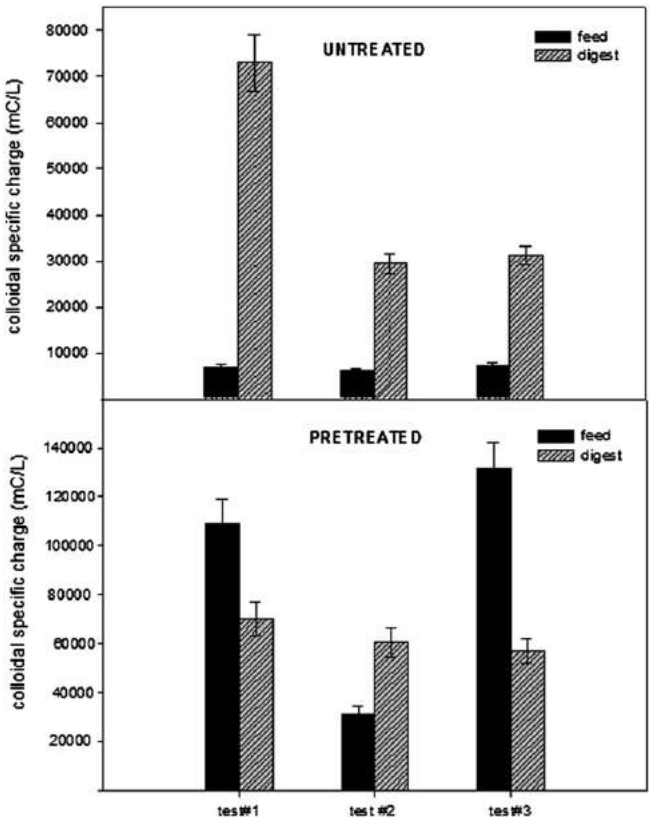

Fig. 3. Colloidal surface charge at steady state conditions for untreated and pre-treated sludge.

3.4. Effect of pre-treatment on biogas production

Cumulative biogas production increased in the reactor fed with sonicated sludge (test #1) in comparison with the reference reactor, and the gain was approximately 26%. As regards the pre-treatment with ozone, the test #2 with the same energy input as test #1 did not present gain in biogas, whereas in test #3 the increase was 17% (Table 2). These results obtained with the ozone treatment might be ascribed to different causes, as for example the formation of refractory compounds, a not well-adapted inoculum or uncontrolled ozone consumption by reduced compounds of the sludge (Bougrier et al., 2006).

The specific biogas production of sonicated sludge was noticeably higher with respect to the one obtained with the same untreated sludge (Table 2), whereas no significant gain was

Table 3
Dewaterability – comparison between untreated and pre-treated sludge before and after digestion.

Test #	1				2				3			
	untreated		sonicated		untreated		ozonized		untreated		ozonized	
	feed	digested	feed	digested	feed	digested	feed	digested	feed	digested	feed	digested
Capillary Suction Time [sec*L/gTS]	1	7	15	11	0.6	9.5	1	14.8	0.4	8.9	1.4	11.9
Particle Charge Density [mC/gTS]	209	2643	3283	2780	200	2250	1090	2670	180	650	3100	1750

Table 4
Heat (kcal/kg VS_{fed}) balance for the three digestion tests at steady state conditions.

	Test #1		Test #2		Test #3	
	untreated	sonicated	untreated	ozonized	untreated	ozonized
Heat demand	1775	1775	1741	1773	1741	1565
Heat from biogas	1074	1406	1160	1413	1160	1341
Balance	−701	−369	−582	−360	−582	−224

obtained with the ozonized sludge. Therefore, only sonication seemed to stimulate conversion into biogas from VS probably due to the presence of specific enzymes released during the sonication treatment.

Nevertheless it must be pointed out that the digestion conversion rate depended also on the feed characteristics. In fact, as reported in Table 2, the specific biogas production of the untreated sludge in the three tests, carried out at the same operating conditions and at the same HRT, were slightly different (from 0.66 to 0.80 $Nm^3/kg\ VS_{degr}$) depending principally on the sludge age of the feed and consequently on the chemical composition of the organic solids. For a correct evaluation of the potential of the disintegration methods a control reactor fed with untreated sludge was always necessary.

3.5. The heat balance of the digestion tests

The anaerobic digestion of waste activated sludge might be hindered by the negative heat balance of the digesters, due to the low specific biogas production and the low concentration of volatile solids in the digester feed (Bolzonella et al., 2005). The thermal balance of the digestion process was evaluated considering the total amount of sludge fed to digester during the steady state conditions in the three tests, and the correspondent cumulative biogas production. The heat demand included the heat to take the influent sludge from its temperature to the digestion temperature and the heat losses from digester (De la Rubia et al., 2006). The heat produced from the biogas has been evaluated considering the methane content measured during the test (60% for the untreated sludge and 70% for the pre-treated one). Data of Table 4 showed that the biogas produced was never sufficient to supply the energy requirement, in fact the heat requirement overcame always the available heat and the balance became negative. For the untreated sludge, in the three tests, the biogas produced supplied 60–67% of the required heat, whereas for the pre-treated sludge this proportion increased to 80–86%, but no significant differences between ultrasound and ozone were observed.

4. Conclusions

Both ultrasound and ozone led to solids solubilisation, and the sludge disintegration degree depended linearly on the specific energy supplied. Most significant differences between the two pre-treatments regarded the anaerobic digestion performance. In fact, by spending the same specific energy, ultrasound induced an improvement as regards volatile solids and soluble COD removal, whereas no beneficial effect was noted with ozone. In terms of biogas, the ultrasound pre-treated sludge produced 26% more biogas with respect to the untreated one, while the ozonized sludge produced even less biogas with respect to the untreated one ascribed probably to a not well-adapted inoculum. This hypothesis was confirmed from the results obtained with ozonized sludge at higher ozone dose. Feeding the same digester with more ozonized sludge, positive effects on the anaerobic digestion process were observed (+26% of VS removal and +17% of biogas production with respect to untreated sludge). The digestion of the sonicated sludge, by the consumption of the released organic matter, attenuated the negative effects of disintegration pre-treatment improving sludge filterability whereas the removal of the colloidal charge during the digestion of the ozonized sludge did not imply a gain in the filterability. Thermal balance of anaerobic digesters evidenced that pretreating waste activated sludge at low disintegration degrees permit to supply up to 86% of the required heat.

Acknowledgements

The authors are pleased to acknowledge Acea S.p.A., the Municipal Agency for Electricity and Environment, for its kind cooperation during the experimental activity. This work was financed by the EU project Neptune.

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Journal of Environmental Management 95 (2012) S144–S148

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Release and conversion of ammonia in bioreactor landfill simulators

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ARTICLE INFO

Article history:
Received 30 September 2009
Received in revised form
15 July 2010
Accepted 23 August 2010
Available online 29 September 2010

Keywords:
Landfills
Ammonia production
Ammonia removal
Anammox

ABSTRACT

Bioreactor landfills are an improvement to normal sanitary landfills, because the waste is stabilised faster and the landfill gas is produced in a shorter period of time in a controlled way, thus enabling CH_4 based energy generation. However, it is still difficult to reach, within 30 years, a safe status of the landfill due to high NH_4^+ levels (up to 3 g/L) in the leachate and NH_4^+ is extremely important when defining the closure of landfill sites, due to its potential to pollute aquatic environments and the atmosphere.

The effect of environmental conditions (temperature, fresh versus old waste) on the release of NH_4^+ was assessed in experiments with bench (1 L) and pilot scale (800 L) reactors. The NH_4^+ release was compared to the release of Cl^- and BOD in the liquid phase. The different release mechanisms (physical, chemical, biological) of NH_4^+ and Cl^- release from the solid into the liquid phase are discussed. The NH_4^+ level in the liquid phase of the pilot scale reactors starts decreasing after 100 days, which contrasts real-scale observations, where the NH_4^+ level increases or remains constant. Based on the absence of oxygen in the simulators, the detectable levels of hydrazin and the presence of Anammox bacteria, it is likely that Anammox is involved in the conversion of NH_4^+ into N_2.

Nitrogen release was shown to be governed by physical and biological mechanisms and Anammox bacteria are serious candidates for the nitrogen removal process in bioreactor landfills. These results, combined with carbon removal and improved hydraulics, will accelerate the achievement of environmental sustainability in the landfilling of municipal solid waste.

1. Introduction

The application of bioreactor landfills has been the most recent development in the disposal of municipal solid waste. The advantages are: shorter waste stabilisation periods, higher biogas production and recovery, reduced leachate organic strength and higher volume recovery due to enhanced waste settlement (Pohland, 1980; Warith, 2002). In previous studies, Valencia et al. (2009a,b) showed improved stabilisation of the solid waste reaching *Final Storage Quality* (Valencia et al., 2009a) much faster in bioreactor landfills than in sanitary landfills and the biogas, pH, and BOD followed similar trends as reported by Pohland (1980) and Warith (2002) with faster responses in those simulators with improved hydraulic conditions (Valencia et al., 2009b).

An important parameter to consider the safe closure of landfill sites is the NH_4^+ content of the leachate (Barlaz et al., 2002; Burton and Watson-Craik, 1998). NH_4^+ tends to accumulate since there are no removal mechanisms under strict anaerobic conditions, especially in landfills with leachate recirculation (Onay and Pohland, 1998; Price et al., 2003). NH_4^+ can be removed from the leachate of landfills via methods such as nitrification/denitrification, precipitation and even irrigation schemes (Jokela et al., 2002; Li and Zhao, 2003; Ohlinger et al., 1998). However, these approaches are likely to produce NO_x and N_2O, which are significant pollutants for their contribution to climate change (Price et al., 2003).

Despite all efforts to reduce the levels of ammonia emissions from landfill leachate, little information is available about the origin, evolution and fate of ammonia in bioreactor landfills. Huber et al. (2004) suggested that 4% of N leaves the landfill via the leachate pathway, while 96% of N remained in the landfill body. Therefore, the objective of this study was to investigate the mechanisms involved in the ammonia release from the solid phase into the liquid phase and possible *in-situ* removal mechanisms under anaerobic conditions in bioreactor landfills.

2. Materials and methods

2.1. Pilot scale reactors

Seven bioreactor landfills were simulated using high density polyethylene sewage pipes (0.75 m^3 working volume). The simulators

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0301-4797/$ – see front matter © 2010 Published by Elsevier Ltd.
doi:10.1016/j.jenvman.2010.08.030

Table 1
Waste characteristics.

a. Municipal solid waste composition			b. Moisture and VS content of waste		
Component	Fresh waste	Old waste[a]	Waste	Moisture	VS
	% of weight			% of weight	
Organics	24.5[b]	24.2	Old waste	53	70
Paper/cardboard	13.4	13.2	Fresh, minus food	4	10
Plastic	6.4	6.3	Fresh + food, uncooked	19	39
Ferrous/non-ferrous	0.8	0.8	Fresh + food, cooked	45	44
Wood	3.2	3.2			
Sand, stone	42.6	43.7			
Glass	8.7	8.6			
Textile	0.4	0.01			

[a] 4 weeks old waste provided by ESSENT Milieu, Wijster, The Netherlands.
[b] Only food waste.

were filled with shredded (particle size max. 4 cm) municipal solid waste (MSW), see Table 1 for the composition. The operational features of the simulators were described elsewhere (Valencia et al., 2009a,b), but the control simulators had no gravel and were filled with 450 kg of MSW or 350 kg of MSW, but compacted with less density. The operational conditions of the two aerated reactors were changed after day 250 as intermittent forced aeration was introduced at the bottom of the reactors (240 L air week^{-1}) aiming for *in-situ* nitrogen removal. Therefore, these two simulators served as control until that day. The amount of waste added was based on the determination of the field capacity of the material collected following the methodology described by Orta de Velázquez et al. (2003).

Buffered tap water (124 L, 0.1 M $NaHCO_3^-$) was added to stimulate leaching. The leachate was recycled 3 times per week ($\pm$60 L week^{-1}) in order to maintain a dynamic leachate flow and at least 45% moisture content (field capacity) on a wet weight basis (Vroon et al., 1999). Buffer (0.3 M $NaHCO_3^-$) was added to the leachate prior to recycling during a period of 6 weeks (day 50–100) to reduce the negative impact of the VFA on the pH. The internal temperature of the simulators was within the range of 30 $\pm$ 4 °C.

2.2. Laboratory scale (1 L)

Specific questions arising from the pilot scale reactors were studied at smaller scale. Six laboratory-scale landfill bioreactors were assembled using 1 L capacity plastic containers (diameter 160 mm, height 210 mm) for all series. Each reactor had three sample ports: for leachate sampling, for leachate recirculation and for gas exhaust. The sampling was carried out with a plastic syringe. About 350 mL of demineralised and deoxygenated water was used to initiate leachate formation. The water was slowly poured into the bioreactor and as soon as the leachate had reached the bottom about 10 mL was withdrawn with a 30 mL plastic syringe. Leachate was recirculated (30 mL) at the top of the reactor and 10 mL samples were withdrawn for analysis. About 30 mL of the leachate was recirculated to the reactor prior to the collection of the sample.

About 30 kg of shredded municipal solid waste, comparable to the composition of normal Dutch solid waste, from ESSENT Milieu (a Dutch solid waste handling company) was used, packed in 1 kg units and stored at 5 °C, ready for use. The C/N ratio was 16, a little bit below the optimal range for the AD process (20–30); the initial moisture content was 20% (wet weight basis) and VS content 44.9%.

The fresh waste was prepared manually, following ESSENT Milieu waste composition (Table 1a). The fresh waste was used with or without food waste; the food waste was either cooked or uncooked. Each reactor contained about 300 mg of waste. The moisture and volatile solids (VS) content of the wastes are shown in Table 1b. Experiments were carried out at 5 °C, 20 °C and 30 °C in controlled temperature rooms.

2.3. Analytical methods

Leachate samples were analysed immediately or stored in the freezer and were analysed for pH and temperature with portable meters WTW pH 340 and LF 340. BOD was analysed according to Standard Methods (APHA, 2005). NH_4^+ was analysed according to NEN (1983). Chloride – used for comparison because it is a very easily released ion – was analysed using an Ion Chromatography system DIONEX ICS-1000 attached with an automated sample injector DIONEX ASI-100. All liquid samples were filtered with glass fibre filters GF 52 (Schleicher & Schuell). Hydrazine was measured using detector tubes (MSA, range 0.1–5 ppm) and a thumb-pump sampler (100 cc sample volume/stroke). Samples for the Anammox bacteria determination were taken from the 4 cores extracted (0.60 cm length), these cores were extracted from the bottom and the middle part of each simulator. Anammox identification in the residue was carried out using fluorescent in situ hybridisation (FISH) techniques employing the following probes: Pla46 for planctomycetales, AMX820 covering all Anammox organisms, specially *Kuenenia* sp. and *Brocadia* sp. and DHI820 for *Anammoxoglobus* sp. Probes and hybridisation procedures are described by Schmid et al. (2003) and Kartal et al. (2007).

3. Results

3.1. Ammonia in the pilot plants

The process parameters of the 800 L bioreactor landfill simulators are shown in Fig. 1. Due to the accumulation of hydrolytic products the pH decreased in the first months of operation, but

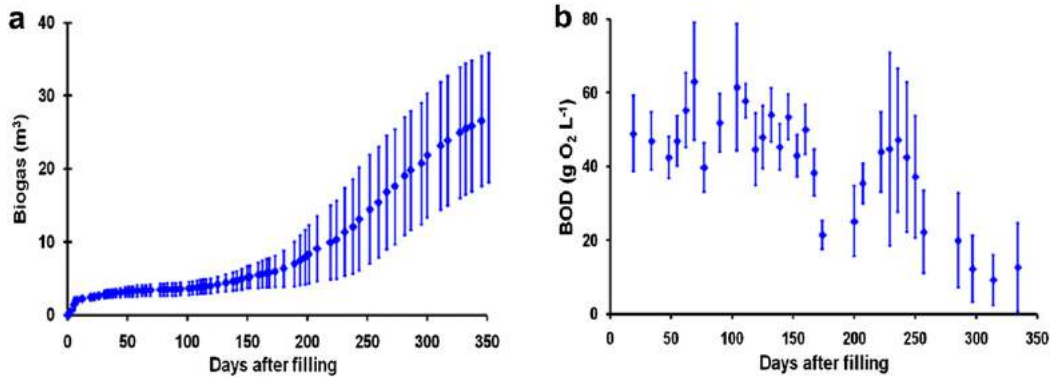

Fig. 1. a) Cumulative biogas production from the bioreactor landfill and b) BOD in the leachate.

increased as soon as a more acclimatised methanogenic population developed, which converted these hydrolytic products into biogas.

During the first 150 days of operation the biogas production (Fig. 1a) was minimal (<5 m^3) and contained mainly CO_2 (±85%). However, as more favourable conditions (i.e. neutral pH) for methanogens were reached, biogas production increased exponentially during the following 200 days, coinciding with a rapid decrease of BOD during the same period (Fig. 1b).

After an initial increase of the NH_4^+ levels (Fig. 2a) in the leachate during the first 100 days (up to 2.5 g L^{-1}), the NH_4^+ concentration remained stable during the period between days 100 and 200.

From day 200 onwards NH_4^+ levels gradually decreased to 1.7 g L^{-1} (Fig. 2a). In a preliminary experiment with longer duration, the same tendency was observed and the final NH_4^+ levels were even below 1 g L^{-1} (Fig. 2). Since none of the normal options for the reduction of ammonia could explain the N removal under anaerobic conditions (nitrification/denitrification, volatilisation, struvite precipitation, bacterial uptake), we were looking for the presence of Anammox bacteria. Samples of the solid material from the 800 L reactors, taken on day 300 revealed the presence of Anammox bacteria (Fig. 3); 5–7% of the bacterial mass was consisting of Anammox bacteria. In addition, high levels of hydrazine (>6 mg L^{-1}), an intermediate product of the Anammox metabolism (Kartal et al., 2007; Schmid et al., 2003), were detected in the gas phase during the last 100 days of operation.

The release of NH_4^+ into the leachate was found to have two phases (Fig. 2): a sharp increase to about 1 g L^{-1} within 10 days and a gradual increase between day 10 and 100 up to 2.5 g L^{-1}. To have a better understanding of the ammonium release processes laboratory-scale reactors (1 L) were set up.

3.2. Temperature effects on the release of ammonia

Fig. 4 shows the effect of temperature on the release of NH_4^+ and Cl^- in the 1 L reactors. For comparison, the release of chloride is shown, since Cl^- is very easily released. While all Cl^- is released within 4 h, independent of the temperature, the release of NH_4^+ is much more gradual and is influenced very much by temperature (highest release at 30 °C).

To investigate the influence of the age and the type of the waste, four different types of wastes were used: waste delivered by ESSENT (old waste) and 3 types of fresh wastes, with the addition of cooked or uncooked food waste and without food waste. There was hardly any NH_4^+ release from fresh wastes, maximally 50 mg L^{-1}, as compared to the old waste, 1500 mg L^{-1} (Fig. 5). If Cl^- was available in the waste, it was released immediately (old waste 1500 mg L^{-1}, fresh waste including cooked food waste 600 mg L^{-1}).

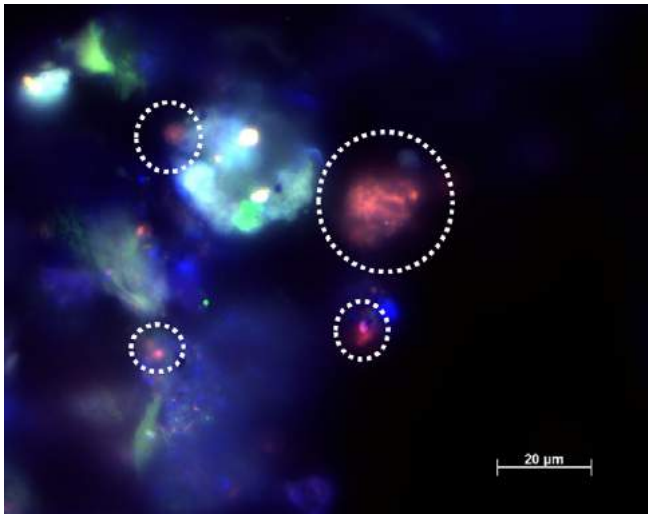

Fig. 3. FISH analyses of red Anammox clusters (encircled). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

4.1. Ammonia in the pilot plants

The release of NH_4^+ into the leachate during the first hours (up to 0.5 g L^{-1}) (Fig. 2a) was completely governed by a physical mechanism: the contact between solids and the liquid percolating through the waste mass. The subsequent increase of NH_4^+ (up to 1 g L^{-1}) in the leachate during the first 10 days was due to constant recirculation, which helped to washout the NH_4^+ salts present in the simulators. The second NH_4^+ increase from day 10 to 100 (Fig. 2) can be attributed to the enhanced microbiological conversion of organic matter (Berge et al., 2005; Jokela et al., 2005), while no NH_4^+ was removed under the prevailing anaerobic conditions.

From day 200 the NH_4^+ levels decreased, not only caused by depletion of the readily degradable organic matter (Fig. 1c) converted into biogas (Fig. 1c), but also due to NH_4^+ removal processes. Similar decreases of NH_4^+ levels were observed (but not explained) in comparable bioreactor landfill experiments (Ağdağ and Sponza, 2005a,b).

During (manual) recirculation of the liquid, some O_2 could have entered into the simulators and nitrification/denitrification could have taken place. From day 300 onwards minimal quantities of NO_3^- (up to 30 mg L^{-1}, data not shown) were indeed detected in the leachate, which suggests that nitrification might have occurred at

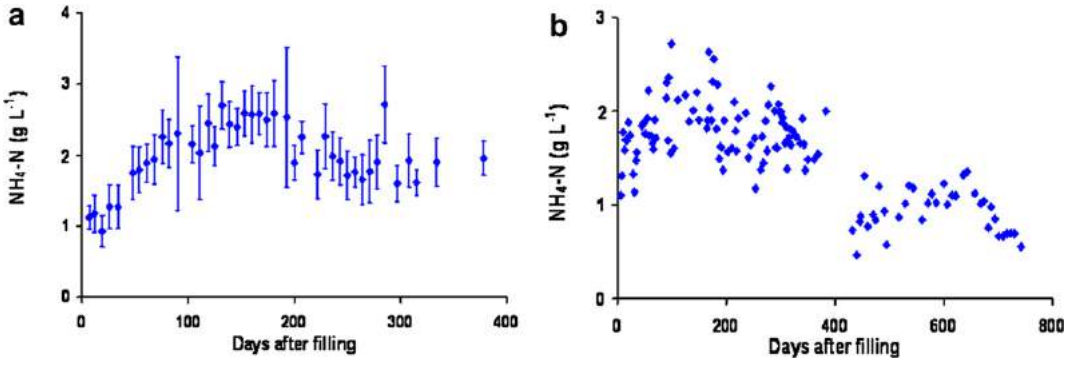

Fig. 2. Evolution of ammonium in the leachate in a) one year experiments with 7 simulators and b) two years experiments with 2 simulators preceding (a).

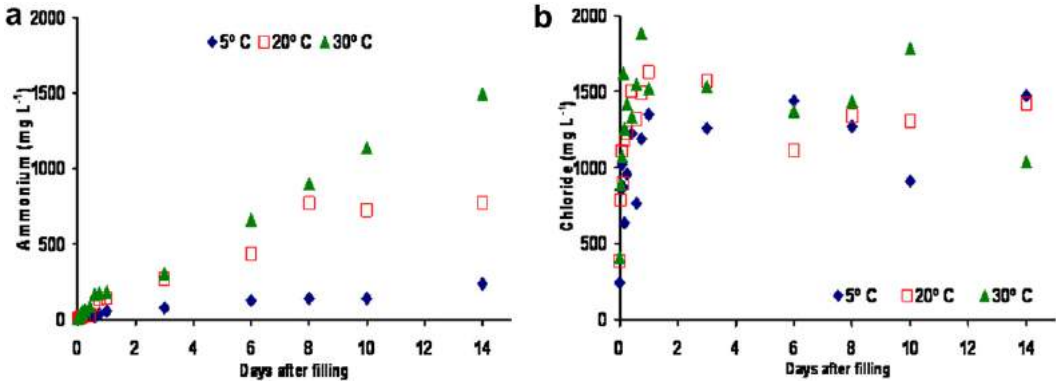

Fig. 4. Effect of temperature on the release of ammonia and chloride.

earlier stages, but the produced NO_3^- was immediately denitrified, as long as sufficient organic matter was available. However, calculations revealed that the amount of O_2 introduced was not sufficient to reduce NH_4^+ levels significantly in this way. The biomass extracted from the simulators without active aeration revealed the presence of Anammox bacteria (Fig. 3), with an estimated bacterial density of 5–7% of the total bacterial density. Partial nitrification to NO_2^- and sufficient NH_4^+ in the leachate, supported by the optimal pH (±7.5–8.0) and temperature (±30 °C) (Strous et al., 1997), were the key factors for the development and activity of the Anammox bacteria. No Anammox bacteria were found in the two aerated simulators. Besides the FISH analyses on the biomass the presence of high levels of hydrazine supported the Anammox activity.

Nitrogen balances (Fig. 6) showed that 40% of the total N was released from the solid waste and transferred either into the liquid or the gas phase in 380 days. At the end of the experimental period, only 2.3 kg of the initial 3.2 kg of N added to the simulators was recovered: 1.9 kg as residual N and 0.4 kg of N in the liquid mainly composed of NH_4^+ (±80%) and organic N (±20%). The unaccounted N, if totally converted into N_2, would account for 0.8 m^3 of gas, which was less than 3% of the total biogas production; this N_2 value is within the ranges suggested in the literature (Tchobanoglous and Kreith, 2002).

Based on the approximated Anammox bacterial density and the removal efficiencies suggested by Strous et al. (1997) it could be possible to explain the removal of about 100 g NH_4^+, which is more than removal via precipitation as struvite, volatilisation of NH_3, biological uptake or nitrification/denitrification.

Theoretically, about 10 g N could be removed via struvite precipitation, although the conditions are good (Ohlinger et al., 1998); in addition, struvite was not detected, probably due to low PO_4^{3-} concentrations. Approximately 30 g N could have been volatilised as NH_3 during the last 180 days, because the volatilisation rate is 2.5% at the prevailing pH of 7.5. Maximally 40 g N could have been used for assimilation by micro-organisms for cell growth, similar to figures reported by Burton and Watson-Craik (1998). Therefore, bacterial uptake cannot explain the total consumption of N as suggested by Ağdağ and Sponza (2005a). Removal of N due to full nitrification (up to NO_3^-) can only account for 2.3 g N, considering that maximally 3.25 mg O_2 L^{-1} was introduced every time the leachate was recycled into the simulators. Denitrification was not a limiting factor for N removal, since there was sufficient organic matter present in the simulators at least during the first 200 days.

However, it is not clear how the necessary NO_2^- for Anammox was produced. According to the properties of the high density ethylene pipes maximally 8.6 g O_2 could have penetrated through the wall during the entire length of the experiment, which was about 2.5% of the required O_2 to nitrify the necessary 100 g NH_4^+. Alternatively, NO_2^- could have been produced externally in the leachate reservoirs and introduced via recirculation without being noticed.

The presence of Anammox bacteria in landfill environments has been suspected before (Burton and Watson-Craik, 1998; Price et al., 2003; Berge et al., 2005), but never confirmed.

4.2. Temperature effects on the release of ammonia

The effect of temperature on the release of chloride and NH_4^+ is different (Fig. 4). The removal of Cl^- is instantaneous and not affected by temperature, suggesting that the highly soluble Cl^- is

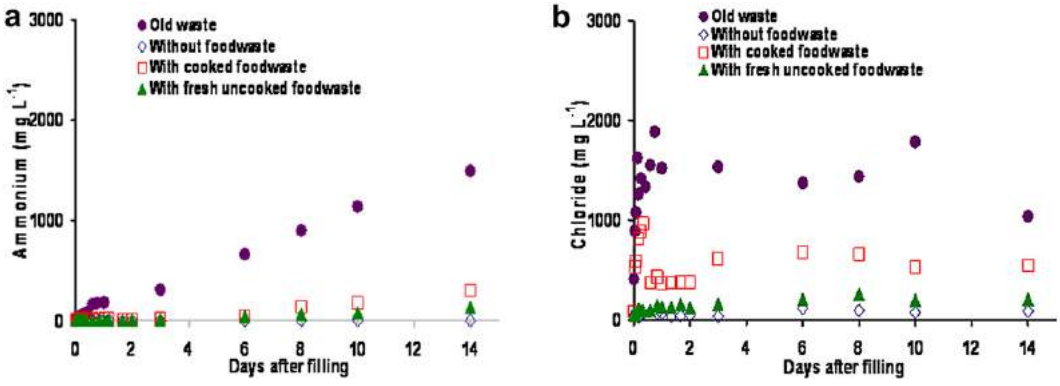

Fig. 5. Effect of age and type of waste on the release of ammonia and chloride.

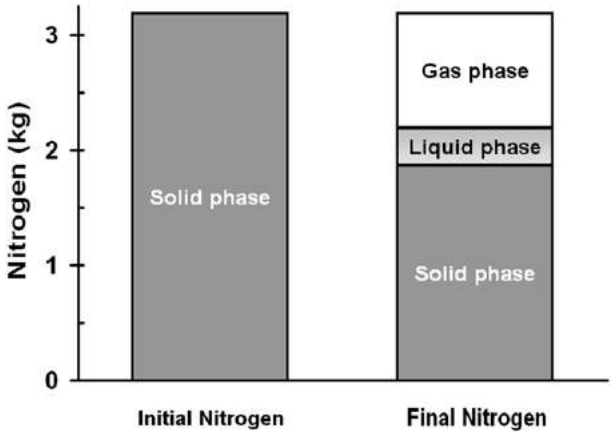

Fig. 6. Nitrogen mass balance of the bioreactor landfill simulators.

removed by a physical/chemical process. Chloride is a conservative ion and hence not involved in oxidation or reduction reactions, forms hardly complexes, does not form salts with low solubility, and is not significantly adsorbed to mineral surfaces. On the contrary, NH_4^+ is not immediately available and its release is more gradual, which is comparable to the NH_4^+ evolution during pre-treatment of municipal solid waste (De Gioannis and Muntoni, 2007). The effect of temperature suggests that the process is of biological origin and that the protein-hydrolysing bacteria are already present in the waste. NH_4^+ release from fresh waste, to which food was added, started after one week, suggesting that it took some time before the bacteria in this waste had hydrolysed some protein (Fig. 5a), which is in contrast with the immediate production of NH_4^+ in old waste (Figs. 4a and 5a).

Chloride was removed immediately, at least if it was available (Fig. 5b): from old waste and from fresh waste including cooked food, because normally, when food is cooked, some salt is added. From fresh waste without food or with uncooked food hardly any Cl^- is released. These findings are comparable with the NH_4^+ and Cl^- release from fresh and old waste from real landfills (Khattabi et al., 2002).

5. Conclusions

- In anaerobically maintained landfills for municipal solid waste, Anammox bacteria will develop and may play an important role in ammonium removal.
- Ammonia is released gradually from solid waste by physical and biological processes, while chloride is released instantaneously in a physical way.

Acknowledgements

The authors would like to thank Markus Schmid and Huub Op den Camp (Radboud University, Nijmegen) for their assistance in the assessment of Anammox.

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Journal of Environmental Management 95 (2012) S149–S153

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Occurrence of high-tonnage anionic surfactants in Spanish sewage sludge

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ARTICLE INFO

Article history:
Received 17 August 2009
Received in revised form
12 May 2011
Accepted 20 May 2011
Available online 8 June 2011

Keywords:
Linear alkylbenzene sulphonate
Soap
Surfactants
Sewage sludge
Soil
Biosolids

ABSTRACT

Agricultural application has become the most widespread method of sewage sludge disposal, being the most economical outlet for sludge and also recycling beneficial plant nutrients and organic matter to soil for crop production. As a matter of fact, the European Sewage Sludge Directive 86/278/EEC seeks to encourage the disposal of sewage sludge in agriculture applications and regulate its use to prevent harmful effects on the soil environment. At the present time, the sewage sludge Directive is under revision and a possible cut-off limit for some organic chemicals may be implemented. Linear alkylbenzene sulphonate (LAS), the main synthetic anionic surfactant, has been included in the draft list of chemicals to be limited. The present research work deals with the monitoring of LAS and soap in Spanish sewage sludge. The average concentration of LAS found in anaerobic sewage sludge samples was 8.06 g/kg, higher than the average values for European sludge. Besides, it has been also found that more than 55% of Spanish anaerobic sludge would not fulfil the limit proposed by the 3rd European Working paper on sludge. As a consequence, the implementation of the limit for LAS would make the disposal of most Spanish biosolids for agricultural applications almost impossible. Regarding the mechanisms why anionic surfactants are found in sludge, two surfactants are compared: LAS and soap, both readily biodegraded in aerobic conditions. Irrespective of the anaerobic biodegradability of soap, its concentration found in sludge is higher than LAS (only anaerobically biodegradable under particular conditions). The relevance of anaerobic biodegradation to assure environmental protection is discussed for this case.

1. Introduction

The implementation of the Urban Waste Water Treatment Directive 91/271/EEC in all Member States is increasing the quantities of sewage sludge requiring disposal. The annual sewage production for the 25 countries of the European Union (EU) was estimated to be higher than 10 million tons of dry sludge (Laturnus et al., 2007) and this quantity is expected to increase in the future due to the strong demand of cleaner sewage water and the increasing population. Moreover, the Sewage Sludge Directive 86/278/EEC seeks to encourage the disposal of sewage sludge in agriculture applications and regulate its use to prevent harmful effects on the soil environment. As a matter of fact, the EU 3rd Working paper on sludge also suggested that sludge should be used when there is an agronomic interest for the crops or the quality of soil can be improved (EU Working paper on sludge, 2000). The treated sludge contains large quantities of organic matter (60–70% of dry matter during aerobic digestion, 40–50% during anaerobic digestion) and nutrients (around 3% of phosphorus and 1.5% nitrogen) (Laturnus et al., 2007). Hence, agricultural application has become the most widespread method of disposal (i.e. more than 55–60% in Spain or more than 65% in Denmark), being the most economical outlet for sludge and also recycling beneficial plant nutrients and organic matter to soil for crop production (Smith, 1996). Agricultural use of raw sludge is encouraged by national authorities as the best way for recycling (Bresters et al., 1997) and it is widely recognised that valorisation of sludge is desirable (Otero et al., 2003). Other disposal routes are incineration and land-filling. For both cases, the organic matter and nutrients are lost and the waste management problem is transformed but not solved (carbon dioxide and methane emissions, respectively). Considering the United States law framework, the EPA regulations for sewage sludge disposal and use (the Standards for the Use or Disposal of Sewage Sludge at Section 40 of the Code of Federal Regulations Part 503) establish numeric limits, management practises, and operational standards to protect public

Abbreviations: LAS, Linear alkylbenzene sodium sulphonate; HERA, Human and environmental risk assessment; EPA, US Environmental Protection Agency; WWTP, Waste water treatment plant; SS, Sum of squares; DF, Degrees of freedom; MSS, Mean squares.

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doi:10.1016/j.jenvman.2011.05.027

health and the environment from adverse effects of chemical and microbiological pollutants in sewage sludge. EPA has conducted three national surveys for purposes of identifying contaminants in sewage sludge coming from sewage facilities treating more than one million gallons of waste water per day. Although surfactants were not included in these surveys; the EPA is encouraged to review existing sewage sludge regulations at least every two years (Targeted National Sewage Sludge Survey, EPA. 2009). Moreover, Harrison et al. (2006) found more than 516 organic compounds in US sludge samples. Similarly, Cai et al., (2007) investigated the presence of organic compounds by GC–MS in sewage sludge during composting in China.

To our knowledge, no limit has been particularly proposed for LAS in these countries. Moreover, the EPA reports that about 50% out of the total treated sludge is being recycled to land and recommends its use as biosolids to fertilize fields for raising crops. Additionally, Fulazzaky et al. (2009) reviewed the problems of sludge pressure in Indonesia due to the potential increase in practises of legal and illegal logging as well as the increasing land and water demands. They concluded that application of sludge on the agricultural lands would promote sustainable development.

With respect to the current European legislation, the sewage sludge Directive established a maximum value for the amount of heavy metals in sludge and also the proper dose of sludge to avoid pollution of soils. At the present time, the sewage sludge Directive is under revision and a possible cut-off limit for some organic chemicals may be implemented even though whether they may provoke adverse effects on the environment via plant-uptake or soil leaching still remains a controversial discussion. Considering the case of LAS, which is the main synthetic anionic surfactant used in the formulation of laundry detergents and cleaning products, it is not clear whether a limit will be finally implemented (2.6 and 5 g/kg have been proposed in several drafts). In fact, there is profuse scientific literature dealing with its environmental fate, behaviour and risk assessment for many environmental compartments: water, sediments, soil or sewage treatment plants (HERA report, 2007). Regarding the fate of LAS in waste water treatment plants, LAS is a ready-biodegradable chemical and it is well-known that high removal of LAS is achieved in sewage treatment plants (Temmink and Klapwijk, 2004). Additionally, LAS tends to precipitate in the presence of calcium ions and to adsorb into solid matrices. As a result, the physical–chemical properties of most surfactants may result in a significant partitioning between aqueous and solid phase in the aquatic environment (Berna et al., 2007). Since sewage sludge is often anaerobically stabilised and considering that LAS is not degraded under anaerobic conditions in laboratory tests, LAS has been misleadingly seen as a potential risk for soil organisms. Besides, much research work has been done in order to monitor the presence of LAS in environmental solid matrices (Jensen and Jepsen, 2005; Schowanek et al., 2007) and to study the risk associated to the application of LAS-containing sludge to agricultural soil and terrestrial environments (De Wolf and Feijtel, 1998; Jensen et al., 2001, 2007; Krogh et al., 2007). According to the latter risk assessment literature, the predicted environmental concentration (PEC) for LAS is lower to the non-effect concentration (PNEC), giving a risk quotient (RQ) lower to 1. That means the concentration of LAS does not pose a risk for fauna and flora of environmental compartments. For instance, the risk quotient for LAS in sewage treatment plants is only 0.08. Considering the risk in soils (including sludge-amended soils), the RQ is 0.68. However, as the main mechanism for the presence of anionic surfactants in environmental solid matrices is the precipitation and adsorption in presence of salts (Berna et al., 2007) rather than the lack of anaerobic biodegradation, the environmental and legal consequences of the presence of surfactants in sewage sludge should be discussed. Therefore, the main assumption of this research paper is that the potential limit could render agricultural use of sludge almost unviable and that the inherent properties (water solubility, precipitation or adsorption) rule the presence of surfactants in solid compartments rather than the anaerobic biodegradability of the molecule. Thus, this paper is focused on the determination of high-tonnage anionic surfactants (LAS and metallic salts of long-chain fatty acids, i.e. soap) in Spanish sewage sludge samples.

2. Materials and methods

2.1. Sludge samples

Fifty one waste water treatment facilities were monitored during 2006–2007. The monitoring comprised four sampling periods and included several sludge treatment modes: aerobic, anaerobic, chemical, composting, drying, no treatment, etc. All WWTP were monitored in one of the sampling periods and only selected WWTP were monitored during the whole sampling periods. LAS was determined for all sludge samples while soap was analysed in a number of samples for comparison purposes.

2.2. Determination of LAS in sewage sludge samples

Commercial LAS (a complex mixture of homologues and isomers with alkyl chain length C10–C13) was used for calibration purposes. 2-phenyl C8 LAS was used as internal standard.

The analysis of LAS comprises the following stages: i) microwave-assisted extraction (MARS5, CEM Corp., New Jersey, USA) of LAS with 25 mL methanol (5 g of sludge, 1600 W at 70 bar, 15 min); and ii) RP-HPLC (Agilent 1100, Agilent Technologies, Santa Clara, USA) determination using C18 columns (RP-C18 Licrhospher 250 mm × 4.6 mm i.d. × 5 μm film thickness) as stationary phase and being water/acetonitrile (95:5) and acetonitrile the mobile phases (flow 1.0 mL/min, initial flow of acetonitrile 70%, then 50% at 20 min and 70% at 25 min). Triethylamine and acetic acid (both 5 mM) were used as buffer in the water mobile phase. The injection volume was 100 μL and a fluorescence detector (excitation 232 nm, emission 290 nm) was used. HPLC determination. It must be pointed out that no pre-concentration/purification stage was required, so the analysis time was reduced in comparison to previous analytical methods (López et al., 2003).

2.3. Determination of soap in sewage sludge samples

Fatty acids with alkyl chain length C8–C20 and tridecanoic acid (source of the internal standard) were obtained from Merck (Darmstadt, Germany). All other reagents and solvents were analytical grade: potassium carbonate, potassium chloride, ethylenediaminetetraacetic tripotassium salt (EDTA-3K), calcium chloride, acetone, 2-propanol, petroleum ether and methanol. The derivatization reagent 2,4-dibromoacetophenon (DAP) was also purchased from Merck. Solutions of calcium soap homologues from C8 to C20 (50 mg/mL in 2-propanol) and internal standard (potassium tridecanoate, 100 mg/mL in methanol) were prepared and stored at 4 °C. All the devices were similar to LAS determination (microwave solid-liquid extraction. However, The HPLC was equipped with RP-C8-Lichrospher columns (250 mm, 4 mm i.d., 5 μm film thickness; Agilent Technologies, Santa Clara, USA).

Due to their inherent physical–chemical properties, soap easily precipitates if calcium or magnesium ions are present in water. Consequently, the water-insoluble calcium soap is found in environmental solid matrices rather than soluble sodium or potassium salts. Therefore, the analytical methodology must be aimed at the determination of insoluble salts of fatty acids: calcium soap. A brief

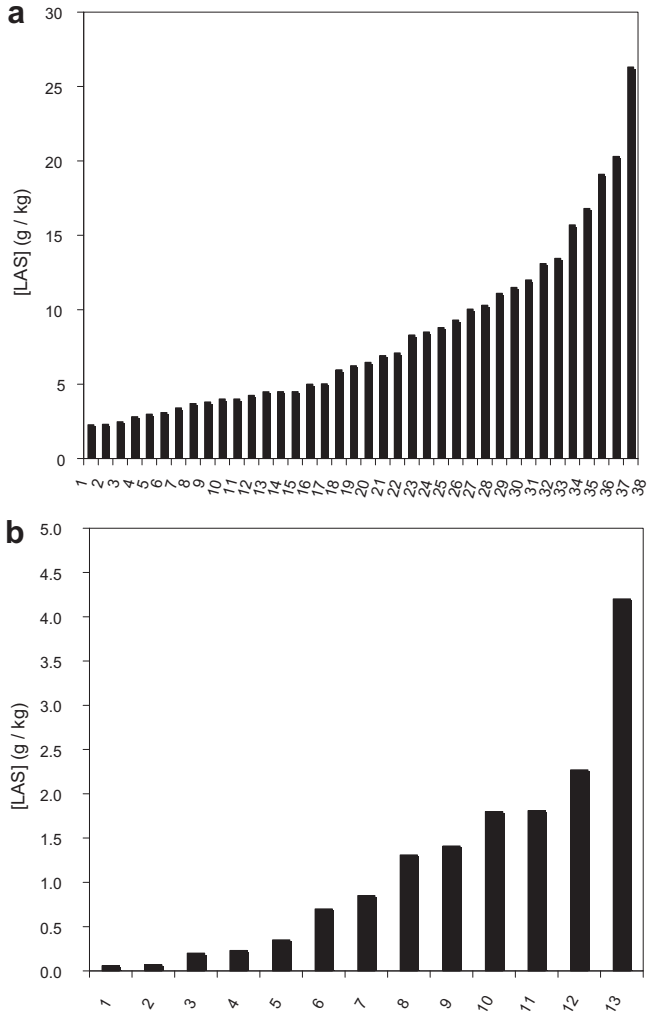

Fig. 1. Concentration of LAS in: a) anaerobic sludge and b) aerobic sludge.

description of the procedure is given below (for a comprehensive review, see Cantarero et al., 2008).

Standard homologues of calcium soap were prepared by reaction of fatty acids with potassium chloride to yield potassium soap and then, precipitation in organic medium (acetone). The precipitated potassium soap is solved in water and then calcium chloride is added. As a result, precipitated calcium soap is obtained. The calcium soap is filtered and rinsed with water to remove excess reagents. Calcium soap homologues from C8 to C20 were synthesised and used for calibration and validation purposes.

The analytical determination of soap consists of several stages, namely: i) sludge cleaning, ii) reaction of calcium soap to yield potassium soap, iii) extraction of soap from sludge, iv) derivatization to yield UV-absorbing species and v) HPLC analysis. The cleaning of sludge allows removing fatty materials (lipids) other than soap. It is carried out through microwave extraction with petroleum ether (5 g of dried sludge and 50 mL of petroleum ether, 15 min at 1600 W at 70 bar). Then, calcium soap reacts with EDTA-3K (50 mg) and K_2CO_3 (25 mg) in water medium (50 mL) to yield potassium soap (reaction time 4 h at 105 °C). Afterwards, a second microwave extraction step takes place in order to extract K-soap with methanol (50 mL, 10 min at 1600 W at 70 bar). Once the methanol soluble K-soap are obtained, they react with 2,4 − dibromoacetophenon (DAP) to yield UV-absorbing species. The final derivative is suitable for HPLC separation and diode array detection. Regarding the chromatographic methods, the analysis of soap was performed by RP-HPLC using OS columns (RP-8) as stationary phase and being water and acetonitrile the mobile phases (flow 1.5 mL/min, initial flow of acetonitrile 70%, then 85% at 15 min and 100% at 25 min). The injection volume was 30 μL and a diode array detector (DAD) at 258 nm was used.

Table 1
Mean and dispersion of linear alkylbenzene sulfonate concentrations for aerobic and anaerobic sludge.

Treatment	Frequency	Mean [LAS] (g/kg)	Standard deviation
Aerobic	13	1.27	1.19
Anaerobic	38	8.06	5.78

2.4. Calibration and statistical validation

The method calibration, optimisation and validation for LAS and soap analysis were previously reported (Bengoechea and Cantarero, 2008; Cantarero et al., 2008). The influence of the different factors on the concentration of surfactants found in sludge has been evaluated by one-way analysis of variance (ANOVA). Statistical calculations were made with Statgraphics Plus 5.0 (Manugistics, Inc, Rockville, USA).

3. Results and discussion

3.1. Determination of LAS in sludge

In all cases LAS present the typical environmental fingerprint enriched in longer chain LAS homologues (C12 and C13) compared to the parent distribution where C11 is the main isomer. The LAS concentration for all sludge samples is shown in Fig. 1. The mean concentration of LAS is around 1.27 g/kg in the case of aerobically treated sludge, while 8.06 g/kg for anaerobically treated samples (Table 1). Please note that the standard deviation shown is not linked to the analytical uncertainty but to the dispersion found on the different sewage plants. According to Table 1, it can be deduced that the sort of treatment (aerobic vs. anaerobic) may show strong influence on the concentration of LAS. A one-way analysis of variance analysis (ANOVA) has been performed in order to assess this correlation (Table 2). Since the p-value <0.05, there is significant difference between the mean values of both treatments at a confidence level of 95%. The percentage of variability explained by the ANOVA comparison is higher than 99%, showing that the treatment of sludge significantly influences on the amount of LAS found in the final biosolids. The relationship between equivalent inhabitants and concentration of LAS has been studied separately for aerobic and anaerobic samples. No fit between equivalent inhabitants and LAS concentration has been found by means of one-way ANOVA (at 95% confidence), since the p-values calculates are above 0.05 (Table 3). Nonetheless, earlier researchers reported good correlation between both variables (Fraunhofer report, 2003).

Other qualitative trends were analysed (for instance influence of water hardness data) however it should be pointed out that no

Table 2
Influence of the kind of sludge stabilisation on LAS concentration: one-way ANOVA.

Source	SS	DF	MSS	F	p-value
Inter-groups (aerobic/anaerobic)	446.227	1	446.227	17.48	0.0001
Intra-group	1251.04	49	25.5314		
TOTAL	1697.26	50			

Table 3
Influence of the equivalent inhabitants of WWTPs on LAS concentration: one-way ANOVA.

Treatment	DF	F	*p*-value
Aerobic	13	6.21	0.1466
Anaerobic	38	1.44	0.2632

statistical analysis has been carried out due to the limited information available. Generally, it can be remarked that the highest LAS concentrations have been found in areas of very high water hardness (eastern coastal areas of Valencia and Catalonia). On the other hand, the lowest LAS levels have been generally detected in areas of very low water hardness (Madrid and Castile).

The distributions of LAS in anaerobic in the European Union sludge has been previously reported on the literature, based on literature data over the time period 1988–2006 (Schowanek et al., 2007). The result of the distribution for the anaerobic sludge (155 records) was a mean of 5.56 g/kg (being 0.49–15.07 g/kg; 5th to 95th percentile, respectively). Based on the data presented on this research work (38 data), the mean concentration of LAS is 8.06 g/kg and the 5th and 95th percentile are 2.10 and 19.3 g/kg, in that order. Hence, it seems that the content of LAS in anaerobic sludge in Spanish biosolids is higher than the European one. The gap between both studies may be explained by the highest detergent consumption *per capita* in the southern Europe countries (Spain, Portugal and Italy). The introduction of an upper limit of 5 g/kg of LAS has been proposed and would not allow the disposal of these biosolids for agricultural uses. Therefore, our data have been fitted to a Beta probabilistic distribution in order to know the probability of exceeding the limit (Fig. 2). The predicted probability according to the Beta distribution is around 55%. As a consequence, 55% of the Spanish anaerobically stabilised sludge might not fulfil the limit.

Considering the total amount of biosolids produced in Spain (1.2 million tons in 2005; report on Spanish strategy to reduce the biodegradable waste disposed to landfill, 2006), putting into force a limit for the content of LAS in sewage sludge would make unviable the disposal of sludge for agricultural purposes (more 600,000 tons just in Spain, Spanish Ministry of Environment, 2005).

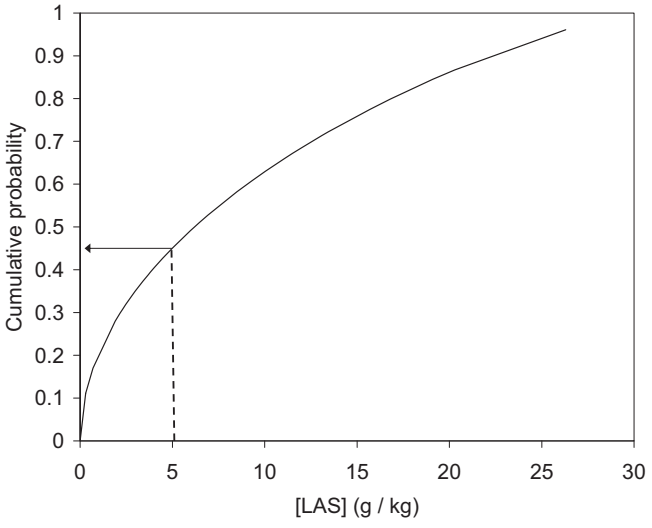

Fig. 2. Fitting of experimental concentrations of LAS in sludge (g//kg) to a Beta distribution ($\alpha = 0.5$, $\beta = 1.2$, lower limit = 0, upper limit = 30).

Table 4
LAS vs. soap concentration in selected sewage facilities.

Sludge	Sludge treatment	[LAS] (g/kg)	[Soap] (g/kg)
A	Anaerobic	5.96	8.18
A composted	Anaerobic + Composted	1.41	2.43
B	Anaerobic	2.27	83.85
C	Anaerobic	0.85	50.11
D	Anaerobic	10.07	212.29
E	Anaerobic	4.50	29.00
F	Anaerobic	1.75	103.64
G	Anaerobic	0.71	11.19
H	Aerobic	0.059	9.71
I	Aerobic	0.20	14.8

3.2. Determination of soap in sludge

Soap (metallic salt of dodecanoic to linoleic acids) has been determined in a number of sludge samples and it has been compared to the concentration of LAS. The most abundant homologues are the longest ones, calcium palmitate, oleate and stearate since they are the most sensitive homologues towards precipitation and show more sorption behaviour. A similar profile has been found in most samples, with palmitate around 30–40% and saturated and unsaturated C18, 50–60%. The C12–C14 content is lower than 20% in all cases. The Table 4 shows a comparison between the LAS and the soap content in ten sludge samples. In all cases, the soap content has been higher than that of LAS. In fact, it ranges from 1.4 to 164 times higher than LAS. The soap concentration in sample D is higher to 200 g/kg. It must be highlighted that soap concentration is quite elevated in anaerobically treated sludge in spite of the fact that soap can be biodegraded under anaerobic conditions at laboratory scale. These data are consistent with previous literature (Folke et al., 2003). Regarding the statistical assessment of the results, the validation of the analytical methods has been published elsewhere (see Methods and material section for references).

3.3. Mechanisms of anionic surfactants occurrence in sludge

The fate of fatty acid salts in aqueous systems is complicated by the fact that there are a numbers of water soluble and water-insoluble groups and combinations of these. In practise whilst the use of Na salts are by far the most common use of soap in finished products, the predominance of calcium and magnesium ions in waste water leads to rapid formation and predominance of relatively insoluble calcium and magnesium salts (Prats et al., 1996). Therefore, the fate of fatty acid salts is strongly influenced by the poor water solubility of some salts. This same statement is valid for other anionic surfactants like LAS, depending on their sensitivity and tolerance to cations. Furthermore, it is well-known that both surfactants are readily biodegraded under aerobic conditions. The main difference between LAS and soap is based on their biodegradation under anaerobic conditions: both sodium and calcium salts of fatty acids (C10–C18) have been shown to exhibit significant removal through under anaerobic conditions (BKH, 1994). On the contrary, LAS does not degrade under anaerobic conditions, except under particular conditions (HERA report, 2007).

Despite the different biodegradation behaviour, the experimental data presented show that soap concentrations are higher than LAS on environmental solid matrices, consistently with previous research (Prats et al., 1996; Folke et al., 2003). Therefore, it cannot be inferred that the lack of anaerobic biodegradation is linked to the presence of surfactants in biosolids. The relevance of anaerobic biodegradability cannot be separated from other properties such as adsorptive behaviour, ecotoxicity profile and above all, aerobic biodegradation rate (Berna et al., 2007). Finally,

according to literature data, LAS had a maximum half-life of one week in sludge-amended soils and monitored concentrations were around 1 mg/kg soil (maximum 1.4 mg/kg soil) at harvesting time. No accumulation in soil and no bioaccumulation in plants could be detected experimentally (HERA report, 2007).

4. Conclusions

The concentration of LAS in sewage sludge has been monitored in fifty one Spanish WWTPs, being 8.06 g/kg the mean concentration for anaerobic sludge. The average concentration of LAS in sewage sludge from Spanish WWTPs was upper that the average value of a European-wide study.

Regarding the revision of the EU Sludge Directive, putting into force a limit for the content of LAS in sewage sludge would make unviable the disposal of sludge for agricultural purposes (more 600,000 tons just in Spain) because most of sewage sludge cannot pass the limit values (more than 55%). In addition, the limit proposal is not substantiated on a risk-basis insofar as updated risk assessments for LAS showed it does not pose a risk for environmental solid compartments. Soap content has been determined for a number of sludge samples and compared to that of LAS. Regardless the sludge treatment (aerobic, anaerobic and composting) the soap content found has been higher than that of LAS. That is to say both surfactants can be found in treated sewage sludge even though soap is biodegraded under laboratory anaerobic conditions while LAS cannot be degraded, indicating that anaerobic biodegradation is not the main factor linked to the occurrence of anionic surfactants in sewage sludge.

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Journal of Environmental Management 95 (2012) S154–S157

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Used battery collection in central Mexico: Metal content, legislative/management situation and statistical analysis

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ARTICLE INFO

Article history:
Received 29 September 2009
Received in revised form
1 September 2010
Accepted 9 September 2010
Available online 8 October 2010

Keywords:
Batteries
Campaign
Metal
Management
Mexico

ABSTRACT

A statistical analysis of a used battery collection campaign in the state of Tlaxcala, Mexico, is presented. This included a study of the metal composition of spent batteries from formal and informal markets, and a critical discussion about the management of spent batteries in Mexico with respect to legislation.

A six-month collection campaign was statistically analyzed: 77% of the battery types were "AA" and 30% of the batteries were from the informal market. A substantial percentage (36%) of batteries had residual voltage in the range 1.2–1.4 V, and 70% had more than 1.0 V; this may reflect underutilization. Metal content analysis and recovery experiments were performed with the five formal and four more frequent informal trademarks. The analysis of Hg, Cd and Pb showed there is no significant difference in content between formal and informal commercialized batteries. All of the analyzed trademarks were under the permissible limit levels of the proposed Mexican Official Norm (NOM) NMX-AA-104-SCFI-2006 and would be classified as not dangerous residues (can be thrown to the domestic rubbish); however, compared with the EU directive 2006/66/EC, 8 out of 9 of the selected battery trademarks would be rejected, since the Mexican Norm content limit is 20, 7.5 and 5 fold higher in Hg, Cd and Pb, respectively, than the EU directive. These results outline the necessity for better regulatory criteria in the proposed Mexican NOM in order to minimize the impact on human health and the environment of this type of residues.

1. Introduction

Environmental pollution produced by disposal of Spent Cells and Batteries (SCB) is a major concern due to the fast growing portable electronic equipment industry that generates thousands of tons of dangerous residues per year. Based on 1992 and 1998 studies, household batteries accounted for approximately 90% of the Hg and 52% of the Cd in Municipal Solid Wastes (MSW) in the US, though that level is projected to decline greatly as manufacturers remove mercury from alkaline batteries (Richard and Woodbury, 1992, 1998). In the EU, the environmental risks related to the disposal of the Cd batteries were assessed in the draft Targeted Risk Assessment Report, "Cadmium (oxide) as used in batteries" (TRAR, 2003). According to the report, the Cd emissions of portable NiCd batteries due to landfill were calculated at 131–655 kg of Cd per year. In 2006, the European Commission required a closed cycle system for all the SCB (Directive 2006/66/EC, 2006) with the purpose of reduce the quantity of spent batteries and accumulators and to set targets for collection and recycling. The US Department of Health and Human Services' Agency for Toxic Substances and Disease Registry states that the metals in batteries can have serious health effects if not managed correctly. Hg at high levels can damage the brain, kidneys and a developing fetus. Pb can harm the nervous system, kidneys and reproductive system. Cd can damage the lungs and kidneys, and irritate the digestive tract. Exposure to large amounts of Zn can cause stomach cramps, anemia and changes in cholesterol levels. And each metal can have a direct harmful effect on the environment (ATSDR, 2010).

The first legal precedent for regulation of SCB in México arose in 1988 with the general law of the ecological balance and the protection of the environment (LGEEPA). This law classifies the SCB as potential dangerous residues given the toxicity risk of some of its components, although the inadequate handling of SCB has continued being a common practice. Mexico's National Institute of Ecology (INE) estimates that from 1960 to 2003, the following residues have been deposited in MSW: 145,918 ton of MnO_2;

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doi:10.1016/j.jenvman.2010.09.019

1232 ton of Hg; 22,063 ton of Ni; 20,169 ton of Cd and 77 ton of Li compounds (Castro-Díaz and Díaz-Arias, 2006). The Mexican Association of Manufacturers and Commercial Dealers of Batteries (Amexpilas) claims their batteries do not pollute the environment and have made efforts to convince public opinion only informal batteries (which have questions regarding legal registrations and corresponding business procedures) are polluting (Vega-Vieyra, 2006). The total market (single use and rechargeable) is approximately 650 million batteries per year. This corresponds to about 500 and more than 200 million dollars per year for formal and informal batteries, respectively (Aguilar and García-Camargo, 2006). In 2006, the federal government made public the proposed Mexican Official Norm (NOM) NMX-AA-104-SCFI-2006. There are important differences between the EU directive 2006/66/EC and the proposed Mexican NOM: the maximum permissible levels for Hg, Cd, and Pb are 20, 7.5, and 5 times, respectively, higher than the EU directive, and approved legal batteries are permitted to be discarded in landfills.

Since the article was first submitted, the Mexican Republic Senate urged the Secretariat of Environment and Natural Resources to implement a comprehensive management program of SCB, and requested that the head of the Department of Environment and Natural Resources report the situation of the proposal PROY-NMX-AA-104-SCFI-2006, asking for its publication if it had completed the consultation process (Mexican Senate, 2009). However, the proposal has not advanced, and some specialists are afraid that the legislation, rather than encouraging the recycling of batteries with the responsible participation of consumers, producers and local authorities, became a disincentive to do so (Cortinas de Nava, 2009). Other Latin-American countries have similar situations: official policies and regulations only establish limits to the potentially hazardous metal content used on batteries' composition but do not mandate the participation of producers and importers. In Argentina, an environmental organization demanded the publication of a law which provides for extended producer responsibility, but it was delayed in the Senate (Greenpeace-Argentina, 2010). In Colombia, the proposed law No. 69 (2009) of the Republic Senate contemplates repurchase of batteries, and electrical and electronic waste by the manufacturers (Gladis, 2009). In Brazil, the CONAMA regulations (2001) prohibited the marketing of batteries with concentrations higher than the stipulated limits of Hg, Cd and Pb, but batteries with lower content can be landfilled. A broader and potentially more effective regulation was established by the Brazilian State of Rio Grande do Sul, where law 11.187 (1998) prohibits the disposal of any material containing heavy metals together with MSW (Soares Tenório, 2003).

In this work, an official program to collect SBC in Central Mexico was used to: 1) conduct a statistical evaluation of the incidence of informal commerce batteries compared to the formal ones; 2) study the residual voltage of SBC to help assess optimal use of this energy source and the potential inclusion of residual energy recovery as part of recycling technology; and 3) study the metal composition of used batteries from formal and informal market to determine if there is a significant difference in their metal content. The objective of this paper was to use these results to evaluate the potential effectiveness of proposed Mexican NOM by classifying both types of SCB (formal and informal market) under this NOM and the EU directive 2006/66/EC.

2. Materials and methods

2.1. Compilation of spent batteries

The collection of spent batteries was carried out from June 2007 to January 2008 in the city of Tlaxcala and its municipal territory (area of 52.449 Km^2 and 83,748 inhabitants). A collection point was installed in every one of the eleven auxiliary municipal presidential offices.

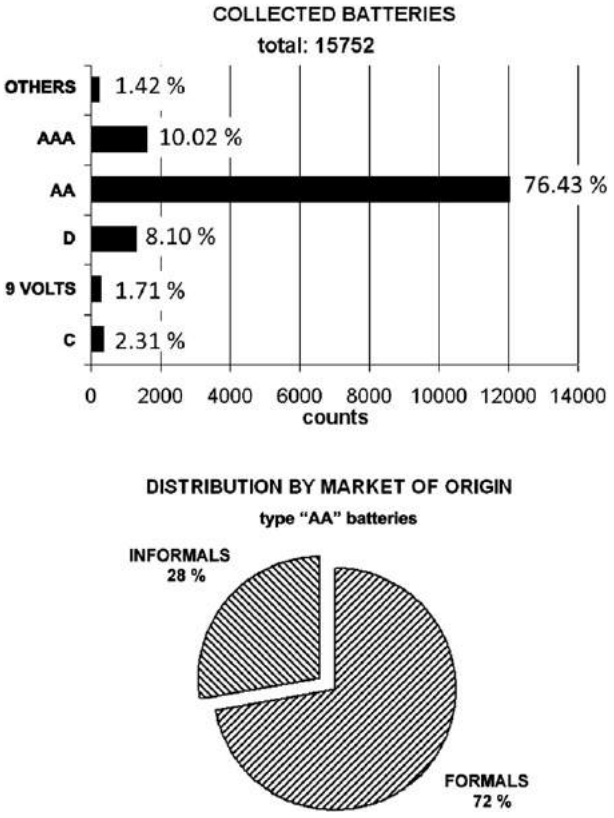

Fig. 1. Statistical results for the used batteries collection campaign. Above: Distribution by type of battery; Below: Cake plot for market of origin of type "AA" batteries. A cake plot for distribution of trademarks (formals and informals) can be consulted in the Supplementary material.

The exhausted portable batteries from the collection points were concentrated and separated in trademarks, models, and the market of origin (formal or informal). Residual voltage of random selected batteries type "AA" from the four major trademarks was measured. Statistical analysis was made with the program Origin V6.1. The collected batteries reflected what the local population voluntarily brought to the collection centers. Although we did not

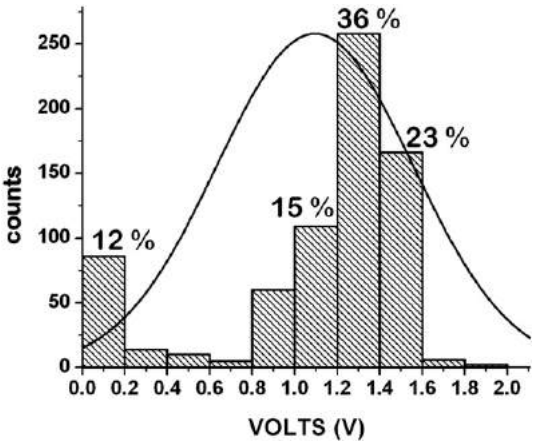

Fig. 2. Distribution of voltages frequency for Duracell type "AA" batteries collected.

Table 1
Metal composition (%) for the electrolyte paste/graphite mixture in batteries.[a]

Trademark	Zn	Mn	Hg	Cd	Pb
Formal market batteries					
Duracell	27.79 ± 4.46	22.61 ± 0.53	0.58 ± 0.03	0.17 ± 0.07	0.44 ± 0.12
Eveready	21.75 ± 0.20	12.52 ± 0.15	0.31 ± 0.01	0.30 ± 0.02	0.10 ± 0.01
Kodak	16.46 ± 8.11	38.30 ± 3.00	0.84 ± 0.01	0.46 ± 0.12	0.27 ± 0.06
Heavy duty	26.06 ± 19.04	36.82 ± 1.06	0.88 ± 0.02	0.11 ± 0.06	0.52 ± 0.07
Panasonic	17.60 ± 0.65	26.80 ± 0.07	0.65 ± 0.02	0.68 ± 0.31	0.42 ± 0.07
Informal market batteries					
Power cell	21.32 ± 5.52	25.09 ± 0.83	0.62 ± 0.03	0.32 ± 0.07	0.66 ± 0.30
Glip 2000	17.24 ± 2.82	27.34 ± 0.67	0.64 ± 0.02	0.46 ± 0.34	0.21 ± 0.01
Tectron	13.01 ± 0.11	21.70 ± 0.97	0.51 ± 0.01	0.32 ± 0.14	0.11 ± 0.01
Rocket	21.12 ± 3.32	25.05 ± 0.89	0.60 ± 0.03	0.47 ± 0.071	0.50 ± 0.09
ANOVA analysis for formal and informal market groups					
f	0.29	0.22	0.25	0.01	0.01
p	0.60	0.64	0.62	0.92	0.90

[a] Every entry represents the arithmetic media of nine determinations and is presented with ±1 standard deviation.

evaluate how this might reflect the actual proportions of various batteries that were purchased, we have no reason to believe that the collected batteries over-represent the proportion of informal market batteries or that differences between our sampling and the actual purchased batteries would significantly alter the conclusions of this study.

2.2. Analysis of metal content

90 spent size "AA" batteries (alkaline and Zn/C) were selected at random from nine brands: five formal and four informal market type. The batteries were manually dismantled, and the electrolyte paste and the graphite electrode were recovered and quantified and then ground by hand with a mortar and sieved using a 500 μm standard sieve. From the electrolyte/graphite dust mixture, 10 mg was digested in 10 mL of concentrated nitric acid (Fluka TraceSelect for trace analysis) using a PTFE Parr microwave acid digestion bomb 4782 (45 mL) and digested according to the USEPA Method 3052 (USEPA, 1995a). The solutions were analyzed for Zn, Mn, Hg, Cd, and Pb content according to the USEPA Method 6010B (USEPA, 1995b), using a Perkin–Elmer ICP-OES, model Optima 2000DV equipped with hydride generator. Conditions for experiments: axial plasma, 1500 W forward power, 15 L/min argon coolant flow, 1.5 L/min argon nebulizer flow, 1.8 mL/min sample pumping rate with a 1 min preflush time. The analyses were performed in five replicates of the same digest. The water used in the solutions was reagent grade from a Millipore Simplicity/Sim-Pak water purification device.

3. Results and discussion

3.1. Battery collection campaign statistics

A total of 15,752 spent batteries, almost 1.5 tons, were collected. Type "AA" had the highest incidence of consumption and informal market batteries almost reached 30% of the total "AA" type (Fig. 1). Trademarks of greater incidence were Duracell (18%), Tectron (informal) (15.9%), Sony (14.2%), and Panasonic (12.4%).

The residual voltage of 700 batteries type "AA" selected at random from the four highest incidence trademarks was determined. In Fig. 2, the distribution of residual voltage in the collected Duracell "AA" batteries is shown. It was observed that 36% of the batteries had a residual voltage in the range 1.2–1.4 V, whereas 70% of the batteries had voltages greater than 1.0 V. Findings were similar in the other three brands (Supplementary material).

It is to note that residual voltage did not follow a normal frequency distribution. Nowadays, batteries' energy is used in electronic devices of high current demand in most cases. Discharge reaction takes place in the outer zones of the electrode, where mass transport is fastest. With an increasing demand, the electrode reactions take place within the electrode structure, leading to diffusion overpotential, poorer electron transfer kinetics and to concentration polarization (Hamann et al., 2007). Batteries cannot supply sufficient power after a while and the user likely discard them. An estimate of 420 million type AA batteries would be commercialized in Mexico every year (NMX-AA-104-SCFI-2006), 189 million of them would contain sufficient residual energy available for other applications, projecting our statistical data. Recovery of residual energy from SCB have not been taken into account until now (Bernardes et al., 2004; Ferella et al., 2008) and could be economically attractive.

3.2. Analysis of metal content

The Zn, Cd, Hg, Mn, and Pb composition of the electrolyte/graphite dust mixture of the nine battery trademarks analyzed is shown in Table 1. An ANOVA analysis for formal and informal market group batteries shown there is no difference between the two groups at 95% significance level. Therefore, there is no support for the argument that pollution comes only from informal market batteries.

Legal market batteries normally have a robust housing seal, generally of steel, which reduces the probability of internal material release for more than 10 years. The informal market batteries are less robustly sealed – cardboard covered with plastic and their components can be released more easily in a shorter time frame. Some studies in countries with inadequate standards indicates that direct disposal of spent household batteries into MSW landfills increase the heavy metal contents in the landfill leachate (Panero et al., 1995; Puetpaiboon et al., 2001; Sohn et al., 2002; Selvapathy and Madhavan, 2003; Karnchanawong and Limpiteeprakan, 2009). Hence, the battery wastes have to be considered as hazardous wastes and should not be mixed with the municipal solid waste, a prevision observed in the EU Battery Directive Extended Impact Assessment (2003) (Kierkegaard, 2007).

4. Conclusions

Based on electrolyte paste/graphite content, under the proposed Mexican NOM regulations, all nine battery trademarks analyzed meet the permissible limit levels and would be considered as

having no dangerous residues. However, eight of nine of the battery trademarks would not meet the EU directive 2006/66/EC standard, which for the reasons described previously, better controls human health and environmental contamination risks.

These results stress the urgent need to review the proposed Mexican NOM to prevent the disposal of formal and informal market SCB, stimulate R&D in recycling technology, require that manufacturers and commercial dealers participate in the collection and recycling of SCB, and establish goals for collection, recycling, and to reduce the levels of toxic metals in batteries.

Acknowledgements

To Dr. Timothy Landry for his technical advice and valuable discussion. To Ing. Ramiro de la Cruz, head of Tlaxcala Attorney's Office of Protection to the Environment (PROFEPA), for his valuable collaboration. VMC acknowledge the fellowship from CONACYT to M.Sc. studies, Reg. No. 227771.

Appendix. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.jenvman.2010.09.019.

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Journal of Environmental Management 95 (2012) S158–S164

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Influence of plants on microbial activity in a vertical-downflow wetland system treating waste activated sludge with high organic matter concentrations

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ARTICLE INFO

Article history:
Received 29 September 2009
Received in revised form
26 February 2011
Accepted 15 March 2011
Available online 21 April 2011

Keywords:
Constructed wetlands
Macrophytes
Organic pollution
Phosphatase activity
Phytopurification
Plant growth stages
Waste activated sludge
Soil respiration

ABSTRACT

The rhizosphere is a key zone for pollutant removal in treatment wetlands; therefore, studies on microbial activity may provide helpful information for a better understanding of purification processes. We studied microbial activity in a vertical-downflow constructed wetland system treating waste activated sludge with high organic matter concentrations, under Mediterranean climate. The aims of the work were to study the influence of (i) the presence of plants, (ii) the plant species (*Phragmites australis* Cav., *Typha latifolia* L., *Iris pseudacorus* L.), and (iii) the plant growth stage (plant senescence and plant fast growing stage) on total respiration rate and phosphatase activity in the substrate (intented here as the solid support on which the plants grow). The presence of plants had a positive influence on microbial activity, since substrate respiration and both acid and alkaline phosphatase activity were always higher in planted than in unplanted mesocosms. Among the three tested species, *Phragmites* was the one that most stimulated both substrate respiration rate and phosphatase activity, followed by *Typha* and *Iris*. These differences of microbial activity between mesocosms were corresponding to differences of removal efficiency. Substrate respiration and phosphatase activity were of similar magnitude at the two growth stages, while the stimulating effect of plants seemed to have been delayed and microbial activity showed higher fluctuations at plant fast growing stage than at plant senescence.

1. Introduction

Constructed wetlands are phytopurification technologies based on natural processes, involving vegetation, soils, and associated microbial assemblages. The rhizosphere is the most active reaction zone where physical, chemical and biological processes of pollutant removal take place. Microorganisms play a key role in the mineralization and transformation of organic matter and nutrients of the wastewater (Stottmeister et al., 2003), and plants have a positive influence for several reasons, mainly via oxygen (Gagnon et al., 2007) and organic compound (Tong and Sikora, 1995) exudates by roots.

These positive effects of plants on the microbial community could explain the higher removal efficiency of planted wetland systems compared to unplanted, and differences between plant species such as we found (Wang et al., 2009). In addition, we showed that the presence of plants stimulated humification process and that some plants species improved organic matter degradation in treatment wetland system (Wang et al., 2010). Larue et al. (2010) found different root peroxidase concentrations in wetlands planted with different plant species. We suppose that these effects of plants and plant species in the rhizosphere may be closely related to soil microbial activity. Therefore, studies on microbial activity may provide helpful information for a better understanding of purification processes in phytopurification systems such as treatment wetlands.

Soil respiration and enzyme activity are indicators for microbial activity. They are positively correlated with soil organic matter content, and often with microbial biomass (Alef, 1995). Soil respiration is one of the most frequently used indicators and measures of overall microbial activity, while enzyme activity measures an activity on the metabolism of one element, such as phosphatases, which are the main enzymes for organic phosphorus mineralization in the soil. Phosphatases hydrolyse organic P compounds and transform them into inorganic forms, which are thus made available to plants. Plant roots constitute an important source of acid phosphatases (APH) in soils, but are devoid of alkaline

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doi:10.1016/j.jenvman.2011.03.021

phosphatases (BPH) that are ascribed to soil bacteria and fungi. In the present study alkaline phosphatase may provide an invaluable indication of microbial activity in treatment wetland systems, while both types of phosphatases play a role in the decomposition of organic P compounds in treatment wetlands.

As part of a mesocosm experiment, we studied microbial activity in the substrate of a vertical-downflow constructed wetland system treating waste activated sludge with high organic matter concentrations, under Mediterranean climate (substrate is intented here as the solid support on which the plants grow). The aims were to study the influence of the following three factors on respiration rate of a plant growth substrate and on phosphatase activity: (i) the presence of plants by comparing with unplanted wetlands, (ii) the plant species (*Phragmites australis* Cav., *Typha latifolia* L., *Iris pseudacorus* L.), and (iii) the plant growth stages (fast growing stage and senescence which is the process of becoming old and showing the effects of being old).

2. Materials and methods

2.1. Experimental design

The experiment was set up in the vicinity of Marseille, South-East France. The site has a typical Mediterranean climate, characterised by a hot dry summer and low annual rainfall mainly occurring during autumn and spring.

Outdoor mesocosms (Fig. 1) were set up to simulate mono-specific vertical-downflow wetlands (mesocosms were designed to conduct more extensive experiments than can be accomplished using laboratory based systems or microcosms, but smaller than most pilot studies). Polypropylene tanks (1 m^3: 95 × 115 × 100 cm) were filled from bottom to top with 15 cm of large cobblestones (diameter: 50–150 mm), 20 cm of small cobblestones (diameter: 4–16 mm), and 20 cm of an organic substrate used as plant growth medium (mixture of peat and crushed pine bark: 1/1; Table 1). Young macrophyte plants of common reed (*Phragmites australis* Cav.), broadleaf cattail (*Typha latifolia* L.) and yellow flag (*Iris pseudacorus* L.) were planted as mono-species in spring 2006 (6 plants/m^2) with three replicates. Two unplanted mesocosms were also set up; however, one more sample was collected from the unplanted mesocosms during substrate samplings for microbial analyses, which allowed the same replicate samples ($n = 3$) for the unplanted modality for comparison with the planted ones.

All plants were cut down at 20 cm height from the substrate in the autumn 2006, and before the first application of waste activated sludge. The above ground parts were allowed to re-grow every spring and to dieback in autumn afterward.

All mesocosms regularly received waste activated sludge from the wastewater treatment plant of a local food processing factory producing soft drinks from fruit concentrates (Table 1). Volume and frequency of irrigation with this waste activated sludge varied according to climatic conditions and water requirement of plants: 0.2 m^3 per mesocosm (1 m^2) every two months in winter; 0.15 m^3 every month in spring and autumn; 0.15 m^3 every half month in summer. This sludge was first supplied in January 2007 (0.2 m^3 per mesocosm); from January 2007 to June 2008, a total of eighteen irrigations (2.85 m^3) were carried out. At all irrigations, sludge was applied in one go, the mesocosms therefore received 0.15 or 0.2 m^3 in one go and in approximately 15 min. Water outflow was hand-controlled using a tap at the bottom of mesocosms.

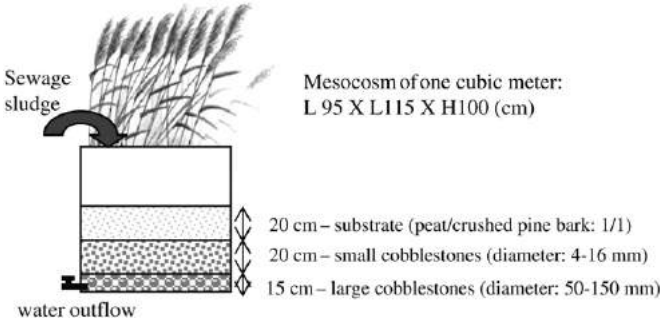

Fig. 1. Set up of mesocosms simulating vertical-downflow wetland system.

Table 1
Characteristics of initial substrate and the waste activated sludge (mean ± Standard Error, $n = 20$).

	Substrate[a]	Sludge[b]
pH	6.5 ± 0.06	7.3 ± 0.04
Temperature	–	4–7 °C
Total suspended solids (TSS)	–	7360 ± 460 mg/L
Chemical oxygen demand (COD)	–	6055 ± 785 mg/L
Biological oxygen demand in 5 days (BOD_5)	–	1910 ± 464 mg/L
Total organic carbon (TOC)	354 ± 7 mg/g	2640 ± 184 mg/L
Total Kjeldahl nitrogen (TKN)	5.4 ± 0.1 mg/g	211 ± 24 mg/L
C/N	65.9 ± 1.7	19.3 ± 2.2
Total phosphorus (TP)	0.32 ± 0.007 mg/g	37 ± 3 mg/L
C/P	1130 ± 28	69.4 ± 4.0
P-Olsen (available P)	0.014 ± 0.001 mg/g	–

[a] The substrate corresponds to the 20 cm at the top of the mesocosms (mixture of peat and crushed pine bark).
[b] The sludge contained neither heavy metals nor organic persistent pollutants, nor at ultra-trace levels.

2.2. Plant growth substrate characterization

Substrate was sampled for chemical characterization before the microbial studies (5 Nov. 07 and 20 May 08): five randomized cores of substrate were collected and thoroughly mixed for one representative sample per mesocosm. Samples were air dried and then sieved (2 mm mesh) before analyses.

Total Kjeldahl nitrogen (TKN) was determined by ammonium measurement with an auto-distillation analyzer (2300 Kjeltel Analyzer Unit Tecator) after acid (H_2SO_4) digestion (ISO-11261). Total organic carbon (TOC) was measured according to the ANNE method (Aubert, 1978) normalized (ISO-14235). Available phosphorus (PO_4^{3-}) was extracted with Olsen's reagent ($NaHCO_3$ 0.5 M pH 8.5) at a ratio of 1/20 (m/v), then determined by an Ionic Chromatograph (Dionex DX-120) (ISO 11263). Normalized methods are described in AFNOR (AFNOR, 1999a; AFNOR, 1999b).

2.3. Below ground biomass of plants

Below ground biomass was collected over the whole depth range (including in cobblestones) of three mesocosms (one for each species) in June 2008, at the end of the experiment. At the same time, maximum depth of roots was measured. Sampling was conducted in a 20 × 20 cm quadrat. Roots and rhizomes were cleaned under tap water, and then dried at 70 °C until constant weight.

2.4. Microbial activity

Two sampling campaigns were done at two plant growth stages: the first from 5 to 19 Nov. 2007, at plant senescence; the second from 20 May to 3 June 2008, at fast re-growing stage. Daily air mean temperature and rainfall data of these two periods are shown in Fig. 2. Surface layer substrate temperature was always measured before sampling, in the morning between 8 h 30 min and 9 h.

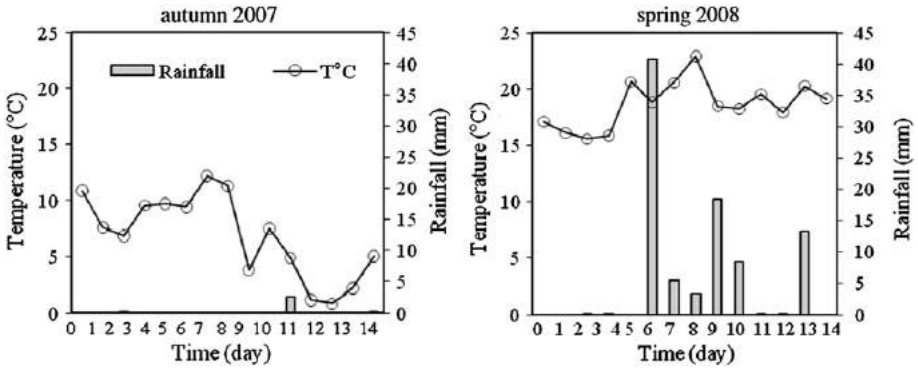

Fig. 2. Air mean temperature and rainfall during the two sampling campaigns: from 5 to 19 Nov. 2007 (left) and from 20 May to 3 June 2008 (right).

Substrate samplings were performed just before the sludge irrigation (Day 0) and 7 times in the two following weeks. Two substrate samples per mesocosm were collected at the surface layer (0–4 cm), where the accumulation of organic matter and biomass and productivity of bacteria were the highest as cited by Tietz et al. (2008). Samples were stored at 4 °C until microbial analyses.

Substrate respiration was determined by measuring O_2 consumption rate with an Oxitop® (WTW) equipment. One fresh sample per mesocosm (~20 g) was mixed with ceramic Raschig rings in an airtight vessel of 1 L. Ceramic Raschig rings increased substrate contact surface for aeration. A beaker with 40 ml of 0.5 M NaOH was placed in the vessel to trap initial CO_2 in air and the one produced by substrate respiration. Vessel without substrate sample was set as control. Incubation lasted 24 h at 20 °C. Oxitop® Control system continuously measured air pressure in the vessel. Oxygen consumption can be calculated according to air pressure changes. Results were expressed in mg of O_2 consumed by 1 g of dry organic substrate in 1 h (mg O_2 h^{-1} g^{-1}DM).

Phosphatase activity (alkaline BPH and acid APH) was measured by a direct procedure using *p*-nitrophenol phosphate (Eivazi and Tabatabai, 1977). Two fresh substrate samples per mesocosm (1 g) were incubated in 5 ml of buffer with 10 mM *p*-nitrophenol phosphate at 37 °C for 1 h. NaOH–glycine buffer 0.1 M, pH 9.0 was used for BPH and acetate buffer 0.1 M, pH 5.0 for APH. Then, 1 ml of $CaCl_2$ 0.5 M and 4 ml of NaOH 0.5 M were added to stop enzymatic activity. The absorbance of supernatant was measured at 405 nm using a spectrophotometer (BioMate 3/THERMO). Results were expressed in units defined as μmoles of *p*-nitrophenol released in 1 min (U) by 1 g of dry organic substrate (U g^{-1}DM).

2.5. Statistical analyses

Kruskal–Wallis tests (StatView 5.0) were performed for differences between treatments. This one-way analysis of variance by ranks is a non-parametric method for testing equality of population medians among groups. When significant differences were found, a Student–Newman–Keuls test was performed as a posteriori pairwise comparison. Mann–Whitney tests were performed to assess differences between two stages intra-treatment. Significance was defined by $p < 0.05$.

3. Results and discussion

3.1. Plant growth substrate characteristics

Substrate chemical characteristics were similar in all mesocosms, and no differences were found between treatments at each date (Kruskal–Wallis, $0.1 < p < 0.9$; Table 2). The substrate chemical characteristics will not therefore be a direct factor causing differences of microbial parameters between treatments for each stage. Comparison between the two dates shows higher available P-Olsen on 5 Nov. 2007 than on 20 May 2008 (Mann–Whitney, $p = 0.03$), that may be the result of higher uptake by plants in their fast growing stage than at their senescence.

Substrate surface layer temperature was in close proximity to air temperature (±1 °C) and always similar for all mesocosms at each date (variation < 0.5 °C) over the two observation periods. However, in the first period (November 2007) substrate surface temperatures were lower, between 5 and 10 °C, as compared to the second period (May–June 2008) with temperatures between 15 and 20 °C.

3.2. Microbial activity

Substrate respiration rates, in this experiment, varied between 0.2 and 1.4 mg O_2 h^{-1} g^{-1}DM (Fig. 3), and phosphatase activity (both BPH and APH) between 0.2 and 1.8 U g^{-1}DM (Fig. 4). These values are similar to values found in soils rich in organic matter (KizIlkaya and BayraklI, 2005; Danon et al., 2007). All the three tested factors, the presence of plants, plant species and plant growth stages, showed an influence on microbial activity (Kruskal–Wallis, $p < 0.05$). Among them the presence of plants was the most significant.

3.2.1. Influence of the presence of plants

The presence of plants had a positive influence on microbial activity in this treatment system, whatever the plant species and whatever their growth stages. Indeed in both stages, planted mesocosms had over 2-fold higher respiration rates (Kruskal–Wallis, $0.01 < p < 0.05$; Fig. 3) and 2–8-fold higher phosphatase (both BPH and APH) activity, than unplanted mesocosms (Kruskal–Wallis, $0.0005 < p < 0.05$; Fig. 4). Furthermore, just after

Table 2
Chemical characteristics of substrate (mean ± Standard Error, $n = 3$) in different mesocosms before each campaign of microbial activity measurements (5 November 2007 and 20 May 2008).

	Phragmites	Typha	Iris	unplanted
5 Nov. 2007				
TOC mg/g	380 ± 19	386 ± 3	395 ± 15	375 ± 3
TKN mg/g	11.5 ± 0.3	10.9 ± 0.8	12.0 ± 0.8	12.1 ± 1.7
C/N	33.1 ± 1.0	35.9 ± 2.6	33.1 ± 1.2	31.6 ± 4.7
P-Olsen mg/g	0.111 ± 0.008	0.118 ± 0.018	0.101 ± 0.009	0.136 ± 0.015
20 May 2008				
TOC mg/g	368 ± 30	382 ± 26	375 ± 12	363 ± 3
TKN mg/g	12.9 ± 2.6	10.3 ± 1.0	13.7 ± 1.0	12.9 ± 0.3
C/N	29.7 ± 3.1	37.7 ± 3.7	27.6 ± 2.2	28.1 ± 0.5
P-Olsen mg/g	0.080 ± 0.016	0.075 ± 0.011	0.078 ± 0.020	0.097 ± 0.016

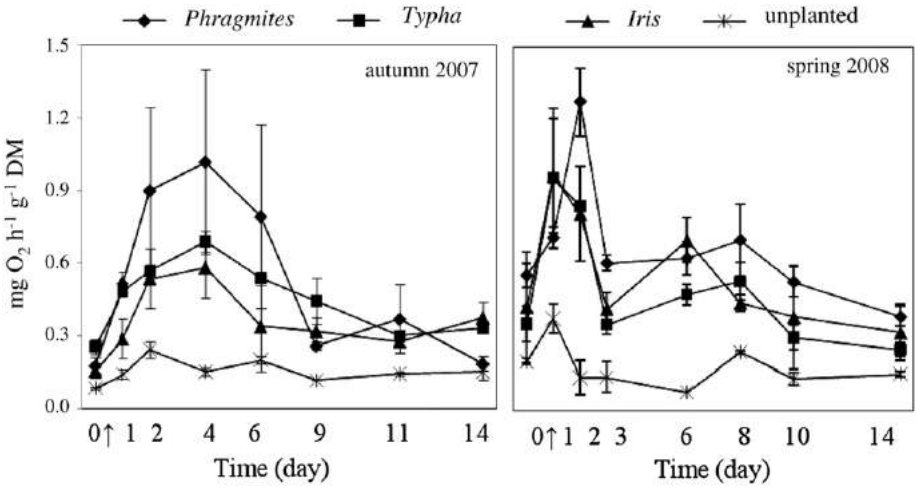

Fig. 3. Substrate respiration (mean ± Standard Error, $n = 3$) in different mesocosms at plant senescence, from 5 to 19 Nov. 2007 (left) and at fast growing stage, from 20 May to 3 June 2008 (right). Waste activated sludge was applied just after the sampling at day 0 (arrow).

sludge irrigation, microbial activity increased in planted mesocosms and reached peak values within 3–10 days, while it remained almost stable in unplanted mesocosms (Figs. 3 and 4). This confirmed the beneficial effects of plants on microbial processes in treatment wetlands (Brix, 1997; Münch et al., 2005). Two main mechanisms could explain the results of the present study. First, as roots present a large surface area for microbial attachment, this could have favored microbial development (Kyambadde et al., 2004). Secondly, roots can stimulate microbial activity through root releases (Sturz and Christie, 2003). Wetlands

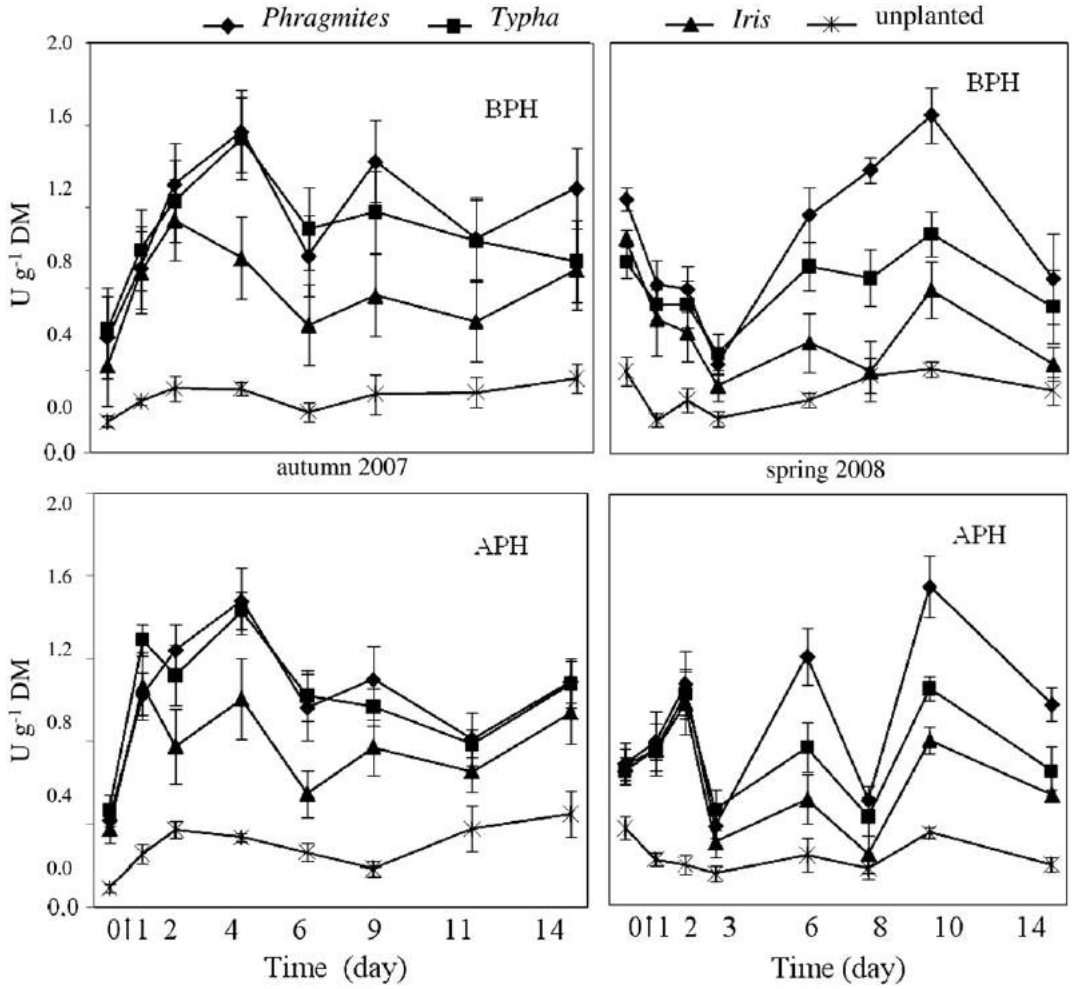

Fig. 4. Alkaline phosphatase (BPH) and acid phosphatase (APH) activity (mean ± Standard Error, $n = 6$) in different mesocosms at plant senescence, from 5 to 19 Nov. 2007 (left) and at fast growing stage, from 20 May to 3 June 2008 (right). Waste activated sludge was applied just after the sampling at day 0 (arrow).

plants translocate oxygen from leaves to roots, release part into the rhizosphere (Wießner et al., 2002a) and affect the redox status (Jespersen et al., 1998; Nikolausz et al., 2008), in agreement with the higher rate of microbial respiration in planted mesocosms in the present study. Besides oxygen, roots release a wide range of organic compounds (Rovira, 1965, 1969; Barber and Martin, 1976; Grayston et al., 1997) corresponding to 5–25% of the photosynthetically fixed carbon (Brix, 1997) that are under the form of enzymes, organic acids and amino acids. This carbon-based compounds released, that can reach up to 10–100 mg soluble compounds/g roots of soluble compounds (Grayston et al., 1997), could have enhanced enzyme activity in planted mesocosms. However, in unplanted mesocosms, microbial activity was relatively stable and did not increase with extra organic matter input; this suggests that microbial activity could be saturated without plants.

Differences of microbial activity had different impacts on changes in substrate characteristics and on treatment efficiency between mesocosms. The higher microbial activity in planted mesocosms increased the rate of organic matter degradation that stimulated the humification process (Wang et al., 2010) and as a consequence improved the treatment performance. Indeed, after the first months of stabilisation, COD concentrations of outflow water from unplanted mesocosms were always the highest, around 700 mg/L and often above 1000 mg/L with peak values at 1400 mg/L, while for outflow water from planted mesocosms peak values reached only 500, 900, and 1200 mg/L, respectively for *Phragmites*, *Typha*, and *Iris* (Wang et al., 2010). On the whole, COD removal efficiency was the highest in the mesocosms planted with *Phragmites* ($>$ 94%), followed by those planted with *Typha* ($>$ 85%), and then those planted with *Iris* ($>$ 76%); all planted units showed higher COD removal efficiency than unplanted mesocosms (removal efficiency $>$ 67%) (input–output differences in COD loads over a period of 8 months).

Some authors have reported no significant effects of the presence of plants on soil bacterial biomass and productivity (Tietz et al., 2008) or microbial communities (Ahn et al., 2007) in wetland treatment systems. Indoor experiments, small experimental systems ($\sim$10 L), short waste application periods (one month), or slow plant growth as well as low plant biomass may be the main explanations for their results in contradiction with ours. Moreover, we evaluated microbial activity and changes in this activity are not always related to an effect on the microbial communities.

3.2.2. Influence of plant species

All plant species stimulated microbial activity in this treatment system: overall *Phragmites* was the one that most stimulated both substrate respiration rate and phosphatase activity, followed by *Typha* and *Iris* (Kruskal–Wallis, $p < 0.05$). Differences were due to plant species' particular morphology (Gagnon et al., 2007), which influences many physiological functions of plants (such as release in the rhizosphere of oxygen, carbon-based components) and in return influenced enzyme types and microbial activity in soils (Renella et al., 2007).

On the one hand, as it has previously been observed (Vymazal and Kropfelova, 2005; Ennabili et al., 1998), *Phragmites* has a dense rooted system. Indeed, we observed for this species the highest below ground biomass compared to the other two species, with 6200, 3500 and 1600 g per mesocosm ($= g/m^2$) for *Phragmites*, *Typha* and *Iris*, respectively. This result is in accordance with findings of Tong and Sikora (1995) in similar system or Lenssen et al. (1999) in natural conditions. Moreover, root systems are more shallow for *Typha* and *Iris* in our mesoscosms, with maximum depth of roots of 65, 50 and 40 cm for *Phragmites*, *Typha* and *Iris* respectively. Such result has been already obtained by Gersberg et al. (1986) and explains the higher removal efficiency with *Phragmites* compared to other species.

On the other hand, *Phragmites* had the highest above ground biomass, followed by *Typha* and then by *Iris* in the present experiment (Wang et al., 2009); this may suggest greater oxygen release by *Phragmites*, as it has been reported that above ground biomass is positively correlated to root oxygen release (Wießner et al., 2002b).

3.2.3. Influence of plant growth stages

Substrate respiration and phosphatase activity each had a magnitude at plant senescence (from 5 to 19 Nov. 2007) similar to that at plant fast growing stage (from 20 May to 3 June 2008) (Figs. 3 and 4). Some studies found microbial biomass to remain stable over time (Rogers and Tate, 2001), whereas many others reported the highest microbial activity during the fast growing stage of plants (Bardgett et al., 1999; Myers et al., 2001). The different patterns observed in microbial activity should be related to the interactions between plant growth, climatic conditions, and substrate properties (Zak et al., 1994).

At plant senescence (from 5 to 19 Nov. 2007), all the microbial parameters showed roughly similar dynamics in all planted mesocosms, especially for respiration activity, i.e. an increase of activity just after sludge irrigation up to a peak reached on the fourth day before a progressive decrease down to a level slightly higher than the initial one (Figs. 3 and 4). This similarity suggests little specific influence of plant species on the dynamic of microbial activity at plant senescence. The positive plant effect on microbial activity at this stage is mostly due to the presence of a root system in comparison with the unplanted mesocosms.

In plant fast growing stage (from 20 may to 3 June 2008), the dynamic was different for the three microbial parameters. Substrate respiration increased after sludge irrigation and rapidly reached its peak value (Mann–Whitney test between day 0 and day 1, $p = 0.0023$ for planted mesocosms and $p = 0.52$ for unplanted mesocosms); then it decreased to nearly the initial level on the third day (between day 1 and day 3, $p = 0.0013$ for planted mesocosms and $p = 0.302$ for unplanted mesocosms) and remained stable (Fig. 3). The increase was faster (1 day) and decreased more rapidly than at the plant senescence. This may be due to the lower content of easily biodegradable organic matter in the waste activated sludge in May 2008 than in November 2007, since the rapid increase of oxygen consumption results from easily biodegradable organic matter (Tusseau-Vuillemin et al., 2003). This is in accordance with results on the patterns of change of humic substances of the substrate in mesocosms (Wang et al., 2010), with an increase of the total humic substance concentrations over time in planted mesocosms (Wang et al., 2010), probably through a transformation of organic matter in the form of molecules that are not easily biodegradable. The rapid increase of substrate respiration may also be related to the faster growth of plants and to the higher temperature of the substrate surface (15–20 °C) in summer 2008 compared to autumn 2007 (5–10 °C). This argument is in accordance with a field study (Imberger and Chiu, 2002), which found soil respiration in the topsoil to be significantly higher in summer than in winter. In this respect, soil temperature represents another factor affecting soil microbial activity (Scott-Denton et al., 2003) and especially microbes' speed of reaction.

Overall, in the faster growing stage of plants, both BPH and APH showed more fluctuations as compared to those at plant senescence. BPH activity decreased during three days after sludge irrigation, and then it increased progressively until the peak value on the tenth day (Kruskal–Wallis test between dates, $p = 0.0001$, 0.0495, 0.013 and 0.010 for *Phragmites*, *Typha*, *Iris,* and unplanted respectively) (Fig. 4). APH activity also showed strong fluctuation

over time (Kruskal–Wallis test between dates, $p = 0.0001$, 0.0021, 0.0007 and 0.03 for *Phragmites*, *Typha*, *Iris*, and unplanted respectively): slight increases and decreases alternately appeared; the lowest value was on the third day and the peak value on the tenth day (Fig. 4). Faster growth of plants probably caused these fluctuations of microbial activity through their higher influence on soil properties (Waldrop and Firestone, 2006). Moreover, the relatively lower temperature of the sludge (4–7 °C; Table 1) compared to the environmental temperature in this period (> 15 °C; Fig. 2) may be the critical factor influencing phosphatase activity in the three first days, since BPH activity is positively correlated with the temperature, while APH activity is negatively correlated with it (Criquet et al., 2004).

4. Conclusion

This study revealed that the presence of plants had a positive influence on microbial activity in a vertical-downflow wetland treating waste activated sludge with high organic matter concentrations. Substrate respiration and both acid and alkaline phosphatase activity were always significantly higher in planted than in unplanted mesocosms. The beneficial effects of plants on microbial activity may contribute to the high removal efficiency of organic matter and nutrients found in wetland treatment systems and so to better quality of water outflow.

In addition, the indicators used distinguished *Phragmites* as significantly more effective than the other two plant species, *Typha* and *Iris*, which may be attributed to the plant's development of below and above ground parts and to function specificity.

Results showed that the main factor influencing the microbial activity in the wetland system was the presence of plants. The plant species was the second factor, and the plant development stages the third. As microbial activity is a key process in treatment of wastewater and sludge, the results of the present study provide some explanation of differences observed between these types of treatment systems.

Acknowledgments

This study was financially supported by the Conseil Régional Provence Alpes Côte d'Azur, Phytorestore®, and Oseo anvar. We thank S. Dupouyet, C. Lecareux, S. Greff, and C. Rolando for their technical and logistical assistance, and Mr. Brahic and all the staff from the Pépinière Départementale DDAF (Bouches-du-Rhône) for looking after the mesocosms. Special thanks are due to Professor P. Francour of the University of Nice for his statistical software support (Student–-Newman–Keuls), and to Mr. Michael Paul for his improvement of the English.

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Journal of Environmental Management 95 (2012) S165–S169

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Nitrogen potential recovery and concentration of ammonia from swine manure using electrodialysis coupled with air stripping

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ARTICLE INFO

Article history:
Received 21 August 2009
Received in revised form
10 May 2011
Accepted 20 May 2011
Available online 11 June 2011

Keywords:
Electrodialysis
Swine manure
Ammonia
Stripping

ABSTRACT

The practice of intensive animal production in certain areas has resulted in excessive manure production for the available regional land base. Consequently, there is a need to develop treatment technologies to recover the valuable nutrients that manure contains so that the resulting product can be transported and used as fertilizer on agricultural land. The project presented here used electrodialysis in a dilution/concentration configuration to transfer the manure ammonia in the diluate solution by electromigration to an adjacent solution separated by an ion-exchange membrane under the driving force of an electrical potential. Then, air stripping from the electrodialysis-obtained concentrate solution without pH modification was used to isolate the ammonia in an acidic solution. An optimal process operating voltage of 17.5 V was first determined on the basis of current efficiency and total energy consumption. During the process, the swine manure pH varied from 8.5 to 8.2, values favourable for NH_4^+ electromigration. Total ammonia nitrogen reached 21 352 mg/L in the concentrate solution, representing approximately seven times the concentration in the swine manure. Further increases in concentration were limited by water transfer from the diluate solution due to electroosmosis and osmosis. Applying vacuum to the concentrate reservoir was found to be more efficient than direct concentrate solution aeration for NH_3 recuperation in the acid trap, given that the ammonia recuperated under vacuum represented 14.5% of the theoretical value of the NH_3 present in the concentrate solution as compared to 6.2% for aeration. However, an excessively low concentrate solution pH (8.6–8.3) limited NH_3volatilization toward the acid trap. These results suggest that the concentrate solution pH needs to be raised to promote the volatile NH_3 form of total ammonia nitrogen.

1. Introduction

The practice of intensive animal production in certain areas has resulted in excessive manure production for the available regional land base. Manure, which is often more than 90% water, sometimes has to be transported over long distances at great cost. Moreover, feed producers are often reluctant to apply manure on their land because of low and poorly balanced nutrient concentrations. Environmental problems have also given a bad reputation to manure, which should be considered a valuable fertilizer source that is part of sustainable agriculture, not disposable waste. There is a need to develop treatment technologies that will help manage the large amount of manure generated and recover the valuable nutrients that manure contains.

Diverse approaches based on the physicochemical properties of manure have been investigated to isolate and concentrate the ammonia contained in swine manure. Liehr et al. (2006) carried out ammonia stripping with the liquid fraction from an in-house belt separation system for swine manure. Using direct aeration and tower air stripping, Liao et al. (1995) investigated the influence of total solids concentration, manure pH, air-to-liquid flow ratio, and temperature on methods for removing ammonia from swine wastewaters. Arogo et al. (1999) correlated the ammonia mass transfer coefficient with environmental conditions, such as air temperature, air velocity, and relative humidity, and with liquid properties, such as liquid temperature and total solids concentration. Bonmati and Flotats (2003) studied the effect on ammonia air stripping of pig slurry type and initial manure pH in pre- or post-treatment for mesophilic anaerobic digestion. Bonmati et al. (2003) studied the quality of the condensate obtained as a function of slurry type and initial pH.

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doi:10.1016/j.jenvman.2011.05.026

Mondor et al. (2008, 2009) studied electrodialysis (ED), which is a membrane separation process in which ions in solution are transferred to an adjacent solution through a perm-selective membrane owing to an externally applied electrical potential gradient, in a dilution/concentration configuration to isolate and concentrate ammonia from swine manure (Fig. 1). A maximum total ammonia nitrogen (TAN) concentration of 14.25 g/L was obtained in the concentrate solution, and those authors projected that a maximum of approximately 16 g/L could be reached. Electroosmosis, osmosis, and volatilization from the open reservoir were identified as problems associated with ED. Electrodialysis was also investigated by Fukumoto and Haga (2004) for swine wastewater treatment. Those authors were able to recuperate and concentrate ammonia as nitrate after a nitrification process followed by ED.

The present article reports a process in which ED was used in a dilution/concentration configuration to transfer ammonia to the concentrate solution, followed by either direct aeration or vacuum application to isolate the transferred ammonia volatilizing from the concentrate solution in an acidic trap (Fig. 2). The main objective of this project was to produce a concentrated nitrogen fertilizer from liquid swine manure without pH modification by chemical addition in order to avoid the reported scaling problem in stripping towers.

2. Materials and methods

2.1. Swine manure

The raw manure was collected from the transfer storage tanks on a typical farrow-to-finish swine operation in Quebec, Canada. The manure contained 40.0 g of dry matter (DM) and 32.9 g of suspended solids (SS) per litre, amounts that are representative of manure concentrations on Canadian farms. Solid-liquid separation of the raw manure by vacuum filtration through wood flour reduced SS, phosphorus, and nitrogen concentrations by approximately 95%, 85%, and 25%, respectively. The liquid fraction represented 75% of the original volume. The liquid fraction (pH 8.5) used as a feed for the ED process contained 3200, 14 000, and 2500 mg of TAN, alkalinity, and potassium per litre, respectively. Dry matter, volatile matter, SS, and volatile SS contents were 8.76, 2.5, 1.65, and 1.04 g/L, respectively.

2.2. Electrodialysis

Electrodialysis was carried out as a batch process, using a modified Stackpack from Ionics, Watertown, Mass., USA with an MK1 stack (Ionics) in a dilution/concentration configuration (Fig. 1). The configuration consisted of 10 cell pairs using AR204SZRA anionic and CR67HMR cationic membranes (Ionics) with an active area of 220 cm^2 per membrane. Using this configuration, the main ionic compounds in the liquid manure (in the diluate cells), i.e. NH_4^+, K^+, and HCO_3^-, were transferred and concentrated in a 0.2 N KCl solution in the adjacent concentrate cells (Fig. 1). The cathode and anode were 316 stainless steel electrodes.

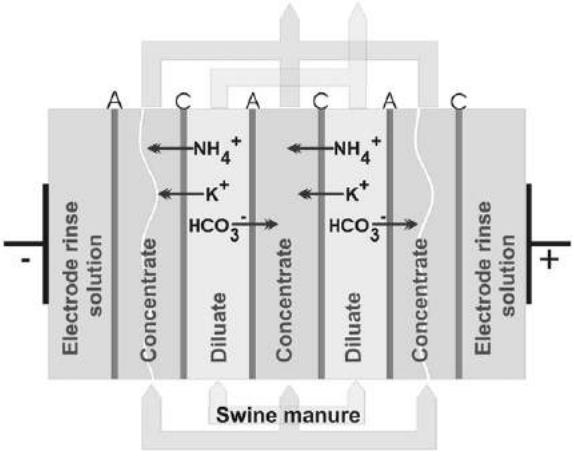

Fig. 1. Electrodialysis cell. C, cationic membrane; A, anionic membrane.

The diluate stream linear flow rate was set at 36 cm/s. The concentrate and electrode rinse solution stream flow rates were adjusted to provide pressure equal to that of the diluting stream. Each circuit was connected to a separate external reservoir under a Plexiglas cover to prevent exchange with the atmosphere, allowing for continuous recycling (Fig. 2). Voltage and current were monitored using on-line data acquisition systems. Temperature was allowed to rise and reached a maximum of 35 °C. The conductivities and pH of the diluate and concentrate solutions were monitored on-line and normalized to 25 °C. The membranes were handwashed to remove any surface deposits and stored in a 0.1 N KCl solution between replicates.

To isolate the ammonia accumulated in the concentrate solution from the other transferred ions, two approaches were used. First, air was recirculated in a closed loop (direct aeration), in which the air, after passing through the acid traps, was returned at the bottom of the concentrate reservoir in the form of fine air bubbles that passed through the concentrated solution (Fig. 2, valve in position 1). In the second case, a partial vacuum was applied to the reservoir, and air was removed from the system after it had passed through the acid traps (Fig. 2, valve in position 2). In both cases, a Thomas 1/20-HP pump (Sheboygan, Wi., USA) with a theoretical air flow rate of 46.7 LPM was used, and the air was circulated through two acid traps each containing 1 L of 2 N HNO_3.

The solution volumes were recorded at the beginning and end of each manure (diluate) batch and, for the concentrate, electrode rinse, and acid trap solutions, at the beginning and end of the replicate. Water transfer from the diluate solution was proportionally distributed between the concentrate and electrode rinse solutions after each batch, suggesting linear transfer during a batch. Samples of the diluate, concentrate, and electrode rinse solutions were collected at the beginning and end of each batch. The samples were analyzed for TAN concentration with a Kjeltec 2400 analyzer (Tecator AB, Höganäs, Sweden).

2.2.1. Determination of optimal operating voltage

The first series of experiments was aimed at determining the most efficient operating voltage, in order to prevent membrane damage, overheating, and excessive power consumption under the process conditions (Ionics, personal communication). Four consecutive manure batches of 8 L each were electrodialyzed at constant voltages of 7.5, 12.5, 15, and 17.5 V until manure conductivity was reduced by 80%. The initial volumes of the concentrate (0.2 N KCl) and electrode rinse (20-g/L Na_2SO_4) solutions were 8 L. The optimal operating voltage was selected on the basis of total energy consumption (sum of the energy used for ion transfer toward the concentrate plus pumping energy), current efficiency, and the maximum operating voltage recommended by the manufacturer. Current efficiency was defined as the ratio of the equivalents of ammonia nitrogen transferred from each manure batch to the faradays of electricity passed, using the equation $e = F^*(N_1V_1 - N_2V_2)/(It)n$, where e is current efficiency, F is Faraday's constant (1608.3 A min/equivalent), N is swine manure TAN (equivalent/L), V is swine manure solution volume (L), I is current (A), and t is time (min); subscripts 1 and 2 refer to the beginning and the end of the run, respectively; and n refers to the number of cell pairs.

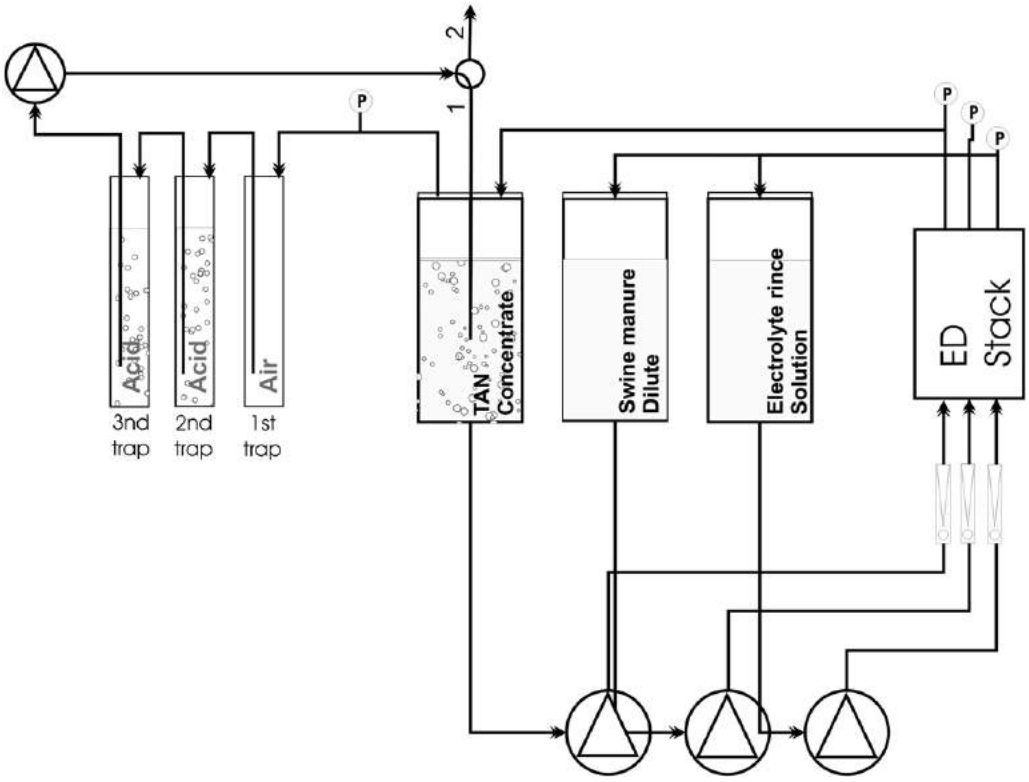

Fig. 2. Experimental set-up.

2.2.2. Determination of the maximum achievable ammonia concentration in the concentrate

The second series of experiments was aimed at determining the maximum achievable TAN concentration in the concentrate solution. The concentrate solution was produced by the ED of swine manure (17 consecutive batches of 8 L), with an initial volume of 8 L of 0.2 N KCl in the concentrate and 5 L of 20-g/L Na_2SO_4 as the electrode rinse solution. In these experiments, TAN-enriched concentrate and electrode rinse solutions were produced for use in all subsequent experiments.

2.2.3. Ammonia stripping with direct aeration or vacuum

The third series of experiments used direct aeration in a closed circuit to isolate ammonia in acid traps. The experiment was done in triplicate, with each replicate consisting of seven consecutive batches of 8.5 L of swine manure, electrodialyzed to a difference of 10 times between the conductivity of the concentrate and diluate solutions, therefore preventing loss of current efficiency due to current leaking through the manifold. The concentrate solution for each replicate had an initial volume of 5 L and an initial conductivity of approximately 110 mS/cm (corresponding to an initial TAN concentration between 17 600 and 19 100 mg/L). The electrode rinse solution for each replicate had an initial volume of 4.5 L and an initial TAN concentration between 3500 and 5700 mg/L (initial TAN concentration variations are due to the continuous recycling of the concentrate and electrode rinse solutions).

In the fourth series of experiments, the experimental conditions were the same as in the third series, with the exception that volatile ammonia was continuously extracted from the concentrate reservoir by exerting a partial vacuum (−45 kPa) in the air space above the concentrate reservoir and sequestering ammonia in the acid trap (Fig. 2, valve in position 2). The experiment was conducted in triplicate, and for each replicate, five consecutive batches of 8 L of swine manure were electrodialyzed with a linear flow rate of 25 cm/s.

2.3. Statistical analysis

For the first series of experiments, analysis of variance (ANOVA) was carried out to establish whether there was an effect of operating voltage on mean total energy and mean current efficiency, as indicated in Montgomery (1991). Duncan's multiple range test was then used to locate any difference between the voltages (Montgomery, 1991). For the third and fourth series of experiments, an ANOVA was carried out to establish whether there was an effect of batch number (with all other experimental parameters remaining constant) on the transfer energy (amperage times minutes) per milligram of transferred TAN. Duncan's multiple range test was then used to locate any difference between the batches. The *t*-test (0.05) was used to determine whether a difference in transfer energy per milligram of transferred TAN existed between the replicates of every series of experiments and between the vacuum conditions used.

3. Results and discussion

3.1. Determination of optimal operating voltage

According to Zhang et al. (1994), at an initial pH of 8.5 and a temperature of 35 °C, ammonium ions represent 96.5% of the TAN in manure. Ammonium ions are transported by the current through the cationic perm-selective membrane toward the cathode from the swine manure (diluate) to the concentrate solution, where the ions are retained by an anionic perm-selective membrane (Fig. 1). In contrast, ammonia, a neutral ion, will only diffuse in the stack. As demineralization progressed, the swine

manure pH decreased to an average value of 8.2. Transferred carbonate and ammonia from the swine manure resulted in a buffered concentrate solution with pH value between 8.6 and 8.4. These concentrate solution pH values are within the range of those reported by Mondor et al. (2008). A constant water transfer of 1.03 ± 0.10 L per batch was found at all of the tested voltages, and an average total transferred TAN from the swine manure of 84 000 ± 6500 mg was obtained for the four batches of the tested voltages. The swine manure TAN content had decreased by 87% at 80% demineralization.

An ANOVA on total energy used during each of the four batches indicated that the applied voltage had a significant effect ($P < 0.01$) on the total energy used to reach 80% manure demineralization. Duncan's multiple range test (Table 1) indicated a decrease in total energy used with an increase in voltage ($P < 0.01$), except between 15 and 17.5 V, for which no significant difference was observed. These results are associated with a decrease in pumping energy, which represents the major portion of total energy. An ANOVA on current efficiency indicated that the applied voltage had no significant effect ($P > 0.05$) on current efficiency. A decrease in current efficiency resulting from excessive applied voltage would have resulted in water dissociation at the surface of the anion-exchange membrane in the concentrate, thereby causing an increase in pH, which was not observed. According to the manufacturer's recommendations, an initial swine manure electrical conductivity of 28 Ω cm means that a maximum voltage of 19.6 V can be applied. The maximum current density used during these tests reached 13.6 and 40 mA/cm^2 for applied voltages of 7.5 and 17.5 V, respectively. These values are well under the maximum current density recommended by Ionics, which is 50 mA/cm^2 for solutions with conductivities similar to those found in these experiments. A voltage of 17.5 V was selected as the optimal operating voltage. On average, 96.5% of the TAN removed from the swine manure was recovered in the first acid trap, concentrate, and electrode rinse solutions.

3.2. Determination of the maximum achievable ammonia concentration in the concentrate

During the second series of experiments, a concentrate solution with approximately six times the original swine manure TAN concentration (18 000 mg/L) was obtained after the ED of 10 batches of swine manure. Further increases in TAN concentration were limited by water transfer, associated with osmosis and electroosmosis, from the swine manure to the concentrate solution (water transfer was found to average 0.88 ± 0.11 L per batch). Mondor et al. (2008) projected a maximum achievable TAN concentration of 16 000 mg/L in the concentrate solution. In the present study's third series of experiments, however, a final TAN concentration of 21 352 mg/L was reached in the concentrate solution, a value 33% higher than the extrapolated value for the open-to-the-atmosphere system used by Mondor et al. (2008). On average, 95% of the TAN removed from the swine manure was recovered using a closed-to-the-atmosphere system. Mondor et al. (2008) reported a TAN recuperation rate of 83% in an open system at a similar pH.

Table 1
Mean total energy used and mean current efficiency determined in the first series of experiments. Values followed by the same letter are not significantly different as determined by Duncan's multiple range tests at 95%.

Operating voltage (V)	Mean total energy (Amperage times minutes)	Mean current efficiency
7.5	175 965 ± 6694^a	0.64 ± 0.02^a
12.5	93 580 ± 5758^b	0.67 ± 0.04^a
15	74 187 ± 5931^c	0.65 ± 0.06^a
17.5	79 142 ± 4607^c	0.71 ± 0.01^a

3.3. Ammonia stripping with air recirculation or vacuum

3.3.1. Ammonia stripping with air recirculation

During the third series of experiments, the concentrate solution was continuously recycled. The initial TAN concentration in the concentrate solution varied between 16 200 and 19 150 mg/L and reached values between 20 970 and 21 352 mg/L. An ANOVA indicated a significant effect ($P < 0.01$) between the batches of a replicate for the transfer energy per milligram of transferred TAN. Duncan's analysis revealed that the first batch of a replicate always required a significantly ($P < 0.01$) lower transfer energy per milligram of transferred TAN than the remaining six batches, which were all similar. This difference can be associated with membrane surface fouling during the first batch of a replicate, with the fouling remaining constant for the rest of the replicate. No difference for transfer energy was observed between the replicates, with an average of 0.3571 W min per milligram of transferred TAN ($P < 0.05$). Water transfer by osmosis and electroosmosis was constant over the seven batches of each replicate at 0.81 ± 0.03 L per batch (9.6% of the original volume, for a total of 5.67 L per replicate), reducing the TAN concentration achieved in the concentrate solution (total volume increase of 51% during a replicate). A mass balance revealed that more than 99% of the total volume used and 104.9 ± 4.1% of the TAN transferred during a replicate were recovered. However, this represented a residual ammonia concentration of 1520 ± 133 mg/L or approximately 41%, on a mass basis, of the original TAN content in the swine manure. The ammonia recuperated in the first acid trap accounted for 6.2% of the theoretical value of the NH_3 present in the concentrate solution, representing 0.22% of the total transferred TAN from the swine manure. No ammonia was detected in the second acid trap. These results suggest that TAN volatilization was limited by the concentrate solution pH, which ranged from 8.6 to 8.3 during a replicate and whose value was controlled by the buffering action of carbonate and ammonia.

3.3.2. Ammonia stripping with vacuum

During the fourth series of experiments, the initial TAN concentration in the concentrate solution varied between 11 690 and 13 315 mg/L and reached values between 12 780 and 13 550 mg/L. As in the third series, Duncan's analysis showed that only the first batch of a replicate required a significantly ($P < 0.01$) lower amount of transfer energy per milligram of transferred TAN. No difference for transfer energy was observed between the replicates, with an average of 0.2964 W min per milligram of transferred TAN ($P < 0.05$). However, the *t*-test (0.01) indicated a significant difference between the third and fourth series of experiments, given that the application of vacuum is more favourable for ammonia transfer. Water transfer by osmosis and electroosmosis was constant at 1.53 ± 0.32 L per batch (18.6% of the original volume, for a total of 7.65 L per replicate) and reduced the TAN concentration achieved in the concentrate solution (139% volume increase during a replicate). A mass balance revealed that more than 99% of the total volume used and 99.7 ± 9.5% of the TAN transferred during a replicate were recovered. Residual ammonia concentration in the swine manure was 1200 ± 189 mg/L or approximately 30%, on a mass basis, of the original TAN content. The ammonia recuperated in the acid trap accounted for 14.5% of the theoretical value of the NH_3 present in the concentrate solution, representing 0.46% of the total transferred TAN from the swine manure. Ammonia volatilization was again limited by the

concentrate solution pH, whose value varied between 8.5 and 8.3 during a replicate.

4. Conclusions

The present study showed the limits of ED in a dilution/concentration configuration coupled with an acid trap applied to recover the TAN present in swine manure. The maximum achievable TAN concentration in the concentrate solution was not limited by swine manure pH but by water transfer toward the concentrate solution by osmosis and electroosmosis. Nevertheless, the result was a seven-fold increase in TAN, at 21 352 mg/L, compared to the initial swine manure content.

The required energy for TAN transfer was lower only for the first batch of all replicates, indicating that membrane surface fouling does not represent a problem given that energy efficiency remained constant for the remaining replicates. Efficient energy utilization limiting the difference in concentration by a factor of 10 between the concentrate and diluate solutions resulted in a significant fraction of swine manure TAN remaining in the diluate solution when the process was stopped. The concentrate solution pH was not high enough to promote the volatile NH_3 form of TAN, and therefore only a minor fraction of the transferred TAN was found in the acid trap.

Improvement of the process would include increasing the concentrate solution pH to promote the volatile NH_3 form of TAN and a lower swine manure residual TAN content by the end of the process. Experiments with an ED process using bipolar membranes are in progress with a view to avoiding the limitations observed in the present study.

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Journal of Environmental Management 95 (2012) S170–S174

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Short- and long-term effects of ammonium and nitrite on the Anammox process

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ARTICLE INFO

Article history:
Received 21 August 2009
Received in revised form
20 July 2010
Accepted 19 October 2010
Available online 11 November 2010

Keywords:
Anammox
Free ammonia (FA)
Free nitrous acid (FNA)
Inhibition
Specific Anammox activity (SAA)
Sequencing batch reactor (SBR)

ABSTRACT

Autotrophic anaerobic ammonium oxidation (Anammox) is a biological process in which Planctomycete-type bacteria combine ammonium and nitrite to generate nitrogen gas. Both substrates can exert inhibitory effects on the process, causing the decrease of the specific activity of the biomass and the loss of the stable operation of reactors. The aim of the present work is to evaluate these effects in short- and long-term experiments. The short-term effects were carried out with two different types of Anammox biomass, biofilm on inorganic carriers and flocculent sludge. The effects of ammonium on both kinds of biomass were similar. A decrease of the Specific Anammox Activity (SAA) of 50% was observed at concentrations about 38 mg NH_3-N·L^{-1}, while 100 mg NH_3-N·L^{-1} caused an inhibition of 80%. With regards to nitrite, the SAA was not affected at concentrations up to 6.6 μg HNO_2-N·L^{-1} but it suffered a decrease over 50% in the presence of 11 μg HNO_2-N·L^{-1} in the case of the biofilm. The flocculent biomass was much less resistant and its SAA sharply decreased up to 30% of its initial value in the presence of 4.4 μg HNO_2-N·L^{-1}.

The study of the long-term effects was carried out in lab-scale Sequencing Batch Reactors (SBR) inoculated with the biofilm biomass. Concentrations up to 20 mg NH_3-N·L^{-1} showed no effects on either reactor efficiency or biomass activity. However, when free ammonia concentrations reached values between 35 and 40 mg NH_3-N·L^{-1}, the operation turned unstable and the efficiency was totally lost. Nitrous acid concentrations around 1.5 μg HNO_2-N·L^{-1} caused a loss of the efficiency of the treatment and a destabilization of the system. However, a total restoration of the SAA was observed after the stoichiometric feeding was applied to the SBR.

1. Introduction

The Anammox process, in combination with a previous step of partial nitrification, is a suitable alternative in order to treat effluents with high content of ammonia and low carbon to nitrogen (C/N) ratios (Fux et al., 2002). In the partial nitrification step the 50% of the ammonium is oxidized into nitrite. This could be done by means of several different processes like SHARON (Hellinga et al., 1999), oxygen-limited bioreactors (Bernet et al., 2001; Pollice et al., 2002), inhibition of nitrite-oxidizers by free ammonia or free nitrous acid (Anthonisen et al., 1976; Villaverde et al., 1997), or the recent aerobic granular reactors (Vázquez-Padín et al., 2006). In the Anammox process the remaining ammonium is oxidized by autotrophic bacteria using the nitrite as the electron acceptor (Strous et al., 1999). This option allows the reduction of costs compared to the traditional nitrification–denitrification system because less oxygen is required and the addition of organic matter is not necessary. Besides, the low amount of surplus sludge would also lead to a reduction in the operational costs (Jetten et al., 1997).

In order to achieve the successful operation of the Anammox process, the potentially negative effects of the compounds present in the wastewater should be studied. Among these compounds, the substrates were reported to be responsible of losses in the Anammox activity (Strous, 2000; Dapena-Mora et al., 2007; Tang et al., 2009). Taking into account that during start-up or overload periods both substrates could not be completely consumed, their presence in the reaction medium could cause the decrease of biomass activity and the destabilization of the reactor. A new start-up or the recovery of the biomass activity might take long time, especially in the case of industrial-size reactors (van der Star et al., 2007), due to the very slow growth rate of Anammox bacteria (Strous et al., 1999).

Some studies reported data about the inhibitory effect of ammonia and nitrite (Fux et al., 2004; Strous, 2000; Jetten et al., 2005; Dapena-Mora et al., 2007). However, these works were sometimes carried out under very different operational conditions (pH, temperature, continuous/batch tests…) which entails that these results are difficult to extrapolate and to use to design an operational strategy. Moreover,

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doi:10.1016/j.jenvman.2010.10.044

the different works about the effects of nitrite did not agree about the concentration threshold that would not be exceeded. In the literature different ranges for this threshold (50–150 mg $N \cdot L^{-1}$, Strous et al., 1999; 30–50 mg $N \cdot L^{-1}$, Fux et al., 2004) can be found. The knowledge about safe levels would be very important for the operators of the wastewater treatment plants with Anammox stages in order to maintain the performance of the system.

Therefore the objective of the present work is to determine the short- and long-term effects of nitrite and ammonium on the Anammox process in order to know the suitable conditions to operate the Anammox reactors. Results will be analyzed in terms of the unionized compounds, which are well known to be responsible for the inhibition of nitrifying bacteria (Anthonisen et al., 1976; Vadivelu et al., 2007) and poly-phosphate accumulating denitrifiers (Zhou et al., 2007).

2. Materials and methods

2.1. Specific Anammox activity (SAA) tests

To determine the short-term effects of ammonia and nitrite on the Anammox biomass, batch experiments were performed according to the methodology described by Dapena-Mora et al. (2007). The tests consisted of the measurement along the time of the overpressure generated in closed vials by the produced nitrogen gas. They were performed at least by triplicate and with an initial pH value of 7.8 provided by the use of phosphate buffer (0.14 g L^{-1} KH_2PO_4 and 0.75 g L^{-1} K_2HPO_4), which also prevented significant pH variations during the tests. In order to assess the short-term effects of ammonium, batch assays were performed with a fixed initial nitrite concentration of 70 mg NO_2^--$N \cdot L^{-1}$ and ammonium concentrations of 70, 700, 1400, 2100 and 2800 mg NH_4^+-$N \cdot L^{-1}$. To evaluate the effects of nitrite, the tests were performed with a fixed initial ammonium concentration of 70 mg NH_4^+-$N \cdot L^{-1}$ and nitrite concentrations of 70, 140, 210, 280, 350 and 420 mg NO_2^--$N \cdot L^{-1}$.

The biofilm biomass employed in the tests was taken from the SBR systems used to study the long-term effects of ammonia and nitrite. Flocculent biomass was also tested in order to assess the influence of the biomass type and it was collected from an Anammox reactor used for Anammox biomass enrichment (Dapena-Mora et al., 2004a). The short-term tests performed with both types of biomass were done under similar conditions.

SAA tests were also used in order to monitor the reactor by assessing the maximum removal capacity. This variable was calculated by multiplying the maximum SAA obtained in batch tests and the concentration of biomass in the system.

2.2. Experimental set-up

Long-term experiments were carried out in two Sequencing Batch Reactors of 5 L (SBR1) and 3 L (SBR2) of useful volumes, respectively. Temperature was controlled at 33 °C (SBR1) and 30 °C (SBR2) by using thermostatic jackets. The complete mixture inside both reactors was achieved using mechanical stirrers with rotating speed of 150 rpm. The control of the pumps and different periods of the operational cycles was performed with a PLC system (CPU224, Siemens). Both reactors were operated in cycles of 6 h distributed in four periods: mixed fill (300 min), mix (30 min), settle (15 min) and draw (15 min) according to Dapena-Mora et al. (2004b). The exchange volume was fixed at 25% and the Hydraulic Retention Time (HRT) was maintained at 1 day in both cases.

To prevent the oxidation of the excess of ammonium or nitrite, the headspace of both reactors was continuously flushed with 200 mL min^{-1} of Ar.

Table 1
Operational strategy.

Reactor	Periods	Days	NH_4^+-N_{inf} (mg L^{-1})	NO_2^--N_{inf} (mg L^{-1})
SBR1	I	0–25	180	250
	II	26–39	250	250
	III	40–74	375	250
	IV	75–103	425	250
	V	104–172	500	250
	VI	173–200	750	400
SBR2	I	0–48	150	185
	II	49–69	150	200
	III	70–95	150	220
	IV	96–118	150	240
		119–132	150	200
	V	133–160	150	240
		161–166	150	280
		167–221	150	200
	VI	222–257	150	300
	VII	258–281	150	400

Note: Shaded cells correspond to recovery periods.

2.3. Feeding media and operational strategy

Both reactors were fed with a synthetic autotrophic medium described by Dapena-Mora et al. (2004a). The operational strategy in both cases was fixing an initial ammonium to nitrite molar ratio approximately at the stoichiometric value (1.32 NO_2^--N/NH_4^+-N according to Strous (2000)). Then the concentrations of ammonium (SBR1) or nitrite (SBR2) were stepwisely increased (Table 1), while the limiting substrate concentration (nitrite for SBR1 and ammonium for SBR2) was not changed. This strategy allowed maintaining the effective nitrogen loading rate applied constant. The effective nitrogen loading rate was calculated as follows: the sum of the concentration of limiting substrate in the feeding plus the stoichiometric concentration of the substrate in excess, divided by the hydraulic retention time.

2.4. Inocula

SBR1 was inoculated with enriched Anammox biofilm biomass from a laboratory scale SBR operated at the University of Santiago of Compostela (Fernández et al., 2008). The support material was natural zeolite. This zeolite was clinoptilolite (ZeoCat, Spain) with particle size between 0.5 and 1.0 mm. The initial average particle (biofilm plus support) size was 1.4 mm. SBR2 was inoculated with biomass taken from SBR1 at the end of its operation. The average particle size at the inoculation of SBR2 was 1.1 mm. The initial concentrations of biomass were 1.2 and 2.0 g $VSS \cdot L^{-1}$ for SBR1 and SBR2, respectively. The initial SAA values were 0.49 g N $(g\ VSS \cdot d)^{-1}$ and 0.21 g N $(g\ VSS \cdot d)^{-1}$ for the biomass of SBR1 and SBR2, respectively.

2.5. Analytical methods

Ammonium was determined spectrophotometrically (APHA, 1998) while nitrate and nitrite were determined by capillary electrophoresis (Waters Capillary Ion Analyzer). The concentrations of solids, determined as Total Suspended Solids (TSS), the fraction corresponding to the biomass as Volatile Suspended Solids (VSS), were determined according to the Standard Methods (APHA, 1998). As zeolite particles were not affected by calcination at 550 °C, the concentration of biomass in the biofilm was assessed as VSS determined according to the Standard Methods (APHA, 1998).

The distribution of particle size was measured using an Image Analysis procedure (Tijhuis et al., 1994; Jeison and Chamy, 1998). Images of the granules were taken with a digital camera (Coolsnap,

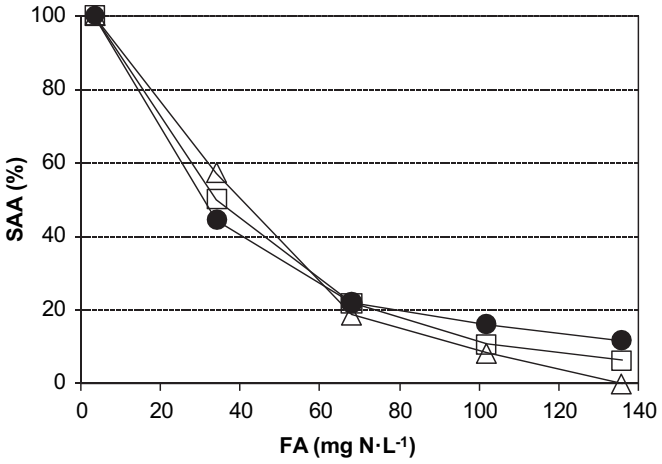

Fig. 1. Short-term effects of FA (□ biofilm biomass, ammonium chloride; Δ biofilm biomass, ammonium sulphate; ● flocculent biomass, ammonium chloride).

Roper Scientific Photometrics) combined with a stereomicroscope (Stemi 2000-C (Zeiss)). For the digital image analysis the programme Image ProPlus was used. Specifically the programme was employed to calculate the average feret diameter of the granules.

3. Results and discussion

3.1. Short-term effects of ammonium and nitrite on the Anammox process

The average results obtained from short-term tests to evaluate the effects of ammonium are presented on the basis of the Free Ammonia (FA) concentration, calculated according to Anthonisen et al. (1976), due to the fact that unionized ammonia is considered to be the true inhibitor compound (Fig. 1).

For biofilm biomass, tests were performed both with ammonium chloride and sulphate, in order to evaluate the possible effect of the counterion. However this hypothetical effect was not observed. On the other hand, results obtained with flocculent biomass showed no difference with those of the biofilm biomass (Fig. 1). In both cases, FA concentration that caused an inhibition of 50% (IC_{50}) was around 38 mg NH_3-N·L^{-1}.

The average results obtained from short-term tests to evaluate the effects of nitrite are presented in terms of the Free Nitrous Acid (FNA) concentration, calculated according to Anthonisen et al. (1976) (Fig. 2).

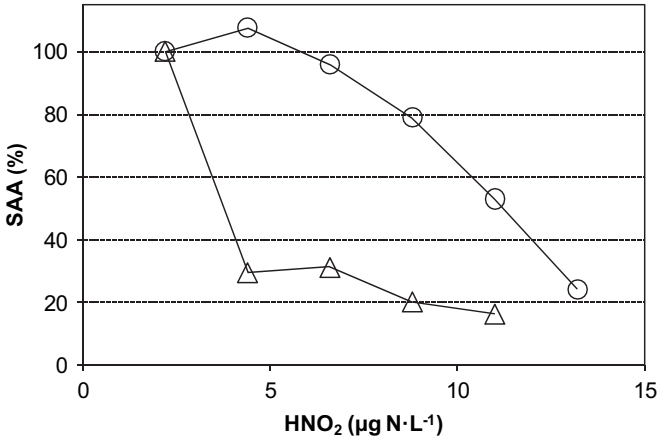

Fig. 2. Short-term effects of FNA (○ biofilm biomass; Δ flocculent biomass).

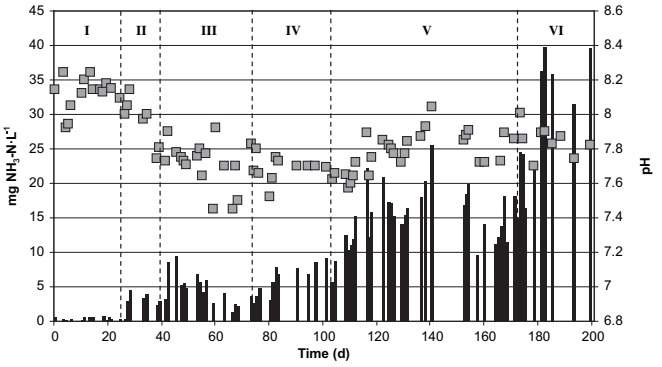

Fig. 3. FA concentrations (bars, mg NH_3-N·L^{-1}) and pH (■) inside the reactor.

No significant loss of SAA was observed up to 6.6 μg HNO_2-N·L^{-1} with biofilm biomass while its IC_{50} was 11 μg HNO_2-N·L^{-1}. On the contrary, the flocculent biomass was much more seriously affected by the FNA, with 70% of inhibition at only 4.4 μg HNO_2-N·L^{-1} and no measurable activity was found at concentrations over 11 μg HNO_2-N·L^{-1}.

3.2. Long-term effects of substrates on the Anammox process

3.2.1. Long-term effects of ammonium

The reactor was operated for 200 days under different inlet ammonium to nitrite molar ratios. During Period I the reactor was fed with a stoichiometric ammonium to nitrite molar ratio and the presence of FA in the media was negligible (Fig. 3). From day 26 the concentration of ammonium was increased stepwise along the different operational periods. During Periods II and III average ammonium concentrations in the reactor were 40 and 92 mg NH_4^+-N·L^{-1}, respectively. Nevertheless, in both periods, FA concentrations were only around 4 mg NH_3-N·L^{-1} due to a decrease of the pH value inside the reactor, which favoured the displacement of the equilibrium to the ionized specie. From Period IV, pH was maintained at a value around 7.8 and FA concentrations inside the reactor increased up to 7 mg NH_3-N·L^{-1} according to the increase of ammonia concentration. During the first five periods of the operation, the efficiency of the reactor in terms of limiting substrate (nitrite) consumption was very close to the 100% (Fig. 4). However in the Period VI, when the FA concentration was in the range of 35–40 mg NH_3-N·L-1, the operation became unstable. On day 200 of operation, when the efficiency was around 40%, the experiment was stopped.

Along the first three periods, the maximum capacity of the system increased (Fig. 4) due to the growth of the biomass (from the initial 1.2 g VSS·L^{-1} to 2.5 g VSS·L^{-1} at the end of Period III). In

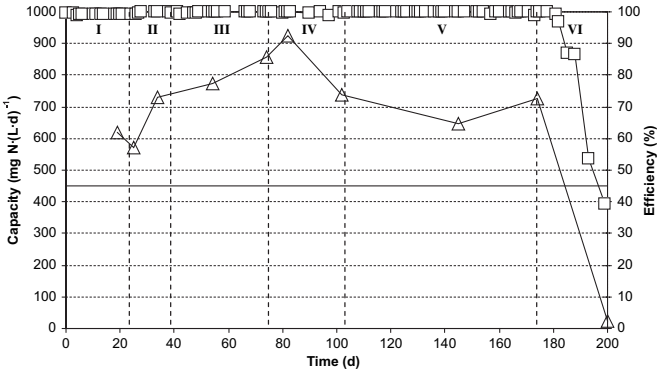

Fig. 4. Efficiency (□) and maximum capacity (Δ) of the system. Effective nitrogen loading rate applied is marked with a continuous line.

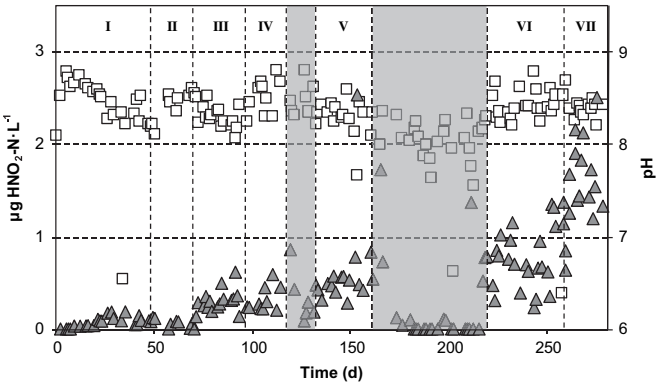

Fig. 5. Free nitrous acid concentration (▲) and pH (□) inside the system.

Period IV, the system lost a 25% of its capacity. Nevertheless, this fact did not affect the efficiency of the reactor because the effective nitrogen loading rate applied was under the maximum capacity (around 740 mg N $(L \cdot d)^{-1}$). Finally, in Period VI, a total loss of the system capacity took place, due to the loss in the SAA of the biomass. The obtained results agree with those reported by Jung et al. (2007) who found a total loss of the ammonium removal rate in the presence of FA concentrations higher than 32 mg NH_3-$N \cdot L^{-1}$. Tang et al. (2009) also observed the loss of the Anammox process stability due to an increase of FA concentrations up to 70 mg NH_3-$N \cdot L^{-1}$ caused by an increase of pH.

Despite the descent in the average particle size (calculated by means of the volume of particles) from 1.43 mm at day 6 to 1.16 mm at day 187, the high relative density of the particles with inorganic carrier (Fernández et al., 2008) led to good settling of the biomass. Therefore, the presence of the aforementioned levels of FA in the system did not cause problems to the biomass retention and the concentration of biomass in the effluent remained below 10 mg $VSS \cdot L^{-1}$, which implied solids retention times higher than 130 d.

At the end of the experimental period, the reactor was again fed with a stoichiometric ammonium to nitrite molar ratio to restore the system capacity. This restoration took place within 1 month (data not included in Fig. 4).

3.2.2. Long-term effects of nitrite

FNA concentrations during the first two operational periods (Fig. 5) were almost negligible. In Period III the system was operated in presence of 0.3 μg HNO_2-$N \cdot L^{-1}$ but no effects on the maximum capacity were observed (Fig. 6).

A failure of the temperature controller occurred during Period IV and the inlet nitrite concentration was decreased to 200 mg NO_2^--$N \cdot L^{-1}$ (days from 119 to 132) in order to achieve a fast restoration of the system. Once this problem was solved, inlet nitrite concentration was increased to 240 mg NO_2^--$N \cdot L^{-1}$ (Period V) and an average FNA concentration of 0.5 μg HNO_2-$N \cdot L^{-1}$ was achieved. No inhibitory effects took place at this FNA concentration, the capacity of the system being similar to that measured during Periods II and III. The period between days 160 and 222 corresponds to another unstable stage due to operational problems and its corresponding restoration time.

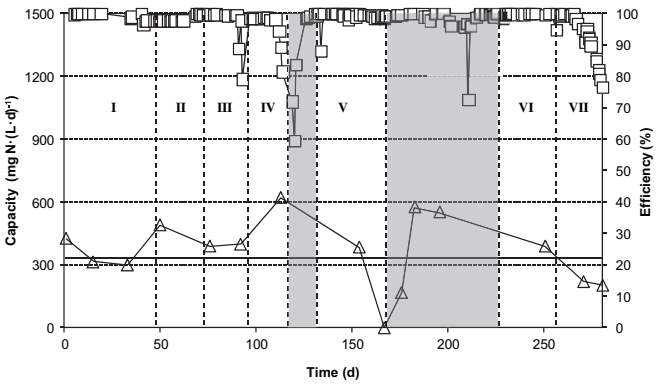

Fig. 6. Efficiency (□) and maximum capacity (Δ) of the system. Effective nitrogen loading rate applied is marked with a continuous line.

During Periods VI and VII, the reactor was operated in the presence of average FNA concentrations of 0.7 and 1.5 μg HNO_2-$N \cdot L^{-1}$, respectively. For these concentrations a decrease of the capacity was observed. Furthermore, the efficiency of the treatment in terms of limiting substrate (ammonium) removal felt to 77% at the end of Period VII, when the capacity was well below the effective nitrogen loading rate applied. Despite that loss of efficiency and the corresponding nitrite accumulation, flotation events like those reported by Dapena-Mora et al. (2004c) were not observed. Therefore the biomass retention was good with a concentration of biomass in the effluent below 13 mg $VSS \cdot L^{-1}$ which implied solids retention times longer than 187 d (calculated with an average value for biomass concentration in the reactor of 2.44 g $VSS \cdot L^{-1}$). Furthermore, the average particle size suffered an increase from 1.05 mm at day 42 to 1.48 mm at day 274.

From day 280 the reactor was again fed with a stoichiometric ammonium to nitrite molar ratio and less than 1 month was necessary in order to restore the SAA to values similar to those measured at the beginning of the Period VI (data not shown).

The results of the present work are similar to those found by Fux et al. (2004). These authors reported 80% of loss in the activity when they operated a fixed bed reactor in the presence of 80 mg NO_2^--$N \cdot L^{-1}$ (according to the operational conditions in their reactor, this nitrite concentration corresponds to around 4 μg HNO_2-$N \cdot L^{-1}$). Moreover, a recent work by Jung et al. (2007) reported a significant long-term inhibition in the presence of 0.8–1.2 μg HNO_2-$N \cdot L^{-1}$ (calculated from data reported by the authors), a range that agrees very well with the results of the present work.

4. Conclusions

Both substrates are inhibitors for Anammox process at long and short-term exposition. The inhibitory effects caused by FA and FNA were stronger at long-term experiments. At short-term tests, flocculent biomass was more affected by FNA than biofilm biomass.

Concentrations higher than 20–25 mg NH_3-$N \cdot L^{-1}$ and 0.5 μg HNO_2-$N \cdot L^{-1}$ should be avoided to maintain the stable operation of Anammox systems. Activity assays are a useful tool in order to predict unstable episodes.

Inhibition caused by both substrates is reversible, the restoration time being around 1 month. The physical properties of the Anammox biofilm biomass are not substantially affected by the presence of FA or FNA.

Acknowledgments

This work was funded by the Spanish Government (TOGRANSYS project CTQ2008-06792-C02-01/PPQ and NOVEDAR_Consolider project CSD2007-00055). Authors want to express their gratitude to Mar Orge, Mónica Dosil and Miriam Vieites for their support with the analytical techniques. I. Fernández wants to thank the Xunta de Galicia for his predoctoral grant.

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Journal of Environmental Management 95 (2012) S175–S181

Contents lists available at ScienceDirect

Journal of Environmental Management

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The performance of natural clay as a barrier to the diffusion of municipal solid waste landfill leachates

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ARTICLE INFO

Article history:
Received 30 September 2009
Received in revised form
7 January 2011
Accepted 23 February 2011
Available online 21 March 2011

Keywords:
Clay barriers
Landfill
Contaminant transport
Anion diffusion

ABSTRACT

In this paper, the diffusion of solutes in natural clay from a concentrated solution consisting primarily of ammonium, sodium and chloride ions at a pH level of 8 was studied and was based on an existing 20-year-old landfill. Contaminant transport through clay liners was predicted using transport and reaction geochemical codes to help explain the experimental data. The model predicted the chloride anion diffusion and cation exchange processes for three different experiments: (1) small-scale interactions in compacted clay, (2) 1:1 European Union (EU) Directive demonstration experiments (0.5-m-thick clay barrier), and (3) analysis of a bore hole with core recovery drilled in an old landfill located above a similar type of clay as that studied in (1) and (2). Orders of magnitude between 10^{-10} and 10^{-9} m^2 s^{-1} were used for the apparent diffusion coefficient to fit the chloride profiles at the different scales; however, at larger space and time scales, diffusion was retarded due to the presence of more consolidated, non-mechanically disturbed clay materials at large depths in a natural clay-rock emplacement.

1. Introduction

The technical requirements for municipal waste landfills in the European Union (EU) are given in the Council Directive 1999/31/EC and decision (2003/33/CE), which established the requirement of a geological barrier thickness of at least 1 m, with a hydraulic conductivity of $1 \cdot 10^{-9}$ m s^{-1}. If a geological barrier does not naturally meet the conditions, a geo-synthetic barrier thickness of at least 0.5 m must be artificially created. Geological barriers are important because they confine waste and buffer the hazardous leachates; thus, they are a key factor for upholding the protection and safety objectives of waste disposal (Bilitewski et al., 1997; Savage, 1995; Astudillo, 2001).

Natural clay materials have a very low permeability because of their small pore sizes and complex porous structures. Their high specific surface areas allow for strong physical and chemical interactions to occur between fluids and dissolved species that are subjected to electrostatic repulsion, sorption and specific cation exchange reactions, and these interactions are responsible for retaining leachate components in landfills (Davis and Kent, 1990; Sposito, 1990; Stumm, 1992; Rowe et al., 1995; Sawney, 1996; Michael et al., 2002). Furthermore, clays impede fluid transport and retard the migration of solutes, which can be confirmed by correlating the predominance of clay under a landfill vessel with a natural pollution attenuation; for example, ammonium attenuation as a function of depth illustrates the role of clays in retardation of cations (National Groundwater and Contaminated Land Center, 2003).

The objective of our work was to perform a preliminary study of the transport of contaminants through natural clay material in municipal solid waste (MSW) landfills. A multi-scale experimental program was designed to help create the models and to compare the relevant parameters from the models, such as the diffusion coefficients, with the field data taken from a 20-year-old municipal landfill. In this study, the behavior of chloride, ammonium and sodium ions is discussed.

2. Materials and methods

2.1. Materials

Plastic clay from El Papiol (Barcelona, Spain) was selected because it has previously been used as a landfill liner in this region (Rogel et al., 2008). El Papiol clay has a heterogeneous mineralogy that is composed primarily of the main sheet silicates in the clay

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doi:10.1016/j.jenvman.2011.02.014

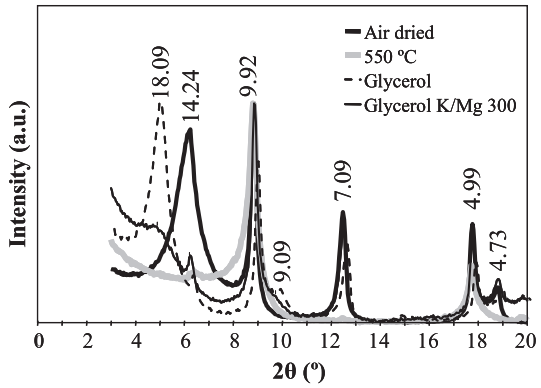

Fig. 1. XRD patterns (λ = Cu Kα) of <2 μm size fraction (clay size) of El Papiol clay. Several characterization treatments were performed (Moore and Reynolds, 1989; Malla and Douglas, 1987). Numbers are XRD reflection positions in Å (transformed from the reflection angle (θ) by the Bragg law (λ = 2d(Å)Sinθ). d: structural distance between parallel layers in the sheet-silicate structure; Glycerol: Glycerol solvated; Glycerol K/Mg 300: homoionized in K^+, heated to 300 °C and re-hydrated in Mg^{2+}-homoionized form; 550 °C and Air dried: heated or dried in the Mg^{2+} form.

mineral family (Brindley and Brown, 1984): illite (primarily), chlorite, kaolinite and smectite. The mineralogy was studied by X-ray diffraction (XRD) and was analyzed by random powder and oriented clay fraction (<2 μm) methods. Illite exhibited a 001 reflection at 10 Å and remained unchanged by glycerol solvation. However, the long d-spacing (14 Å) of the expandable clay (smectite) was modified upon solvation with glycerol (18 Å). The sharpness of the reflection and the collapse of the 14–18 Å structure under K^+ homoionization at 300 °C indicate that smectite is a highly charged, large-sized beidellite crystal, as shown in Fig. 1 (Malla and Douglas, 1987). The relatively low specific surface area determined for this clay (19 $m^2\ g^{-1}$) is in agreement with the sharpness of the clay mineral reflections (large-sized crystals imply a small surface area). Furthermore, the destruction of the 7-Å structure at 550 °C indicates the existence of kaolinite or iron-rich chlorite (Moore and Reynolds, 1989). Non-clay minerals, such as quartz, calcite, feldspars and iron oxide (hematite), represent approximately 40% of the bulk material (Table 1). The clay, which had a hydraulic conductivity of $5.2 \cdot 10^{-10}$ m s^{-1} for the optimum water content at the maximum density (10% H_2O; 2.0 g cm^{-3} dry density), was tested to satisfy the technical EU Directive requirements. Also, the total organic carbon content (TOC) was less than 0.01%.

Table 1 shows the mineralogical composition, the distribution of the exchangeable cations and the major ions extracted in a 1:5 solid/water ratio. The geochemical code PHREEQC 2.14 was used to fit the initial pore water of the clay and the initial distribution of the exchangeable cations. The code was run at fixed pH/Eh conditions, 8.5/pe = −4, in the clay. This pH level was measured at the clay-leachate interface in both the small- and EU demonstration-scale experiments. Also, the redox potential remained constant to prevent the bicarbonate reduction to methane, which occurs at pe < −5 at a pH level of 8.5.

The initial pore water composition was measured in a 1:5 aqueous extract with El Papiol Clay. A factor of 20 to concentrate the main ions (Cl^-, K^+ and Na^+) was calculated by accounting for the naturally compacted water in saturated clay (0.25 l H_2O/kg, saturation at 2.0 g/cm^3 dry density), and the aqueous extraction was obtained in a 1:5 solid to liquid ratio. The alkalinity was related to the calcite solubility, and the Mg^{2+} concentration was related to dolomite solubility. The cation Ca^{2+} was used to satisfy the charge balance. Then Na^+ was varied to approach the initial cation exchange equilibrium, and the model also included the main minerals present in the clay: montmorillonite, illite, quartz, calcite, dolomite and hematite.

A portion of the calcium and magnesium content was dissolved from carbonates when the aqueous extraction was performed. These cations, with a higher electric charge, displace Na^+ from the exchange complex. Thus, the Na^+ concentration in the pore water should be lower than the measured concentration.

2.2. Experiments

2.2.1. Small-scale diffusion experiments

The clay was compacted at its optimum water-saturated maximum density in a Teflon cylinder cell that had a height of 2.1 cm and a diameter of 7 cm. The cell was confined in a stainless steel mold, where fluid was forced to flow at either side of the disc through the Teflon porous membranes (<1 mm thickness under compaction, one filter at the top of the mold and the other filter at the bottom). The small thickness and large-sized pores of this membrane minimized the artifacts introduced in the diffusion experiments through the use of porous plates in the compacted clays (González et al., 2009). The fluid at each side was recycled to configure the pure diffusion experiment. Distilled water (2 L) flowed through the bottom filter for 2 months in one side. After 2 months, a synthetic leachate was recirculated through the top porous membrane for another 2 months. Electrical conductivity was measured on-line in the distilled water deposit. After 2 months of leachate diffusion, the clay sample was divided into 4-mm sections to determine the mineralogy and physical-chemical

Table 1
Mineralogical composition (weight %) and physical–chemical properties of El Papiol clay.

Mineralogical composition									
Clay minerals				Q	Fn	Fk	Cal	Dol	Other
T	K	I	S						
58	9	33	16	30	1	1	6	2	H, C
Physical–chemical properties									
	SUP	ΣEC	Exchangeable cations				CEC		
			Na^+	K^+	Mg^{2+}	Ca^{2+}			
Exp.	19 ± 1	20 ± 2	0.25 ± 0.05	0.3 ± 0.1	3.4 ± 0.3	14 ± 1	16 ± 1		
Model	–	–	2.0	0.1	2.5	11.0	15.6		
	Soluble ions							pH	
	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	SO_4^{2-}	HCO_3^-		
Exp.	1.56 ± 0.44	0.11 ± 0.01	–	0.69 ± 0.11	0.56 ± 0.29	0.19 ± 0.08	–	8.5	
Model	20.00	2.43	1.00	0.5	12.39	–	0.5	8.5	

T: Total clay; K: kaolinite; I: Illite; S: Smectite; Q: quartz; Fn, Fk: sodium, potassium feldspar; Cal: calcite; Dol. Dolomite; H: Hematite; C: Chlorite. SUP: Specific surface measured by the BET N_2 adsorption method ($m^2\ g^{-1}$). Σ EC: sum of exchangeable cations. Na^+, K^+, Mg^{2+} and Ca^{2+}: exchangeable cations (cmol(+)/Kg). CEC: Cation exchange capacity (cmol(+)/Kg). Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, SO_4^{2-} and HCO^{3-} : soluble cations (mmol/l) in a 1:5 solid/water aqueous extract. Model: initial conditions fitted with PHREEQC (Pakhurst and Appello, 1999) modeling.

properties of the samples, as was reported for the natural clay in Table 1. The synthetic leachate (L1 in Table 2, Cuevas et al., 2009) was a simplified formulation of the landfill leachate obtained from the El Garráf (Barcelona) Landfill (L2 in Table 2, Cuevas et al., 2009). The synthetic leachate had a composition of an aged landfill leachate reacting at the methanogenic stage (Williams, 1998; Pivato and Raga, 2006).

2.2.2. EU Directive (0.5-m-) scaled diffusion experiments

Compacted clay columns with lengths of 0.5 m were packed in 0.25-m diameter, 1-m length methacrylate cells, which resulted in a dry density of 2.0 g/cm^3. Two cells were prepared: (1) one with distilled water and (2) one with the addition of a landfill leachate obtained from a collector pipe in the El Garráf (Barcelona) Landfill (L2 in Table 2). The clay was held in contact with the solutions for six months. The contact between the clay and the liquid displayed an altered zone of 2–5 cm that had expanded in relation to the bulk column. Samples were taken at 2-(M01), 4-(M02) and 6-(M03) cm depths, measured from the point of liquid contact (Fig. 2). Another sample was taken from a section at 40–45 cm, measured from the cell bottom with an average depth of 10 (M09) cm from the point of liquid contact. These samples were analyzed to determine their mineralogy, physical–chemical properties and heavy metal ions. In addition, the entire column was tested every 5 cm for chlorides and sulfates to study the ionic transport through the clay.

2.2.3. Sampling of a clay bed under an old landfill

A 20-year-old landfill, which lay on El Papiol geological formation, was drilled up to a 3-m depth from the clay-waste contact. The clay was located below 16 m of waste. The leachate in the waste-clay contact was primarily inorganic, with a sodium chloride composition in the range of 0.4 mol l^{-1} (L3 in Table 2) and a lower organic content and a lower pH level (7.2) than the El Garráf Landfill.

2.3. Analytical methods

The mineralogy was studied by XRD after dismantling the experiments or from the samples from the drilled cores. To semi-quantify the minerals from the bulk sample, the procedure proposed in the UNE 22-161-92 was used. The specific surface area was determined by the Brunauer, Emmett and Teller method (BET) by multipoint N_2 adsorption using a Micromeritics® GEMINI V, after degassing under a N_2 flow for 18 h at 90 °C (UNE 22-164/94). For the surface chemistry analysis, the exchangeable cations were extracted from the clay at room temperature as described by Thomas (1982). The cations were analyzed with the following tools: Na^+ and K^+ using a Buck Scientific™ PFP-7 flame photometer; Ca^{2+} and Mg^{2+} by an ICP-MS (ELAN 6000, PERKIN ELMER™); and NH_4^+ by ion selective potentiometry (ORION™ 9512 Ammonia Gas Sensing Electrode). The cation exchange capacities (CEC) of the original clays were determined at room temperature by Na^+ homoionization (Na–COO–CH_3 1 M, pH = 8) and Mg^{2+} displacement ($MgNO_3 \cdot 5H_2O$ 0.5 M, pH = 5), which was used by Rhoades (1982).

Chloride and sulfate anions were measured by ion chromatography (Metrohm™ 761 Compact IC) in aqueous extracts (1:10 solid:liquid ratio). The suspension was soaked for 2 h and was then left to settle for 24 h. The supernatant was extracted and filtered. To determine the heavy-metal ion content in the uppermost zone of the clay column (EU Directive experiments), the dried clays were digested in aqua regia (6 ml of HCl and 2 ml of HNO_3) inside a microwave oven (12 min at 1000 W in PFA vials). The drill cores were treated in the same way. The suspensions were filtered and analyzed by atomic absorption spectrometry (AAS) (Unicam™ Solaar M). The same analysis was also performed in the leachate collected before the clay columns were sampled.

Table 2
Characteristics of the different leachates used as reference for modelling calculations.

	Synthetic Leachate (L1)	El Garraf Leachate (L2)	Old Landfill Leachate (L3)
pH	7.78	8.60	7.20
CH_3COO^-	0.10	0.039	0.025
HCO_3^-	0.15	n.d.	n.d.
Cl^-	0.25	0.253	0.400
NH_4^+	0.25	0.254	0.025
Na^+	0.25	0.196	0.450
K^+	–	0.059	0.012
Ca^{2+}	–	1.25×10^{-4}	–
Mg^{2+}	–	0.005	0.012
Cu	–	9.45×10^{-7}	2.36×10^{-6}
Zn	–	4.89×10^{-6}	4.89×10^{-6}
Pb	–	1.01×10^{-6}	2.08×10^{-6}
Cd	–	$< 1.78 \times 10^{-7}$	1.78×10^{-7}
Fe	–	4.83×10^{-4}	–

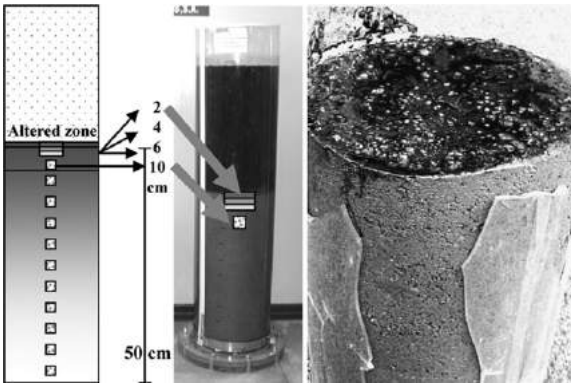

Fig. 2. Detail of "Biofilm" formation and sampling of the column experiments, with "El Garráf" leachate.

3. Results and discussion

3.1. Small-scale diffusion experiments

A significant diffusion of soluble salts was detected after the beginning of the synthetic leachate circulation (Fig. 3). A diffusion reaction model was used to simulate the diffusion of chloride and the cation exchange processes. The initial conditions for the model were described for the pore-water calculation in the natural clay (Table 1). The code PHREEQC (Parkhurst and Appelo, 1999) was used in the simulation. The simulation was carried out in two steps during a 2 month duration; first, distilled water was forced to circulate at the bottom of the cell, and second, the synthetic leachate circulated at the top. The fluid volumes in the simulation were 0.125 L for the leachate container, 0.01 L for the pore water (25% porosity in the clay) and 1 L for the water deposit. A trend line was obtained to explain the increase of the electrical conductivity in the water deposit with an apparent diffusion coefficient of $2 \cdot 10^{-9}$ m^2 s^{-1}, which is within the order of magnitude of the values obtained by Barone et al. (1989) (7.5 10^{-10} m^2 s^{-1}) in similar experiments performed with a compacted, carbonate-rich, clay-like soil (51% clay minerals).

In addition to the diffusion of the soluble components in the solution, other changes were determined for the solutions and the clay material. The pH level increased from 7.8 to 9.2 in the leachate deposit, and subtle mineralogical changes were observed in the clay slice in contact with the leachate port. When upon contact with the leachate, the amounts of calcite ($CaCO_3$) and dolomite

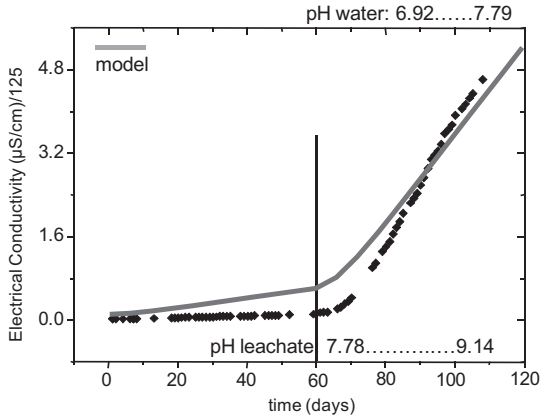

Fig. 3. Electrical conductivity (EC) profile in the small cells diffusion experiment (dots) transformed to chloride concentration (NaCl equivalence using molar electrical conductance = 125 Ω^{-1} cm^2 mol^{-1}). Modeling of chloride concentration with an apparent diffusion coefficient: $2 \cdot 10^{-9}$ m^2 s^{-1} pHs correspond to the initial and final pHs front stage 1 to stage 2 of the diffusion experiment.

($CaMgCO_3$) increased and decreased around 1%, respectively. This result can be explained by the formation of acetate complexes with calcium (Hatch, 2008) that would maintain the calcium in the solution which favors the precipitation of calcite. Alternatively, the precipitation of carbonates can be caused by the fermentation of acetate, which adds carbonate and increases the pH level (VanGulck et al., 2003), but the concentration of acetate was not measured. The physical–chemical properties, namely the CEC and specific surface area (SUP), did not change, which made ammonium the major exchangeable cation with a mole fraction greater than 0.8 of the CEC throughout the entire clay disc. In fact, chemical gradients were not observed inside the clay disc due to the long diffusion time of the experiment, which allowed the leachate fluid to equilibrate within the clay.

Fig. 4a shows the constant chloride profile across the thickness of the clay. The concentration in the clay was lower than predicted by the diffusion model, which predicted a sharp decrease of chloride in the leachate. The exclusion of chloride from this smectitic-rich clay can explain this result (Derrington et al., 2006). However, the diffusion of chloride into water was properly modeled.

The resulting ammonium concentration in the exchange complex was set to 2 cmol(+)/kg by the model (Fig. 4b). This low value, compared with the experimental value (10 cmol(+)/kg), was a result of the significant amount of Ca-montmorillonite (7%) or dolomite (1%) dissolution needed for the equilibrium calculations between the clay and the pore water. This calculation cannot be avoided in an equilibrium reaction model when the actual mineralogy must be considered. Calcium was incorporated into the exchangeable positions rather than ammonium. To overcome this problem, kinetic constraints must be used to control the mineral dissolution. The concentration of sodium was maintained near the initial values in the clay.

Table 3
Values of depth (*D*, m), pH, chloride concentration in fluid and fluid pores (Cl^-), exchangeable cations cmol(+)·Kg^{-1}): Na^+, K^+, Mg^{2+} and Ca^{2+}, specific surface (SUP, m^2 g^{-1}) and total organic carbon (TOC, C %) in the 0.5 m UE directive experiments with El Papiol clay. L1: distilled water; L2: landfill leachate.

	D	pH	Cl^-	Σ EC	Na^+	K^+	Mg^{+2}	Ca^{+2}	NH_4^+	SUP	TOC
L1											
Fluid		7.8	0.3								
M01	0.01	8.7	2.7	28	0.3	0.6	5.2	22	0.1	21	0.2
M02	0.03	8.7	3.2	31	0.9	1.4	6.7	22	–	19	0.2
M03	0.05	8.7	3.7	33	0.7	1.2	6.5	25	0.1	19	0.2
M09	0.1	8.7	3.5	19	0.5	0.4	4.5	13	–	20	0.2
L2											
Fluid		8.2	253.0								
M01	0.01	8.8	260.0	31	6.0	5.1	1.7	17	0.8	9	1.5
M02	0.03	9.0	216.0	34	6.1	6.1	4.4	13	4.7	12	1.0
M03	0.05	8.8	19.24	33	3.6	5.2	3.7	12	8.6	15	0.4
M09	0.1	8.7	14.67	40	1.8	2.1	2.9	28	6	16	0.4

Note: Additional information and data can be obtained from the reports summarized in Rogel et al. (2008).

3.2. EU Directive diffusion experiment

After six months, the physical–chemical parameters measured in the clay-leachate contact region from the experiments displayed strong similarities with those observed in the previous small cells. The pH value was above 8 in the leachate contact, and calcite also precipitated in small amounts at depths of 2–4 cm. Despite a high concentration of ammonium in the El Garráf leachate, this cation was not significant in the exchange complex of the M01 sample. This result was detected to a small extent in the small diffusion experiments and can be related to the formation of the clay-organic complexes at this contact region. In this region, a critical effect was observed where the specific surface decreased in the first 4 cm, which is related to the presence of organic cements (see TOC in Table 3). This result produces organic and inorganic clog particles and decreases the available surface area (Cuevas et al., 2009; Islam and Singhal, 2004; VanGulck and Rowe, 2004).

A diffusive chloride (major anion) front was produced, but its shape could not be modeled exactly; however, an apparent diffusion coefficient of $5 \cdot 10^{-9}$ m^2 s^{-1} (Fig. 5a) resulted in the best profile.

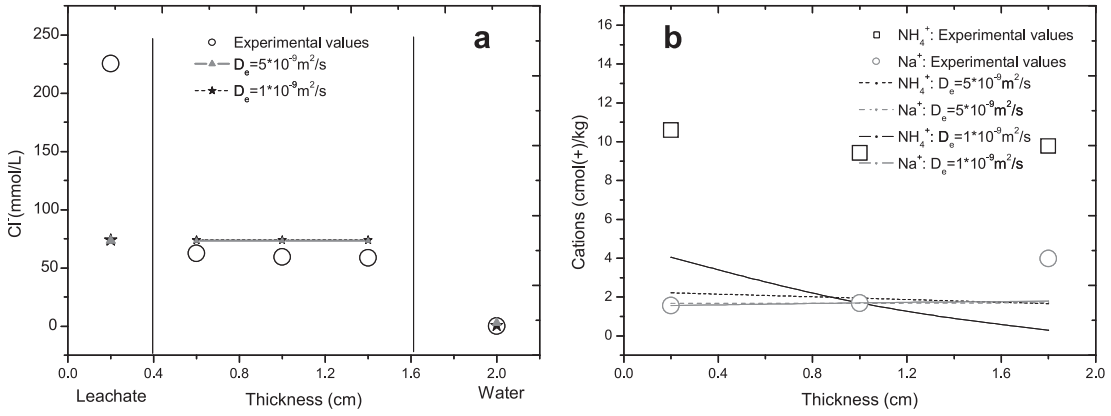

Fig. 4. Ions profiles *vs.* thickness of clay: a) chloride, b) ammonium and sodium.

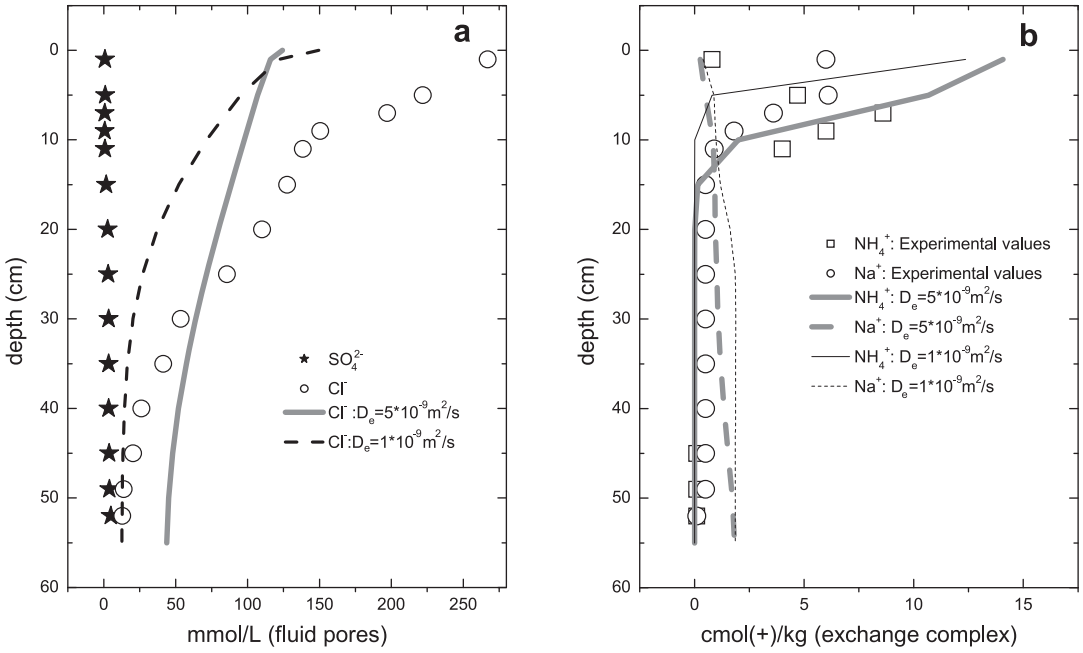

Fig. 5. Ions profiles *vs.* depth of UE directive column experiments: a) chloride and sulfate (concentration in pore water, mmol/L), b) ammonium and sodium (concentration in the exchange complex on a dry solid mass basis, cmol(+)/kg).

This diffusion coefficient was similar to the value from the small-scale experiments. However, the high content of chloride in the first 10–15 cm should be related to the density change produced by the overall 5- or 6-cm expansion of the 50-cm column, which affects the upper part of the column because higher quantities of the chloride solution are absorbed. The hydraulic conductivities of $1–10\cdot10^{-10}$ m s^{-1} at the hydraulic gradient equal to 1 should produce a solute front penetration of less than 3 cm in 10 months, which means that contaminant penetration in landfills should be studied where all the transport mechanisms are considered, not just the permeability.

An important detail in the column, although not modeled, is the sulfate reduction effect in the first 15 cm of depth (Fig. 5a). This effect is normally a reaction mediated by bacteria and precedes the methanogenic stage of the landfill organic matter degradation (Williams, 1998; Brun and Engesgaard, 2002), which establishes the basic pH level and the predominance of the inorganic ammonium-sodium chloride leachates. The basic pH levels and the precipitation of minerals can explain the low mobility of the heavy metal ions analyzed (Table 4). Heavy metals, such as Cu, Zn and Pb, migrate less than the soluble species due to the precipitation of carbonates (Yanful et al., 1988a,b). For example, in the Sarnia Landfill site, metals have diffused only 20 cm into the clay compared with 130 cm for chloride and sodium (Yanful et al., 1988b).

The modeling of exchangeable ammonium (Fig. 5b) predicted the increase of ammonium to a depth of 4–10 cm, but a strong buffering of the carbonate equilibrium produced a predominance of calcium and magnesium at greater depths. This buffering affected the sodium content in the exchange positions in the entire column because sodium has less selectivity than both ammonium and divalent cations. This case should also be studied in future works with the aid of a kinetics-based code that controls the mineral dissolution.

Table 4
Heavy metal ions (μg g^{-1}) in the clay-liquid contact (M01 samples).

Sample	[Zn]	[Cd]	[Pb]	[Cr]
Clay untreated	101.8	1.67	36.2	38.5
L1M01	107.8	1.35	38.6	38.5
L2M01	105.3	0.72	48.9	32.2

3.3. Clay bed under an old landfill

Regarding the pH level, the ammonium depletion at both the waste contact, the salt gradient and the observed physical–chemical parameters in the landfill clay (Table 5) displayed obvious similarities with the observed values of these parameters in the EU Directive 0.5-m experiments and even in the small-scale cells. However, different values of several parameters, such as the mineralogy and the specific surface, were observed. This difference is linked to the existence of an unconsolidated, less clay-rich material in the upper part of the base landfill clay. In this case, the upper clay-organic layer was not significant, as all the clays were less than 0.1% in the TOC.

The migration of sodium and ammonium up to 1.5 m below the waste occurred in the landfill after 20 years (Table 5). Similar data were found for Na and K migration in the Sarnia Landfill site after 16 years (Rowe, 1989).

Table 5
Selection of parameters tested in the drill cores form the old landfill with a base clay similar to El Papiol.

D	pH	EC	Cl^-	NH_4^+	Na^+	CEC	F	Cal	Do	SUP
1.25	8.6	11.30	201.8	0.38	6.88	18.65	46.7	28.1	0.3	16
3.75	8.7	11.22	150.4	0.36	7.52	14.91	50.4	27.2	0.4	20
6.25	8.6	11.22	158.0	0.58	7.20	13.80	55.1	24.6	0.6	17
8.75	8.6	10.70	116.1	0.57	6.99	20.34	53.4	26.6	0.8	20
17.5	8.7	10.47	126.2	0.66	5.82	18.30	41.9	30.5	1.2	18
65.0	8.9	10.00	124.6	0.41	8.13	21.77	62.2	12.8	3.6	28
115.0	8.7	6.10	50.4	0.24	3.91	25.39	75.5	7.9	3.0	31
155.0	9.2	0.807	6.0	0.10	1.46	22.83	59.9	14.1	5.0	31
225.0	9.2	0.815	2.8	0.13	2.03	24.45	74.3	9.7	3.5	39

D: Depth (cm). EC: electrical conductivity (mS cm^{-1}). NH_4^+ and Ca^{2+}: exchangeable cations (cmol(+)·Kg^{-1}). CEC: Cation exchange capacity (cmol(+)·Kg^{-1}). F: Total clay minerals; Cal: Calcite; Dol: Dolomite (%). SUP: Specific surface (m^2 g^{-1}). The complete data set obtained in the drilled cores is reported in the final report of the project: MMA: I+D+i A113/2007/3_02.6. La difusión de contaminantes en las barreras de vertederos urbanos y su evolución en el tiempo. Junio 2010.

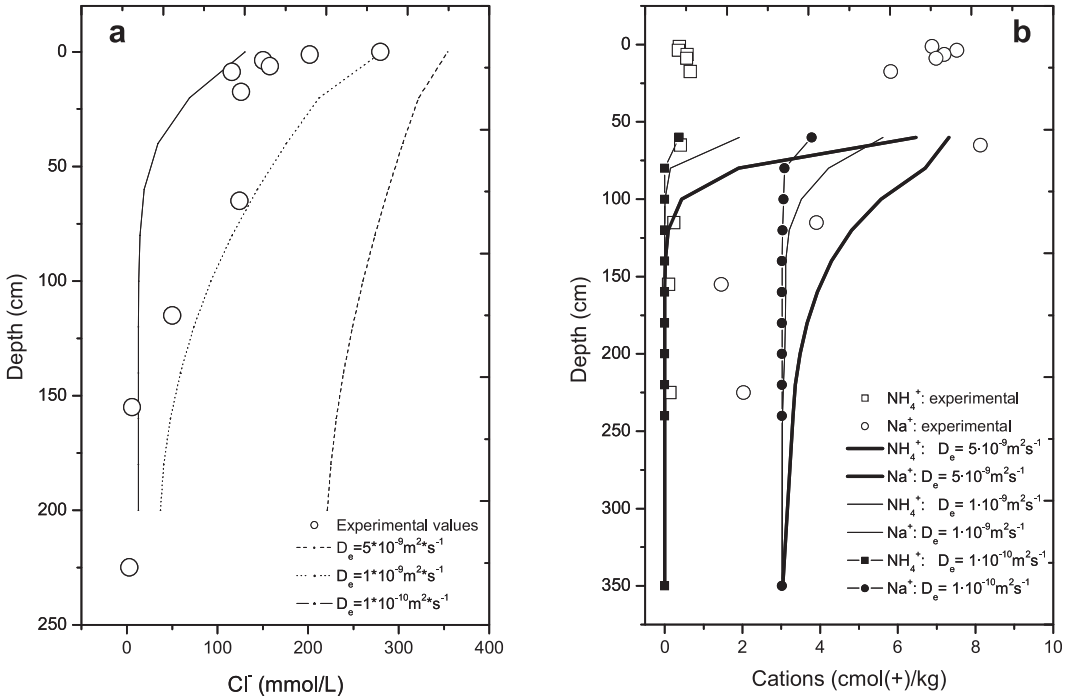

Fig. 6. Ions profiles *vs.* depth of landfill clay: a) chloride (concentration in pore water, mmol/L), b) ammonium and sodium (concentration in the exchange complex on a dry solid mass basis, cmol(+)/kg).

The modeled chloride diffusion at 20 years introduced a reduction of one order of magnitude for the apparent diffusion coefficient (10^{-10} m^2 s^{-1}, Fig. 6a). For the modeled cations (Fig. 6b), the diffusion coefficient was more consistent with the same order of magnitude modeled for the ions in the small cells and the EU Directive experimental data sets (10^{-9} m^2 s^{-1}). The relatively higher velocity of the cations at this scale was related to the different pathways for the cations associated with the interlayer exchange positions of the smectites (Van Loon et al., 2007). In the landfill, sodium exchange was better simulated than the columns of the EU Directive. The higher concentration of sodium in the landfill leachate compared with the El Garráf leachate and the lower concentration of ammonium were responsible for the observed behavior. However, in addition to the occurrence of pure diffusion, the distribution of the solutes agreed with the significant mineralogical and physical changes in the landfill clay with increasing depth. The upper zone of the profile (<0.5-m depth) contained less clay and more carbonates, and the lower part (0.5-m depth) contained a more clay-like, compacted material. Apart from the scale effect, the natural and non-reworked clay at depths greater than 0.5 m performed better as a true natural barrier for the solutes that were highly concentrated in the upper part of the clay barrier.

4. Conclusions

Solute transport processes through a natural clay barrier were studied using a multi-scale approach. Conservative anion diffusion (chloride) was modeled at different scales. An order of magnitude of 10^{-10}–10^{-9} m^2 s^{-1} for the apparent diffusion coefficient was used to fit the chloride profiles at the different scales. However, at larger space and time scales, the transport velocity was retarded due to the presence of more consolidated, non-mechanically disturbed clay materials in the natural clay at different depths. The modeling of the cation transport was studied through cation exchange distribution variations. The modeling should be complemented using kinetic constraints to control the mineral dissolution. Calcium primarily dissolved from carbonates and silicates to achieve the chemical equilibrium conditions, which strongly buffered the distribution of the exchangeable cations. Thus, in most cases, it is difficult to accurately predict the migration profiles of sodium and ammonium.

The mixing of materials in the landfill base and the artificial compaction may produce a specific skin under the landfill, where the solutes are transported or retained. It is important to understand why this occurs for further studies of the clay materials so that these differences can be included in future models. The next step in this study is to better model the cation exchange and other reactions, such as the redox potential, which affects the dissolution-precipitation of minerals and organic evolution, to test the behavior of a more extended family of dissolved contaminants, including heavy metal ions.

Acknowledgments

This work was supported by the Spanish Environmental Ministry (MMA: I+D+i A113/2007/3_02.6) in cooperation with Geotecnia y Cimientos S.A. (GEOCISA), Centro de Experimentación de Obras Públicas (CEDEX), and the Instituto de Ciencias de la Tierra Jaume Almera (ICTJA).

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Journal of Environmental Management 95 (2012) S182–S187

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Interaction of organic contaminants with natural clay type geosorbents: Potential use as geologic barrier in urban landfill

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ARTICLE INFO

Article history:
Received 31 August 2009
Received in revised form
4 January 2011
Accepted 7 February 2011
Available online 3 March 2011

Keywords:
Clay
Adsorption isotherm
Organic contaminants

ABSTRACT

The aim of this work is to characterize the capability of several clay materials as preservative of organic pollution for use as landfill barrier. Interaction of representative organic pollutants with different polarity and water solubility (atrazine, benzamide, methomyl, paraquat and toluene) with several clay materials coming from several locations of Spain were studied. Batch suspension method was used to study the pesticide adsorption onto the clay sorbents in solution conditions that simulate the composition of a young leachate in its aerobic acetogenic stage (pH = 5 and $I = 0.15$) The obtained data of the analytes sorption were modelized by several sorption isotherm models, and the best fitted data were got with a generalized Langmuir adsorption isotherm. The higher maxima adsorptions were observed for paraquat (50–62 mmol kg^{-1}) and toluene (19–34 mmol kg^{-1}) whereas more hydrophobic compounds present lower adsorption (0.7–2.5 mmol kg^{-1}). Paraquat is the compound that presents the higher bonding coefficients. Therefore these clays could be used as components of the multibarriers in controlled urban landfill.

1. Introduction

The great amount of solid wastes generated by the cities can be processed in different ways, incinerated, sent to composting plants or simply deposited in landfills. Municipal solid waste (MSW) disposal in landfill may cause both mechanical and geochemical perturbation in the underlying geological substrate. In order to avoid this risk, the technical requirements for the landfill of MSW ought to fulfil the technical requirements of the Landfill EU directive (2003/33/CE). This legislation establishes that a geological barrier of at least 1 m thick ought to give a hydraulic conductivity of 10^{-9} m s^{-1}.

Although the urban landfills are included within the landfills of non-dangerous wastes, due to the own characteristics of the spill and the water percolation thorough the waste, mainly rainwater (Abbas et al., 2009), leachates are generated with characteristic physicochemical properties. These leachates can cross the barrier of the garbage dump, contaminating the surroundings and the ground waters. The leachates are a complex and variable mixture of soluble organic and inorganic compounds, microorganisms and suspended solids in aqueous media. The composition and volume of leachate also vary not only between different urban landfills but with the age of the garbage dump (Chen, 1996). A young leachate, between two and five years of antiquity, is characterized by a high relation between biological oxygen demand (BOD) and dissolved organic carbon (DOC), low pH and high organic load, due to volatile fatty acids (Tchobanoglous et al., 1993). In old leachates, the BOD/DOC ratio is smaller than 0.1, the pH value is greater than 7.0, and the organic load decreases due to the volatile fatty acid absence (Banar et al., 2006).

Prediction and assessment of risk of soil and ground water contamination from landfills by organic compounds require an understanding of contaminant transport through the geological barrier of the landfill (Rodriguez-Cruz et al., 2007; Xie et al., 2009). Sorption/desorption onto solid phase of barrier is one of the most important processes influencing movement of organic pollutants in natural systems. Sorption with reference to a pollutant is the transfer from an aqueous phase to the solid phase in contact with it (Ehlers and Loibner, 2006; Abelmann et al., 2005; Gramatica et al., 2000).

There are several chemical characteristics of the soil such as pH, organic matter concentration, clay mineralogy, CEC and Eh which

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doi:10.1016/j.jenvman.2011.02.011

have effect on sorption (and degradation) of pesticides (Dorado et al., 2003; Lesan and Bhandari, 2004) A soil with more clay and/or organic matter usually will adsorb more quantity of organic pollutant from leachates and retard the chemical in its downward movement (He et al., 2006; Abate and Masini, 2005; Inoue et al., 2004).

The global objective of the investigation project developed in our laboratories is to evaluate the effectiveness of clay materials as mineral barrier for urban landfills, to establish the suitability of the usual properties measured in these materials for clay characterization and selection, and to establish the critical parameters to guarantee that the clay material is functional as geologic barrier. Within this project in this work we evaluate the capability of several clay materials for restrain contaminant organic compounds. The behavior of five selected Spanish clay materials of different characteristics is studied. As organic chemical models atrazine, benzamide, methomyl, paraquat and toluene were selected. In order to simulate the conditions of solution present in a leachate, the sorption study was performed in presence of a young leachate model, characterized by an elevated organic and inorganic charge and an acid (5.0) pH value.

2. Theory

Sorption isotherms are used for quantifying the maximum amount of chemical that can be sorbed onto the solid phase, and the affinity between solutes and the solid phase (Aboul-Kassim and Simoneit, 2001; Schwab et al., 2006). The most common sorption isotherms used for organic compounds are represented by Langmuir and Freundlich equations. The Langmuir isotherm model is expressed as:

$$q = \frac{QK_L C_e}{1 + K_L C_e} \quad (1)$$

where q is the amount sorbed per unit mass of sorbent and C_e is the equilibrium solution concentration of sorbate. Two parameters, maximum sorption (Q) and bonding energy coefficient (K_L) are used to describe the sorption capacity of material. However, the theoretical assumption of Langmuir model is that the energy of sorption is the same for all surface sites and independent on degree of coverage. Other authors have generalized the Langmuir equation (GL) in order to take into account the degree of heterogeneity of sorbent surface (Sohn and Kim, 2005; Tsai et al., 2005). This isotherm is the most promising extension of the Langmuir and Freundlich isotherms (Cheng et al., 2010). The isotherm is expressed as

$$q = \frac{Q(K_{min} C_e)^{\beta}}{1 + (K_{min} C_e)^{\beta}} \quad (2)$$

where K_{min} is the minimum value of bonding energy coefficient K_L and β is a constant related to the degree of heterogeneity, ranging from 0 to 1 (corresponding to very flat to very sharp distribution) when no competition for adsorption points is present.

3. Materials and methods

3.1. Materials

Five clays were selected from significant ceramic raw material quarried in Spain. The selection criteria was to have a minimum clay content of non expandable clays (<5% smectite) to reach mechanical stability and to assure $k < 1 \times 10^{-9}$ m s^{-1} required from Landfill EU directive. Their physical and mineralogical characteristics are shown in Table 1. All materials were quarried in situ (500 kg) and stored. 100 kg of each material was dried in a rotary kiln at 60 °C, then ground to <5 mm size, homogenized and packaged in polyethylene bags.

The Ariño (Teruel) quarry from the Cretacic age (González-López et al., 2005) provides high grade kaolinitic clay. The Miocene clays from the Pantoja (Toledo) quarry (García-Calleja, 1991) are illitic (dioctahedral), with significant contents of kaolinite and low content of smectite (di- and tri-octahedral). Illite–smectite mixed-layer minerals are not significant in this clay. The quarry of Bailén, in the province of Jaén, also contains clays that come from the Miocene in the Tertiary period (Gonzalez et al., 1998). The black clay minerals consist essentially of illite and smectite, in addition with some kaolinite. It is the material with less phyllosilicate content. Clays from the Carboneros (Jaén) quarry (Vazquez and Jimenez-Millan, 2004) are Triassic and are only illite. Finally, the Papiol clay material from Barcelona has a quarry with red clays too, but

Table 1
Physical and chemical properties of clay samples.

Parameter	Samples				
	Ariño	Bailen	Carboneros	Pantoja	Papiol
Clay, %	60	30	26	50	44
S_{BET}, m^2 g^{-1}	20.1	29.7	38.7	42.3	19.5
CEC, cmol g^{-1}	12.3	13.2	12.7	15.7	15.3
C org, %	1.18	0.77	0.01	0.06	0.01
C inorg, %	1.7	11.0	8.2	1.8	8.7
Quartz, %	20	29	17	14	26
Phyllosilicates, %	74	47	69	72	60
Kaolinite, %	62	6	<1	11	9
Illite, %	12	31	69	60	34
Smectite, %	<1	10	–	1	17
Microcline, %	1	3	4	4	4
Albite, %	1	3	<1	10	2
Dolomite, %	2	4	7	<1	<1
Calcite, %	1	13	<1	<1	7
Other minerals (presence)	Siderite	Pyrite	Hematite	–	Hematite
Fe_2O_3 amor, %	0.2	0.3	1.7	0.4	1.5
k, m s^{-1}	1.93×10^{-10} (1.79)	4.07×10^{-10} (1.76)	8.21×10^{-10} (1.93)	1.69×10^{-10} (1.67)	5.22×10^{-10} (1.95)

Mineralogical composition, in mass percentage of crystalline fraction.
<1: detected at concentrations lower than 1%.
k: hydraulic conductivity measured in a triaxial cell (sample dry density in g cm^{-3}).

coming from the Pliocene, in the Tertiary period. This last clay has the most diverse mineralogy. It presents significant quantity of the three most common phyllosilicates: illite, smectite and kaolinite.

3.2. Analytical methods

Semi-quantitative mineralogical analysis of crystalline fraction of sludge and sediments was carried out by X-ray diffraction (XRD), using a SIEMENS D-5000 diffractometer equipped with a copper cathode, a nickel filter, operating at a speed of 0.48° per min from 3° to 70° and a current and voltage of 40 mA and 40 kV, respectively. Bulk sample was analyzed by the random powder method and the oriented slides method was applied for the <2 μm size fraction (Moore and Reinolds, 1989). To semi-quantify the minerals in the bulk sample the procedure proposed by Schultz (1964) was used (UNE 22-161-92 standard method for mineralogical quantification of clay samples containing sepiolite).

The specific surface was determined by BET analysis using a Micromerits® GEMINI V instrument (multipoint N_2 adsorption after degassing under N_2 flow during 18 h at 90 °C). The cation exchange capacity (CEC) was determined at room temperature by Na^+ homoionization (1 M sodium acetate at pH = 8) and Mg^{2+} displacement (0.5 M $MgNO_3 \cdot 5H_2O$ at pH = 5) (Rhoades, 1982).

3.3. Sorption experiments

The organic compounds used have been selected ranging in a broad interval of water solubility from the highly soluble paraquat, due to its cationic character, to sparingly soluble atrazine or toluene. Their physicochemical properties are summarized in Table 2. The classical batch suspension method was used to measure the organic compound adsorption onto the clay material. In the adsorption experiments, unless stated otherwise all organic solvents were HPLC grade and all other reagents were ACS grade. Stock solutions of organic sorbates of about 1000 mg L^{-1} were used to make a series of solutions in a young synthetic leachate. The composition of this leachate is shown in Table 3 and is characterized by an acidic pH (pH = 5.0) and high organic and inorganic loads (I = 0.15 mol L^{-1}) that simulate the composition of a leachate in its aerobic acetogenic stage.

A 25 mL aliquot of solution containing the studied organic compounds was added to 0.250 g of clay in a 30 mL glass centrifuge tube. The ranges of initial concentrations of organic compounds were 5–40 mg L^{-1} for atrazine, 5–60 mg L^{-1} for benzamide and methomyl, 5–150 mg L^{-1} for paraquat and 20–50 mg L^{-1} for toluene. Within each set of samples, a group of soil-free blanks as control was also monitored. The control series did not indicate any significant degradation or sorption losses during the course of experiments. Toluene solutions can present losses for evaporation and their adsorption studies were performed with the minimum air chamber in the tube as possible. All soil–chemical combinations were replicated two times. The tubes were shaken mechanically on an orbital shaker (Selecta model Rotavit, Spain) at 220 min^{-1} for 24 h thermostatized at 30 °C in the dark. Preliminary studies indicate that 24 h is enough time to reach an apparent equilibrium.

Table 2
Selected physicochemical properties of adsorbates at 25 °C and pH 5.0.

Compound	M.W.	Molar vol., $cm^3\ mol^{-1}$	Solubility, g L^{-1}	K_{ow}
Atrazine	215.68	169.8	0.07	639
Benzamide	121.14	108.1	8.4	60.2
Methomyl	162.21	137.9	11	50.5
Paraquat	257.16		700	–
Toluene	92.14	105.7	0.30	682

M.W. Molecular weight; K_{ow}, n-octanol/water partition coefficient.

Table 3
Composition of synthetic leachate.

Compound	Concentration, g L^{-1}
$CaCl_2 \cdot 6H_2O$	3.92
$MgCl_2 \cdot 6H_2O$	1.48
$Mg(CH_3CO_2)_2 \cdot 4H_2O$	1.14
$NH_4CH_3CO_2$	3.51
KCH_3CO_2	1.35
$NaCH_3CO_2$	3.47
$Na_2SO_4 \cdot 10H_2O$	0.709
HCH_3CO_2	3.29

After equilibration, the suspensions were decanted and filtered through syringe acetate membranes of 0.45 μm pore size (Scharlab, Spain). The filtrate was placed in a 50 mL vial at 4 °C until analysis. Except paraquat, which is highly soluble in water, all other solutes were added via methanol carrier, keeping the methanol percentage in the range of 0.05–2%. Preliminary experiments have shown that although methanol content varied slightly over an isotherm, there is not effect on bulk solution properties if it is kept lower than 2% (Kan et al., 1998).

The analysis of pesticides in the solutions was performed with an HPLC Jasco system consisted of a plus quaternary gradient pump model PU-2089, a column oven model CO-2067, an UV-DAD detector type MD-2010 Plus programmable and an automatic sample Injector Model AS-2055. The data were collected and analyzed with a Jasco computing system (ChromPass Chromatography Data System, version 1.8.6.1). The chromatographic column was a Kromasil $100C_{18}$ column (5 μm, 4.6 × 150 mm, Scharlab Science, Spain). The separations were performed at 40 °C. The flow rate was maintained at 1 ml min^{-1} by a gradient controller and solvent delivery system with continuum degassing. The injection volume was 20 μL. Other chromatographic conditions are depicted in Table 4.

3.4. Data analysis

The data of batch experiment were modelled by using both Langmuir and generalized Langmuir models. Langmuir isotherm was used for comparison of homogeneity and heterogeneity based isotherm models. Nonlinear regression was used to determine the parameters of models. The nonlinear regression was performed using the utility Solver of Excel 2002 Microsoft® package software. The residual root mean square error (RMSE) was used as performance function

$$\mathrm{RMSE} = \sqrt{\frac{1}{n}\sum_{i=1}^{m}(Q_i - q_i)^2} \quad (3)$$

where Q_i are the data of batch experiments, q_i are the estimated values associated with Q_i, m is the number of data of batch experiments, and n is the degree of freedom. In order to estimate the

Table 4
Wavelength and mobile phases employed for individual analysis of pesticides.

Compound	Wavelength, nm	Mobile phase
Atrazine	254	Methanol/water (70 + 30, v/v)
Benzamide	220	Methanol/water (50 + 50, v/v)
Methomyl	240	Methanol/water (30 + 70, v/v)
Paraquat	257	3.0 g Heptanesulfonic acid, 13.5 mL orthophosphoric acid, 10.3 mL ethylenediamine and up to 1000 mL of water.
Toluene	254	Methanol/water (70 + 30, v/v)

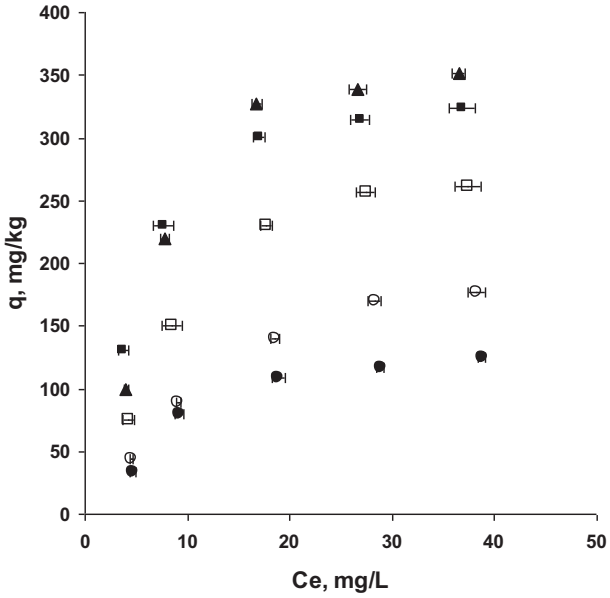

Fig. 1. Adsorption isotherms of atrazine from synthetic lixiviate solution on studied clay adsorbents at 30 °C. (●) Ariño clay, (■) Bailen clay, (□) Carboneros clay, (▲) Pantoja clay and (○) Papiol clay.

fitting degree, the value of the correlation coefficient, R^2, was estimated for the two approximations.

4. Results and discussion

4.1. Quarry material characterization

The quarries were selected with different clay contents, from 26% in Carboneros quarry to 60% in Ariño quarry. The mineralogical composition (Table 1) shows that clays from Pantoja, Carboneros and Ariño are the quarries with higher content of phyllosilicates, round 70%. All materials also contain a wide spectrum of accessory minerals with the presence or absence of carbonates, and a low amount of amorphous iron oxides.

Pantoja and Carboneros clays presented the high specific surface because their richness in phyllosilicates of illite type. In the case of Ariño clay, even being the richest in phyllosilicates, it has poor specific surface, since it has great amount of kaolinite, which is a phyllosilicate with a higher crystalline size. The cation exchange capacity, does not present significant differences among clays, locating itself between 12 and 15 cmol kg^{-1} because they have a relative low amount of smectite, and these phyllosilicates are those that more affect this property. In relation to their hydraulic properties, all materials fulfilled the technical requirements of the landfill EU directive (10^{-9} m s^{-1}).

Papiol, Carboneros and Bailen quarries have an important amount of carbonates. In the quarries selected the organic matter is low. Only Ariño and Bailen quarries have an appreciable quantity of organic carbon. Pantoja quarry presents neither organic nor inorganic carbon. This clay has a great quality and purity.

4.2. Adsorption of pollutants onto clays

In order to characterize this interaction, sorption isotherms for all compounds and clay materials were constructed. As an example of the obtained isotherms, Fig. 1 shows the experimental data obtained for atrazine interactions with all clay materials. The experimental data were modelled by the isotherm models. Langmuir and the generalized Langmuir equations present the best correlation values. R^2 values for Langmuir model are lower than GL model (Table 5), indicating that GL model is more suitable than L one for modelling the sorption of organic compounds on geological sorbents. The data of batch experiments and the fitting to L and GL models for benzamide-Pantoja isotherm are shown in Fig. 2. The

Table 5
Parameters in Langmuir and generalized Langmuir adsorption isotherm models of organic compound studied onto clay adsorbents.

Compound	Clay	Langmuir isotherm			Generalized Langmuir isotherm			
		Q_L, mmol kg^{-1}	K_L, L mg^{-1}	R^2	Q_{GL}, mmol kg^{-1}	K_{min}, L mg^{-1}	β	R^2
Atrazine	Ariño	0.79 ± 0.10	0.080 ± 0.027	0.927	0.58 ± 0.02	0.137 ± 0.060	2.11 ± 0.23	0.998
	Bailen	1.80 ± 0.10	0.166 ± 0.033	0.986	1.08 ± 0.01	0.209 ± 0.016	1.72 ± 0.05	0.999
	Carboneros	1.71 ± 0.17	0.078 ± 0.020	0.987	1.31 ± 0.03	0.130 ± 0.027	1.75 ± 0.12	0.999
	Pantoja	2.19 ± 0.29	0.100 ± 0.035	0.972	1.67 ± 0.03	0.160 ± 0.043	2.21 ± 0.15	0.999
	Papiol	1.28 ± 0.12	0.052 ± 0.011	0.999	0.96 ± 0.05	0.091 ± 0.021	1.48 ± 0.14	0.999
Benzamide	Ariño	0.67 ± 0.06	0.100 ± 0.025	0.983	0.54 ± 0.03	0.147 ± 0.053	1.77 ± 0.09	0.993
	Bailen	1.19 ± 0.28	0.040 ± 0.019	0.979	0.72 ± 0.03	0.098 ± 0.045	2.20 ± 0.22	0.999
	Carboneros	1.11 ± 0.15	0.031 ± 0.008	0.990	0.86 ± 0.02	0.053 ± 0.010	1.62 ± 0.08	0.999
	Pantoja	1.40 ± 0.23	0.026 ± 0.008	0.990	0.91 ± 0.02	0.057 ± 0.010	1.71 ± 0.08	0.999
	Papiol	1.82 ± 020	0.029 ± 0.006	0.998	1.31 ± 0.06	0.056 ± 0.012	1.48 ± 0.10	0.999
Metomil	Ariño	2.90 ± 0.26	0.049 ± 0.011	0.993	2.274 ± 0.066	0.081 ± 0.015	1.52 ± 0.09	0.999
	Bailen	3.32 ± 0.38	0.031 ± 0.007	0.995	2.41 ± 0.14	0.058 ± 0.015	1.47 ± 0.13	0.999
	Carboneros	1.72 ± 0.12	0.043 ± 0.007	0.996	1.382 ± 0.012	0.069 ± 0.003	1.38 ± 0.02	0.999
	Pantoja	1.67 ± 0.72	0.013 ± 0.009	0.984	0.768 ± 0.043	0.050 ± 0.033	2.16 ± 0.26	0.999
	Papiol	1.12 ± 0.17	0.024 ± 0.007	0.994	0.741 ± 0.024	0.052 ± 0.009	1.57 ± 0.08	0.999
Paraquat	Ariño	42.25 ± 0.67	0.218 ± 0.036	0.974	59.9 ± 1.1	0.190 ± 0.004	0.26 ± 0.01	0.999
	Bailen	59.83 ± 0.68	0.435 ± 0.037	0.995	58.60 ± 0.40	0.382 ± 0.056	1.32 ± 0.10	0.999
	Carboneros	35.78 ± 0.32	0.197 ± 0.022	0.987	34.74 ± 0.03	0.103 ± 0.015	1.85 ± 0.05	0.999
	Pantoja	55.10 ± 1.37	0.371 ± 0.077	0.968	69.4 ± 9.4	0.272 ± 0.032	0.38 ± 0.11	0.997
	Papiol	60.94 ± 0.22	0.343 ± 0.009	0.999	61.59 ± 0.28	0.351 ± 0.014	0.92 ± 0.03	0.999
Toluene	Ariño	89 ± 39	0.013 ± 0.008	0.969	34.3 ± 2.7	0.053 ± 0.085	2.80 ± 0.58	0.995
	Bailen	42.8 ± 3.9	0.026 ± 0.007	0.936	23.31 ± 0.20	0.054 ± 0.032	4.21 ± 0.20	0.996
	Carboneros	71 ± 38	0.011 ± 0.008	0.956	25.8 ± 2.2	0.046 ± 0.011	3.21 ± 0.82	0.992
	Pantoja	35.0 ± 3.9	0.031 ± 0.007	0.983	79.39 ± 0.70	0.042 ± 0.030	0.64 ± 0.33	0.985
	Papiol	62 ± 31	0.011 ± 0.007	0.977	19.9 ± 1.3	0.056 ± 0.068	2.77 ± 0.46	0.997

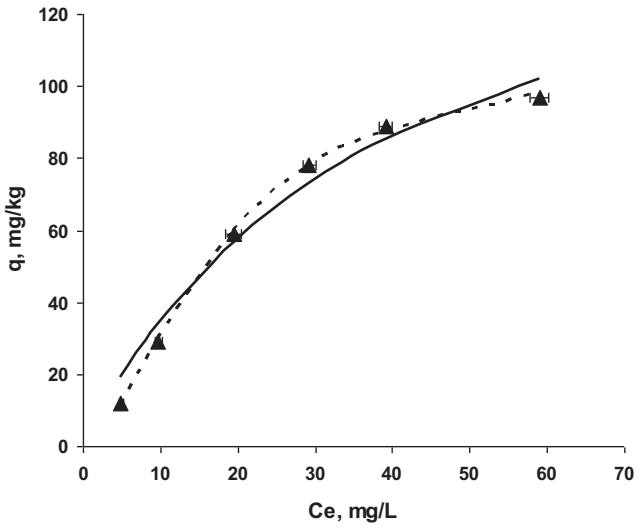

Fig. 2. Isothermal adsorption of benzamide from synthetic lixiviate solution onto clay adsorbent from Pantoja at 30 °C. (▲) Experimental data; (—) simulation of Langmuir model; (–) simulation of generalized Langmuir model.

curve of GL model is fitted closely to the data of batch experiment. It can be observed in the figure that the sorbate presents low sorption at all concentration levels. This result is observed in all isotherms and it is due the high organic load present in synthetic lixiviate solution used as model. Several studies have reported that the increase of dissolved organic carbon in the solution causes a decrease of organic pollutant adsorption (Ben-Hur et al., 2003 and Tsai and Lai, 2006) The competition between the organic load and sorbates in the lixiviate solution causes β values higher than 1 in the curve fitting. Paraquat, because of its cationic character, presents a different behavior observing β values less than 1 for Ariño, Pantoja and Papiol clay materials. These values indicate surface heterogeneity.

Comparing the calculated data obtained for all organic compounds, the higher maxima adsorptions were observed for paraquat, methomyl and toluene. Their different chemical characteristics indicate different sorption mechanisms. Paraquat is mainly adsorbed by ionic exchange to clays (Seki and Yurkaoc, 2005; Tsai and Lai, 2006). Otherwise, toluene and other hydrophobic organic compounds are preferably absorbed onto soil organoclays and organic matter present in the solid material (Sharmasarkar et al., 2000) although in general the low organic content of the studied sorbents cannot explain the high adsorption observed. The sorption of hydrophobic organic compounds by natural inorganic surfaces can be an important uptake route where the organic carbon content is low (Ehlers and Loibner, 2006). Various mechanisms by which organic chemical may sorb onto inorganic solids include adsorption to specific surface sites due to electron donor–acceptor interaction or adsorption of charged molecules from the aqueous phase to complementary charged surface by electrostatic interaction (Schwarzenbach et al., 2003).

We study the possible correlation between the maxima adsorption of each organic pollutant and the clay material characteristics, using the software SPSS 16, and we have observed few correlations. Only maxima adsorption of methomyl correlates with the organic matter concentration in clay materials ($R = 0.887$). Otherwise atrazine adsorption correlates with specific surface and illite concentration in the adsorbents ($R = 0.914$ and 0.918, respectively). Benzamide, methomyl and toluene adsorption do not present evident correlation with some clay characteristics. These results indicate that several clay components contribute to retain the organic compounds on the clay material, especially phyllosilicate minerals.

The data of bonding energy coefficients present differences from the maximum adsorption observed for the compound. Although paraquat is again the compound that presents the higher bonding coefficients, now the compounds with less affinity for the clay adsorbents are the more hydrophobic compounds: methomyl and toluene. These results are mainly due to the low organic concentration presents in the adsorbents and indicate that the adsorption occurs by an electron donor–acceptor interaction. Atrazine and benzamide present medium bonding affinity.

5. Conclusions

From the results obtained in this work, the isotherm data of organic pollutant adsorption onto clay material used in this study are best fitting with generalized Langmuir adsorption isotherm. This isotherm considers the competition between sorbate and organic matter dissolved in the leachate and the inhomogeneity of the sorbent which presents several sorption organic and inorganic sites for organic pollutant adsorption. The results of the sorption experiments show that the clay material present adequate maximum adsorptions, bonding energy coefficients and hydraulic conductivities for organic compound restrain, especially the clay materials from Pantoja, Bailen and Ariño. Therefore these clay types of materials could be used as components of the multibarriers in controlled urban landfill.

Acknowledgments

The authors are grateful to GEOCISA and Spanish Environmental Ministry for financial support of this work (Project A113/2007/3-02.6)

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Journal of Environmental Management 95 (2012) S188–S192

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Chemical properties and biological activity in soils of Mallorca following twenty years of treated wastewater irrigation

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ARTICLE INFO

Article history:
Received 29 September 2009
Received in revised form
8 June 2010
Accepted 14 August 2010
Available online 19 September 2010

Keywords:
Wastewater reuse
Agricultural soils
Chemical parameters
Soil microbial biomass
Enzymatic activities

ABSTRACT

On the Mediterranean island of Mallorca, the use of secondary-treated municipal wastewater in irrigation was introduced with the construction of the first wastewater treatment plants in the 1970s. In this study, the chemical properties and biological activity of 21 arable soils, irrigated for more than 20 years with secondary-treated wastewater, were tested in order to assess their quality. Soil quality was evaluated by measuring cation exchange capacity, pH, calcium carbonate equivalent, soil organic matter, total nitrogen, available phosphorus, water-soluble organic carbon, soil microbial biomass, soil basal respiration, and the activities of the enzymes dehydrogenase, β-glucosidase and alkaline phosphatase. No negative effects of the irrigation treatment were observed on the measured soil parameters. Indeed, soil water-soluble organic carbon, soil microbial biomass and β-glucosidase and alkaline phosphatase activities increased under treated wastewater irrigation. Biological activity of soils irrigated with treated wastewater was affected mainly by soil organic matter content. Although the typical crop management of alfalfa, and other forage crops associated with treated wastewater irrigation, may have contributed to the increase of these parameters, the results suggest that irrigation with treated wastewater is a strategy with many benefits to agricultural land management.

1. Introduction

The use of wastewater for irrigation is well established in arid and semiarid areas around the world (Hamilton et al., 2007; Qadir et al., 2007). The main advantage of wastewater irrigation, in addition to the implied nutrient input, is the constant availability of this water resource (Toze, 2006; WHO, 2006). Irrigation with untreated wastewater may increase soil organic matter, nitrogen and concentrations of major cations (Siebe, 1998; Angin et al., 2005). However, it has been associated with negative impacts on health (Gantzer et al., 2001; Rutkowski et al., 2007). Moreover, long-term irrigation with untreated wastewater could lead to a heavy metal accumulation, and a consequent loss of soil quality, depending on the origins of the wastewater (Lucho-Constantino et al., 2005; Mapanda et al., 2005). For these reasons, treatment of wastewater is generally recommended before its use in irrigation (Jiménez-Cisneros, 1995; Toze, 2006).

The effects on soil properties of irrigation with treated wastewater over different lengths of time have been studied by several authors. Qian and Mecham (2005) and Rusan et al. (2007) each reported an increase in soil salinity and Na accumulation, with higher values associated with longer periods of treated municipal wastewater irrigation. Such increases in salinity can lead to a decrease in aggregate stability and soil hydraulic conductivity (Qian and Mecham, 2005), however, the presence of Ca and Mg in calcareous soils can mitigate this deleterious effect (Lado and Ben-Hur, 2009). Schipper et al. (1996) did not observe any changes in soil biological and biochemical parameters after 3 years of irrigation with a tertiary-treated domestic effluent, while Chen et al. (2008) found an enhancement of soil enzyme activities following 10 years of treated municipal wastewater irrigation.

On the Mediterranean island of Mallorca, irrigation of arable soils with treated municipal wastewater was introduced in the seventies, when the first wastewater treatment plants were built. The use of secondary-treated wastewater made the support of traditionally irrigated lands possible in areas where the groundwater had become saline as a result of seawater intrusion into the aquifers (Mateos-Ruiz and Lopez-Garcia, 2003). Such areas have been cultivated with crops destined for animal feed; principally alfalfa (*Medicago sativa*), a pluriannual crop, usually cultivated for 5–7 years, but also maize (*Zea mais*) and other forage crops. Irrigation is typically carried out via a process of flooding.

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doi:10.1016/j.jenvman.2010.08.017

Soil management has an important influence on soil biological activity: microbial biomass and enzymatic activities are sensitive to intensive cultivation (Riffaldi et al., 2002), but are stimulated by organic matter supply (Pascual et al., 2002). For these reasons, soil biological parameters are considered to be good indicators of soil quality (Bandick and Dick, 1999).

The objective of this study was to evaluate the quality of soils which had been subjected to irrigation by secondary-treated municipal wastewater over a period of more than 20 years using selected soil chemical and biological indicators.

2. Material and methods

In July of 2006, one composite sample (consisting of four randomly collected sub-samples) was taken from the plough layer (0–20 cm) at 21 sites on the island of Mallorca which had been irrigated with secondary-treated wastewater for more than 20 years. All of the sites had been farmed conventionally, according to local practice. Although some of the fields were not under cultivation at the moment of sampling, alfalfa had been the main crop in all of them, and had been rotated with other crops such as maize, barley and oats.

The study sites were located in the four areas of Mallorca where irrigation with treated wastewater was first introduced. In these areas, excessive extraction of local groundwater had led to the intrusion of seawater into the aquifers (Lopez-Garcia and Mateos-Ruiz, 2003), and secondary-treated wastewater is the only available source of water, consequently there were no other non-wastewater irrigated sites available which shared similar management and could therefore have been utilized as controls.

Calcisols, sometimes associated with Regosols and Luvisols, were the predominant soil types in all the areas of study. Sites were irrigated by flooding with secondary-treated wastewater from the closest municipal wastewater treatment plant. Treatment of wastewater was performed with an activated-sludge system in all cases. Selected physical and chemical characteristics of the secondary-treated wastewater are reported in Table 1.

Field-moist soil samples were immediately sieved (2 mm), moistened to water holding capacity and incubated at 25 °C for 10 days to permit uniform rewetting before the analysis of soil microbial biomass, basal respiration and soil enzymatic activities. In addition, sub-samples of each soil were air dried and ground for chemical analysis.

Cation exchange capacity was measured by the ammonium acetate method (Rhoades, 1982). Soil pH was determined in a soil:-water suspension (1:2.5) and percentage of calcium carbonate equivalent was measured using a Bernard calcimeter. Soil organic matter was determined by the wet oxidation method with dichromate (Nelson and Sommers, 1982), total nitrogen by the Kjeldahl method (Bremmer and Mulvaney, 1982) and available phosphorus measured using the extraction method of Olsen et al. (1954). Water-soluble organic carbon was measured after shaking 8 g of soil with 40 ml of distilled water (soil:water; 1:5) for 30 min in an end-to-end shaker. The resulting suspension was centrifuged and the extract filtered to <0.45 μm. The filtrate was used to determine soluble organic carbon using a Shimadzu TOC-5000A analyser. Soil microbial biomass was determined using a fumigation-extraction procedure (Vance et al., 1987). Basal respiration was estimated using the method described by Alef (1998), while dehydrogenase, β-glucosidase and alkaline phosphatase activities were determined as reported by Tabatabai (1982). Dehydrogenase activity was measured using triphenyltetrazolium chloride as a substrate; samples were incubated for 24 h at 37 °C. β-Glucosidase and alkaline phosphatase activities were measured using p-nitrophenyl-β-D-glucoside and p-nitrophenyl phosphate as substrates, respectively; samples were incubated for 1 h at 37 °C for both analyses. All measured parameters were calculated on a dry matter basis.

Table 1
Physical and chemical characteristics of secondary-treated wastewater.

	Mean	Standard deviation
pH	7.8	0.4
EC 25 °C (dS m^{-1})	2.32	0.77
SS (mg l^{-1})	17.0	16.6
NH_4-N (mg l^{-1})	8.67	11.47
NO_3-N (mg l^{-1})	5.42	5.25

EC, electrical conductivity; SS, suspended solids.

The data generated from the analyses of soils irrigated with treated wastewater for more than 20 years were compared with data from 23 non-wastewater treated agricultural soils that were sampled and analyzed in July of 2003 for a previous study (Farrús et al., unpublished data). These agricultural soils included a variety of soil types, mainly Calcisols, but including Regosols and Luvisols, and were generally cultivated with herbaceous crops. Soil samples from both studies were processed following the same methodologies.

Descriptive statistics and Pearson's correlations were calculated using SPSS 15.0 for Windows. A *Student's independent sample t-test* was used for comparisons between means when variances were equal and an unequal variance *t*-test was used when variances were unequal (Ruxton, 2006). The Levene's test was performed to test for homogeneity of variances.

3. Results and discussion

Data from non-wastewater treated soils were collected from different soil types and cropping systems and can be considered as reference values for a representative range of soils from the island of Mallorca. However, the sampling of soils irrigated with treated wastewater was limited to the few locations where this type of irrigation had been established for more than 20 years. Consequently, less variation was observed in the chemical and biological soil parameters of the soils irrigated with treated wastewater than in those of the non-wastewater treated soils (Table 2).

With the exception of soil pH, available phosphorus and water-soluble organic carbon, the chemical properties of soils irrigated with treated wastewater were generally within the same ranges as the non-wastewater treated soils (Table 2). Previous studies, concerning soils under long-term irrigation with untreated wastewaters, have reported an increase in soil C and N contents (Siebe, 1998; Friedel et al., 2000), and even an increase in cation exchange capacity (Angin et al., 2005) which can be attributed to the high contents of organic compounds in the applied wastewater. However, no such increase was observed when the wastewater was treated before use (Qian and Mecham, 2005).

Soil pH was significantly higher in soils irrigated with treated wastewater, compared to non-wastewater treated soils. Significantly higher values of available phosphorus and water-soluble organic carbon were observed in the former group of soils, compared to the latter. Such an observed increase in soil pH, following treated wastewater irrigation, concurs with the findings of other authors (Schipper et al., 1996; Qian and Mecham, 2005), and can be attributed to the additional input of exchangeable cations, mostly sodium, created by the irrigation water (Gelsomino et al., 2006). An increase in available soil phosphorus was reported by Mohammad and Mazahreh (2003), and also by Mandal et al. (2008), in treated wastewater irrigated soils, reflecting the high phosphorus content in the wastewater used. Siebe (1998) and Angin et al. (2005) obtained similar results with untreated wastewater. The increased content of water-soluble organic carbon in the soils irrigated with treated wastewater may be related to the

Table 2
Descriptive statistics of biological activity and soil properties. Comparison between treated wastewater (1) and non-wastewater irrigated soils (2).

	T	*n*	Mean	Standard deviation	Sig
CEC	1	21	15.3	4.7	
	2	23	17.2	6.1	
pH	1	21	8.5	0.2	<0.001
	2	23	8.2	0.2	
$CaCO_3$	1	21	39.6	14.7	
	2	23	41.0	20.7	
SOM	1	21	3.28	0.75	
	2	23	3.14	1.66	
N	1	21	0.20	0.04	
	2	23	0.18	0.08	
C/N	1	21	9.5	0.5	
	2	23	9.9	1.5	
P	1	21	86.2	36.0	0.034
	2	23	55.1	55.0	
WSOC	1	21	44.8	16.2	0.037
	2	23	35.1	13.9	
MB	1	21	658	200	0.017
	2		508	200	
BR	1	21	5.57	1.90	
	2	23	4.99	1.96	
DH	1	21	1.60	0.55	
	2	23	1.15	0.95	
GL	1	21	2.44	1.13	<0.001
	2	23	1.04	0.62	
AP	1	21	7.66	2.99	<0.001
	2	23	3.33	1.63	

T, treatment; *n*, number of samples; Sig, significance; CEC, cation exchange capacity (cmol kg^{-1}); $CaCO_3$, calcium carbonate equivalent (%); SOM, soil organic matter (%); N, total nitrogen (%); P, available phosphorus (mg kg^{-1}); WSOC, water-soluble organic carbon (mg kg^{-1}); MB, soil microbial biomass (mg C kg^{-1}); BR, basal respiration (mg CO_2 kg^{-1} h^{-1}); DH, dehydrogenase activity (mmol tryphenylformazan kg^{-1} 24 h^{-1}); GL, β-glucosidase activity (mmol PNP kg^{-1} h^{-1}); AP, alkaline phosphatase activity (mmol p-nitrophenol kg^{-1} h^{-1}).
All values based on soil dry weight.

presence of dissolved organic matter (cell fragments and macromolecules) in the water (Shon et al., 2006). However, land use and management practices can also have a considerable effect on this parameter (Chantigny, 2003). For example, water-soluble organic carbon tends to increase under perennial crops such as alfalfa (Wu et al., 2003), which is one of the most common crops in the soils irrigated with treated wastewater on Mallorca.

Soil microbial biomass was significantly higher in soils irrigated with treated wastewater, when compared to that found in the other soils, although no differences were observed in basal respiration and dehydrogenase activity between the two treatments. In addition, there were strong differences in β-glucosidase and alkaline phosphatase activities between treated wastewater irrigated soils and the soils of the other treatment (Table 2). These differences could be attributed to the wastewater irrigation, as was observed by Filip et al. (1999) who found higher enzymatic activities in soils irrigated with untreated wastewater over 100 years compared to non-irrigated soils. Friedel et al. (2000) observed a similar increase in microbial biomass and dehydrogenase activity in Vertisols which had been irrigated on a long-term basis with untreated wastewater. Brzezinska et al. (2006) and Truu et al. (2009) reported a significant increase of alkaline phosphatase in soils irrigated with treated wastewater over shorter periods of time (4 and 3 years, respectively) and Chen et al. (2008) observed an enhancement of various enzymatic activities in soils irrigated with treated wastewater over 10 years. The positive effect of treated wastewater irrigation on soil microbial biomass and its associated activities can be attributed to the addition of easily decomposable organic matter and nutrients (Friedel et al., 2000; Chen et al., 2008). Nevertheless, the effect of crop type on microbial biomass and enzymatic activities must also be taken into consideration. According to Dodor and Tabatabai (2005), cropping systems that leave residues on the soil surface will enhance enzyme activity. The highest values of β-glucosidase activity obtained by these authors were found in soils directly below an alfalfa crop. Similarly, Truu et al. (2009) suggested a co-effect of municipal wastewater and vegetation on the soil microbial community.

Some basic chemical properties of the soils irrigated with treated wastewater were statistically correlated. The highest correlation was found between soil organic matter and nitrogen content (Table 3) and this was also highly correlated in the non-wastewater treated soils (Table 4). Soil organic matter was also strongly correlated with cation exchange capacity and with all the parameters related to biological activity in both groups of soils (Tables 3 and 4). However, high correlations of soil organic matter with available phosphorus and water-soluble organic carbon were observed only in the non-wastewater treated soils (Table 4). This suggests that the available phosphorus and water-soluble organic carbon in these soils are the result of soil organic matter mineralization, while the soils irrigated with treated wastewater may receive these elements directly from the irrigation water itself.

Negative correlations between pH and calcium carbonate equivalent with soil organic matter and biological parameters were found in soils irrigated with treated wastewater (Table 3). These results indicate a soil type effect and can be explained by the low biological activity of Calcareous Regosols (Adrover et al., 2007).

Soil microbial biomass was highly correlated with dehydrogenase and β-glucosidase activities. Dehydrogenase is an intracellular enzyme which is involved in microbial oxidoreductase metabolism. The high correlation of this enzyme with soil microbial biomass has been widely reported (García-Gil et al., 2000; Taylor et al., 2002). β-

Table 3
Correlations between soil properties and biological activity in long-term treated wastewater irrigated soils of Mallorca.

	pH	$CaCO_3$	SOM	N	P	WSOC	MB	BR	DH	GL	AP
CEC	−0.608**	–	0.851***	0.821***	–	–	0.860***	0.707***	0.521*	0.860***	0.685**
pH		0.457*	−0.540*	−0.513*	−0.509*	–	−0.470*	−0.614**	–	−0.593**	–
$CaCO_3$			–	−0.525*	–	–	–	−0.511*	−0.578**	−0.493*	–
SOM				0.978***	–	–	0.783***	0.737***	0.725***	0.802***	0.730***
N					–	–	0.778***	0.714***	0.759***	0.791***	0.740***
P						–	–	–	–	–	–
WSOC							–	–	–	–	–
MB								0.650**	0.735***	0.735***	0.473*
BR									0.682**	0.745***	–
DH										0.636**	0.610**
GL											0.611**

CEC, cation exchange capacity; $CaCO_3$, calcium carbonate equivalent; SOM, soil organic matter; N, total nitrogen; P, available phosphorous; WSOC, water-soluble organic carbon; MB, soil microbial biomass; BR, basal respiration; DH, dehydrogenase activity; GL, β-glucosidase activity; AP, alkaline phosphatase activity.
***, **, *Correlations are significant at $p < 0.001$, $p < 0.01$, $p < 0.05$, respectively.

Table 4
Correlations between soil properties and biological activity in non-wastewater treated agricultural soils of Mallorca.

	pH	$CaCO_3$	SOM	N	P	WSOC	MB	BR	DH	GL	AP
CEC	–	−0.812***	0.669***	0.670***	–	0.503*	0.581**	0.629**	0.547*	–	–
pH		0.466*	–	–	–	–	–	–	–	–	–
$CaCO_3$			−0.454*	−0.519*	–	–	–	–	−0.500*	–	–
SOM				0.961***	0.646**	0.845***	0.553**	0.812***	0.734***	0.501*	0.772***
N					0.704***	0.839***	0.525*	0.770***	0.659**	–	0.679***
P						0.676***	–	0.506*	–	–	–
WSOC							0.496	0.767***	0,454*	–	0.662**
MB								0.746***	0.660**	0.601**	0.715***
BR									0.760***	0.666**	0.734***
DH										0.754***	0.777***
GL											0.636**

CEC, cation exchange capacity; $CaCO_3$, calcium carbonate equivalent; SOM, soil organic matter; N, total nitrogen; P, available phosphorous; WSOC, water-soluble organic carbon; MB, soil microbial biomass; BR, basal respiration; DH, dehydrogenase activity; GL, β-glucosidase activity; AP, alkaline phosphatase activity.
***, **, *Correlations are significant at $p < 0.001$, $p < 0.01$, $p < 0.05$, respectively.

Glucosidase is an enzyme involved in the degradation of cellulose, the main component of plant residues. The high correlation of β-glucosidase and soil microbial biomass in both groups of soils, also reported by Turner et al. (2002), suggests that the total and extracellular activity of this enzyme are mainly associated with microbial biomass in soils (Dodor and Tabatabai, 2005). β-Glucosidase was also strongly correlated with cation exchange capacity and soil organic matter (Table 3). These results are in agreement with Turner et al. (2002), who suggested that β-glucosidase activity can provide a meaningful integrative measure of physico-chemical and biological soil quality parameters, and who proposed the use of this parameter in monitoring soil biological quality. Alkaline phosphatase was strongly correlated with soil organic matter and total nitrogen content (Tables 3 and 4). Deng and Tabatabai (1997) obtained significant correlations between organic carbon and phosphatases; suggesting that organic matter plays an important role in protecting and maintaining soil enzymes in their active forms.

The use of treated wastewater for agriculture has been implemented in other areas affected by seawater intrusion into the aquifers (Anderson, 2003; Bixio et al., 2006), because, in addition to implying a reduction in groundwater extraction for agricultural uses, it provides a groundwater recharge via percolation through the soil and the vadose zone (Durham et al., 2002; Asano and Cotruvo, 2004). Moreover, treated wastewater is not only a low cost source of water, but also a source of nutrients for plant growth (Vazquez-Montiel et al., 1996). Irrigation with treated wastewater may reduce the demand for synthetic fertilizers and may contribute to a decrease in the level of nutrients in rivers. Moreover, it may eliminate the need for expensive tertiary treatment and facilitate the sustainable development of landscape and agricultural irrigation (Angelakis et al., 1999). For these reasons, the use of treated wastewater for irrigation should be expanded in order to preserve fresh water resources and increase waste recycling. The forage crops associated with treated wastewater irrigation contribute to the sustainability of an acceptable soil quality, however, more information is needed concerning the consequences of utilizing this type of water in the irrigation of soils cultivated with other crops.

4. Conclusions

After more than 20 years of treated wastewater irrigation, no negative changes have been observed in the evaluated soil properties of the island of Mallorca, with the exception of an increase in soil pH caused by greater sodium supply. The enhancement in soil microbial biomass, β-glucosidase activity and alkaline phosphatase activity, in addition to relatively higher values of water-soluble organic carbon and available phosphorus, can be attributed to a combination of the irrigation regime and the agricultural management at each location. Thus, treated wastewater use in irrigation can have positive effects, not only in aspects of soil quality, but also in social terms, as it allows the maintenance of irrigated agriculture in areas where groundwater has been polluted by seawater intrusion.

Acknowledgements

The authors thank Dr. B. Jiménez and Dr. M. Truu for their constructive review, Dr. E. Baraza for her contribution, and Mr. K. MacMillan for the English language revision.

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Journal of Environmental Management 95 (2012) S193–S198

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Effect of pulp mill sludge on soil characteristics, microbial community and vegetal production of *Lolium Perenne*

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ARTICLE INFO

Article history:
Received 1 October 2009
Received in revised form
9 October 2010
Accepted 1 November 2010
Available online 30 December 2010

Keywords:
Andisol
Kraft mill sludge
Lolium perenne
Microbial community

ABSTRACT

The effect of pulp mill sludge addition (10–30 Mg/ha) to soil derived from volcanic ash (Andisol) on soil characteristics, microbial community and *Lolium perenne* L. cv quartet. biomass production was evaluated in field assays. Soil without sludge was used as a control treatment. The sludge addition improved the chemical properties of the soil. Organic matter and phosphorous content increased in the soil with increasing amounts of sludge, obtaining 35% more organic matter content with the application of 30 Mg/ha than the control soil. The phosphorous was accumulated into the soil after the end of cultivation improving the phosphorous pool in the soil. When 30 Mg/ha sludge was added to the soil, a biomass of *Lolium perenne*, was 60% more than the control soil at the end of the experiment. The analysis of soil microbial community showed that the application of sludge did not modify greatly the microbial community of fungi and bacteria even when high doses were applied.

1. Introduction

The biological wastewater treatment plants in the pulp and paper industry produce larges quantities of sludge that require safe disposal. The production of solid waste in pulp and paper production generates around 45% wastewater sludge (0.2–1.2 kg dry matter (DM)/kg of biological oxygen demand (BOD) removed), 25% ash, which can be used as pH corrector in acid soils (Zambrano et al., 2003), 15% wood cuttings and waste, and 15% other solid waste.

Chemical properties of pulp mill sludge (high organic matter content, pH, buffer capacity, nitrogen and phosphorous level, and low concentrations of heavy metals and organic pollutants) suggest that this material may represent a valuable resource as a soil amendment, improving soil fertility (Zhang et al., 2004; Gallardo et al., 2007; Ribeiro et al., 2010). The controlled disposal of pulp and paper mill sludge improves the physical, chemical and biological properties of soil (Foley and Cooperband, 2002; O'Brien et al., 2002; Aravena et al., 2007; Gallardo et al., 2007; Nunes et al., 2008; Price and Voroney, 2007; Gallardo et al., 2010), decreases soil acidification (Battaglia et al., 2007; Gallardo et al., 2007) and serves as a partial replacement of the most expensive chemical fertilizers (Snyman et al., 1998). Despite the beneficial effects of the application of sludge in the soils, some considerations must be taken into account. For example, its use requires good application practices and periodic monitoring of the quality of the soils, residues and water resources near the application area (Ribeiro et al., 2010). Land application of sludge with a low N concentration may lead to a temporary immobilization of soil N conversely, large amounts of N applied with paper mill sludge could potentially cause nitrate leaching (Feldkinchner et al., 2003). Therefore, restrictions on sludge applications based on nutrient content and plant needs or in other criteria must be established in the regulations of each country.

In Chile, soils derived from volcanic ash occupy approximately 3.1 million ha, of which nearly one million are cultivable lands. These soils are characterized by a well developed structure, high cationic exchange capacity, high water retention, and low apparent density. These soils also show high adsorption capacity of phosphate, low percentage of base saturation, high levels of exchangeable Al, and moderately acidic to strongly acidic pH levels (Gallardo et al., 1999). It is important to emphasize that high contents of phosphorus in these soils are usually present in a non-available form for plants, requiring incorporation of phosphorous fertilization to obtain an increase in crop yield (Mora et al., 2004).

The application of sludge from the pulp mill industry in laboratory scale has modified and improved some physical and chemical properties of Andisols (Gorbea series) in the south of Chile

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doi:10.1016/j.jenvman.2010.11.001

(Aravena et al., 2007; Gallardo et al., 2007), however, fields assays have not yet been performed with this type of soil. Taking all of this into consideration, the aim of this study was to evaluate the effect of adding Kraft pulp mill sludge to volcanic soil (Andisol), Temuco series, on soil characteristics, microbial community, and vegetal production of *Lolium perenne* in field assays.

2. Materials and methods

2.1. Soil and sludge

The soil used for this study was an Andisol, belonging to the Temuco Series, located in southern Chile (38° 42′ S, 73° 35′ W). The sludge used was a secondary sludge from the bleached Kraft mill wastewater treatment plant (aerated pond) and was collected from a landfill after one year disposal. In this condition the sludge becomes stable naturally and fulfills with the requirements established in the Chilean Normative (NCh 2880) ((INN, 2004)). The soil sample for the analyses was taken from 0 to 20 cm depth, air dried at room temperature, sieved through a 2 mm mesh and stored in plastic bags under refrigeration (4 °C).

2.2. Field assay

Field assays were conducted on an Andisol soil. Each plot was of 5 × 2 m with three replicates (totaling 12 plots). Stabilized sludge was added to soil (0, 10, 20 and 30 Mg/ha), incorporating it into the first upper 10 cm. This soil was incubated for 1 month in natural conditions before putting seeds of *Lolium perenne* L. cv quartet. The seeds were applied in each plot in a quantity equivalent to 25 kg/ha. The experiment lasted 105 days, and at days 45, 75 and 105 the plants were cut to evaluate the vegetal production (biomass). At the end of the experiment, the physico-chemical characteristics of the soil were determined. The effect of sludge addition on soil microbial community using molecular techniques (DGGE) was also evaluated.

2.3. Analytical methods

The physico-chemical analysis was carried out using the methodology described by Sadzawka et al. (2004). The organic carbon content was determined by the dichromate oxidation method and colorimetric determination of the reduced chromate (Cr^{+3}). pH was measured in 1:2.5 (w/v) soil/water mixture; available mineral nitrogen ($N{-}NH_4^+ + N{-}NO_3^-$) by extraction with KCl 2 M and quantification by titration with HCl and specific electrode, respectively; and available P by extraction with sodium bicarbonate (0.5 M, pH 8.5) and determined colorimetrically with the molybdate-ascorbic acid method. Available macro and microelements were determined by atomic absorption spectrophotometry (Shimadzu GBC SensAA). Ca, Mg, K, and Na were quantified after extraction with ammonium acetate 1 M, pH 7.0; Fe, Mn, Cu and Zn, were quantified after extraction with a solution composed of DTPA (diethylenetriaminepentacetic acid), calcium chloride and TEA (triethanolamine) buffered at 7.3 pH; and Al was quantified after extraction with KCl 1 M (1:10 soil/solution ratio) by shaking for 24 h. Total metals (Cd, Cr, and Ni) were determined using the nitric/perchloric (1:1 v/v) acid digestion method and analyzed by atomic absorption spectrophotometry.

The dry material (foliar and radical) was weighed after drying the plant samples at 65 °C in a forced air furnace for 24 h. All the analyses were done in triplicate.

Changes in microbial community composition in soil after treatment with increasing doses of sludge were evaluated by denaturing gradient gel electrophoresis (DGGE) by using specific primer sets for bacteria and fungi. Briefly, soil DNA extraction was carried out by using UltraClean Soil DNA Isolation Kit (Mo-Bio Laboratories, Inc., Carlsbad, CA, USA). For bacterial community analysis, fragments of 16S rRNA gene were amplified by touchdown polymerase chain reaction (PCR) with two different primer sets EUBf933-GC/EUBr1387 (454 bp of variable regions V6–V8) and 358F-GC/907R (549 bp of variable regions V3–V4) (Cea et al., 2010). For fungal community analysis, fragments of 18S rRNA gene were amplified by nested PCR. Firstly, fragments were obtained by touchdown PCR using the primer set NS1/NS8, followed by a second PCR with the primer set F1Ra/NS7-GC (400 bp of variable region V9) (Cea et al., 2010). All PCR amplifications were carried out with reagents supplied with GoTaq® DNA Polymerase (Promega, Co. Madison, WI, USA). The DGGE analysis was performed using a DCode system (Bio-Rad Laboratories, Inc.). Twenty microliters of PCR product was loaded onto a 9% (w/v) polyacrylamide gel with 50% and 70% gradient (urea and formamide). The electrophoresis was run for 16 h at 100 V. The gel was then stained with SYBR Gold (Molecular Probes, Invitrogen Co.) for 30 min and photographed on an UV transilluminator. Modifications in the microbial community composition of each sample were identified through the ImageJ 1.43u program (Wayne Rasband National Institutes of Health, USA). Hierarchical analysis and clustering representation was performed on the data set obtained from DGGE analysis with the software package TMEV (Saeed et al., 2003).

2.4. Statistical analysis

All the experiments were carried out in triplicate. The data were statistically analyzed by one-way analysis of variance (ANOVA). Where statistical differences were observed, means were separated using Tukey's minimum significant difference test ($P < 0.05$).

3. Results and discussion

3.1. Soil and sludge characteristics

Table 1 shows the physico-chemical characterization of the Temuco soil and the sludge, before planting *L. perenne* seeds. The soil presents basal levels of N, P, and K for crop production therefore no supplements of these elements were considered in

Table 1
Chemical characterization of Temuco soil and Kraft mill sludge at the beginning of the experiment. Results are the means of three replicates.

Parameters	Unit	Temuco soil	Sludge
Nitrogen ($N{-}NH_4^+ + N{-}NO_3^-$)[a]	(mg/kg)	70	586
Phosphorous[a]	(mg/kg)	14.67	313
pH (1:2.5 H_2O)		6.11	6.97
Organic Carbon	(%)	6.28	44.12
Organic Matter	(%)	10.82	76.07
Sodium[a]	(cmol+/kg)	0.08	41.55
Calcium[a]	(cmol+/kg)	11.19	27.95
Magnesium[a]	(cmol+/kg)	2.3	13.68
Potassium[a]	(cmol+/kg)	0.96	3.62
Aluminium[a]	(cmol+/kg)	0.11	0.03
CEC	(cmol+/kg)	14.53	86.83
Aluminium saturation	(%)	0.75	0.035
Zinc[a]	(mg/kg)	0.64	376.3
Manganese[a]	(mg/kg)	3.76	111.05
Cupper[a]	(mg/kg)	2.1	5.04
Iron[a]	(mg/kg)	30.89	18.47
Cadmium[b]	(mg/kg)	–	1.77
Chromium[b]	(mg/kg)	–	22.25
Nickel[b]	(mg/kg)	–	26.30

Organic matter = Organic carbon × 1.724 factor.
CEC = Cation exchange capacity (∑Ca, Mg, K, Na).
Aluminium saturation (%) = [Al / (∑Ca, Mg, K, Na and Al) × 100].
[a] Available elements.
[b] Total elements.

this study. In addition, exchangeable aluminium was low, thereby avoiding the toxic effect over the plants. High levels of exchangeable aluminium can mean markedly stunted roots and reduced growth in sensitive plants (Gallardo et al., 1999). Sludge presented a high content of organic matter, macronutrients (N, P, K, Ca and Mg) and micronutrients (Mn, Cu and Zn). Total content of metals (Cd, Cr and Ni) was low, below the concentration established in the Chilean Regulation (NCh 2880) and in other international Regulations (Price and Voroney, 2007; Nunes et al., 2008). Conversely, the contents of Fe and Al are lower compared with Temuco soil (Table 1). According to the sludge properties, it is expected that its addition to the Temuco soil could contribute to improving in the characteristics of this soil and therefore its fertility.

3.2. Organic matter and phosphorous content

The incorporation of sludge into the soil before planting *L. perenne* seeds increased organic matter content with the increase in the sludge dose (0–30 Mg/ha) from 11.5 to 14.3% (Fig. 1a). After the end of cultivation (105 days) the level of organic matter in the soil decreased compared to the unplanted plots. Nevertheless, the organic matter content in the amended soil was higher than in the control with no sludge addition (Fig. 1a). The decrease in the organic matter content can be explained by the absorption of nutrients by the plants from the organic matter degraded by the microorganisms as well as by a temporal retention of some organic compounds released by organic matter decomposition that are retained by some soil particles (Haynes and Mokolobate, 2001). Soil amended with pulp mill sludge increases the microbial activity and enzymatic activity of the soils significantly (Gallardo et al., 2010). Organic matter contribution is more significant when the soil has a low level of organic matter (Price and Voroney, 2007; Nunes et al., 2008). Newman et al. (2005) found that the carbon and nitrogen content increased in a sandy soil after 4 years of applying fresh and composted kraft mill sludge. In two soils (Argisol and Espodosol) in Pernambuco, Nascimento et al. (2004), observed significant increases in organic matter up to 53% and 62% after 60 Mg/ha of sewage sludge incorporation.

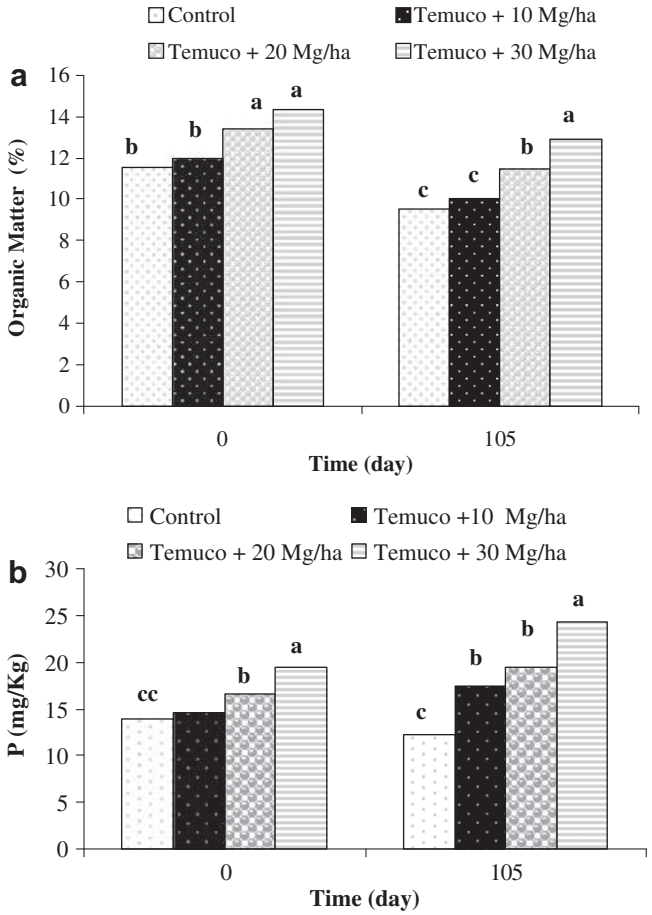

Fig. 1. Organic matter (a) and phosphorous (b) content before (0 days) and after planting *L. perenne* seeds (105 days). Different letters indicate significant differences according to the Tukey test ($P < 0.05$).

Before planting *L. perenne* seeds, the P content increased from 13.9 to 19.4 mg/kg after 30 Mg/ha sludge had been incorporated into the soil. At the end of the experiment (105 days), the P content in the soil remained higher than the values measured before planting seeds except for the control soil (Fig. 1b). This may suggest that the P had accumulated in the soil after sludge application, reaching the maximum value of 24.37 mg/kg at 30 Mg/ha. This is a relevant aspect because Andisols have a great capacity for fixing P, mainly Andisols with acidic pH (Gallardo et al., 2007), and the application of sludge can represent an important input of P in the soil. The incorporation of organic residues into the soil increases the availability of P and will depend on the capacity of the residue to reduce the adsorption of P in the soil, on the contribution of different species of P and on the microbial capacity to degrade the organic compounds of P with the subsequent release of phosphate (Haynes and Mokolobate, 2001; Pypers et al., 2005). The reduced P adsorption and increased P availability following applications of organic residues to soil can be a consequence of several mechanisms (Iyamuremye and Dick, 1996). These include the release of inorganic P from decaying residues, blockage of P adsorption sites by organic molecules released from the residues, a rise in soil pH during decomposition and the complexation of soluble Al and Fe by organic molecules (Haynes and Mokolobate, 2001).

3.3. Microelements content

Fig. 2 shows the effect of sludge incorporation on the content of microelements (Cu, Mn, Fe and Zn) in the soil before planting *L. perenne* seeds and at the end of cultivation. For all treatments, significant differences ($P < 0.05$) were obtained between the control and 30 Mg/ha sludge application. The greatest increase was for Zn which increased from 2.7 to 9.3 mg/kg with the addition of 30 Mg/ha. This increase is associated with the high level of Zn present in the sludge (Table 1). In spite of the increase in the concentration of these heavy metals in the soil, they did not exceed the levels allowed by the Chilean regulation for this type of soil (CONAMA, 2001).

At the end of cultivation (105 days), the level of all microelements decreased due to the absorption process for the plants, except for the Fe content, which increased up to 57.1 mg/kg with the addition of 30 Mg/ha of sludge. The Mn presented the lowest content in the soil after plant cultivation, with values of 1.7 for the control and 2.5 mg/kg for the soil with 30 Mg/ha, with no significant differences showing between the treatments. Among the microelements measured in this study, the Zn has major potential of pollution when it is present at high concentrations, due to its high mobility and assimilation by plants. Nevertheless, an inversely proportional correlation has been found between this metal and the pH of the soil, reducing its availability with the increase in pH values (Beyer et al., 1997).

3.4. L. perenne biomass production

The biomass obtained in each cut (45, 75 and 105 days) rose with the increase in sludge addition and mainly when 30 Mg/ha were added (Fig. 3). The total biomass of *L. perenne*, obtained at the

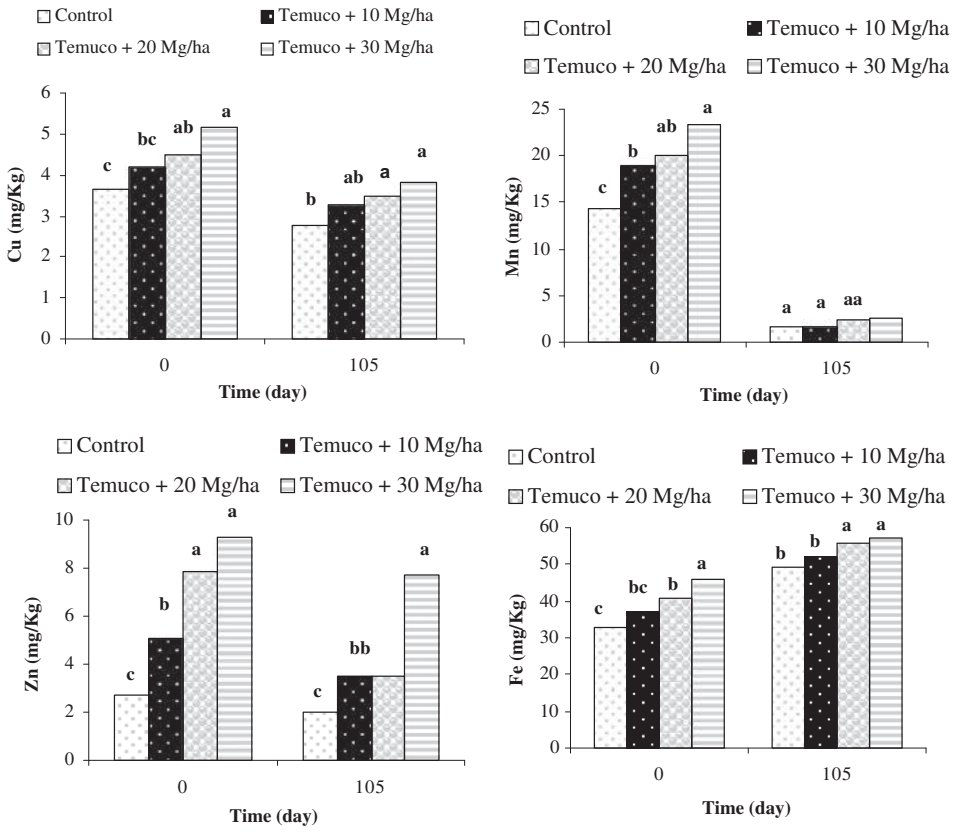

Fig. 2. Microelements (Cu, Mn, Fe and Zn) content before (0 days) and after planting *L. perenne* seeds (105 days). Different letters indicate significant differences according to the Tukey test ($P < 0.05$).

end of the experiment was approximately 60% more than the control soil when 30 Mg/ha sludge was added to the soil.

In the first cut significant differences were obtained ($P < 0.05$), which produced an increase in the biomass production with the increasing sludge application. The production changed from 1.41 ton/ha in the control to 3.19 ton/ha in the soil with 30 Mg/ha of sludge, i.e. a 126% increase. In the second cut all treatments showed significant differences ($P < 0.05$) with the application of the sludge. A similar increase percentage (128%) was achieved changing the production from 1.22 ton/ha in the control to 2.79 ton/ha in the soil with 30 Mg/ha of sludge. A reduced increase to only 65% was measured in the last cut (105 days), where the production changed from 1.01 ton/ha in the control to 1.67 ton/ha in the soil with 30 mg/ha of sludge. These results are similar to those observed in other studies (Navas et al., 1999; Gallardo et al., 2007).

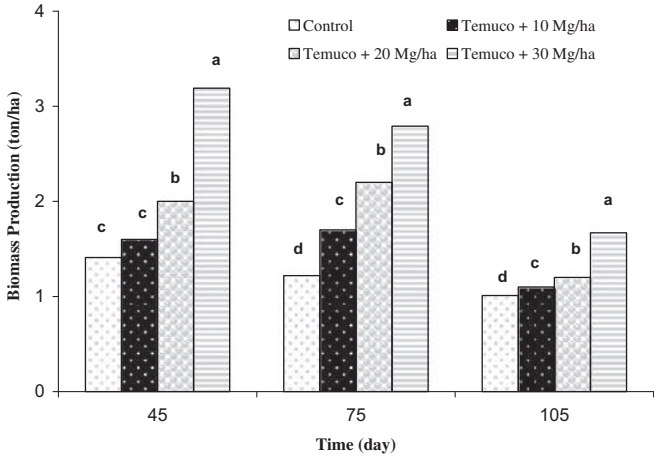

Fig. 3. Dry Matter production (ton/ha) of *Lolium perenne* in Temuco soil under different doses of sludge (0, 10, 20 and 30 mg/ha), in the field assay after 105 days of cultivation. Different letters indicate significant differences according to the Tukey test ($P < 0.05$).

3.5. Microbial community

The analysis of the microbial community by DGGE analysis showed that the application of sludge did not modify the microbial community of fungi (Fig. 4a) or bacteria (Fig. 4b) greatly even with high doses applied. However, the intensity of some bands slightly increased in both fungal and bacterial species, mainly when 10 and 20 Mg/ha of sludge were applied. Cluster analysis of fungi revealed the presence of two major groups (Fig. 4a). One group corresponds to strains that were always present in the samples with and without sludge (dark color in the hierarchical analysis in Fig. 4a) and the strains that were appearing with the addition of sludge (strains 2, 3, 6 and 10 in Fig. 4a).

Respect to bacteria the cluster analysis from DGGE obtained with EUBf933-GC/EUBr1387 (454 bp of variable regions V6–V8) primer (Fig. 4b) showed that the most bacteria strains were not affected by sludge addition (dark color in the hierarchical analysis in Fig. 4b). However, bacteria strains 2, 7 and 4 disappeared with the addition of 20 and 30 Mg/ha of sludge, instead of strain 3 appeared with the addition of 10 Mg/ha. The cluster analysis from DGGE obtained with 358F-GC/907R (549 bp of variable regions V3–V4) primer showed the same behavior but the number of strains negatively affected was minor (strain 7 in Fig. 4c). These results suggest that in this type of soil the application of sludge does not necessarily involve competition between the different populations of microorganisms due to the

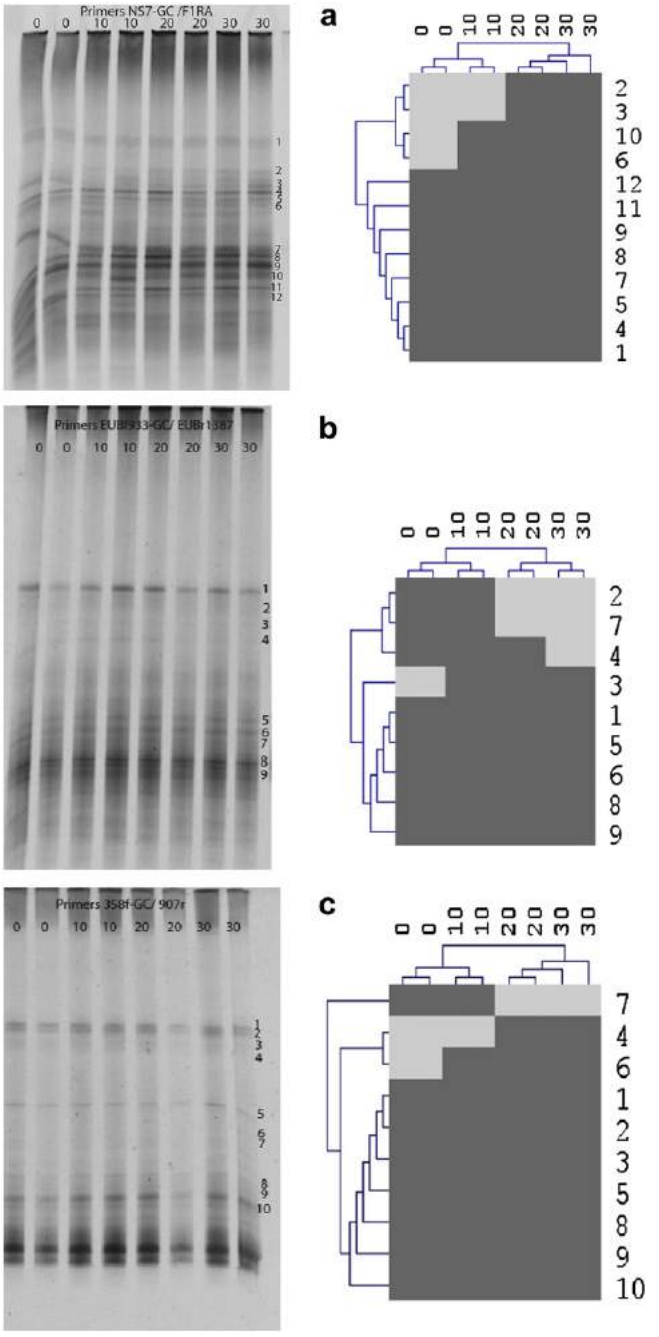

Fig. 4. DGGE profile, hierarchical analysis and clustering representation of the fungi 18S rDNA (a), bacterial 16S rDNA primer set EUBf933-GC/EUBr1387 (b) and bacterial 16S rDNA primer set 358f-GC/907r (c) gene amplified from soil samples under different doses of sludge (Mg/ha). In the hierarchical analysis, dark color represents the presence of the strain.

adequate nutrient contents that stimulate the microorganism populations. Different results were obtained in our Laboratory with Andisols from the Gorbea and Collipilli series (unpublished data). Indeed, the addition of increasing doses of pulp mill sludge determined differences in the structure of microbial communities. In contrast to the Temuco soil series, the Gorbea and Collipulli series soils present a low level of nutrients and an acid pH. Therefore, the addition of sludge not only increase the nutrient levels, but also influences the biological properties of the soil like respiration and enzymatic activities (Gallardo et al., 2007, 2010), thereby improving the microbial community.

4. Conclusions

This study confirms a range of beneficial effects exerted on soil fertility induced by pulp mill sludge addition. The addition of sludge improved the Temuco soil characteristics, increasing all micro as well as macronutrient content. Moreover, it increased the availability of nutrients in the soil, and consequently its productivity. The highest production of biomass of *L. Perenne* was obtained with 30 Mg/ha of sludge addition to the Andisol (Temuco series). The analysis of soil microbial community showed that the application of sludge did not modify greatly the microbial community of fungi and bacteria even when high doses of sludge were applied.

Acknowledgements

Investigation supported by FONDECYT 1080427 and DIUFRO GAP-2007 projects.

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Journal of Environmental Management 95 (2012) S199–S202

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Effect of organic amendments on microbial activity in chlorpyrifos contaminated soil

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ARTICLE INFO

Article history:
Received 26 September 2009
Received in revised form
14 September 2010
Accepted 7 October 2010
Available online 28 October 2010

Keywords:
Chlorpyrifos
Biostimulation
DHA
Organic amendments (Biogas slurry
Farmyard manure
Mushroom spent compost
Vermicompost)

ABSTRACT

The aim of this research was to study the inhibitory effect of chlorpyrifos (CPF) on soil microbial activity and to evaluate the efficacy of different organic amendments as a biostimulation agent for sustaining the microbial activity and thereby assisting in the remediation of CPF (10 ppm) contaminated soil. Experiments were carried out under controlled conditions (37 °C) up to 74 days; CPF was analyzed by GC-ECD while dehydrogenase activity (DHA) was measured as one of the indices of soil microbial activity. Throughout the experiment, there was higher microbial activity in uncontaminated soil (S) as compared to CPF contaminated soil (SP) and overall a considerably high reduction (63.51%) in average DHA was noticed in CPF contaminated soil. Organic amendments enhanced the microbial activity over unamended CPF contaminated soil. The trend of DHA on 24th day was MS (SP + 1% Mushroom Spent) >VC (SP + 1% Vermicompost) >BS (SP + 1% Biogas Slurry) >SP (Soil spiked with 10 ppm CPF) >FM (SP + 1% Farmyard Manure). The enhancement in pesticide dissipation over the unamended soil showed the following trend VC (37%)>MS (24%) >FM (1.9%). In spite of sufficient DHA, BS could not enhance pesticide dissipation over the unamended soil (SP). These results indicate the potential of vermicompost and mushroom spent compost as suitable biostimulation agents to sustain the microbial activity in CPF contaminated soil.

1. Introduction

Chlorpyrifos (O, O-diethyl-O-3, 5, 6-trichloro-2-pyridyl phosphorothioate) is a broad spectrum systemic phosphorothioate ester insecticide widely used against agricultural and residential (termites) pests. Due to extensive usage environmental and food matrix has become contaminated with CPF (Kumari et al., 2008). Several reports demonstrating potential toxicity of CPF are available (Pandey and Singh, 2006). The effects of CPF on soil microbial characteristics have been studied by a number of researchers (Singh et al., 2002; Menon et al., 2004, 2005; Shan et al., 2006). It had been reported that soil microbial biomass was reduced by 25% and 50% after CPF treatment at concentrations of 10 and 50 mg/kg, respectively, in an Italian biobed (Vischetti et al., 2007). Shan et al. (2006) also indicated the inhibition in soil bacterial, fungal and actinomycete populations at a concentration of 10 ppm CPF. CPF not only affects the soil microbial system but also disturbs the nutrient level in the soil. Menon et al. (2004) reported that nitrogen mineralization in the loamy sand and sandy loam soil was significantly inhibited after CPF application. Its application also significantly reduced new root initiation and free-living nematode populations, and altered microbial community structure and function in a range of managed grasslands (Singh et al., 2003).

Organic amendments play a very important role in enhancing the soil fertility and soil microbial activity (Moreno et al., 1999). Therefore, the same may also alleviate the inhibitory effects of residual pesticides on soil microbes. Organic amendments enrich the matrix with a diverse microflora and nutrients and hence act as efficient biostimulating agent. Hence, a number of researchers has investigated the potential of compost, farmyard manure, urban waste, public green compost etc. to accelerate the pesticide dissipation in different biobeds and soil (Vischetti et al., 2004; Coppola et al., 2007; Kadian et al., 2008). Recently CPF biodegradation using different type of composts in biobeds (Coppola et al., 2007) and various agro-residues such as coconut husk, peat moss, peanut shell, rice husk (Romyen et al., 2007) has been reported. Hazardous affects of CPF can be minimized with the application of organic amendments however systematic investigations on CPF contaminated soils in this direction are lacking. Hence, in the present study, four different organic amendments (biogas slurry, farmyard manure, mushroom spent compost and vermicompost) commonly used in sustainable agriculture were selected to see the efficacy of

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doi:10.1016/j.jenvman.2010.10.023

these in sustaining the microbial community in CPF contaminated soil.

2. Materials and methods

2.1. Soil and organic amendment

2.1.1. Soil

Field soils were collected from a selected plot in IIT, New Delhi, from top 2–15 cm after removing the upper 2–3 cm organic layer following the standard procedure (Gupta, 2000). It was analyzed for some selected physico-chemical properties (pH, EC, organic matter, organic carbon, nitrogen, phosphorous and potassium) using standard methods (Gupta, 2000). For this laboratory scale study, the grounded sieved (2.0 mm sieve) soil samples were taken to investigate the effect of organic amendments on microbial activity in CPF contaminated soil for a period of 74 days.

2.1.2. Organic amendments

Biogas slurry, Farmyard manure, Mushroom spent were used as reported in Kadian et al. (2008). Additionally, Vermicompost obtained from Teri Gram, near Delhi, India was used. It was air dried and characterized as described earlier (Kadian et al., 2008).

2.1.3. Experimental setup

The experiments were conducted in plastic plantation pots (1 kg capacity, 135 mm dia). For the biostimulation study calculated amount of commercially available CPF (Lethal TC Chlorpyrifos 20% EC manufactured by Insecticides Limited, Bhiwadi, Rajasthan, India) was added to soil samples, to make the final concentration of CPF at 10 ppm.

Detailed investigation using 4 organic amendments was carried out under controlled conditions. The calculated quantity i.e. 10 g (1%) of different soil amendments was added in respective treatment pot having 10 ppm spiked soil, mixed well and kept under incubation at 37 ± 0.5 °C up to 74 days. Different treatments were planned as: 1 kg Soil only (control)-(S) (without CPF application), 1 kg Soil spiked with 10 ppm CPF – (SP), SP + 1% Farmyard Manure (FM), SP + 1% Mushroom Spent Compost (MS), SP + 1% Biogas Slurry (BS), SP + 1% Vermicompost (VC). No chemical nutrient supplement was supplied during the experimental period.

2.2. Extraction and analysis of chlorpyrifos residues

Soil samples (15 g) were extracted for residual CPF using the method as described by Kumari et al. (2008). Finally the extracted samples were analyzed by GC-ECD using a Nucon gas chromatograph model 5765 equipped with electron capture detector (ECD) and a BP-5 capillary column (30 m × 0.59 mm ID) (Kadian, 2010).

2.3. Dehydrogenase activity

Method described by Friedel et al. (1994) was followed for the determination of DHA of the incubated soil samples. Triplicate soil samples (3 g) were taken from six pots comprising of different soil amendments on predetermined intervals (0, 12, 24, 38, 48, 60 and 74th day). Finally soil sample's absorbance was noted at 485 nm on spectrophotometer (Perkin Emnler, Labda 25 Model).

3. Results and discussion

The characterization of soil used in the present study is shown in Table 1. It is a sandy loam soil having sufficient macro and micronutrients. Physico-chemical characteristics of vermicompost are following: pH 7.4, EC 0.55 ds/m, OM 24.1%, phosphorus 0.11%, C/N ratio 10.85. The characteristics of other organic amendments (biogas slurry, farmyard manure and mushroom spent) were reported earlier (Kadian et al., 2008).

Table 1
Physico-chemical properties of the soil.

Characteristics	
Texture	Sandy Loam (58% Sand, 30% Silt, 12% Clay)
pH	8.5
EC(dS/m)	0.13
Organic Carbon (%)	0.50
Available phosphorus (kgP/ha)	17.7
Available potash (kgK/ha)	106
Calcium carbonate	Nil
Zn(ppm)	5.56
Cu(ppm)	4.12
Fe(ppm)	15.9
Mn(ppm)	11.4

3.1. Effect of CPF on microbial activity in soil

Fig. 1 shows the comparison of DHA in CPF contaminated soil (SP) and uncontaminated soil (S). Throughout the experiment, there was higher microbial activity in uncontaminated soil as compared to CPF contaminated soil and overall a considerably high reduction (63.51%) in average DHA was noticed in CPF contaminated soil. Significant reduction in DHA in the contaminated soil was observed on the zero day itself (Fig. 1). Hence the inhibitory effect of CPF on microbial activity was immediately visible. The DHA in contaminated soil decreased till 12th day beyond which a gradual increase was recorded. There was significant pesticide dissipation in the initial 12 days and the CPF concentration in the soil decreased to ~3 ppm. Hence, the decrease in pesticide concentration might have reduced the toxicity effect thereby restoring the microbial activity in the soil. Several scientists have reported the negative effect of CPF on microflora (Sardar and Kole, 2005; Pandey and Singh, 2006; Xiaoqiang et al., 2008; Dutta et al., 2010). Present study confirmed the finding that CPF has negative effect on the microflora of soil.

3.2. Role of organic amendments

Different organic amendments (1%) enhanced the microbial activity as compared to SP, especially in the initial phase (Fig. 2). Statistical interaction between days and treatments was found to be significant ($p = 0.05$). Critical difference calculated through two-way ANOVA revealed that the dehydrogenase activity is significantly different among the treatments except being at par

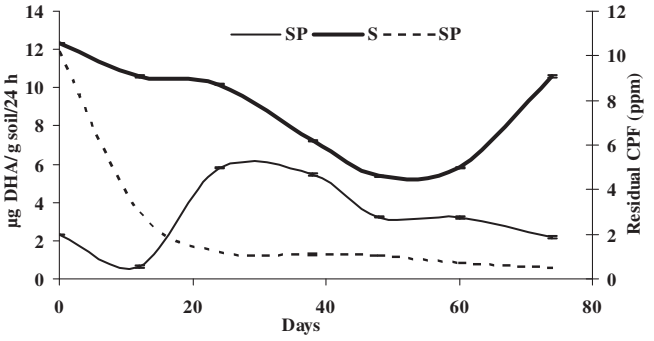

Fig. 1. Co-variations in dehydrogenase activity (DHA, solid lines) and chlorpyrifos concentration (CPF, dashed line) in soils. SP = Soil spiked with 10 ppm chlorpyrifos, S = Uncontaminated soil (Bars represent standard error of three treatments).

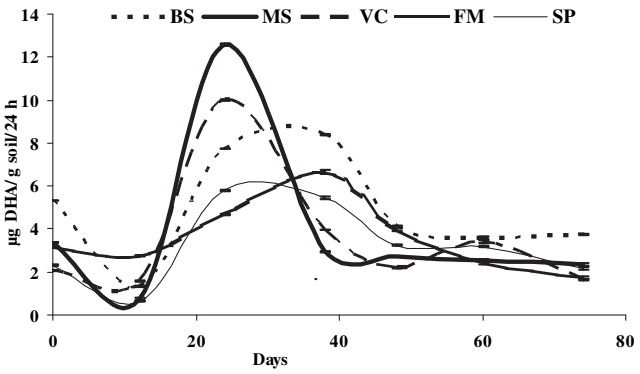

Fig. 2. Variation in trends of dehydrogenase activity (DHA) with different organic amendments in chlorpyrifos contaminated soil. SP (soil spiked with 10 ppm chlorpyrifos); BS (SP + 1% biogas slurry); MS (SP + 1% mushroom spent compost) VC (SP + 1% vermicompost); FM (SP + 1% farmyard manure); Bars represent standard error of three treatments.

on 74th day in case of VC and FM treatments. Although there was significant fluctuation in DHA in the later phases, overall average DHA in amended soils was higher than SP. Earlier, Moreno et al. (1999) and Masciandaro et al. (2000) studied DHA under the influence of organic matter and reported an increase in DHA immediately following the organic matter amendment. However, they reported unchanged DHA during extended period of incubation suggesting that the enhancement was transient.

This is an interesting observation that even in the presence of CPF; organic amendments have stimulatory effect on microflora. Nevertheless, all the amendments could not enhance the DHA at par with the uncontaminated soil (S). This reflects the ability of organic amendments to partially prevent the microflora from the toxicity of CPF and sustain the microbial activity in the matrix. Earlier researchers observed increase in microbial population with the addition of different kind of organic manure in atrazine and carbamate contaminated soil (Gupta and Baummer, 1996; Kuo and Regan, 1998).

As discussed earlier, there was considerable fluctuation in the DHA throughout the 74 days study (Fig. 2). Such variations in DHA have been previously reported (Pandey and Singh, 2006). Lowest microbial activity was observed on 12th day in all the treatments. Such a trend was not observed in uncontaminated soil. After an initial reduction, microbial activity started to recover when pesticide level was lower than 70% and gradually reached the highest level on 24th day. This could be attributed to fast growth of microbial strains able to tolerate/degrade CPF or due to reduced toxicity effects accompanied with dissipation of the pesticide. It is reported that certain microbial populations (especially fungal populations in case of CPF) which are able to degrade the pesticides get stimulated by the pesticide application (Vischetti et al., 2008; Pandey and Singh, 2004). The same has also been observed during our separate studies on remediation of CPF contaminated soils (data not shown).

3.3. Effect of type/nature of organic amendments

As shown in Fig. 2, highest microbial activity was observed on 24th day, and a clear difference between various organic amendments could be seen. The trend of DHA on this day was MS > VC > BS > SP > FM. Out of all four amendments, MS and VC produced very high DHA peaks and comparison of the data revealed that microbial activity in these amendments was even higher (MS) or almost equal (VC) than the uncontaminated soil (Fig. 3). Pesticide dissipation trends were also almost analogous to DHA trends (except BS) and reported as VC > MS > FM > SP > BS. Hence, the higher CPF dissipation efficacy of MS and VC compared to FM and SP could be attributed to their higher DHA. Further, both the trends of CPF dissipation and DHA could be very well correlated with the C/N ratio of organic amendments which was: vermicompost (10.85) < biogas slurry (25.23) < mushroom spent (28.93) < farmyard manure (38.89). Therefore, VC in view of optimum C/N ratio seems to support profuse microbial growth resulting in highest dissipation (96.6%). A number of previous researchers observed that CPF dissipation increased with catalyzation by metal ions or bacterial soil enzymes (Meikle and Youngson, 1978). In comparison with other organic amendments, vermicompost is a product that is rich in chelating and phytohormonal elements (Tomati et al., 1995). Hence, out of the tested materials, vermicompost seems to be the most efficient organic amendment for the remediation of CPF in the sandy loam soil used in the present study. The DHA in case of MS also showed similar trend to VC and reported high pesticide dissipation i.e., 96.03%.

SP and FM both have lower microbial activity as compared to MS, VC and BS. Almost comparable DHA and CPF dissipation was observed in FM and SP (Fig. 3). Farmyard manure used in this experiment (C/N ratio: 38.9) is a nutrient poor material as compared to other organic amendments. In a previous study,

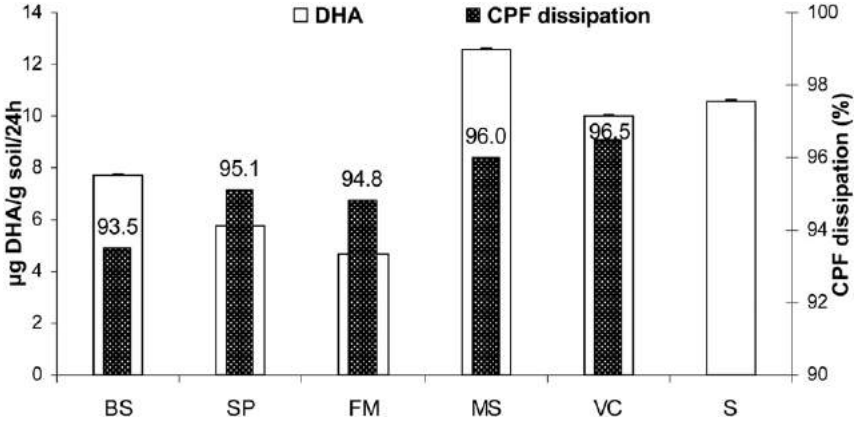

Fig. 3. Dehydrogenase activity (DHA, 24th day) and chlorpyrifos (CPF) dissipation with different organic amendments in CPF contaminated soil. SP (soil spiked with 10 ppm chlorpyrifos); BS (SP + 1% biogas slurry); MS (SP + 1% mushroom spent compost) VC (SP + 1% vermicompost); FM (SP + 1% farmyard manure); (Bars represent standard error of three treatments).

Kadian et al. (2008) reported lowest atrazine dissipation potential of farmyard manure as compared to mushroom spent compost. In the present study also, out of all the amendments, farmyard manure has demonstrated least significant effect on the dissipation of CPF.

BS demonstrated a relatively prolonged period of high DHA values rather than high peaks for short duration as in case of VC and MS. However, it showed the lowest CPF dissipation (93.6%). These results indicate that while microflora of BS was stable and metabolically active throughout the experimental period, it was not helpful in degradation of CPF. Although this performance is quite abnormal; it can be attributed to very different nature of BS as compared to other organic amendments, owing to the microbial flora of anaerobic origin. A study by EI-Shinnawi et al. (1988) demonstrated that anaerobically processed manures show higher DHA. Out of all the organic amendments, biogas slurry contains highly decomposed material. Researchers suggest that less decomposed materials rich in straw and lignin etc. support more fungal growth as well as degradation of pesticides such as CPF and its metabolites (Coppola et al., 2007).

4. Conclusions

The present study indicated that CPF (10 ppm) inhibits the microbial activity (measured as DHA) in soil. A strong inhibition is observed soon after the CPF application which is relieved in the later phase as CPF dissipates and its residual concentration gets reduced below 3 ppm. The organic amendments to the CPF contaminated soil help in reducing the inhibitory effect of CPF. All the amendments brought about considerable enhancement in average DHA activity over the 74 days experimental period. The trends in DHA and pesticide inhibition were analogous indicating that higher DHA activity supports higher dissipation of CPF from soil. Vermicompost and mushroom spent compost which displayed a higher DHA as well as pesticide dissipation as compared to other organic amendments have a good potential as a cost-effective tool for CPF dissipation in the alkaline sandy loam soil. Although use of organic manure has been an integral part of sustainable agriculture practices; the present findings reveal its unique characteristics for sustenance of microbial activity and removal of persistent pesticides from soil thus leading to enhanced food safety.

Acknowledgements

One of the authors (Ms. Neeru Kadian) is grateful to the Department of Science and Technology for Women Scientist Fellowship.

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Journal of Environmental Management 95 (2012) S203–S209

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Quantitative and nutritional characterization of fruit and vegetable waste from marketplace: A potential use as bovine feedstuff?

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ARTICLE INFO

Article history:
Received 18 August 2009
Received in revised form
27 August 2010
Accepted 26 September 2010
Available online 1 February 2011

Keywords:
Environmental impact
Organic waste
Protein
Recycling
Ruminal degradability

ABSTRACT

There are different sources for the generation of solid waste, and marketplaces are considered one of them. Fruit and vegetable waste (FV) from a marketplace in Colombia was quantitatively and nutritionally characterized to contribute to its use in bovine feeding and to contribute minimizing its environmental impact. The evaluation was carried out 7 days per week during 4 periods of the year. FV was grouped by cluster analysis using SAS® 2006. FV was composed of 43% fruit, 30% vegetables and 27% stems, leaves, leaf wrappers, corncobs, roots, refuse and others. FV was defined in four main groups. On average, FV contained 10% crude protein (CP), 36.6% neutral detergent fiber (NDF), 29.6% acid detergent fiber (ADF), 87.8% ruminal degradability at 24 h, 3657 kcal/kg, 0.59% calcium (Ca^{+2}), and 0.21% phosphorous (P). There were no statistical differences between days or between periods of evaluation ($p > 0.05$) for CP or for Ca^{+2}. As for NDF and ADF, there were statistically significant differences between periods but not between days. The microbiological parameters only increased when the humidity was up to 12%. FV represents a potential feedstuff for bovine feeding, and its recycling could avoid the discharge of a large amount of waste to landfills, which would minimize its environmental impact.

1. Introduction

The world produces around 1600 million tons of solid waste per year (Cardona et al., 2004), and the generation and inappropriate management of this waste is considered one of the main environmental problems associated with emissions of methane and carbon dioxide, the emission of odors from landfill sites (Qdais et al., 2010), and with damage of water and air surface quality (United Nations, 2010). Solid waste has increased significantly in the world in the last few years. Total annual municipal solid waste (MSW) generation in the U.S. has increased more than 67% since 1980 to a current level of 254 million tons per year (U.S. Environmental Protection Agency, 2008). Similar increases in solid waste generation have occurred in India (Taylan et al., 2008), China (Chen et al., 2010), and Colombia (Nieves, 2009). Medellín is one of the major cities of Colombia and is located in the Metropolitan Area of the Aburrá Valley. This area has 3.4 million people and is made up by 10 municipalities, being Medellín the most important and largest (CORANTIOQUIA, 2006). According to CORANTIOQUIA (2006), the population is projected to increase about 534 128 people per year (1.42%) by 2018. Likewise, MSW is reported to increase about 500 000 ton per year (Jaramillo, 1999). It is a current problem since about 2000 tons of waste per day from Aburrá Valley is thrown in the only landfill of this area known as "La Pradera environmental park disposal site" and this site has a limited working life projected until 2020 (Posada, 2008). Beede and Bloom projected that in developing countries, MSW would increase at an annual rate of 2.7% from 1995 through the year 2010. Although developing countries have different solid waste management problems than those found in fully industrialized countries, future problems are projected to become a serious matter in both (Zerbock, 2003; Rathi, 2006).

There are different sources for the generation of solid waste, and marketplaces are considered one of them on a global scale (Buenrostro et al., 2000). Consequently, these places not only contribute to the environmental problems mentioned above but also create economical problems due to the high costs of compilation, transportation, and disposal (Cardona et al., 2004). Social problems also result due to the fact that places for waste treatment (landfills and incinerators) are often located in economically depressed, minority areas, which results in the generation of more poverty and health risks (Bullard, 2004; Qdais et al., 2010). For

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0301-4797/$ – see front matter © 2010 Published by Elsevier Ltd.
doi:10.1016/j.jenvman.2010.09.022

these reasons, there is a need to seek sustainable alternatives for solid waste recycling in the world. Though composting has been one of the most evaluated uses for recycling organic waste originating from urban places (Martínez-Blanco et al., 2009), an interest in linking waste management and sustainable animal food production as a complementary method for recycling has increased significantly in the world. The use of fruit and vegetable waste (FV) has been considered for this purpose (Garcia et al., 2005; Esteban et al., 2007; Katongole et al., 2008; Marquez et al., 2010), and FV's use looks like an obvious recovery option; however, before strategies can be developed regarding how to proceed, it is necessary to know its composition and nutritional value in different marketplaces.

The amount of MSW generated daily in Colombia is about 30 886 tones (Nieves, 2009), of which about 52.3% is organic (16 153 tones) (Jaramillo and Zapata, 2008). Currently, about 92.54% of MSW is thrown in landfills, and 7.46% has an inappropriate final disposal (Nieves, 2009). Moreover, the recycling of FV from marketplaces is minimal, and there is a lack of information about its potential for animal feeding because there has not been a published quantitative or nutritional characterization of the generated FV from the main marketplaces.

The aim of this study was to perform a quantitative and nutritional characterization of FV from a main marketplace in Medellín (Colombia) during different periods of the year to determine its potential use as feedstuff for bovine feeding, which would contribute to minimizing its environment impact.

2. Materials and methods

2.1. Location

This study was carried out in the second biggest marketplace of Medellin (Colombia), José Maria Villa, known traditionally as Minorista. The habitual waste compilation was done daily throughout special collector cars from the place where the products were sold to the place where waste was deposited (stationary boxes). The hours of major flow of FV to the compilation places were from 7 am to 2 pm (determined in a pilot evaluation).

2.2. Methodology followed for the characterization of FV

The process was carried out 7 days per week during 4 periods of the year. Characterization of FV was done every day between 7 am and 2 pm. The collector cars were selected randomly in the whole marketplace; therefore, the material was deposited in a special place chosen for this study instead of being normally discarded in stationary boxes. Every product included in FV was separated, classified, weighed, and registered by hand by 5 people according to the kind of fruit or vegetable that it contained and according to its condition (acceptable for animal feeding or not). The final waste that contained a mixture of small pieces of the different products and rinds, which was difficult to classify, was considered as refuse. Finally, the material selected as product serviceable was deposited individually in a fiber sack, and the gross weight, date of characterization, and collector car number were written on its individual card. Additionally, the inorganic material that arrived in each collector car was quantified, registered, and rejected. Once separated and characterized, every product was cut manually or mechanically in accordance with the product. Subsequently, it was packed in plastic, hermetic wastebaskets for dehydration.

2.3. Analysis of nutritional quality

Before the evaluation of its nutritional quality, the gathered material was dehydrated on the same day or one day after its recollection by reducing its humidity to 10–16%. A special oven was used for organic materials (PREMAC® Combustion and heating solutions, Medellin, Colombia), which was handled at 60–65 °C with propane gas. FV was dehydrated using special trays (25 kg capacity), and each tray was processed for 3 h, changing position in the oven every hour. On average, 175 kg of fresh FV was processed every 9 h, and a sample of 500 g was recollected in plastic bags from the final dry product of each characterization. The amount of crude protein (CP) was determined by Kjeldhal's method [described by the Association of Official Agricultural Chemists AOAC (A.O.A.C., 1990)]; neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were determined by the method of Van Soest et al. (1991); calcium (Ca^{+2}) content was determined by the ethylenediaminetetraacetic acid (EDTA) complexometry method (West, 1969); phosphorus (P) content was determined by a photocolorimetry method; gross energy was determined through use of a calorimetric bomb [as described by McDonald et al. (2002)]; and ruminal degradability of the dry matter (DMRD) was determined by the nylon bag technique described by Orskov and Shand (1997). The DMRD was measured at 2, 4, 6, 8, 12, and 24 h.

2.4. Microbiological analysis

Some samples of the obtained product were collected to measure its content of aerobic microorganisms and mesophylls/g, total colliforms/g, fecal colliforms/g, number of molds and yeasts, and Salmonella spp./25 g of each sample was determined. In accordance with Lanyasunya et al. (2005), mold is one of the principal problems for the conservation of feed used for animal ration formulation due to its propitious proliferation on feeds containing 12–13% moisture. Considering this fact, the obtained samples were classified into two groups: a less than 12% humidity group and a more than 12% humidity group. The microbiological results were contrasted by the humidity content of the products.

2.5. Statistical analysis

FV was grouped by cluster analysis to determine the relationship between them as well as their relevancy in the general composition of waste through time. The FASTCLUS program of SAS® 2006 was used. Cluster analysis was done considering only the products that were present in at least three of the four evaluated periods. Additionally, descriptive statistical analysis was applied to determine the average composition of FV. To determine differences in the nutritional composition of FV between periods and days, a variance analysis and a Tukey test (at $p \leq 0.05$) were used. Only descriptive statistical analysis was carried out to analyze the microbiological results.

3. Results and discussion

3.1. Amount of FV per day

The weight of the main products included in FV at the Minorista market is shown in Table 1. The total material characterized per day was 584 936 g, which amounts to 83 562 g per collector car (7 cars/day). If this number is inferred with a total of 100 collector cars per day (average per day calculated for this marketplace), the production capacity of FV in this marketplace would be approximately 8 356 200 g/day (8.35 ton/day, 250.5 ton/month). Considering a possible daily intake of 500 g by an adult bovine, FV could be

Table 1
Average weight of FV components.

Product	Average amount per day in every period (grams)				Total per week	Average/day	%
	P1	P2	P3	P4			
Cabbage	131 979	76 400	65 550	84 821	627 813	89 688	15.333%
Orange	64 057	21 907	87 671	144 479	556 700	79 529	13.596%
Refuse	207 450	6821	42 614	13 350	472 913	67 559	11.550%
Stems	82 150	44 986	21 771	30 750	314 400	44 914	7.678%
Papaw	31 000	42 743	28 079	45 000	256 938	36 705	6.275%
Mango	126 071	807	1936	6914	237 525	33 932	5.801%
Banana	59 214	26 221	10 879	15 693	196 013	28 002	4.787%
Lettuce	38 721	15 036	28 300	18 957	176 775	25 254	4.317%
Lemon	0	50 293	7393	4000	168 363	24 052	4.112%
Leaf wrappers and corncobs	21 775	5700	21 107	11 793	129 813	18 545	3.170%
Rinds	56 064	0	0	13 667	123 021	17 574	3.005%
Tomato	8586	10 161	19 771	8293	81 919	11 703	2.001%
Swiss Chard	7061	13 079	3242	14 136	66 040	9434	1.613%
Cassava	11 271	5121	12 036	5586	59 525	8504	1.454%
Plantains	22 336	1686	7086	1000	56 188	8027	1.372%
Citrus fruits	22 214	643	8421	500	55 613	7945	1.358%
Pineapple	11 257	2579	8243	6179	49 450	7064	1.208%
Sapote	15 029	5821	1500	3064	44 475	6354	1.086%
Yellow passion fruit	126 050	6793	0	429	44 150	6307	1.078%
Bean rinds	13 521	3050	0	3743	35 550	5079	0.868%
Coriander	15 114	971	2043	0	31 725	4532	0.775%
Soursop	8221	3293	3971	2400	31 303	4472	0.765%
Cucumber	5479	4036	3536	3014	24 413	3488	0.596%
Potatoes	3243	1897	1814	5950	22 583	3226	0.552%
Watermelon	1100	2786	3821	6100	24 163	3452	0.590%
Broccoli	2629	5186	893	3014	20 513	2930	0.501%
Carrot	5729	3004	1671	0	18 208	2601	0.445%
Common Pepper	4100	2089	2193	1579	17 431	2490	0.426%
Pumpkin	5607	321	786	1629	14 600	2086	0.357%
Beetroot	5014	1071	2043	0	14 225	2032	0.347%
Celery	1336	500	1086	4607	13 175	1882	0.322%
Onion bulb	4400	536	0	1086	10 538	1505	0.257%
Ochuva leafs	5586	179	0	0	10 088	1441	0.246%
Onion	1550	1236	1700	536	8788	1255	0.215%
Mandarin	4600	0	0	171	8350	1193	0.204%
Guava	0	393	1957	2043	7688	1098	0.188%
Spinach	729	1393	1121	371	7588	1084	0.185%
Other vegetables	0	0	3.386	586	6.950	993	0.170%
Tree tomato	1300	1214	171	457	5500	786	0.134%
Mustard	0	2757	0	286	5.325	761	0.130%
Avocado	250	0	714	1679	4625	661	0.113%
Different fruits	500	393	1714	0	4563	652	0.111%
String beans	800	207	1164	193	4138	591	0.101%
Radish	93	0	0	1857	3413	488	0.083%
Cauliflower	0	143	1371	264	3113	445	0.076%
Others	1764	0	0	0	3088	441	0.075%
Arracacha	86	1264	171	0	2663	380	0.065%
Sweet passion fruit	0	707	700	0	2463	352	0.060%
Muskmelon	0	964	0	336	2275	325	0.056%
Parsley	364	0	0	857	1888	270	0.046%
Honeyberry	529	0	0	200	1275	182	0.031%
Eggplant	300	93	164	0	975	139	0.024%
Patata	236	29	0	236	875	125	0.021%
Green pea leafs	0	429	0	0	750	107	0.018%
Green pea	386	0	0	0	675	96	0.016%
Banana guineo	0	257	100	0	625	89	0.015%
Grapes	71	0	0	200	475	68	0.012%
Kale	100	0	0	0	175	25	0.004%
Peach	0	0	71	0	125	18	0.003%
Red pepper	0	0	29	0	50	7	0.001%
Total FV/day	1 137 022	377 194	413 992	472 002	*4 094 554*	584 936	100%

potentially used for feeding approximately 17 000 animals per day, which is an amount that might otherwise be discharged as waste in landfills. Additionally, according to Themelis (2003), 1 ton of MSW would produce approximately 62 m^3 of methane via the anaerobic decomposition of the biodegradable art of the waste. If the amount of FV quantified in the present study that was 100% biodegradable was placed in a landfill, it would produce approximately 1035.4 m^3 of methane per day, contaminating the environment. The Minorista market represents a large marketplace in a major city in the world, which indicates that marketplaces like this have a potential capacity to produce almost 1% of FV that is usually thrown in landfills. It would be important to use the same methodology in other marketplaces of the city and of the world to quantify the real positive environmental impact that would result if FV were used as bovine feedstuff. On the other hand, the values of FV produced per day in the Minorista market indicate clearly that the problem of FV

management in marketplaces is not only a matter of garbage collection but also of education for recycling. In this way, Minorista market supported this study and has begun to organize its structure to look for new recycling alternatives. Another notable approach is the progress that Colombia has made in recent years in terms of environmental education and legislation. Currently, Medellín has a specific plan for the integral management of MSW which has allowed an important advance in educational programs of recycling (CORANTIOQUIA, 2006). However, a lack of comprehensive facilities for the treatment and final disposal of many types of waste still remains problematic, and improvements in the exploitation and use of recoverable waste and enhancing efforts to minimize waste generation by promoting responsible consumption still need to be resolved (United Nations, 2010).

3.2. Principal components of FV

FV was composed of 43% fruit, 30% vegetables and 27% stems, leaves, leaf wrappers, corncobs, roots, refuse, and others. In accordance with the cluster analysis (Fig. 1), and considering the quantities and the frequencies during all periods, FV of the Minorista market was divided into four main groups: Group 1 contained oranges and cabbage, which were the products that were found in the largest quantity and frequency in the waste; Group 2 contained Swiss chard, tomatoes, bananas, lettuce, lemons, papaws, stems, leaf wrappers and corncobs, which were found second most frequently in amount and frequency in the waste; Group 3 contained mangos and refuse, which were products of high quantity but of sporadic frequency in the organic waste; and Group 4 contained avocados, eggplant, patata, cauliflower, different fruits, string beans, pumpkins, onion bulbs, arracacha, tree tomatoes, onions, spinach, common pepper, beetroot, carrots, coriander, celery, guava, potatoes, watermelons, broccoli, soursop, cucumbers, bean rinds, yellow passion fruit, sapote, citrus fruit, plantains, pineapples, and cassava, which were products found in the least amount and frequency. There are different aspects that could influence the kind of product present in FV, including the geographical location of the marketplace, the harvest period, the demand of the products, some specific behaviors in the marketplaces as well as characteristics of the products and their handling.

The geographical location of the Minorista market, which was close to production places of different kinds of fruit, and its high demand of fruit as well as the results found by Holmann et al. (2005), which showed that fruit production was the most important land use in Antioquia, could have influenced the fact that FV was composed more of fruit than of vegetables. This market is different from other main marketplaces in Colombia, such as in Corabastos (Bogotá city), where the waste contained a higher proportion of vegetables (67.6%) than fruit (14.1%) (DAGMA, 1999). The Minorista market is also different from results found in other countries, such as India, where waste from marketplaces was composed of 85% vegetables (Mukherjee and Kumar, 2007). Another factor that must be considered for the variation in FV composition is the harvest period. According to the results presented above, FV of the Minorista market is composed of some basic products (especially products of group 1 and 2), which had a constant harvest period in the same region or in close regions to the marketplace during all evaluation periods (Statistical Yearbook, 2004), while there are other products that could be present during certain periods of the year (products of group 3 and 4) depending on their specific harvest period. However, it is important to consider that sometimes the demand of the product or some specific behavior in the marketplace can influence FV composition more than the harvest period. For example, potatoes (group 4) had the biggest entries in the marketplace during all periods of evaluation but had a low constant presence in FV, which can be explained by its high demand in this marketplace as well as by the recollection observed during this study, i.e., the potatoes are thrown to the garbage in a good condition and are recollected by some people. Finally, it is necessary to consider that some characteristics of the products and their handling make them more susceptible to damage; therefore, they are present more often in the waste. A clear example is the orange, which was the fruit most included in FV. Oranges had a constant harvest period during the evaluation and had a high demand in the marketplace, but oranges were the most damaged fruit reported in this study. The orange is very susceptible to post-harvest mechanical damage, which increases its water loss and decreases its shelf life (FAO, 1989). Moreover, mechanical damage releases oil from the oil glands of the fruit, and it results in oleocellosis, a physiological rind disorder

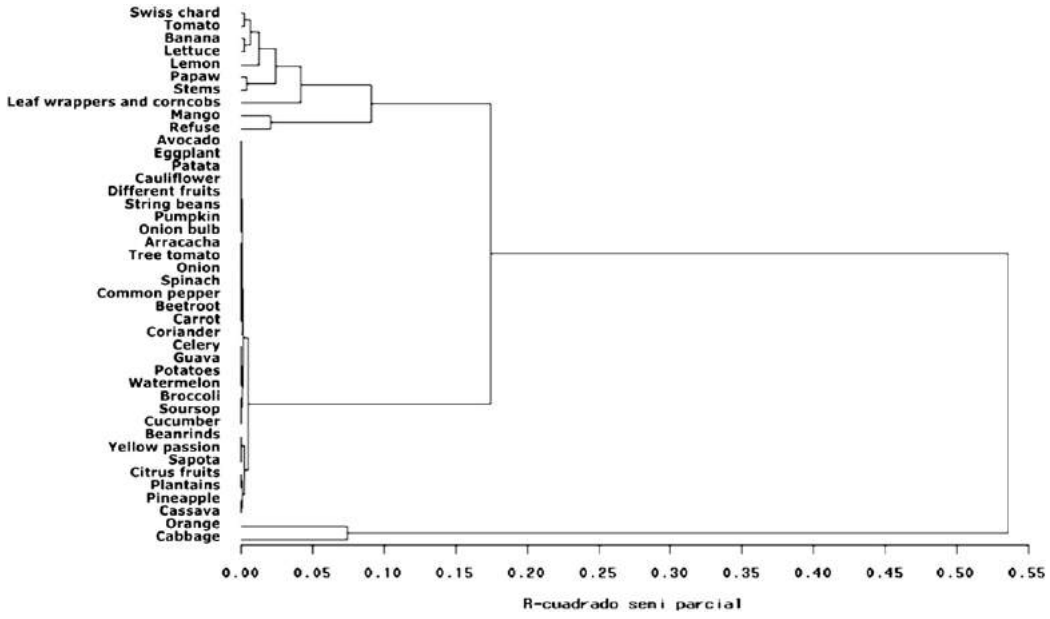

Fig. 1. Cluster analysis of the principal sources of FV in the Minorista Market.

Table 2
Nutritional composition of FV in the Minorista market during different periods and days.

	Periods				Days						
	P1	P2	P3	P4	D1	D2	D3	D4	D5	D6	D7
	August	September	October	November	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Protein %	9,05	10,2	9,95	10,7	10,0	10,7	10,3	9,5	8,9	8,9	11,6
DNF %	43.3bc	39.1ac	32.3a	31.6c	37.6	39.8	41.9	33.2	32.4	32.8	38.5
DAF %	32.7b	33.2ac	25,3ac	27,2bc	31.4	31.9	34.1	26,7	25,1	27,5	30,7
Degradability (24 h)	89.82	84.78	87.53	89.05	86.62	88.6b	89.61	86.04	89.64	82.94	86.78
Gross energy Kcal/kg	3553b	3569b	3717 ab	3789a	3643ba	3711ba	3592ba	3772a	3803a	3576a	3502b
Ca %	0.53	0.99	0.47	0.36	0.49	0.61	1,05	0.87	0.27	0.30	0.51
P %	0.20bc	0.27a	0.2bc	0.17c	0.21	0.2	0.21	0.22	0.20	0.23	0.21

Different letters among periods or days in the same row indicate significant statistical differences $p \leq 0.05$.

caused by the action of phytotoxic rind oils on the rind tissue (Knight et al., 2002). These findings indicate that it is necessary to do a previous characterization of each marketplace to know the basic composition of the waste as well as its possible variations before beginning their utilization for animal feeding on a large scale.

3.3. Nutritional composition

The nutritional analysis of FV is shown in Table 2. No statistical differences were found in CP or in Ca^{+2} content, either between days or between periods of evaluation ($p > 0.05$), while P was significantly different between periods but not between days. CP had a great stability among periods and days. The protein values found in this study are in agreement with those of Esteban et al. (2007) and Garcia et al. (2005), who found that FV contained 12% CP. However, our results differs from those of Ulloa et al. (2004), who mentioned that most of the tropical agricultural studied waste contained low levels of protein, which may limit their use for animal feeding, especially in fish feeds. The Ca^{+2} values obtained in this study (0.36–0.53%) were higher while P values (0.17–0.27%) were in the same range as the values found in FV by Garcia et al. (2005) (0.19–0.25% and 0.17–0.21% for Ca^{+2} and P, respectively). NDF and ADF were significantly different between periods but not between days. The ruminal degradability of the dry matter was significantly different between periods at 2 and 4 h but not at 6, 8, 12, and 24 h (Fig. 2). According to Esteban et al. (2007) fiber percentage of organic waste is around 13%, which contradicts data found in this study, where NDF reached values between 22.6 and 47.7%, and ADF reached values between 19.8 and 37.1%, with high dry matter degradability in all periods. Thus, more than 50% of FV was degraded at 2 h, and more than 80% was degraded at 24 h for all periods (Fig. 2), which demonstrates that there were components with a high digestibility during all evaluated periods. The differences found between periods could be attributed to the smaller degradability of the product during the first 2 h of period 1 and could be due to the smaller amount of soluble fraction as well as to the higher amount of the insoluble, potentially degradable fraction. During period 1, there were a large number of fibrous products, such as cabbage, stems, mangos, leaf wrappers, corncobs, bananas, plantains, and citrus fruit. Despite the fact that these products are fibrous, they had a fast degradability from 4 h. Gross energy was significantly different between periods and days. The average level of gross energy value was 3728 kcal/kg which corroborates the value reported by Martínez et al. (1990), who argued that the energy level was quite low for this kind of organic waste compared

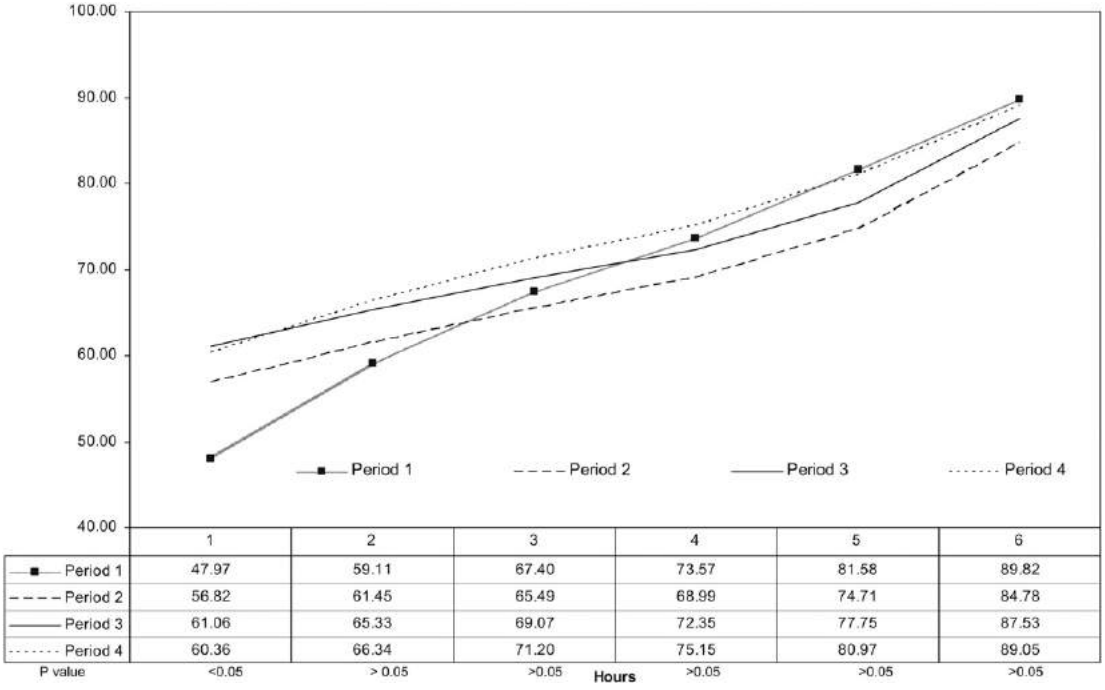

Fig. 2. Degradability of the dry matter during each period.

Table 3
Microbiological analysis of FV in the Minorista market (average/period).

Analysis	Samples with more than 12% humidity	Samples with less than 12% humidity	Reference value in feed destined for animals[1]
Mesophylls aerobic microorganisms/g	761 900	27 775	<1 000 000
Total colliforms/g	<226	<3	<1000
Fecal colliforms/g	<5	Absent	Absent
Molds and yeasts	110 805	943	<100.000
Salmonella spp/25g of food	Absent	Absent	Absent

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to some pastures. Nevertheless, our results are opposite of those reported by Zapata (1999) in the Fusagasuga market (Colombia) (4333 kcal/kg).

The National Research Council NRC (2001) recommends between 17.5 and 19.5% CP, between 1.24 and 1.46 Mcal/kg of net energy, between 0.60 and 0.67% Ca^{+2}, and between 0.32 and 0.38% P for dairy cows in early lactation (average milk yield between 55 and 120 lb/d); between 14.1 and 16.7% CP, between 1.88 and 2.02 Mcal/kg of net energy, between 0.74–0.79/Ca^{+2}, between 0.38 and 0.42% P at 90 days in milk (average milk yield 55–77 lb/d); and 10.8% CP, 0.96 Mcal/kg of net energy, 0.45% Ca^{+2}, and 0.23% P for dry, pregnant 270 days in gestation cows. Likewise, the NRC (1996) recommends between 7.5 and 10.6% CP, between 0.98 and 1.18 Mcal of net_m/kg, between 0.19 and 0.29% Ca^{+2}, and between 0.14 and 0.19% P for beef lactating cows, with an average 15 lb/d of milk at peak and 1000 lb mature weight. According to these recommendations, FV might be used as a potential source for beef lactating cows' diets supplemented with P, while it might be used as feedstuff for lactating dairy cows only if it is balanced with other feedstuffs that allow covering the nutritional requirements of animals, especially CP. However, it is necessary to consider that the nutritional composition of FV only gives an idea of its potential use for bovine feeding, but its real use could be given after evaluation with animals, where acceptability, voluntary intake, and percentage of inclusion in diets are factors that must be carefully considered.

3.4. Microbiological analysis

An increase was observed in the content of analyzed microorganisms, especially in mesophylls aerobic microorganisms, when the humidity of the samples was over 12% (Table 3). In a general way, FV with a maximum humidity level of 12% should be used for animal feeding to avoid microorganism proliferation and to allow the best product preservation. The microbiological determinations indicated no apparent health threat for animals, which agreed with data found by Myer et al. (1999) and by Sancho et al. (2004). When the dehydration process allows having no more than 12% DM (dry matter) in FV, it is still enough for an appropriate microbiological state. In the future, it will be necessary to organize the marketplaces to separate FV from the place where the products are being sold. Thus, it would be possible to reduce contamination and to get a product with better microbiological quality for use on a large scale.

4. Conclusions

The results found in this study indicate that the Minorista market produces approximately 8 356 200 g of FV per day (8.35 ton/day), which is composed of 43% fruit, 30% vegetables and 27% leaves, stems, leaf wrappers, corncobs, roots, refuse, and others. The forty-two products included in FV were classified into four groups, with the basic composition found in the products of groups 1 and 2 (oranges, cabbage, swiss chard, tomatoes, bananas, lettuce, lemons, papaw, stems, leaf wrappers, and corncobs), which changed according to different factors, such as the harvest period, the demand of the product, and its handling. In reference to the nutritional and the microbiological composition, FV represents a potential resource that might be used in bovine diets. Thus, recycling FV from the marketplaces for animal feeding is an important waste disposal alternative that might reduce the amount of biodegradable urban waste going to landfills; consequently, it might mitigate the environmental impact that organic solid waste generates in the world. However, it is important to highlight the need to first determine the FV's amount, components, and nutritional value for each case (using the same methodology) before assuming that in other marketplaces/cities the results will be similar to those presented in this manuscript.

"Prevent and minimize waste and maximize reuse, recycling and use of environmentally friendly alternative materials, to minimize adverse effects on the environment and improve resource efficiency is a necessity in the world" (United Nations, 2010).

Acknowledgments

The authors gratefully acknowledge a grant from Universidad de Antioquia (Colombia) (Project GRICA 026) and the economical support from Orgánicos Urbanos S.A. E.S.P as well as the statistical consultancy of Dr. Mario Fernando Cerón Muñoz (Universidad de Antioquia). Sincere thanks are given to the Minorista market "José Mª Villa" of the Medellin city, Antioquia, Colombia, for the logistic collaboration.

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Journal of Environmental Management 95 (2012) S210–S214

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Nutritional evaluation of fruit and vegetable waste as feedstuff for diets of lactating Holstein cows

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ARTICLE INFO

Article history:
Received 18 August 2009
Received in revised form
15 June 2011
Accepted 24 June 2011
Available online 23 July 2011

Keywords:
CLA
Environmental impact
Fatty acids
Linolenic acid

ABSTRACT

Organic waste from markets represents about 10–20% of the total waste of a city. A large proportion comes from the overproduction of fruit and vegetables, turning them into potential pollutant. The nutritional value found for fruit and vegetable waste (FV) from a marketplace, in a previous work, showed that this product might be considered as a potential alternative for animal feeding. This study evaluated the use of FV as feedstuff for diets of lactating Holstein cows with an emphasis on milk yield and quality. FV was included in 0, 6, 8, 12, and 18% of the concentrate. A 4 x 4 Latin squares model was used to analyze data (4 animal groups, 4 periods of evaluation, and 4 treatments). No statistical differences in milk yield per kilogram of eaten concentrate or concentrate intake were recorded between groups fed FV and the control group. There was a significant effect of the treatment on *cis*-9,*trans*-11 CLA and α-linolenic acid content in milk. These results showed that FV can be used as a dietary ingredient for high-yield lactating cows without detriment in the milk yield and with improvement in the milk quality. FV could be included at proportions of between 6% and 18% in the concentrate, as long as the animal's dietary requirements are covered. The main impact of these results is the alternative generated for the improvement of the environment.

1. Introduction

Thousands of years ago, the human population was small and faced few environmental problems. Humans' subsistence depended on the consumption and use of natural resources; the residues of the human activities were rapidly decomposed in the environment and did not cause many problems. However, the development of human settlements and with them the increase of urban agriculture has brought not only environmental problems but also economic and social impacts. According to McMichael (1994), "the life support systems of the biosphere are coming under stress because of the aggregate impact of human numbers and economic activity". In this way, marketplaces have become one of the main sources of fruit and vegetable waste (FV) in the world (Katongole et al., 2008; Buenrostro et al., 2000). Some alternatives such as composting and energy production have been used to reduce their long-term impact (Suthar, 2009; Kamaraj, 2008; Hossain and Fazliny, 2010). However, every day the problems increase because the amount of waste produced is higher than the amount reused. Therefore, it is necessary to seek more alternatives. In recent years, there has been substantial interest in linking waste management and sustainable animal food production. Consequently, the use of FV for animal feeding has also been proposed (Garcia et al., 2005; Esteban et al., 2007; Katongole et al., 2008). Using different products of fruit and/or vegetable waste as animal feed is not new. Some crop waste from rural places have been evaluated for cattle supplementation (Gaztambide, 1975; Montoya et al., 2004), for fish feed (Ulloa et al., 2004), and for layers' diets (Rehman et al., 2006) and is also a common informal practice at a low scale for developing countries (Furedy, 2004). Likewise, the use of vegetable subproducts from greenhouses (VSG) for sheep and goat feed has been recently evaluated by Márquez et al. (2010). However, there has been little research regarding the use of FV from urban marketplaces for animal feeding. Ruiz et al. (2000) have evaluated its use in rat diets, while Ngu and Ledin (2005) and Esteban et al. (2007) have conducted similar studies on its effect on the growth of goats and on its use in pig diets, respectively. However, we are not aware of any reports of studies made with dairy cows.

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doi:10.1016/j.jenvman.2011.06.050

The aim of this study was to know whether FV could be used as feedstuff for diets of lactating Holstein cows and how much FV these cows could make use of. This is a preliminary study focused on the effect of diets, including different percentages of FV on voluntary intake and the yield and quality of the milk produced.

Table 1
Average components and nutritional composition of fruit and vegetable waste from a marketplace.

Product	%
Cabbage	15.333%
Orange	13.596%
Refuse	11.550%
Stems	7.678%
Papaw	6.275%
Mango	5.801%
Banana	4.787%
Lettuce	4.317%
Lemon	4.112%
Leaf wrappers and corncobs	3.170%
Rinds	3.005%
Tomato	2.001%
Swiss Chard	1.613%
Cassava	1.454%
Plantains	1.372%
Citrus fruits	1.358%
Pineapple	1.208%
Sapote	1.086%
Yellow passion fruit	1.078%
Bean rinds	0.868%
Coriander	0.775%
Soursop	0.765%
Cucumber	0.596%
Potatoes	0.552%
Watermelon	0.590%
Broccoli	0.501%
Carrot	0.445%
Common Pepper	0.426%
Pumpkin	0.357%
Beetroot	0.347%
Celery	0.322%
Onion bulb	0.257%
Ochuva leafs	0.246%
Onion	0.215%
Mandarin	0.204%
Guava	0.188%
Spinach	0.185%
Other vegetables	0.170%
Tree Tomato	0.134%
Mustard	0.130%
Avocado	0.113%
Different fruits	0.111%
String beans	0.101%
Radish	0.083%
Cauliflower	0.076%
Others	0.075%
Arracacha	0.065%
Sweet passion fruit	0.060%
Muskmelon	0.056%
Parsley	0.046%
Honeyberry	0.031%
Eggplant	0.024%
Patata	0.021%
Green pea leafs	0.018%
Green pea	0.016%
Banana guineo	0.015%
Grapes	0.012%
Kale	0.004%
Peach	0.003%
Red pepper	0.001%

FV had on average 10% protein (PC), 36.6% NDF (Neutral detergent fiber), 29.6% ADF (Acid detergent fiber), 87.8% ruminal degradability at 24 h, 3657 Megacal/kg, 0.59% Ca (Calcium), and 0.21% P (Phosphorous).Adapted from Angulo et al. (2011).

2. Materials and methods

2.1. Location

The experiment was performed at the El Cofre Farm located in San Pedro de los Milagros (Antioquia, Colombia) at 2400 m above sea level, with an average temperature of 16 °C, a mean annual rainfall of 2500 mm, and a relative humidity of 72%.

2.2. Animals and experimental design

Twelve multiparous, lactating Holstein cows (≥3 births; between 120 and 140 postpartum days) were arranged in a 4 x 4 Latin square design that included four groups of three cows and four periods of evaluation of 25 days per group. The first five days of each period were set for adaptation. At the beginning of the experiment, the average milk yield of each group was 26, 25, 25, and 26 L/cow/day. The animals grazed in pastures of *Pennisetum clandestinum* under a rotational grazing system and were milked twice a day with an 11-hour interval. They were supplemented with maralfalfa grass (*Pennisetum purpureum*) cut at 90 days, molasses, 6% salt, and a specific dry mixed food (concentrate) according to the treatment. Maralfalfa grass mixed with molasses was supplemented before milking, while two equal portions of concentrate were given individually during each milking. For each cow, concentrate was supplemented at a level of 1 kg for each 3 L of milk yield. All animals had *ad libitum* access to water and salt.

Treatments were composed of concentrate including different levels of FV:

T1: (control group) concentrate without FV (0% FV).
T2: concentrate including 6% of FV (6% FV).
T3: concentrate including 12% of FV (12% FV).
T4: concentrate including 18% of FV (18% FV).

All treatments were prepared in the farm and were balanced considering the nutritional requirements of the animals (NRC) and the nutritional offer given by molasses and grass. The balance of diets was made by Colanta S.A. company (Medellín, Colombia) using NRC software ® (USA). The feedstuffs and nutritional composition of each treatment are presented in Tables 2 and 3, respectively. FV included in all treatments was taken from a marketplace located in Antioquia (Colombia) known as the Minorista market. The process of recollecting and selecting FV in the marketplace as well as its composition is described in detail by Angulo et al. (2011). Briefly, the collector cars were selected randomly in the entire marketplace, therefore the material, instead of being discarded as usual in the stationary boxes, was deposited in a special place chosen for this study. Every product included in FV was separated, classified, weighed, and registered by hand by 5

Table 2
Composition of each treatment.

Feedstuffs, % DM	Treatments			
	0% FV	6% FV	12% FV	18% FV
Milled corn	49.23	49.42	46.57	41.30
FV	0.00	6.00	12.00	18.00
Cotton seed	20.00	14.32	6.67	9.50
Energetic supplement	9.00	9.10	12.97	10.23
Cotton cake	8.35	2.00	2.00	2.00
Soybean cake	8.00	14.36	16.08	15.31
Sodium chloride (marine salt)	2.00	2.00	1.00	1.00
Calcium carbonate	0.40	0.40	0.40	0.40
Fertimin (mineral mix)	3.00	2.41	2.30	2.27

Table 3
Nutritional composition of treatments.

	Treatments			
	0% FV	6% FV	12% FV	18% FV
Dry Matter %	89.60	89.70	89.70	89.60
Crude protein (%DM)	16.60	16.60	16.60	16.60
TDN[a] (%DM)	79.00	79.00	79.50	77.30
NFE[b] (M cal/Kg)	1.79	1.78	1.78	1.74
NSC[c] (%DM)	46.70	46.40	52.80	51.30
Fat (%DM)	7.00	7.30	5.70	5.30
Ashes (%DM)	7.30	7.90	8.20	8.60
NDF (%DM)	22.40	21.80	16.80	18.10
ADF (%DM)	14.20	14.10	10.50	11.90
Lignin (%DM)	4.60	4.50	3.10	3.50
Ca^{+2} (%DM)	1.20	1.20	1.21	1.20
P (%DM)	0.66	0.59	0.51	0.52

[a] TDN = total digestible nutrients.
[b] NFE = nitrogen free extract.
[c] NSC = non structural carbohydrates.

people according to the kind of fruit or vegetable it contained and according to its condition (acceptable for animal feeding or not). The final waste contained a miscellany of small pieces of the different products, and rinds, which are difficult to classify, were considered as refuse. Finally, the material determined to be serviceable product was dehydrated. The average components and nutritional composition of FV is shown in Table 1.

Individual milk yields including all cows were measured daily throughout the experiment. Additionally, the ratio between the milk yield and the intake of concentrate (milk yield per kg of concentrate) was considered. Milk samples were collected for two consecutive milkings (morning and afternoon) on the same day at the end of each period for the determination of fatty acid composition by gas chromatography (GC) according to the methodology described by Mahecha et al. (2009). The methylation of fatty acids was made by BF_3/methanol (boron trifluoride-methanol). A total of 300 mg of fat residue were dissolved in 0.5 N NaOH/methanol and heated and refluxed for 10 min. After adding 5 ml of BF_3 20%, and heating and refluxing for 2 min, 4 ml of heptanes were added, and the heating and refluxing was continued for 2 min. The hexane layer was washed with sodium chloride-saturated solution. Fatty acid methyl esters were evaluated on a GC Hewlett Packard 5590 with an FID detector, a capillary column (Supelco wax-10 fused-60 m long × 0.32 mm id) equipped with an automatic split/splitless injector. An initial temperature of 150 °C was used. The temperature was then raised to 220 °C at 3 °C/min and held at this temperature for 15 min. H_2 was the carrier gas at a constant flow rate of 1.2 ml/min. Individual fatty acids were identified by comparing retention times with known FAMES. The fatty acid composition included linoleic acid (C18:2 *n*-6), *α*-linolenic acid (C18:3 *n*-3), oleic acid (C18:1*cis*-9), conjugated linoleic acid *cis*-9,*trans*-11 (CLA 1), conjugated linoleic acid *trans*-10,*cis*-12 (CLA2), myristic acid (C14:0), palmitic acid (C16:0), and stearic acid (C18:0). Myristic and palmitic acid standards were purchased from Aldrich; linoleic acid methyl ester, linoleic acid methyl ester isomers, and linolenic acid methyl ester were purchased from Supelco; oleic and stearic acids were obtained from Sigma.

2.3. Statistical analysis

All of the measurements were analyzed by a triplicate (2df) Latin square using 12 cows at 4 treatments (3df), 4 periods (3df), and 4 groups of cows (3 df). Analyses were performed using GLM of the Statistical System, SAS (2006). The differences between treatments were analyzed using a Tukey test at a significance level of 5%. The results are reported as least-squares means.

3. Results and discussion

This study was carried out to test the hypothesis that FV from marketplaces could be used as a dietary ingredient for lactating Holstein cows. In general, treatment diets including FV were successful in ensuring milk yields and concentrate intakes that are similar to that used in the control diet without FV. Therefore, compared with the control group, the milk yield did not change significantly when FV was included up to 12% of concentrate, and there were no significant differences in the concentrate intake up to an inclusion of 18%. Likewise, when the milk yield was analyzed based on a ratio of milk in litters per kilogram of concentrate, there were no significant differences up to an inclusion level of 18% (Table 4). These results indicate that the inclusion of FV in the concentrate did not present problems for its consumption. In the same way, considering that concentrate was supplemented according to the milk yield of each cow, it is necessary to focus on the results of milk yield in litters per kilogram of eaten concentrate, which indicates that FV could be incorporated into the concentrate up to a level of 18% for high-yield lactating cows without negative effects on their production. To our knowledge, there are no studies that report the intake and milk yield of dairy cows consuming concentrate including FV coming from a marketplace. It makes it difficult to contrast these results with literature. Nevertheless, there are some results obtained with pigs and goats using some FV from markets and others with dairy cows using individual agricultural waste that support the results observed in this study. Thus, Esteban et al. (2007) evaluated the inclusion of 20% FV collected from shops in the city of Salamanca (Spain) as potential feedstuff for growing-finishing pigs; FV was added to the swine diet at a level of 20%, and the results showed that it could be managed as alternative feedstuff. Ngu and Ledin (2005) found that feeding goat bucks with *Brassica* vegetable market waste did not affect the body weight gain. Katongole et al. (2008) found that sweet potato vine waste from markets was sufficient to provide the CP and metabolizable energy required by growing goats under tropical conditions in Kampala (Uganda). In the same way, other reports suggest that the use of fruit and vegetables from crops independently or in any combination of some of them does not cause any detriment in the milk yield. According to Gaztambide (1975), the inclusion of banana, plantain, potato, cassava, grapefruit, pineapple, and orange pulp from crops in diets for dairy cattle allows obtaining good results in the milk yield without affecting the smell or taste of the milk. Likewise, Montoya et al. (2004) found significant increases in milk yield after supplementation with 6 kg per day of potato waste,

Table 4
Milk yield, concentrate intake and milk yield per kg of eaten concentrate of lactating Holstein cows fed FV.

	Treatments				Significance *p* value
	0% FV	6% FV	12% FV	18% FV	
Milk yield (L/cow/period)	416.47[ab]	421.05[a]	398.97[bc]	393.75[c]	0.01
Concentrate intake (kg/cow/period)	113.81[ab]	118.00[ab]	106.82[b]	106.84[b]	0.05
Milk yield in litters per kg of eaten concentrate	3.65[a]	3.57[a]	3.83[a]	3.72[a]	0.53

Different letters among treatments in the same row indicate significant statistical differences $p \leq 0.05$.

Table 5
Milk fatty acids composition of lactating Holstein cows fed FV (g/100g fatty acids) (LSM ± SEM).

Treatments	Unsaturated fatty acids					Saturated fatty acids		
	Oleic	Linoleic	Linolenic	CLA 1	CLA 2	Miristic	Palmitic	Estearic
0% FV	24.98 ± 2.28[a]	1.20 ± 0.18[a]	0.28 ± 0.05[b]	1.01 ± 0.19[b]	0.16 ± 0.02[a]	16.77 ± 1.36[a]	39.97 ± 2.8[a]	16.14 ± 2.54[a]
6% FV	24.56 ± 2.03[a]	1.35 ± 0.18[a]	0.34 ± 0.04[a]	1.06 ± 0.29[bc]	0.17 ± 0.03[a]	16.27 ± 1.86[a]	39.85 ± 2.74[a]	16.03 ± 2.43[a]
12% FV	25.02 ± 3.10[a]	1.33 ± 0.36[a]	0.35 ± 0.1[a]	1.15 ± 0.32[ac]	0.18 ± 0.05[a]	16.84 ± 2.24[a]	39.63 ± 2.10[a]	15.50 ± 1.30[a]
18% FV	24.92 ± 2.45[a]	1.23 ± 0.90[a]	0.31 ± 0.06[ab]	1.18 ± 0.23[ac]	0.17 ± 0.03[a]	16.94 ± 1.56[a]	39.76 ± 2.15[a]	15.49 ± 2.27[a]

Different letters among treatments in the same column indicate significant statistical differences $p \leq 0.05$.

corresponding to 15% of non structural carbohydrate requirements in Holstein cattle.

In reference to fatty acid composition, there were significant differences between treatments for α-linolenic acid, whose presence was shown to be higher when FV was included as 6% and 12% (T2, T3) of concentrate, and for *cis*-9,*trans*-11 CLA, whose presence was shown to be higher after treatments with higher FV inclusion (12% FV, T3; and 18% FV, T4) ($p < 0.05$). No significant statistical differences between treatments were found for the other fatty acids ($p > 0.05$) (Table 5). These results highlight the viability of using FV as a dietary ingredient for lactating Holstein cows, not only because there is no negative effect on milk yield but also because the FV could improve milk quality. Although that the percentage of fatty acid in the FV was not measured, these results could be explained due to some fruit and vegetables are reported as a good source of α-linolenic acid (Bere, 2007; Simopoulos, 2004). It is well known that part of dietary C18:3 *n*-3 fatty acids can escape ruminal biohydrogenation (BH) and go on to be deposited in the tissues. However, most of them are extensively metabolized and biohydrogenated in the rumen resulting in different products according to different ruminal and diet conditions (Bauman and Griinari, 2003). Our results suggest that part of the possible higher amount of α-linolenic acid in treatment diets including FV could have been transferred at higher proportions to the milk of cows fed FV, and part could have been biohydrogenated producing vaccenic acid (*trans*-11 C18:1, VA). α-linolenic acid is a source for VA in rumen (Chilliard et al., 2007), which then goes to the mammary epithelial cell being transformed by delta-9 desaturase to *cis*-9,*trans*-11 CLA (Angulo et al., 2009). Several studies have investigated possible feeding strategies to increase PUFA and *cis*-9,*trans*-11 CLA content in meat and milk via the manipulation of ruminal BH. Results found by Vasta et al. (2009) indicates that including tannins in their diet could be a useful strategy for increasing the content of *cis*-9,*trans*-11 CLA and polyunsaturated fatty acids (PUFA) in ruminant meats; tannins inhibited the last step of the BH to a larger extent than the previous steps, leading to the accumulation of VA. Considering that citrus fruit contain tannins (Oluremi et al., 2007) and that orange was the second main component of FV in the present study, the possible higher presence of tannins in diets including FV could have influenced the increase of *cis*-9,*trans*-11 CLA and α-linolenic acid in milk. Because this is a preliminary study, there remain a number of nutritional questions to solve, and more research is necessary. Altering milk fatty acid composition with the potential to improve long-term human health includes enhancing the concentrations of several bioactive lipids (McKain et al., 2010). It is suggested that *cis*-9,*trans*-11 CLA might have anticarcinogenic and antiatherogenic effects (Palmquist et al., 2005; Lee, 2008) while *n*-3 fatty acids are reported to be very important in balancing the *n*-6/*n*-3 ratio, which is increasingly negative in the actual human diet (Simopoulos, 2008), and they also have a positive effect on heart health and potentially other diseases such as cancer, diabetes, and neurological disorders (Sretenović et al., 2009). Therefore, the increase of *cis*-9,*trans*-11 CLA and α-linolenic acid obtained in this study is very important for human health and indicates that the use of FV in dairy cows diets might increase the nutritive and therapeutic value of milk.

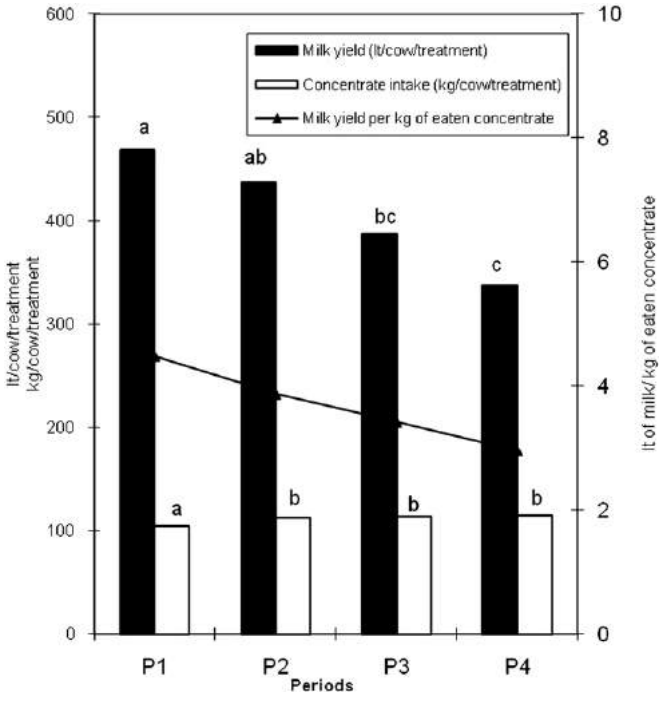

Fig. 1. Milk yield, concentrate intake and milk yield per kg of eaten concentrate of lactating Holstein cows during each period of evaluation. Different letters above the bars indicate a significant difference between periods with *p* value ≤0.05.

All of the traits showed significant differences between periods ($P < 0.05$). The milk yield and the milk yield per kilogram of eaten concentrate showed expected decreases in response to all cows after their peak of lactation and the milk yield gradually declines after the peak is attained (Silvestre et al., 2009). In reference to the intake of the concentrate, the differences observed between the first period and the others could be explained by a period of adaptation to the different diets (Fig. 1).

4. Conclusion

The inclusion of FV in the concentrate of lactating Holstein cows increased the proportion of α-linolenic acid and *cis*-9,*trans*-11 CLA in milk without affecting concentrate intake nor milk yield per kg of eaten concentrate. Our results indicate that FV is a good alternative feedstuff to balance concentrate for high-yield lactating cows. The level of its inclusion in the concentrate depends on the maintenance of the nutritional balance being possible its inclusion between 6% and 18%. These results show an important alternative for sustainable animal food production. However, the main impact of them is the alternative for the improvement of the environment. In general, if FV from marketplaces begins to be used for bovine

feeding, it might represent a significant alternative for their reuse at a high scale, since these animals have a greater capacity to consume these products than other species do. Therefore, they might be particularly useful in converting vast amounts of FV from marketplaces into food that is edible for humans and in this way might add value to this waste. It is currently very important but will become especially significant in the future, as the variety and quantity of FV from marketplaces and other urban places is expected to increase, and disposal options for many of these wastes, such as landfills, will become more limited and costly.

Acknowledgments

The authors would like to especially acknowledge the logistical contribution and all support from Juan Manuel Cerón from Colanta S.A. for the development of this study; likewise, they gratefully acknowledge the grant from Universidad de Antioquia (Colombia) (Project CIAG 026), Organicos Urbanos S.A. E.S.P., and Colanta S.A., as well as the statistical consultancy of Dr. Mario Fernando Cerón Muñoz (Universidad de Antioquia). Sincere thanks are given to Jaime Arturo Perez (owner of the farm) and Ramiro Gonzalez (worker) for the logistic collaboration.

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Journal of Environmental Management 95 (2012) S215–S222

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Almond tree and organic fertilization for soil quality improvement in southern Italy

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ARTICLE INFO

Article history:
Received 26 August 2009
Received in revised form
26 August 2010
Accepted 22 October 2010
Available online 13 November 2010

Keywords:
Degraded soil restoration
Organic amendment
Soil-plant system
Enzyme activities
Physical properties

ABSTRACT

The semi-arid Mediterranean region, characterized by long dry periods followed by heavy bursts of rainfall, is particularly prone to soil erosion.

The main goal of this study is to evaluate the soil quality under different practices of bio-physical amelioration which involve the soil-plant system (almond trees) and microorganism-manure. This study, carried out in the South of Italy (Basilicata Region- Pantanello farm), considered two types of fertilization (mineral and organic) and three slope gradients (0, 2 and 6%), in order to evaluate the effects of management practices in resisting soil erosion.

Chemical (organic carbon and nitrogen), physical (soil shrinkage and bulk density) and biochemical (dehydrogenase activity and hydrolytic enzyme activities) parameters were selected as markers to follow agro-ecological changes with time. The organic treatment affected soil microbiological and physico-chemical properties by increasing soil nutrient availability, microbial activity, and improving soil structure. The consistently higher values of the hydrolytic enzyme activities (β-glucosidase, phosphatase, urease and protease) often observed in the presence of plants and on the 0 and 2% slopes, suggested the stimulation of nutrient cycles by tree roots, which improve the conditions for soil microorganisms in carrying out their metabolic activity. In the 6% slope and, in particular, in the mineral fertilizer treatment, soil metabolism was lower as suggested by the dehydrogenase activity which was 50% lower than that found in the 0 and 2% slopes, this seemed to be related to a slowdown in the nutrient cycling and organic carbon metabolism. However, on this slope, in both mineral and organic treatments, a significant stimulation of hydrolytic enzyme activities and an improvement of soil structure (reduction of bulk density of about 10% and increase in total shrinkage from 20 to 60%) were observed with plants compared to the control soil. The combination of organic fertilization and almond trees resulted effective, also in the highest slope, in mitigating the degradation processes through the improvement of chemico-nutritional, biochemical and physical soil properties.

1. Introduction

Soil degradation refers to a decline in soil productivity through deterioration of the physical, chemical and biological properties of the soil. A key factor in the degradation of soils, in particular in arid and semi-arid areas, is the loss of plant cover, resulting in erosion and salinization problems (Albaladejo et al., 1994).

Soil erosion, which is caused by the impact of raindrops on bare soil and by the power of running water on the soil surface, has reached a very high level of importance in some areas, such as those around the semi-arid Mediterranean zone. In this area, inappropriate agricultural practices, together with adverse environmental and climatic factors, make the soil ecosystems very susceptible to these processes. Erosion affects the properties of soils mainly in three important ways: (i) loss in soil organic matter and nutrients, (ii) exposure of subsoil material with low fertility and/or high acidity, and (iii) degradation of soil structure (Smith et al., 2001).

As an important topographic factor, slope gradient has often been the focus of research into soil loss. In previous studies, slope gradient has been considered an important parameter in predicting

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doi:10.1016/j.jenvman.2010.10.050

soil losses (Valmis et al., 2005; Cheng et al., 2008). In general, a steeper slope gives a higher potential for runoff (Cheng et al., 2008). In fact, the effect of slope gradient on soil erosion influenced soil infiltration which decreased with the increase of slope gradient, producing runoff and soil losses (Jin, 1996).

The importance of vegetal cover in the physical protection of the soil from degradation and sediment transport has been clearly recognized (Morgan, 2005). In fact, the presence of vegetation reduces water-caused erosion by intercepting rainfall, increasing water infiltration, intercepting runoff at surface level, and mechanically stabilizing the soil with roots (Bochet et al., 2000). Moreover, vegetation contributes also to a significant enhancement of organic matter in the soil, thus increasing soil water holding capacity and soil biological fertility (Garcia et al., 1994). This is particularly important in arid and semi-arid zones, where SOM content is frequently low and climatic conditions leads to continuous losses (Madejón et al., 2007).

Vegetal cover may better sustain both the biological and physical characteristics of soil when an external source of organic manure is added. The addition of organic matter, in fact, is a current and efficient method for improving soil bio-physical properties. This occurs through the enhancement of root development, resistance to erosion, improvement of soil porosity and water infiltration and a decrease in soil crusting (Fernandes et al., 1997). This method has also resulted suitable for recovering degraded soils in semi-arid areas (Pascual et al., 1998; Ros et al., 2003).

The soil physico-chemical parameters generally used to evaluate soil quality (Parr and Papendick, 1997) change very slowly. In contrast, soil biological and biochemical parameters are very sensitive even to small changes occurring in soil (Smith and Papendick, 1993).

The aim of this study is to evaluate the efficacy and suitability of almond tree cultivation under organic fertilization as an environmental approach for improving the chemical (organic carbon and nitrogen), physical (soil shrinkage and bulk density) and biochemical (dehydrogenase activity and hydrolytic activities) conditions in semi-arid degraded soil at different slope gradients.

2. Material and methods

2.1. Experimental layout

The field experiment was located in the Metapontino area (province of Matera, Basilicata region) in the South of Italy (latitude 40° 23′N and longitude 16° 46′E). The climate in this area is predominantly Mediterranean with dry hot summers and cold winters, an average annual temperature of 16.6 °C and an average rainfall of 46.2 mm. The soil was classified as a Vertic Cambisol (European Soil Bureau Network, 2005). It was a sandy clay loam soil (USDA texture classification) with a low content of organic matter (1.07%). Three different fields of about 0.3 ha (85 m × 35 m) each were set up on Pantanello farm. The different fields were characterized by different slopes (0, 2, and 6%). Each field was split up into two parts, one assigned to organic fertilization and the other assigned to mineral fertilization. The almond variety TUONO in the rootstock of GF677 was chosen for its superior pedological and climatic adaptability. The planting was carried out in March 2006 with a distance of 4 m × 5 m. The organic fields were fertilized only once a year (April 2006) using 1.5 t ha^{-1} of commercial cow-manure (in pellets). This organic fertilizer, containing 25% organic carbon, 3% organic nitrogen and 3% phosphorus oxide, was applied to the soil surface and incorporated into the soil at 0–15 cm. The mineral fertilization was carried out using a mixture of ammonium nitrate (15%), phosphorus oxide (7.5%) and potassium oxide (20%). The mineral fertilizer was spread on the soil surface without incorporation three times during the spring-summer period and with a total quantity of 0.3 t ha^{-1}. A soil tillage (harrowing) was performed at 10–15 cm depth in interrow soils every 40–50 days from March to June, retaining the plant residues and weeds on the land. A drip irrigation system with about 2000 m^3 ha^{-1} of water per year was established.

The soil samplings were carried out in November 2006 (t1) and November 2007 (t2). To evaluate the effect of plants for each experimental factor (fertilizer and slope), the samples were collected in the rhizosphere area (plant) within 1 m from the trunk, and in the plant interrow (interrow) 2 m away from the trunk. For each slope a plot close to each field without fertilizers and plants was also used as a control (control soil).

Therefore, the experimental site was established in order to verify the effects of different management and environmental factors: fertilization, plant, slope and time.

Each sample (three samples for each experimental factor), consisting of five subsamples (150 cm^3 soil cores), was taken from the upper soil layer (0–15 cm depth). The subsamples were mixed, homogenised, and sieved (2 mm) and the resulting samples stored at room temperature until analysis.

2.2. Chemical, physical and biochemical analyses

The particle size analysis was calculated by a pipette procedure (Indorante et al., 1990). Total organic C (TOC) and N (TN) were determined by dry combustion with a RC-412 multiphase carbon and a FP-528 protein/nitrogen determinator, respectively (LECO corporation). Total extractable carbon (TEC) was measured in pyrophosphate 0.1 M pH 11 extract (1:10 w:v). Water soluble carbon (WSC) and carbohydrates (WSCA) were measured in an aqueous extract (1:10 w:v). WSC and TEC were determined by the Yeomans and Bremner (1989) method. WSCA were determined as reported by Brink et al. (1960). NH_4^+ was measured with an ammonia-selective electrode (ORION 95-12), and NO_3^- was measured with a DIONEX chromatograph. Available P (avP) was determined by the method of Murphy and Riley (1962). Soil shrinkage was determined by the Petruzzelli et al. (1976) method. Soil bulk density was measured on undisturbed cores (Blake and Hartge, 1986). Urease and N-α-benzoyl-L-argininamide (BAA) hydrolysing protease activities were determined following the Nannipieri et al. (1980) methods. β-glucosidase and phosphatase activities were determined by the Masciandaro et al. (1993) methods. Dehydrogenase activity was determined by the method of Masciandaro et al. (2000a).

2.3. Statistical analysis

All results are the means of three replicates ($n = 3$). All numerical parameters before statistical analysis were normalized and autoscaled: the result for each variable is a zero mean and a unit standard deviation (Latorre et al., 1999). The STATISTICA 6.0 software (StatSoft Inc., Tulsa, Oklahoma, USA) was used for all statistical analysis.

A statistical correlation between the data was calculated. The reported significant levels ($P < 0.05$) are based on Student's distribution.

Analysis of variance (ANOVA) was used to evaluate the differences ($p < 0.05$) between time (t1 and t2), fertilizers (organic and mineral) presence or absence of plants (P and I), slopes (0%, 2% and 6%), and their interactions. Differences between treatments and control soil within each slope and time were tested using Dunnett's comparison test.

The results were also studied using principal component analysis (PCA). The PCA is a multivariate statistical data analysis technique which reduces a set of raw data to a number of principal

Table 1
Soil chemical and biochemical properties at two sampling dates (November 2006 - t1; November 2007 - t2) in the plot at 0% slope.

	t1					t2				
	C	OF-P	OF-I	MF-P	MF-I	C	OF-P	OF-I	MF-P	MF-I
TOC/%C	0.57	0.91*	0.83*	0.85*	0.86*	0.52	0.91*	0.75*	0.80*	0.80*
TN/%N	0.08	0.13*	0.11*	0.11*	0.12*	0.06	0.13*	0.13*	0.10*	0.11*
WSC/μg C g^{-1}	203	183	154*	185	183	190	187	201	204	155*
WSCA/μg C g^{-1}	20.9	27.5*	28.6*	37.9*	38.9*	13.9	14.5	9.37*	10.1*	8.51*
TEC/μg C g^{-1}	1999	3488*	3884*	3485*	3582*	2164	3325*	3540*	3948*	3207*
NH_4^+/μg g^{-1}	1.19	1.28	1.82*	0.61*	0.44*	1.54	0.55*	0.61*	2.18*	1.55
NO_3^-/μg g^{-1}	11.0	14.7*	13.5*	13.3*	14.2*	8.08	39.5*	45.8*	24.6*	23.4*
avP/μg g^{-1}	15.9	22.6*	28.9*	11.9*	14.2	9.57	3.66*	9.5*	1.44*	5.37*
β-Glucosidase/μg PNP g^{-1} h^{-1}	35.7	84.7*	68.8*	74.9*	70.7*	29.9	109*	103*	95.9*	75.1*
Phosphatase/μg PNP g^{-1} h^{-1}	31.6	78.5*	67.1*	51.1*	26.6	45.5	98.7*	81.3*	101*	66.7*
Urease/μg NH_4^+ g^{-1} h^{-1}	6.45	12.11*	12.96*	8.48*	12.32*	6.31	9.12*	9.84*	11.34*	9.27*
Protease/μg NH_4^+ g^{-1} h^{-1}	2.98	4.10*	3.87*	5.62*	4.65*	1.91	6.02*	4.53*	2.37*	1.93
Dehydrogenase/μg INTF g^{-1} h^{-1}	0.97	1.52*	1.17*	0.77*	0.69*	1.09	1.96*	1.25	1.53*	1.21
β-Glucosidase/C/g PNP kgC^{-1} h^{-1}	6.26	9.31*	8.29*	8.81*	8.22*	5.75	12.0*	13.7*	12.0*	9.39*
Phosphatase/C/g PNP kgC^{-1} h^{-1}	5.54	8.63*	8.08*	6.01	3.09*	8.75	10.8*	10.8*	12.6*	8.34
Urease/C/g NH_4^+ kgC^{-1} h^{-1}	1.13	1.33	1.56*	1.00	1.43*	1.21	1.00*	1.31	1.42	1.16
Protease/C/g NH_4^+ kgC^{-1} h^{-1}	0.523	0.41	0.466	0.661*	0.541	0.367	0.662*	0.604*	0.296*	0.241*
Dehydrogenase/C/g INTF kgC^{-1} h^{-1}	0.170	0.167	0.141*	0.091*	0.080*	0.210	0.215	0.167*	0.191	0.151*

* Dunnett's test ($p < 0.05$), comparing separately mean value ($n = 3$) of each treatment with its control at each time. C, control; OF, organic fertilization; MF, mineral fertilization; P, plant; I, interrow. TOC, Total Organic Carbon; TN, Total Nitrogen; WSC, Water Soluble Carbon; WSCA, Water Soluble Carbohydrates; TEC, Total Extractable Carbon; avP, available phosphorus.

components that retain the most variance within the original data in order to identify possible patterns or clusters between objects and variables (Carroll et al., 2004).

3. Results and discussion

The effects of plants, farming system and slope were evaluated with one year of experiment from November 2006 to November 2007.

Total organic carbon (TOC) and nitrogen (TN) content of treated soil samples increased significantly with respect to the control soils (Table 1–4). A possible explanation for these increases could be the release of root exudates and plant remains by almond trees and vegetal ground cover naturally established in the interrow soils after fertilization. Also Ramos et al. (2010), in a study on different almond orchard managements under semi-arid conditions, reported a greater soil organic matter due to the presence of vegetation cover. The increase in TOC and TN in the organic treatment may also be due to the incorporation of organic matter added as amendment, confirming the finding of previous works (Liebig and Doran, 1999; Derrick and Dumaresq, 1999). The significant decrease in these parameters with experimental time, especially in the 2% and 6% slopes, indicated the mineralization of the easily degradable soil organic matter (Table 1–4).

On the other hand, the more resistant pool of organic matter (humic substances), evaluated by the assay of total extractable carbon (TEC), showed a tendency to decrease with time in each slope. However, it was significantly higher than that found in the control soils, thus meaning a great contribution of the treatments in enriching the soil with the more stable component of organic matter. In addition, after one year from soil organic amendment application, TEC content in the organic fertilization was not different with respect to the mineral fertilizer (Table 1–3), confirming the results of a previous study (Marinari et al., 2007), in which the soil organic matter dynamics in organically and conventional managed fields was evaluated.

The WSC fraction, which is an important pool of organic matter readily decomposable for soil microorganisms (Caravaca et al., 2002), showed little variation among treatments, slopes and time of sampling.

Table 2
Soil chemical and biochemical properties at two sampling dates (November 2006 - t1; November 2007 - t2) in the plot at 2% slope.

	t1					t2				
	C	OF-P	OF-I	MF-P	MF-I	C	OF-P	OF-I	MF-P	MF-I
TOC/%C	0.63	0.92*	1.06*	0.96*	0.83*	0.56	0.81*	0.75*	0.56	0.63
TN/%N	0.08	0.13*	0.12*	0.13*	0.15*	0.08	0.16*	0.14*	0.10*	0.09
WSC/μg C g^{-1}	108	235*	246*	212*	203*	123	249*	236*	102*	179*
WSCA/μg C g^{-1}	23.6	34.4*	32.5*	29.7*	33.3*	18.9	14.8*	9.94*	14.0*	7.36*
TEC/μg C g^{-1}	2729	3967*	3811*	3768*	3385*	2875	3551*	3444*	2918	3132
NH_4^+/μg g^{-1}	2.44	1.33*	1.61*	1.29*	1.15*	1.98	0.95*	0.61*	0.73*	2.68*
NO_3^-/μg g^{-1}	16.2	9.81*	10.1*	7.09*	13.6	14.9	38.8*	36.4*	9.82*	6.52*
avP/μg g^{-1}	10.0	4.5.0*	7.32*	10.3	21.8*	6.88	0.94*	1.23*	8.09	3.66*
β-Glucosidase/μg PNP g^{-1} h^{-1}	45.3	72.6*	66.1*	39.7	66.1*	37.2	111*	81.1*	70.5*	63.7*
Phosphatase/μg PNP g^{-1} h^{-1}	50.5	60.8*	76.4*	54.2	50.7	40.8	123*	106*	56.2*	48.1
Urease/μg NH_4^+ g^{-1} h^{-1}	6.24	8.17*	7.02*	5.51	9.61*	6.93	12.4*	9.52*	11.8*	5.06*
Protease/μg NH_4^+ g^{-1} h^{-1}	2.17	3.52*	2.95*	2.88*	2.90*	1.87	6.59*	4.63*	3.15*	1.97
Dehydrogenase/μg INTF g^{-1} h^{-1}	0.77	1.68*	0.8	0.44*	0.78	1.15	2.11*	1.46*	1.07	1.03
β-Glucosidase/C/g PNP kgC^{-1} h^{-1}	7.19	7.89	6.24	4.14*	7.96	6.64	13.7*	10.8*	12.6*	10.1*
Phosphatase/C/g PNP kgC^{-1} h^{-1}	8.02	6.61*	7.21	5.65*	6.11*	7.29	15.2*	14.1*	10.0*	7.63
Urease/C/g NH_4^+ kgC^{-1} h^{-1}	0.990	0.888	0.662*	0.574*	1.16	1.24	1.53*	1.27	2.10*	0.803*
Protease/C/g NH_4^+ kgC^{-1} h^{-1}	0.344	0.383	0.278*	0.300	0.349	0.334	0.814*	0.617*	0.563*	0.313
Dehydrogenase/C/g INTF kgC^{-1} h^{-1}	0.122	0.183*	0.075*	0.046*	0.094*	0.205	0.260*	0.195	0.191	0.163*

* Dunnett's test ($p < 0.05$), comparing separately mean value ($n = 3$) of each treatment with its control at each time. C, control; OF, organic fertilization; MF, mineral fertilization; P, plant; I, interrow. TOC, Total Organic Carbon; TN, Total Nitrogen; WSC, Water Soluble Carbon; WSCA, Water Soluble Carbohydrates; TEC, Total Extractable Carbon; avP, available phosphorus.

Table 3
Soil chemical and biochemical properties at two sampling dates (November 2006 - t1 and November 2007 - t2) in the plot at 6% slope.

	t1					t2				
	C	OF-P	OF-I	MF-P	MF-I	C	OF-P	OF-I	MF-P	MF-I
TOC/%C	0.69	0.80	0.76	0.90*	0.89*	0.63	0.86*	0.68	0.62	0.61
TN/%N	0.08	0.15*	0.12*	0.13*	0.12*	0.07	0.10*	0.12*	0.09*	0.06
WSC/μg C g^{-1}	204	253*	244*	199	174	179	225*	182	220*	238*
WSCA/μg C g^{-1}	21.4	35.8*	33.9*	33.8*	34.1*	18.6	29.2*	13.0*	20.3	26.5*
TEC/μg C g^{-1}	2084	3843*	3292*	4399*	3453*	2087	3261*	3315*	3272*	3529*
NH_4^+/μg g^{-1}	1.43	1.63	0.53*	0.39*	0.23*	1.95	0.59*	1.18*	0.55*	3.49*
NO_3^-/μg g^{-1}	28.4	14.3*	15.1*	15.9*	8.93*	30.8	58.3*	25.8	17.7*	13.8*
avP/μg g^{-1}	9.49	6.11*	7.92*	8.59	6.11*	7.96	0.84*	4.86*	3.76*	3.45*
β-Glucosidase/μg PNP g^{-1} h^{-1}	33.4	48.9*	47.4*	33.2	24.0*	45.7	116*	90.5*	60.9*	42.1
Phosphatase/μg PNP g^{-1} h^{-1}	39.7	58.9*	50.1*	51.8*	35.6	56.4	99.2*	85.1*	68.7*	54.4
Urease/μg NH_4^+ g^{-1} h^{-1}	7.90	6.10*	5.08*	6.02*	6.39*	7.20	8.60	5.65*	9.81*	7.13
Protease/μg NH_4^+ g^{-1} h^{-1}	2.11	5.05*	3.01*	3.52*	4.04*	1.70	7.30*	2.27*	3.12*	1.93
Dehydrogenase/μg INTF g^{-1} h^{-1}	0.54	0.73*	0.75*	0.74*	0.64	0.70	1.10*	1.12*	0.64	0.51*
β-Glucosidase/C/g PNP kgC^{-1} h^{-1}	4.84	6.11*	6.24*	3.69*	2.70*	7.25	13.5*	13.3*	9.82*	6.90
Phosphatase/C/g PNP kgC^{-1} h^{-1}	5.75	7.36*	6.59	5.76	4.00*	8.95	11.5*	12.5*	11.1*	8.92
Urease/C/g NH_4^+ kgC^{-1} h^{-1}	1.14	0.763*	0.668*	0.669*	0.718*	1.14	1.00	0.831*	1.58*	1.17
Protease/C/g NH_4^+ kgC^{-1} h^{-1}	0.306	0.631*	0.396*	0.391*	0.454*	0.270	0.849*	0.334*	0.503*	0.316
Dehydrogenase/C/g INTF kgC^{-1} h^{-1}	0.078	0.091	0.099*	0.082	0.072	0.111	0.128	0.165*	0.103	0.084*

* Dunnett's test ($p < 0.05$), comparing separately mean value ($n = 3$) of each treatment with its control at each time. C, control; OF, organic fertilization; MF, mineral fertilization; P, plant; I, interrow. TOC, Total Organic Carbon; TN, Total Nitrogen; WSC, Water Soluble Carbon; WSCA, Water Soluble Carbohydrates; TEC, Total Extractable Carbon; avP, available phosphorus.

Table 4
ANOVA, effect of time, slope, fertilization and plant and their interaction in chemical, biochemical and physical parameters (*, $p < 0.05$; ns, not significative).

Effect	TOC	TN	WSC	WSCA	TEC	NH_4^+	NO_3^-	avP	TS	BD	β-Glu	Pho	Ure	Pro	DH	β-Glu/C	Pho/C	Ure/C	Pro/C	DH/C
Time (1)	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Slope (2)	*	*	*	*	ns	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Fertilization (3)	*	*	*	ns	ns	ns	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Plant (4)	*	*	ns	*	*	*	*	*	*	ns	*	*	*	*	*	*	*	*	*	*
1 × 2	*	*	*	*	*	*	*	*	*	ns	*	*	*	*	*	*	ns	*	*	*
1 × 3	*	*	ns	*	ns	*	*	*	*	ns	*	ns	ns	*	ns	ns	*	*	*	*
2 × 3	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	ns	*	ns	ns
1 × 4	ns	ns	*	*	ns	*	*	*	*	*	*	ns	*	*	*	*	ns	*	*	ns
2 × 4	ns	*	*	*	ns	*	*	*	*	ns	*	*	*	*	*	*	*	*	*	*
3 × 4	*	ns	*	*	ns	*	ns	*	ns	ns	ns	*	*	*	*	ns	*	ns	*	*
1 × 2 × 3	*	*	*	*	ns	*	*	*	*	ns	ns	*	*	*	*	*	*	*	*	*
1 × 2 × 4	ns	*	*	*	*	*	*	*	ns	ns	*	*	*	*	ns	*	*	*	*	ns
1 × 3 × 4	*	*	*	*	ns	*	ns	*	*	ns	*	*	*	ns	*	*	ns	*	*	*
2 × 3 × 4	*	*	*	*	*	*	ns	*	*	ns	*	*	ns	*	*	*	*	*	*	*
1 × 2 × 3 × 4	*	*	*	*	ns	*	*	*	*	ns	*	ns	*	*	*	*	ns	*	*	*

TOC, Total Organic Carbon; TN, Total Nitrogen; WSC, Water Soluble Carbon; WSCA, Water Soluble Carbohydrates; TEC, Total Extractable Carbon; avP, available phosphorus; TS, Total Shrinkage; BD, Bulk density; β-Glu, β-Glucosidase; Pho, Phosphatase; Ure, Urease, Pro, Protease; DH, Dehydrogenase, β-Glu/C, β-Glucosidase/TOC; Pho/C, Phosphatase/TOC; Ure/C, Urease/TOC, Pro/C, Protease/TOC; DH/C, Dehydrogenase/TOC.

Table 5
Correlation matrix between chemical, biochemical and physical parameters of soil samples.

	TOC	TN	WSC	WSCA	TEC	NH_4^+	NO_3^-	avP	TS	BD	β-Glu	Pho	Ure	Pro	DH	β-Glu/C	Pho/C	Ure/C	Pro/C
TN	0.75*																		
WSC	0.41*	0.31																	
WSCA	0.43*	0.17	0.21																
TEC	0.70*	0.69*	0.28	0.24															
NH_4^+	−0.35	−0.47*	−0.13	−0.24	−0.24														
NO_3^-	0.09	0.17	0.18	−0.32	−0.02	−0.20													
avP	−0.02	−0.07	−0.35	0.40*	−0.18	0.06	−0.51*												
TS	0.05	0.14	−0.17	−0.27	0.20	0.15	−0.24	0.20											
BD	−0.17	−0.40*	0.09	0.29	−0.30	−0.08	−0.11	0.18	−0.59*										
β-Glu	0.34	0.47*	0.13	−0.33	0.40*	−0.06	0.56*	−0.40*	0.39*	−0.52*									
Pho	0.30	0.39*	0.28	−0.40*	0.39*	0.03	0.62*	−0.61*	0.18	−0.52*	0.75*								
Ure	0.20	0.26	−0.11	−0.11	0.21	−0.15	0.37*	−0.05	0.47*	−0.40*	0.60*	0.36*							
Pro	0.57*	0.67*	0.33	0.29	0.47*	−0.64*	0.35	−0.23	0.09	−0.27	0.50*	0.37*	0.42*						
DH	0.09	0.31	−0.05	−0.50*	0.14	0.00	0.39*	−0.37*	0.44*	−0.56*	0.66*	0.62*	0.46*	0.33					
β-Glu/C	−0.11	0.15	−0.05	−0.54*	0.10	0.09	0.55*	−0.41*	0.39*	−0.47*	0.90*	0.65*	0.54*	0.27	0.66*				
Pho/C	−0.21	0.01	0.08	−0.63*	0.04	0.21	0.58*	−0.62*	0.15	−0.44*	0.59*	0.87*	0.27	0.08	0.59*	0.72*			
Ure/C	−0.43*	−0.21	−0.35	−0.36	−0.23	0.08	0.29	−0.03	0.41*	−0.27	0.35	0.15	0.80*	0.04	0.38*	0.57*	0.38*		
Pro/C	0.14	0.39*	0.17	0.12	0.18	−0.57*	0.38*	−0.27	0.08	−0.23	0.42*	0.28	0.40*	0.89*	0.35	0.38*	0.21	0.28	
DH/C	−0.36	−0.04	−0.23	−0.65*	−0.18	0.15	0.33	−0.34	0.39*	−0.45*	0.47*	0.45*	0.35	0.06	0.90*	0.66*	0.64*	0.54*	0.27

* Correlation coefficients statistically significant at $p < 0.05$ ($n = 3$). TOC, Total Organic Carbon; TN, Total Nitrogen; WSC, Water Soluble Carbon; WSCA, Water Soluble Carbohydrates; TEC, Total Extractable Carbon; avP, available phosphorus; TS, Total Shrinkage; BD, Bulk density; β-Glu, β-Glucosidase; Pho, Phosphatase; Ure, Urease, Pro, Protease; DH, Dehydrogenase, β-Glu/C, β-Glucosidase/TOC; Pho/C, Phosphatase/TOC; Ure/C, Urease/TOC, Pro/C, Protease/TOC; DH/C, Dehydrogenase/TOC.

On the other hand, in all treatments the significant decrease in carbohydrates (WSCA) over time indicated a higher utilisation of these compounds as a primary C available energy source by microorganisms (Table 1–4). WSC and WSCA often resulted higher in the presence of plants, suggesting inputs from the root exudates and an amount of fresh residues released by the almond trees into the soil (Garcia et al., 1997) (Table 1–4).

As regards the soluble forms of N, at the end of the experiment, in agreement with previous findings (Gelsomino et al., 2006), soil nitrate was significantly higher in the organic than in the mineral fertilization, while ammonia resulted lower (Table 1–4). A possible explanation is a direct addition of different microorganism species and a stimulation of activity of the microbial nitrifying population (Chao et al., 1996), probably due to a better chemico-physical condition induced by organic amendment (Giusquiani et al., 1995).

It is also well known that organic fertilization in semi-arid regions improves the microbiological and biochemical conditions in soil (Masciandaro et al., 2000b; Tejada et al., 2007). Dehydrogenase activity, often used as a measurement of the effects induced by management systems on the entire microbial metabolism, even in semi-arid conditions (Garcia et al., 1997), resulted higher in the organic treatments (Table 1–4). A significant increase in dehydrogenase activity over time was also observed in the mineral fertilization. This enzyme activity was not generally significantly different between plant and interrow samples in the mineral treatments, while the association of plants and organic manure showed higher dehydrogenase activity in the 0 and 2% slopes. The lower dehydrogenase activity found in all treatments in the 6% slope, in particular in the mineral treatment, suggested worse conditions for microbial activity.

The complete enzymatic metabolic picture was followed by testing hydrolytic enzyme activities linked to the C (β-glucosidase), N (urease, protease), and P (phosphatase) cycles (Table 1–4). The β-Glucosidase activity, which represents the soil potential to hydrolyze low molecular weight carbohydrates (Eivazi and Tabatabai, 1990), and phosphatase, which catalyses the hydrolysis of organic P with the release of free phosphate (Condron et al., 2005), showed, as seen for the dehydrogenase enzyme, a significantly higher activity for the organic treatment than for the mineral one, with increasing values over time (Table 1–4). These enzymes were also generally higher in the rhizosphere soils, suggesting that the plant roots could induce their synthesis. In addition, the phosphatase activity was negatively correlated with available phosphorus, as expected (Table 5) (Nannipieri et al., 1990).

On the other hand, the protease activity, an enzyme involved in the nitrogen cycle, showed a different trend in the two types of fertilizations. In fact, it was activated by the addition of organic matter to the soil, while it was inhibited in the mineral treatment (Table 1–4). This was probably due to the higher content of ammonia, as suggested by the negative correlation between these two parameters (Table 5). In fact, several studies have reported repression of protease synthesis by high levels of NH_4^+ (Bascaran et al., 1990; Beg et al., 2002). However, the highest activity was always found in the presence of plants for both treatments with respect to the control soils (Table 1–4).

Similarly, the activity of urease, another enzyme linked to the N cycle, which generally increased over time in both treatments (Table 1–4), resulted higher in the presence of plants in both treatments at the end of the experiment. In agreement with our results, Garcia et al. (2002) reported an increase in urease activity in the planted soil under a semi-arid climate as being attributable to root exudates.

Finally, to summarize the trend of all the hydrolytic enzyme activities, it is possible to see a clear tendency to decrease as the slope increases (Table 1–4).

In order to verify if the increase of enzyme activities was mainly due to the direct addition of organic matter or to the influence of other environmental factors, the specific enzyme activity was calculated as the ratio between each enzyme and TOC (Barriuso et al., 1988). Our results showed that the specific activities reflected the trend of general enzyme activities within the treatments. This suggests that biochemical properties besides being affected by organic farming, in this case, were probably more influenced by the improvement of other soil environmental conditions, such as physical properties. The physical effects of the treatments were evaluated through measurement of bulk density and surface

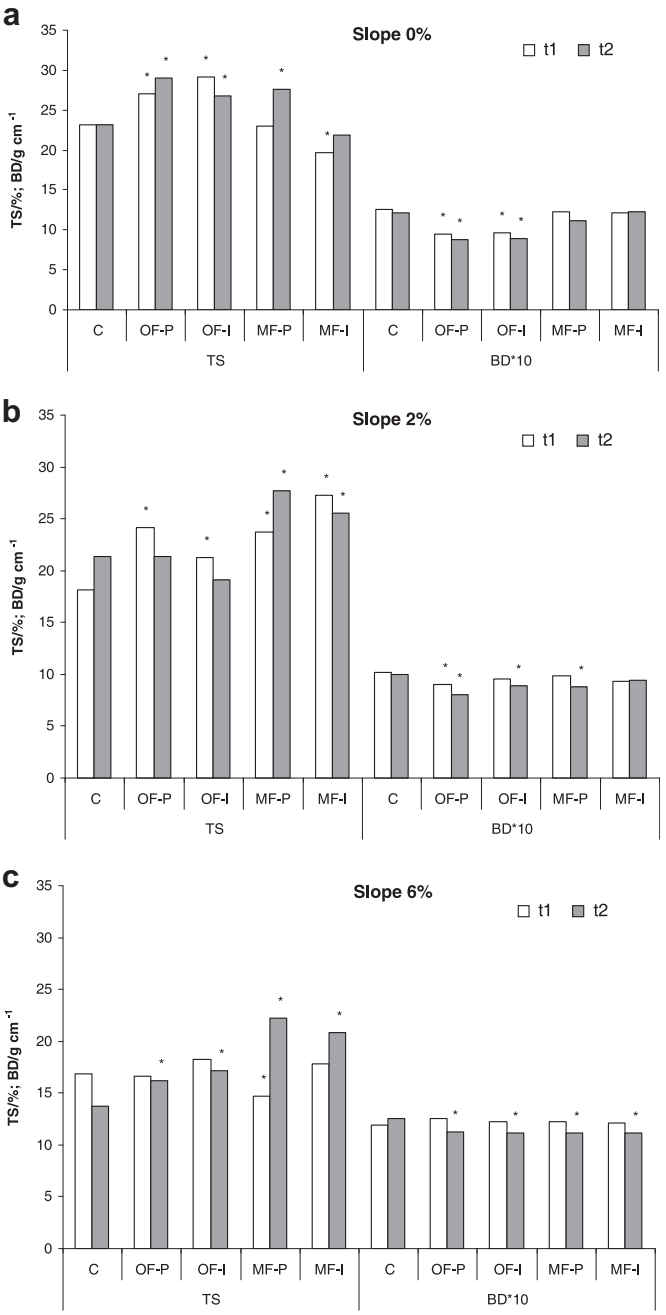

Fig. 1. Mean ($n = 3$) of total shrinkage and bulk density measured in November 2006 (t1) and November 2007 (t2) in 0% (a), 2% (b) and 6% (c) slopes. The values of bulk density were multiplied for 10. C, control; OF, organic fertilization; MF, mineral fertilization; P, plant; I, interrow; t1, November 2006; t2, November 2007. * Dunnett's test ($p < 0.05$), comparing separately each treatment with its control at each time.

Table 6
Principal components (PC) and component loadings.(ns, not significative).

	Principal Components		
Variables	1	2	3
TOC	ns	0.872	ns
TN	ns	0.805	ns
WSC	ns	ns	ns
WSCA	ns	0.640	ns
TEC	ns	0.761	ns
NH_4^+	ns	ns	ns
NO_3^-	0.820	ns	ns
P available	−0.736	ns	ns
β-Glucosidase	0.690	ns	ns
Phosphatase	0.800	ns	ns
Urease	ns	ns	0.757
Protease	ns	0.693	ns
Dehydrogenase	0.670	ns	ns
Total shrinkage	ns	ns	0.893
Bulk density	ns	ns	−0.708
Var. Sp.	3.538	3.752	3.001
Prp.Tot.	0.236	0.250	0.200

TOC, Total Organic Carbon; TN, Total Nitrogen; WSC, Water Soluble Carbon; WSCA, Water Soluble Carbohydrates; TEC, Total Extractable Carbon., Var. Sp, explained variance; Prp.Tot., total proportionality.

shrinkage of soils (Fig. 1). A decrease in bulk density, meaning an increase in total porosity, is especially important for plant development since it may have a direct effect on soil aeration and could enhance root growth (Sugiyanto et al., 1986).

In all treatments, at the end of the experiment, a significant decrease in bulk density was observed (Fig. 1 and Table 4). However, the lower values were found in the 0 and 2% slopes with organic treatments, which resulted the lowest even at the first soil sampling. An improvement of soil physical structure with the application of organic amendment has usually been found (Caravaca et al., 2002).

Moreover, both fertilizers, in particular in soil with plants, were capable of increasing the total shrinkage area, which represents the whole pool of surface cracks (Fig. 1). A greater shrinkage in soil treated with mineral and organic fertilizers with respect to the control soil, was also observed by Marinari et al. (2000).

The increase in total shrinkage area is of great agronomic relevance, in that it improves drainage (Bouma et al., 1979), infiltration (Swartz, 1966), and soil structure (Bronswijk, 1989), thus reducing soil erodibility. The improvement of soil physical properties positively affects biological and biochemical properties, including enzymatic activities (Giusquiani et al., 1995). In fact, in our study, total shrinkage area resulted positively correlated with microbial activity (dehydrogenase, $r = 0.44$, $p = 0.015$) and other hydrolytic enzyme activities (β-glucosidase, $r = 0.39$, $p = 0.034$; urease, $r = 0.47$, $p = 0.008$) (Table 5).

Lower values of shrinkage were measured in the 6% slope, thus confirming a worse soil response with respect to the 0 and 2% slopes.

The PCA multivariate statistical analysis gives a clearer picture of the relationship between parameters, the influence of the treatments on soil properties, and the interactions among the different factors (time, slope, fertilization, plant). PCA analysis isolated three principal components (PC) (total variance explained: 68.6%) covering variables related to chemical, physical and biochemical parameters at the t1 and t2 samplings (Table 6). The 1st PC (23.6% of the total variance) included all the enzyme activities except for urease and protease and the soluble form of nitrogen and phosphorus (NO_3^- and avP). The 2nd PC loading (25.0% of the total variance) included total organic carbon and nitrogen, the soluble form of carbon (WSCA and TEC) and protease activity. Total shrinkage, bulk density and urease activity were included in the 3rd PC, with 20.0% of the total variance.

The biplot of 1st PC to 2nd PC (Fig. 2) indicated that the treatments (plant and fertilizer) had affected positively the chemical soil characteristics at the first sampling (t1): in fact, all the samples were shifted with respect to the control soils along PC2, which was associated with nutrients (TOC, TEC, TN and WSCA).

After one year of experimentation (t2) the mineral treatments, generally resulting very close to the control soils (shift along PC2), indicated, as expected, the short term effect of the mineral fertilizer on chemical soil properties, i.e. a loss of nutrients (TOC, TN). On the contrary, the organic treatments were shifted mainly along PC1, which represents the variation of enzymatic activities, thus indicating an improvement in the soil metabolism over time and the capability of storing mineral nutrients. Moreover, at t2 the organic fertilizer associated with the plants (OF-P) resulted more efficient with respect to the other treatments in mitigating the negative slope effects. This was shown by the fact that the OF-P treatments at different slopes (0%, 2% and 6%) resulted grouped together in the biplot, thus highlighting their similar chemical and biochemical soil properties.

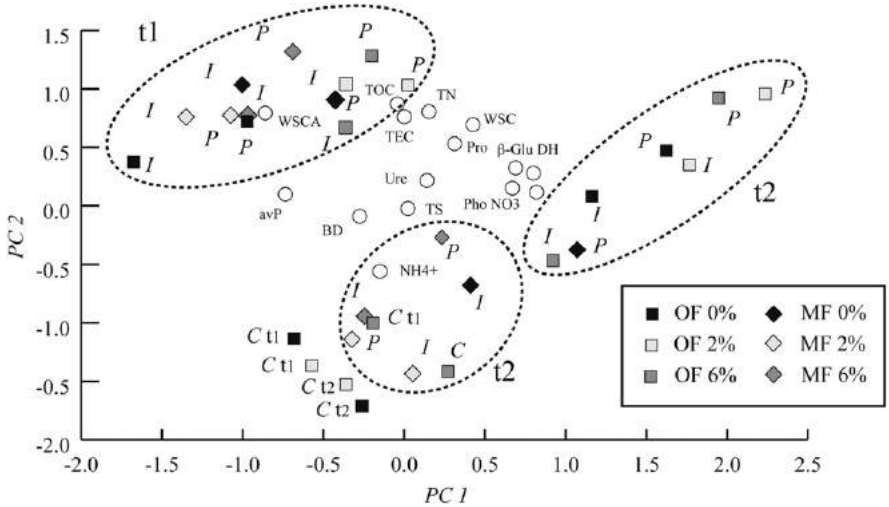

Fig. 2. Biplot of factor scores and loadings. C, control; OF, organic fertilization; MF, mineral fertilization; P, plant; I, interrow; t1, November 2006; t2, November 2007. TOC, Total Organic Carbon; TN, Total Nitrogen; WSC, Water Soluble Carbon; WSCA, Water Soluble Carbohydrates; TEC, Total Extractable Carbon; avP, available phosphorus; TS, Total Shrinkage; BD, Bulk density; β-Glu, β-Glucosidase; Pho, Phosphatase; Ure, Urease, Pro, Protease; DH, Dehydrogenase.

4. Conclusions

The results show that almond tree cultivation represents an efficient and suitable environmental approach for improving physical, chemical and biochemical properties in semi-arid degraded soils.

The plants mainly affected soil properties through the activation of C, N and P nutrient cycles and the stimulation of hydrolytic enzyme activities related to the degradation of the organic compounds. Moreover, the presence of roots increased soil shrinkage, reducing the potential for runoff production and soil loss.

Among the fertilizers, even if both were effective in contrasting carbon loss, the organic fertilizer was more efficient in increasing soil porosity and, as a consequence, in enhancing dehydrogenase enzyme activity, which is indicative of the whole soil microbial metabolism.

By considering the slope gradient, the 6% slope, at a high risk of soil erosion, showed lower enzyme activities, thus reflecting slower nutrient cycling.

Finally, the combination of organic fertilization and almond trees resulted effective, also in the highest slope, in mitigating the degradation processes through the improvement of chemico-nutritional, biochemical and physical soil properties.

Acknowledgments

The study was carried out within the framework of the EU project “Soil Protection in Mediterranean Areas Through Cultivation of New Varieties of Almond Tree” ALMOND PRO-SOIL (LIFE 05-E-000288 2005/2008).

We would like to thank Giovanni Rocco Quinto for his assistance in the field management and soil sampling and Chris Powell for the English revision of the manuscript.

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Journal of Environmental Management 95 (2012) S223–S227

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Desorption of two organophosphorous pesticides from soil with wastewater and surfactant solutions

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ARTICLE INFO

Article history:
Received 29 August 2010
Accepted 26 September 2010
Available online 29 October 2010

Keywords:
Diazinon
Dimethoate
Dissolved organic carbon
Solubilisation

ABSTRACT

A batch test was used to evaluate the extent of desorption of diazinon and dimethoate, preadsorbed on a calcareous agricultural soil, representative of the Mediterranean area. Urban wastewater from a secondary treatment and seven surfactant solutions, at concentrations ranging from 0.75 mg L^{-1} to 10 g L^{-1}, were used. The surfactants assayed were cationic (hexadecyl trimethyl ammonium bromide (HD)), anionic (sodium dodecyl sulfate (SDS), Aerosol 22 (A22) and Biopower (BP)), and nonionic (Tween 80 (TW), Triton X 100 (TX) and Glucopon 600 (G600)). Desorption of dimethoate was either not affected or only slightly by the nonionic and anionic surfactants tested, while desorption of diazinon from the soil was only enhanced by A22, BP and TW. This desorption increase correlated significantly with the surfactant concentration of the solution used for desorption and with the concurrent increase in the supernatant of the dissolved organic carbon, in particular that originating from the surfactant. This parameter did not vary with the use of SDS, G600 and TX. The cationic surfactant HD was retained on the soil surface, as confirmed by an increase in soil organic carbon, resulting in a fall in desorption rate for both pesticides. Comparing treatment by wastewater with control water, there was no difference in desorption rate for either pesticide. Mixed TW/anionic surfactant solutions either did not modify or slightly increased desorption of both pesticides in comparison with individual surfactant solutions.

1. Introduction

The contamination of soils, groundwater, and surface waters by pesticides used in agriculture is currently a significant concern throughout the world. Many of these compounds are a threat to both human health and the environment. Organophosphorous pesticides, which are widely used in agriculture for the control of sucking and chewing insect pests in a wide range of crops, can be toxic for different life forms despite their low persistence and relatively short preharvest interval (NPIC, 2009). They often appear in studies aimed at monitoring groundwaters (Hernández et al., 2008).

Irrigation with wastewater (WW), which is becoming an alternative in regions with scarce water resources, can modify the fate and transport of these pollutants in soil. In municipal WW surfactants, originating mainly from detergents, are the most abundant organic chemicals (Abu-Zreig et al., 1999).

Some studies have addressed the effects of surfactants on the fate of pesticides in soils (Iglesias-Jimenez et al., 1996; Sánchez-Camazano et al., 2003), focusing on the influence of the nature (anionic or non-anionic) and concentration of the surfactants considered. Other reports have indicated that surfactants favour the degradation of hydrophobic organic compounds in the soil, facilitating their desorption and rendering them more accessible to microorganisms (Christofi and Ivshina, 2002). However controversial results showing increases or no variation of the sorption/desorption of organic compounds from soil can also be found in the literature (Payá-Perez et al., 1996; Beigel et al., 1998).

The aim of the present work is to evaluate the influence of WW and several aqueous surfactant solutions with a wide range of properties (Rosen, 2004) on the desorption rate of two organophosphorous insecticides, diazinon (DZN) and dimethoate (DMT), relating the results with the physicochemical properties of insecticides and surfactants, as well as with some properties of the solid and solution soil phases.

2. Materials and methods

2.1. Soil

A calcareous silt loam soil (0–25 cm) from an agricultural zone of intensive activity was sampled and sieved to 2 mm. Its main

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doi:10.1016/j.jenvman.2010.09.025

characteristics (Hernandez-Soriano et al., 2007) are: 31% sand; 58% silt; 11% clay; 12.1 g kg^{-1} organic carbon (OC) content; 7.9 $cmol_q$ kg^{-1} CEC; 76 mg L^{-1} dissolved organic carbon (DOC); pH 7.8 (5:20 soil/water ratio) and 214 μS cm^{-1} electrical conductivity (EC).

The pesticide-amended soil (100 mg kg^{-1}) was prepared by adding a mixture in acetone of DZN and DMT to a bulk soil sample, mixing thoroughly and allowing the solvent to evaporate. Then, the soil sample was aged for 2 weeks at 10 °C.

2.2. Insecticides

We used two insecticides, DZN and DMT, with purity ≥97.5% (Dr. Ehrenstrofer, Augsburg, Germany). Their octanol/water partition coefficients (Log K_{ow}) are 3.3 and 0.70 and their water solubilities 0.06 and 23.3 g L^{-1}, respectively (Tomlin, 2003).

2.3. Desorption solutions

2.3.1. Wastewater

Urban WW, corresponding to a secondary treatment, was supplied by the WW treatment plant from Granada (South of Spain). The average surfactant concentration is 0.75 mg L^{-1}, pH 7.7, EC 1110 μS cm^{-1}, DOC 20 mg L^{-1}, SAR 5.91 and total dissolved solids 575 mg L^{-1}.

2.3.2. Surfactants

Aqueous solutions of seven surfactants (Table 1) were prepared between 0.75 mg L^{-1} (as in WW) and 10 g L^{-1}. In addition, binary mixtures were prepared containing the nonionic TW at 50 mg L^{-1} plus each one of the anionic surfactants (A22, BP, SDS) between 50 and 500 mg L^{-1}.

2.4. Pesticide desorption batch test

Two grams of pesticide-amended soil were placed into a centrifuge tube, to which 20 mL each of surfactant solutions were added. A control desorption was run with MilliQ water (MQ). Additionally, four consecutive extractions were performed with WW.

The tubes were then shaken end-over-end for 24 h at 15 °C and then centrifuged at 3000 rpm for 15 min. Insecticide content, EC, DOC_{soil} and DOC_{surf} were determined in the supernatants. Soil OC was analyzed in the pellet. DZN and DMT results are expressed as percentage of pesticide desorbed relative to the results obtained with MQ. Duplicate test was performed in all cases.

2.5. Analytical procedures

DZN and DMT were quantified with a high performance liquid chromatograph (HITACHI, LaChrom Classic) equipped with a monochannel UV–visible detector (L-7420) and an automatic injector (L-7200). A 50 μl volume was injected on a Purospher®STAR column (RP-18e, 5 μm; 125–4 mm), detecting DZN at 247 nm and DMT at 205 nm. The mobile phase (0.8 mL min^{-1}) consisted of a water–methanol gradient: 99/1 for the first 3 min, then to 30/70 from 3 to 5 min (3 min), and to 99/1 from 8 to 10 min (6 min). Retention times were 5.09 min for DZN and 9.19 min for DMT.

DOC in supernatants and OC in soil were measured with a TOC-analyzer (Analytical Sciences Thermalox). Partition of DOC_{surf} and DOC_{soil} was performed by spectroscopic measurement of absorptivity at 254 nm (Amery et al., 2007). Supernatant EC was also measured (CON 510, Eutech/Oakton Instruments).

2.6. Data analysis

Comparison between two groups of means was conducted by the *t*-Student test, while the Dunnet test was used for multiple comparison, i.e., to determine differences between pesticide desorption from the control group (MQ) and the remaining treatment groups. Relationships between variables were assessed by regression analysis. A factor analysis was applied to detect how a set of variables were related. Principal component was used as extraction method and varimax rotation to maximize the variance of each factor. In all cases, values for $P < 0.05$ were considered significant. SPSS v.17 was used for statistical analyses of the data.

3. Results and discussion

3.1. Desorption with individual surfactant solutions

Two factors were found to explain 75% of total variance in desorption data. The first factor takes into account the greatest amount of variance between the variables (53%) and is made up of EC (0.914) + DOC_{surf} (0.829) + DZN (0.798) + DOC_{soil} (0.656). The second one accounts for less variance (22%) and is associated with DMT (0.830) and DOC_{soil} (0.538). Therefore the DOC_{soil} fraction has the least effect, this being true for desorption of both DMT and DZN.

3.1.1. Dimethoate

The desorption rate of DMT was not greatly affected compared to that corresponding to MQ, with either the various non-ionic surfactants (TW, TX and G600) or the anionic surfactants A22 and BP (Fig. 1).

Table 1
Surfactant properties.

Type	Trade name (supplier)	Abbreviation	Chemical name	MW	CMC[a] (g L^{-1})	OC[b] (%)	pH_{init}[c] (5 g L^{-1})	pH_{fin}[d] (5 g L^{-1})
Cationic	HDTMA (Aldrich)	HD	Hexadecyl trimethyl ammonium bromide	364	0.35	65	7.7	8.0
Anionic	Aerosol 22 (Sigma)	A22	Tetrasodium N(1,2-dicarboxyethyl)-N-octadecyl sulfosuccinamate	653	0.653	48	7.7	8.6
	SDS (Scharlau)	SDS	Sodium dodecyl sulfate	288	2.38	45	7.1	9.2
	Biopower[e] (Bayer)	BP	Sodium alkylethersulfate	–	2.7	50	4.0	7.8
Non-ionic	Triton X 100 (Merck)	TX	Octylphenol-polyethylene glycol ether	650	0.158	75	7.8	7.9
	Glucopon 600 (Fluka)	G600	Alkylpolyglucoside	386	0.111	29	8.9	7.8
	Tween 80 (Sigma)	TW	Polyoxyethylene sorbitan monooleate	1310	0.016	50	3.9	7.6

[a] Critical micelle concentration.
[b] Organic carbon content in the surfactant molecule.
[c] pH of the solution before the desorption process.
[d] pH of the solution after the desorption process.
[e] Biopower is a commercial wetting agent for pesticide application.

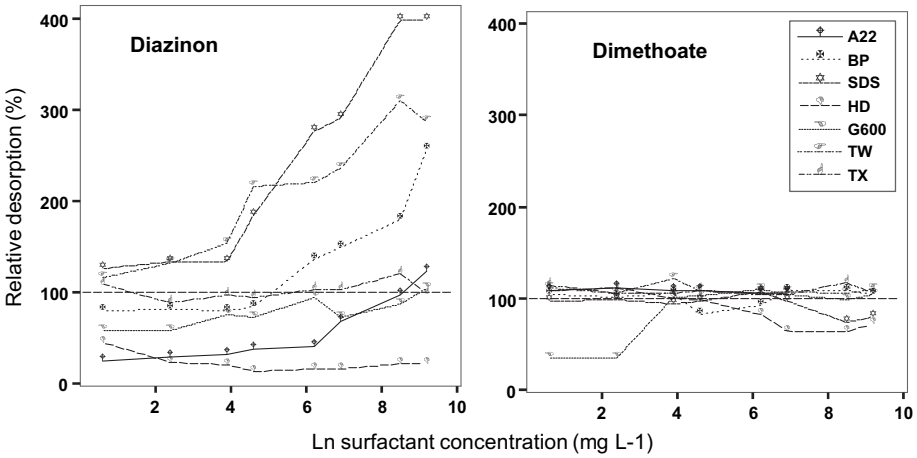

Fig. 1. Relative dimethoate and diazinon desorption rates (%), compared to desorption with MilliQ water, for single aqueous surfactant solutions. For surfactant abbreviations see Table 1.

With the cationic surfactant HD, DMT desorption was similar to MQ at surfactant concentrations $\leq 100\ mg\ L^{-1}$, and significantly decreased for higher concentration values ($P < 0.05$). This behaviour was explained by an increase of the soil OC content (from 1.4% at $0.75\ mg\ L^{-1}$ to 4.8 and 3.7% for the two higher surfactant concentration values, 5 and $10\ g\ L^{-1}$) as well as by the lack of variation of DOC_{surf}, DOC_{soil} and solution EC with surfactant concentration (Fig. 2). This result confirms the covering of the soil surface by the surfactant, providing more sites for pesticide retention, which would diminish DMT desorption, in agreement with previous studies (Hernandez-Soriano et al., 2007).

For SDS a significant decrease ($P < 0.05$) of DMT desorption only at high concentrations may be related with an increase of soil OC up to 70%, probably as a result of release of Ca^{2+} ions from the calcareous soil, which can complex with SDS and induce the precipitation of the SDS calcium salt. This polar pesticide, which is very soluble in water, seems not to be affected by the increase of DOC_{surf} (Fig. 2).

3.1.2. Diazinon

The desorption pattern when using the cationic surfactant HD was similar to that observed with DMT (Fig. 1), but the decrease higher for DZN. As already indicated, HD would bind to the soil by a cation exchange mechanism, enhancing the retention of this more hydrophobic pesticide (González et al., 2008).

DZN desorption rate from soil was significantly affected ($P < 0.05$) in an exponential way ($y = e^{ax-b}$) by the concentration of the anionic surfactant solutions (R^2 0.945 for SDS, 0.857 for BP and 0.943 for A22).

A significant linear regression ($P < 0.05$) was found between relative DZN desorption and DOC_{surf} concentration (R^2 0.955 for BP and 0.761 for SDS) (Fig. 2). Both surfactants significantly ($P < 0.05$)

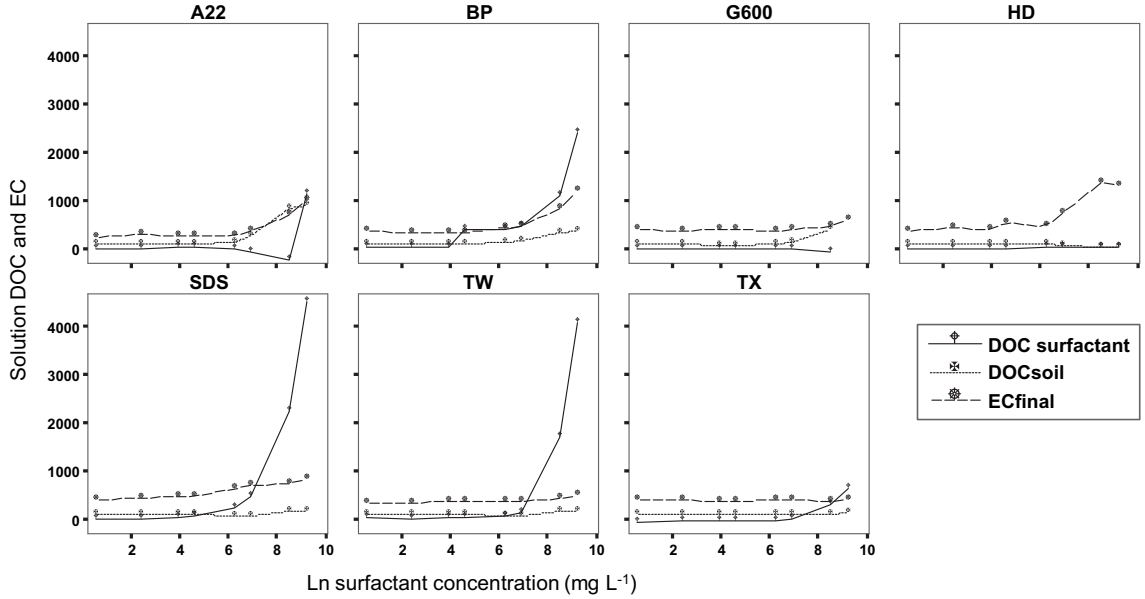

Fig. 2. Dissolved organic carbon ($mg\ L^{-1}$) and electrical conductivity ($\mu S\ cm^{-1}$) in the supernatant after pesticide desorption. DOC from soil (DOC_{soil}) and from the surfactant (DOC_{surf}) are differentiated.

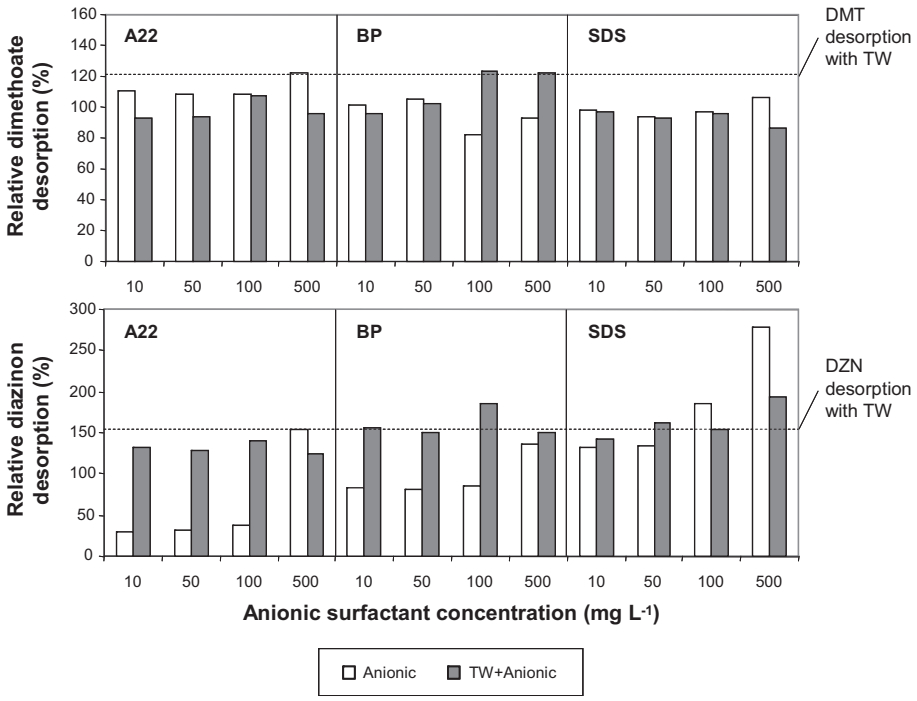

Fig. 3. Relative dimethoate and diazinon desorption rates for mixed anionic TW surfactant solutions. The horizontal line (- - -) indicates relative desorption rate achieved with the nonionic surfactant, TW, at a fixed concentration (50 mg L^{-1}).

enhanced DZN desorption from soil compared to that obtained with MQ.

The third anionic surfactant studied, A22, may exchange the Na^+ in its molecule for Ca^{2+} from the calcareous soil, precipitating as a calcium sulfosuccinamate salt (Payá-Perez et al., 1996; Hernandez-Soriano, 2009). Accordingly the precipitated salt would not be in solution, in agreement with the low DOC_{surf} and EC in the supernatant (Fig. 2). Additionally the desorbed DZN would be trapped in the precipitate thus resulting in an apparently lower desorption rate than that from MQ (Fig. 1).

The desorption behaviour of DZN when nonionic surfactants were assayed did not follow a general trend. The soil OC confirms that, in general, nonionic surfactants (60–127% OC increase) are better retained on soil than the anionic surfactants (20–74%) (Beigel et al., 1998; Rodríguez-Cruz et al., 2005).

However, only TW, which is acid (Table 1), significantly increased DZN desorption from soil ($P < 0.05$) which was linearly correlated with DOC_{soil} (R^2 0.537, $P = 0.039$). For TX we observed neither a significant difference in relative DZN desorption ($P > 0.05$) (Fig. 1), nor a variation of DOC_{surf}, DOC_{soil} or solution EC (Fig. 2). For G600 a decrease ($P < 0.05$) in relative DZN desorption occurred for surfactant concentrations below 1 g L^{-1} (Fig. 1), while for higher concentration values no differences with MQ ($P > 0.05$) were detected, coinciding with a slight DOC_{surf} increase (Fig. 2). Surfactant retention on soil did not occur to a high degree, due to the low soil OC variation at $\leq$1 g L^{-1} concentration. More research is needed to understand the mechanisms involved in the pesticide desorption process mediated by surfactants, and to explain the complex interactions between pesticides, soil matrix and surfactants.

3.2. Desorption with wastewater and surfactant mixtures

Four consecutive extractions were performed with both WW and MQ. No significant differences ($P > 0.05$) were observed in desorption rates due to the presence of WW, with most of both pesticides being desorbed in the first step (approximately 22% of DZN and 70% of DMT).

The pesticide desorption ability of some surfactant mixtures was evaluated by selecting a fixed concentration for the nonionic surfactant TW, which provided high relative desorption rates for DZN when used individually (Fig. 3). Mixtures of anionic and nonionic surfactants were tested because both are usually present in WW, and because enhanced solubilisation of organic compounds by surfactant mixtures has been reported (Mohamed and Mahfoodh, 2006; Xu et al., 2006).

Compared with the individual anionic surfactant solutions, only mixtures of TW with BP increased DMT desorption. For DZN desorption increased for A22 and BP but not for SDS. However, only the TW + BP mixtures desorbed both pesticides at similar or slightly greater rates than TW alone. These preliminary results show that further studies are required to evaluate desorption and solubilisation of pesticides from soil, both in the laboratory and in the field.

4. Conclusions

The data have shown that the type of surfactant is the main parameter affecting DMT and DZN desorption. The highly polar DMT was on the whole not affected by the use of anionic or nonionic surfactants. On the contrary anionic surfactants increased DZN desorption in all cases: BP and SDS by bringing DZN directly into the solution and A22 by entrapping the pesticide into the surfactant-Ca salts that precipitated due to a cation exchange process. The nonionic surfactants each behaved in a different way, ranking as follows: G600 < MQ = TX < TW, for the whole surfactant concentration range. The cationic surfactant provided new sites for pesticide sorption, reducing desorption of both pesticides from soil.

DOC_{surf} and accordingly, EC, strongly modified DZN desorption while DOC_{soil} only slightly altered desorption of both pesticides.

The application of WW, which contains several kinds of surfactants, as well as other sources of dissolved organic matter or

some ions, seems not to modify insecticide desorption rates. On the other hand, the combination of TW with anionic surfactants, simulating the surfactant composition of WW, did not affect DMT desorption as expected, while DZN desorption was higher than that achieved with MQ but lower than the desorption corresponding to the nonionic TW.

Further research will be necessary with additional pesticides and soils with a wide range of physicochemical properties to establish more detailed trends.

Acknowledgments

This research takes part of a research project (CGL2007-60355/HID) co-financed by the Spanish Ministry of Science and Innovation and European FEDER funds. The municipal EDAR (Emasagra) is acknowledged for providing the WW samples. We thank Prof. E. Smolders and B. Horemans, Division of Soil and Water Management of KU Leuven (Belgium), for the assistance with some analytical determinations.

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Journal of Environmental Management 95 (2012) S228–S232

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Influence of pine or oak wood on the degradation of alachlor and metalaxyl in soil

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ARTICLE INFO

Article history:
Received 3 September 2009
Received in revised form
17 March 2010
Accepted 19 October 2010
Available online 13 November 2010

Keywords:
Pesticide
Metabolite
Degradation
Soil
Wood residue
Remediation

ABSTRACT

The objective of this work was to study the influence pine or oak wood added to soil as an amendment (5% w/w) had on the degradation rate of two pesticides, alachlor and metalaxyl, with different hydrophobic character. The formation of pesticide metabolites and the soil dehydrogenase activity in non-amended and amended soil samples were also monitored. The degradation of metalaxyl followed first-order kinetics, while the degradation of alachlor followed first-order or biphasic kinetics in the soil samples studied. The results indicated that the degradation rate was slower for metalaxyl than for alachlor, and for both pesticides followed the order: pine amended soil < oak amended soil < non-amended soil. The faster degradation rate in non-amended soil was attributed to the higher sorption of pesticides by wood amended soils. The alachlor ethane sulfonic acid (ESA), and two metalaxyl metabolites (2-[(2,6-dimethylphenyl)-methoxyacetylamino]-propionic acid and N-(2,6-dimethylphenyl)-2-methoxy-acetamide) were detected during the incubation period. Soil dehydrogenase activity recorded close values in non-amended and amended soil treated with alachlor, but it was higher in wood amended soil treated with metalaxyl. Pine and oak wood increase the immobilization of the pesticides studied, but they also limit their bioavailability in soil by decreasing their degradation rate in amended soil.

1. Introduction

Alachlor is an herbicide used in pre-emergence to control perennial grasses and many broad-leaved weeds in corn and other crops. Given its widespread use, alachlor has been found in soils and in both surface water and groundwater (Sánchez-Camazano et al., 2005; Vryzas et al., 2009). Soil organic matter (OM) is the major factor for alachlor adsorption, and it is sorbed to a lesser extent by clay colloids (Guo et al., 1993). Biodegradation is the single most significant mechanism for controlling the dissipation of alachlor in agricultural soils. Biological degradation by dechlorination in soil leads to the formation of the ethane sulfonic acid (ESA) metabolite (Stamper and Tuovinen, 1998). Alachlor ESA is a more polar compound than alachlor and has been found in groundwater and in surface water more often and in higher concentrations than the parent herbicide.

Metalaxyl is a systemic acylanilide fungicide widely applied in different crops to eliminate different fungal species. Metalaxyl is highly soluble in water and has a low hydrophobicity, indicating low adsorption by soils and the possibility of leaching into groundwater. Metalaxyl has recently been found in groundwater at concentrations of up to 0.49 μg L^{-1}, which exceeds the 0.1 μg L^{-1} EU limit (Hildebrandt et al., 2008). Several authors have indicated the importance of OM and clay content in the adsorption of metalaxyl by soils (Andrades et al., 2001). Its degradation in soil has been reported mainly as biodegradation (Sukul and Spiteller, 2000). Metalaxyl is degraded in soil by cleavage of the methyl ester group, forming the main acid metabolite (CGA 62826), although biodegradation of metalaxyl could occur by benzylic hydroxylation of the methyl chain or aromatic hydroxylation. A second metabolite (CGA 67868 or CGA 92370) is formed either directly from metalaxyl or from the metabolite CGA 62826 by N dealkylation. (Pesaro et al., 2004).

Recent work has shown that the water pollution caused by pesticides from point sources (spills, uncontrolled disposal, equipment washing water, etc.) can be more serious than that due to agricultural practice (De Wilde et al., 2007; Fait et al., 2007). The use of organic materials or wastes may prevent the mobility of pesticides from these point sources of contamination and enhance their biodegradation (Rodriguez-Cruz et al., 2007a). In recent years, different low-cost adsorbent systems (biobed, biomassbed, biofilter, etc.) have been developed to minimize point sources of pesticide pollution by retaining and degrading pesticides (Castillo et al., 2008; De Wilde et al., 2007; Fait et al., 2007). In particular, the use of wood residues as low-cost adsorbents has recently been developed as a new technology for the immobilization of heavy metals, dyes, pesticides, other organic compounds, etc., in soil (Gupta and Suhas,

Abbreviations: S, soil; SS, sterilised soil; S + P, soil amended with pine wood; S + O, soil amended with oak wood.

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doi:10.1016/j.jenvman.2010.10.043

2009; Rodríguez-Cruz et al., 2007b; Shukla et al., 2002). In a recent study, Rodríguez-Cruz et al. (2007b) found that oak and pine wood can be effectively used as adsorbents of pesticides. The Freundlich constants (Kf) for alachlor and metalaxyl adsorption by oak and pine were related to the lignin content of these woods. However, the influence the addition of wood residues had on the degradation of pesticides in soils has been less studied (Grenni et al., 2009). The addition of an organic amendment to the soil affects the biodegradation of pesticides because the OM and nutrients added can strongly affect the structure and activity of bacterial and fungal populations as a result of the increased metabolism of the readily available nutrients (Briceño et al., 2007). Some organic amendments may stimulate biodegradation, but others can reduce it (Moorman et al., 2001; Rodríguez-Cruz and Lacorte, 2005).

The main objective of this work was to investigate the effects pine and oak wood added to soil as amendments had on the rates of degradation of alachlor and metalaxyl. These pesticides have different hydrophobic characters and water solubility and are widely used in agriculture. Soil dehydrogenase activity was monitored in soil treated with alachlor or metalaxyl and amended with either pine or oak residues or non-amended in order to analyze the effect of the amendment and the pesticide on the microbial activity in the soil. The findings of this study provide information regarding the use of wood amendments for preventing soil and water contamination by pesticides with different characteristics.

2. Materials and methods

2.1. Soil and wood samples

Soil samples were collected from the surface layer (0–15 cm depth) of an agricultural field located in Aldearrubia (Salamanca, Spain). The soil was left overnight at room temperature to reduce moisture content and then sieved (<2 mm). The soil was sandy-loam (11.8% clay, 13.6% silt and 74.5% sand), with 0.85% organic matter content, a pH of 6.3, and a cation exchange capacity of 4.8 cmol kg^{-1} (Rodríguez-Cruz et al., 2007a).

Pine and oak sawdust (<1 mm) were obtained from a local industry in Salamanca (Spain). They had different lignin contents: 18.2% for oak and 24.4% for pine (Rodríguez-Cruz et al., 2007b).

The amended soils were prepared by uniformly mixing soil with oak or pine sawdust (5% w/w), similarly to other organic residues (Moorman et al., 2001). Sub-samples were analyzed to assess both the total organic carbon (TOC) and the soluble carbon contents, as described elsewhere (Rodríguez-Cruz et al., 2007b). The TOC content in the soil amended with pine (S + P) or oak (S + O) was 2.89% and 2.79%, respectively, and about fourfold greater than in non-amended soil (S) (0.72%). Moreover, the soluble carbon content was higher in S + P (0.047%) and S + O (0.037%) than in S (0.008%). Sample pH varied between 5.9 (S + O) and 6.6 (S + P).

2.2. Chemicals

Alachlor (99.5% purity) was supplied by Chem Service (West Chester, USA). It is an herbicide with a water solubility of 240 μg mL^{-1} (pH 7, 20 °C) and a log K_{ow} of 2.63. The alachlor ESA was supplied by Monsanto Chemical Co. (St. Louis, MO, USA). Metalaxyl (>98% purity) was supplied by Novartis Crop Protection AG (Basel, Switzerland). It is a fungicide with a water solubility of 8400 μg mL^{-1} (22 °C) and a log K_{ow} of 1.75 (Tomlin, 2003). The two metalaxyl metabolites studied (>99% purity), 2-[(2,6-dimethylphenyl)-methoxyacetylamino]-propionic acid (CGA 62826) and N-(2,6-dimethylphenyl)-2-methoxyacetamide (CGA 92370), were supplied by Syngenta Crop Protection AG (Münchwilen, Switzerland).

2.3. Degradation experiments with amended and non-amended soil

The pesticide degradation experiment was conducted in duplicate in accordance with SETAC guidelines (Lynch, 1995). The standard compound was added to soil (200 g) to obtain a final pesticide concentration of 1 mg kg^{-1}. The final moisture content of the soils was adjusted to 60% of their maximum water holding capacity. Some soil samples were first sterilised by autoclaving at 120 ± 2 °C for 20 min on two consecutive days (SS), and then treated with alachlor or metalaxyl, other soil samples (S) were treated only with alachlor or metalaxyl and others were treated with both pesticide and pine (S + P) or oak (S + O) sawdust. The soils were maintained in Erlenmeyer flasks plugged with sterilised cotton wrapped in gauze to allow air exchange. Soil moisture was kept constant throughout the experiments by periodic weighing and the replacement of any losses with sterile water. Samples were incubated at 20 ± 0.5 °C in the dark. Solutions and instruments were sterilised and all steps were performed in a sterile cabinet.

2.4. Chemical analysis

Alachlor and metalaxyl and their metabolites were measured immediately after treatment and at different sampling times (0, 1, 2, 3, 6, 8, 10, 14, 20, 28, 51, 70, and 98 days). Two soil replicates (1 g) were taken from each microcosm and shaken with 5 mL of methanol for 24 h at 20 °C for residue analysis. Samples were centrifuged and 4 mL of each supernatant were evaporated under an air stream

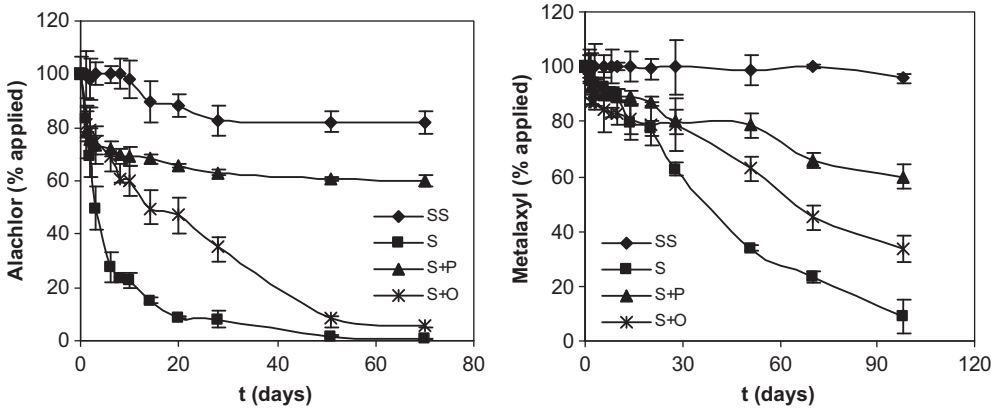

Fig. 1. Degradation kinetics of alachlor and metalaxyl in sterilised soil (SS), non-sterilised soil (S) and soil amended with pine (S + P) or oak (S + O) woods. Bars indicate the standard deviation of the replicates ($n = 4$).

Table 1
Kinetic equation, correlation coefficient and half-life values of alachlor and metalaxyl in non-amended (sterilised-SS and non-sterilised-S) and pine (S + P) or oak (S + O) amended soils.

Pesticide/treatment	Kinetic equation	r^2	$t_{1/2}$ (d)
Alachlor			
SS	$y = 98.935\ e^{(-0.0035x)}$	0.78	198
S[a]	$y = 101.65\ e^{(-0.2187x)}$	0.99	3.17
S + P	$y = 76.54\ e^{(-0.0046x)}$	0.53	151
S + O	$y = 90.738\ e^{(-0.0414x)}$	0.98	16.7
Metalaxyl			
SS	–	–	nd[b]
S	$y = 108.75\ e^{(-0.0238x)}$	0.98	29.1
S + P	$y = 95.073\ e^{(-0.0048x)}$	0.95	144
S + O	$y = 94.613\ e^{(-0.0101x)}$	0.96	68.6

[a] First-order equation calculated for the first degradation phase (0–6 days).
[b] nd, no degradation.

using an Evaporator EVA-EC2-L (VLMGmbH, Bielefeld, Germany) and re-dissolved in 0.5 mL of methanol for analysis. The quantitative determination of alachlor, metalaxyl and their metabolites was performed by HPLC–DAD–MS (Waters Assoc., MilfordMA, USA). A Waters Symmetry C18 (75 mm × 4.6 mm I.D., 3.5 μm) column was used at ambient temperature. The mobile phase was 70:30 acetonitrile/water for metalaxyl and its metabolites and 80:20 acetonitrile/water for alachlor, and the flow rate of the mobile phase was 0.4 mL min^{-1}. The mobile phase was 90:10 acetonitrile/water for alachlor ESA with a flow rate of 0.5 mL min^{-1}. The sample injection volume was 10 μL. Detection by HPLC-DAD was at 196 nm for alachlor, 205 nm for alachlor ESA, and 194 nm for metalaxyl and its metabolites, and detection by HPLC-MS to confirm the identity of these compounds was carried out by monitoring the positive molecular ion (m/z) 238.2 for alachlor, 280.3 for metalaxyl, 266.2 for CGA 62826 and 194.2 for CGA 92370 and the negative molecular ion (m/z) 314 for alachlor ESA.

2.5. Soil dehydrogenase activity

Soil dehydrogenase activity considered as overall soil microbial activity was measured using the Tabatabai method (Tabatabai, 1994).

2.6. Statistical analysis of the data

The data obtained were subjected to analysis of variance. Standard deviation (SD) was used to indicate variability among replicates in the determination of pesticides or their metabolites and the least significant difference (LSD), at a confidence level of 95%, was determined to evaluate the effects of different soil treatments on dehydrogenase activity. The statistical software Statgraphics Plus version 5.1 (Statgraphics Plus Corp., Princenton, NJ, USA) was used.

3. Results and discussion

3.1. Degradation kinetics of alachlor and metalaxyl and metabolite formation

Fig. 1 shows the degradation kinetics for alachlor and metalaxyl in non-amended soil (S), non-amended and sterilised soil (SS) and soil amended with pine (S + P) or oak (S + O). The data are plotted as residual concentrations of the pesticide (percentage of pesticide initially applied) against the time of incubation of each soil.

The degradation of alachlor followed first-order kinetics in the SS, S + O and S + P samples, but followed a biphasic pattern in the S sample with a rapid first phase and a slow second phase of degradation (Fig. 1). The half-life ($t_{1/2}$) of alachlor in non-amended soil was

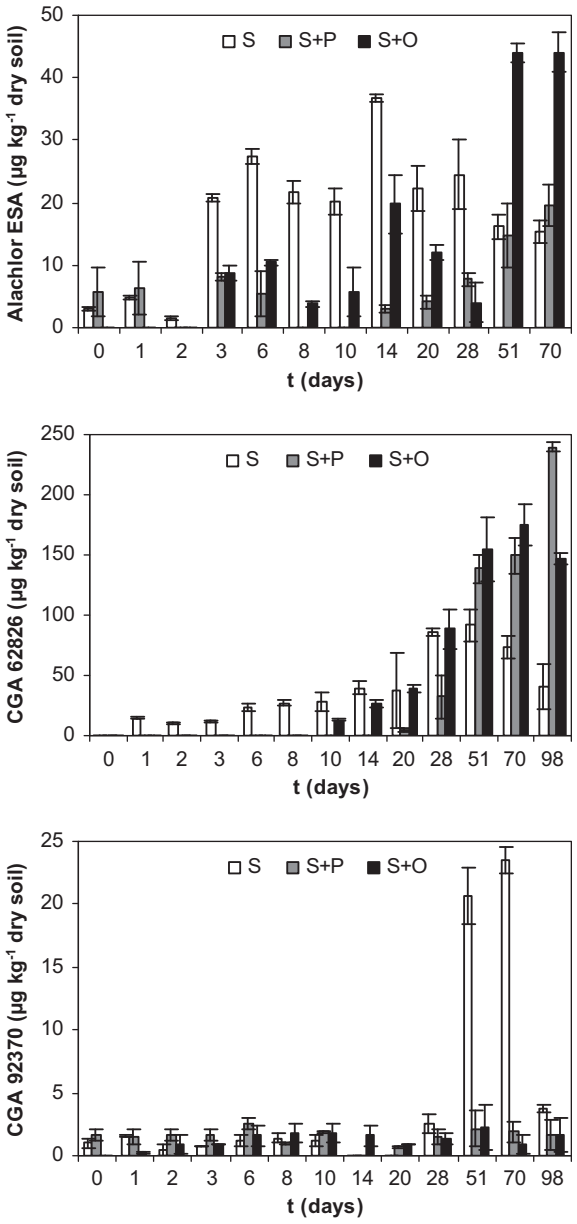

Fig. 2. Formation of alachlor ESA and metalaxyl metabolites CGA 62826 and CGA 92370 in non-sterilised soil (S) and soil amended with pine (S + P) or oak (S + O) woods. Bars indicate the standard deviation of the replicates ($n = 4$).

calculated in the first phase because degradation was rapid (>90% in this first phase). The $t_{1/2}$ was 3.2 days and increased to a lesser extension in oak amended soil (16.7 days) and to a greater extension in pine amended soil (151 days) (Table 1). The degradation of alachlor in S + P was slower (<50% at the end of the experiment) and the $t_{1/2}$ value should be considered with caution given that only an r^2 of 0.53 was reached. A slight degradation was observed in the sterilised soil, which might be caused by chemical and abiotic factors other than photodegradation, as the soil samples were kept in the dark during the incubation period.

The higher $t_{1/2}$ observed in the amended soils could be due to the higher TOC content of these soils compared to the non-amended soil. Given that alachlor is adsorbed mainly by the soil OM, its degradation is expected to depend on the OC content of the soil. Several authors have suggested that OM could play an important role in enhancing alachlor sorption by an amended soil (Dorado et al., 2005). Wood residues could enhance herbicide adsorption,

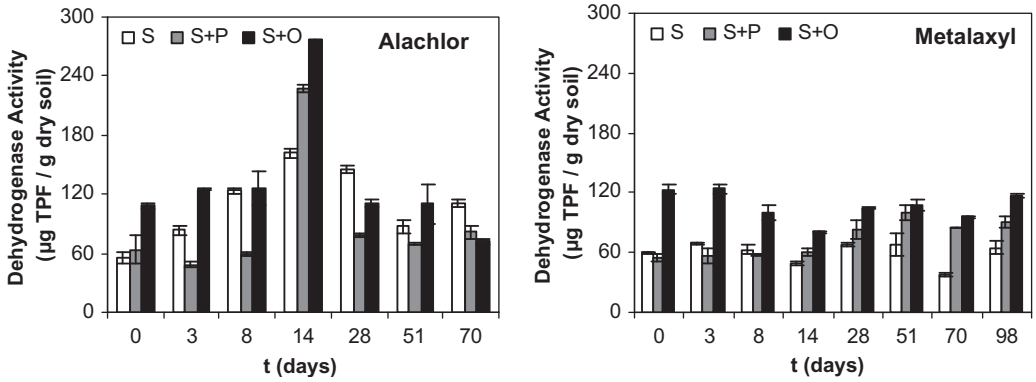

Fig. 3. Dehydrogenase activity of non-amended (S) and amended soils (S + P and S + O) treated with alachlor and with metalaxyl at different incubation times. Bars indicate the standard error of the replicates ($n = 2$).

decreasing the bioavailability and increasing the $t_{1/2}$ of alachlor in amended soils. In addition, a higher adsorption of alachlor by soil amended with pine occurs due to the lignin content of the wood, which influences the sorption of pesticides by soils (Kf values were 22.4 and 41.4 for the adsorption of alachlor by oak and pine wood, respectively), as reported previously (Rodriguez-Cruz et al., 2007b). Other authors have also indicated that the addition of amendment (sewage sludge) to soil led to a decrease in alachlor degradation (Rodríguez-Cruz and Lacorte, 2005).

Concentrations of alachlor ESA were detected simultaneously to parent compound in non-sterile conditions. The evolution of this metabolite during the incubation period is included in Fig. 2. The maximum amount detected was 36.7 μg kg^{-1} dry soil in S after 14 days. The amount then decreased, possibly due to the degradation of alachlor ESA and the low input of new metabolite in accordance with the residual parent compound (less than 20% of the original pesticide applied). In the S + O sample, a relative increase in alachlor ESA was recorded at 50 days of incubation after a rapid degradation of the parent compound (close to 30%) occurred between 28 and 70 days of incubation. This compound was not subsequently degraded at the end of the experiment (70 days). Lower amounts of this metabolite were found in the S + P sample according to a lower degradation of alachlor in this soil (Fig. 1).

The degradation of metalaxyl in the non-amended and amended non-sterilised soil samples studied followed first-order kinetics (Table 1). The results indicated that no metalaxyl degradation occurred in the non-amended and sterilised soil (Fig. 1). This suggests a microbial role in degrading this fungicide. The $t_{1/2}$ values indicated a slower degradation of metalaxyl in the S + P (144 days) or S + O (68.6 days) samples compared with the S (29.1 days) sample. As indicated for alachlor, an increase in metalaxyl adsorption by the amended soils occurs, with the fungicide being less available to degradation. The adsorption by pine amended soil could be higher than by oak amended soil according to the Kf values of metalaxyl by pine (8.28) and oak (4.95) wood (Rodriguez-Cruz et al., 2007b). Similarly, Fernandes et al. (2006) reported an increase in the $t_{1/2}$ of metalaxyl in a sandy soil with different organic amendments due to the increase in sorption, with the fungicide being protected from degradation.

The two metalaxyl metabolites studied (CGA 62826 and CGA 92370) were detected in the non-sterilised non-amended and amended soils (Fig. 2). Concentrations of CGA 62826 (acid metabolite) were higher than those of CGA 92370. The maximum amounts of CGA 62826 metabolite were 91.5 (S), 175 (S + O) and 240 (S + P) μg kg^{-1} dry soil at 51, 70 and 98 days, respectively. For CGA 92370 metabolite, the maximum amounts of 23.5 (S), 2.25 (S + O) and 2.61 (S + P) μg kg^{-1} dry soil were recorded at 70, 51 and 6 days, respectively (Fig. 2). The faster degradation of metalaxyl in the non-amended soil gave rise to a higher production of CGA 92370 in comparison with the amended soils.

3.2. Soil dehydrogenase activity

The dehydrogenase activity of non-amended and amended soils treated with alachlor or metalaxyl for different incubation times was monitored as an indicator of the overall microbial activity of the soils to determine the pesticide's possible side effects on microbiological activity in the soil (Sukul, 2006) (Fig. 3).

Soil dehydrogenase activity recorded a similar behaviour in non-amended and amended soil for soil samples treated with alachlor. The values were not significantly different (LSD = 36.0, $p < 0.1$). However, significant differences were noted for time (LSD = 55.0, $p < 0.05$). A maximum value of dehydrogenase activity was detected on day 14 for all soils and this was followed by a decrease from day 28 up to the end of the experiment on day 70. The increase was sharper in wood amended soils than in non-amended soil reaching values of 162, 227 and 277 μg TPF g^{-1} dry soil for S, S + P and S + O, respectively (Fig. 3). The increase in the dehydrogenase activity observed at 14 days may correspond to a maximum activity of microorganisms to degrade alachlor in non-amended soil. A peak in the amount of its metabolite alachlor ESA was detected at this time (Fig. 2), indicating that microbial growth was stimulated by the use of alachlor as a source of energy. Sukul (2006) reported that soil dehydrogenase activity is likely to be affected by pesticides. However, the activity of microorganisms in amended soils might also be affected by the wood amendment as an additional carbon source. Some authors have noted the positive influence of organic amendments on the dehydrogenase activity of the microbial community (Moorman et al., 2001; Delgado-Moreno and Peña, 2007; Grenni et al., 2009).

The soil dehydrogenase activity in amended and non-amended soils treated with metalaxyl increased in the order S ≤ S + P < S + O (LSD = 14.8, $p < 0.001$). However, this activity was not significantly different over the entire incubation period for either non-amended or amended soils (Fig. 3). In a previous study, Pesaro et al. (2004) reported that changes in specific activities (e.g., pesticide degradation) are not necessarily reflected by bulk microbial activities such as dehydrogenase activity.

4. Conclusions

The use of wood residues to immobilize pesticides in soils affects the degradation kinetics of these compounds as shown in this study. The addition of a pine or oak wood amendment to the

soil caused a significant reduction in the degradation rate of alachlor and metalaxyl. The specific activity of the microorganisms degrading the pesticides was negatively affected by the wood amendments due to the increased adsorption of pesticides by woods and the decreased bioavailability of the pesticide to be degraded. The results indicate that the use of wood amendments could be effective for limiting the leaching of pesticides in the soil, although the adsorption capacity of the amended soils should be taken into account, since it could decrease the degradation rate of these compounds. Further knowledge on the role wood residues play in influencing the degradation of pesticides in the soil will provide a better understanding of the bioavailability and potential toxicity of these contaminants and their metabolites.

Acknowledgements

This work was financially supported by the Spanish Ministry of Education and Science and the regional government, the Junta de Castilla-Leon as part of projects CTM2004-00381/TECNO and CSI02A06. The authors thank Syngenta for kindly supplying the metalaxyl metabolite standards and Monsanto for kindly supplying the alachlor ESA standard.

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Journal of Environmental Management 95 (2012) S233–S237

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Mercury uptake by *Silene vulgaris* grown on contaminated spiked soils

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ARTICLE INFO

Article history:
Received 30 September 2009
Received in revised form
7 April 2010
Accepted 12 July 2010
Available online 12 August 2010

Keywords:
Mercury
Polluted soil
Silene vulgaris

ABSTRACT

Mercury is a highly toxic pollutant with expensive clean up, because of its accumulative and persistent character in the biota. The objective of this work was to evaluate the effectiveness of *Silene vulgaris*, facultative metallophyte which have populations on both non-contaminated and metalliferous soils, to uptake Hg from artificially polluted soils. A pot experiment was carried out in a rain shelter for a full growth period. Two soils (C pH = 8.55 O.M. 0.63% and A pH = 7.07 O.M. 0.16%) were used, previously contaminated with Hg as $HgCl_2$ (0.6 and 5.5 mg Hg kg^{-1} soil). Plants grew healthy and showed good appearance throughout the study without significantly decreasing biomass production. Mercury uptake by plants increased with the mercury concentration found in both soils. Differences were statistically significant between high dosage and untreated soil. The fact that *S. vulgaris* retains more mercury in root than in shoot and also, the well known effectiveness of these plants in the recovering of contaminated soils makes *S. vulgaris* a good candidate to phytostabilization technologies.

1. Introduction

Mercury is a global pollutant, highly toxic, which can cross international borders. The toxicity varies depending on the element speciation, from the least toxic elemental form that is less bioavailable, to the highly toxic organomercurial compounds that can become concentrated as they move up the food chain (Dowling and Doty, 2009). The natural sources of mercury, independent of man's action, occur as a general cycle (Patra and Sharma, 2000). It is transported to surface waters by soil erosion and is circulated into the atmosphere by natural degassing of the earth's crust and oceans. Natural emissions account for two thirds of the input, man-made release forms about one-third. The annual anthropogenic release of mercury on a global basis was about 3×10^6 kg around the year 1900 and increased during the 1970s. A considerable amount is emitted to the air (45%), water (7%) and land (48%).

Cleaning up soils polluted by mercury is expensive, but in some cases it is a legal obligation required by the environmental policies. Phytoremediation involves the use of plants to extract, detoxify and/or sequester environmental pollutants from soil and water and it offers the benefit of being *in situ*, low cost and environmentally sustainable (McGrath, 1998). A plant suitable for phytoremediation should have the ability to accumulate toxic metal, preferably in above ground parts, tolerance to the metal concentrations accumulated, fast growth and highly effective biomass and be easily harvestable (Kärelampi et al., 2000). Although phytoremediation has been successful in cleaning up sites contaminated by a number of organic contaminants and heavy metals, it has not been so successful with mercury because it is toxic for most plants (Atwood and Zaman, 2006).

The accumulation of mercury in terrestrial plants has been reported to be related to soil characteristics, including concentration of the element (Adriano, 2001), but also the uptake of Hg has been found to be plant-specific (Crowder, 1991; Molina et al., 2006). Soil characteristics such as high pH value, abundant lime and accumulated salt reduce its uptake by plants. A highly significant correlation exists between mercury and organic matter content in the top layer of forest soils (Lag and Steinnes, 1978). In general, the availability of soil mercury to plants is low, and there is a tendency to accumulate mercury in roots, indicating that roots serve as a barrier to mercury uptake. This fact and the high mercury toxicity make it difficult to find plants to decontaminate mercury-polluted soils. It should also be considered that the total concentrations of mercury in the contaminated soil do not indicate the amount of mercury taken up by plants. Firstly, because transport, bioavailability and physico-chemical and toxicological properties are highly

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doi:10.1016/j.jenvman.2010.07.018

depend on the mercury chemical forms in the environment (Millán et al., 2006). Secondly, due to the affinity and tolerance shown by the plant to mercury concentration (Molina et al., 2006).

Silene vulgaris Garcke (Moench) is a facultative metallophyte which has populations on both metalliferous and non-metalliferous soils. This plant is capable of colonizing contaminated sites due to its fast and vigorous growth by producing seeds and rhizomes. In addition, Wierzbicka and Panufnik (1998) have described lead stimulation of root elongation, biomass gain of roots, and formation of root hairs in *S. vulgaris* populations growing on calamine waste heaps. This plant shows multiple tolerance and co-tolerance to heavy metals (Chardonnens et al., 1998; Schat and Voojijs, 1997; Verkleij et al., 1998; Sneller et al., 1999). As a perennial plant, biomass production and season cutting offers an excellent opportunity to analyse the impact during a full life cycle. Roots are directly exposed to metal concentration of the soil and benefits for the host by mycorrhizal fungi can be excluded. In contrast to many other metal-tolerant plants, *S. vulgaris* has nearly no symbiosis with arbuscular mycorrhizal fungi (Pawloska et al., 2000). Previous studies with the population used in this study, from "Santos de la Humosa" show its tolerance to As and Cd (Carpena et al., 2008) and to multipolluted soil with Cr, Cd, Zn and Pb (Pérez-Sanz et al., 2007). Other authors also suggested that the effectiveness of *S. vulgaris* in the revegetation of contaminated soils seemed to result in a different reduction of heavy metal toxicity on soil bacteria (Martinez-Iñigo et al., 2009).

The objective of this work was to evaluate the ability of *S. vulgaris* Garcke (Moench) from the described population of "Santos de la Humosa" to grow on soils with different physico-chemical properties and artificially polluted by Hg during a full cycle of living.

2. Materials and methods

2.1. Soil characteristics

Degradation of soil caused by mercury anthropogenic activities was simulated using samples of two soils with different physical and chemical properties. About 100 kg of agricultural clean soils were collected from the locations of Valdeolmos (A Typic Haploxeraft, Soil Taxonomy USDA, 1999) and Alcalá de Henares (C Typic calcixerept, Soil taxonomy USDA, 1999), both sites in the Madrid Region, Spain. Fresh soil samples were taken from the upper 0–20 cm, air dried and sieved (<2 mm) before analysis. Electrical conductivity (EC) and pH were measured in a 1:2.5 soil to water ratio. Organic matter content and total nitrogen were determined using Walkley–Black and Kjeldahl methods (Ministerio de Agricultura, 1994) respectively. Carbonates in soil were measured according to Bernard calcimeter. Soil texture was analyzed according to Day (1965). Macronutrients were extracted with NH_4–Ac (pH = 7). Heavy metals were measured after acid digestion of soil samples with HCl–HNO_3 following the digestion programs for the Anton Paar microwave system (Multiwave 3000, Anton Paar Gmbh, Graz, Austria). Total concentrations of Cu and Pb were analyzed by Inducible Conductivity Plasma Atomic Emission Spectrometry (Varian Liberty AX Victoria, Australia) and Ca, Mg, Na, K and Zn concentrations were measured by Atomic Absorption Spectrometry (Varian EspectrAA-600, Victoria, Australia).

2.2. Plant experiments

Plant experiments were carried out in a rain shelter which was a greenhouse from which all side glass panels above 40 cm had been removed. The central part of the roof was folded back to allow ventilation throughout the crop cycle, so that temperature and radiation closely resembled ambient conditions. Plastic pots were filled with 1.6 kg of clean soil and kept moist in a rain shelter. Soils were artificially contaminated with Hg as $HgCl_2$ (solved in water) at either a low dosage HgL (0.6 mg Hg kg^{-1} soil) or a high dosage HgH (5.5 mg Hg kg^{-1} soil). The salts were dissolved in a small amount of Millipore water and added to the plastic pots at the start of February. Control pots were left uncontaminated. Pot moisture was brought to 50% of field capacity and metal contamination was consolidated for 40 days.

Seeds of *S. vulgaris* were obtained from a wild population growing in soil from Santos de la Humosa (Alcalá de Henares, Spain). Germination was made by placing 20 seeds on filter paper in Petri dishes soaked with Millipore water. When the radicle had emerged at least 1 mm, seeds were removed to a compressed turf container (or a peat-based degradable pot) until plants showed six or eight developed leaves. Small plants were transplanted in mid-March to the plastic pots of 1.6 kg spiked with Hg as described above. In order to sample a full pot in each sampling time, eight independent pots were used per treatment. Plants were grown during a vegetative period from March to July. After transplanting, pots received the same amount of water (16 mm) with the following frequencies: once every ten days in February and three times a week in April and May. The total amount of water applied in each irrigation cycle was based on the normal rainfall in Mediterranean conditions. Two samples were taken, the first one 60 days after transplanting and at the end of the life cycle, 130 days after transplanting (May and July). Soil was air dried and sieved at <2 mm.

Aerial parts were washed with tap water thoroughly and rinsed twice with Millipore water. Roots were washed with tap water, shaken in Millipore water three times for three cycles of five minutes in an ultrasonic bath, dried with filter paper and weighed. Approximately 500 mg of roots were frozen in liquid N_2 and stored at −20 °C. Aerial parts and the rest of the roots were dried at $T \leq 30$ °C to constant weight, and then dry weights were recorded.

2.3. Mercury determination in soils and plant material

Soil samples and plant material were dried at $T \leq 30$ °C in order to minimise Hg loss due to volatilization. Soil samples were disaggregated and sieved, and the fine fraction (<2 mm) was homogenised.

Mercury concentration was determined using an Advanced Mercury Analyser (AMA-254, LECO Company; detection limit 0.5 μg kg^{-1}) according to Sierra et al. (2009). This equipment is based on an atomic absorption spectrophotometer and specifically designed for mercury determination. Certified reference materials (CRM) were used to determine the accuracy and precision of the measurements and to validate the applied methods. They were obtained from the Community Bureau of Reference (BCR), nowadays called as Standard Measurements and Testing. These reference materials are denominated as CRM027 (EEUU contaminated soil, 3.80 ± 0.65 mg kg^{-1}), SRM 2709 (San Joaquin agricultural soil, 1.40 ± 0.08 mg kg^{-1} of Hg), BCR-CRM 62 (olive leaves, 0.280 ± 0.020 mg kg^{-1} of Hg), BCR-CRM 151 (skim milk powder, 0.101 ± 0.010 mg kg^{-1} of Hg), SRM1573A (Tomato leaves 0.034 ± 0.004) BCR-CRM 150 (spiked skim milk powder, 0.0094 ± 0.0017 mg kg^{-1} of Hg). The mean value determined for ten measurements using the AMA-254 equipment was 3.49 ± 0.25 mg kg^{-1}, 1.34 ± 0.05 mg kg^{-1}, 0.316 ± 0.013 mg kg^{-1}, 0.097 ± 0.005 mg kg^{-1}, 0.037 ± 0.002 mg kg^{-1} and 0.0113 ± 0.0010 mg kg^{-1}, respectively.

2.4. Determination of acid-soluble thiols

Total thiols were assayed according to Vazquez et al. (2006), using 100 mg fresh weight of frozen pulverised plant material with 0.4 ml of

NaOH (1 M) containing $NaBH_4$ (1 mg ml^{-1}) and 0.2 ml of deionised water. After centrifugation (11,000g, 10 min), 0.5 ml of supernatant was added to 0.5 ml of 5,5′-dithiobis (2-nitrobenzoic acid) (300 mM) dissolved in neutralizing buffer (0.5 M potassium phosphate, pH 7.2). Absorbance was measured at 412 nm (Jocelyn, 1987).

2.5. Statistical treatment

All data from the plant and soil analysis were analyzed by GLM–ANOVA, followed by a post-hoc multiple comparison of means using the Duncan test ($P < 0.05$) using the statistical software SPSS 16.0. Values in the tables and figures indicate mean values ± standard error (S.E.).

3. Results and discussion

Table 1 shows the mean values of the original soil properties and total element concentrations of the soils used in the present study. The main soil characteristics at the end of the experiments are shown in Table 2. According to the results in Tables 1 and 2, P concentration did not vary from the beginning to the end of the experiment for soil C, while a 50% decrease was observed in soil A. These losses can be explained based on the lixiviation caused by frequent irrigations in pots and soil characteristics. In natural systems, the P lixiviation is limited at 50 cm depths in the soil. In our experimental conditions, soil characteristics as shown by soil A (loam texture and low organic matter content) can also improve the physico-chemical process of dispersion and contributes to P losses (Quinton et al., 2001).

Electrical conductivity (EC) value increased in soil C at the end of the experiment, but the levels still remain <2 dS m^{-1} which is considered normal for the plant development (Smagin et al., 2006). In both cases, there were no significant differences between mercury treatments and control.

The initial concentrations in the artificially polluted soil were 0.6 mg Hg kg^{-1} soil for the low dosage, and 5.5 mg Hg kg^{-1} for the high one. They were based on the values proposed by regional Spanish legislation (ORDEN 761/2007, BOCM, 26/04/2007) for land uses other than urban and industrial (under 5 mg kg^{-1}) and normal levels found in agricultural soils which range 0.3 mg kg^{-1} (Adriano, 2001). The concentrations added in soil were measured in the lab to check that the levels in the soil experiment were 0.6 and 5.5 mg Hg kg^{-1} for the low and high doses respectively (data not shown). Table 2 also shows the concentrations of this element in soil at the end of the experiment. One important point is the mercury losses produced during the plant experiment, especially in pots treated with high dosage. Approximately only 15% of the metal remains in soil in treatments with high dosage of pollutant, and it is unlikely that *S. vulgaris* was able to uptake 85% of the mercury applied in soil. Experiment was made from February to July following the *S. vulgaris* natural cycle. Average temperatures along the experiment are given in Table 3. Temperatures rose during the experiment as a result of seasonal changes, exceeding values of 35 °C from the middle of June. In these conditions, mercury volatilization is expected. A review about mercury evaporation from soils (Schlüter, 2000) concludes that the rates from background soils are usually smaller than 0.2 μg m^{-2} h^{-1}. About 5.2% of total deposited atmospheric Hg can be expected to evaporate, independent of seasonal variation. Temperature and soil radiation can influence in this rate. The evaporation rate increases only slightly with increasing temperature from 10 °C to 15–20 °C and exponentially from 15 to 20–35 °C in soils contaminated with inorganic Hg(II) (Lindberg et al., 1979; Rinklebe et al., 2010). In another laboratory experiment, using amended soils with 1 ppm of mercury (mercuric nitrate), Rogers and Mc Farlane (1979) showed that during the first week, 20–45% of the applied mercury was lost respectively from a silt clay-loam soil and loamy sand soil. In our experiment, the metal was added as $HgCl_2$, highly soluble in water, as consequence, mobility by leaching and volatilization was expected.

Plant growth was healthy and no visual differences appeared. According to Patra and Sharma (2000), the effect of mercury on root length, stem length, leaf area and dry matter production of higher plants such as *Brassica oleracea* L. var. *capitata*, *Chinese cabbage*, *Beta vulgaris* or *Pisum sativum*, is inversely proportional to the concentration of mercury. In some cases there was no inhibition and occasionally some stimulation of growth was observed. Tables 4 and 5 give dry matter content for both aerial part and root of *S. vulgaris* grown in two soils and sampling times of May and July, respectively. The plants came originally from soil C, and probably for that reason they grew more in soil C than in soil A. No statistically different values were found in the values of aerial part dry matter for both soils and Hg content in soil. However, the dry matter content in root significantly decreased when increasing concentrations of Hg for the highest mercury content, being statically significant with relation to the highest mercury content in soil C at the end of the experiment.

Table 1
Original soil characteristics.

	C	A
pH	8.5 ± 0.6	7.1 ± 0.2
E.C. (dS m^{-1})	0.12 ± 0.03	0.56 ± 0.05
$CaCO_3$ (%)	5.6 ± 0.3	n.d.
N (%)	0.05 ± 0.01	0.02 ± 0.01
OM (%)	0.63 ± 0.04	0.17 ± 0.03
Porosity (%)	19.25	64.05
Texture	Sandy clay-loam	Loam
Element (mg kg^{-1} soil)		
P Olsen	21 ± 0.9	12 ± 0.6
Ca	4787 ± 540	4052 ± 956
Mg	146 ± 24	440 ± 80
Na	42 ± 4	113 ± 20
K	355 ± 74	244 ± 54
Cu	53 ± 5.6	88 ± 8.6
Zn	44 ± 1	54 ± 9
Cd	0.90 ± 0.03	0.25 ± 0.09
Hg	<0.005	<0.005

Table 2
Soil characteristics from the pots at the end of the experiment.

Soil	Treatment	pH	P Olsen (mg kg^{-1})	Hg (mg kg^{-1})	EC (dS m^{-1})
C	Control	$8.3 \pm 0.1^{ns,A}$	$20.5 \pm 0.5^{ns,A}$	$0.035 \pm 0.004^{c,C}$	$0.41 \pm 0.02^{ns,B}$
	HgL	8.4 ± 0.1^{A}	22.0 ± 0.5^{A}	$0.335 \pm 0.038^{b,B}$	0.38 ± 0.03^{B}
	HgH	8.2 ± 0.1^{A}	19.5 ± 3.5^{A}	$0.681 \pm 0.152^{a,A}$	0.44 ± 0.01^{B}
A	Control	$7.9 \pm 0.1^{ns,B}$	$7.5 \pm 0.5^{ns,B}$	$0.016 \pm 0.003^{c,C}$	$0.64 \pm 0.06^{ns,A}$
	HgL	7.7 ± 0.1^{B}	6.0 ± 2.0^{B}	$0.377 \pm 0.022^{b,B}$	0.59 ± 0.07^{A}
	HgH	7.8 ± 0.1^{B}	6.0 ± 1.0^{B}	$0.910 \pm 0.120^{a,A}$	0.66 ± 0.04^{A}

Significant differences among Hg treatments for each treatment and soil are indicated by different small letters and differences among soil with different capital letters ns not significant differences at $p < 0.05$ (Duncan's test, mean ± SE, $n = 6$).

Table 3
Monthly minimum, maximum and average temperatures along the experiment.

Month	TM (°C)	tm (°C)	*T* average (°C)
February	20.0	−4.0	7.3
March	20.0	−4.0	8.2
April	25.0	−3.5	11.2
May	28.0	1.0	13.5
June	34.5	8.0	19.0
July	39.0	11.0	24.0

TM: maximum temperature, tm: minimum temperature, *T*: average temperature.

Table 4
Effect of treatments on *Silene vulgaris* grown in soils with two different Hg concentrations in the sampling time of May.

Soil	Treatment	Fresh weight (g)		Dry weight (g)		Hg aerial part (mg kg^{-1} DW)	Thiols (nmol−SH g^{-1})
		Aerial part	Root	Aerial part	Root		
C	Control	$8.4 \pm 0.8^{a,A}$	$2.5 \pm 0.5^{ns,NS}$	$1.40 \pm 0.14^{ns,A}$	$0.41 \pm 0.10^{ns,NS}$	$0.03 \pm 0.005^{b,B}$	$207 \pm 41^{ns,NS}$
	HgL	$6.9 \pm 0.5^{ab,A}$	2.4 ± 0.2	1.40 ± 0.48^{A}	0.48 ± 0.04	$0.31 \pm 0.07^{b,B}$	246 ± 26
	HgH	$4.9 \pm 0.8^{b,B}$	2.0 ± 0.5	1.47 ± 0.52^{A}	0.52 ± 0.11	$0.6 \pm 0.2^{a,B}$	189 ± 26
A	Control	$4.6 \pm 0.4^{a,BC}$	2.7 ± 0.4^{ns}	$0.83 \pm 0.08^{ns,B}$	0.54 ± 0.10^{ns}	$0.15 \pm 0.09^{b,B}$	209 ± 15^{ns}
	HgL	$5.0 \pm 0.5^{a,B}$	2.8 ± 0.3	0.84 ± 0.10^{B}	0.46 ± 0.04	$0.33 \pm 0.05^{b,B}$	204 ± 22
	HgH	$3.1 \pm 0.4^{b,C}$	1.7 ± 0.4	0.56 ± 0.07^{B}	0.33 ± 0.08	$3.17 \pm 1.07^{a,A}$	156 ± 21

Fresh and dry weight (g), Hg concentration in aerial part (mg Hg kg^{-1} DW^{-1}) and total thiol groups in roots (nmol−SH (g FW)$^{-1}$).
Significant differences among Hg treatments for each soil are indicated by capital letters, and among soils are indicated by small letters different letters ns NS not significant differences (mean ± SE, $n = 6$; Duncan's test $p < 0.05$).

One point is the fresh weight of aerial part decreased with the increasing of Hg concentration in both soils. Data from May sampling time are given in Table 4. The differences were statically significant in control plants, which showed weight increments up 70% in soil A and 40% in soil C with relation to high dosage of mercury. Previous studies in sunflowers (Van Heeswijk and van Os, 1986; Ye and Verkman, 1989) show that mercury application to both sides of sunflower leaves caused a greater closure of the stomata on the upper side but increased cuticular resistances to gas diffusion only in leaves, suggesting that mercury decreases the permeability of nonstomatal epidermal cells. Maggio and Joly (1995) described the results of experiments carried out to test the effects of $HgCl_2$, a known inhibitor of water channels on the water permeability of intact tomato (*Lycopersicon esculentum*). The $HgCl_2$ induced the reversible inhibition of water flux. The fact was reported later to be consistent with the presence of a protein-mediated path for trans-membrane water flow in plant roots. The $HgCl_2$ used in this experiment to pollute the soil could play as an inhibitor of the water channel, especially at the beginning of the experiment where it was in major proportion.

With relation to Hg in plants of *S. vulgaris*, the concentration of this element increased both in aerial part and roots positively to the concentration in soil. Tables 4 and 5 also give the mercury concentration (mg kg^{-1} DW) of aerial part in the experiment. The highest concentration is found in the sampling time corresponding to May (Table 4), when the mercury was more available because originally it was added as $HgCl_2$. It is positively correlated with the metal concentration in soil. Differences are statistically significant between control plants and treatments. In general, the availability of soil mercury to plant is low and it is related among other factors to soil characteristics. High pH value, abundant lime, and accumulated salt in soil reduced its uptake by plants, which are in agreement with in both tables. The highest mercury concentrations in aerial part were obtained in plants grown in soil A, which had the lowest pH values and loam texture.

Organic compounds such us thiols have been used as biomarkers, which can be useful in the early diagnosis of metal toxicity and especially before biomass reduction (Prasad, 2003; Vazquez et al., 2006). For that reason total thiol concentrations were measured in fresh roots of *S. vulgaris* only in the sampling time of May (Table 4). The thiol concentrations in roots of *S. vulgaris* did not increase Hg with concentration in soil. Although, mercury concentrations in aerial part of *S. vulgaris* grown in soils polluted by mercury was over fifteen times higher than in control, significant differences were just found in plants grown with soils polluted by high dosage of mercury. No differences were found among other treatments and between soils. In addition, no mercury stress symptoms are observed.

Regards to mercury concentration in roots, data included in Table 5 are from the plants sampled at the end of the experiment. Due to scarce of plant material, the data obtained in the May sampling time were inconclusive and consequently not included in Table 4. At the end of the experiment, Hg concentrations in roots increased significantly in plants treated with high dosages (Table 5). The differences are significant between soils and also for the high dosages. Roots from plants grown in calcareous soils increased mercury concentration higher than when the roots came from plants grown in soil A.

The efficiency of phytoextraction is determined by two key factors: biomass production and the metal bioconcentration factor (McGrath and Zhao, 2003). The bioconcentration factor is defined as the ratio of metal concentration in plant shoots to metal concentration in soil. In order to quantify mercury in soil, the initial value added to soil (0.6 and 5.5 ppm) should be corrected, because the high mobility of $HgCl_2$ caused losses previously described. The magnitude of this correction is not easy to predict because it is involved to Hg dosage and soil characteristics. At the end of this experiment mercury was added in low doses about 60% of metal remains in soil C, slightly lower percentage than in soil A. This percentage increases but not as same proportion as high doses. Another parameter for identifying the accumulation of heavy

Table 5
Effect of treatments on plant of *Silene vulgaris* growth in pots with soil C (pH > 7) and soil A (pH ≈ 7) with two different Hg concentrations at the end of the experiment.

Soil	Treatment	Dry weight (g)		Hg (mg Hg kg^{-1} DW^{-1})		TF
		Aerial part	Root	Aerial part	Root	
C	Control	$2.43 \pm 0.27^{ns,A}$	$1.26 \pm 0.03^{a,A}$	$0.024 \pm 0.002^{b,C}$	$0.010 \pm 0.001^{b,C}$	
	HgL	2.33 ± 0.14^{A}	$1.01 \pm 0.20^{a,AB}$	$0.07 \pm 0.01^{b,C}$	$0.38 \pm 0.20^{b,C}$	0.27^{ab}
	HgH	2.36 ± 0.16^{A}	$0.70 \pm 0.06^{b,DC}$	$0.55 \pm 0.08^{c,B}$	$3.7 \pm 0.9^{a,A}$	0.15^{b}
A	Control	$0.97 \pm 0.06^{ab,B}$	$1.06 \pm 0.40^{ns,CB}$	$0.052 \pm 0.009^{b,C}$	$0.003 \pm 0.007^{b,C}$	
	HgL	$1.14 \pm 0.02^{a,B}$	0.81 ± 0.20^{C}	$0.11 \pm 0.03^{b,C}$	$0.58 \pm 0.05^{b,C}$	0.20^{ab}
	HgH	$0.76 \pm 0.07^{b,B}$	0.87 ± 0.01^{D}	$0.98 \pm 0.21^{a,A}$	$2.9 \pm 1.3^{a,B}$	0.50^{ab}

Biomass (g), Hg concentrations in aerial part and root (mg Hg kg^{-1} DW^{-1}) and Translocation Factor TF = [Hg_{shoot}/Hg_{root}].
Significant differences among Hg treatments for each soil are indicated by capital letters, and among soils are indicated by small letters different letters (mean ± SE, $n = 6$; Duncan's test $p < 0.05$).

metals and to provide an insight into their mobilization sequences is the Translocation Factor. This ratio was defined as the concentration of Hg in shoot divided by the concentration of Hg in root. With this parameter it is also possible to evaluate the phytoextraction feasibility based on Hg mobilization in plant, although it is not possible to assess the plant net performance to mobilize the metal from the soil. The translocation factor at the end of the experiment is included in Table 5. Most of the Hg content is stored in root, avoiding that heavy metals reach the aerial parts of the plant as can be concluded from the translocation factor values.

Mercury concentrations in *S. vulgaris* plants grown in the conditions described here were in the low range reported by Millán et al. (2006) in the Almaden mining district and Moreno-Jimenez et al. (2007) in hydroponic conditions. Mercury uptake is related to soil characteristics. The best growth was found in the calcareous original soil. Good yields were shown in the soil A where the *S. vulgaris* was able to uptake more Hg than in the calcareous soil. The good biomass production, Hg uptake including translocation to shoot and root and the lack of differences found in thiols in roots with Hg content suggest that *S. vulgaris* could be interesting in remediation phytotechnologies. The fact that *S. vulgaris* retains more mercury in root than in shoot, and also the effectiveness of this plants in the recovering of contaminated soils, related to the reduction of heavy metal toxicity on soil bacteria (Martinez-Iñigo et al., 2009) makes *S. vulgaris* a good candidate to phytostabilization technologies. In this context, possible alternatives are to manipulate the plant rizhosphere to enhance metal bioavailability, and increase the metal uptake by shoots.

Acknowledgements

This work has been supported by Programme EIADES, S-0505/AMB/0296 from Consejería de Educación, Comunidad de Madrid and Ministerio de Ciencia e Innovación-INIA (Ayuda para la contratación de Doctores – CCAA).

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Journal of Environmental Management 95 (2012) S238–S244

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Use of urban composts for the regeneration of a burnt Mediterranean soil: A laboratory approach

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ARTICLE INFO

Article history:
Received 29 September 2009
Received in revised form
2 September 2010
Accepted 19 October 2010
Available online 15 December 2010

Keywords:
Forest fire
Municipal wastes compost
Sewage sludge
Green wastes compost
Mineralisation
Total microbial biomass
Fungal biomass
Soil characteristics
Runoff
Infiltration

ABSTRACT

In Mediterranean region, forest fires are a major problem leading to the desertification of the environment. Use of composts is considered as a solution for soil and vegetation rehabilitation. In this study, we determined under laboratory conditions the effects of three urban composts and their mode of application (laid on the soil surface or mixed into the soil) on soil restoration after fire: a municipal waste compost (MWC), a compost of sewage sludge mixed with green waste (SSC) and a green waste compost (GWC). Carbon (C) and nitrogen (N) mineralisation, total microbial biomass, fungal biomass and soil characteristics were measured during 77-day incubations in microcosms. The impact of composts input on hydrological behaviour related to erodibility was estimated by measuring runoff, retention and percolation (i.e. infiltration) of water using a rainfall simulator under laboratory conditions. Input of composts increased organic matter and soil nutrient content, and enhanced C and N mineralisation and total microbial biomass throughout the incubations, whereas it increased sporadically fungal biomass. For all these parameters, the MWC induced the highest improvement while GWC input had no significant effect compared to the control. Composts mixed with soil weakly limited runoff and infiltration whereas composts laid at the soil surface significantly reduced runoff and increased percolation and retention, particularly with the MWC.

1. Introduction

The Mediterranean climate is characterized by a long dry summer and strong winds favouring recurrent forest fires (Bagnouls and Gaussen, 1957; Scarascia-Mugnozza et al., 2000; De Luis et al., 2001). Fires induce major alterations to the ecosystem. Their frequency, duration and intensity are important factors determining the impact on biological, chemical and physical properties of the ecosystem: the more fires are recurrent and serious, the more their marked impact (Boerner, 1982).

Fire can produce a partial or total destruction of the vegetal cover and the soil organic horizons (Guerrero et al., 2001). Subsequently, burned soils are prone to erosion and could decline in stability (Kutiel and Inbar, 1993; Hart et al., 2005). For example, part of the nutrients are oxidised and volatilised by fire (Grogan et al., 2000; Hart et al., 2005) and can easily be lost by wind erosion and runoff (DeBano and Conrad, 1978; Boerner, 1982; De Luis et al., 2001) aggravated by an increase of soil hydrophobicity (DeBano, 2000). Thus, Mediterranean soils are often deficient in organic matter (OM) (Archibold, 1995). Another biological change due to fire is the activity and structure of the microbial community, with a shift to communities in which heat-resistant microorganisms dominate (Vásquez et al., 1993; Hart et al., 2005).

The use of composts as an amendment for soil restoration and forest regeneration in frequently burnt or degraded Mediterranean ecosystems is increasing (Navas et al., 1999; Martinez et al., 2003; Román et al., 2003; Curtis and Claassen, 2009; Kowaljow and Mazzarino, 2007). The spreading of biosolids stabilized by composting, can improve the low fertility of soils and constitutes an alternative to landfill disposal. Moreover, this stabilization decreases risks of heavy metal leaching (Garcia et al., 1990; Planquart et al., 1999).

Compost amendment improves physical, chemical and biological properties of soils, in particular by increasing available

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0301-4797/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jenvman.2010.10.042

nutrients mainly in the organic soil fractions (Larchevêque et al., 2005a). This induces an increase of soil microbial biomass (Borken et al., 2002) and positively affects plant cover by an improvement of plant nutrition (Villar et al., 1998; Guerrero et al., 2000, 2001; Caravaca et al., 2003; Larchevêque et al., 2005b, 2006; Larchevêque et al., 2010). The increase of microbial activity can induce a better aggregate stability (Guerrero et al., 2001; Caravaca et al., 2003), and, combined with the development of plant biomass, reduces the risk of erosion (Guerrero et al., 2000).

The objective of this study was to i) compare the effects of different types of composts and the mode of application on some chemical and microbial properties under laboratory conditions in a Mediterranean burned soil, and ii) to determine how these amendments modify the soil's hydrologic response using rainfall simulations.

2. Materials and methods

2.1. Soil and composts characteristics

The soil was collected in a burnt *Pinus halepensis* (Mill.) ecosystem on calcareous substrate under Mediterranean climate in south-eastern France (43°28′47″N–5°27′55″E, 245 m altitude). The sampling was realised on 30 sampling points in the burnt area over 20 cm depth (including surface ashes) in February 2006, 6 months after the fire. The fire was intense (high calorific power) but rapidly progressed because of a strong wind. Fire affected crown and soil surface. Consumption of litter layer could be observed, organo-mineral layer had been eroded or was missing, but there was no visible alteration of the surface of the mineral soil. Burned trees were still standing but no vegetation had begun to grow. The soil was homogenized by sieving (<4 mm) for the experiments. Three urban composts sieved at 10 mm were studied: green waste compost (GWC), sewage sludge (1/5 volume) mixed with green waste compost (SSC) and municipal solid waste compost (only organic wastes, MWC). Levels of heavy metals in composts studied were below the minimum current standards (NFU 44-095 AFNOR, 2002; NFU 44-051 AFNOR, 2006). Soil and compost were stored at 4 °C before incubations. According to the self-heating test depending on the maximum temperature reached (T_{max}) (FCQAO, 1994), GWC was stabilized whereas SSC and MWC were unstabilized (Table 1).

Soil and compost initial characteristics are presented in Table 1. Soil is a Haplic Cambisol (Calcaric) (FAO, 1998).

Table 1
Initial microbiological, chemical and physical characteristics of composts and soil. Values of the Dewar indice.

	Soil	MWC	SSC	GWC	Methods
pH	8.1	7.8	6.6	8.0	NF EN 12176
Water content (%)	27.09 ± 0.86	21.94 ± 4.83	22.03 ± 5.00	26.52 ± 0.88	
Conductivity (mS/m)	15	3.02	0.85	1.38	Extr. Water 1/5 (V/V) and Conductivity NF EN 13039
OM (g/kg)	84.4	712	454	357	NF EN 12879
Total N (g/kg)	2.4	15.1	22.1	14.2	Attack Kjeldahl + colorimetry
C/N	17.0	23.0	10.0	12.0	Organic C/total N
$N\text{-}NH_4^+$ (mg/kg)	0.06	3419.0	3407.7	147.9	Extraction KCl M & dosage, Berthelot
$N\text{-}(NO_3 + NO_2)$ (mg/kg)		3.48	0.80	12.67	Extraction KCl M & dosage, Griess and Ilossay's
Total phosphorus (g/kg)	0.04	0.32	0.08	0.34	ISO 11-263-1 adapted
K_2O (g/kg)	3.4	8,0	7.91	12.6	NF EN 13346, Dosage ICF AES NF EN ISO 11885 extraction with *aqua regia*
Total microbial biomass (mg C/kg)	269	4788	2481	2324	Vance et al., 1987
Fungal biomass (mg/kg)	4.61	12.17	0,0	4.64	Gessner and Schmitt, 1996
T_{max} (°C)	–	71	58.5	22	FCQAO, 1994
Dewar indice	–	I	II	V	

Units are related to dry matter of soil.

2.2. Laboratory incubations

Soil–compost mixtures were incubated in 2 L jars hermetically closed at 28 ± 1 °C, in the dark. Composts were mulched or mixed with soil at field capacity. The rate of compost used was 10 g of compost in each jar corresponding to 27 Tm ha^{-1} of fresh matter. The amount of soil in each jar was 150 g and corresponded to 20 cm depth of soil sampling. Soil OM, total N, organic C, total phosphorus, K_2O contents, soil C/N ratio, C and N mineralisation and biological activities were measured periodically during 77 days. Measurements were made only on the soil fraction for the mulched composts.

2.3. Carbon mineralisation

C mineralisation during incubation was based on the amount of CO_2 produced during the incubation time. A flask with 25 ml of NaOH 1 N and another with 10 ml of water were introduced into each 2 l jar. The jars were opened to change the flasks and to renew the atmosphere on days 2, 4, 7, 10, 14, 21, 28, 35, 49, 63 and 77. Five replicates for the 2 modes of application were made for the 3 composts. 5 controls of soil without compost were also done. $C\text{-}CO_2$ trapped in NaOH was analysed by colorimetry at 550 nm with a continuous flux analyser (SKALAR, Netherlands) after acidification with H_2SO_4 solution (100 mg/L), and addition of phenolphthalein (pH = 8.6, 50 °C).

2.4. Nitrogen mineralisation

The measurements of N mineralisation were destructive and were carried out on days 0, 14, 28, 49 and 77. At each date, 5 controls of soil without compost and 5 replicates for each mode of application and for the 3 composts were performed. For mulch application, the compost layer was removed before the sampling. Mineral N was extracted with KCl 1 N (1/4, soil mass/KCl volume) by agitation during 1 h and decantation. Mineral N was measured on the filtered supernatant (Whatman, GF/C) by colorimetric methods (Berthelot's method for $N\text{-}NH_4^+$ at 660 nm and Griess and Ilossay's method for $N\text{-}(NO_3 + NO_2)$ at 540 nm; SKALAR, Netherlands).

2.5. Total microbial and fungal biomass

The measurements of total microbial and fungal biomass were destructive and were carried out on days 14, 28, 49 and 77 for 5 controls of soil without compost, 5 replicates for each mode of application and for the three composts. As for nitrogen mineralisation, only the soil below compost was analysed for mulch application. Total microbial biomass was measured on 24 g of the soil–compost mixture or the soil under mulch by fumigation-extraction (Vance et al., 1987). Microbial extractable C was estimated from the difference in C released between fumigated and unfumigated samples (Wu et al., 1990). Fungal biomass in soil was determined from ergosterol concentrations using solid-phase extraction and high-performance liquid chromatography (HPLC; Gessner and Schmitt, 1996).

Table 2
Chemical characteristics of soil after 49 days of incubation.

	Control	Incubation with MWC		Incubation with GWC		Incubation with SSC	
		Mulched	Mixed	Mulched	Mixed	Mulched	Mixed
pH	7.88 ± 0.04^{bc}	7.86 ± 0.05^{bc}	7.74 ± 0.05^{d}	7.90 ± 0.00^{ab}	7.98 ± 0.04^{a}	7.84 ± 0.05^{bc}	7.80 ± 0.00^{cd}
Conductivity (mS/cm)	1.28 ± 0.10^{c}	1.31 ± 0.08^{bc}	1.44 ± 0.02^{ab}	1.23 ± 0.00^{c}	1.29 ± 0.08^{c}	1.49 ± 0.06^{a}	1.59 ± 0.02^{a}
Total phosphorus (g/kg)	0.03 ± 0.00^{cd}	0.02 ± 0.00^{d}	0.03 ± 0.00^{cd}	0.03 ± 0.00^{cd}	0.04 ± 0.00^{b}	0.03 ± 0.00^{c}	0.12 ± 0.01^{a}
OM (g/kg)	66.48 ± 3.19^{c}	70.02 ± 3.37^{bc}	80.72 ± 6.39^{a}	68.46 ± 3.91^{bc}	74.86 ± 4.63^{abc}	69.56 ± 6.66^{bc}	78.52 ± 7.16^{ab}
C/N	14.40 ± 0.89^{a}	13.60 ± 0.55^{a}	14.20 ± 1.10^{a}	13.60 ± 0.89^{a}	13.40 ± 1.34^{a}	14.00 ± 1.22^{a}	10.67 ± 0.58^{b}
$N\text{-}NH_4^+$ (mg/kg)	11.34 ± 2.58^{bc}	22.40 ± 5.18^{a}	14.20 ± 1.92^{b}	7.44 ± 1.35^{c}	8.20 ± 1.85^{c}	7.18 ± 1.76^{c}	14.00 ± 1.87^{b}
K_2O (g/kg)	0.31 ± 0.01^{f}	0.38 ± 0.00^{e}	0.47 ± 0.01^{c}	0.43 ± 0.01^{d}	0.61 ± 0.02^{a}	0.39 ± 0.03^{e}	0.54 ± 0.01^{b}

Mean ± SD, $N = 5$. Units are related to dry matter of soil. Results of the comparison are given by an exponent letter: values that do not differ at the 0.05 level are noted with the same letter (one-way ANOVA and Tukey test, a > b > c > d > e > f).

2.6. Rainfall simulation: runoff and infiltration (retention and percolation)

Compost effects on runoff and percolation were studied in laboratory by using a rainfall simulator, on another set of soil samples. Amended soils were placed in pierced bottom containers of 31.5 cm in length, 24.6 cm width and 6 cm height. From the bottom to the top of the container, we placed respectively: a geotextile to avoid matter loss through holes, 996 g of sand (depth = 1 cm), 2500 g of homogenized soil, and 167 g of compost homogenously distributed on soil surface for mulch treatment or mixed with soil. We performed 3 controls of soil without compost and 3 replicates for each mode of application and for the 3 composts. Containers with soil and composts were placed under the rainfall simulator with a 10° slope. The rainfall simulator, similar to the one used by Foster et al. (1979), was equipped with oscillating nozzles allowing a rainfall equivalent to 45 mm h^{-1} during 40 min, at a pressure of 0.9 bar. Runoff and percolation were measured by weighing the water respectively from runoff and from percolation after rainfall. Retention was estimated by the difference between the weight of the soil before and after the rainfall. We also determined dissolved organic carbon (COD) (NF EN 1484), suspended solids (SS) (NF T 90-105-2), total phosphorus (NF EN ISO 11885 before mineralisation with *aqua regia*), chloride and $N\text{-}NO_3^-$ (NF EN ISO 10304-2), $N\text{-}NH_4^+$ (NF T 90-015-1), SO_4^{2-} (NF EN ISO 10304-2), Ca^{2+}, K^+ and Na^+ (NF EN ISO 11885 before mineralisation with *aqua regia*) contents of the water collected from runoff and percolation.

2.7. Statistical analyses

The effect of compost type, mode of application and incubation duration were analysed by one-way ANOVA combined with Tukey tests (Zar, 1984). Previously, normality and homoscedasticity were verified by Shapiro–Wilks and Bartlett tests respectively (Zar, 1984). We used Kruskal–Wallis analyses and post-hoc Student–Newman–Keuls when these conditions were not met (for C and N mineralisation). Significance was defined by $p < 0.05$. The software MINITAB® (Minitab Inc., 2000) was used.

3. Results

3.1. Soil–compost characterization after 49 days of incubation

After 49 days of incubation, all treatments increased OM, total N and K content (Table 2; one-way ANOVA, $p < 0.05$). Only SSC input induced an increase of P content and MWC of NH_4^+ content (Table 2; one-way ANOVA, $p < 0.001$). Total phosphorus, OM, total N, organic C and total potassium content were greater for mixed compost than for mulched compost (one-way ANOVA, $p < 0.05$).

3.2. Carbon mineralisation

Input of compost induced an increase of C mineralisation in comparison with the control except for the GWC (Fig. 1; Kruskal–Wallis, $p < 0.001$). C mineralisation of amended soil was the

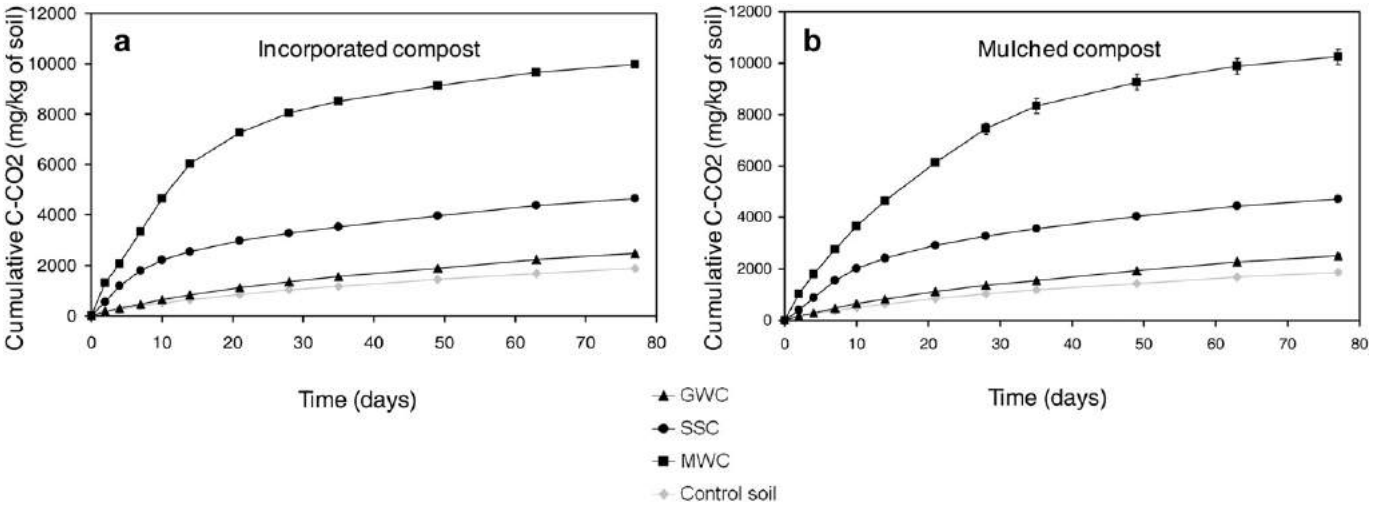

Fig. 1. Carbon mineralised during soil–compost incubation (mean ± SD, $N = 5$).

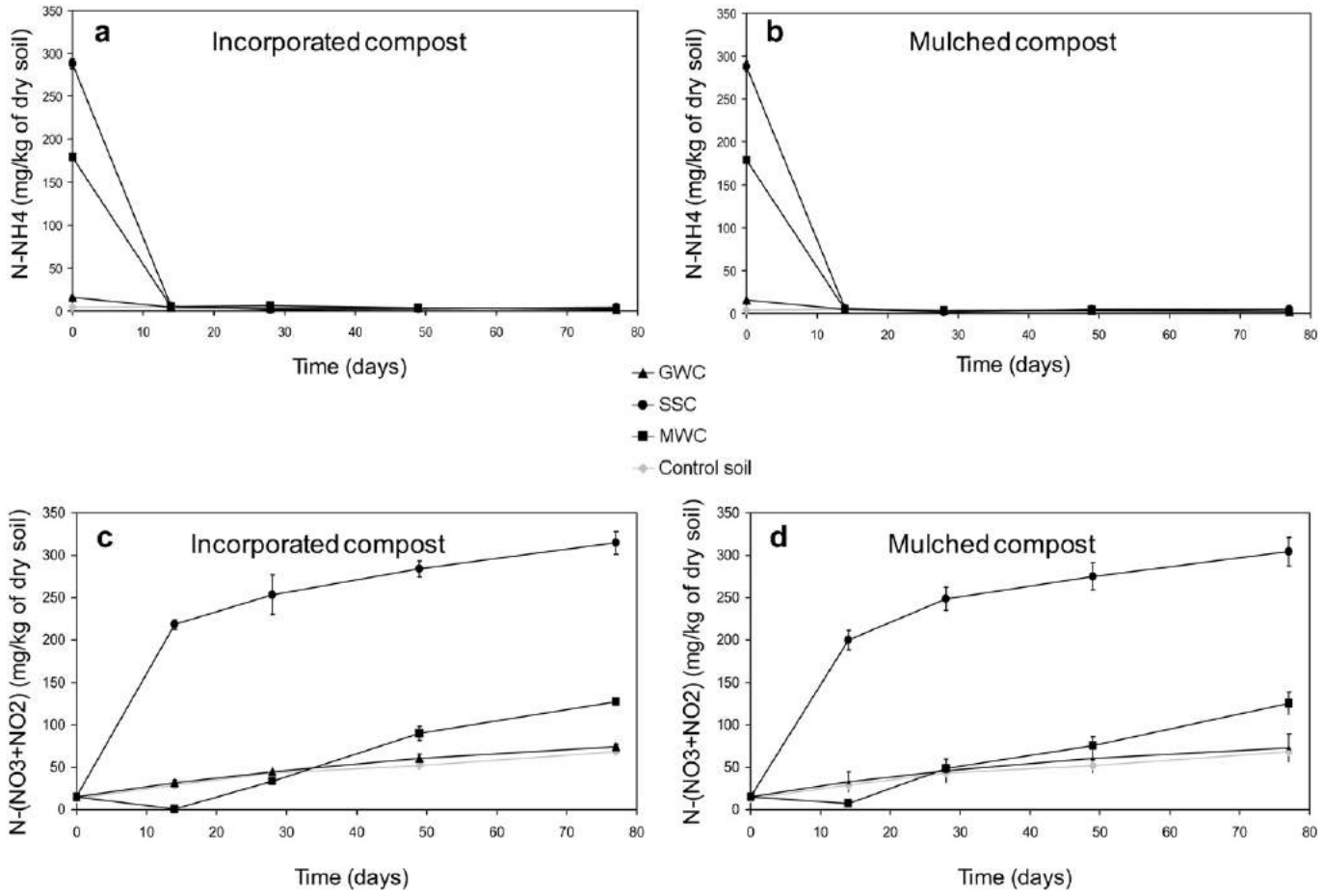

Fig. 2. Nitrogen mineralised during soil–compost incubation (mean ± SD, $N = 5$).

highest with MWC, the lowest with the GWC and intermediate with SSC (Fig. 1a and b; Tukey test, $p < 0.05$).

Differences between mulched and mixed compost modes were significant but depended on the type of compost (Kruskal–Wallis, $p < 0.05$). C mineralisation was higher with mixed mode compared to mulched mode at the beginning of the incubation for MWC and SSC (Kruskal–Wallis, $p < 0.05$), but was lower with the mixed mode for GWC from 4 to 77 d of incubation (Kruskal–Wallis, $p = 0.009$).

3.3. Nitrogen mineralisation

All compost types induced an increase of NH_4^+ content immediately after input (Fig. 2a and b; Kruskal–Wallis, $p < 0.001$), with

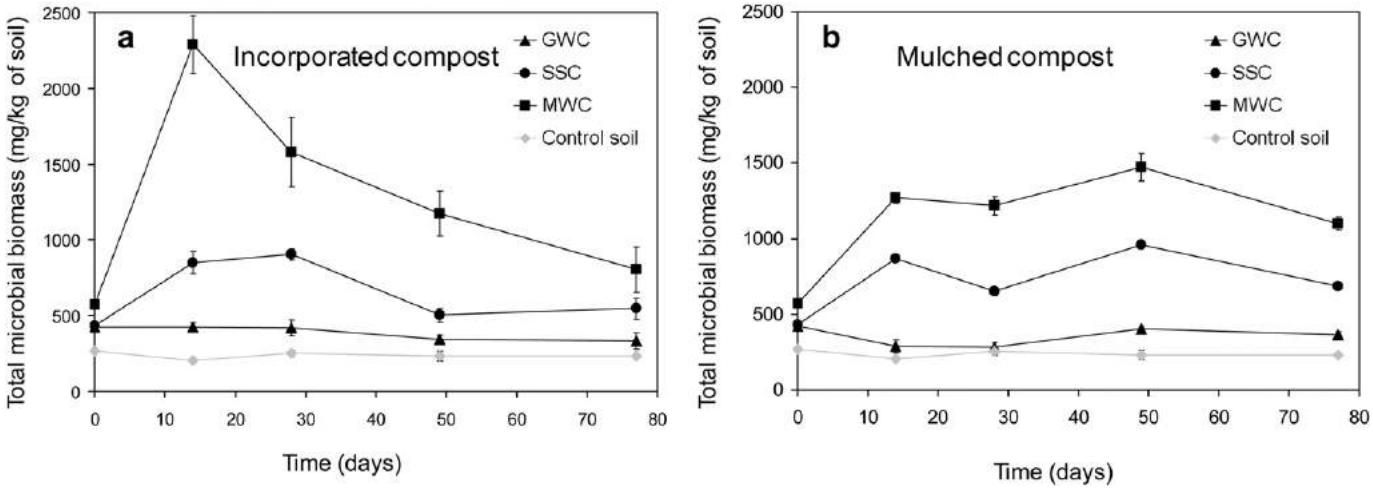

Fig. 3. Dynamics of total microbial biomass during soil–compost incubation (mean ± SD, $N = 5$).

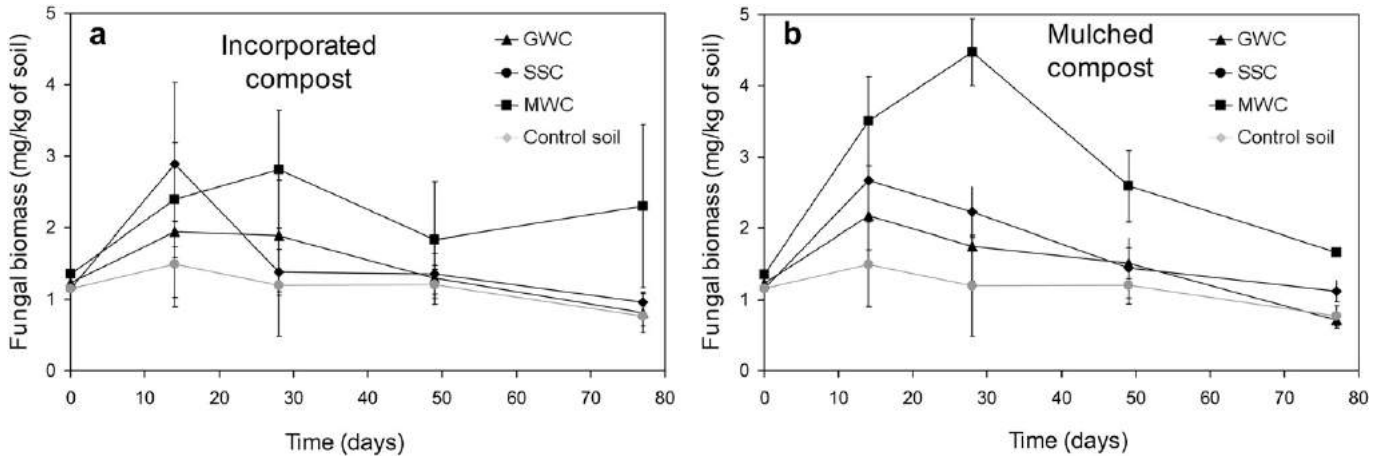

Fig. 4. Dynamics of fungal biomass during soil–compost incubation (mean ± SD, $N = 5$).

the highest ammonium content observed for MWC and SSC (Fig. 2a and b). Then ammonium strongly decreased to a value close to zero and remained constant until the end of incubation whatever the compost (Fig. 2a and b; Kruskal–Wallis, $p > 0.05$).

Except for MWC during the first 14 days and GWC throughout the incubation time, we observed an increase of N-($NO_3^- + NO_2^-$) for amended soil compared to control (soil) with maximum values reached at the end of the incubation for SSC (Fig. 2c and d; Kruskal–Wallis, $p < 0.001$; Student–Newman–Keuls, $p < 0.05$).

No effect of the mode of application was observed on N mineralisation (NH_4^+ and N-($NO_3^- + NO_2^-$); Kruskal–Wallis, $p > 0.05$).

3.4. Total microbial biomass

Amendment increased total microbial biomass especially for MWC (Fig. 3a and b; Kruskal–Wallis, $p < 0.001$). For this compost, the microbial biomass was lower with the mulched mode than with the mixed mode between days 0 and 49 and higher from the 49th until the 77th day (Fig. 3a and b; Kruskal–Wallis, $p < 0.05$).

3.5. Fungal biomass

Fungal biomass increased just after composts were applied except for SSC (Fig. 4a and b; Kruskal–Wallis, $p < 0.001$). There were no significant differences between mulched and mixed modes except for MWC from the 14th to the 49th day.

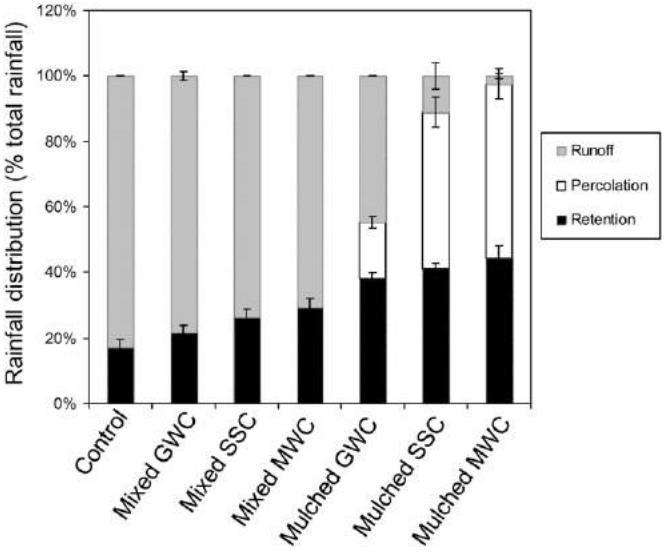

Fig. 5. Water rainfall distribution (mean ± SD, $N = 3$).

3.6. Composts effects on runoff and infiltration

Only two ways of distribution were observed for control treatment: runoff and retention. Mixed composts weakly decreased runoff and increased retention except for mixed GWC and no percolation was observed with this mode (Fig. 5; one-way ANOVA, $p < 0.001$). Mulched composts induced an important decrease of runoff, and an increase of retention, and some water from percolation may have been observed. Effects were the most marked for MWC and the less for GWC (Fig. 5; one-way ANOVA, $p < 0.001$).

In water from runoff, input of GWC induced an increase of dissolved organic carbon (DOC) (Table 3; one-way ANOVA, $p = 0.005$). MWC and SSC reduced exported NH_4^+ and NO_3^- (Table 3; one-way ANOVA, $p < 0.05$). Mulched application decreased exportation of SO_4^{2-} and chloride (Table 3; one-way ANOVA, $p < 0.05$).

In water from percolation, MWC induced the most important exportation of NH_4^+, SO_4^{2-}, Ca^{2+}, K^+, Na^+, and, chloride (Table 3; one-way ANOVA, $p < 0.001$). With SSC, this was observed for NH_4^+, total P, K^+ and chloride (Table 3; one-way ANOVA, $p < 0.001$) and only for NO_3^- with GWC (Table 3; one-way ANOVA, $p < 0.001$).

4. Discussion

4.1. Effect of compost characteristics on chemical and microbiological soil properties

As it has been previously observed (Larchevêque et al., 2005a, 2006), compost input led to an increase of soil nutrients and then could enhance vegetation recolonization after fire (Navas et al., 1999; Martinez et al., 2003; Larchevêque et al., 2005a). However, according to the different initial characteristics of composts, soil nutrient and biological responses to amendment differed: MWC induced the greatest effect, and GWC the lowest. The stability level of the composts explains those results. MWC was not stabilized and therefore rich in easily degradable OM which induced a strong mineralisation of C. In contrast, GWC presented a high stability level, and then induced low mineralisation. The dynamics of C mineralisation also varied over time whatever the type of compost, with the highest rate of mineralisation at the beginning of incubation, when labile OM is still available (Bernal et al., 1997).

The three composts induced different dynamics in levels of N-NH_4^+ and N-($NO_2 + NO_3$), due to initial compost C/N ratio and

Table 3
Chemical analysis of runoff and percolation water collected after the rainfall simulation.

	Runoff							Percolation		
	Control	MWC		SSC		GWC		MWC	SSC	GWC
		Mixed	Mulched	Mixed	Mulched	Mixed	Mulched	Mulched	Mulched	Mulched
SS (g)	9.33 ± 3.83^{a}	6.62 ± 1.69^{ab}	0.00^{c}	7.27 ± 2.25^{ab}	0.00^{c}	8.17 ± 2.04^{a}	0.97 ± 0.88^{bc}	0.18 ± 0.04^{A}	0.41 ± 024^{A}	–
DOC (mg C)	23.9 ± 4.8^{b}	27.3 ± 5.2^{b}	0.00^{c}	24.1 ± 5.2^{b}	0.00^{c}	30.0 ± 12.5^{b}	53.6 ± 22.91^{a}	–	0.00^{A}	0.00^{A}
N-NH_4^+ (mg)	11.8 ± 2.8^{a}	2.5 ± 0.7^{bc}	0.00^{c}	7.4 ± 2.8^{ab}	0.00^{c}	10.8 ± 2.5^{a}	8.5 ± 2.9^{ab}	71.9 ± 21.5^{A}	67.7 ± 9.5^{A}	1.2 ± 0^{B}
N-NO_3^- (mg)	2.03 ± 0.65^{a}	0.87 ± 0.09^{bc}	0.00^{c}	0.93 ± 0.06^{abc}	0.00^{c}	1.37 ± 0.66^{ab}	0.58 ± 0.11^{bc}	0.62 ± 0.03^{B}	0.57 ± 0.06^{B}	5.72 ± 0.85^{A}
Total P (mg)	2.5 ± 1.0^{ab}	2.8 ± 0.8^{ab}	0.4 ± 0.5^{b}	4.4 ± 2.1^{a}	1.3 ± 1.2^{ab}	2.6 ± 0.5^{ab}	4.2 ± 0.8^{a}	3.2 ± 0.3^{B}	6.6 ± 1.3^{A}	0.81 ± 0.15^{C}
SO_4^{2-} (mg	59.0 ± 13.8^{a}	43.4 ± 4.6^{ab}	0.00^{c}	44.2 ± 1.9^{ab}	0.00^{c}	49.3 ± 4.5^{ab}	29.2 ± 5.5^{b}	226.7 ± 16.7^{A}	208.5 ± 16.6^{A}	12.9 ± 11.3^{B}
Ca^{2+} (mg)	558 ± 190^{a}	415 ± 113^{a}	26.5 ± 31.5^{b}	483 ± 73^{a}	17.8 ± 16.2^{b}	533 ± 73^{a}	73.4 ± 12.0^{b}	631 ± 145^{A}	186.4 ± 25.4^{B}	68.2 ± 3.5^{B}
K^+ (mg)	38.0 ± 11.1^{a}	30.7 ± 4.6^{a}	15.6 ± 20.92^{a}	33.0 ± 5.3^{a}	5.4 ± 5.3^{a}	167.7 ± 218.4^{a}	94.1 ± 32.0^{a}	136.0 ± 28.4^{A}	71.1 ± 4.1^{B}	18.3 ± 1.0^{C}
Na^+ (mg)	3.7 ± 0.9^{a}	8.6 ± 1.7^{a}	21.7 ± 29.85^{a}	3.5 ± 0.7^{a}	1.1 ± 1.2^{a}	3.5 ± 0.3^{a}	4.1 ± 1.4^{a}	284.1 ± 26.8^{A}	39.0 ± 3.4^{B}	4.5 ± 0.3^{B}
Chloride (mg)	45.7 ± 3.8^{a}	37.8 ± 2.8^{ab}	0.00^{c}	38.7 ± 2.9^{ab}	0.00^{c}	41.8 ± 5.0^{ab}	30.1 ± 0.1^{b}	485.5 ± 39.0^{A}	204.2 ± 16.1^{B}	32.1 ± 27.8^{C}

Mean ± SD, N = 3. Units are related to dry matter of soil. Results of the comparison are given by an exponent letter: values that do not differ at the 0.05 level are noted with the same letter (one-way ANOVA and Tukey test, a > b > c to compare runoff water for the control and the 3 composts and their 2 application modes; A > B > C to compare percolation water for the three composts).

OM matter stability (Barbarika et al., 1985; Trinsoutrot et al., 2000; Parnaudeau et al., 2004).

Input of compost initially increased fungal and microbial biomass (Perucci, 1992; Albiach et al., 2001; Debosz et al., 2002) corresponding to the decomposition of easily biodegradable OM (Bernal et al., 1997). Maximum value of microbial biomass observed for MWC occurred at the same time as the lowest value of N-($NO_2 + NO_3$) showing N immobilization. This compost showed the highest values of microbial biomass and C mineralisation related to its labile OM content (Annabi et al., 2007). In contrast, microbial biomass remained stable and low for GWC, as the labile OM of this mature compost has already been degraded during composting.

4.2. Effect of the application mode on chemical and microbiological soil properties

Composts increased soil nutrient content whatever the mode of application, indicating a migration of nutrients from the mulched composts to the soil (Larchevêque et al., 2006). However, composts mixed with soil induced a greater C mineralisation and microbial biomass increase compared to mulched composts during the first stage of incubation. OM of mixed compost was rapidly degraded through a greater surface contact between soil and compost, enhancing biological activity (Schomberg et al., 1994; Coppens et al., 2007). Differences of C mineralisation and microbial biomass between the two modes of application were no longer observed during the second stage of incubation, because of the decrease of microbial biomass due to less available feeding substrate for microorganisms during the second stage.

In contrast, the development of the fungal biomass was affected by the mode of application but it was greater with mulch mode than buried in the case of MWC. We hypothesize that this development may be explained by a fungal community specific to compost favoured by the greater availability of the substrate in the case of mulch mode.

4.3. Effect of composts on infiltration and runoff and their potential implications for soil erosion risk

Unlike the findings of Tejada and Gonzalez (2006), no significant effects of mixed composts on water infiltration and runoff were observed, probably because the rainfall intensity was different between the studies: they applied a 140 mm h^{-1} rainfall while ours was 45 mm h^{-1}. According to Albaladejo et al. (1994) and Agassi et al. (2004), mulching is a method to limit runoff and, thus, erosion. Our study under controlled conditions confirmed this assertion, as mulched composts (particularly MWC) reduced runoff by increasing water retention and percolation, and thus infiltration in the soil (Giusquiani et al., 1995). The compost formed a protective layer on the surface and absorbed the kinetic energy of rain responsible for soil erosion (Agassi et al., 1985). This effect was more marked with MWC, probably due to its more fibrous texture than others that allowed better absorption of rainfall. Moreover, mulched composts allowed water percolation and the high decrease of runoff also led to a decrease of exported nutrients in surface water.

When composts were mixed into the soil, no significant effects were observed on exportation of elements.

Owing to these results, use of compost type and mode of application could affect in a different way water quality. Mulched mode compared to mixed mode lead to less surface water export.

5. Conclusion

Under laboratory conditions, input of compost on burned soil induced an increase in organic matter and nutrient soil content. Thus it increased the microbial biomass and its activity, depending on stability: the more the compost is unstable the more the effect is marked. The application mode had an effect on the physical protection of soil: mulched composts allow the absorption of kinetic energy of rainfall and thereby can limit losses by erosion and favour water infiltration.

Although compost effects could differ considerably under field conditions, the results obtained could help for generalizations through some implications for practice.

The use of composts for restoring burnt ecosystems shows promise as a method to speed up Mediterranean soil regeneration. Due to the high initial nutrient content, compost amendment after fire can accelerate vegetation recolonization. Unstable composts are the most efficient for rapidly enhancing soil biological activity. Their mulch application seems suitable to decrease nutrient exportation in surface water, reducing the risk of eutrophication. Moreover, as this mode of application increased water retention in the soil, it may increase water availability to plants.

Acknowledgements

This study was financially supported by Véolia Environnement. We thank M. Jolly, J.-N. Rampon of and C. Lecareux for their help during the laboratory experiment and F. Darboux, B. Renaux and L. Prud'Homme for their assistance in the use of the rainfall simulator (INRA, Orléans). We also thank Mr. Michael Paul for revision of English.

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Journal of Environmental Management 95 (2012) S245–S249

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Effects on soil organic matter mineralization and microbiological properties of applying compost to burned and unburned soils

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ARTICLE INFO

Article history:
Received 29 September 2009
Received in revised form
23 September 2010
Accepted 7 October 2010
Available online 4 November 2010

Keywords:
Carbon mineralization kinetics
Organic amendment
Burned soil
Compost dose
Incubation experiment

ABSTRACT

This study was undertaken in the context of a project of reclamation of a burned forest area applying municipal waste compost (MWC) and it consisted of an incubation experience carried out under laboratory conditions. The objectives of this research were to asses the effect of three doses of MWC added to burned and unburned calcareous soils on a) SOM mineralization and b) soil microbiological parameters. The laboratory incubation experience was carried out with three compost doses (1, 2 and 4% w/w) on a burned soil and another unburned one from an adjacent plot, besides the corresponding control samples. The mineralization kinetics of the organic matter was studied for 92 days. The kinetics data were adjusted to a double exponential model, showing two C pools of different degrees of resistance to mineralization and concentration, with half-life times of 1.9–4.9 and 34–76 days, respectively.

In the unburned soil, the initial potential mineralization rate of the labile and stable C pools showed an opposed behavior, increased and decreased with the MWC dose, respectively. However in the burned soil no significant tendencies were observed. Although applying compost tended to increase the size of more labile pool with respect to total mineralizable C, however most of the soil or compost OM did not result mineralizable in the short and medium term. The compost amendment did not increase soil microbial activity.

1. Introduction

Soil organic matter (SOM) is universally recognized to be among the most important factors responsible for soil fertility, crop production, and land protection from contamination, degradation, erosion and desertification, especially in semiarid and arid areas (Senesi et al., 2007). Forest fire is considered the main disturbance in Mediterranean areas (Whelan, 1995) and constitutes a serious environmental problem, due to the destruction of vegetation and also because of the soil degradation (Hernández et al., 1997; Guerrero et al., 2001; Turrión et al., 2010). Fire can consume part or all of the standing plant material and litter, as well as the SOM in the surface horizons (Guerrero et al., 2001). The loss of organic matter (OM) through fire, as well as the effect of fire on microbiota and the decrease in vegetation and soil cover, will favor surface soil erosion, particularly in Mediterranean forests, causing alterations in biological cycling of nutrients (Prichett and Fisher, 1987; Hernández et al., 1997). In recent years, the need to protect soils from degradation and/or erosion has spurred a series of efforts to find alternative practices aimed at restoring and/or improving SOM content and functions. As a result, recycling large amounts of organic residues, by-products, wastes and effluents (such as municipal sewage sludges and urban solid wastes, food industry and wood processing wastes, agricultural crop residues and animal wastes) as soil organic amendments has become a very popular and efficient agricultural practice (Senesi, 1989; Senesi et al., 2007). Increasing SOM content also enhances soil quality, reduces soil erosion (Guerrero et al., 2001), improves water quality, increases biomass and agronomic productivity and improves environmental quality by adsorbing pollutants from natural waters and reducing atmospheric CO_2 concentration (Lal and Kimble, 1999).

Applying organic wastes to soil could represent a useful tool in maintaining and increasing amounts of SOM (Mondini et al., 2007). Effective recycling of organic residues in soil requires the optimization of soil and organic waste management in order to minimize CO_2 emissions and optimize soil C sequestration efficiency. Most studies, however, have been conducted for evaluating the effects

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doi:10.1016/j.jenvman.2010.10.020

of organic amendments on total and available amounts of nutrient elements added to soil, of the danger of phytotoxicity to crops, of potential modifications of soil microbial populations and of activities and effects of toxic trace metals and organic chemicals on crops and waters (Mondini et al., 2007). In contrast, relatively little attention has been applied to study the effects that organic amendments may exert on stabilization of soil organic matter and chemical status, environmental role and fertility functions of the soil humic substances (Senesi et al., 2007).

From an environmental point of view, correct management of the exogenous OM addition to soil relies on two main aspects: efficient SOM increase and adequate match of the release of mineral nutrients to plant demand. Therefore, knowledge of C mineralization dynamics in amended soils is of intrinsic interest. Using laboratory methods involving incubation of soil-waste mixtures under controlled conditions can supply accurate information about C mineralization dynamics (Fernández et al., 2007) and efficiency of soil C sink (Mondini et al., 2007). Rates of organic matter decomposition depend upon type of organic amendment and soil properties, so exogenous material added to burned and to unburned soils could be expected to behave differently. Soil microorganisms play an important role in the decomposition of organic matter acting as agents in nutrient cycling and energy flow, and they are extremely sensitive to environmental changes (Vázquez et al., 1993).

This study was an incubation assay carried out under laboratory conditions and it was undertaken in the context of a project of reclamation of a burned forest area that includes a field experience of soil recovery using municipal waste compost (MWC, Olalla et al., 2008). The objectives of this research were to asses the effect of three doses of MWC added to burned and unburned calcareous soils on a) SOM mineralization and b) soil microbiological parameters.

2. Material and methods

2.1. Location

The study area, called *Monte de la Abadesa* (42°19′14″ N y 3°41′11″ W), is located at 897 m above sea level in a calcareous moor next to Burgos city in the region of *Castilla y León* (Northwest Spain). Mean annual rainfall is 564 mm and mean annual temperature is 10.5 °C. Soils are *leptic Cambisols* (*eutric*) over calcareous bedrock (IUSS Working Group WRB, 2006). The area was forested during the 60's with *Pinus sylvestris* Mill. and *Pinus pinaster* Aiton, and was burned in October 2004. Fire severity (Pausas et al., 2003) can be considered as moderate, consumption of litter layer could be observed, but no visible alteration of the mineral soil surface. The fire affected only a part of the forested area. At present, unburned and burned forests coexist in adjacent plots.

2.2. Soil sampling and incubation experiments

Soil sampling was performed in April 2006, eighteen months after fire. For laboratory incubation experience, 0–5 cm depth soil samples from the burned and unburned areas were used, obtaining a composite sample from each area by mixing five samples. Soil texture was sandy clay loam with a 26.4% of clay. The burned and unburned points of sampling were 100 m away each other. Visible plant residues and roots were removed and soil samples were sieved (<2 mm). For soil and MWC characterization, pH, electric conductivity, total N, total C, carbonate and organic C (SOC) and SOC/N were determined. Total concentrations of soil C and N were determined in an automated C/N analyzer (CHN-2000, Leco). Organic carbon was calculated as the difference between total and carbonate C. Soil total carbonates were determined using 1 M HCl titrated with 0.5 M NaOH (FAO, 2007). Some properties of the materials used for the mineralization assay are shown in Table 1.

Table 1
Some properties of the materials used in the incubation assay.

	pH	EC	Carbonates	SOC	N	SOC/N
		[mS cm^{-1}]	[gCaCO$_3$ kg^{-1}]	[g kg^{-1}]		
UBS	5.8	0.305	17	108	3.1	35
BS	7.4	0.380	54	53.8	2.6	21
MWC	8.0	15.4	192	187	9.3	20

Note: UBS: unburnt soil; BS: burnt soil, MWC: municipal waste compost, EC: electric conductivity SOC: soil organic carbon, N: total nitrogen.

Three compost (MWC) doses were added to each soil sample (1, 2, and 4% dry weight); control and compost samples were used as well. Soil samples (50 g of soil sieved by 2.0 mm) were thoroughly mixed with 0.50 g, 1.0 g or 2.0 g of MWC (sieved by 2 mm), and for MWC incubation an amount of 50 g was also used. Compost doses were chosen in accordance with a parallel field experience of reclamation (Olalla et al., 2008) and they were equivalent to 15, 30 and 60 Mg ha^{-1} (for 10 cm of soil depth), which can be considered usual organic amendment doses (Kaschl et al., 2002; Pedra et al., 2007; Barral et al., 2009). Five replicates were considered, obtaining 45 samples for the mineralization assay and microbiological analysis.

2.3. C mineralization

C mineralization was determined according to Isermayer (1952) in closed chambers and under laboratory-controlled conditions. Each sample was analyzed in five parallel incubations. Dry samples were wetted to 75% of water holding capacity and incubated in 1 L jars at 29 °C for 92 days. The moisture content was kept constant by weighing at each sampling date. The CO_2 evolved was collected, after 2, 6, 9, 12, 15, 19, 22, 26, 29, 34, 40, 44, 48, 51, 54, 61, 68, 75, 81 and 92 days of incubation, in 10 ml 0.5 M NaOH and determined by titration with 0.5 M against a phenolphthalein indicator after precipitation with $BaCl_2$ (0.5 M). The quantities and rates of labile and stable C mineralized during the course of the incubation were calculated by fitting the cumulative CO_2–C curves to the double exponential model (Andrén and Pauskian, 1987). This model separates the mineralizable organic C into active and slow pools and can be presented as

$$C_m = C_1\left(1 - e^{-k_1 t}\right) + C_2\left(1 - e^{-k_2 t}\right) \quad [1]$$

where C_m is the known cumulative amount of C respired at sampling period t; C_1 and C_2 are the sizes of the active and slow pools of mineralizable C, respectively; and k_1 and k_2 are the corresponding mineralization rate constants for each pool.

2.4. Microbiological analyses

Microbial biomass C (MBC) was determined by the chloroform fumigation extraction method, using 0.5 M K_2SO_4 as extractant (Vance et al., 1987) after 92 days of incubation. C contents in the fumigated and non-fumigated extracts were determined using an SKALAR FormacsHT Total Organic Carbon (TOC) analyzer for liquid samples.

The total respiration in 92 days ($C_{\min\ 92d}$) was determined. The metabolic quotient (q_{CO_2}) represents the respiration per mass unit of microbial biomass C, and was calculated as reported by Anderson and Domsch (1993). The microbial quotient (MBC/SOC) represented the fraction of MBC with respect to SOC (Anderson and Domsch, 1993).

2.5. Statistical analyses

The STATISTICA 7.0 software package was used to apply an ANOVA to test the effect of compost amendments after verifying normal distribution with the Kolmogorov–Smirnov test and variance homogeneity of residual with the Levene test. The factor considered was compost dose at four levels (control, dose 1, dose 2 and dose 3). The ANOVA was performed for both fire regimes separately (burned and unburned). When there were significant effects, means were compared using the Bonferroni test at level $p < 0.05$. A polynomial contrast (first and second degree) was carried out to ascertain the tendency with the increase of compost doses for each parameter studied.

3. Results and discussion

3.1. C mineralization

For soils, compost, and soils amended with compost, the accumulated amount of organic C mineralized was fitted to a double exponential model (Equation (1)) with high determination coefficients ($r^2 > 0.999$). This model appears to be consistent with some mechanistic models that divide soil organic matter into active, slow and resistant pools (or other synonyms) with different mineralization rates (Jenkinson, 1977; Kätterer et al., 1998). The resistant pool is not included in the equation, assuming that it does not contribute significantly to C mineralization in a relatively short period (Wang et al., 2004). In their study on utilization of MSW compost for organic matter conservation in agricultural soils in Northwestern Spain, Barral et al. (2009) found that a simple first-order kinetic model described compost mineralization adequately, whereas soil mineralization and soil amendment were best described by a two-compartment first-order model. Table 2 shows the model parameters obtained in the present study from the regression of the C–CO_2 emission data, the half-life times and the initial potential rates of C mineralization ($C_n \times k_n$) for each pool, and the percentage of C active pool (C_1) with respect to total mineralizable C.

The mineralization rates of the labile organic pool (k_1) showed values between 0.15 and 0.36 d^{-1} and this pool had half-life times of 1.9–4.9 d. The k_1 values were higher than those obtained by Bernal et al. (1998), but closer to those found by Pedra et al. (2007). The C_1 values for the control soils were higher than those obtained by Pedra et al. (2007) for a *Haplic Podzol* and a *Calcic Vertisol,* but the values were similar for the amended soils. Our results showed that the initial potential rate of C mineralization and the half-life times for the active or labile C pool, for burned and unburned soils, were similar suggesting that, in both soils, this pool presents organic compounds of similar decomposability.

The low values obtained for the percentage (% C_1) of the more active pool relative to total mineralizable C ($C_1 + C_2$) showed that a small part of SOC has a half-life time of only few days. Some authors have indicated that an initial mineralization flush is produced by the sample handling (drying, rewetting, etc.) in mineralization studies and that it corresponds to the active pool that appears in the equation (Molina et al., 1980). The % C_1 was higher in the burned soil than in the unburned, which could be related to the modification of the soil conditions that can favor microbiota development, such as high soil pH and increase in nutrient availability, as can be seen in Table 1 (Marion et al., 1981; Prieto-Fernández et al., 1993; Fernández et al., 1999). As can be seen in supplementary material (Table 3), in unburned soils, k_1 showed a significant linear tendency to decrease with the MWC dose, whereas half-life time of the labile C pool had a significantly quadratic tendency to increase with the MWC dose. In the burned soil not significant tendencies were observed for these parameters. For C_1 in both soils (burned and unburned) the tendency was to increase with the increment of MWC addition.

For the mineralization of the stable C pool, which mainly consisted of compounds more resistant to microbial attack that broke down slowly during a second mineralization phase, the mineralization rate values varied between 0.009 and 0.020 d^{-1} and the half-life times between 34 and 76 d (Table 2). The compost mineralization showed intermediate values between the two studied soils for these parameters (k_2 and C_2). Bernal et al. (1998) found that more than 88% of TOC in mature compost added to calcareous silt loam soil was slowly mineralizable with rate constants ranging from 0.0030 to 0.0010 d^{-1}, which is very similar to the mineralization rates obtained in the present study.

The mineralization rates of the stable pool (k_2) and concentrations of this pool (C_2) were higher in the unburned soil than in the burned one. During the second phase of mineralization, the burned soil SOM was more stable than that of the unburned soil, as is reflected by the lower $C_2 \times k_2$ product and the longer half-life time of C_2 in the burnt soil. These results indicate that the burnt soil SOM shows lower decomposability than that of the unburned (González-Pérez et al., 2004). Several researchers have suggested that the product $C_n \times k_n$ (initial potential mineralization rate) is more accurate to explain and understand SOM quality than either one separately (Fernández et al., 2007).

The half-life time of the stable mineralizable C pool showed no significant differences among doses in both soils (unburned and burned). The initial potential mineralization rate of the stable pool

Table 2
Mineralization rate constants (k_1 y k_2), labile and stable C pool concentrations (C_1, C_2), half-life time of the labile and stable pools ($t_{1/2}$ C_1 and $t_{1/2}$ C_2), initial potential rate ($C_1 \times k_1$, $C_2 \times k_2$), percentage of labile C with respect to total mineralizable C ($C_1 + C_2$) and microbiological parameters for compost, unburned and burned soils.

	C_1	C_2	% C_1	k_1	k_2	$t_{1/2}$ C_1	$t_{1/2}$ C_2	$C_1 \times k_1$	$C_2 \times k_2$	MBC	$C_{min\ 92d}$	MBC/SOC	q_{co_2}
	[g C kg^{-1} soil]			[day^{-1}]		[day]		[g kg^{-1} day^{-1}]		[g C kg^{-1} soil]		[gC_{mic} kg^{-1}SOC]	[gC_{min92d} g^{-1}MBC]
MWC	1.85	24.2	6.4	0.19	0.013	3.5	54.3	0.339	0.309	6.97	17.85	55.0	2.56
UBS	0.51b	12.4a	4.0b	0.36a	0.016b	1.9b	42.3a	0.198a	0.203a	1.51a	10.15a	14.8a	7.67a
UBSC1	0.47b	12.0a	4.0b	0.35a	0.018b	2.1b	38.2a	0.164b	0.218a	1.42a	10.15a	14.4a	7.37a
UBSC2	0.76b	10.0b	6.5b	0.30a	0.020a	2.6b	34.1a	0.185b	0.204a	1.49a	9.13b	15.1a	6.18 ab
UBSC3	1.47a	10.3b	12.4a	0.15b	0.016b	4.9a	42.4a	0.209a	0.169b	1.72a	9.43b	17.1a	5.30b
BS	0.82b	7.03a	11.0b	0.24a	0.010a	2.9a	75.8a	0.196a	0.062a	1.18a	4.65a	24.1a	3.99a
BSC1	1.05a	5.75b	15.3a	0.16b	0.009a	4.1a	75.5a	0.161a	0.054a	1.03 ab	4.66a	19.6 ab	4.81a
BSC2	1.08a	5.12c	15.5a	0.18b	0.011a	4.1a	63.2a	0.203a	0.058a	1.09 ab	4.40a	21.5 ab	4.11a
BSC3	1.08a	5.59bc	16.1a	0.18b	0.011a	3.8a	66.2a	0.196a	0.061a	0.80b	4.68a	16.2b	5.50a

Note: MBC: microbial biomass C; $C_{min\ 92d}$: basal respiration after 92 days of incubation; q_{CO2}: metabolic quotient. MWC: municipal waste compost; UBS: unburned soil; BS: burned soil. Values in one column followed by the same letter are not significantly different ($p < 0.05$) among doses within the same material (burned or unburned) with the Bonferroni test.

was significantly lower for the highest dose than for the others in the unburned soil, and as can be seen in supplementary material (Table 3) the tendency was to decrease with the MWC dose increase, probably due to an inhibitor effect (Borken et al., 2002); there were no significant differences in burned soils. The decrease observed in the amount of C mineralized in samples with added compost would be consistent with that established by Blagodstkaya and Kurzyakov (2008), who determined that substrate additions to the soil such that the rate of added C with respect to microbial biomass C exceed 200–500% cause a priming effect (short-term change in the turnover of soil organic matter) that tends towards zero and can be negative. Considering that MBC usually varies in 1–5% range of total organic C (Alef and Nannipieri, 1995), and it varied between 1.5 and 2.4% in our results (Table 2), the added compost doses exceed those rates. Mechanisms for this effect may be different and simultaneous (Blagodstkaya and Kurzyakov, 2008): changes in microbial community structure, preferential microbial substrate utilization, demand for other nutrients (such as N), etc.

The incubation assay of compost samples showed that the mineralizable C ($C_1 + C_2$) was only 14% of MWC C, so a large amount of added C was in recalcitrant forms. The low biodegradation rate of compost in the incubation experiment might thus suggest that these materials really contribute to long-term accumulation of OM in soil. Ribeiro et al. (2010) indicated that the addition of three different combinations of hen manure and stabilized compost to a *Cambic Arenosol* promoted an initial faster mineralization of the OM and consequently a faster release of nutrients, without affecting the total amount of C sequestered in soil.

3.2. Microbiological and biochemical parameters

Microbial biomass C reflects the size of the soil microbial community and basal respiration reflects the activity of this biomass (Nannipieri et al., 1990). Our results showed similar values of MBC for the soils studied and lower values of $C_{\text{min 92d}}$ in the burned control soil than the unburned one (Table 2). Compost addition showed significant decrease on MBC for burnt soil with the highest dose and significant decrease on $C_{\text{min 92d}}$ for unburnt soil with dose 2 and 3 (Table 2). Calbrix et al. (2007) did not observe significant differences on MBC after amending a cultivated soil with three types of organic materials (sewage sludge, turkey manure and compost made of turkey manure and ligneous waste); the absence of influence was justified by the use of organic amendment levels no higher than normal recommendations and by the assay with a single soil type and over a short length of time. Generally, compost additions cause a significant increase in soil respiration and soil microbial biomass carbon in relation to the control (Pedra et al., 2007; Tejada et al., 2009). These increases are attributed to the incorporation of easily degradable organic C, which stimulates the zymogenous microbial activity of the soil (Bernal et al., 1998; Stemmer et al., 2000) and to the incorporation of exogenous microorganisms (Blagodatsky et al., 2000). However, some authors (Borken et al., 2002) observed a reduction in microbial biomass following compost treatment of degraded temperate forest soils, suggesting that the initial salt content of the compost could have contributed to the decline in the original microbial biomass.

The MBC/SOC quotient has been used by many authors as an indicator of changes in SOM (Hernández et al., 1997). The metabolic quotient (q_{co_2}) is an index of microbial efficiency in the utilization of C resources and greater efficiency results in a low metabolic quotient (Anderson, 2003). This parameter has been successfully used to detect disturbance or stress of the soil microbial biomass due to external inputs of organic matter (Anderson and Domsch, 1993) or the presence of toxic substances such as heavy metals (Brookes and McGrath, 1984), which were usually reflected as an increase of q_{co_2}. Killham (1985) suggested that microbial biomass under stress diverts more energy from growth into maintenance, so an increased proportion of carbon taken up by the biomass is respired as CO_2.

The burned control soil showed lower q_{co_2} and larger MBC/SOC than the unburned control soil, suggesting that microbial communities in burned soils were more efficient in C use than communities in unburned soil. The effect of compost addition on the microbiological parameters studied was different in the burned and unburned soil as can be seen in supplementary material (Table 3), where significant polynomial contrast and different tendency with the MWC dose are showed. The MBC/SOC quotient showed a non-significant increase with the compost dose in the unburned soil, and a significant linear decrease in the burned soil supplementary material (Table 3). In the unburned soil, a significant increase in metabolic efficiency was observed with the increase of compost dose; however, no significant differences among doses were observed in the burned soil (Table 2), but the tendency to linear increase of the metabolic quotient with the MWC dose was significant in this soil supplementary material (Table 3). These different effects of compost amendment on microorganisms depended on the soil characteristics. These findings are in agreement with those reported by Bernal et al. (1998) who found, in an incubation experiment of soil amended with compost with different stabilization degrees, more efficient C conservation with mature compost.

4. Conclusions

Many of the parameters studied showed different tendencies with compost addition when the amendment was applied to burned or unburned soil. For the unburned soil, compost tended to decrease the initial potential mineralization rate in the stable SOM pool and to increase it in the labile SOM pool. However, it showed no significant effects on these pools in the burned soil. Adding compost tended to increase the size of more labile pool with respect to total mineralizable C ($C_1 + C_2$).

The compost amendment carried out did not increase soil microbiological activity, which even decreased in some cases. Most of the soil or compost OM did not result mineralizable in the short and medium term. For these soils and this compost, it could be considered that additions such as those tested may contribute to increasing long-term SOM levels. What is more, from this perspective, compost application constitutes an interesting option for sequestering C in soil. Behavioral differences of different composts and different soils require further investigation to obtain more advanced models that, integrating soil and compost features and conditions, facilitate making reasonable forecasts on OM evolution.

Acknowledgements

This study was funded by the Spanish Ministry of Education and Science; Reference CGL2006-13505-C03-03/BOS. We are indebted to Laura Heras for assisting with the laboratory analysis. We would also like to thank Cinnamon Nolan checked the English version and provided generous linguistic advice.

Appendix. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.jenvman.2010.10.020.

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Journal of Environmental Management 95 (2012) S250–S255

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Isolation and characterization of aerobic culturable arsenic-resistant bacteria from surfacewater and groundwater of Rautahat District, Nepal

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ARTICLE INFO

Article history:
Received 24 September 2009
Received in revised form
29 June 2011
Accepted 1 August 2011
Available online 24 August 2011

Keywords:
Arsenate
Arsenite
Arsenic-resistant bacteria
Bioaccumulation

ABSTRACT

Arsenic (As) contamination of groundwater is a serious Environmental Health Management issue of drinking water sources especially in Terai region of Nepal. Many studies have reported that due to natural abundance of arsenic in the environment, various bacteria have developed different resistance mechanisms for arsenic compound. In this study, the culturable arsenic-resistant bacteria indigenous to surfacewater as well as groundwater from Rautahat District of Nepal were randomly isolated by standard plate count method on the basis of viable growth on plate count agar amended with arsenate ranging from 0, 0.5, 10, 40, 80 to 160 milligram per liter (mg/l). With respect to the morphological and biochemical tests, nine morphologically distinct potent arsenate tolerant bacteria showed relatedness with *Micrococcus varians, Micrococcus roseus, Micrococcus luteus, Pseudomonas maltophilia, Pseudomonas* sp., *Vibrio parahaemolyticus, Bacillus cereus, Bacillus smithii* 1 and *Bacillus smithii* 2. The isolates were capable of tolerating more than 1000 mg/l of arsenate and 749 mg/l of arsenite. Likewise, bioaccumulation capability was highest with *M. roseus* (85.61%) and the least with *B. smithii* (47.88%) indicating the potential of the organisms in arsenic resistance and most probably in bioremediation.

1. Introduction

In Nepal, safe drinking water supply is a major problem. Groundwater is the main source of drinking water in Terai region of Nepal. Though household tube wells are the nearest water source for people far from river water, are contaminated with arsenic. With Nepal arsenic standard of 50 μg/l and 10 μg/l by World Health Organization (WHO) for drinking water, Rautahat district is categorized as a high risk districts to be looked after for the Arsenic problem where the arsenic concentration was substantially above the safe limit. The prevalence of arsenicosis was also high around 10% among middle aged people (Maharjan et al., 2006).

Arsenic can occur in the environment in several forms but in natural waters, and in drinking water, it is mostly found as trivalent arsenite (As III) or pentavalent arsenate (As V). Arsenate generally is the dominant form in oxic waters. In contrast, arsenite dominates in sulfidic and methanic waters including most geothermal water. Both forms are toxic; comparatively arsenite is the most toxic form. While arsenic is a well-known poison, a number of taxonomically diverse microorganisms have evolved biochemical mechanisms that either prevent arsenic from entering cells in the first place or rapidly extrude it back to the environment if it does enter (Welch et al., 2006). These detoxification reactions are mostly centered on redox changes between the As (III) and As (V) oxidation states, and can alter the speciation of arsenic found in the surrounding aqueous medium. Many bacteria have been isolated that exhibit resistance to lethal concentrations of arsenic (Jackson et al., 2005). Microorganisms and microbial products have been reported to efficiently remove soluble and particulate forms of metals, especially from dilute solutions through bioaccumulation and therefore microbe-based technologies provide an alternative to the conventional techniques of metal removal/recovery (Ozdemir et al., 2004).

This paper presents preliminary findings of isolation and characterization of arsenic-resistant bacteria from arsenic enriched as well as arsenic free or low background level aquifers of Rautahat, Nepal. The basic objective was to screen out potential microbes, characterize them and explore their properties for arsenic mitigation.

2. Materials and methods

2.1. Site description

Rautahat district situated in Narayani zone is one of the seventy-five districts of Nepal, a landlocked country of South Asia (Fig. 1). The district with Gaur as headquarter covers an area of 1126 km^2

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doi:10.1016/j.jenvman.2011.08.001

Fig. 1. Map showing the sampling sites location (marked with black stars).

and has a population of 545,132 with annual growth rate of 2.75% (CBS, 2002). According to National arsenic steering committee (NASC), July 2003 about 70% of the groundwater tube wells in Rautahat were above the WHO guideline value while 9% were above the Nepal arsenic standard (Panthi et al., 2006).

2.2. Sample collection

The water samples were collected randomly from different water sources located at Toribari Jungle, Toribari non-Jungle, Bagahi village, Mardhar village in Rautahat District of Nepal. Water samples (100 ml) were collected aseptically in sterile sampling bottles from each site and brought to laboratory at Kathmandu University stored in ice box. For arsenic elemental metal estimation and total organic carbon (TOC), separate water samples were collected preserved in concentrated hydrochloric acid. Similarly, for nitrate samples were collected preserved in concentrated sulphuric acid. Hydrogen ion concentration (pH) and temperature were checked on site with the help of portable pH meter (Jenway, UK).

2.3. Screening and isolation of arsenic-resistant microorganisms

The samples were serially diluted to 0, 10^{-1} and 10^{-2} dilutions. 1 ml from each dilution was taken for enumeration of arsenic tolerant bacteria by pour plate technique in plate count agar amended with different concentrations of sodium arsenate ($Na_2HAsO_4.7H_2O$) i.e. 0, 0.5, 10, 40, 80 and 160 mg/l respectively. The tests were carried out in triplicate for each dilution and arsenic concentrations and incubated for 24–48 h at 30 °C. Viable counts of bacteria capable of tolerating the concentrations of arsenic were determined as colony forming unit (CFU) per ml. Number of viable cells per milliliter reported represents the mean of triplicate samples. Standard deviation was analyzed using Microsoft excel 2007. Nine morphologically distinct potent arsenate tolerant bacteria were selected and characterized.

2.4. Chemical analysis

Water samples collected from each site were analyzed for total arsenic concentration using Hydride Generation atomic absorption spectrophotometer (HG-AAS, Thermo Co., UK). Total organic carbon was analyzed by total organic carbon analyzer (TOC $-V_E$, Shimadzu, Japan) and nitrate was estimated by Brucine absorptivity method using UV- Spectrophotometer (Genesys 10_{VV}, Thermo Spectronic, UK) (Clesceri et al., 1998).

2.5. Identification and characterization of arsenic-resistant bacteria

Arsenic tolerant bacteria obtained from different water sources of Rautahat were initially characterized in terms of colony morphologies (color, shape, size, elevation, margin, consistency, opacity) and basic microscopic observations (gram stain, spore stain, dimension). Isolates were then biochemically analyzed for the activities of oxidase test, catalase test, IMViC test, O/F test, Nitrate reduction, alkaline phosphatase test, Arginine utilization test, ornithine decarboxylase test, Deaminase test, Urease test, Starch hydrolysis, Gelatin liquefaction test, carbohydrate utilization tests like maltose, xylose, dextrose, galactose, trehalose, Melibiose, L-arabinose, D-arabinose, Mannose, Salicin, Glucosamine, Dulcitol, Inulin, Inositol, Sorbitol, Mannitol, Adonitol, L methyl D glucoside, sodium gluconate, arabinose, raffinose, Glycerol, Rhamnose, Cellobiose, Melezitose, a-Methyl-D-Mannoside, Xylitol, ONPG, Esculin, Citrate, Malonate and Sorbose etc. Then, these tests were used to identify the isolates referring to the Bergey's manual of Systematic Bacteriology (Garrity et al., 2005), Bergey's manual of Determinative Bacteriology (Hensyl and Forlifer, 2000) and probabilistic identification matrix (Willcox et al., 1973). The Willcox probability (P) matrix was used to assign and test the isolates where P scores of 0.8 and above indicated a positive identification.

2.6. Influence of physicochemical parameters

2.6.1. Effect of temperature, hydrogen ion concentration (pH) and salinity variation on bacterial growth

Each isolate was inoculated in 5 ml of the nutrient broth in water bath shaker at temperatures 25 °C, 30 °C, 35 °C, 45 °C, 55 °C and 60 °C for 48 h. For optimum pH determination, depending upon the optimum temperature they were incubated in 5 ml of the nutrient broth with different pH of 4.5, 5.5, 6.5, 7.5, 8.5 and 9.5. Likewise, the effect of salinity was studied in nutrient broth amended with

Table 1
Physical and chemical characteristics of the water samples.

Location	Depth in meter (m)	Arsenic Conc^{n} (μg/l)	pH	Temperature (°C)	Total organic carbon (TOC) (mg/l)	Nitrate (mg/l)
Toribari jungle	16.77	19	7.5	24.2	58.04	9.43
Toribari non-jungle	16.77	42	7.3	24.4	151.6	0.93
Bagahi well	8.53	25	7.3	24.1	69.77	0.93
Mardhar river	surface	6	8.4	22	61.78	0.44

different concentrations of sodium chloride as 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10%. The optical density of the growing cultures in the above-mentioned conditions were observed at 600 nm using an ultraviolet visible spectrophotometer (Electra, USA) and optimal growth was determined as a function of biomass by measuring the absorbance at 600 nm against blank media. Microsoft excel 2007 was used for statistical analysis.

2.7. Determination of maximum tolerance concentration (MTC)

Resistance to As (V) and As (III) was determined for each isolate by growing them separately in nutrient agar plate and 5 ml nutrient broth amended with increasing concentration of sodium arsenate from 0, 0.5, 10, 40, 80, 160, 200, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1873 to 3746 mg/l or sodium arsenite from 0, 74.92, 374.6, 749.2 to 1498.4 mg/l. The cultures were incubated at 30 °C for 7 days. The maximum tolerance concentration was determined by observing the presence or absence of visible growth detected by colony forming unit/turbidity in agar plate and broth respectively. The result reported represents the result of triplicate tests.

2.8. Sensitivity to antibiotics

Antibiotic sensitivity of the arsenic-resistant bacteria was determined by the standard disc-agar diffusion method. Antibiotic impregnated discs (6 mm diameter, Hi media) were placed on Muller-Hinton agar (Hi media) plates previously swabbed with respective arsenic tolerant bacterial suspension prepared by mixing CFU in sterile distilled water (2 ml). Plates were incubated at 37 °C for 24 h. The diameter of the inhibition zones around the discs was measured and the interpretation was made as per the zone size interpretation chart provided by the manufacturer of antibiotic discs (Himedia, India). The antibiotic concentrations of the discs used were ampicillin (10 μg), penicillin (6.25 μg), tetracycline (30 μg), chloramphenicol (30 μg), cotrimoxazole (10 μg), and gentamycin (10 μg).

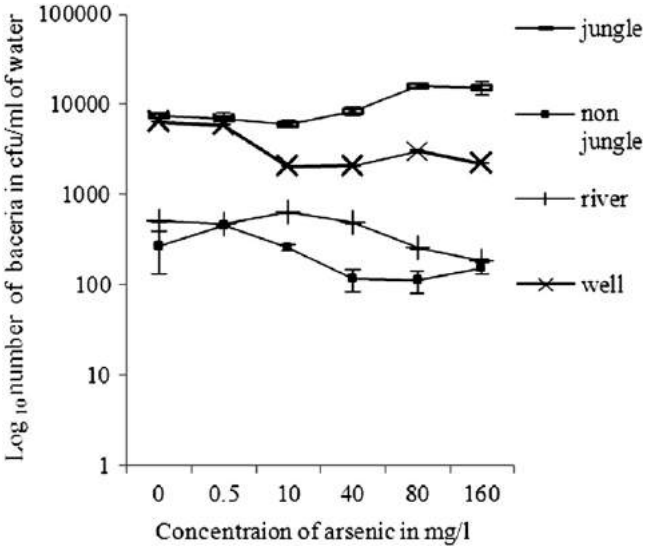

Fig. 2. Enumeration of arsenic-resistant bacteria from different water sources of Rautahat, Nepal. Data are expressed as mean ± standard deviation of triplicate.

2.9. Bioaccumulation assay

Bioaccumulation assay was carried out in 50 ml of nutrient broth supplemented with 200 mg/l of arsenic as sodium arsenate, incubated in water bath shaker at 30 °C for 24, 48, 72 and 96 h. At selected intervals of time, samples were harvested by centrifugation at 10,000 rpm for 10 min. The cell pellet was air dried for a day at room temperature, treated with 500 μl of conc. HNO_3 and bioaccumulation of arsenic in cell mass was estimated using HG-AAS.

3. Results and discussion

3.1. Isolation and identification of arsenic-resistant bacteria

The physical and chemical parameters measured in the study for each contamination monitoring water sources are shown in Table 1. Viable counts of the total heterotrophic and arsenate resistant bacteria in water sources showed that the water samples contained very high numbers of culturable arsenate resistant bacteria, despite the diverse arsenic level and depth (Fig. 2). Jackson et al. (2005) have also reported 20–50% of viable count of bacteria from water at three coastal sites in the Lake Pontchartrain estuary, Louisiana, USA showing appreciable arsenate resistance. The Toribari jungle tubewell water showed the highest viable count probably due to high organic carbon and nitrate content. The jungle tubewell was freshly drilled during the study and Bagahi well was shallow and in use by the villagers, which might be the main reason for more contamination by the microbes. The abundance of heterophs and arsenic-resistant bacteria in rice soil due to high organic carbon content has been reported by Bachate et al. (2009). The pH of the groundwater samples were found to be near neutral while the river water showed alkaline (Table 1).

In terms of colony morphologies (color, shape, size, elevation, margin, consistency, opacity), basic microscopic observations

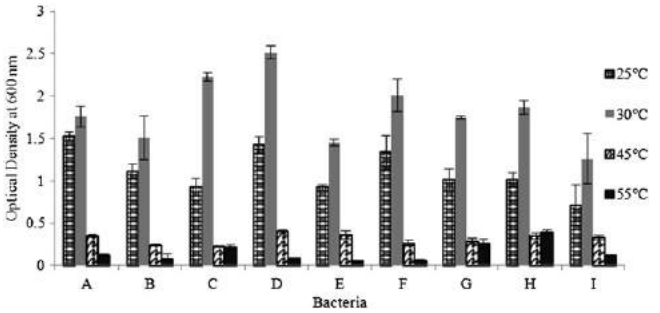

Fig. 3. Growth of arsenic-resistant bacteria in nutrient broth after 48 h of incubation at corresponding temperatures. Data are expressed as mean ± standard deviation. *Pseudomonas maltophilia* (A), *Micrococcus roseus* (B), *Micrococcus luteus*(C), *Micrococcus varians* (D), *Vibrio parahaemolyticus* (E), *Pseudomonas* sp. (F), *Bacillus smithii* 2 (G), *Bacillus cereus* (H), *Bacillus smithii* 1 (I).

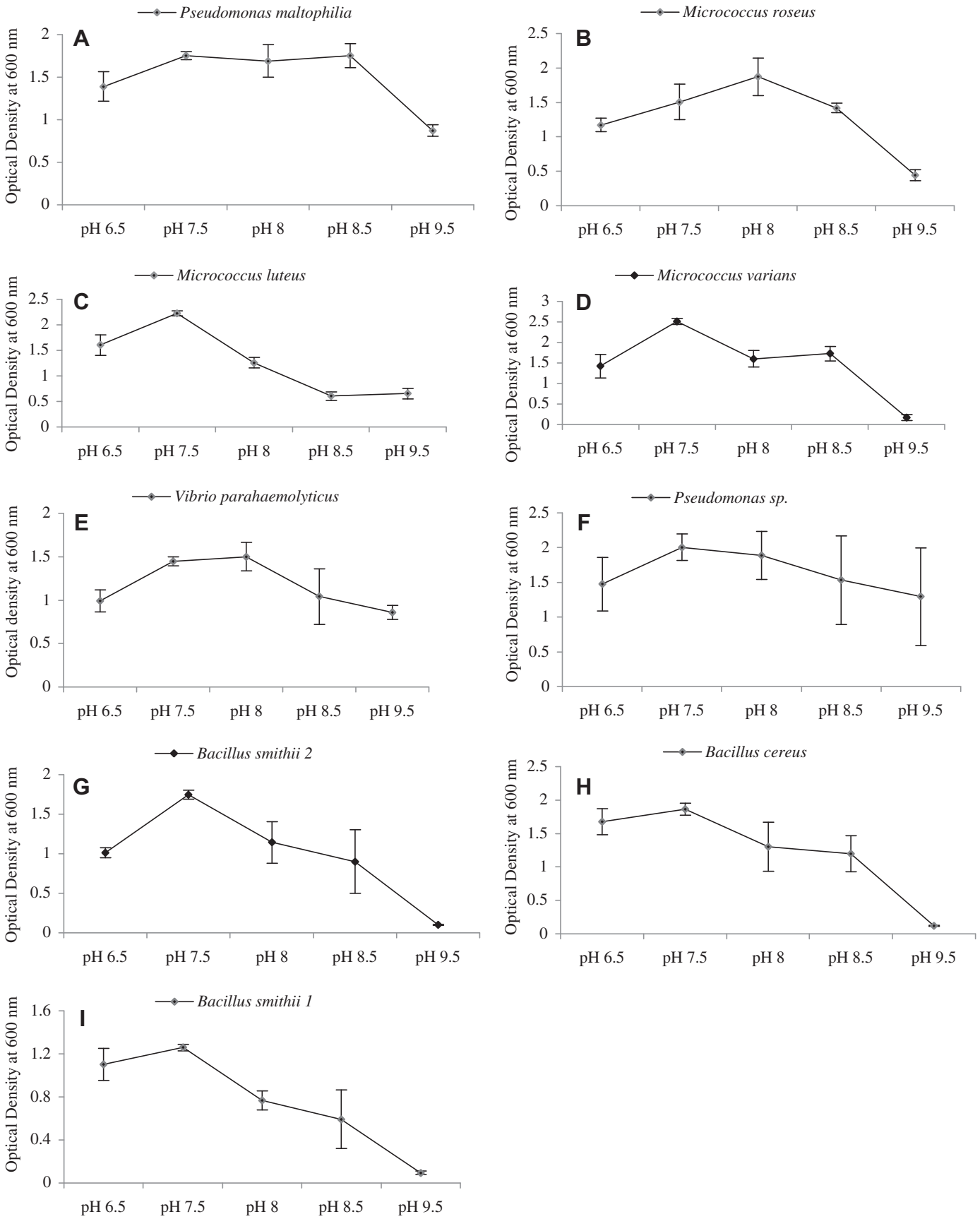

Fig. 4. Growth of arsenic-resistant bacteria in nutrient broth after 48 h of incubation at 30 °C and corresponding pH. Data are expressed as mean ± standard deviation.

Table 2
Maximum Tolerance Concentration to Sodium arsenate (As V) and arsenite (As III).

Organisms	Arsenic conc^n (As V) (mg/l)	Arsenic conc^n (As III) (mg/l)
Pseudomonas maltophilia	1000	749
Micrococcus roseus	3746	749
Micrococcus luteus	1873	749
Micrococcus varians	1873	749
Vibrio parahaemolyticus	1000	1498
Pseudomonas sp.	1000	1498
Bacillus smithii 2	1000	749
Bacillus cereus	1000	>749
Bacillus smithii 1	1000	>749

(gram stain, spore stain, cell size), biochemical tests with reference to the Bergey's manual of Systematic Bacteriology, Determinative Bacteriology and probabilistic identification matrix, the selected arsenic tolerant bacteria were found closely related to *Micrococcus varians*, *Micrococcus roseus*, *Pseudomonas maltophilia*, *Pseudomonas* sp., *Vibrio parahaemolyticus*, *Bacillus cereus*, *Bacillus smithii* 1 and *Bacillus smithii* 2. Other previous studies have reported the presence of the arsenic-resistant genera like *Acidithiobacillus* (Dopson et al., 2001), *Bacillus* (Switzer et al., 1998; Suresh et al., 2004a; Shivaji et al., 2005), *Deinococcus* (Suresh et al., 2004b), *Pseudomonas* (Vicente de et al., 1990; Raja et al., 2006), *Staphylococcus* (Guangyong and Silver, 1992), *Escherichia* (Kostal et al., 2004; Saltikov and Olson, 2002), *Alkaligenes* (Silver and Phung, 2005; Bachate et al., 2009), *Corynebacetrium* (Ordonez et al., 2005), *Aeromonas* (Pepi et al., 2007).

3.2. Effect of physicochemical parameters

Temperature is one of the most important factors influencing the activity of bacterial enzymes. The result showed that most of them are mesophilic, thermotolerant bacteria (Fig. 3). pH is one of the several factors that influence the release of the arsenic from sediments to groundwater. An increase in pH can lead to desorption of arsenic from iron oxides (Welch et al., 2006). The effect of hydrogen ion concentration (Fig. 4) showed that most of the bacteria growing in the pH range from 5.5 to 8.5 with optimum growth at pH 7.5.

According to Kostal et al. (2004), many whole cells sorbent for metal are sensitive to the Na^+ commonly found in contaminated water. To investigate the whole cell binding of arsenic, the effect of NaCl on binding as well as to the organisms is necessary. High arsenic concentration associated with this process has been well documented in central Oklahoma (Welch et al., 2006). In the study, the optimum Na^+ ion concentration for growth was found to be 0.5%.

Table 3
Antibiotic resistance profile.

Antibiotic	Concentration	Growth inhibition zone (mm)								
		A	B	C	D	E	F	G	H	I
Ampicillin	10.00 μg	CI	45	30	30	NZ	NZ	NZ	NZ	30
Penicillin	6.25 μg	CI	47	50	35	NZ	NZ	NZ	NZ	33
Chloramphenicol	30.00 μg	CI	40	43	35	17	26	23	23	27
Cotrimoxazole	23.75 μg	CI	45	45	40	15	19	19	44	46
Tetracycline	30.00 μg	CI	35	40	40	20	21	16	36	31
Gentamycin	10.00 μg	CI	33	25	35	34	23	30	34	30

Note: NZ – No zone, CI – complete inhibition, *Pseudomonas maltophilia* (A), *Micrococcus roseus* (B), *Micrococcus luteus* (C), *Micrococcus varians* (D), *Vibrio parahaemolyticus* (E), *Pseudomonas* sp. (F), *Bacillus smithii* 2 (G), *Bacillus cereus* (H), *Bacillus smithii* 1 (I).

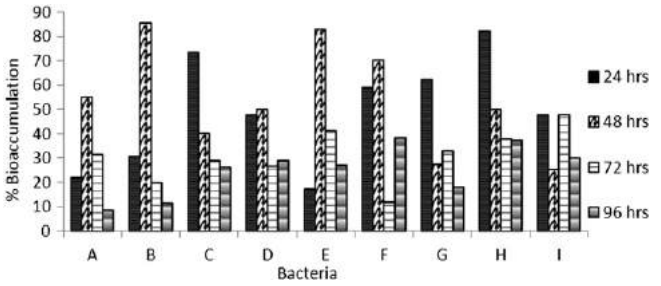

Fig. 5. Arsenic uptake by arsenic-resistant bacteria. *Pseudomonas maltophilia* (A), *Micrococcus roseus* (B), *Micrococcus luteus* (C), *Micrococcus varians* (D), *Vibrio parahaemolyticus* (E), *Pseudomonas* sp. (F), *Bacillus smithii* 2 (G), *Bacillus cereus* (H), *Bacillus smithii* 1 (I).

3.3. Resistance to arsenate and arsenite

Metal resistance has been attributed to number of factors. Major among them are the microbes potential to prevent the entry of metal inside the cell and the second is the ability of the cells to take up metal inside and its safe storage. Microorganisms may increase the mobility of arsenic by reducing As (V) to As (III) (Macy et al., 2000). However, microorganisms may lessen the toxicity of arsenic by immobilizing it via oxidation, methylation, or accumulation (Oremland et al., 2002). In the study, arsenic resistance level was found to be more than 1000 mg/l for arsenate and 749.2 mg/l for arsenite (Table 2). It is observed that the isolates are capable of growing on media with arsenite indicating the potential of the organisms in arsenic tolerance and most probably in bioremediation.

3.4. Sensitivity to antibiotics

Antibiotic resistance profiles for each isolates are summarized in Table 3. *V. parahaemolyticus* (E), *Pseudomonas* sp. (F), *B. smithii* 2 (G) and *B. cereus* (H) exhibited complete resistant to ampicillin as well as penicillin. While *P. maltophilia* (A) was found to be sensitive against all the antibiotics examined.

3.5. Bioaccumulation of arsenic

The accumulation of arsenic by bacteria showed that uptake of arsenic is quite high at 24–48 h of growth and the capability ranged from highest with *M. roseus* (85.61%) and the least with *B. smithii* (47.88%) (Fig. 5). These findings clearly indicated that most of the bacteria isolated have got the potential to accumulate much arsenate.

4. Conclusion

Efficient uptake of metalloid, ability to grow over wide range of arsenic concentration as As (III) and As (V) under aerobic conditions along with antibiotic resistance indicates that these bacteria may offer advantage for bioremediation of contaminated water. The presence of As (III)-resistant bacteria in the water sources implies a possible mechanism for counteracting the mobilization of arsenic if these As (III) tolerant organisms can also oxidize As (III) to As (V). Future studies will include the determination of As (III) oxidation or As (V) reduction and detection of genes associated with arsenic resistance.

Acknowledgment

The authors acknowledge the International Foundation for Science (IFS), Stockholm, Sweden for financial support.

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Journal of Environmental Management 95 (2012) S256–S259

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Screening for thermotolerant ligninolytic fungi with laccase, lipase, and protease activity isolated in Mexico

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ARTICLE INFO

Article history:
Received 30 September 2009
Received in revised form
24 September 2010
Accepted 19 October 2010
Available online 13 November 2010

Keywords:
Fungi
Ligninolytic
Laccase
Protease
Lipase
Strain selection

ABSTRACT

The State of Hidalgo (Mexico) has a large area of forests known as the Huasteca Hidalguense, with a large variety of microorganisms inhabiting it. They represent an important resource from the ecological and technological point of view because they can be used in a broad variety of industrial processes. Due to the climatic conditions of this region, fungi inhabiting it must be thermophile or, at least, thermotolerant, as temperatures can be higher than 45 °C in the summer, declining to 20 °C in the winter. Use of ligninolytic fungi relies on their capacity to produce enzymes of industrial interest, a topic that has been under continuous research by academic and industrial investigators. Among the most important enzymes are proteases that are widely used due to their biotechnological applications with a high economic impact. Other enzymes, laccases, peroxidases, and lipases are of interest for the industries of the state of Hidalgo, especially in the textile industry, specifically in effluent processing. Fungi ($n = 156$) were collected in the Huasteca Hidalguense, of which 100 were isolated in potato-dextrose-agar covered plates and maintained in tilted tubes. Afterwards, enzymatic activity (laccase, protease and lipase) was determined in the plates. The purpose was to select those fungi with the highest potential for biotechnological applications. Fungi generally grew at either 30 °C or 37 °C, and for some isolates enzymatic activities were detected at this higher temperature. Results are presented as the relation between enzymatic activity and growth rate: 60 fungi presented laccase activity, 49 had lipase activity, and none had protease activity. In most cases, enzymatic activity was higher than the growth rate, indicating that the isolated fungi have a great biotechnological potential. Statistical analysis revealed that isolates 31 (*Trametes*) and 8.1 (unidentified) have a larger potential to be studied as laccase-producing fungi. On the other hand, isolates 144.2 (*Fomes*), 154 (*Trametes*), and 147.2 (*Pycnoporus*) are of interest as lipase activity producers, an activity scarcely studied in this type of microorganisms.

1. Introduction

Wood is the most abundant biopolymer in nature and is constituted mainly by cellulose, hemicellulose, and lignin. Lignin confers resistance to plants, and due to its chemical complexity there are very few organisms capable of degrading it. Only ligninolytic organisms and, especially, white rot fungi (Higuchi, 1990) are able to mineralize completely the vegetal residues. Lignin degradation involves extracellular enzymes with multiple industrial applications, such as laccases and peroxidases (Cammarota and Freire, 2006; Duráan and Espósito, 2000; Hasan et al., 2006; Morozova et al., 2007; Rao et al., 1998). Another important enzymes for the industry are lipase and protease that are not involved in the lignin degradation but they are produced for fungi and its industrial application is extensive (Leonowicz et al., 1999).

Mexico is known for its large biological diversity, and the State of Hidalgo occupies the third place with the largest surface covered by forests, particularly the Huasteca Hidalguense. This region has a warm-humid climate because it lies at 127 m above sea level, its mean yearly temperature is 31.1 °C (reaching 40–50 °C in the summer), rainfall amounts to 1500 mm/year, and is almost constant throughout the year. These features are ideal for the growth of thermotolerant fungi, hence there must be a large amount of non-identified microorganisms in that region, among them fungi, that produce and secrete enzymes and can be used in a large variety of industrial processes. Currently, there is an ever increasing interest in the isolation and study of thermophile organisms, capable of producing thermostable enzymes resistant to high temperatures (Hilden et al., 2009).

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0301-4797/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jenvman.2010.10.045

It is estimated that there are approximately 1.5 million fungi species in the world, of which around 4.6% are known (Hawksworth, 2001). Just in Mexico, there might be 200,000 species, of which only 3.5% is known (Guzman, 1998), indicating the lack of knowledge about this group of microorganisms and, hence, the need to perform studies on these organisms (Hawksworth and Kalin-Arroyo, 1995).

Based on the afore mentioned, the objective of the present study was to isolate, identify, and detect the enzymes (laccase, lipase, and protease) secreted by these ligninolytic fungi collected in the Huasteca Hidalguense, Mexico, and, thereby, be able to choose those fungi with the greatest biotechnological potential.

2. Experimental methods

2.1. Isolation of the microorganisms

Ligninolytic fungi were collected in Huejutla (the most important municipality in the Huasteca Hidalguense). Isolation was made on potato-dextrose-agar (PDA, 39 g/L) plates supplemented with ampicillin (100 μg/mL) and chloramphenicol (200 μg/mL) to avoid growth of bacteria and Benomyl (3 μg/mL) to inhibit growth of yeasts and ascomycetes fungi. The plates were incubated at 37 °C, performing successive isolations until reaching pure cultures.

Identification was performed with molecular methods, using ITS (Internal Transcribed Spacer) sequences as molecular marker. For it, fungi were grown at 37 °C in liquid culture with malt extract (20 g/L) and peptone (10 g/L) to obtain mycelia, which were then used for DNA extraction by the method described by Raeder and Broda (1985). ITS region was amplified using universal primers, ITS4 and ITS5 (White et al., 1990), ITS sequenced was compared with the sequences reported in GeneBank.

2.2. Enzymatic activity

Enzymatic activity was analyzed in plates and reported as potential index, defined as the relation of the activity and growth rate. To determine the activity of each isolated strain, inoculates were made in triplicate, taking a fraction (approx. 0.5 cm^2) of the mycelium under aseptic conditions and placing it in the center of the plate, containing the corresponding medium for the activity to be analyzed; then, they were incubated at 37 °C ± 1 °C, growth was measured in each plate following the Valera and Morales (1996) method.

2.2.1. Laccase activity

To demonstrate laccase activity, the PDA medium (39 g/L) supplemented with ABTS (0.5 mM 2,2′-azino-di-3-ethylbenzotiazol-6-sulfonate acid) was used. Enzymatic activity was reported by measuring the greenish-colored halo produced by the oxidation of ABTS (Chairattanamanokorn et al., 2006).

2.2.2. Lipase activity

To demonstrate lipolytic activity, the PDA medium (39 g/L), supplemented with reagents, NaCl (5 g/L), $CaCl_2$, (0.1 g/L), Tween 20 (10 mL/L), was used. Activity was reported by measuring the halo of calcium laureate formation when the lipase enzyme reacted with Tween (Nikoleit et al., 1995; Córdova et al., 2003)

2.2.3. Protease activity

We used bacteriological grade agar supplemented with 0.3% powdered milk. Activity was determined by the halo formed by the degraded milk proteins (Mayerhofer et al., 1973)

2.3. Statistical analysis

To establish significant differences between growth rates and the respective enzymatic activity, the SPSS 15.0 software (2006) was used for the variance analysis (ANOVA) to compare the means by the Duncan method with a 95% confidence interval.

3. Results and discussion

3.1. Isolation and identification

The Huasteca Hidalguense depicts ideal environmental conditions for the growth of fungi, we collected a total of 156 carpophores from live trees and dead wood, it is known that ligninolytic fungi can be parasites, but they are preferentially saprobes. All collected fungi were inoculated on plates with PDA supplemented with an antibiotic, after their incubation at 37 °C, 100 of them grew; and the capacity to produce laccase, lipase, and protease activity was measured in every isolate.

Identification by molecular methods (Table 1) determined the genus of most of fungal isolates with a 95% similitude, which is the minimum required to establish the genus of microorganisms (Rossello Mora and Amman, 2001).

The obtained fungi belong to the basidiomycetes genera: *Coprinellus*, *Polyporus*, *Trametes*, *Fomes*, *Coriolopsis*, *Phanerochaete*, *Pycnoporus*, *Schizophyllum*, all causing spoilage of wood and described in other studies (Chairattanamanokorn et al., 2006; Solarska et al., 2009).

Ascomycete fungi of different genera were also obtained: *Chaetomium*, *Cochliobolus*, *Rhizomucor*, and *Botryosphaeria*, they were probably among the collected carpophores.

3.2. Enzymatic activity

Table 1 shows the growth and activity results. In all cases, linear regression had a minimal R^2 of 0.98 and a less than 5% standard deviation. The growth rate and the enzymatic activity, expressed as potential index, are shown (relation between activity and growth). An important feature of this study is that all analyses were performed at 37 °C, although the optimal temperature for ligninolytic fungi is 30 °C. In addition, we confirmed that most of them are able to grow at 42 °C (data not shown), granting them their thermotolerant character, an interesting feature for their possible biotechnological application (Hilden et al., 2009). The environmental conditions of the Huasteca Hidalguense foster the growth of this type of microorganisms, since its temperature can reach up to 45 °C in the summer.

3.2.1. Laccase activity

Ligninolytic fungi are characterized for possessing laccase activity as part of their constituent enzymatic pool to mineralize lignin (Morozova et al., 2007). Of the 100 fungi isolated in this study, 60 had laccase activity (Table 1). All genera of known basidiomycetes fungi have been reported previously for their capacity to secrete laccase, the most important of them is the genus *Trametes* and this was the genus isolated most frequently (Arana-Cuenca et al., 2004; Hilden et al., 2009; Mikolasch and Schauer, 2009; Morozova et al., 2007). We isolated three microorganisms of the *Coprinellus* genus, which has been described as laccase producer (Dritsa et al., 2007), but this was not found in the present study, probably because the tests were made in a culture medium without any laccase activity inducer. The same occurred with the *Rhizomucor* genus, which has been described as a good microorganism for the discoloration of effluents from paper bleaching processes due to its laccase activity (Driessel and Christov, 2001). The rest of the isolated ascomycetes

Table 1
Isolated fungi with lipase and laccase activity.

Num.	Genus	Lipase		Laccase		Num.	Genus	Lipase		Laccase		Num.	Genus	Lipase		Laccase	
		G.r.	P.I.	G.r.	P.I.			G.r.	P.I.	G.r.	P.I.			G.r.	P.I.	G.r.	P.I.
3.1	*Hypocreaceae*	0.432	1.094	1.707	0.540	47	*Trametes*	1.241	1.007	1.237	1.105	104	*Polyporales*	wo/a		1.515	0.527
8	*NI*	0.556	1.000	0.481	0.434	49	*Trametes*	1.147	1.031	1.116	1.128	105.1	*NI*	wo/a		0.616	0.391
8.1	*NI*	wo/a		0.586	1.352	49.1	*NI*	wo/a		0.709	1.014	105.2	*Cochliobolus*	wo/a		0.687	0.847
9	*Coprinellus*	0.826	1.000	wo/a		49.2	*Trametes*	1.014	1.195	0.886	1.166	110	*NI*	0.52	1.000	0.837	0.882
10	*NI*	0.477	1.014	0.474	0.877	50	*Phanerochaete*	1.353	1.000	1.983	0.437	119	*Trametes*	0.315	1.402	0.852	1.16
12.1	*Polyporus*	0.498	1.004	1.154	0.990	51	*Trametes*	0.747	1.009	1.059	1.022	123	*Trametes*	1.006	1.012	1.227	1.068
12.2	*Hypocreaceae*	1.031	1.002	wo/a		52.1	*Cochliobolus*	wo/a		0.048	1.186	129	*Trametes*	0.398	1.402	0.86	0.708
22	*NI*	1.027	1.023	1.001	1.112	52.2	*Trametes*	1.165	1.068	1.185	1.043	134	*Trametes*	0.891	1.174	1.043	1.016
24	*NI*	0.704	1.002	0.722	1.001	60	*NI*	0.686	1.000	0.835	1.220	139.1	*Pycnoporus*	wo/a		0.99	0.403
27	*Coprinellus*	0.450	1.000	wo/a		64	*Polyporus*	0.863	1.000	1.082	0.433	139.3	*NI*	wo/a		0.453	0.912
29.1	*Coprinellus*	0.666	1.071	wo/a		65	*Chaetomium*	0.564	1.000	0.526	0.535	139.4	*Polyporus*	wo/a		0.892	0.504
29.2	*Polyporus*	0.618	1.071	0.404	1.209	66	*NI*	0.647	1.000	0.773	0.94	140	*Trametes*	wo/a		0.922	1.077
30	*Polyporus*	0.834	1.000	1.044	1.002	75	*Rhizomucor*	0.222	1.000	wo/a		141.1	*NI*	wo/a		0.29	0.837
31	*Trametes*	0.735	1.050	0.611	1.419	77	*Botryosphaeria*	wo/a		2.042	0.39	144.2	*Fomes*	0.653	1.307	1.065	1.018
36	*Fomes*	0.571	1.000	0.641	0.892	79	*Polyporus*	0.858	1.000	1.18	0.813	147.1	*Pycnoporus*	0.585	1.088	0.95	0.59
40	*Trametes*	0.964	1.085	1.099	1.231	84	*NI*	wo/a		0.322	2.348	147.2	*NI*	0.84	1.276	1.115	1.121
43	*Coriolopsis*	0.680	1.000	0.653	0.863	85.1	*Fomes*	0.052	1.000	0.782	0.815	150.1	*Trametes*	wo/a		0.757	1.123
44	*Trametes*	1.015	1.199	1.139	1.253	89	*Schizophyllum*	1.021	1.000	0.439	0.556	150.2	*Cochliobolus*	0.753	1.120	0.951	1.055
45	*Trametes*	1.261	0.910	1.142	1.065	90	*Pycnoporus*	0.829	1.060	0.87	1.006	151	*NI*	0.558	1.000	1.303	0.245
46	*Trametes*	0.980	1.000	1.142	1.128	100	*Pycnoporus*	wo/a		1.041	1.003	152	*Trametes*	0.644	1.177	0.964	0.464
46.1	*Coprinellus*	0.562	1.000	0.502	0.394	102	*NI*	0.642	1.220	0.823	1.255	153	*Trametes*	wo/a		0.593	0.598
46.2	*Trametes*	1.060	1.008	1.152	1.020	103	*Trametes*	0.617	1.300	1.178	1.004	154	*Trametes*	0.634	1.283	1.308	0.306

G.r. (Growth rates in mm/h); P.I. (Potential index); NI (unidentified); wo/a (without activity).

fungi depicted laccase activity, as described previously: *Botryosphaeria* (Castilho et al., 2009); *Chaetomium* (Chefetz et al., 1998) and *Cochliobolus* (Burke and Cairney, 2002) with a potential index of 0.390, 0.535 and 1.055, respectively.

3.2.2. Lipase activity

Lipase activity has been studied in this type of microorganisms; the results of this study reveal a relevant capacity of the studied fungi to secrete this type of enzymes, making their further study very interesting. Of the analyzed strains, 49 depicted lipase activity, being present in most of the isolated genera (Table 1). There are reports showing that *Phanerochaete chrysosporium* can present three lipases, according to a computational analysis performed on proteins secreted in complex cultures (Vanden Wymelenberg et al., 2006). In *Rhizomucor* the capacity to synthetize this type of enzymes is well characterized (Broadmeadow et al., 1994), which was also found in this study.

3.2.3. Protease activity

The presence of extracellular protease activity in basidiomycete fungi, such as *Trametes troggi* (Caporale et al., 1996) and *Trametes versicolor* (Staszczak and Nowak, 1984), has been well known for decades and is a current topic of interest as revealed by recent reports on ligninolytic and protease enzymes in *Phanerochaete chrysosporim* (Xiong et al., 2008). Notwithstanding, in the present study we did not observe protease activity in the tested isolates, probably because we did not use the optimal medium for the production of this enzyme, or because these fungi are unable to degrade milk proteins and, therefore, their activity could not be observed.

3.3. Selection of strains

To enable us to select those strains with the greatest biotechnological potential, we performed an ANOVA test. We obtained a total of 13 homogeneous groups for laccase activity and 16 for lipase activity, showing a greater variability in the obtained data (Table 2). Growth rate analysis revealed that there are isolates, as in the case of several *Trametes* (isolates 123 and 47) with a growth rate higher than 1.2 mm/h, so that they invade completely the plate in less than 48 h, although this type of microorganisms usually needs several days to grow.

The isolate with the greatest potential in the laccase study could not be identified with the techniques used in this study and corresponds to number 84 with a potential index of 2.35, but with

Table 2
Selection of fungi with enzymatic activity from statistic program SPSS with 95% confidence.

Determination	Laccase activity				Lipase activity			
	Value	Homogenous	Analyzed	Half	Value	Homogenous	Analyzed	Half value
Growth speed (mm/h)	Higher	13	123, 47	1.235	Higher	16	50	1.350
		12	103, 52.2	1.180		15	47	1.240
		11	7 fungi	1.128		14	52.2, 3.2, 49	1.156
	Lower	3	8, 52.1	0.485	Lower	3	129	0.400
		2	29.2	0.400		2	119	0.320
		1	84	0.320		1	75	0.220
Potential index	Higher	7	84	2.350	Higher	8	119, 129	1.400
		6	8, 31	1.425		7	144.2	1.315
		5	8.1	1.380		6	154, 147.2	1.280
	Lower	3	119, 49.2, 52.1	1.170	Lower	3	147.1	1.105
		2	6 fungi	1.120		2	6 fungi	1.070
		1	15 fungi	1.021		1	31 fungi	1.000

a low growth rate of 0.3 mm/h, therefore we chose isolates 31 (*Trametes*) and 8.1 (not identified), because they possess a potential index of 1.38 but a growth rate of approximately 0.6 mm/h.

For lipase activity, results were similar, fungi with the highest potential index were isolates 119 and 129, both belonging to the *Trametes* genus with a 1.4 value, but a growth rate of less than 0.4 mm/h. Therefore, we consider that the fungi with the greatest potential are isolates 144.2 (*Fomes*), 154 (*Trametes*), and 147.2 (*Pycnoporus*) with a potential index above 1.2 and a growth rate of approximately 0.6 mm/h.

4. Conclusion

We collected a total of 156 carpophores, obtaining growth in 100 of them on plates at a temperature of 37 °C. The genus of most isolates was determined, corresponding to basidiomycetes fungi, except in four cases: *Chaetomium*, *Rhizomucor*, *Botryosphaeria*, and *Cochliobolus*, the latter with two isolates. Enzymatic activity studies revealed that 60 fungi had laccase activity, 49 had lipase activity, and none had protease activity in the assayed conditions. Statistical analyses revealed that isolates 31 (*Trametes*) and 8.1 (not identified) have a high potential for their further study as laccase-producing fungi. On the other hand, isolates 144.2 (*Fomes*), 154 (*Trametes*), and 147.2 (*Pycnoporus*) are of interest as producers of lipase activity, which has been scarcely studied in this type of microorganisms. In all of case, the enzymatic activity measured in this work is considered as basal level.

It is important to point out the interesting features of the obtained isolates as they grow rapidly at high temperatures, which are important characteristics for possible biotechnological applications.

Acknowledgments

To CONACYT and the State of Hidalgo (Mexico) government for the financial support to the project “Estudio poblacional de hongos ligninolíticos de la Huasteca Hidalguense con aplicaciones biotecnológicas” [Population study of ligninoolytic fungi of the Huasteca Hidalguense with biotechnological applications] FOMIX-HGO-2006-C01-45552.

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Journal of Environmental Management 95 (2012) S260–S264

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Effect of distance and depth on microbial biomass and mineral nitrogen content under *Acacia senegal* (L.) Willd. trees

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ARTICLE INFO

Article history:
Received 29 September 2009
Received in revised form
1 March 2011
Accepted 29 March 2011
Available online 22 April 2011

Keywords:
Acacia senegal
Rhizosphere
Microbial biomass
Inorganic-N
Senegal

ABSTRACT

The relations between plants and soil biota involve positive and negative feedbacks between soil organisms, their chemical environment, and plants. Then, characterization of microbial community functioning is important to understand these relations. An experiment was conducted in a field system in the north of Senegal for two years (2005 and 2006) in order to investigate the effect of depth and distance from *Acacia senegal* tree stem on soil microbial biomass and inorganic-N content. Soils were sampled during dry season (April, T_0) and wet season (August, T_1) along transects (R_0, foot tree; $R_{/2}$, approximately 0.50 m distance from the stem; and R, approximately 1 m distance from the stem) and at different layers: 0–25 cm, 25–50 cm and 50–75 cm of *A. senegal* trees rhizosphere. Total microbial biomass and inorganic-N content were negatively correlated to the distance from tree stem and the depth. The highest values of microbial biomass and mineral nitrogen were found at the foot tree (R_0) and at 0–25 cm layer. Inorganic-N was mostly in nitrate form (NO_3^-) during the dry season. In contrast, during the wet season, inorganic-N was dominated by ammoniac form (NH_4^+). Soil total microbial biomass and inorganic-N ($NH_4^+ + NO_3^-$) were negatively correlated. Our results suggest a positive influence of *A. senegal* rhizosphere on soil microbial biomass and inorganic-N content.

1. Introduction

Acacia senegal (L.) Willd. is widely distributed through arid and semiarid areas of Africa and the Middle East. *A. senegal* produces the internationally-traded commodity 'gum-arabic'. This leguminous tree improves soil fertility of degraded areas through its ability to fix atmospheric nitrogen in symbiosis with rhizobia. Several authors showed a high genetic diversity of rhizobia associated to *A. senegal* (Nick et al., 1999; Sarr et al., 2005; Fall et al., 2008).

Relations between plants and soil biota involve positive and negative feedbacks. The rhizosphere, defined as the volume of soil adjacent to and influenced by the plant roots (Sørensen, 1997), is of great importance to plant health and soil fertility. Roots are known to excrete several forms of organic compounds. Microbial population in the vicinity of the roots is influenced by root exudates (Brant et al., 2006) and could be different in composition and density (Bowen and Rovira, 1991).

Soil microbial biomass is an essential component of most terrestrial ecosystems because it regulates nutrient cycling, and acts as a highly labile source of plant-available nutrients (Singh et al., 1989). Soil microbial biomass is most sensitive to changes in organic matter status than the total amount of organic C (Sparling, 1992). The microbial biomass has been used as an index of soil fertility (Staddon et al., 1999), which depends primarily on rates of nutrient fluxes. Environmental factors such as geographical location, vegetation, land use, land cover, soil type can influence soil microbial biomass (Black et al., 2003). An increase in the size of soil microbial biomass is essential for the improvement of soil fertility. Plant roots have been shown to affect the microbial growth by reducing soil available N or soil moisture, or by providing C substrates for microbial growth (Jackson et al., 1989).

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doi:10.1016/j.jenvman.2011.03.038

Nitrogen (N) mineralization is of a crucial importance in natural forest ecosystems where N has been reported to be a limiting nutrient for plant growth (Clein and Schimel, 1995). The initial product of organic-N heterotrophic mineralization is ammonium, which is further oxidized by autotrophic microbes to form nitrate through nitrification. N mineralization is influence by soil microclimate (Wang et al., 2006) and the amount and quality of organic matter (Sall et al., 2003). Water availability controlled soil microbial activity and thus the rates of N net mineralization (Nicolardot et al., 2001). However, Zaman and Chang (2004) reported that soil temperature and soil moisture were most important than substrate quality in controlling mineral N in agroforestry systems.

Soil microbial biomass and mineral nitrogen content are the most important indicators of soil fertility (Staddon et al., 1999; Adrover et al., 2012). Thus the main objective of this work was to evaluate the effect of legumes rhizosphere in particular *A. senegal* on these soil components. We assessed the spatial and seasonal variations of soil total microbial biomass and inorganic-N content in natural conditions under mature *A. senegal* trees during dry and wet seasons of two years 2005 and 2006 in the north part of Senegal.

2. Material and methods

2.1. Soils and samplings

Soil samples were collected at Kamb (an arid savannah with 400–500 mm of annual rainfall), 300 km north of Dakar (Senegal). In this part of Sahel, temperatures values rank between 25 °C and 39 °C with an annual average temperature of approximately 29 °C. Soils were sampled at two periods: T_0 (dry season, April), T_1 (wet season, August) during two years 2005 and 2006 in a plantation of *A. senegal*, 13 years old. The plantation was separated into two blocks with 48 plants each block. For each sampling period, soils were collected from three trees of each block. For each tree, soil samples were taken along transects from the stem up to 1 m distance (R_0, foot tree; $R_{/2}$, 0.50 m distance from the foot tree and R, 1 m distance from the foot tree). Soil samplings were replicated in four directions around the tree stem (East, West, North and South), at depths of 0–25 cm, 25–50 cm, and 50–75 cm. For each tree, the four soils samples collected at the same distance to the tree stem and at the same depth were pooled to obtain a homogenous soil sample around the tree. Then, for each tree, nine soils samples were collected. Thus for each block, we have 27 soils samples. During the second year (2006), soils samples were taken only at 0–25 cm depth because in the first season (2005), we found that most of the mineral nitrogen content and microbial biomass were found in layer 0–25 cm. The soil characteristics for each soil depth and distance from tree stem are presented in Table 1. For each sampling period, soil moisture was determined.

2.2. Determination of gravimetric soil moisture

For each sampling period, 10 g from soil samples were dried in an air-forced oven at 105 °C during 72 h for determining soil moisture content.

2.3. Soil microbial biomass

Microbial biomass N was determined by the fumigation-extraction method (Amato and Ladd, 1988) by measuring ninhydrin reactive N compounds extracted from soils with 1 M KCl after a 10-days fumigation period. Fumigated and unfumigated soil samples were suspended in KCl solution, shaken at 25 °C for 1 h and then filtered (Whatman 0.45 μm) and stored frozen for further analysis. Ninhydrin reactive N content was determined colorimetrically by flow injection analysis (Evolution II, Alliance-Instruments, France). Microbial biomass C was estimated from the gain in ninhydrin reactive N after fumigation, multiplied by 21 (Amato and Ladd, 1988). Microbial biomass was expressed in μg C g^{-1} dry soil.

2.4. Soil inorganic-N content

Soil inorganic-N content was determined colorimetrically in the KCl 1 M extract by flow injection analysis according to the method of Bremner (1965). The results were expressed as μg N (NH_4^+ or NO_3^-) g^{-1} dry soil.

3. Results

3.1. Soils chemical characteristics

Results showed that soils were acids with pH ranging between 5.3 and 6.6. A weak variation of pH in relation to distance from tree stem and the depth was noted (Table 1). Regarding the nutrient elements, we observed that mineral nitrogen, organic carbon and soluble phosphorus decreased with distance from tree stem. Mineral nitrogen content decreased with depth whatever the distance. However, no variation in relation to depth was observed at R for the organic carbon and soluble phosphorus.

3.2. Soils moisture

Soils moisture was determined for the two sampling periods. We presented only soil moisture recorded only at R_0 and 0–25 cm layer. Results showed that soil moisture was higher during the wet season (August) for the two years. The soil moisture was 0.41% and 9.40% during respectively the dry season and the wet season of the year 2005 against 0.35% and 7.45% respectively in dry and wet seasons of the year 2006. A decrease of soil moisture was observed during the second year 2006 comparatively to the first year (2005) of the experiment.

Table 1
Chemical characteristics of soils collected in dry season of April 2005 (T_0, 2005) at different layers (0–25 cm; 25–50 cm and 50–75 cm) and different distances to tree stem (R_0; $R_{/2}$ and R).

Layers (cm)	[a]R_0				$R_{/2}$				R			
	pH_{H2O}	N	C	P	pH_{H2O}	N	C	P	pH_{H2O}	N	C	P
0–25	5.3	14.4	0.23	29.8	6.4	8.2	0.17	8.3	6.6	4.9	0.13	5.2
25–50	5.9	11.6	0.14	11.8	6.5	7.0	0.11	7.4	6.5	3.7	0.10	5.8
50–75	6.0	10.3	0.12	9.2	6.0	5.8	0.11	4.8	6.3	3.2	0.10	5.6

[a] R_0, foot tree; $R_{/2}$, 0.50 m distance from the stem and R, 1 m distance from the stem. The six soils sampled at the same distances and depths were pooled to get one composite soil sample. N, NH_4^++NO_3^- (mg/kg of soil); C, organic carbon (%) and P, soluble phosphorus (mg/kg of soil).

Table 2
Microbial biomass (in μg C g^{-1} dry soil) of soils collected at different layers (0–25 cm; 25–50 cm and 50–75 cm) and different distances to *A. senegal* tree stem (R_0; $R_{/2}$ and R) during the different sampling periods of the two years (2005 and 2006) of the experiment. In 2006, only layer 0–25 cm was sampled.

Layers (cm)	2005						2006					
	Dry season April (T_0)			Wet season August (T_1)			Dry season April (T_0)			Wet season August (T_1)		
	[a]R_0	$R_{/2}$	R	R_0	$R_{/2}$	R	R_0	$R_{/2}$	R	R_0	$R_{/2}$	R
0–25	[b]^{A}29a[c]	^{A}16a	^{A}12a	^{A}39b	^{A}33b	^{A}37b	^{A}15	^{A}7	^{A}3	^{A}60	^{A}59	^{A}61
25–50	^{B}22a	^{A}7a	^{A}4a	^{B}21a	^{B}19 ab	^{A}9a	–	–	–	–	–	–
50–75	^{A}10a	^{A}4a	^{A}3a	^{A}16a	^{A}13a	^{A}7a	–	–	–	–	–	–

[a] R_0, foot tree; $R_{/2}$, 0.50 m distance from the stem and R, 1 m distance from the stem.
[b] For each season, values within of line followed by same upper case letter comparing distance effect are not significantly different at $P < 0.05$ (Student–Newman and Keuls test).
[c] For each season, value within of column followed by same lower case letter comparing depth effect are not significantly different at $P < 0.05$ (Student–Newman and Keuls test).

3.3. Spatial and seasonal variations of soil microbial biomass

Soil microbial biomass decreased with increasing depth and distance from tree stem, whatever the soil sampling period and year except the wet season (T_1) of the second year (Table 2). During the dry season of 2005, the highest value of microbial biomass was found at the foot tree (R_0) and at 0–25 cm soil layer (29 μg C g^{-1} dry soil) and the lowest value was obtained in the R position and at 50–75 cm soil layer (3 μg C g^{-1} dry soil). No significant difference ($P > 0.05$) was noted on the effect of distance on microbial biomass of soils collected at 0–25 cm and 50–75 cm layers for all the sampling periods. However, a significant effect of distance ($P < 0.05$) was observed on microbial biomass of soils sampled at 25–50 cm layer. Considering the depth effect, results showed no significant difference ($P > 0.05$) was observed in soil microbial biomass during the dry season of 2005. Nevertheless, in the wet season, microbial biomass of soils collected at 0–25 cm layer was significantly greater ($P < 0.05$) than the one of soils coming from 25 to 50 cm and 50–75 cm layers except for $R_{/2}$.

Microbial biomass increased during the wet season compared to the dry season (Table 2). For 2005 and at 0–25 cm layer, soil microbial biomass content was 39 and 29 μg C g^{-1} dry soil, respectively for the wet season and the dry season. This increase was more marked during the second year of the experiment. Indeed, soil microbial biomass was multiplied by 4, 8 and 20 respectively at R_0, $R_{/2}$ and R distances at 0–25 cm layer.

3.4. Inorganic-N content

As soil microbial biomass, inorganic-N content decreased with the depth and the distance from tree stem (Table 3). Whatever the sampling period, the highest amount of mineral nitrogen content was recorded in layer 0–25 cm and at foot tree (R_0). During the first year (2005), the amount of mineral N measured around the foot tree (R_0) was significantly ($P < 0.05$) higher than that measured at R at 0–25 cm layer. No significant difference ($P > 0.05$) was noted on the effect of distance on inorganic-N content of soils collected at 0–25 cm and 50–75 cm layers for all the sampling periods of 2005 and the wet season of 2006.

Soil mineral nitrogen content was reduced during the wet season (T_1) by comparison to the dry season (T_0) (Table 3) for the two years of experience. For the soil layer 0–25 cm, mineral N content was 9.1 μg N g^{-1} dry soil at T_0 against 3.7 μg N g^{-1} dry soil at T_1 during the first year (2005). Hence, influence of the tree stem was less evident.

3.5. Seasonal variations of ammonium (NH_4^+) and nitrate (NO_3^-)

Table 4 showed that during the dry season (T_0), the highest amount of mineral nitrogen was in nitrate form (NO_3^-) in contrast during the wet season (T_1) it was mainly in ammoniac form (NH_4^+) for the two years. Significant difference ($P < 0.05$) was observed between ammoniac form and nitrate form for the most of soil samples. For example in 2006, mineral nitrogen content amount of 6.7 μg N–NO_3^- g^{-1} dry soil and 3.0 μg N–NH_4^+ g^{-1} dry soil were recorded at T_0 against 1.2 μg N–NO_3^- g^{-1} dry soil and 3.9 μg N–NH_4^+ g^{-1} dry soil at T_1 (Table 4).

3.6. Comparative evolution of soil microbial biomass and inorganic-N content

Fig. 1 showed that soil microbial biomass and mineral nitrogen content (NH_4^++NO_3^-) were negatively correlated. For the two years of the experiment, soil microbial biomass was higher during the rainy season (T_1) while the lowest mineral nitrogen contents were recorded at this same period. Inorganic-N content was higher during the dry season (T_0) at the lowest microbial biomass value.

4. Discussion

The decrease in soil microbial biomass in relation to the depth and the distance from tree stem could be attributed to the amount of nutrient elements such as carbon, nitrogen. Hence, this decrease in microbial biomass could also correlate to root biomass. *A. senegal* root biomass was highest at R_0 and 0–25 cm layer (data not shown). As reported in several studies, the diversity and numbers of microorganisms in rhizosphere are, to a large extent, determined by the composition and concentration of root exudates excreted by plants (Lynch, 1990; Yang et al., 2001; Marschner et al., 2004). However, the most pronounced aspect of this 'rhizosphere effect' is quantitative with microbial population sizes and activities increasing closer to the root (Castro-Sowinski et al., 2007). Root

Table 3
Inorganic-N content (in μg NH_4^++ μg NO_3^- g^{-1} dry soil) of soils collected at different layers (0–25 cm; 25–50 cm and 50–75 cm) and different distances to *A. senegal* tree stem (R_0; $R_{/2}$ and R) during the different sampling periods of the two years (2005 and 2006) of the experiment. In 2006, only layer 0–25 cm was sampled.

Years	Layers (cm)	Dry season (T_0)			Wet season (T_1)		
		[a]R_0	$R_{/2}$	R	R_0	$R_{/2}$	R
2005	0–25	[b]^{B}9.1b[c]	^{A}4.3b	^{A}4.1a	^{B}3.7b	^{A}1.6a	^{AB}2.6b
	25–50	^{A}4.4a	^{A}4.0b	^{A}4.5a	^{A}2.1ab	^{A}1.4a	^{A}1.3a
	50–75	^{B}4.1a	^{A}1.7a	^{B}3.7a	^{A}1.5a	^{A}1.3a	^{A}1.3a
2006	0–25	^{B}9.7	^{B}8.1	^{A}4.7	^{A}5.1	^{A}3.5	^{A}4.1

[a] R_0, foot tree; $R_{/2}$, 0.50 m distance from the stem and R, 1 m distance from the stem.
[b] For each season, values within of line followed by same upper case letter comparing distance effect are not significantly different at $P < 0.05$ (Student–Newman and Keuls test).
[c] For each season, value within of column followed by same lower case letter comparing depth effect are not significantly different at $P < 0.05$ (Student–Newman and Keuls test).

Table 4
Different forms of inorganic-N (in μg NH_4^+ and μg NO_3^- g^{-1} dry soil) of soils collected at different layers (0–25 cm; 25–50 cm and 50–75 cm) and different distances to *A. senegal* tree stem (R_0; $R_{/2}$ and R) during the different sampling periods of the two years (2005 and 2006) of the experiment. In 2006, only layer 0–25 cm was sampled.

Years (cm)	Layers	Dry season (April, T_0)						Wet season (August, T_1)					
		[a]R_0		$R_{/2}$		R		R_0		$R_{/2}$		R	
		NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-
2005	0–25	2.1a[b]	7.0b	1.8a	2.5a	1.0a	3.1b	3.1b	0.6a	1.3b	0.3a	1.9b	0.7a
	25–50	0.8a	3.6b	1.1a	2.9b	1.5a	4.0b	1.4b	0.7a	1.4b	0.0a	1.3b	0.0a
	50–75	0.7a	3.4b	0.7a	1.0a	1.6a	2.1a	1.1b	0.4a	1.3b	0.0a	1.3b	0.0a
2006	0–25	3.0a	6.7b	1.2a	6.9b	2.3a	2.4a	3.9b	1.2a	2.8b	0.7a	3.4b	0.7a

[a] R_0, foot tree; $R_{/2}$, 0.50 m distance from the stem and R, 1 m distance from the stem.
[b] For each depth, values within of line followed by same letter comparing ammonium (NH_4^+) and nitrate (NO_3^-) content in the same distance are not significantly different at $P < 0.05$ (Student–Newman and Keuls test).

exudates mainly serve as nutrient sources for microorganisms (De Troch and Vanderleyden, 1996). Legume species rhizosphere stimulated the growth of microorganisms by their rhizodeposition, rich in amino acids (Jones, 1999) and soluble sugars (Jensen, 1996). Similar results were obtained by Raubuch and Beese (2005).

The reduction in microbial biomass of soils sampled in dry season (T_0) could be explained by the decrease of water availability. Several studies suggest that seasonal variation in microbial biomass is related to variation in soil water potential (Piao et al., 2000; Ford et al., 2007) and substrate (i.e., C) availability (Srivastava, 1992). Changes in soil moisture status can markedly affect the magnitude of the soil microbial biomass (Schnürer et al., 1986) because many soil microorganisms are known to be intolerant of low soil moisture contents (Harris, 1981). In our study, soil moisture was weak during the dry season and high during the wet season.

Our results showed that mineral nitrogen content decreased with the depth and distance from tree stem. Inorganic-N content was higher at R_0 (foot tree) and 0–25 cm soil layer. Similar results were obtained by Maithani et al. (1998) and Iyyemperumala et al. (2007). Inorganic-N pools variation could be attributed to variation in mineralization rates, to the plants and microbes uptake, and losses through soil erosion, leaching, run-off and denitrification. The highest value of inorganic-N content obtained at R_0 and at 0–25 cm layer could be explained by the fact that nitrogen mineralization in the rhizosphere are strongly influenced by plant root exudates, which consist of easily degradable organic carbon compounds (Lynch, 1990).

Comparing the inorganic-N pool between seasons, our results showed that inorganic-N content decreased during the wet season. This phenomenon was mainly due to the immobilization of inorganic-N by the microorganisms (Sall et al., 2007) and the demand for this nutrient by weeds which grow vigorously during the wet season (Arunachalam et al., 1996). Conversely, the increase in inorganic-N content during dry season may be attributed to the mineralization of the dead microorganisms by the microbial community that survived desiccation (Kieft et al., 1987) and a decrease in demand by plants owing to slow growth. During dry season, plant uptake was reduced and the microbial activity was lower due to the lack of humidity resulting in an increase in pool of mineral N during these periods in comparison with wet periods.

Considering the seasonal variation of the different forms of mineral nitrogen, we observed that inorganic-N was mostly in nitrate form (NO_3^-) during the dry season and in ammoniac form (NH_4^+) during the wet season. The decrease of NO_3^- form during the wet season could be mainly due to high soil moisture content, plant uptake (Jackson et al., 1989; Schimel et al., 1989) or reduction in ammonium-N (NH_4^+) availability (Montangnini et al., 1986).

Results showed that soil microbial biomass and mineral nitrogen content were negatively correlated. Soil inorganic-N decreased in wet season when microbial biomass was high. The

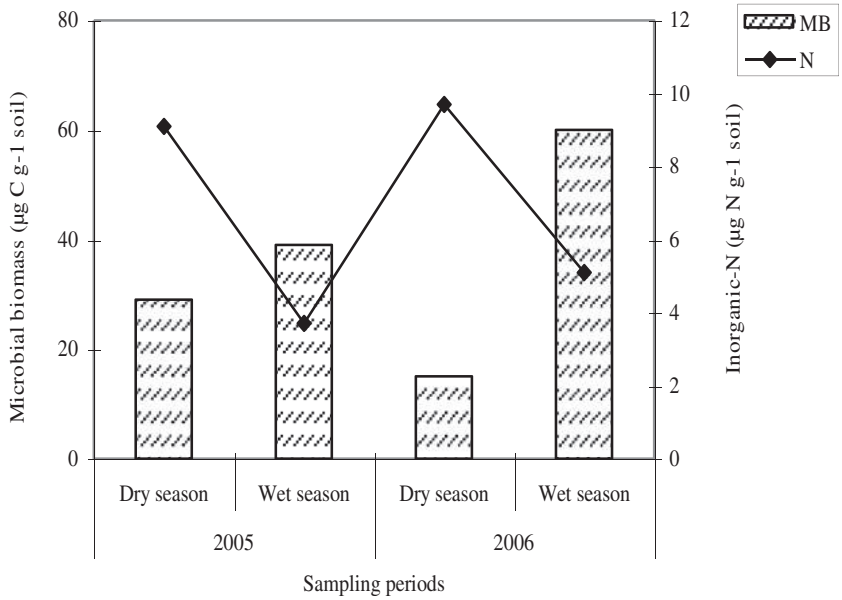

Fig. 1. Comparative evolution between soil microbial biomass and inorganic-N content at R_0 and 0–25 cm layer during the two years of the experiment. MB = microbial biomass, N = inorganic nitrogen.

increase of soil microbial biomass induced an increase of inorganic-N immobilization by microorganisms and then provokes a decrease in soil mineral nitrogen content (Sall et al., 2007).

5. Conclusion

This study showed that soil microbial biomass and inorganic-N were higher near *A. senegal* foot tree. Our results suggest a positive influence of *A. senegal* rhizosphere on soil microbial biomass and inorganic-N content. The significance of these findings is that due to mineral enrichment of the soil, the association with *A. senegal* might be beneficial for crops in agroforestry systems in N-deficient soils of the Sahelian zone. Soil total microbial biomass and inorganic-N content were negatively correlated. Soil microbial biomass increased during the rainy season while mineral N content decreased. Inorganic-N was mainly in nitrate form during the dry season and as ammonium during the wet season. In further investigations, it will be important to study the functional diversity of microorganisms in the rhizosphere of *A. senegal*.

Acknowledgments

This work was financially supported with funds from the CORAF/WECARD (Grant N FC/2003/15/UCAD). We thank the local populations of Kamb for their help and the LEMSAT technicians.

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Journal of Environmental Management 95 (2012) S265–S268

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Effects of a rock phosphate on indigenous rhizobia associated with *Sesbania sesban*

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ARTICLE INFO

Article history:
Received 30 September 2009
Received in revised form
22 September 2010
Accepted 1 November 2010
Available online 18 December 2010

Keywords:
Phosphorus
Rhizobia
Multipurpose leguminous tree

ABSTRACT

Tilemsi rock phosphate (TRP) of Mali is one of the most promising rock phosphate in West Africa for soil fertilization, but it is little used because of its insoluble form. The main objective of this study is to investigate TRP effects on rhizobia associated with the multipurpose leguminous tree *Sesbania sesban* grown on a sandy soil, poor in phosphorus and not sterilised. The experiment included treatments with and without TRP and was conducted during 105 days. At the end, 114 nodules have been collected and analysed by PCR/RFLP of 16S-23S intergenic spacer. Sixteen different RFLP profiles corresponding to different genomic groups of rhizobia have been detected. Five were dominant and present in both treatments. Five groups appear only in treatments without TRP whereas the six others are only in nodules of plants with TRP, suggesting a different capacity of natural phosphates solubilization by these strains.

1. Introduction

Compared with the other major nutrients, phosphorus (P) is by far the least mobile and is, after nitrogen (N_2), the most limiting factor for plant growth (Bieleski, 1973; Vance et al., 2000). The bioavailability of soil inorganic phosphorus in the rhizosphere varies considerably with plant species and soil conditions. Tropical and subtropical soils are often extremely phosphorus-deficient, mainly due to high phosphorus sorption capacities. Importance of P in plants nutrition has been extensively reported. In particular, P is needed for plant growth, nodule formation and development on legumes, and synthesis of ATP (source of energy necessary for the cleavage and reduction of N_2 into ammonia), each process being vital for biological nitrogen fixation (Waidyanatha et al., 1979; Islam et al., 1980). Leguminous trees required P to assure a good growth and a better N_2-fixation, and their effectiveness in soils improving may be hindered by a P deficiency (Giller and Cadisch, 1995). P fertilization is often necessary to circumvent phosphorus deficiency. In Mali, imported P fertilizers are expensive, and the locally produced Tilemsi rock phosphate (TRP) deposits supply the farmers with a cheaper alternative (Bationo et al., 1997). This low cost insoluble phosphate has demonstrated is usefulness in agroforestry systems (Bâ and Guissou, 1996; Bâ et al., 2001; Babana and Antoun, 2005).

The shrub leguminous Sesbania sesban is an important agroforestry species (Odee et al., 2002) which is often used as cover tree by farmers (Desaeger and Rao, 2001) and as green manure (Giller, 2001). In some dry lands of West Africa, *S. sesban* is indiscriminately used as timber wood and building, as fodder for livestock and also as fertilizer. This fallow species, like other legumes, may form symbiosis with wide indigenous rhizobial populations (Odee et al., 1995), but is among the least promiscuous when compared to other tropical trees like *Leucaena leucocephala* and *Gliricidia sepium* (Bala and Giller, 2001) and nodulated most effectively only with its homologous strains (Odee et al., 2002). Its N_2-fixation capacity may be about of 20 kg N^{-1} ha^{-1} (Kang et al., 1999; Ndoye and Dreyfus, 1988). Previous studies showed the lack of compatible rhizobia and of nodulation of *S. sesban* in different African, Asian and South American soils (Bala et al., 2003a, b). However, *S. sesban* presents a low response to inoculation by rhizobia and arbuscular mycorrhizal fungi (Ndoye and Dreyfus, 1988; Habte and Manjunath, 1991). It has been shown that P deficiency is an important constraint to the use of inoculation technology to increase *S. sesban* productivity in the Ethiopian highlands ecosystem for increased and sustainable crop-livestock productivity (Haque et al., 1996). However reports

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doi:10.1016/j.jenvman.2010.11.002

about phosphorus often focus on its impact on nutrition and/or trees growth, with the aim of having a good yield. Ndiaye et al. (2009) for example showed that natural rock phosphate can significantly increase the shoot biomass and mineral (N, P and K) content of *S. sesban* seedlings. On the other hand, there is little information about micro-organisms behaviour in soil in the presence of such important nutrient as phosphorus. A better knowledge of phosphorus-micro-organisms interaction may therefore be a major trump for a sustainable growth of this tree.

The global aim of our study was to explore the effects that phosphorus may have on the rhizobial behaviour in *S. sesban* rhizosphere, in particular under the influence of Tilemsi rock phosphate.

2. Materials and methods

2.1. Soil sampling

The soil used in this experience has been taken in the ISRA (Institut Sénégalais des Recherches Agricoles) station in Nioro (Senegal), 13°44' N and 15°47' W, with 700 mm annual rainfall. Soil sample was collected between 0 and 25 cm depth. It is a sandy soil with neutral pH and 23.40 ppm available P. It contains 2.06% clay, 0.62% thin limon, 7.74% rough limon, 49.2% thin sand, 36.8% rough sand, 2.04 mg kg^{-1} soil total C, 0.14 mg kg^{-1} soil N, 106 ppm total P. The pH (H_2O) and (KCl) are, respectively, 7.23 and 5.63. The soil has been crushed and passed through a 1 mm sieve.

2.2. Fertilization and experimental design

The rock phosphate used as fertilizer (TRP) originated from Bourem, in the Tilemsi valley (Mali). It is one of the most soft and receptive phosphate in West Africa (Truong et al., 1978). It is used as pulverized form with 30% P_2O_5. TRP, actually commercialized, presents 0.007% of solubility in water. Its chemical contents are reported in Table 1. The experience has been carried out during 105 days in Laboratoire Commun de Microbiologie IRD/ISRA/UCAD in Dakar (Senegal). TRP has been applied and mixed to 1 kg of soil per bag to a final concentration of 50 mg P kg^{-1}, corresponding approximately to 150 kg P_2O_5 ha^{-1}. Each treatment (with or without TRP) was repeated 15 times.

2.3. Plant material

S. sesban seeds were scarified into 98% H_2SO_4 for one hour. They were after plentifully rinsed out with sterile distilled water, soaked overnight into last rinsed water and transferred on medium agar during 2 days at 28 °C temperature. Pre-germinated seeds had been planted out in plastic bags at the rate of two seedlings per bag. After a week, only one seedling was preserved into each bag. Seedlings in bags have been put into greenhouse in ambient conditions and plants were watered daily.

Table 1
Chemical composition of different natural rock phosphates from West Africa (after Truong et al., 1978).

Rock phosphates (country of origin)	Total content (%)		Solubility (% of total P_2O_5)			CO_3/PO_4 substitution
	P_2O_5	CaO	Citrate	Citric acid	Formic acid	
Arli (Burkina Faso)	30.8	47.6	5.4	19.2	38.7	0.098
Kodjari (Burkina Faso)	27.16	44.8	6.1	18.8	37.1	0.093
Tahoua (Niger)	34.5	44.8	8.3	19.3	34.0	0.112
Taiba (Senegal)	37.8	44.8	5.0	19.8	38.7	0.098
Tilemsi (Mali)	30	43.1	10.4	29.7	47.3	0.210
Hahotoe (Togo)	35.4	36.4	4.3	19.1	36.7	0.088
Gafsa (Tunisia)	30.2	31.9	20.5	37.8	78.6	0.254

2.4. Bacterial molecular characterization

One-hundred and fourteen nodules (58 on plants growing on soil without TRP, 56 with TRP) have been collected randomly at the end of the experiment (105 days after seedling). They were surface sterilised by immersion in 3.3% (w/v) $Ca(OCl)_2$ for 3 min, and rinsing in sterile water. This was followed by a second immersion in 96% ethanol for 2–3 min and rinsing in sterile water. From this stage the nodules were manipulated aseptically. Each nodule was crushed in 300 μl of sterile water with plastic pestle sterilised in 96% ethanol in a 1.5-ml Eppendorf tube 150 μl of 2× CTAB/PVPP buffer sterilised in 96% ethanol in a 1.5-ml Eppendorf tube 150 μl of 2× CTAB/PVPP buffer (0.2 M Tris–HCl, pH 8; 0.04 M EDTA pH 8; 2.8 M NaCl; 4% w/v CTAB (hexadecyltrimethylammonium bromide); 2% w/v PVPP (polyvinylpolypyrrolidone)) was added to 150 μl of crushed nodule for DNA extraction. The homogenate was incubated at 65 °C for 60 min and centrifuged for 10 min at 15,000 × *g* to remove cellular debris. Supernatant was then extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1 v/v/v) and centrifuged for 15 min at 15,000 × *g*. DNA from the aqueous phase was purifed from phenol with 300 μl of chlorophorm isoamyl alcohol (24:1 v/v) and centrifuged for 15 min at 15,000 × *g*. Supernatant was centrifuged one more time for 5 min. DNA from the aqueous phase was precipitated overnight at −20 °C with the addition of 0.1 volume of sodium acetate and 2.5 volumes of absolute ethanol. The samples were centrifuged for 30 min at 13,000 rpm at +4 °C. The resulting DNA pellet was washed with 70% v/v ethanol by centrifugation for 15 min at 13,000 rpm at +4 °C, vacuum dried, and solubilized in 20 μl of ultrapure water. The purity and the quantity of DNA extracted were estimated by spectrophotometry (Pharmacia Biotech) in the range 200–340 nm. Bacterial genomic DNA was extracted from 1.5 ml of stationary phase bacterial cultures grown in YM (Yeast extract Mannitol medium). Cells were pelleted by centrifugation and resuspended by homogenisation in one volume of 1× CTAB/PVPP buffer. Total genomic DNA was recovered and purified as described above for crushed nodule DNA. PCR amplification of 16S-23S rDNA spacer region was performed using two primers: FGPS1490-72 (5'-TGCGGCTGGATCCCCTCCTT-3') (Normand et al., 1996), and FGPL132-38 (5'-CCGGGTTTCCCCATTCGG-3') (Ponsonnet and Nesme, 1994). PCR was carried out in 25 μl reaction volume containing 50 ng of pure total DNA extract, one dried bead (Ready-to-Go PCR beads, Pharmacia Biotech) containing 1.5 U of Taq polymerase, 10 mM Tris–HCl, (pH 9 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP and 1.0 μM of each primer. PCR amplification was performed in GeneAmp PCR System 2400 (Perkin Elmer) thermal cycler adjusted to the following temperature profile: initial denaturation at 95 °C for 5 min; 35 amplification cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, and extension 72 °C for 1 min; and final extension at 72 °C for 3 min. After electrophoresis of 3 μl on a 1% (w/v) agarose gel in TBE buffer (1.1 w/v Tris–HCl; 0.1% w/v $Na_2EDTA{\cdot}2H_2O$; 0.55% w/v boric acid), the gel was stained for 30 min in an aqueous solution of ethidium bromide (1 μg/ml) and photographed under UV illumination with Gel Doc (BIO-RAD) software. Restriction fragment analysis of 16S–23S intergenic spacer region was performed on aliquots (6–10 μl) of PCR products digested with restriction endonucleases *Msp*I as specified by the manufacturer (Amersham Pharmacia Biotech) with an excess of enzyme (10 U per 20 μl reaction volume) for 2 h. Restricted DNA was analysed by horizontal electrophoresis in 2.5% (w/v) agarose MetaphorR (FMC BioProducts, Rockland, Marine USA). Electrophoresis was carried out at 80 V for 3 h in 11 × 14-cm gels. Gel was stained and photographed as described above.

Table 2
Number of nodules in the different IGS groups, with or without TRP.

IGS groups	Without TRP	With TRP
I	18	15
II	14	12
III	5	1
IV	8	16
V	1	
VI	6	4
VII		2
VIII		1
IX		1
X		2
XI		1
XII	1	
XIII	1	
XIV		1
XV	2	
XVI	2	
Total	58	56

2.5. Statistical analysis

The data were analysed as unordered rows by columns contingency tables using the Likelihood ratio test implemented by the StatXact statistical analysis software package (CYTEL Software, Cambridge, MA), with IGS RFLP groups in rows and frequency of each IGS type in columns.

3. Results and discussion

A total of 114 nodules have been analysed for the two treatments (56 with and 58 without TRP). Sixteen different *Msp*I RFLP profiles have been discriminated (Table 2). The more frequent are showed in Fig. 1. Several authors have demonstrated the discriminating power of PCR-RFLP analysis of 16S–23S IGS regions for studying natural bacterial diversity and grouping genetically related strains (Navarro et al., 1992; Jensen et al., 1993; Laguerre et al., 1996; Doignon-Bourcier et al., 1999; Krasova-Wade et al., 2003; Diouf et al., 2007). It appears therefore that a remarkable diversity exist among rhizobia nodulating *S. sesban* in a same sandy Senegalese soil. This observation confirmed those of Odee et al. (1995) in Kenya, of Wolde-Meskela et al. (2004) in Ethiopia and of Sharma et al. (2005) in the semi-arid Delhi region where different species of *Sinorhizobium* nodulating *S. sesban* dominate. This large rhizobial diversity has already been shown in West African soils for other legume species such as cowpea (Krasova-Wade et al., 2003), *Pterocarpus erinaceus* and *Pterocarpus lucens* (Sylla et al., 2002), *Acacia seyal* (Diouf et al., 2007), *Acacia senegal* (Sarr et al., 2005; Fall et al., 2008). Despite of this diversity, a group of five profiles (I, II, III, IV and VI) is present in more than 85% of all the nodules that have been analysed, and are encountered in both treatments (Table 2). Even more, two profiles (I and II) are dominant, with half of the observed profiles, bringing to light a clear competitiveness for nodulation in such conditions, whatever the addition or not of TRP. On the other hand, it is interesting to observe that eleven profiles are found only in one treatment, and not in the other. Six profiles (VII to XI, and XIV) are encountered only in presence of TRP, whereas five profiles (V, XII, XIII , XV and XVI) are only in nodules of plants without TRP (Table 2). The exact *P* value (0.044) indicates that the null hypothesis can be statistically rejected and that there is altogether a significant difference in the genetic nodule population structure linked to the treatment with or without TRP. However this difference is slight, and is mainly due to two of the dominant profiles (IGS groups III and IV) and to the small groups specific to each treatment. It would be interesting to precise if these specific profiles correspond to closely related strains, and if they are strongly different from one treatment to the other. If yes it could mean that the presence of TRP really influence strains behaviour and induce significant changes in population structure. It is well known that rhizobia are often good phosphate-solubilizing bacteria (Abd-Alla, 1994; Abril et al., 2007; Taiwo and Ogundiya, 2008), but that differences exist between strains. Rosas et al. (2006) for example have shown that *Sinorhizobium meliloti* strain 3DOh13 solubilized iron and phosphate while *Bradyrhizobium japonicum* strain TIIIB was a poor phosphate solubilizer. The solubilization ability may be dependant on the form of P: from a total of 446 rhizobial isolates tested for P solubilization by the formation of visible dissolution halos on agar plates, 198 (44% of the isolates)

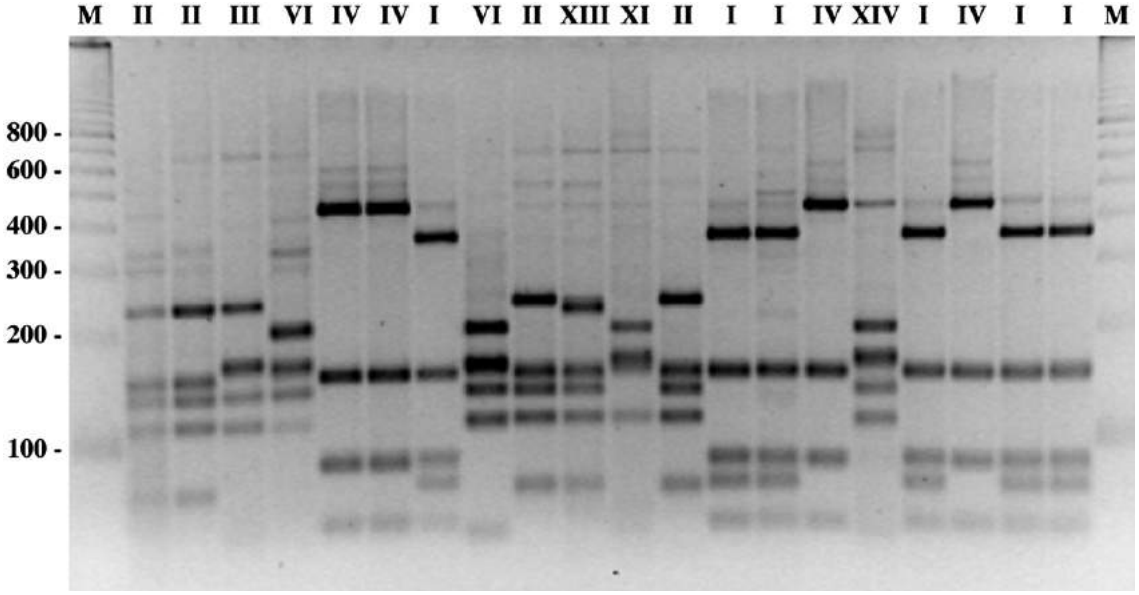

Fig. 1. Major restriction patterns of PCR-amplified 16S–23S rDNA IGS of crushed nodules DNA of *S. sesban*, obtained with *Msp*I. Lanes M, 100 bp DNA size marker (Pharmacia Biotech).

solubilized $Ca_3(PO_4)_2$ and 341 (76%) inositol hexaphosphate (Alikhani et al., 2006). The differences that were observed may be the result of a differential ability to solubilize TRP.

In conclusion, it appears that a great diversity exists among strains of rhizobia able to nodulate *S. sesban* in a given soil. Some of them are dominant for competition for nodulation. The addition of Tilemsi rock phosphate induce significant modifications of rhizobial populations. However the differences are slight and must be confirmed by further analyses. TRP has no effects on most of the major IGS groups encountered, but can have on the other hand an impact on the behaviour of minority groups. It could be due to a difference of capacity to solubilize phosphates, which would deserve to be studied, in particular in the optics of the inoculation with these strains, if they turn out besides good nitrogen fixers in symbiosis with *S. sesban*.

Acknowledgements

This study was supported by grants from INCO-ENRICH Project No. 1CA4-CT-2000-30022 and Agence Universitaire de la Francophonie (AUF). The authors thank G. Laguerre for helpful advices.

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Journal of Environmental Management 95 (2012) S269–S274

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Ectomycorrhizal fungi as an alternative to the use of chemical fertilisers in nursery production of *Pinus pinaster*

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ARTICLE INFO

Article history:
Received 4 September 2009
Received in revised form
25 March 2010
Accepted 12 July 2010
Available online 10 August 2010

Keywords:
Ectomycorrhiza
Forest nursery inoculation
Improved plant growth
Maritime pine
Selected ectomycorrhizal fungi

ABSTRACT

Addition of fertilisers is a common practice in nursery production of conifer seedlings. The aim of this study was to evaluate whether ectomycorrhizal (ECM) fungi can be an alternative to the use of chemical fertilisers in the nursery production of *Pinus pinaster*. A greenhouse nursery experiment was conducted by inoculating seedlings obtained from seeds of *P. pinaster* plus trees with a range of compatible ECM fungi: (1) *Thelephora terrestris*, (2) *Rhizopogon vulgaris*, (3) a mixture of *Pisolithus tinctorius* and *Scleroderma citrinum*, and (4) a mixture of *Suillus bovinus*, *Laccaria laccata* and *Lactarius deterrimus*, using forest soil as substrate. Plant development was assessed at two levels of N–P–K fertiliser (0 or 600 mg/seedling). Inoculation with a mixture of mycelium from *S. bovinus*, *L. laccata* and *L. deterrimus* and with a mixture of spores of *P. tinctorius* and *S. citrinum* improved plant growth and nutrition, without the need of fertiliser. Results indicate that selected ECM fungi can be a beneficial biotechnological tool in nursery production of *P. pinaster*.

1. Introduction

Pinus pinaster Ait. (maritime pine) represents approximately 23% of the Portuguese forest area and is widely used on reforestation practices (Autoridade Florestal Nacional, 2009). Reforestation efficiency relies on the ability of seedlings to adjust to unfavourable conditions. By increasing their resistance, not only seedlings have a higher growth performance, but also post-transplantation mortality can be reduced. Reforestation using container-grown seedlings of *P. pinaster* produced in nurseries is a common practice in many countries.

Fertilisers are often used in nurseries since they enhance seed germination and root growth and development, resulting in a faster transplantation as desired in management practices for reforestation (Rincón et al., 2007; Walker, 2001). However, problems can arise from transplanting fertilised seedlings into forest soil as they can resent the dramatic change of nutrient availability and may not be able to adjust to the new and often adverse conditions (Castellano and Molina, 1989). Moreover, the use of chemical fertilisers can constitute a threat to the environment. A significant share of nutrients applied may be left in the soil, altering its ecology, and can be lost by leaching leading to eutrophication of surface waters (Entry and Sojka, 2007; Steinfeld et al., 2006; Syers et al., 2008). Another relevant aspect is the fact that some of the nutrients used in fertilisers, such as phosphorus, are not renewable sources and its use must therefore be well managed (Syers et al., 2008).

Ectomycorrhizal (ECM) fungi are known to form symbiotic relations with *P. pinaster* (Nieto and Carbone, 2009; Pera and Alvarez, 1995). Root colonisation by ECM fungi often has a beneficial effect on plant survival and growth. Their network of exploring hyphae or rhizomorphs brings water and nutrients from distant sites to the sites of utilisation by the host plant, in exchange for its photosynthetic carbohydrates (Chalot et al., 2002; Conjeaud et al., 1996). However, the association of host–ECM fungi is not always beneficial for the plant, since the demand for carbohydrates is increased when colonisation occurs, resulting in less carbon for its development (Conjeaud et al., 1996). Fertilisation practices can also have different effects on the establishment of ectomycorrhizas, as nutrient demand and response to nutrient supplements vary among fungi (Rincón et al., 2007). Fertilisers may enhance fungal associations with benefit for the plant (Liu et al., 2008) or, on the other hand, inhibit colonisation (Castellano and Molina, 1989; Vaario et al., 2009). Other studies have also reported that fertilisation does not affect ECM formation (Castellano and Molina, 1989; Conjeaud et al., 1996; Rincón et al., 2007). These observations point to a high specificity between host and ECM fungal species or isolates and also to a high sensitivity of the fungi regarding fertiliser and soil properties.

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doi:10.1016/j.jenvman.2010.07.016

The aim of this study was to evaluate whether selected ECM fungi can enhance the growth of *P. pinaster* seedlings, without the use of chemical fertilisers, for reforestation purposes. The work was conducted in a forest nursery greenhouse.

2. Materials and methods

2.1. Experimental design

In a forest nursery greenhouse, in Amarante, Northern Portugal, trays with 210 cm^3 cells were filled with non-sterile homogenised forest soil (10 mg l^{-1} N, 325 mg l^{-1} P_2O_5, 10,600 mg l^{-1} K, 6600 mg l^{-1} Mg, 4620 mg l^{-1} Ca, 260 mg l^{-1} Na, pH (H_2O) 6.2, electrical conductivity 0.2 mS cm^{-1}) collected from a forest site in Arcos de Valdevez, Northern Portugal. Fungi were added to the substrate as mycelial suspensions or spores. The fungal isolates used in these experiments belong to the collection of Escola Superior de Biotecnologia, and are referenced in the collection as: ref. TT-00, *Thelephora terrestris* Ehrh; ref. RH-01, *Rhizopogon vulgaris* (Vitt.) M. Lange; ref. SB-00, *Suillus bovinus* (Pers.) Roussel; ref. LL-02, *Laccaria laccata* (Scop.) Cooke; and ref. LD-02, *Lactarius deterrimus* Gröger. The isolates were maintained by successive transfers in Potato Dextrose Agar (PDA, Sigma) and in modified Melin Norkans agar (MMN, Marx, 1969). Spores of *Pisolithus tinctorius* (Pers.) Coker & Couch and *Scleroderma citrinum* Pers were collected in from a forest site in Caminha, Northern Portugal. For each treatment, different fungal inocula were used: mycelium of *T. terrestris* Ehrh. (treatment designated as T), mycelium of *R. vulgaris* (Vitt.) M. Lange (treatment designated as R), a spore mixture of *P. tinctorius* (Pers.) Coker & Couch and *S. citrinum* Pers. (treatment designated as PS), and a mixture of mycelium from *S. bovinus* (Pers.) Roussel, *L. laccata* (Scop.) Cooke and *L. deterrimus* Gröger (treatment designated as SLL). These ECM fungal isolates and mixtures were chosen for their compatibility with *P. pinaster* in previous laboratory studies (Oliveira et al., personal communication). The ECM fungal isolates were isolated from forest ecosystems of Northern Portugal. Only one isolate of each ECM fungal species was used in these experiments. Inoculation was performed either by injecting 6 ml of three weeks old mycelial suspensions (ca. 200 mg of fresh weight) or 10 ml of spore suspension (10^7 and 10^6 spores per seedling of *P. tinctorius* and *S. citrinum*, respectively) to the substrate of each cell. The spore concentration was assessed with a haemocytometer. A control treatment with non-inoculated seedlings was also established. All treatments were replicated six times.

P. pinaster seeds collected in the area of Ponte de Lima, Northern Portugal, from five adult trees classified as plus were rinsed overnight in running tap water, surface sterilised with 10% bleach solution for 15 min and washed three times with deionised sterile water. Two disinfected seeds were placed in each root tray. All cells were covered with autoclaved vermiculite (Verlite, Vermiculita y Derivados S.L., Asturias, Spain). One month after placing seeds and inoculum, plants were trimmed to one seedling per cell and two fertilisation treatments were applied: no fertilisation (0 mg/seedling) and fertilisation (600 mg/seedling). The nutrients were supplied as N–P–K slow release fertiliser (12% N, 12% P_2O_5, 17% K_2O, 2% MgO, 15% SO_3, 0.02% B, 0.1% Fe, 0.01% Zn) (BASF, Germany). Seedlings were watered everyday and maintained under an average photoperiod of 8 h. Greenhouse temperature varied between 1.9 and 41.0 °C and relative humidity between 10 and 80%. Trays of different treatments were periodically rotated to different bench positions to minimise differences due to their location in the greenhouse. With the exception of fungal inoculation, all the above mentioned procedures are currently used in forest nursery production in Portugal.

2.2. Plant sampling and analysis

After six months, all seedlings were gently removed from the trays and transported to the laboratory for further analyses. The shoot height was measured. The root system was separated from the shoot and washed to remove adhered substrate. The % of ECM fungal colonisation and the number of ECM root tips per root length were assessed using a stereomicroscope (SZ30, Olympus, Japan) according to Brundrett et al. (1996). Representative ECM root tips were characterised on the basis of colour, branching, shape, presence of emanating hyphae and inner and outer mantle patterns under a stereomicroscope and by differential interference contrast microscopy (BX60, Olympus, Japan) according to Agerer (1998). The fresh weight of the plants was determined by weighing plant material. Needles were dried at 70 °C for 48 h. Oven-dried needles were finely ground and 0.2 g of material were digested according to Novozamsky et al. (1983). The digested samples were used to determine the total nitrogen (N) concentration in needles by colorimetry (Unicam, Helios Gamma, Cambridge, UK) (Walinga et al., 1989).

2.3. Statistical analysis

The data were tested for normality and analysed using one-way analysis of variance (ANOVA). When a significant *F*-value was obtained ($P < 0.05$), treatment means were compared using the Duncan's multiple range test. Regression analyses were conducted at a significance level of 0.05. All statistical analyses were performed using the SPSS 16.0 software package (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Plant parameters

The effect of fertilisation on the shoot height of *P. pinaster* seedlings varied with fungal inoculation. Fig. 1 shows that fertilisation led to an increase in shoot height of non-inoculated plants. In inoculated plants, the use of fertiliser increased shoot height in plants inoculated with the individual fungi *T. terrestris* and *R. vulgaris*, whereas the opposite effect was verified with the fungal mixtures *P. tinctorius* + *S. citrinum* and *S. bovinus* + *L. laccata* + *L. deterrimus*, where fertilised plants were significantly smaller than non-fertilised ones. Moreover, fertilised *T. terrestris* and *R. vulgaris* treatments and non-fertilised treatments using fungal mixtures,

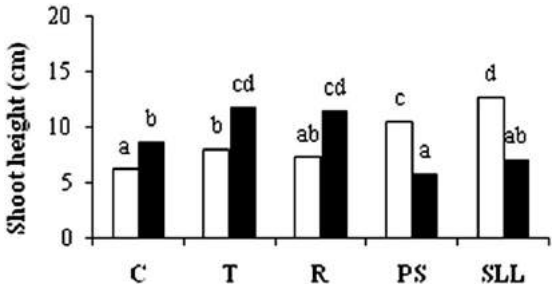

Fig. 1. Shoot height of *Pinus pinaster* seedlings inoculated with *Thelephora terrestris* (T), *Rhizopogon vulgaris* (R), a mixture of *Pisolithus tinctorius* and *Scleroderma citrinum* (PS), a mixture of *Suillus bovinus*, *Laccaria laccata* and *Lactarius deterrimus* (SLL) and non-inoculated control (C) under two fertilisation regimes: non-fertilised (open bars) or fertilised (black bars). Columns marked with different letters differed significantly according to Duncan's Multiple Range test at $P < 0.05$.

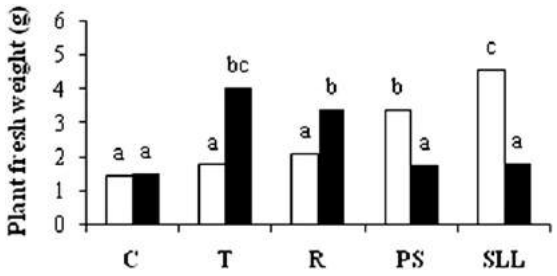

Fig. 2. Plant fresh weight of *Pinus pinaster* seedlings inoculated with *Thelephora terrestris* (T), *Rhizopogon vulgaris* (R), a mixture of *Pisolithus tinctorius* and *Scleroderma citrinum* (PS), a mixture of *Suillus bovinus*, *Laccaria laccata* and *Lactarius deterrimus* (SLL) and non-inoculated control (C) under two fertilisation regimes: non-fertilised (open bars) or fertilised (black bars). Columns marked with different letters differed significantly according to Duncan's Multiple Range test at $P < 0.05$.

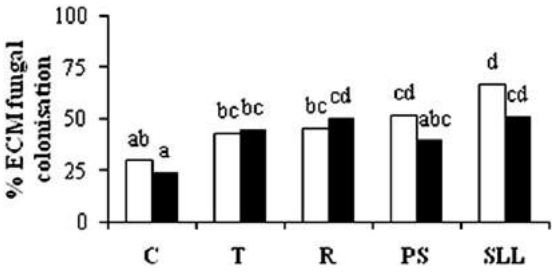

Fig. 4. Percentage of ectomycorrhizal fungal colonisation of *Pinus pinaster* seedlings inoculated with *Thelephora terrestris* (T), *Rhizopogon vulgaris* (R), a mixture of *Pisolithus tinctorius* and *Scleroderma citrinum* (PS), a mixture of *Suillus bovinus*, *Laccaria laccata* and *Lactarius deterrimus* (SLL) and non-inoculated control (C) under two fertilisation regimes: non-fertilised (open bars) or fertilised (black bars). Columns marked with different letters differed significantly according to Duncan's Multiple Range test at $P < 0.05$. ECM, ectomycorrhizal.

resulted in significantly higher plants than in non-inoculated controls.

There was no apparent influence of fertilisation in plant fresh weight of control seedlings. In inoculated plants, fertilisation enhanced plant biomass when *T. terrestris* and *R. vulgaris* were inoculated, whereas in the presence of mixtures of fungi (*P. tinctorius* + *S. citrinum* and *S. bovinus* + *L. laccata* + *L. deterrimus*), non-fertilised plants showed a significantly higher biomass. Also, fertilised *T. terrestris* and *R. vulgaris* and non-fertilised plants inoculated with fungal mixtures, produced greater biomass than non-inoculated ones (fertilised and non-fertilised) (Fig. 2).

In non-inoculated plants and in plants inoculated with *T. terrestris*, fertilisation had no significant effect in N needle concentration. However, a different response was obtained for other fungal treatments. Fertilised plants inoculated with *R. vulgaris* presented a higher N concentration than the non-fertilised ones, whereas the opposite effect was verified for plants inoculated with the fungal mixtures (*P. tinctorius* + *S. citrinum* and *S. bovinus* + *L. laccata* + *L. deterrimus*). Regarding non-fertilised treatments, the N concentration in plants inoculated with *T. terrestris* or *R. vulgaris* was similar to that of non-inoculated plants. The needles of plants inoculated with fungal mixtures, however, showed significantly higher N concentration. Regarding fertilised plants, the opposite effect was obtained. Plants inoculated with fungal mixtures had similar N concentration as control plants whereas the single fungal treatments (*T. terrestris* and *R. vulgaris*) presented higher N concentration (Fig. 3).

3.2. Fungal parameters

Fertilisation did not affect the percentage of root colonisation by ECM fungi (Fig. 4). Seedlings inoculated with fungal mixture *S. bovinus* + *L. laccata* + *L. deterrimus* showed a significantly higher ECM colonisation than non-inoculated controls (both fertilised and non-fertilised). The same was observed in the treatment *P. tinctorius* + *S. citrinum* without fertiliser. Non-fertilised *S. bovinus* + *L. laccata* + *L. deterrimus* and fertilised *R. vulgaris* were the treatments where a significantly higher number of ECM root tips per root length was observed (Fig. 5). The application of fertiliser decreased the number of ECM root tips in seedlings inoculated with *P. tinctorius* + *S. citrinum* and *S. bovinus* + *L. laccata* + *L. deterrimus*.

The ECM morphotypes identified for each fungal treatment under the two fertilisation regimes are presented in Table 1. Non-inoculated plants had the lowest number of different ECM morphotypes while the fungal mixture *S. bovinus* + *L. laccata* + *L. deterrimus* presented the highest. In plants inoculated with *R. vulgaris* and *S. bovinus* + *L. laccata* + *L. deterrimus*, fertilisation decreased the number of morphotypes whereas in the other fungal treatments no difference occurred. Moreover, in non-inoculated plants and in those inoculated with *T. terrestris* and *P. tinctorius* + *S. citrinum*, the same morphotypes were observed in non-fertilised and in fertilised plants. The only three morphotypes occurring in non-inoculated plants were present in all fungal treatments. With the exception of the morphotype EM4, which appears simultaneously in plants inoculated with *R. vulgaris* and *S. bovinus* + *L. laccata* + *L. deterrimus*, the remaining morphotypes were specific to each fungal treatment.

3.3. Correlation between plant development and fungal colonisation

The plant fresh weight and shoot height of *P. pinaster* showed a significantly positive correlation with the percentage of ECM colonisation when no fertiliser was applied (Figs. 6a and 7a).

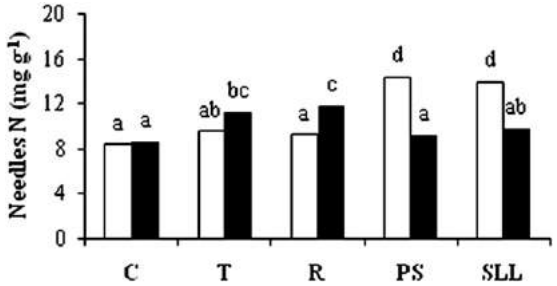

Fig. 3. Needles nitrogen concentration of *Pinus pinaster* seedlings inoculated with *Thelephora terrestris* (T), *Rhizopogon vulgaris* (R), mixture of *Pisolithus tinctorius* and *Scleroderma citrinum* (PS), a mixture of *Suillus bovinus*, *Laccaria laccata* and *Lactarius deterrimus* (SLL) and non-inoculated control (C) under two fertilisation regimes: non-fertilised (open bars) or fertilised (black bars). Values are expressed in mg of N per g of oven-dried needles. Columns marked with different letters differed significantly according to Duncan's Multiple Range test at $P < 0.05$.

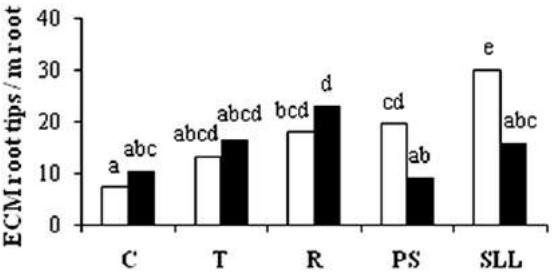

Fig. 5. Number of ectomycorrhizal root tips per root length of *Pinus pinaster* seedlings inoculated with *Thelephora terrestris* (T), *Rhizopogon vulgaris* (R), a mixture of *Pisolithus tinctorius* and *Scleroderma citrinum* (PS), a mixture of *Suillus bovinus*, *Laccaria laccata* and *Lactarius deterrimus* (SLL) and non-inoculated control (C) under two fertilisation regimes: non-fertilised (open bars) or fertilised (black bars). Columns marked with different letters differed significantly according to Duncan's Multiple Range test at $P < 0.05$.

Table 1
Ectomycorrhizal morphotypes found on the roots of *Pinus pinaster* seedlings inoculated with *Thelephora terrestris* (T), *Rhizopogon vulgaris* (R), mixture of *Pisolithus tinctorius* and *Scleroderma citrinum* (PS), a mixture of *Suillus bovinus*, *Laccaria laccata* and *Lactarius deterrimus* (SLL) and non-inoculated control (C) under two fertilisation regimes: non-fertilised or fertilised.

Morphotype code	Non-fertilised					Fertilised				
	C	T	R	PS	SLL	C	T	R	PS	SLL
EM1	+	+	+	+	+	+	+	+	+	+
EM2	+	+	+	+	+	+	+	+	+	+
EM3	+	+	+	+	+	+	+	+	+	+
EM4	−	−	+	−	+	−	−	−	−	+
EM5	−	+	−	−	−	−	+	−	−	−
EM6	−	−	−	+	−	−	−	−	+	−
EM7	−	−	+	−	−	−	−	+	−	−
EM8	−	−	−	−	+	−	−	−	−	+
EM9	−	−	−	−	+	−	−	−	−	−
Total number	3	4	5	4	6	3	4	4	4	5

+, presence; −, absence.
Morphotype description: EM1 − Dark brown, unbranched, long tortuous tips, smooth mantle surface, pseudoparenchymatous outer and inner mantle with angular cells and mounds of flattened cells, few emanating hyphae; EM2 − Dark brown, dichotomous branching, tortuous tips, smooth mantle surface, pseudoparenchymatous outer and inner mantle with angular cells, few emanating hyphae; EM3 − Dark orange, unbranched, straight tips, smooth mantle surface, pseudoparenchymatous outer and inner mantle with angular cells; EM4 − Dark grey, unbranched, smooth mantle surface; EM5 − Brown and whitish, dichotomous branching, tortuous tips, smooth mantle surface, pseudoparenchymatous outer and inner mantle with angular cells and irregularly arranged hyphae, few emanating hyphae; EM6 − Light brown, dichotomous branching, long tortuous tips, smooth mantle surface, pseudoparenchymatous outer and inner mantle with angular cells, few emanating hyphae; EM7 − Dark orange, dichotomous branching, straight tips, grainy mantle surface, pseudoparenchymatous outer and inner mantle with angular cells and mounds of flattened cells; EM8 − Golden yellow, dichotomous branching, straight hairy tips, pseudoparenchymatous outer mantle, plectenchymatous inner mantle with epidermoid cells bearing a delicate hyphal net, abundant emanating hyphae; EM9 − Light grey, monopodial pinnate branching, tortuous tips, smooth mantle surface.

The plant fresh weight and shoot height of non-fertilised *P. pinaster* were also significantly positive correlated with the number of ECM fungal tips per m of root (Figs. 6b and 7b). In fertilised plants there was no correlation between any plant and fungal parameters (data not shown).

4. Discussion

Many studies performed in nurseries have been carried out with single ECM fungal species (Duñabeitia et al., 2004; González-Ochoa et al., 2003; Walker, 2001). However, in nature, forest trees are often colonised by multiple ECM fungi (Parladé et al., 1999). It is suggested that these seedlings are more resistant than those colonised by single species (Parladé and Alvarez, 1993) as fungi can have complementary behaviour, with benefit to the host plant (Reddy and Natarajan, 1997). In this study two single species (*T. terrestris* and *R. vulgaris*) and two mixtures (*P. tinctorius* + *S. citrinum* and *S. bovinus* + *L. laccata* + *L. deterrimus*) were tested as an alternative to the use of fertiliser in nurseries. The development of *P. pinaster*, including biomass and height, was highly affected by fungal inoculation. The highest plant growth was obtained with fertilised *T. terrestris* and *R. vulgaris* and non-fertilised fungal mixtures. All these treatments were more effective in promoting plant growth when compared with non-inoculated controls (both fertilised and non-fertilised).

Without the application of fertiliser, seedlings inoculated with *R. vulgaris* presented no significant growth difference from controls. Similar results were reported for *Rhizopogon* spp. by Rincón et al. (2005) and may be due to their high demand of carbohydrates, which does not allow the plant to obtain the carbon it needs for its growth. All other inoculation treatments (non-fertilised) promoted plant growth as also reported in other studies with *Pinus* spp. (Reddy and Natarajan, 1997; Rigou et al., 1995; Rincón et al., 2007).

When fertiliser was applied, there were also differences in plant development amongst the inoculation treatments. While inocula addition greatly enhanced plant height and weight on treatments with the individual fungi *T. terrestris* and *R. vulgaris*, the opposite was verified with the fungal mixtures *P. tinctorius* + *S. citrinum* and *S. bovinus* + *L. laccata* + *L. deterrimus*. *T. terrestris* is a common nursery seedling colonising ECM fungus. It is considered an early-stage fungus with medium-distance exploration (Agerer, 2001), which does not form a dense mycelium and is not the most efficient in nutrient uptake when external nutrient concentrations are low (Colpaert et al., 1999). These characteristics may explain the improvement in plant performance verified with the addition of fertiliser, since nutrients are more accessible to the fungus and consequently to the plant. Nevertheless, no significant difference was obtained in N needle concentration between fertilised and non-fertilised *T. terrestris* inoculated plants, suggesting an uptake of other nutrients. Fertilised plants in treatments *P. tinctorius* + *S. citrinum* and *S. bovinus* + *L. laccata* + *L. deterrimus* showed a decrease in plant development compared with the non-fertilised seedlings, which may be related to the significant decrease in the number of ECM fungal tips per m of root. Negative effects or no effect of fertilisation on plants inoculated with ECM fungi have been reported in studies with *L. laccata*, *P. tinctorius*, *Rhizopogon* spp. (Castellano and Molina, 1989) and *Hebeloma cylindrosporum*

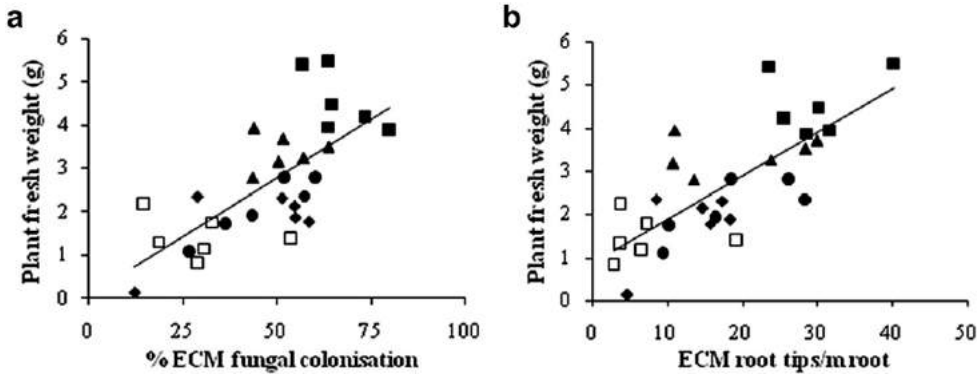

Fig. 6. Relationship between the plant fresh weight of non-fertilised *Pinus pinaster* seedlings and (a) the percentage of ectomycorrhizal fungal colonisation ($y = 0.054x + 0.084$, $R^2 = 0.489$, $P < 0.001$); and (b) the number of ectomycorrhizal root tips per meter of root ($y = 0.101x + 0.872$, $R^2 = 0.579$, $P < 0.001$). Seedlings inoculated with *Thelephora terrestris* (black diamonds), *Rhizopogon vulgaris* (black circles), a mixture of *Pisolithus tinctorius* and *Scleroderma citrinum* (black triangles), a mixture of *Suillus bovinus*, *Laccaria laccata* and *Lactarius deterrimus* (black squares) and non-inoculated control (open squares).

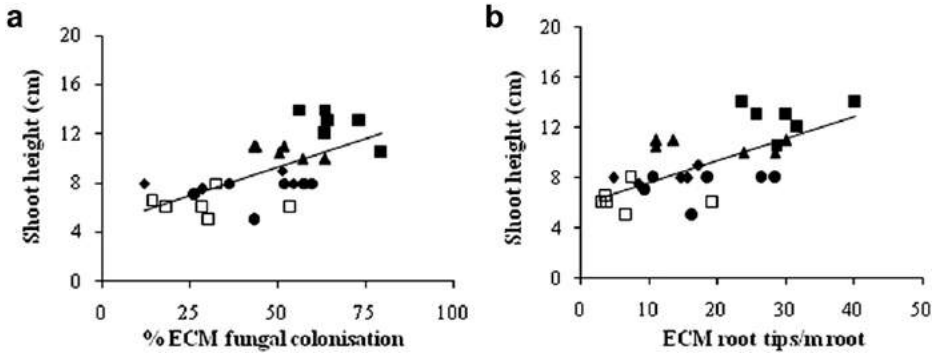

Fig. 7. Relationship between shoot height of non-fertilised *Pinus pinaster* seedlings and (a) the percentage of ectomycorrhizal fungal colonisation ($y = 0.093x + 4.607$, $R^2 = 0.384$, $P < 0.001$) and (b) the number of ectomycorrhizal root tips per meter of root ($y = 0.173x + 5.943$, $R^2 = 0.457$, $P < 0.001$). Seedlings inoculated with *Thelephora terrestris* (black diamonds), *Rhizopogon vulgaris* (black circles), a mixture of *Pisolithus tinctorius* and *Scleroderma citrinum* (black triangles), a mixture of *Suillus bovinus*, *Laccaria laccata* and *Lactarius deterrimus* (black squares) and non-inoculated control (open squares).

(Conjeaud et al., 1996). ECM fungi are especially important for the host plant on soils with low fertility, where the need for their exploring ability promotes plant–fungi association (Castellano and Molina, 1989). Adding nutrients to the soil can implicate a dramatic change on fungal behaviour and also an increase of plant independence towards fungi, not promoting the symbiotic association with a negative effect of fertilisation in plant development.

The majority of morphotypes occurred in both fertilisation treatments. However, plants inoculated with *R. vulgaris* and *S. bovinus* + *L. laccata* + *L. deterrimus* presented one less morphotype than the correspondent non-fertilised ones, indicating that fertiliser might have inhibited ECM formation. These results and the fact that fertilisation had no influence in the percentage of ECM colonisation corroborate the perception that different species respond very differently to fertilisation.

Variation in the percentage of ECM colonisation accounted for 48.9% of the variation in plant fresh weight and 38.4% in shoot height of non-fertilised *P. pinaster*, while variation in the number of ECM fungal tips accounted for 57.9% of the variation in plant fresh weight and 45.7% in plant shoot height of non-fertilised *P. pinaster*. The present data indicate that ECM fungal colonisation influences the growth of *P. pinaster* under nursery conditions. The fact that no correlation was found in fertilised seedlings suggests that when fertiliser is applied growth is more dependent on plant than on fungal mechanisms, since nutrients are more accessible, whereas when no fertiliser is applied, plant development is more dependent on the ECM fungal symbiosis. The overall N needle concentration was strongly consistent with fungal colonisation and plant biomass. Non-fertilised fungal treatments *P. tinctorius* + *S. citrinum* and *S. bovinus* + *L. laccata* + *L. deterrimus*, which presented significantly higher percentage of ECM fungal colonisation than the control plants, also presented higher N needle concentration and higher plant biomass, suggesting that N deficiency was suppressed by the inoculated fungi. Non-fertilised plants inoculated with *T. terrestris* and *R. vulgaris*, although had higher number of morphotypes, presented similar fungal colonisation percentage, similar N needle concentration and similar biomass when compared with the non-inoculated plants. Analogous cohesiveness between the three parameters was observed in fertilised plants, with the exception of the treatment *S. bovinus* + *L. laccata* + *L. deterrimus*, indicating that this fungal mixture is not as efficient in N uptake.

The major advantage of the use of fertilisers in nursery seedlings relies on the supply of nutrients, which speeds their production. However, there are some associated environmental threats, due to the leaching of nutrients, and economical disadvantages, since fertiliser can be an important financial fraction in production costs. In this study plants inoculated with selected ECM fungi had a greater biomass without the application of fertiliser under nursery conditions.

It is also important to investigate whether the behaviour of seedlings will be maintained after transplantation. Field studies conducted on this research topic presented distinct outcomes. Selosse et al. (2000) reported that inoculated *Laccaria bicolor* persisted at least ten years after outplanting and greatly enhanced plant development, whereas the inoculation of Sitka spruce with *L. laccata*, *Hebeloma crustuliniforme* and *Cenococcum geophilum* performed by Shaw et al. (1987) did not promote any nutrient benefits in comparison with non-inoculated seedlings. Quoreshi et al. (2008) and Vosátka et al. (2008) reported that a successful inoculation can be obtained by using more host-specific and site-specific plant–fungal combinations.

5. Conclusion

The application of chemical fertilisers should be minimised as its long-term consequences are unknown. The results from this study show that it is possible to replace chemical fertilisers by ECM fungi in the nursery production of *P. pinaster*, with a significant increase in plant development, and thus the use of selected ECM fungi can be an effective and more environmental friendly approach to plant management in the nursery. Nevertheless, due to the specificity of the ECM associations, further nursery and field studies should be undertaken in order to assess which fungal inoculum and conditions are adequate to produce more resistant and healthier outplanted seedlings.

Acknowledgements

This work was supported by the EU program AGRO (DE&D Action) Projecto AGRO 752, Medida 8 – Desenvolvimento Tecnológico e Demonstração do Programa Operacional Agricultura e Desenvolvimento Rural. The authors wish to thank Fundação para a Ciência e a Tecnologia, POCI 2010 and FSE, Grants SFRH/BD/31250/2006 and SFRH/BPD/23749/2005.

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Journal of Environmental Management 95 (2012) S275–S279

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Differences in nutrient availability and mycorrhizal infectivity in soils invaded by an exotic plant negatively influence the development of indigenous *Acacia* species

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ARTICLE INFO

Article history:
Received 26 August 2009
Received in revised form
23 November 2010
Accepted 29 January 2011
Available online 20 February 2011

Keywords:
Exotic invasive plant
Amaranthus viridis
Soil chemistry
Soil microflora
Arbuscular Mycorrhizal fungi

ABSTRACT

Plant species (exotic invasive *vs* native non-invasive) colonization pattern and the relation with the soil nutrient availability and AM fungi abundance, was investigated. Soil samples were collected from two sites: one invaded by the exotic plant, *Amaranthus viridis*, and one uninvaded site for chemical and AM propagules density analyses. Additionally, we grew five Sahelian *Acacia* species in soil from the two sites, sterilized or not, to test the involvement of soil biota in the invasion process. While nutrient availability was significantly higher in soil samples from the invaded sites, a drastic reduction in AM fungal community density, was observed. Moreover, *Acacia* seedlings' growth was severely reduced in soils invaded by *Amaranthus* and this effect was similar to that of sterilized soil of both origins. The observed growth inhibition was accompanied by reduction of AM colonization and nodulation of the roots. Finally, the influence of soil chemistry and AM symbiosis on exotic plants' invasion processes is discussed.

1. Introduction

The large ecological and economic costs associated with invasion of terrestrial ecosystems by exotic plant species has stimulated great interest in elucidating how invasive plant species influence, and are influenced by, biotic and abiotic interactions (Mummey and Rillig, 2006). While many studies aimed to determine aboveground impacts of exotic plant invasion, it appeared that a wide range of belowground biotic interactions regulate plant distribution and ecosystem function (Bever et al., 1997; Stinson et al., 2006). Hence, understanding interactions between plant and soil microbes are required to fully understand the dynamic of plant community succession in the face of exotic invasive species development (Wolfe and Klironomos, 2005). Recent studies indicate that invasive plant species can impact soil microbial community composition and function, resulting from changes in plant-derivative inputs to the soil (Ehrenfeld et al., 2001; Kourtev et al., 2002; van der Putten et al., 2007), as well as phytochemistry interference (Vivanco et al., 2004; Stinson et al., 2006).

Among soil symbiotic microorganisms, Arbuscular Mycorrhizal (AM) fungi have been found to be essential components of sustainable soil-plant systems. Representing a key interface between the host plant and the soil mineral nutrients, AM fungi is also benefic by enhancing plant resistance to pathogens and other environmental stresses, and improving water relations (Smith and Read, 2008). More recently, evidence of detrimental impact of non-mycorrhizal invasive plants on AM communities has been documented (Vogelsang et al., 2004; Stinson et al., 2006). If persistent, altered microbial community composition and functioning in invaded areas may represent a limiting factor for restoration efforts even after removal of invasive species, i.e. there may be a soil ecological legacy of invasion (Mummey and Rillig, 2006).

Amaranthus viridis L. (Amaranthaceae) is an annual weed native from Central America, that is considered to be an invasive plant (USDA Plant Database), along with nine other *Amaranthus* species. In Senegal, *A. viridis* is found in agrosystems, and increasingly invades fallow lands, areas of pasture, domestic waste deposit areas and its growth is positively correlated to soil fertility (organic matter and nitrogen contents) (Le Bourgeois and Merlier, 1995). In *Amaranthus*-invaded large areas, the survival of native plants (grasses, shrubs and trees including *Acacia* species), is compromised (Sanon et al., 2009). The mechanism underlying *Amaranthus* capacity to enter and proliferate within intact Sahelian native plant

Abbreviation: IRD, Institut de Recherche pour le Développement.

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doi:10.1016/j.jenvman.2011.01.025

communities has not been addressed yet. Moreover, much less is known about the impact of this invasive plant on soil microbiota, particularly AM fungi. *Acacia* species were selected as the bioassay test species because they are leguminous natives from Sahelian regions and are frequently used for soil rehabilitation programs as their rhizobial symbiosis improves soil fertility and, have high economical value.

The aims of this study were: 1) to investigate AM fungal community abundance and nutrient availability in soils invaded or not by *A. viridis*; 2) to study the effect of soil origin on the growth of native *Acacia* species; and 3) to better understand the mechanisms of growth inhibition of native plants by determining whether this inhibition is a microbially-mediated outcome. We hypothesized that *A. viridis* will colonize soil patches with specific properties and that post-alterations of soil properties by *A. viridis* development will ultimately affect the competitive performance of native *Acacia* species.

2. Material and methods

2.1. Field site and sampling design

The study was carried out in the region of Dakar (14°43′ N, 17°26′ W) in Senegal. The climate is sahelian influenced by maritime trade winds alleviating high temperatures and low moisture during dry season. The mean annual temperature is 24 °C and rainfall 300 mm (Gassama-Dia et al., 2003). The soil is sandy (>90% of soil) and representative of a Dior-type tropical ferruginous soil (Alfisol).

The sampling site was located at the IRD experimental station of Bel Air – Dakar. From this site, a sampling area (500 m^2) has been chosen in order to cover the diversity of non-invasive plant species and soil patches completely colonized by the invasive *A. viridis*. Most frequently non-invasive plants recorded were: *Alysicarpus ovalifolius* (Fabaceae; annual herb), *Boerhavia diffusa* L. (Nyctaginaceae; annual herb), *Commelina forskalaei* Vahl (Commelinaceae; annual herb), *Eragrostis tremula* L. (Poaceae; annual herb) with a canopy of hardwood trees including *Acacia* spp, *Leuceuna* spp, *Balanites aegyptiaca*. Six plots (1 m × 1 m each) were randomly chosen in sites entirely colonized by *Amaranthus* for *A. viridis*-invaded soil samples (further called invaded soils) and six other plots in sites dominated by non-invasive plants and without *A. viridis* (further called uninvaded soils). Soil samples (2 kg per plots) were collected from 1 to 15 cm depth layer of the 12 plots and they were sieved (mesh size < 2 mm) to remove coarse roots and debris.

2.2. Chemical properties and chitinase activity

For each type of soil, pH in a soil: water suspension (3/10) was determined. The total organic carbon was measured according to the ANNE method (Aubert, 1978) and the total nitrogen by the Kjeldahl method. The total phosphorus and soluble phosphorus were determined colorimetrically (Murphy and Riley, 1962).

Chitin, a polymer of N-acetyl glucosamine, is an important constituent of fungal structures and its dosage allows estimation of the amount of fungal mycelia in the soil, both viable and non-viable (Plassard et al., 1983), and is commonly used to assess mycorrhizal abundance in soil (Plassard et al., 1983). Enzymatic hydrolysis of chitin is mediated by two hydrolases (chitinase and chitobiase), chitinases being the most common in soil (Rodriguez-Kabana et al., 1983). Chitinase activity was measured following Beam (1971) method. Briefly, the hydrolysis of chitin substrates releases *p*-nitrophenol, which amount was determined by spectrophotometry (Ultraspec 3000, Pharmacia Biotech) at 420 nm and compared with standard curve.

2.3. Mycorrhizal propagules density measurement

Spores of AM fungi were extracted from the soil samples by wet sieving and decanting, followed by sucrose centrifugation (Sieverding, 1991) and recovery of the spores through 50 μm sieving of the supernatant. Spores were counted using a stereomicroscope and grouped according to morphological characteristics: size and color, wall structure and hyphal attachment (Walker, 1983; INVAM, 1997). The different morphotypes were identified to genus.

Hyphae were extracted from the soil samples by aqueous membrane-filtration, and subsequent microscopic examination. The total hyphal length was estimated using the Gridline intersect method (Hanssen et al., 1974). The AM fungi hyphae were distinguished from hyphae of other soil fungi following the morphological criteria described by Nicolson (1959).

2.4. Greenhouse experiments with Acacia species

Soils from the same origin site (invaded *vs* uninvaded) were pooled in the lab and, half of both soil types were sterilized (120 °C, 60 min, 2 cycles) by autoclaving to perform greenhouse experiments with sterile soil as well as native soil from the two origin types. On the four types of soils (invaded sterilized or not and, uninvaded sterilized or not), a single seedling of five different *Acacia* species: *Acacia albida*, *Acacia nilotica* var. *tomentosa*, *Acacia raddiana*, *Acacia senegal*, *Acacia seyal*, was grown in 250 mL PVC tubes. Six replicates for each treatment combination were done (4 × 5 × 6 = 120 tubes). Tubes were randomly placed in the greenhouse and were watered regularly with tap water without fertilizer. After 5 months of growth, shoots, roots, and root nodules were harvested, dried at 60 °C for one week, and weighed to determine biomass. Approximately 1-g subsample of roots from each seedling was extracted, cleared and stained (Phillips and Hayman, 1970) and analyzed for AM colonization percentage.

2.5. Statistical methods

Chemical and biological soil properties data were examined by one-way analysis of variance (ANOVA) and pairwise *t* tests corrected by Bonferroni adjustment method used to compare means (at $P < 0.05$). Computations were performed with the free R software (http://r-project.org).

3. Results

3.1. Soil chemical characteristics and AM inoculum potential

Invaded soils had higher pH, total organic carbon, nitrogen content and phosphorus content than non-invaded sites (Table 1).

Table 1
Chemical characteristics of the soil samples invaded or not by *Amaranthus viridis*.

	Soil origin		Significance of the relations		
	Uninvaded soil	Invaded soil	Df[b]	F value	*P* value
pH	7.8 a[a]	8.4 b	1	33.22	$P < 0.0001$
Total organic carbon (%)	1.2 a	2.5 b	1	247.82	$P < 0.0001$
Total nitrogen (%)	0.1 a	0.2 b	1	33.22	$P = 0.0001$
Total phosphorus (mg kg^{-1})	625.3 a	1675.7 b	1	247.82	$P < 0.0001$
Soluble phosphorus (mg kg^{-1})	107.6 a	211.6 b	1	235.02	$P < 0.0001$

[a] Data in the same line followed by the same letter are not significantly different according to the one-way analysis of variance ($P \leq 0.0001$).
[b] Degree of freedom.

Conversely, we observed significant decrease in all mycorrhizal parameters measured in the soil collected in *A. viridis* stands (Fig. 1). Compared to uninvaded soils, spore numbers were reduced 2.69 fold in invaded ones (Df = 1; F value = 64.25; *P* value = 0.000012). AM hyphal length decreased severely in the invaded soils compared to uninvaded ones, with values decreasing from 3.2 to 0.8 m g^{-1} dry soil for uninvaded and invaded sites, respectively (Df = 1; F value = 53.05; *P* value = 0.000027). The same pattern was observed for chitinase activity (Df = 1; F value = 39.87; *P* value = 0.000087) (Fig. 1).

Four AM species were detected in the soils: *Glomus* sp. strain 1 [81 and 83% of the total number of spores recorded for invaded and uninvaded soils, respectively]; *Glomus* sp. strain 2 [4 and 4.5% for invaded and uninvaded soils, respectively]; *Scutellospora* sp. strain 1 [10.5 and 8.9% for invaded and uninvaded soils, respectively]; and *Scutellospora* sp. strain 2 [5.1 and 3.6% for invaded and uninvaded soils, respectively]. Nevertheless, no significant differences were found between the distributions of AM species within the two soil origins ($\chi^2 = 28.7$ [$P = 0.3$]).

3.2. Acacia species growth in greenhouse experiment

Acacia species development (biomass), root AM colonization and nodulation were strongly affected by the soil origin. Growth of *Acacia albida*, *A. nilotica A. senegal* and *A. seyal* seedlings were significantly higher when plants were grown in uninvaded soils ($P < 0.05$), similarly root AM colonization and nodulation were higher. No significant difference was recorded for *A. raddiana* growth efficiency despite significantly higher AM colonization rates and nodulation for plants grown in uninvaded soil. The negative effect of invaded soil on the growth of these plants was similar to that observed when seedlings were grown in sterilized soil from both uninvaded and invaded soils (Table 2).

4. Discussion

4.1. Evidence for alterations in soil chemistry and AM fungal community

Our results clearly indicate that the exotic plant species, *A. viridis*, exerts a positive effect on soil nutrient content by increasing carbon, nitrogen and phosphorus concentrations. In the present study, changes in plant-derivative inputs and mineralization dynamic could have induced changes in nutrients cycling. Previous results have reported that invasion by exotic plant species could result in higher carbon and nutrient (N and P) pools in soil (Ehrenfeld et al., 2001; Kourtev et al., 2002; Ehrenfeld, 2003). Indeed, exotics have been described to increase standing crop biomass and net primary production (Ehrenfeld, 2003). Differences in the litterfall mass interact with differences in the decomposition rate to affect the net flux of C and nutrient into the soil. Also, the litter of many exotic plants decomposes more rapidly than litter of native plants (Ehrenfeld, 2003). In our experimental site, the similar pattern was observed when comparing decomposition rate (i.e. by comparing the mass of plant litter that remains on the soil surface) between *A. viridis* and other non-invasive plants (Duponnois R., unpublished data).

The higher pH recorded in soil samples collected from *A. viridis* stands could result from soil heterogeneity; but certain processes mediated by plants, including rapid uptake of nitrate (NO_3^-) and/or higher content of base cations in the litter returning to soil (Ehrenfeld et al., 2001) could also increase it. However, the mechanism driving the change remains unclear and further analyses regarding soil chemical properties that may affect soil pH such as [NO_3^-], [NH_4^+], nitrification rates or base cations concentration in the litter must be undertaken to support our hypothesis.

Additionally, important modifications have been recorded in soil bacterial community composition and enzyme activities in soils colonized by *A. viridis* (Sanon et al., 2009). These alterations in microbial functioning could also interact with nutrient cycling and might profoundly modify nutrient availability in soils.

Invasive plants affect the AM fungal community in ways that may create a plant-soil biota feedback facilitating invasion and altering native communities. It has been previously observed that mycorrhizal fungi could facilitate exotic plant invasions by increasing the competitive dominance of mycotrophic invasives (Richardson et al., 1994; Fumanal et al., 2006). Conversely, disruption of these mutualistic associations between native plants and AM fungi has been recorded as the main mechanism facilitating invasion of non-mycorrhizal plants (Mummey and Rillig, 2006; Stinson et al., 2006). In our study, a high reduction in mycorrhizal soil infectivity in soils collected from invaded areas was recorded as well as reduction of AM colonization of *Acacia* species roots grown in the same soil. Accordingly, the genus *Amaranthus* is generally thought as non-mycorrhizal (Vierheilig and Ocampo, 1990) and, in a greenhouse experiment where we assessed the mycorrhizal dependency of *A. viridis* following inoculation with different amounts of AM propagules (0; 3; 10; 30; 100), we observed a negative correlation between seedling growth and quantities of AM propagules inoculated (Sanon et al., 2009).

Therefore, the dominance of *A. viridis* may ultimately result in reduced densities of AM fungal community in invaded sites ('*The Degraded Mutualisms Hypothesis*'; Vogelsang et al., 2004) relative to sites covered by non-invasive mycotrophic plant species, as *A. viridis* may not contribute to multiplication of AM propagules. In addition, reduction in AM community could even result from high phosphorus content in soil (Smith and Read, 2008) as that recorded in invaded sites. AM fungal community degradation by

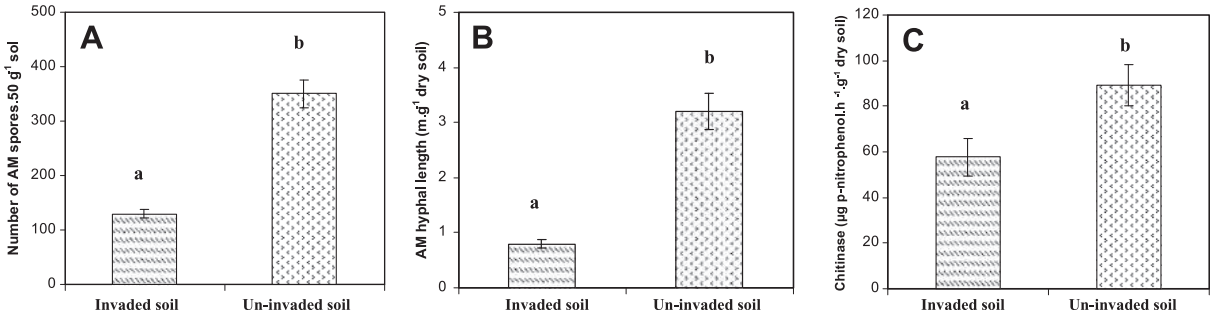

Fig. 1. AM spore density (A), AM hyphal length (B) and chitinase activity (C) in soils from invaded and uninvaded areas. For each soil property, bars indexed by different letters are significantly different ($P < 0.05$).

Table 2
Growth response, AM colonization and nodule biomass of *Acacia* species seedlings grown in soils invaded or uninvaded by *Amaranthus viridis* (adapted from Sanon et al., 2009).

	Invaded by *A. viridis*		Uninvaded by *A. viridis*	
	Sterilized	Un-sterilized	Sterilized	Un-sterilized
A. albida				
Plant total biomass (mg dry weight)	768.5 a[a]	847 a	891.8 a	1065.1 b
Root AM colonization (%)	–	29.8 a	–	83.7 b
Nodule biomass (mg dry weight)	–	6.9 a	–	17.5 b
A. nilotica				
Plant total biomass (mg dry weight)	2125 a	3131 b	2713.4 b	3548.4 c
Root AM colonization (%)	–	28.3 a	–	51.8 b
Nodule biomass (mg dry weight)	–	69.7 a	–	112.8 b
A. raddiana				
Plant total biomass (mg dry weight)	163 a	144.7 a	216.6 a	261.8 a
Root AM colonization (%)	–	9.67 a	–	20.8 b
Nodule biomass (mg dry weight)	–	0 a	–	8 b
A. senegal				
Plant total biomass (mg dry weight)	396.7 a	387.4 a	415.1 a	493.4 b
Root AM colonization (%)	–	33.1 a	–	75.5 b
Nodule biomass (mg dry weight)	–	2.3 a	–	8.8 b
A. seyal				
Plant total biomass (mg dry weight)	2150 a	3000 b	2720 b	3916.8 c
Root AM colonization (%)	–	28.3 a	–	51.8 b
Nodule biomass (mg dry weight)	–	69.7 a	–	112.8 b

[a] Data in the same line followed by the same letter are not significantly different according to the one-way analysis of variance ($P < 0.05$).

chemical inhibition might also be investigated as this pathway has ever been reported for other exotic invasives (Vivanco et al., 2004; Stinson et al., 2006).

Reductions in *Acacia* species nodulation were recorded in the greenhouse experiment. Importantly, when we studied in Petri dishes the effect of *A. viridis* aqueous extract on the growth of 30 strains of rhizobia originating from different areas of Africa, we observed drastic inhibition of rhizobial growth (Sanon et al., 2009). Such inhibition effect has also been observed on *Acacia sephorae* nodulation upon the invasion of bitou bush (*Chrysanthemoides monilifera* spp. *rotundata*) (Vranjic et al., 2000).

4.2. Implications for Acacia species growth reduction

Results of the greenhouse experiment indicated a significant growth reduction when seedlings were grown in invaded soil, sterilized or not, and in sterilized uninvaded soil. Additionally, these growth declines were accompanied by critical reductions of plant root colonization by AM fungi. The reduction in seedlings growth when they were grown in invaded soil is similar to that observed when seedlings were grown in sterilized soil from both invaded and uninvaded sites strongly supporting that the mechanism by which *A. viridis* suppresses the growth of native tree species is microbially-mediated. Our results thus corroborate the previous observations made by Stinson et al. (2006) who documented that the invasive plant, *Alliaria petiolata*, suppresses the growth of native tree seedlings through interference with soil biota.

The initial establishment of the exotic plant could therefore be facilitated by soil disturbance sufficient to reduce AM fungal density, thereby giving invasive species a competitive advantage over indigenous *Acacia* species (Vogelsang et al., 2004), which largely rely on mycorrhizal association for their development (Ducousso and Thoen, 1991).

Afterward, it could be expected that successive growth of *A. viridis* on certain soil patches during several rainy seasons could then generate a '*novel ecological niche*' on these sites, which might favor the exotic invasive plant's own fitness relative to that of native species (Bever et al., 1997; Klironomos, 2002). Additionally, the reduction in *Acacia* seedling nodulation induced by *A. viridis* might also contribute to reduce *Acacia* seedling growth.

These results support the evidence of additional mechanisms by which soil biota could shape plant invasion processes, in addition to the enemy-escape hypothesis, and in addition to other non-microbial mediators (e.g. direct plant–plant allelopathy interference, herbivory, etc.).

5. Conclusion

Our studies suggest that reduced dependence on the mycorrhizal mutualism might be one of the mechanisms underlying the success of the invasive plant, *A. viridis*, within the area studied. This invasive might preferentially colonize certain soil patches or transform soil attributes in such a way that native species are broadly disadvantaged. Importantly, our results suggest tight plant-mediated relationships between soil fertility, mutualistic AM fungi and, invasion processes in a Sahelian ecosystem where man-made disturbances (fertilization, tillage, pesticides, monocultures, …) ultimately result in an increase in nutrient availability and in AM inoculum degradation in ecosystems. These results might be of crucial importance for invaded areas' restoration because they highlight the necessity to protect mycorrhizal symbionts that could promote native plants performance and preserve biodiversity.

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Journal of Environmental Management 95 (2012) S280–S284

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Isolation and identification of actinomycetes from a compost-amended soil with potential as biocontrol agents

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ARTICLE INFO

Article history:
Received 17 August 2009
Received in revised form
28 April 2010
Accepted 29 November 2010
Available online 28 December 2010

Keywords:
Streptomyces
Lechevalieria
Phytopathogenic fungi
Composted two-phase olive mill waste

ABSTRACT

The search for new biocontrol strategies to inhibit the growth of phytopathogenic microorganisms has become widely widespread due to environmental concerns. Among actinomycetes, *Streptomyces* species have been extensively studied since they have been recognized as important sources of antibiotics. Actinomycete strains were isolated from a calcareous soil, 2 two-phase olive mill waste ('alperujo') composts, and the compost-amended soil by using selective media, and they were then co-cultured with 5 phytopathogenic fungi and 1 bacterium to perform an *in vitro* antagonism assay. Forty-nine actinomycete strains were isolated, 12 of them showing a great antagonistic activity towards the phytopathogenic microorganisms tested. Isolated strains were identified by 16S rDNA sequence analysis and phenotypic procedures. Eleven isolates concerned the genus *Streptomyces* and 1 actinomycete with chitinolytic activity belonged to the genus *Lechevalieria*.

1. Introduction

Composted materials have traditionally been applied to agricultural and horticultural soils as means of improving soil fertility and crop growth, mainly by enhancing soil physical and chemical properties. These organic amendments also contribute to improve soil biological characteristics, often providing an effective control of soil-borne diseases (Vargas-García and Suárez-Estrella, 2008).

Compost capability to suppress soil-borne plant pathogens has become an interesting subject as a strategy for reducing the adverse effects of massive fungicides' applications on the environment. Several mechanisms have been proposed to explain plant disease control by compost such as competition, hyperparasitism, activation of disease-resistance genes, or antibiotic production by beneficial microorganisms (Hoitink and Boehm, 1999; Maher et al., 2008).

In this context, actinomycetes have received considerable attention as biocontrol agents, particularly *Streptomyces* species. *Streptomyces* are Gram-positive aerobic members of the order Actinomycetales within the classis Actinobacteria which produce an extensive branching substrate and aerial mycelium (Anderson and Wellington, 2001). Since the first antibiotic discovery in 1942, there have been continued efforts towards screening compounds from the genus *Streptomyces* (Watve et al., 2001) which is known to be the largest antibiotic-producing genus. In fact, about 60% of the antibiotics developed for agriculture and horticulture have been isolated from *Streptomyces* spp. (Hwang et al., 2001). *Lechevalieria* (formerly classified as *Saccharothrix*), like *Streptomyces*, are Gram-positive aerobic members of the order Actinomycetales within the classis Actinobacteria (Labeda et al., 2001). Until now, only one *Lechevalieria* species has been described as antibiotic producer (Onaka, 2009). In addition, no chitinolytic activity has been reported for this genus.

The aim of this investigation was to isolate actinomycetes from a soil, 2 two-phase olive mill waste ('alperujo') composts, and a compost-amended soil to perform an *in vitro* antagonism assay, and to identify the actinomycete strains with the highest inhibitory activity.

2. Materials and methods

2.1. Sample collection

Actinomycetes were isolated from one calcareous soil located at Lliria (Valencia, Spain), −39°45′04″ in latitude and 0°41′10″ in longitude, 2 composts prepared from two-phase olive mill waste (85% by dry weight) mixed with fresh horse manure (15% dry wt) – one irrigated with water and the other with an animal fatty proteinaceous waste sludge during the first 38 days of composting,

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doi:10.1016/j.jenvman.2010.11.023

and the soil amended with each of the composts at two rates (12 Mg ha^{-1} and 24 Mg ha^{-1}).

2.2. Isolation of actinomycete strains

The samples were firstly homogenized in buffered peptone water at 100 rpm for 30 min, serially diluted, and cultured on starch casein agar (SCA), arginine glycerol salts agar (AGSA), and glycerol asparagine agar (International *Streptomyces* Medium No. 5 [ISP-5]) (Shirling and Gottlieb, 1966), all supplemented with cyclohexamide (50 mg L^{-1}) to reduce fungal contamination (Labeda and Shearer, 1990). Sample dilution plates were incubated at 28 °C for 14–21 days until sporulated or non-sporulated actinomycete colonies were observed. Selected colonies were then inoculated onto yeast extract–malt extract agar (ISP-2) for purification and stored at 4 °C in slant agar and in 20% glycerol at −80 °C.

2.3. Phytopathogenic strains

To assess the actinomycete potential suppressive effects, 5 phytopathogenic fungi – *Fusarium oxysporum* f. sp. *melonis* (CECT 20474), *Phytophthora cinnamomi* (CECT 20186), *Pythium debaryanum* (CECT 2362), *Sclerotinia sclerotiorum* (CECT 2823), and *Thanatephorus cucumeris* (CECT 2813) – and 1 bacterium – *Agrobacterium tumefaciens* (CECT 4119) – obtained from the "Colección Española de Cultivos Tipo" (CECT) were included in the *in vitro* antagonism experiment. Previous experiments using six different culture media showed that the maximum antifungal activities of actinomycetes were obtained with the potato dextrose agar (PDA) and the yeast-malt extract agar (YMA) (data not shown). Then, fungi strains were incubated at 28 °C on PDA and YMA media for 5–7 days. The *A. tumefaciens* strain was cultured on nutrient agar (NA) at the same temperature for 24 h.

2.4. Fungal antagonism assay

With the aim of obtaining active growing microorganisms, actinomycete strains were incubated on PDA (Castillo et al., 2002; Getha et al., 2005) and YMA (Gomes et al., 2000) at 28 °C for 7 days or until sporulation was detected. From these media, 4 agar plugs of 6 mm diameter (corresponding to 4 different isolated strains) were transferred at equidistant positions to Petri dishes containing YMA and PDA, and incubated for 7 days at 28 °C. After this period, a plug with fungal mycelia was placed in the centre of the plate and co-cultured at 28 °C for 7–14 days. Antagonism was determined by measuring the distance between the growing edges of actinomycetes and fungi, hence establishing 4 levels of inhibition: maximum (+++), scored as an inhibition distance >2 cm, intermediate (++) as a distance of 2–1 cm, minimum (+) as a measurement <1 cm, and no antagonism (−) when contact between actinomycetes and fungi occurred. Two replicates were carried out for each of the presumptive antagonist strains and the phytopathogenic fungi.

2.5. Bacterial antagonism assay

Prior to the test, *A. tumefaciens* was grown on NA (see Section 2.3) and suspended in sterile nutrient broth (NB) with a concentration adjusted to approximately 1.5×10^8 CFU mL^{-1}. This bacterial inoculum was homogenized and serially diluted to 1.5×10^7, 1.5×10^6 and 1.5×10^5 CFU mL^{-1}, respectively. One millilitre of the resulting suspensions was added to a test tube containing 19 mL liquid sterile NA at ca. 50 °C, vortexed, and transferred to Petri dishes. Once solidified, 6 plugs (6 mm in diameter) of actinomycetes grown on YMA and PDA were equidistantly positioned on each inoculated medium and incubated at 28 °C. After 24 and 48 h of incubation, inhibition zones were measured as the radial distance between bacterial and actinomycete growth.

2.6. Identification of antagonistic actinomycete strains

Actinomycete strains which showed the highest inhibitory effects towards the phytopathogenic microorganisms tested were selected to perform their identification by using molecular and phenotypic procedures.

Total genomic DNA was extracted according to the CTAB (cetyltrimethylammonium bromide, Sigma) procedure (Wilson, 1987) and subsequently subjected to PCR (polymerase chain reaction) amplification using primers 27f and 1525r as described by Lane (1991). Each 50 μL PCR contained 1 μL DNA extract, 1.5 μM $MgCl_2$, 0.2 mM of each dNTPs (Ecogen), 0.4 μM of each primer, and 1.5 U *Taq* DNA polymerase (Ecogen) with 1× PCR buffer. Amplification was performed in a PTC-100 Peltier Thermal Cycler using the program: initial denaturation at 95 °C for 5 min and 30 cycles at 95 °C for 1 min; annealing at 54 °C for 1 min; and, primer extension at 72 °C for 1 min followed by a final extension at 72 °C for 10 min. Controls – where template DNA was replaced by sterile water– were also included in each PCR experiment. The PCR product was purified with the GenElute PCR Clean-up Kit (Sigma) with the same sets of primers. The 16S rDNA gene sequences were obtained using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit (version 3.1) and the automatic sequencer Applied Biosystems 3730xl DNA

Table 1
Antagonistic activity of the actinomycete strains on YMA and PDA culture media.

Isolate	*Fusarium oxysporum* f. sp. *melonis*		*Phytophthora cinnamomi*		*Sclerotinia sclerotiorum*		*Pythium debaryanum*		*Thanatephorus cucumeris*	
	YMA	PDA	YMA	PDA	YMA	PDA	YMA	PDA	YMA	PDA
CO2-9	–[a]	–	++	+	–	–	–	–	++	+++
CO2-16	++	++	+++	+++	+	+++	+++	+++	+++	+++
S-1	+++	+++	+++	+++	++	+++	+++	+++	+++	+++
S-2	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
S-3	+++	+++	+++	+++	++	+++	+++	+++	+++	+++
S-5	++	++	++	++	+	++	+	++	+++	+++
S-6	+++	+++	+++	+++	+++	+++	+	+++	+++	+++
S-7	–	–	–	–	–	++	+++	+++	+++	+++
T2-10	–	–	+++	+++	–	–	–	–	++	++
T2-19	–	–	++	++	+	–	+	+	+	+
T6-32	–	–	++	–	++	–	++	–	+	–
T8-2	–	+	–	+	–	+	–	+	++	++

[a] –, +, ++, +++: no inhibition, minimum, intermediate and maximum antagonism, respectively.

Table 2
Width (mm) of the inhibition zone between *Agrobacterium tumefaciens* (at three concentrations) and actinomycete isolates grown on YMA and PDA media for 48 h.

Isolate	Bacterial inoculum concentration (CFU mL^{-1})					
	1.5×10^7		1.5×10^6		1.5×10^5	
	YMA	PDA	YMA	PDA	YMA	PDA
S-6	3	0	4	0	6	0
T2-10	2	2	3.5	3.5	2	3.5
T8-2	1	3	5	5	1	2

Analyzer. The 16S rDNA gene sequences were manually assembled from the combination of separate fragments generated with forward and reverse sequencing primers using the PHYDIT program (Chun, 1995). The sequences were presumptively identified using the BLAST (Basic Local Alignment Tool) program (NCBI; http://www.ncbi.nlm.nih.gov/). The almost complete sequences were aligned against sequences of reference strains. Phylogenetic trees were inferred using the neighbour-joining algorithm (Saitou and Nei, 1987) from the PHYLIP suite programs (Felsenstein, 1993), and evolutionary distance matrices prepared after Jukes and Cantor (1969). The topologies of the resultant unrooted trees were evaluated in a bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings of the neighbour-joining dataset using the PHYLIP package.

Diaminopimelic acid isomers from whole-cell extracts were determined on ISP-2 cultured actinomycete strains (5 days at 28 °C) according to standard procedures (Staneck and Roberts, 1974). Aerial spore-mass colour, substrate mycelial pigmentation, diffusible pigments, and melanin production were recorded on the International *Streptomyces* Project culture media (Shirling and Gottlieb, 1966) after 14 days of incubation at 28 °C. Spore-chains morphology from cultures incubated for 10–14 days on ISP-5 were observed by light microscopy.

Chitinase activity was determined by streaking actinomycete strains on a culture medium as described by Kawase et al. (2004). Chitinase production was assessed by visual examination of cleared zones developed around colonies incubated for 7 and 14 days.

3. Results and discussion

3.1. Isolation of actinomycetes

A total of 49 actinomycete strains were isolated from the composts, soil and amended soil samples using the selective media SCA, AGSA and ISP-5, and included in the *in vitro* screen against the 6 phytopathogenic strains.

3.2. Fungal antagonism assay

From the *in vitro* assays, 12 actinomycete strains were found to be highly effective in the suppression of most of the 5 reference phytopathogenic fungi tested, thus providing 24.5% isolates with strong inhibitory effects (Table 1). This percentage is similar to those reported by other authors (Larkin and Fravel, 1998). It is remarkable that 7 strains (CO2-16, S-1, S-2, S-3, S-5, S-6, and T8-2) showed high antifungal activity against the 5 pathogenic strains, whereas antagonistic activity towards 2–4 fungal strains was observed in the 5 remaining actinomycete isolates (CO2-9, S-7, T2-10, T2-19, and T6-32).

3.3. Bacterial antagonism assay

The assay with *A. tumefaciens* disclosed that 3 actinomycete strains – S-6, T2-10, and T8-2 – had antimicrobial activity against this phytopathogenic species. Inhibition radial zones controlled after 48 h of incubation were wider than those recorded after incubation during 24 h, thus suggesting that actinomycetes were still producing antibiotic compounds. The distance between actinomycetes and bacterial growth after 48 h is presented in Table 2.

3.4. Identification of antagonistic actinomycete strains

After studying the morphology and pigmentation properties of colonies, all isolates – except the strain T2-19 – were presumptively assigned to the genus *Streptomyces* (Table 3). As shown in Fig. 1, the identification achieved from 16S rDNA gene sequences revealed that 11 actinomycete isolates belonged to the genus *Streptomyces* with 8 different species being represented: 3 strains were identified as *Streptomyces variegatus* (S-1, S-2, and S-3; Fig. 1d), 2 as *Streptomyces griseoruber* (S-5 and T6-32; Fig. 1f), 1 as *Streptomyces lincolnensis* (CO2-9; Fig. 1f), 1 as *Streptomyces lusitanus* (S-6; Fig. 1c), 1 as *Streptomyces aureoverticillatus* (CO2-16; Fig. 1e), 1 as *Streptomyces olivochromogenes* (S-7; Fig. 1b), 1 as *Streptomyces coeruleorubidus* (T2-10; Fig. 1a), and 1 as *Streptomyces albogriseolus* (T8-2; Fig. 1f). Nucleotide similarities between the *Streptomyces* isolated and the corresponding reference strains (Fig. 1) ranged from 99.43% for S-1 to 100% for T2-10 and T8-2. These results support that streptomycetes have been investigated predominantly as biocontrol agents, since they are frequently and easily isolated, and their antibiotics' production arouses significant commercial interest (Anderson and Wellington, 2001).

Strain T2-19 (accession number FN808347) was the only non-streptomycete isolate belonging to genus *Lechevalieria.* Phylogenetic position of strain T2-19 (Fig. 2) was between *Lechevalieria*

Table 3
Identification of actinomycete strains by using 16S rDNA analysis and phenotypic methods.

Isolate	Actinomycete species	DAP isomer	Aerial spore-mass colour	Melanin production	Diffusible pigment production	Spore chains morphology	Chitinase production
CO2-9	*S. lincolnensis*	L	Light green	+	+	RF[a]	–
CO2-16	*S. aureoverticillatus*	L	Dark orange	+	–	RF	–
S-1	*S. variegatus*	L	Dark orange	+	–	RF	–
S-2	*S. variegatus*	L	Orange-red	+	–	RF	–
S-3	*S. variegatus*	L	Light orange	+	–	RF	–
S-5	*S. griseoruber*	L	Blue-green	+	+	S	–
S-6	*S. lusitanus*	L	Dark yellow	–	–	RF	–
S-7	*S. olivochromogenes*	L	Light grey	–	–	S	–
T2-10	*S. coeruleorubidus*	L	White green	+	+	S	–
T2-19	*Lechevalieria* sp.	*meso*	na	–	–	na	+
T6-32	*S. griseoruber*	L	Blue-green	–	+	S	–
T8-2	*S. albogriseolus*	L	Blue-green	+	–	S	–

[a] RF, S: Rectus-Flexibilis or Spira, respectively. na: not applicable.

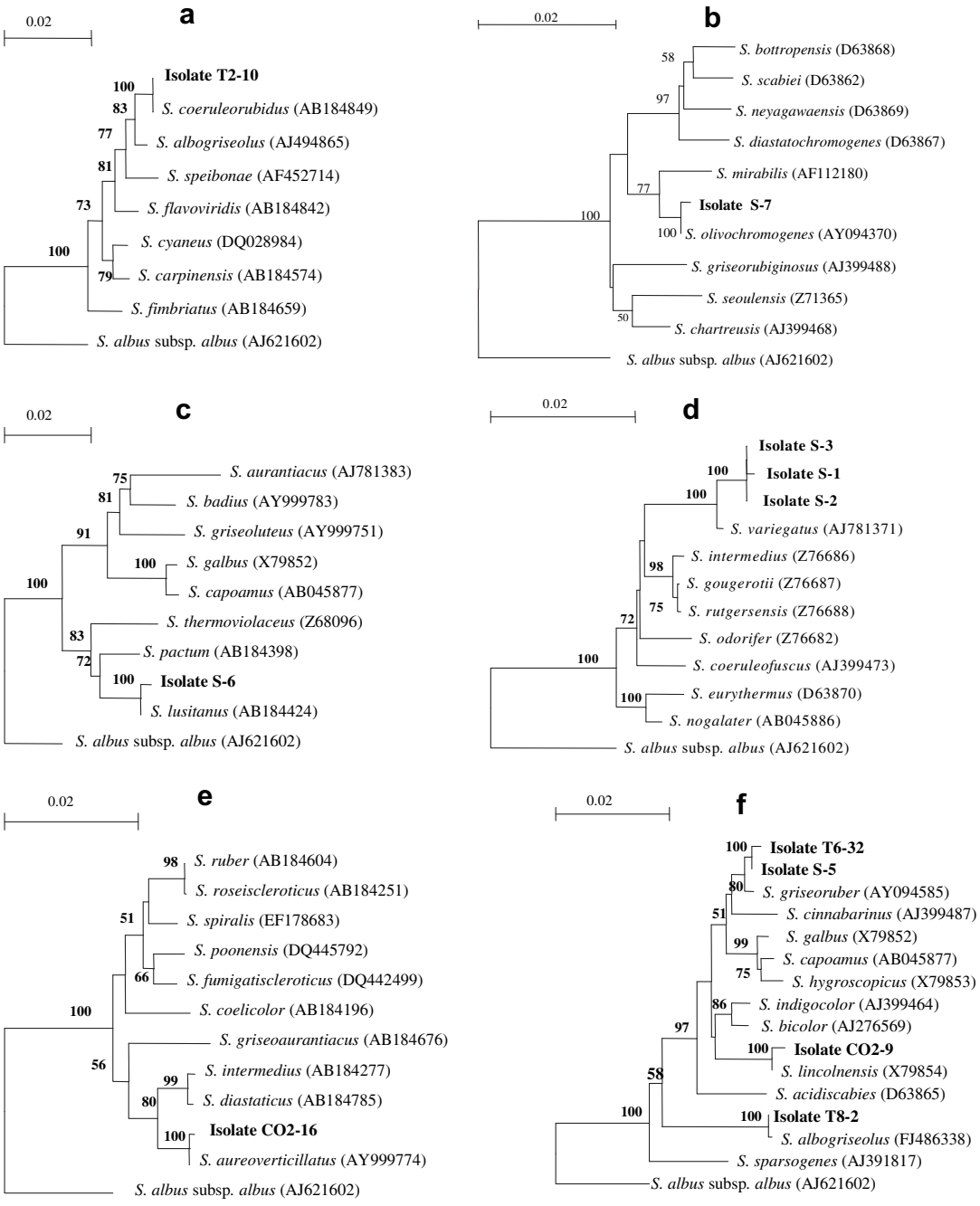

Fig. 1. Neighbour-joining tree based on nearly complete 16S rDNA gene sequences showing relationships between *Streptomyces* isolates and related *Streptomyces* type strain species. Numbers at nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets; only values >50% are given. The scale bar indicates 0.02 substitution position.

xinjiangensis (Wang et al., 2007) and the cluster made up of *Lechevalieria atacamensis*, *Lechevalieria roselyniae*, and *Lechevalieria deserti* (Okoro et al., 2010). Sequence similarities between strain T2-19 and *L. xinjiangensis*, *L. atacamensis*, *L. roselyniae*, and *L. deserti* were 99.19%, 99.41%, 99.19%, and 99.04%, respectively. Although the most related reference strain is *L. atacamensis* (99.41%), DNA:DNA hybridization and accurate polyphasic taxonomy as well as phenotypic descriptions will be carried out in the near future in order to clarify the taxonomic position of T2-19 strain.

Concerning the phenotypic characteristics studied (Table 3), actinomycete isolates were Gram+, aerobic, and with no mycelium fragmentation. *Streptomyces* strains produced moderate to abundant aerial hyphae, whereas isolate T2-19 showed a slight aerial mycelium development. In addition, the peptidoglycan layer in streptomycetes contained mainly L-diaminopimelic acid (cell wall type I), while *meso*-diaminopimelic acid (cell wall type III) was detected in *Lechevalieria* T2-19. Diffusible pigments were produced by strains CO2-9, S-5, T2-10, and T8-2; melanin production was detected by the brown pigmentation of ISP-6 culture medium in 8 strains (CO2-9, CO2-16, S-1, S-2, S-3, S-5, T2-10, and T8-2). According to the morphology of the spore chains observed under light microscopy, CO2-9, CO2-16, S-1, S-2, S-3, and S-6 were grouped as Rectus-Flexibilis (RF), and S-5, S-7, T2-10, T6-32, and T8-2 as Spira (S), whereas strain T2-19 did not produce aerial mycelium.

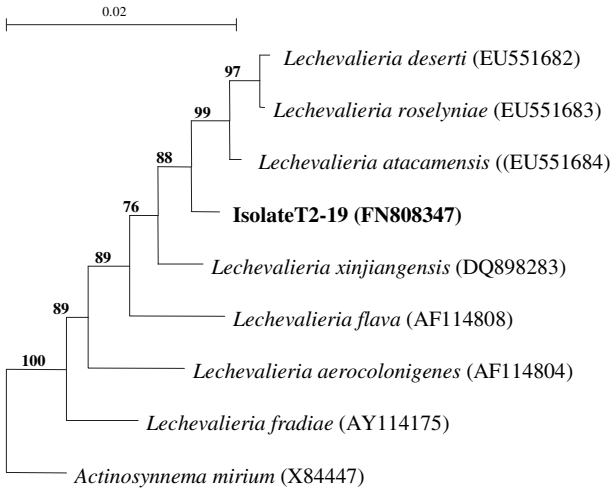

Fig. 2. Neighbour-joining tree based on nearly complete 16S rDNA gene sequences showing relationships between isolate T2-19 and the *Lechevalieria* type strain species. Other details are given in the legend of Fig. 1.

Regarding chitinolitic activity, strain T2-19 was the only one capable to degrade colloidal chitin. Nevertheless, chitinase synthesis seems not to be the main fungi-inhibiting mechanism of *Lechevalieria* T2-19 since it inhibited mainly the oomycete *P. cinnamomi* and *P. debaryanum* (Table 1) which do not have chitin in their cell walls. The antifungal activities showed by the *Streptomyces* and the *Lechevalieria* isolated in this study were probably due to the synthesis of antibiotic compounds.

4. Conclusions

The *in vitro* assay carried out showed that nearly 25% of the actinomycete strains exhibited high activity to suppress the phytopathogenic microorganisms tested, a rate which is similar to the percentages reported by several studies. This rate of effective antagonists indicates that both the isolation media and the culture media used in the *in vitro* experiment were appropriately selected. In addition, the analysis of 16S rDNA sequences provided a rapid and reliable identification of the actinomycete strains, and it also produced results which were in line with the phenotypic properties observed for the isolates. Further research should focus on *in vivo* assays with the potential biocontrol isolates obtained, especially *Lechevalieria* sp. (strain T2-19), to develop an effective biological control strategy in commercial crop production systems.

Acknowledgements

This research was carried out within the framework of the project UPV-PPI-05-05-5542 subsidized by the Polytechnic University of Valencia (UPV) and a PhD fellowship supported by the Valencian Council of Education.

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Journal of Environmental Management 95 (2012) S285–S290

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Influence of xenobiotic contaminants on landfill soil microbial activity and diversity

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ARTICLE INFO

Article history:
Received 30 September 2009
Received in revised form
13 May 2010
Accepted 12 July 2010
Available online 17 August 2010

Keywords:
Landfill pollutants
Microbial activity
DGGE profiles
Phylogenetic analysis

ABSTRACT

Landfills are often the final recipient of a range of environmentally important contaminants such as hydrocarbons, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). In this study the influence of these contaminants on microbial activity and diversity was assessed in a municipal solid waste (MSW) landfill placed in Torrejón de Ardoz (Madrid, Spain).

Soil samples were collected from four selected areas (T2, T2B, T8 and T9) in which the amount of total hydrocarbons, PAHs and PCBs were measured. Soil biomass, substrate induced respiration (SIR) and physiological profiles of soil samples were also determined and used as indicators of total microbial activity.

Highest concentration of total hydrocarbons was detected in T2 and T9 samples, with both PCBs and benzopyrene being detected in T9 sample. Results corresponding to microbial estimation (viable bacteria and fungi, and SIR) and microbiological enzyme activities showed that highest values corresponded to areas with the lowest concentration of hydrocarbons (T2B and T8). It is noticeable that in such areas was detected the lowest concentration of the pollutants PAHs and PCBs. A negative significant correlation between soil hydrocarbons concentration and SIR, total bacteria and fungi counts and most of the enzyme activities determined was established. DGGE analysis was also carried out to determine the microbial communities' structure in the soil samples, establishing different profiles of *Bacteria* and *Archaea* communities in each analysed area. Through the statistical analysis a significant negative correlation was only found for *Bacteria* domain when Shannon index and hydrocarbon concentration were correlated. In addition, a bacterial 16S rRNA gene based clone library was prepared from each soil. From the clones analysed in the samples, the majority corresponded to *Proteobacteria*, followed by *Acidobacteria* and *Actinobacteria*. It is important to remark that the most polluted sample (T9) showed the lowest microbial diversity only formed by six phyla being *Proteobacteria* and *Acidobacteria* the most representative.

1. Introduction

In Spain the most habitual practice for elimination of urban solid wastes has been its storage in municipal solid waste landfills. Although many old landfills are now sealed, these landfills continue to be potentially sources of significant environmental contamination. Moreover, the generation of contaminated leachate remains as inevitable consequence of the practice of waste disposal in landfills (Banar et al., 2006), with groundwater pollution being the most significant concern arising from leachate migration (El-Fadel et al., 1997). Degradation of the old landfill material is a slow process, lasting over 30 years.

Among the most hazardous compounds accumulated in soil and leachate, heavy metals, nitroaromatic compounds (NACs,) polycyclic aromatics (PAHs), and pesticides are the most dangerous. These compounds originated from household and industrial wastes can be found in most municipal landfills. Depending on their composition and on soil characteristics, these products may cause dramatic changes in aquifer geochemistry and landfill microbiology (Röling et al., 2001; Sastre et al., 2003).

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doi:10.1016/j.jenvman.2010.07.017

Table 1
Concentration of total (aliphatic and aromatic) hydrocarbons, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) in soil samples of Torrejón de Ardoz landfill.

Sample	Total hydrocarbons (ppm)	PAHs (ppm)	PCBs (ppm)
T2	165.60 ± 4.28	0.21 ± 0.01	0.22 ± 0.01
T2B	84.52 ± 1.80	0.16 ± 0.01	0.057 ± 0.01
T8	54.48 ± 1.34	0.20 ± 0.01	< 0.04
T9	189.91 ± 4.11	11.06 ± 0.2	4.14 ± 0.12

Through knowledge of microbial community structure in polluted landfills, the capabilities of the microbial populations and their effect on the environment may be used as tools for predicting and monitoring natural degradation (Jain et al., 2005). Although bacteria capable of degrading pollutants usually play central roles in bioremediation, other organisms (*i.e.* fungi, protozoa and plants) can also affect the process (Demmerová et al., 2005). Techniques used to study landfill microbiology include traditional and emerging genetic molecular tools (Arias et al., 2005). The genetic diversity of microbial communities may now be monitored using profiling techniques such as denaturing gradient gel electrophoresis (DGGE). This technique allows the analysis of many samples simultaneously, and provides information relating to microbial communities. The construction of clone libraries and the sequencing of 16S rRNA gene sequences is another molecular technique frequently used to obtain phylogenetic information of the microorganisms present in a sample.

The aim of the present work was to study how the presence of different pollutants, including hydrocarbons, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) affect the microbial activity and diversity in a municipal solid waste landfill at Torrejón de Ardoz (Madrid), Spain.

2. Materials and methods

2.1. Municipal landfill characteristics

Samples were taken from a municipal solid waste (MSW) landfill placed in Torrejón de Ardoz (Madrid), a city located in the Spanish central region. The original landfill received urban and industrial wastes and was capped in 1982. Over the past 10 years new residues have been stored on the site, constituting solid urban, industrial and inert wastes which have not undergone any pretreatment prior to dumping.

2.2. Soil sampling and processing

Four samples, T2 (40° 26′ 18.72″ N, 3° 28′ 30.66″ W), T2B (40° 26′ 12.60″ N, 3° 28′ 37.80″ W), T8 (40° 26′ 17.34″ N, 3° 28′ 36″ W) and T9 (40° 26′ 17.76″ N, 3° 28′ 34.56″ W) were taken from the landfill. All soil samples (2 Kg) were collected from upper layer (0–15 cm) in plastic bags and transported to the laboratory on ice. Then, soils were dried at room temperature and sieved at 2 mm mesh size. For physical and chemical analysis one sub-sample of each soil was maintained at room temperature, another was stored at 4 °C for enzyme analysis and the last one was frozen at −20 °C for genetic analysis.

2.3. Physical and chemical analysis

Soil pH was measured in H_2O slurry (1:2.5 solid:liquid ratio). Soil water holding capacity (WHC) was determined following the standard methods described by Hernández and Pastor (1989). Total hydrocarbons (UNE 77307), polycyclic aromatic hydrocarbons (PAHs, ISO 18287) and polychlorinated biphenyls (PCBs, ISO 10382) were also determined.

2.4. Biological analysis

2.4.1. Bacterial and fungal viable counts

Microorganisms were isolated from soil by blending soil samples (10 g dry weight) with 95 ml of phosphate buffer 0.1 M, pH 7. The number of colony-forming units (cfu) was determined on Tryptic Soy Agar (TSA) for bacteria and on Oxytetracycline-Glucose-Yeast Extract (OGYE) for filamentous fungi after 7 days of incubation at 28 °C. Three plates were inoculated per dilution, and plates with 30–300 colonies were counted. Counts were calculated as the means of three determinations and expressed as colony-forming units per gram of dry soil (cfu/g dwt).

2.4.2. Microbial activity

Substrate induced respiration (SIR) was measured using the method described by Jenkinson and Powlson (1976) with some modifications. Three soil samples (5 g each) were adjusted to 50% WHC and incubated with glucose (0.45 mg/g soil) for 2 days at 30 °C in sealed flasks. CO_2 concentration was measured by an automated method (Bac-Trac) based on the changes of the impedance of a KOH solution (2%) in a μ-Trac 4200 analyser.

2.4.3. Enzyme determinations

Acid and alkaline phosphatases, β-glucosidase and ß-N-acetyl-glucosaminidase activities were analysed following the methods developed by Tabatabai (1982). Invertase and cellulase (Hoffmann and Pallauf, 1965 modified by García Álvarez and Ibáñez, 1994) and urease activities (Kandeler and Gerber, 1988) were also determined in the soil samples.

2.4.4. Genetic analysis

2.4.4.1. DNA extraction. DNA was extracted from 0.5 g of soil samples using a soil DNA extraction kit (Power Soil DNA Isolation kit, MO BIO Laboratories, Carlsbad, CA, USA). Purified DNA samples were resolved by electrophoresis in a 1% agarose gel, stained with ethidium bromide and observed under UV light (λ 254 nm). DNA extraction was performed in triplicate from each soil sample.

2.4.4.2. Bacteria and Archaea communities fingerprinting by DGGE. Bacteria and *Archaea* 16S rRNA genes were amplified from DNA samples by PCR and then subjected to analysis by DGGE. Primers 341f + GC clamp and 907r and 344f + GC clamp and 915r

Table 2
Physical and biological parameters determined in Torrejón de Ardoz landfill.

Sample	pH	WHC (%)	Bacteria (cfu/g)	Fungi (cfu/g)	SIR[a]
T2	7.58 ± 0.37	36.4 ± 1.82	$4.6 \times 10^6 \pm 2.3 \times 10^5$	$5.0 \times 10^4 \pm 2.5 \times 10^3$	46.55 ± 2.32
T2B	7.75 ± 0.31	23.01 ± 1.03	$1.68 \times 10^7 \pm 7.56 \times 10^5$	$2.05 \times 10^5 \pm 4.10 \times 10^3$	76.19 ± 3.04
T8	7.62 ± 0.34	31.30 ± 1.25	$7.9 \times 10^7 \pm 3.16 \times 10^6$	$1.2 \times 10^5 \pm 4.8 \times 10^3$	77.33 ± 3.47
T9	7.8 ± 0.39	20.91 ± 1.04	$1.5 \times 10^7 \pm 6.0 \times 10^5$	$5.5 \times 10^4 \pm 1.65 \times 10^3$	45.05 ± 2.11

[a] SIR: Substrate induced respiration (mg CO_2/h/100 g dry soil).

Table 3
Enzyme activities determined in soil samples of Torrejón de Ardoz landfill. One enzymatic unit (U) = μmoles/h.

	Acid Phosphatase (U/g)	Alkaline Phosphatase (U/g)	ß-glucosidase (U/g)	Invertase (U/g)	Cellulase (U/g)	ß-N-acetyl -glucosaminidase (U/g)	Urease (U/g)
T2	0.61 ± 0.01	1.35 ± 0.07	0.59 ± 0.02	7.15 ± 0.25	0.08 ± 0.05	0.07 ± 0.01	0.80 ± 0.04
T2B	0.76 ± 0.04	4.85 ± 0.16	1.70 ± 0.05	44.02 ± 1.10	0.34 ± 0.02	0.17 ± 0.01	1.90 ± 0.10
T8	2.47 ± 0.05	9.72 ± 0.38	6.66 ± 0.17	53.75 ± 2.15	0.18 ± 0.01	0.32 ± 0.01	2.77 ± 0.10
T9	0.70 ± 0.03	2.90 ± 0.15	1.94 ± 0.10	18.49 ± 0.54	0.18 ± 0.01	0.11 ± 0.01	1.03 ± 0.10

were used to study *Bacteria* and *Archaea* community profiles, respectively. Taq polymerase (FideliTaq PCR Master Mix) from Invitrogen (USA) was used in all PCR amplifications.

DGGE was performed with a D-code Universal Mutation Detection System (Bio Rad laboratories, Hercules, CA, USA). PCR products (between 800–1000 ng) were loaded onto 6% polyacrylamide gels containing a formamide–urea linear denaturing gradient of 55–60% or 50–60% for *Bacteria* and *Archaea*, respectively. The 100% denaturant gradient was defined as 7 M urea and 40% (v/v) deionized formamide. Gels were run in 1 × TAE at a constant voltage of 60 V for 18 h at 60 °C. Bands were visualized by staining the gels with ethidium bromide (50 μg/ml) for 20 min and destaining in deionized water for 40 min. The gels were exposed to UV light to visualize the bands and digitalized in a Gel Doc 2000 (BioRad laboratories, Hercules, CA, USA).

With the obtained DGGE banding profiles a UPGM cluster analysis using the PAST program (http://folk.uio.no/ohammer/past) was performed taking into account the Jaccard's similarity measure obtained from absence–presence of bands. Similarities between the banding profiles were also displayed graphically as a dendrogram. Shannon indexes of general diversity were also calculated using the same program.

2.4.4.3. 16S rRNA gene clone library and sequence analysis. The phylogenetic affiliation of the *Bacteria* present in the samples was examined by partially sequencing the 16S rRNA gene. *Bacteria* 16S rRNA genes were amplified from soil DNA by PCR using the primers 27f and 1492r. The PCR-amplified DNA fragments were cloned into the pCR 2.1 vector of the Topo TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Then, competent *E. coli* cells were transformed and plated. White colonies were screened for inserts of the expected size by using primers M13f and M13r. Clones were purified with Ultraclean PCR clean-up Kit (MO BIO, USA) and selected for sequencing at the Molecular Biology Service of the University of Alcalá de Henares (Madrid, Spain). The sequences obtained were compared to available database sequences using the Ribosomal Database Project for phylogenetic assignment (http://rdp.cme.msu.edu). Sequences with similarities >95% were considered to represent the same taxonomic group. Rarefaction curves (Simberloff, 1972) and Shannon index of general diversity was determined using PAST program.

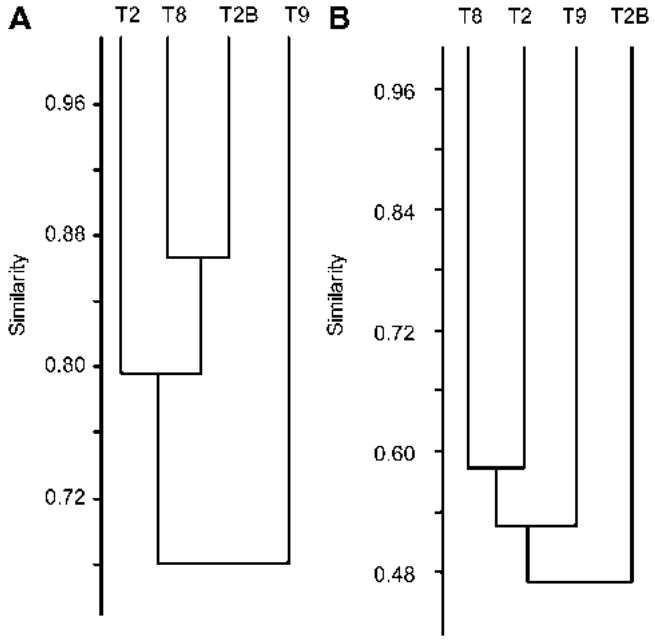

Fig. 1. A dendrogram representation of a hierarchical cluster analysis of the denaturing gradient gel electrophoresis (DGGE) profiles using Jaccard's similarity measure. A: *Bacteria*. B: *Archaea*.

2.5. Statistical analysis

Analysis of the data was performed using SPSS version 17.0 for Windows (SPSS, 2005). Linear correlation coefficients were determined between the physical, chemical, biological and genetic parameters previously determined. The significance of all statistical analysis was accepted at $p < 0.05$.

3. Results and discussion

3.1. Physical, chemical and biological analysis

Soil samples were collected *in situ* at four different zones of a solid waste landfill in which toxic compounds were detected. Organic pollutants were measured in the samples. The concentrations of total hydrocarbons, PAHs and PCBs determined in the T2, T2B, T8 and T9 soil areas are shown in Table 1. As can be observed in the table, although pollutants have been found in the four zones of the landfill, the highest values for hydrocarbons and PCBs were detected in the areas T9 (189.91 ppm and 4.14 ppm respectively) and T2 (165.6 ppm and 0.22 ppm respectively). A high concentration of polycyclic aromatic hydrocarbons (PAHs) was detected in the T9 area (11.06 ppm), consisting mainly of pyrene (1.33 ppm), fluoranthene (1.55 ppm), benzo[b]fluoranthene (1.69 ppm) and benzo[a]pyrene (2.23 ppm) (data not shown). Although the lowest concentration values of hydrocarbons and PCBs were detected in the area T8, low amounts of benzo[b]fluoranthene (0.062 ppm) and benzo[a]pyrene (0.059 ppm) were also found in this sample (data not shown).

Physical (pH and WHC) and biological parameters (total viable bacteria and fungi and SIR) were estimated in the soil samples, and the results are shown in Table 2. Results corresponding to the estimation of microbial biomass by both counting of viable microorganisms and measuring the SIR showed the highest number of microorganisms and the more intensive microbial activity in the zones T8 and T2B, which correspond to the areas less contaminated. From statistical analysis of the data above mentioned we observed that the parameters SIR and total bacteria and fungi counts were significantly negatively correlated with soil hydrocarbon concentration. The correlation coefficients were:

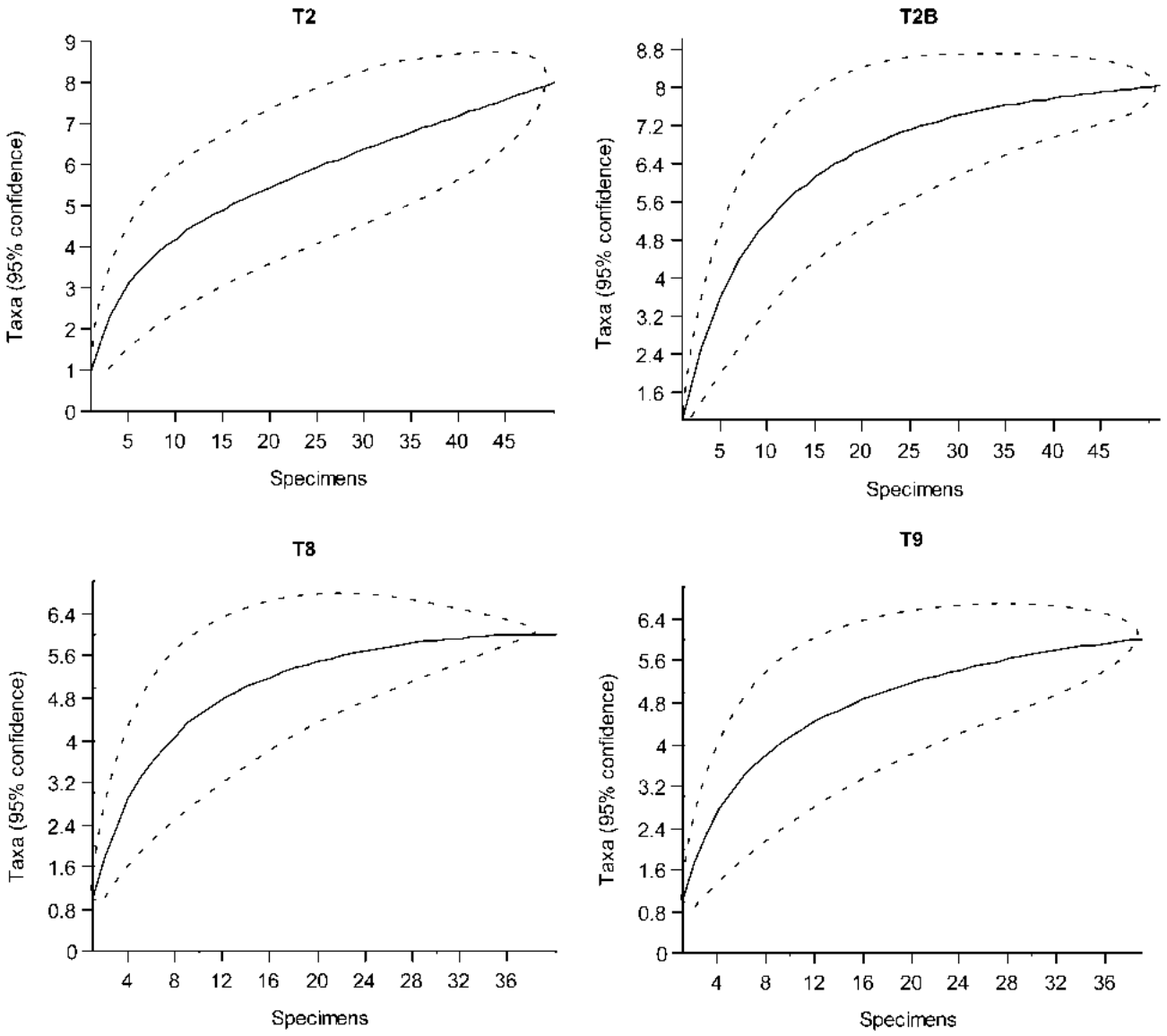

Fig. 2. Rarefaction curves of clone libraries from the soil samples of Torrejón de Ardoz landfill (95% confidence).

$r = -0.957$, $p < 0.001$, $r = -0.745$, $p = 0.034$ and $r = -0.756$, $p = 0.030$, respectively. On the contrary, a positive significant correlation was found between SIR and total fungi count ($r = 0.857$, $p = 0.007$). SIR and total bacteria count also showed a positive correlation ($r = 0.652$), but it was not significant ($p = 0.08$).

The activity of different enzymes related with C, N and P cycles was also measured in soil samples and the results are shown in (Table 3). It can be observed that the enzymatic activities were also lower in the more polluted areas (T2 and T9) than in the areas where the pollutants were in a lesser concentration (T2B and T8). In addition, a significant decrease in the activity of the most of enzymes studied was detected when the total hydrocarbon concentration increased. The analysis of soil enzymes showed a significant negative correlation between soil hydrocarbon concentration and the activities acid phosphatase ($r = -0.734$, $p = 0.038$), alkaline phosphatase ($r = -0.850$, $p = 0.008$), β-glucosidase ($r = -0.708$, $p = 0.049$), ß-N-acetylglucosaminidase ($r = -0.864$, $p = 0.006$), invertase ($r = -0.924$, $p = 0.001$) and urease ($r = -0.938$, $p = 0.001$). From these results, it could be concluded that microbial activity (SIR) and enzyme activities were lower when the concentration of the pollutants detected in the landfill was higher. In contrast, SIR showed significantly positive correlations with the activities of alkaline phosphatase ($r = 0.809$, $p = 0.015$), ß-N-acetylglucosaminidase ($r = 0.824$, $p = 0.012$), invertase ($r = 0.954$, $p = 0.001$), and urease ($r = 0.914$, $p = 0.001$) activities. In the same way, positive and significant statistically correlations were found between viable bacteria and acid phosphatase ($r = 0.990$, $p = 0.000$), alkaline phosphatase ($r = 0.964$, $p = 0.000$), ß-N-acetylglucosaminidase ($r = 0.957$, $p = 0.000$), β-glucosidase ($r = 0.992$, $p = 0.000$), invertase ($r = 0.781$, $p = 0.022$), and urease ($r = 0.898$, $p = 0.002$) activities, and between total viable fungi and activities cellulase ($r = 0.910$, $p = 0.002$) and invertase ($r = 0.762$, $p = 0.028$) activities. From these results a positive correlation between enzyme activities, viable microorganisms and SIR could be established in accordance with that previously reported for other soils (Taylor et al., 2002). Taking into account that enzyme activities and other biological parameters such as microbial biomass and respiration have been suggested as biomarkers in degraded soils (García et al., 1997; Pascual et al., 2000; Trasar Cepeda et al., 2000), T9 and T2 areas can be considered the most stressed and degraded zones into the landfill.

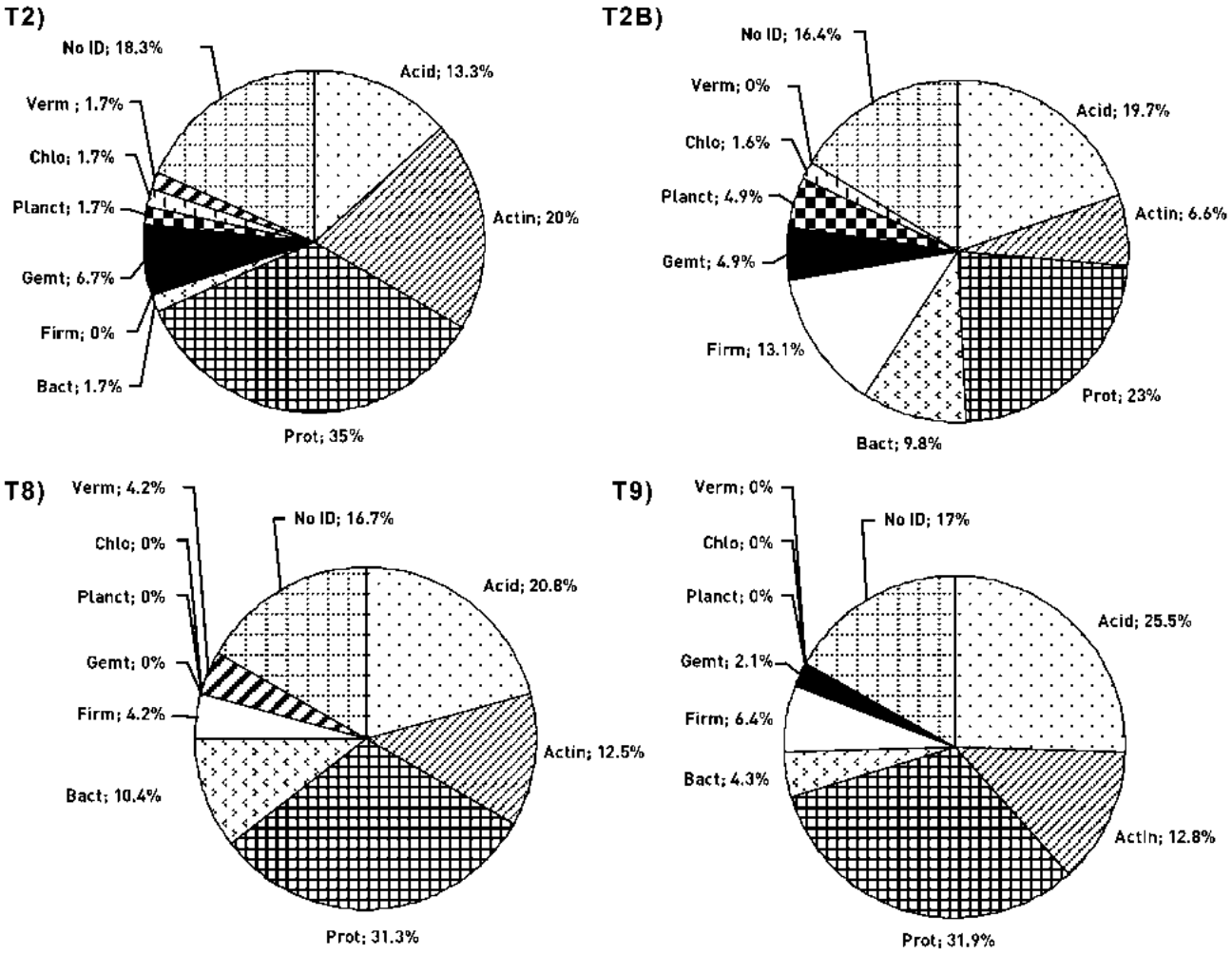

Fig. 3. Distribution (%) of bacterial clones related to phylogenetic groups in clone libraries. *Acidobacteria (Acid)*; *Actinobacteria (Actin)*; *Proteobacteria (Prot)*; *Bacteroidetes (Bact)*; *Firmicutes (Firm)*; *Gemmatimonadetes (Gemt)*; *Planctomycetes (Planct)*; *Chloroflexi (Chlo)*; *Verrucomicrobia (Verm)*; Not identified (No ID).

3.2. Microbial community profiles

Bacteria and *Archaea* communities in each landfill sample were profiled by DGGE of amplified 16S rDNA fragments. The DGGE patterns for *Bacteria* seem to be slightly different for each sample. T9 sample showed the lowest number of bands while T2B showed the highest (data not shown). Cluster analysis was done to study similarities between the banding patterns generated by PCR-DGGE of the soil samples. From the dendrogram representation (Fig. 1) we observed that the landfill soils could be divided in two parts. T9 area seems to be separated from the T2, T8 and T2B areas, being T2B and T8 areas (sample soils with lesser amount of total hydrocarbons) grouped together. Shannon indexes of each soil (T2, 2.485; T2B, 2.639; T8, 2.639; T9, 2.565) were also calculated using PAST program. A significant negative correlation was found between Shannon index and total hydrocarbon ($r = -0.792$, $p = 0.019$). Once again, it could be observed the negative impact of the pollutants on the diversity of microbial communities.

In the *Archaea* profiles, T8 sample presented the highest number of bands and T2 and T9 the lowest (data not shown). On the contrary that occurred with *Bacteria* domain, Shannon indexes of the samples (T2, 2.079; T2B, 2.709; T8, 2.398; T9, 2.398) were not significantly correlated with the total hydrocarbon concentration ($r = -5.08$, $p = 0.198$). The dendrogram of these samples shows the presence of three clusters. Samples T8 and T2 are grouped together, in spite of their difference in hydrocarbon concentration. Results seem to indicate that there was no straight correlation between the *Archaea* diversity and the hydrocarbon content of the soils.

3.3. Clone library analysis

Analysis of clone library was used to characterise the microbial communities of soil landfill samples. This analysis allows getting more detailed phylogenetic information on the microorganisms contained in the samples. DNA was extracted from the four samples and PCR amplifications using 16S rRNA gene primers were carried out only for *Bacteria* domain. Clone libraries of bacterial 16S rRNA genes were constructed. In total, 340 clones were subjected to sequence analysis followed by online homology searches using the Ribosomal Database Project. From those only 214 were valid sequences. Only the sequences that shared more then 95% of identity with the database sequences (T2, 50 clones; T2B, 51 clones; T8, 40 clones; T9, 39 clones) were used for the phylogenetic analysis. For the phyla obtained, rarefaction curves were constructed to determine whether a sufficient number of clones were screened in order to estimate the total diversity in each clone library (Fig. 2). Rarefaction curve of sample T2 showed very little saturation in the curve, suggesting that the diversity in the sample would be higher if more clones were analysed. In contrast, T2B, T8 and T9 curves

showed saturation indicating that in these cases the number of clones were representative enough.

From the bacterial 16S rRNA gene sequences, 9 different phyla were identified. In all the samples analysed, the most of bacterial sequences were classified as *Proteobacteria* (23–35%), followed by *Acidobacteria* (13.3–25.5%) and *Actinobacteria* (6.6–20%). Only 6 phyla were found in T9 area, being *Proteobacteria* (31.9%) and *Acidobacteria* (25.5%) the most representative groups (Fig. 3).

Shannon diversity index was calculated for all communities on the basis of the phyla observed. The diversity indexes were between 1.87 and 1.481 values, corresponding the lowest values to T9 and T2 samples (1.481 and 1.558) and the highest to T2B and T8 (1.87 and 1.579).

As above mentioned, the predominant group in all samples was *Proteobacteria*. Thus, α-, β-, γ- and δ-*Proteobacteria* were found, being α-*Proteobacteria* the most abundant sub-group in all areas (T2: 66.7%; T2B: 66.7%; T8: 33.3%; T9 53.3%). In T8 area, the β-*Proteobacteria* was as abundant as the alpha sub-group (33.3%). T9 area showed a strong presence of bacteria belonging to the δ-*Proteobacteria* sub-group (13.3%) unlike the other studied samples.

Once determined the *phyla* some genera could be identified from the sequences analysis. The most abundant genera corresponding to *Proteobacteria phylum* in the studied areas were: *Sphingosinicella*, *Rhizobium*, *Microvirga*, *Mesorhizobium* and *Herminiimonas* (T2); *Microvirga*, *Dervosia* and *Herbaspirillum* (T9); *Amaricoccus*, *Skermanella* and *Masilia* (T8) and *Dervosia* and *Skermanella* (T2B). In *Actinobacteria phylum*, the following genera were identified: *Conexibacter*, *Arthrobacter Modestobacter Couchioplanes* and *Cryobacterium* (T2); *Marmoricola* and *Conexibacter* (with a 93% of identity in T9); *Pseudonocardina* and *Microbacterium* (94% of identity in T8), and *Arthrobacter* and *Rubrobacter* (T2B). In the four samples analysed, all the clones corresponding to *Acidobacteria phylum* belong to *Acidobacteriaceae* family. Although the sequences obtained could not be assigned to any defined genus, some of them were more abundant in some specific samples. Thus, Gp4, Gp6 and Gp10 were most abundant in T2 and T9 samples; Gp6, Gp7 and Gp10 in T8 and T2B samples; Gp4 sequence was not found in T8 and T2B samples, and Gp7 sequence was not detected in T9.

Although hydrocarbon-degrading strains are phylogenetically related to γ- and δ-*Proteobacteria* (Powell et al., 2003), the data obtained through the 16S sequence analysis did not allow concluding that these clones were precisely involved in the degradation of hydrocarbons. Specific cultures of these strains will be required to determine their metabolic capabilities.

In conclusion, our findings suggest a clear relationship between the presence of pollutants and the microbial activity and diversity (Shannon index). In fact we observed that in the presence of the highest concentration of toxic compounds (T9 and T2 areas), the lowest values of microbial biomass and enzymatic activities were found. However, from this study a clear relation between both phyla and genera identified and pollutant concentration could not be established.

Acknowledgments

This work has been supported by the Comunidad de Madrid EIADES project (S-0505/AMB/0296) and the CICYT project (CGL2006-12730-C03-02).

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Journal of Environmental Management 95 (2012) S291–S299

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Tolerance and growth of 11 *Trichoderma* strains to crude oil, naphthalene, phenanthrene and benzo[*a*]pyrene

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ARTICLE INFO

Article history:
Received 3 September 2009
Received in revised form
8 August 2010
Accepted 14 August 2010
Available online 25 September 2010

Keywords:
Petroleum hydrocarbons
Polycyclic aromatic hydrocarbons
Fungal growth

ABSTRACT

Petroleum hydrocarbons (PHs) are major organic contaminants in soils, whose degradation process is mediated by microorganisms such as the filamentous fungi *Cunninghamella elegans* and *Phanerochaete chrysosporium*. However, little is known about the tolerance and the degradation capability of *Trichoderma* species when exposed to PH. This research evaluated the tolerance and growth of 11 *Trichoderma* strains to crude oil (COil), naphthalene (NAPH), phenanthrene (PHE) and benzo[*a*]pyrene (B[a]P) by using *in vitro* systems. Petri dishes containing solid mineral minimum medium were separately contaminated with COil, with seven doses of either NAPH or PHE (250, 500, 750, 1000, 2000, and 3000 mg L^{-1}), and with six doses of B[a]P (10, 25, 50, 75, and 100 mg L^{-1}). Non-contaminated plates were used as controls. *Trichoderma* strains were exposed to all the contaminants by triplicate, and the growth of each fungal colony was daily recorded. No significant differences were observed among *Trichoderma* strains when they were exposed to COil in which the maximum fungal growth was reached at 96 h. In contrast, *Trichoderma* strains showed variations to tolerate and grow under different doses of either NAPH, PHE or B[a]P. Increasing NAPH doses resulted on significant greater fungal growth inhibition than PHE doses. The exposure to B[a]P did not inhibited growth of some *Trichoderma* strains.

1. Introduction

Crude oil (COil) is composed of four different types of hydrocarbons: saturated, aromatics, asphaltenes and resins (Leahy and Colwell, 1990; Harayama, 1997). Among the aromatic fraction, polycyclic aromatic hydrocarbons (PAHs) are constituted by two or more aromatic rings. The stability and the hydrophobic properties of these compounds confer more persistence and recalcitrance in the environment (Kanaly and Harayama, 2000). In addition, these aromatic compounds are also considered as teratogenic and/or mutagenic agents (Chen and Liao, 2006). Biodegradation of PAHs via microbial activity has been well documented, especially for bacteria such as *Pseudomonas*, *Azoarcus*, *Geobacter*, *Desulfobacterium* and *Metanospinilum*, among others (Widdel and Rabus, 2001), and fungi such as *Cunninghamella elegans* and *Phanerochaete chrysosporium* (Cerniglia and Yang, 1984; Bumpus, 1989; Sutherland et al., 1991; Pothuluri et al., 1992; Cerniglia et al., 1994; Moen and Hammel, 1994). In contrast, the influence of *Trichoderma* species on the degradation of PAH has been scarcely described.

Trichoderma species belong to the group of filamentous fungi classified as Ascomycetes as a part of the Hypocreales Order in which at least 30 species are known (Lieckfeldt et al., 1999). These fungi are characterized for being one of the most distributed fungal groups in terrestrial (agricultural systems, grasslands, forests, and deserts) and aquatic ecosystems (Zhang et al., 2005) since they have high reproductive rate that confers their ability to colonize several environments. Some *Trichoderma* species are free living, opportunists or plant symbionts, and others are mycoparasites (Bissett, 1991; Harman et al., 2004). In addition, the nutritional requirements of this fungal group are considered to be low since they can survive under adverse conditions; however, the fungal growth in soil is also favored by the presence of organic matter and moisture, and the optimal temperature is between 25 and 30 °C (Papavizas, 1985). The genus *Trichoderma* is important for plant species since its fungal species have mycoparasitic and antibiotic capabilities by which they may control the growth and incidence of microorganisms that cause diseases in several horticultural plants (Score and Palfreyman, 1994; Druzhinina and Kubicek, 2005; Ávila-Miranda et al., 2006; Rojo et al., 2007).

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doi:10.1016/j.jenvman.2010.08.011

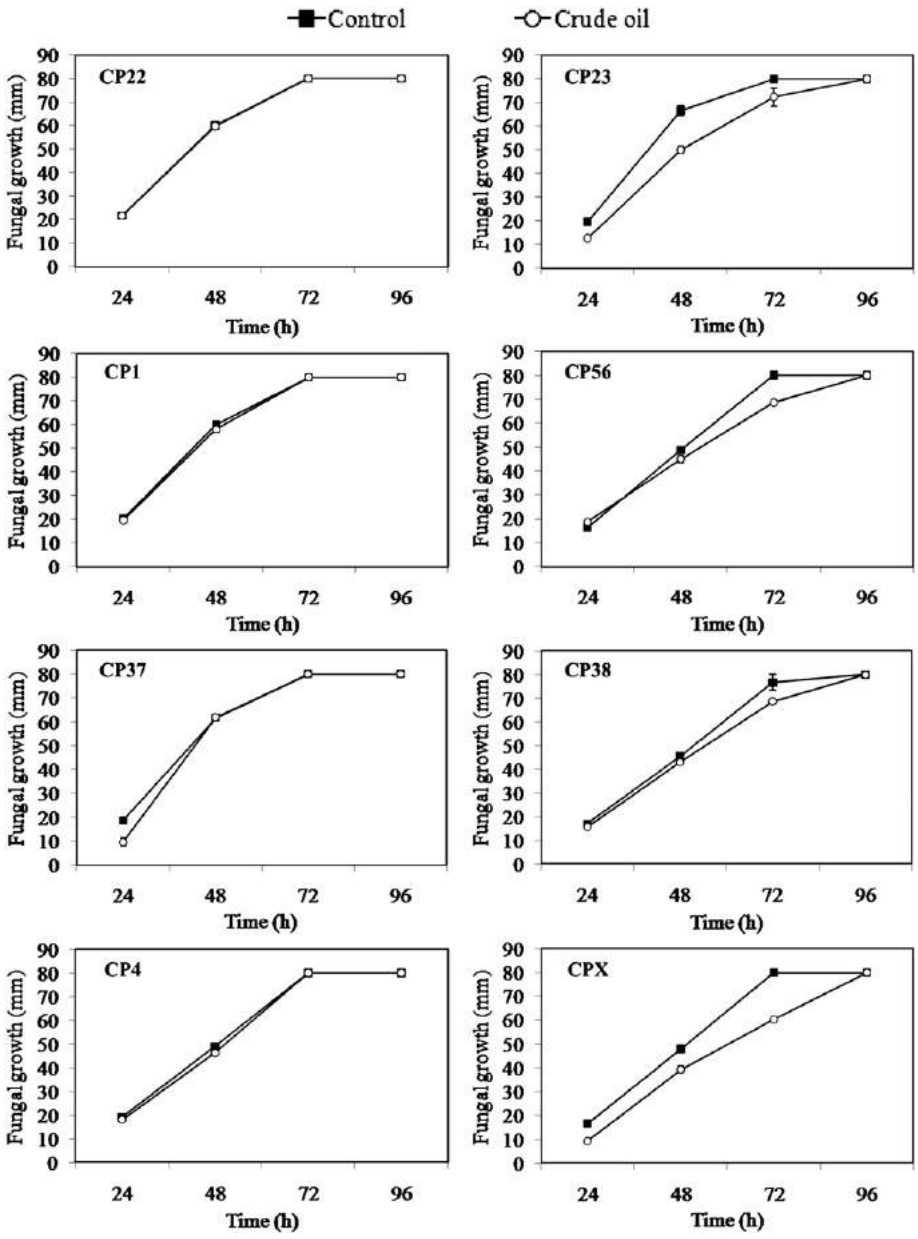

Fig. 1. Growth response of eight *Trichoderma* strains exposed to crude oil (1 mL) on the agar surface. At 24, 48 and 72 h, *Trichoderma* strains CP22, CP1, CP37, and CP4 showed similar growth rate than control. *Trichoderma* strains CP23, CP56, CP38, and CPX had slower growth rate than control ($n = 3$, means ± standard error).

In spite of the significant role of *Trichoderma* species on the biocontrol of plant pathogens and on the enzyme production for textile and food industries (Reese and Mandels, 1989; Galante et al., 1993; Walsh et al., 1993; Cavaco-Paulo et al., 1998), the studies focused on describing their capabilities to tolerate or to degrade petroleum hydrocarbons are scarce. Chaîneau et al. (1999) showed that some *Trichoderma* species contributed to the degradation of some fractions of petroleum hydrocarbons. Thus, saturated hydrocarbons were more easily degraded than PAHs. For instance, the strain of *Trichoderma* S019 is able to degrade 73% of *n*-eicosane when glucose is applied as carbon source (Hadibarata and Tachibana, 2009). Nevertheless, the rate of diesel degradation by *Trichoderma harzianum* is lower than that observed by *Bacillus subtillis* (Nwaogu et al., 2008). On the other hand, the degradation of either saturated or aromatic hydrocarbons by *Trichoderma koningii* was significantly affected by low temperatures (Whyte et al., 1999; Hughes et al., 2007).

Studies have shown the capability of *T. harzianum*, *Trichoderma pseudokoningii* and *Trichoderma viride* to utilize and degrade pyrene (one aromatic ring) (Ravelet et al., 2000; Saraswathy and Hallberg, 2002). Some *Trichoderma* species including *T. harzianum*, *Trichoderma longibrachiatum*, and *Trichoderma inhamatumson*, have been shown to tolerate 100 mg L^{-1} of either phenanthrene (PHE) or pyrene (Silva et al., 2003). In addition, Atagana (2009) reported that the ability of *Trichoderma* for degrading PAHs such as benzo[*a*] anthracene, benzo[*a*]fluoranthene, benzo[*a*]pyrene (B[a]P), chrysene, and PHE is significantly affected by the presence of cadmium and nickel.

In order to obtain more useful information, this research was focused on studying the tolerance and growth of 11 *Trichoderma*

strains exposed to crude oil or to several doses of three PAHs (naphthalene, phenanthrene and benzo[a]pyrene).

2. Materials and methods

2.1. Trichoderma strains

Eleven strains of *Trichoderma* belonging to the Soil Microbiology's microbial collection (Colegio de Postgraduados) were used in this study. The fungal strains were isolated from different soil samples collected from several states of Mexico such as Jalisco, Oaxaca, Veracruz, and Guanajuato.

2.2. Tolerance of the Trichoderma strains to crude oil

The growth of the fungal strains was activated in Petri dishes containing potato dextrose agar (PDA, Baker®) at 28 °C for 5 days. Afterwards, individual PDA disks (7 mm diameter) with each fungal strain were extracted and placed on new Petri dishes with PDA which were previously contaminated with 1 mL of crude oil (COil) by spreading it on the agar surface. The Petri dishes were incubated at 28 ± 2 °C for 10 days, and the fungal growth was measured every 24 h. Non-contaminated Petri dishes were used as controls.

2.3. Tolerance of Trichoderma strains to naphthalene, phenanthrene and benzo[a]pyrene

Fungal growth of each strain was obtained as previously described. Each fungal strain was placed on the center of Petri dishes containing solid mineral medium which consisted on (g L^{-1}): 0.1 $CaCl_2$; 0.2 KCl; 0.5 KH_2PO_4; 0.5 $(NH_4)_2SO_4$; 0.2 $MgSO_4 \cdot 7H_2O$; 10 agar, adjusted at pH 5.5. The carbon source was supplied in the culture medium by adding either naphthalene (NAPH, two aromatic rings) or phenanthrene (PHE, three aromatic rings) at doses of 250, 500, 750, 1000, 2000 and 3000 mg L^{-1}, respectively. In addition, fungal strains were exposed to five concentrations of benzo[*a*]pyrene (B[a]P, with five aromatic rings): 10, 25, 50, 75 and 100 mg L^{-1}. The low B[a]P doses were adjusted on the basis of its high hydrophobicity and its electrochemical stability, chemical properties that make the B[a]P more toxic for microorganisms (Wilson and Jones, 1993; Kanaly and Harayama, 2000). The crystals of each PAH were individually dissolved in a solution of acetone in order to apply each dose of either NAPH, PHE or B[a]P on the agar surface. In addition, non-contaminated Petri dishes for each fungal strain were used as controls.

Petri dishes were incubated at 28 ± 2 °C for 10 days and the fungal growth was assessed by measuring the diameter of each fungal colony every 24 h. The fungal inhibition percentage due to the PAH exposure was calculated utilizing the following equations:

$$\mathrm{FG}(\%) = \frac{D_{\mathrm{PAH}}}{D_{\mathrm{C}}} \times 100 \quad (1)$$

$$\mathrm{FI}(\%) = 100 - \mathrm{FG} \quad (2)$$

where, FG = fungal growth, D_{PAH} = diameter of the fungal colony exposed to PAH (at 240 h for NAPH, and at 72 h for PHE), D_{C} = diameter of the fungal colony of controls, and FI = fungal growth inhibition.

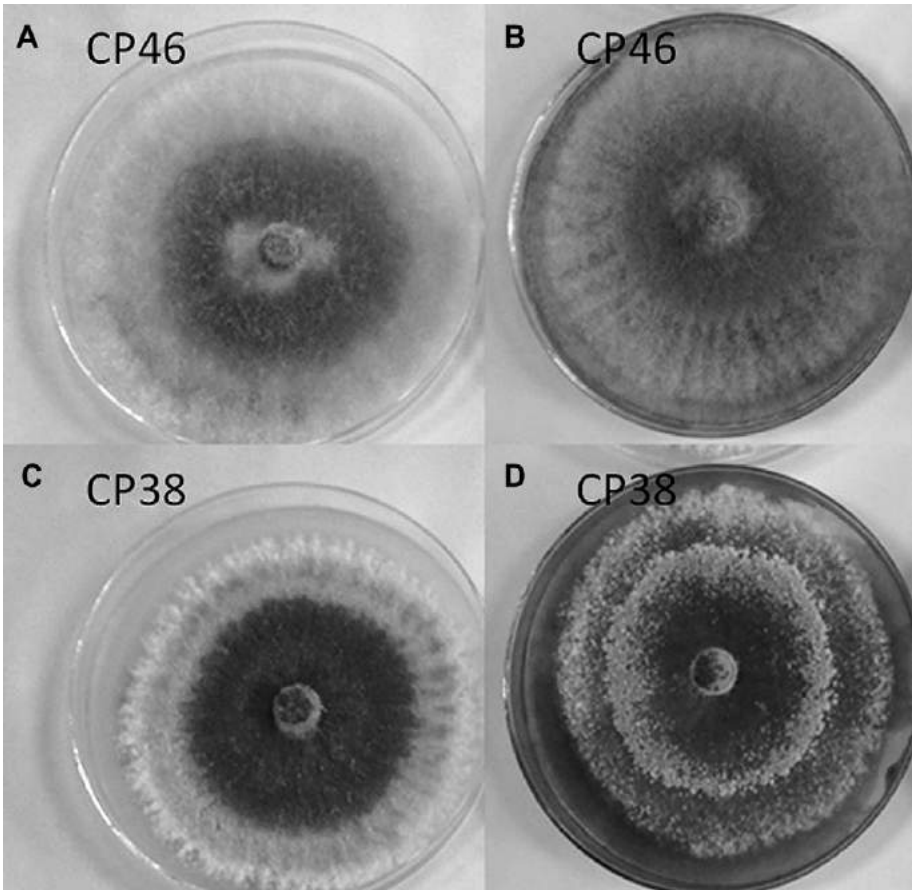

Fig. 2. Tolerance of two *Trichoderma* strains CP46 and CP38 to crude oil spread on the agar surface, after 10 days. (A and C) fungal growth of the control (without contamination); (B and D) fungal growth at contaminated medium (1 mL of crude oil).

2.4. Statistical analysis

The fungal tolerance assay to COil was set as a completely randomized experimental design. For the NAPH and PHE assay an $11 \times 2 \times 7$ factorial experiment was set in a completely randomized experimental design (11 *Trichoderma* strains, two PAHs, and seven doses). The B[a]P assay was established using a 11×6 factorial experiment (eleven *Trichoderma* strains and six doses). Each treatment for each assay had three replicates. Data were analyzed by means of the analysis of variance and the mean comparison test (Tukey, $\alpha = 0.05$) (SAS Institute Inc., 2002).

3. Results

There were no detrimental effects of COil on the growth of any of the 11 *Trichoderma* strains. After 24, 48 and 72 h, the fungal growth of the strains exposed to COil was significantly lower than controls. However, at 96 h, the growth of all fungal strains exposed to COil did not showed significant differences when compared to the controls. The strains with higher tolerance to COil at 24 and 48 h were CP22, CP1 CP37, and CP4, while the strains CP23, CP56, CP38 and CPX had reduced growth at 72 h (Fig. 1). The total agar surface was completely covered by all fungal strains at 96 h. Figure 2 shows the growth of two *Trichoderma* strains (CP46 and CP38) on culture media contaminated with COil after 10 days. Although COil did not significantly inhibit fungal growth after 10 days, some effects were observed on the mycelium abundance, fungal colony growth and sporulation patterns of *Trichoderma* strains (Fig. 2).

By performing the three-way analysis of variance of the data it was observed that the independent factors had significant effects on the fungal growth when exposed to several doses of either NAPH or PHE. These significant effects were observed from 48 h to

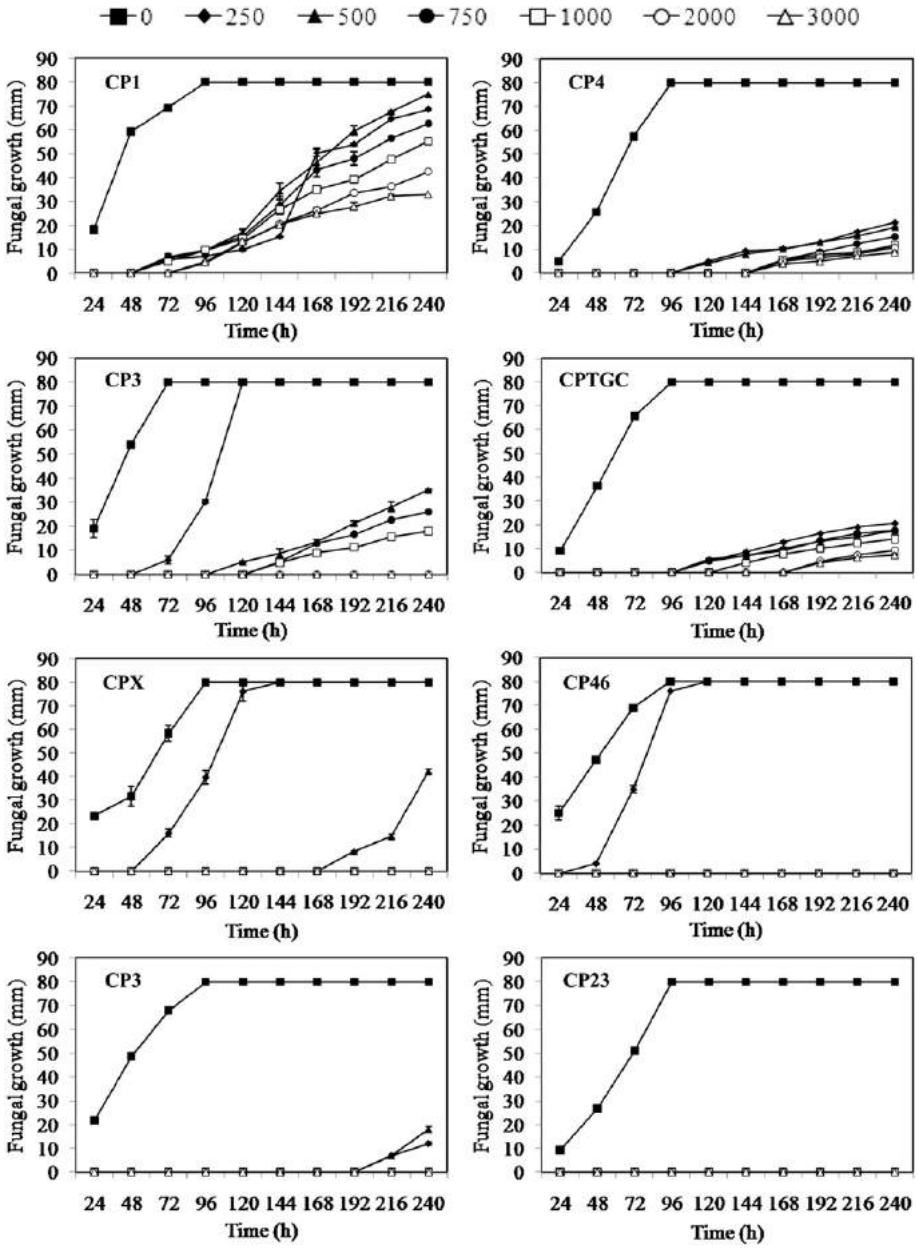

Fig. 3. Growth response of eight *Trichoderma* strains exposed to seven doses of naphthalene (mg L^{-1}). *Trichoderma* strains CP1, CP4, CP37, CPTGC, CPX, and CP46 were more tolerant when compared to strains CP23 and CP3 ($n = 3$, means $\pm$ standard error).

96 h after PAHs exposure. In regards to the type of PAH, NAPH showed significant negative effects ($P < 0.001$) on the growth of the *Trichoderma* strains when compared to PHE, resulting that NAPH had stronger growth inhibition (76%) on the *Trichoderma* strains than PHE (11%). On the other hand, the doses of the PAH's analyzed as independent factors also showed that *Trichoderma* strains had significant ($P \leq 0.001$) reduced growth as the doses of PAH increased.

Due to the number of the treatments (154) achieved by the original factorial experiment ($11 \times 2 \times 7$), results are presented by analyzing the growth for the 11 *Trichoderma* strains obtained in the seven doses of each individual PAH.

The NAPH had more toxic effects than PHE for most of the fungal strains, from which only five of them were able to grow at 3000 mg L^{-1} (CP1, CP22, CP4, CPTGC, and CP56). The fungal growth for controls (0 mg L^{-1}) started after 24 h, while for the most tolerant strains to NAPH, the fungal growth was observed in average after 48 h (Fig. 3). Figure 3 shows the fungal growth of six tolerant and two sensitive strains to NAPH. The strain CP1 showed greater tolerance to all NAPH doses, at 240 h its fungal growth (diameter) was greater than 30 mm at the highest NAPH concentration (3000 mg L^{-1}). In contrast, the strain CP23 did not grow at any of the NAPH concentration (Fig. 3). Phenanthrene was significantly less toxic to the fungal strains than NAPH (data non presented). With the exception of the strain CP1 exposed to 3000 mg L^{-1} (Fig. 4), all the fungal strains tolerate all PHE doses. The strains CP1, CP37, CP46 (Fig. 4) and CP22 completely covered the Petri dish at 96 h, while the strains CP38, CP3 (Fig. 4) and CP23

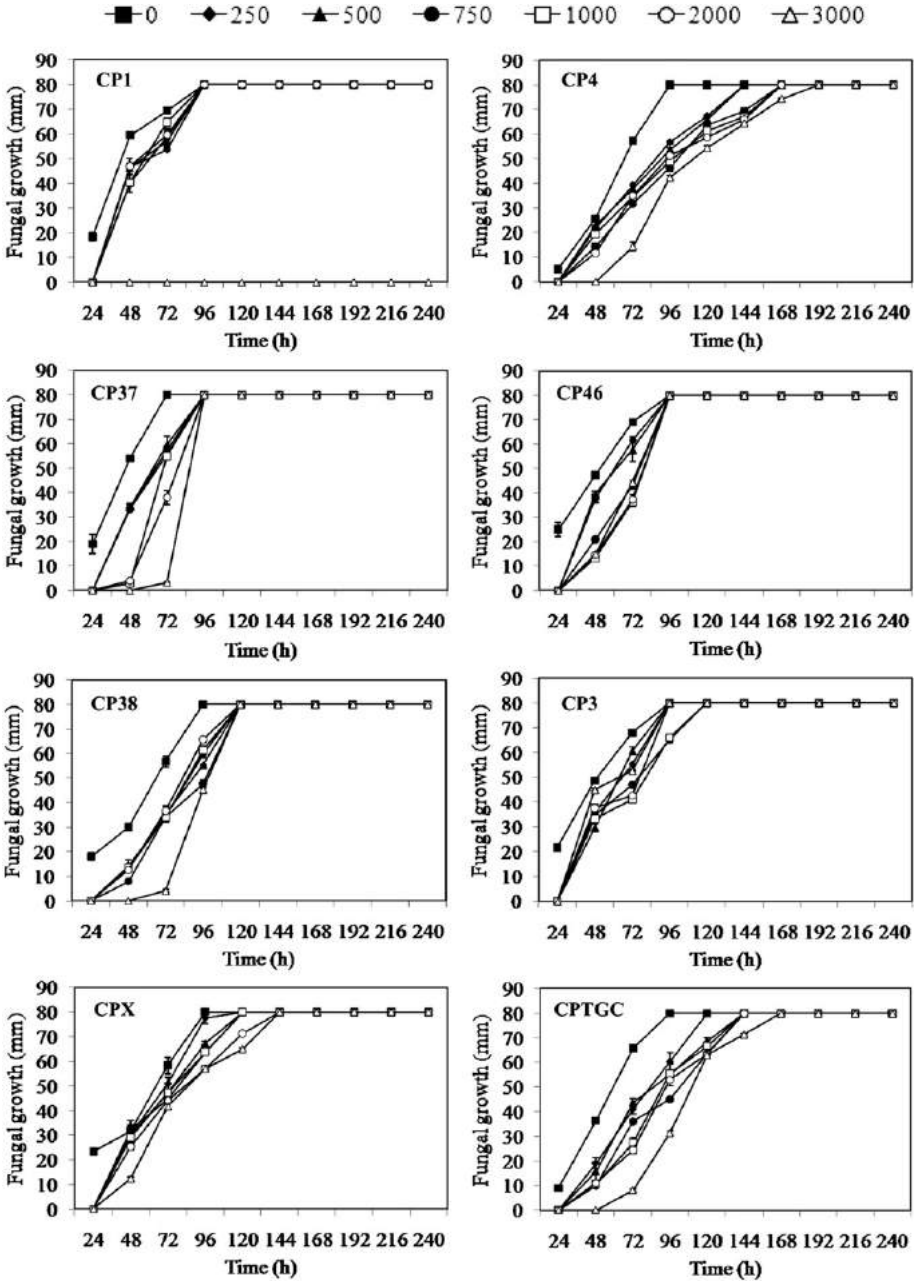

Fig. 4. Growth response of eight *Trichoderma* strains exposed to seven doses of phenanthrene (mg L^{-1}). Strains CP1, CP4, CP37, CP46, CP38, CP3, CPX, and CPTGC showed a similar growth response when exposed to the contaminant ($n = 3$, means ± standard error).

did it at 120 h. In contrast, the strains CPX, CPTGC (Fig. 4), CP56, and CP4 covered the entire agar surface after 144 h.

The inhibition percentage estimated for *Trichoderma* strains due to PHE exposure was lower in comparison to that for NAPH. Figure 5 compares the inhibitory effects of NAPH or PHE on the growth of four strains (CP1, CP4, CPX, and CP23). The average fungal adaptation period to PHE was shorter (72 h) than NAPH (>240 h). The strains CP1 (Fig. 5) and CP22 showed greater tolerance to both NAPH and PHE by showing lower fungal growth inhibition percentages.

For most *Trichoderma* strains at 48 and 72 h, the fungal growth was significantly ($P \leq 0.001$) reduced due to B[a]P doses; however, the fungal strains completely covered the contaminated-agar surface between 72 and 96 h. The strains CP1, CP22 and CP46 were the most tolerant to B[a]P than the rest of the strains (Fig. 6). Although the strains CP3, CP4, CP38, CP46, CP23, CP56, and CPTGC showed growth stimulation under different doses of B[a]P (Table 1); this stimulatory effect was not observed with NAPH or PHE.

4. Discussion

This work represents one of the few studies focused on the evaluation of the tolerance of *Trichoderma* strains to petroleum hydrocarbons such as COil or PAH (with two, three or five aromatic rings), and specially by exposing the fungal strains to extremely high doses of either NAPH, PHE or B[a]P. We demonstrated that *Trichoderma* strains are able to grow on a solid culture medium contaminated with COil but showing certain macroscopic morphological changes on fungal growth. In addition, this study shows that the tolerance of *Trichoderma* is dependent on the fungal strain, on the type of PAH, and also on the concentration of each contaminant in the culture media.

There is scarce information about the genus *Trichoderma* under petroleum hydrocarbon-contaminated systems in order to make comparisons about the tolerance of *Trichoderma* to either crude oil or PAH. Nevertheless, the present study demonstrates that some of the tested *Trichoderma* strains are capable of tolerating doses of

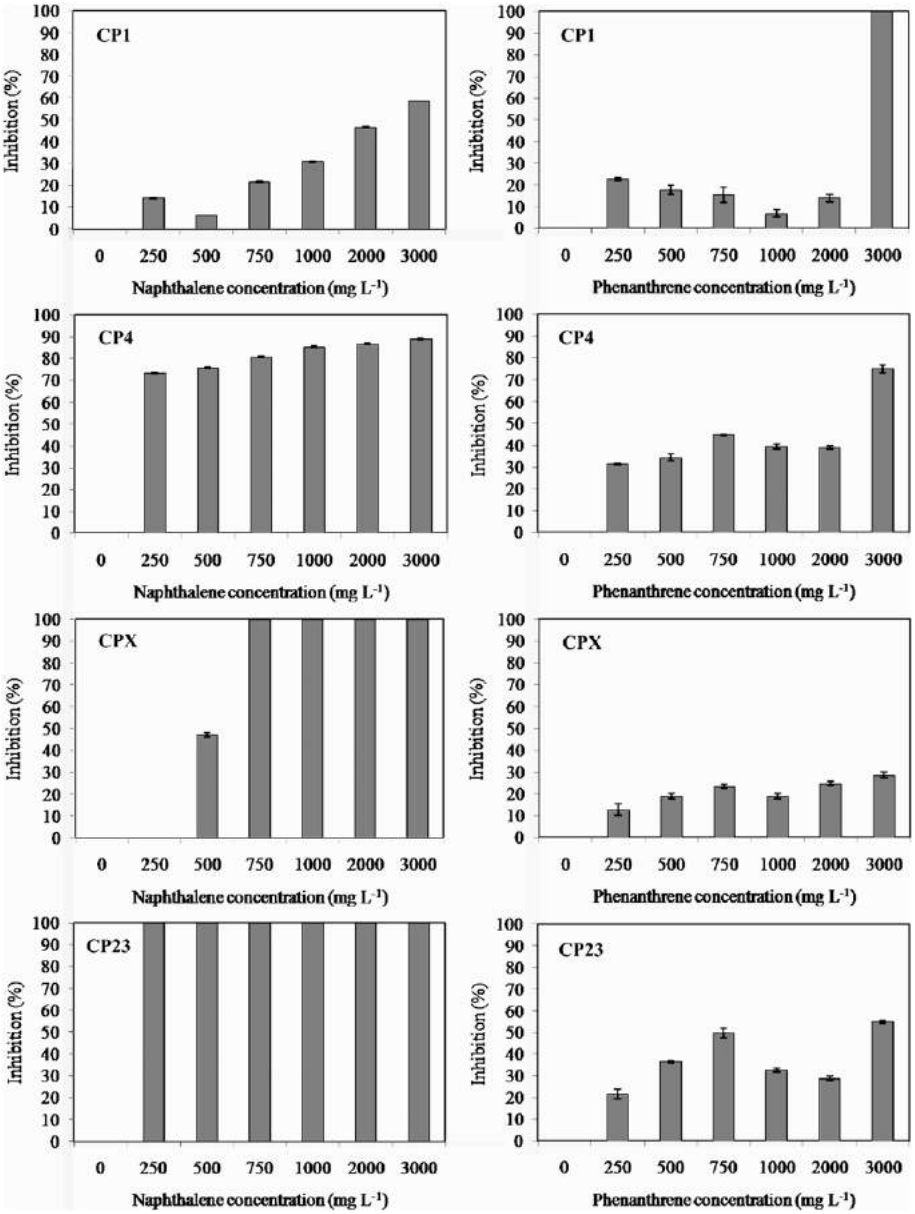

Fig. 5. Growth inhibition of four *Trichoderma* strains due to increased doses of naphthalene after 240 h, or phenanthrene after 72 h ($n = 3$, means ± standard error).

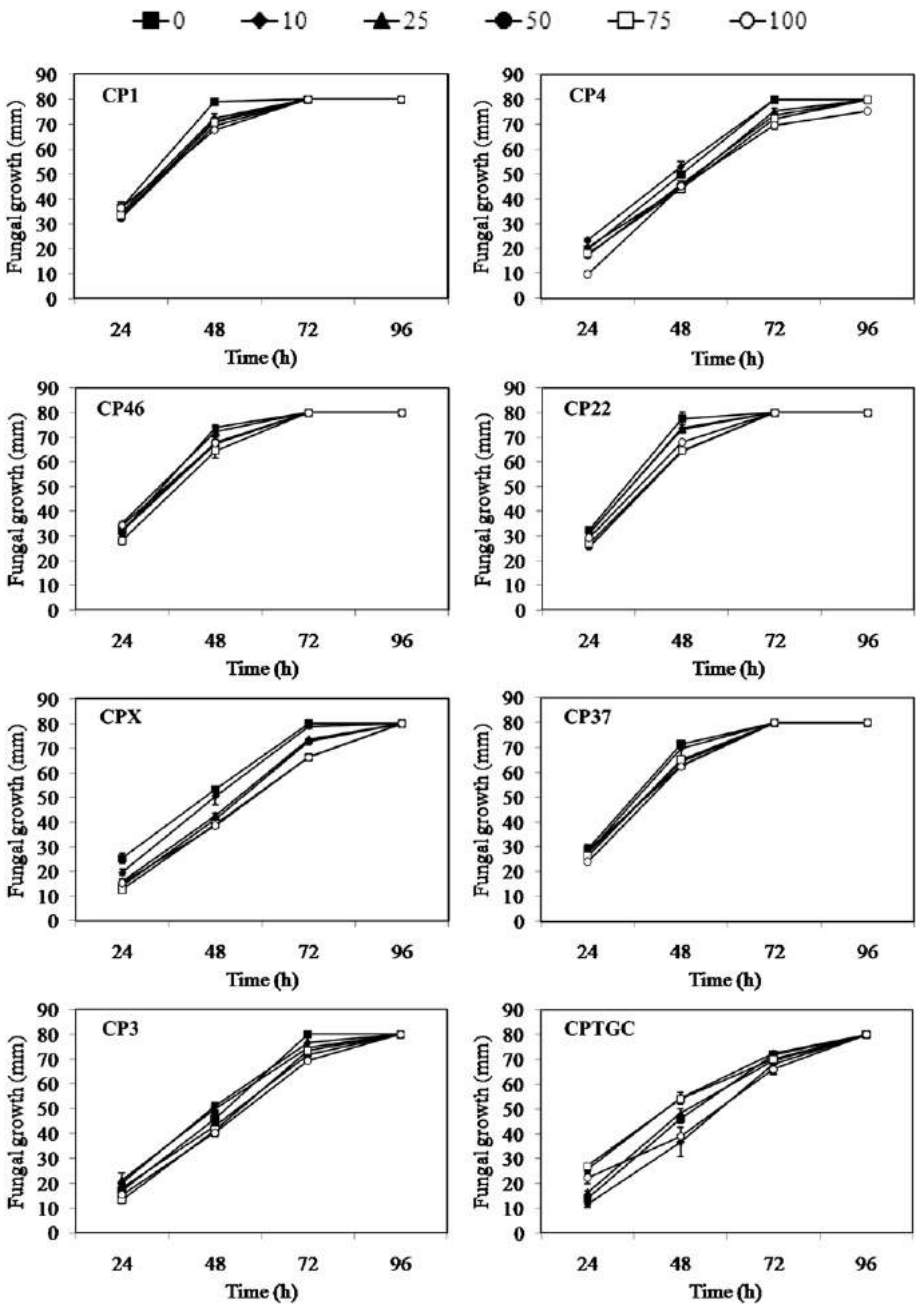

Fig. 6. Growth response of eight *Trichoderma* strains exposed to six doses of benzo[*a*]pyrene (mg L^{-1}). *Trichoderma* strains CP1, CP4, CP46, CP22, CPX, CP37, CP3, and CPTGC showed similar growth response when exposed to the contaminant ($n = 3$, means ± standard error).

PHE or NAPH greater than 250 mg L^{-1}, as well as B[a]P doses lower than 100 mg L^{-1}.

Results showed that NAPH had more toxic effects than PHE or B[a]P for most of the fungal strains. Due to the chemical nature of these three PAHs, it was expected that the compound with the most complex molecule such as B[a]P (with five aromatic rings) might have exerted more toxic and inhibitory effects on the growth of the fungal strains; however, the compound with two aromatic rings (NAPH) resulted on the strongest inhibitory effects of the fungal growth. The negative effects of NAPH on fungal growth may be explained in part due to chemical properties of this compound such as the vapor pressure, which reflects the propensity of a substance to evaporate. The vapor pressure of NAPH is 0.23 mm Hg at 25 °C which is considerable higher than that for PHE (6.8×10^{-4}) and B[a]P (5.6×10^{-9}) (Oja and Suuberg, 1998; Goldfarb and Suuberg, 2008). In addition, the water solubility of NAPH is much greater (30 mg L^{-1}) than that for PHE or B[a]P (Cerniglia and Shuttleworth, 2002). Thus, the toxic effect of NAPH on the fungal growth may correlate with the combined effects of vapor pressure and water solubility of this compound when compared to the effects observed with PHE and B[a]P. The fungal strains were enclosed in a system in which the NAPH vapors may have saturated the air in the Petri dish (Cerniglia and Shuttleworth, 2002) in consequence those vapors may have exerted their negative effects on the growth of all *Trichoderma* strains.

Although *Trichoderma* strains showed high tolerance to COil and PAH, their ability to degrade these organic compounds is controversial since the oxidation of PAHs by the enzymatic activity such as

Table 1
Percentage of growth stimulation of seven *Trichoderma* strains exposed to increased doses of benzo[*a*]pyrene (B[a]P).

Trichoderma strain	Fungal growth stimulation[a] (%)				
	B[a]P concentration (mg L^{-1})				
	10	25	50	75	100
CP3	10.9	8.7	–	–	–
CP4	6.7	–	–	–	–
CP38	–	–	–	–	2.7
CP46	9.9	7.4	1.1	–	0.5
CP23	–	–	–	4.7	–
CP56	–	–	–	0.6	–
CPTGC	–	5.1	18.1	17.5	–

[a] Fungal growth stimulation was estimated with respect to the growth of its own corresponding control (without contamination, 100%).

laccasse and peroxidase (Cerniglia and Yang, 1984; Cerniglia et al., 1989; Sutherland et al., 1991; Dhawale et al., 1992; Hammel et al., 1992; Pothuluri et al., 1992) has not been yet described for the *Trichoderma* group. However, recent findings have reported that some *Trichoderma* strains may contribute to the degradation (72%) of 0.1 mM ($\approx$17.8 mg L^{-1}) of PHE in liquid cultures (Hadibarata et al., 2007) via releasing specific oxidative enzymes which participate at the first steps of aerobic degradation of PAH (Resnick et al., 1996; Meyer et al., 1999). Matsubara et al. (2006) indicated that the growth of *T. harzianum* is negatively affected by pyrene and PHE, resulting in low degradation of these aromatic compounds (<10%) in comparison to two ligninolytic fungi (basidiomycetes) such as *Pycnoporus coccineus* and *Coprinus cinereus* whose degradation percentage of these organic compounds ranged between 65 and 85%. On the other hand, *T. viride* has been showed to degrade 50% of B[a]P when exposed to 0.04 mM ($\approx$10 mg L^{-1}) (Verdin et al., 2004).

From our results, we hypothesized that the tolerant strains (CP1, CP4, CP37 and CPX) may have a significant contribution on the degradation of either NAPH, PHE or B[a]P. In addition the enzymatic activities and the physiological responses of the *Trichoderma* species under PAH-contaminated systems are not well known. Thus, further studies are needed to determine the role of *Trichoderma* species on the degradation of PAHs, and to evaluate the enzymatic activity involved on both detoxification and oxidation of organic compounds based on findings reported for filamentous fungi such as *C. elegans* or *P. chrysosporium* (Cerniglia et al., 1989; Sutherland et al., 1991; Dhawale et al., 1992; Hammel et al., 1992). Furthermore, this research allowed us to select prominent strains for being utilized in bioremediation systems.

5. Conclusions

The 11 *Trichoderma* strains differed on tolerating and growing at COil contaminated culture media, and mainly when exposed to increased doses of either NAPH, PHE or B[a]P. The fungal exposure to increased doses of NAPH caused greater drastic and significant growth inhibition for all *Trichoderma* strains in comparison to the effects observed for PHE or B[a]P doses. Four prominent strains of *Trichoderma* (CP1, CP4, CP37 and CPX) with high tolerance to PAHs were detected.

Acknowledgments

This work was financially supported by CONACYT grant 79456. Special thanks to the CONACyT for financial support to R.A.D., and also thanks for comments and suggestions of two anonymous reviewers who helped improving this manuscript. Thanks to James D. Spiers (Auburn University) for the critical reading to correct and improve the English language of this manuscript.

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Journal of Environmental Management 95 (2012) S300–S305

Contents lists available at ScienceDirect

Journal of Environmental Management

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Influence of microorganisms and leaching on simazine attenuation in an agricultural soil

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ARTICLE INFO

Article history:
Received 2 October 2009
Received in revised form
21 December 2010
Accepted 21 June 2011
Available online 28 July 2011

Keywords:
Simazine
Leaching
Microbial degradation
Bioaugmentation
Pseudomonas sp. MHP41

ABSTRACT

Simazine is an *s*-triazine herbicide world widely used for the control of broadleaf weeds. The influence of leaching and microorganisms on simazine attenuation in an agricultural soil long-term treated with this herbicide was studied. To elucidate the leaching potential of simazine in this soil, undisturbed soil columns amended with simazine were placed in a specially designed system and an artificial precipitation was simulated. To evaluate the simazine removal by soil microorganisms, three soil microcosm sets were established: i) control soil; ii) soil subjected to *gamma* irradiation (γ-soil) and iii) γ-soil inoculated with the simazine-degrading bacterium *Pseudomonas* sp. strain MHP41. The simazine-degrading microorganisms in soil were estimated using an indicator for respiration combined with MPN enumeration. The simazine removal in soil was monitored by GC-ECD and HPLC. In this agricultural soil the leaching of the applied simazine was negligible. The *gamma* irradiation decreased in more than one order of magnitude the cultivable heterotrophic bacteria and reduced the simazine-degrading microorganisms. Simazine was almost completely depleted (97%) in control soil by natural attenuation after 23 d, whereas in γ-soil only 70% of simazine was removed. The addition of the simazine-degrading strain MHP41 to γ-soil restored and upheld high stable simazine catabolic microorganisms as well as increased the simazine removal (87%). The results indicated that simazine is subjected to microbial degradation with negligible leaching in this agricultural soil and pointed out the crucial role of native microbiota in the herbicide removal.

1. Introduction

Pesticides are periodically applied in agriculture and forestry. Only a minor fraction of the pesticides reaches the target, whereas the main fraction contributes to the environmental pollution. *s*-Triazine herbicides has been world widely used for the control of broadleaf weeds in agriculture, forestry and annual grasses in non-crop fields. Simazine (2-chloro-4,6-bis(ethylamine)-*s*-triazine) is one of the most applied *s*-triazine herbicides (Dinamarca et al., 2007; Gunasekara et al., 2007). Reports have revealed that *s*-triazines have a mutagenic and carcinogenic potential (Birnbaum and Fenton, 2003; Hayes et al., 2006). The commercialization of most *s*-triazines such as simazine and atrazine has been forbidden in EU countries (Morgante et al., 2010). However, *s*-triazine herbicides are still widely used in North America, Australia and South America (Flores et al., 2009; Seeger et al., 2010).

The fate of the herbicides in soil is governed by different processes such as retention, transport and degradation (Gunasekara et al., 2007). Adsorption to both organic and inorganic matter of the soil influence the leaching, bioavailability and degradation of the herbicides (Li et al., 2003; Gunasekara et al., 2007; Flores et al., 2009). The *s*-triazines may reach surface- and groundwater by runoff and leaching. The persistence of *s*-triazines in soil is strongly influenced by physical and chemical soil properties. The half-life of *s*-triazines is higher at lower temperature and soil moisture (Di Corcia et al., 1999; Barra Caracciolo et al., 2001) because these factors reduce microbial activity (Barra Caracciolo et al., 2005; Shaner and Henry, 2007). Microorganisms are involved in the removal of *s*-triazine herbicides (Rhine et al., 2003; Gunasekara et al., 2007; Hernández et al., 2008a, 2008b; Morgante et al., 2010). The bacterial metabolic pathways involved in simazine degradation are shown in Fig. 1. The upper catabolic pathway converts simazine into cyanuric acid and its enzymes are encoded by the *atzA*, *atzB* and *atzC* genes. The enzyme atrazine chlorohydrolase AtzA catalyzes the hydrolytic dechlorination of simazine to

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doi:10.1016/j.jenvman.2011.06.045

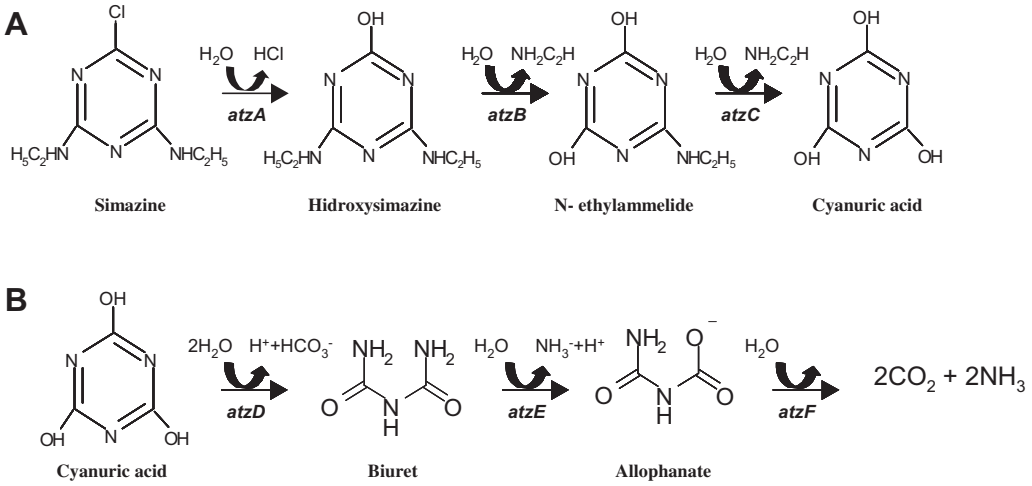

Fig. 1. The bacterial simazine catabolic pathways. A) Upper catabolic pathway. B) Lower catabolic pathway. The catabolic *atz* gene encoding the respective enzyme is indicated at each metabolic step.

yield hydroxysimazine, which is further deaminated by the AtzB hydrolase into N-etilammelide and N-etilamine. N-etilammelide is hydrolyzed by AtzC hydrolase producing cyanuric acid and N-etilamine. In addition, the initial hydrolase TrzN that has broader substrate specificity than AtzA has been described (Santiago-Mora et al., 2005; Devers et al., 2007; Satsuma, 2009). The lower pathway mineralizes cyanuric acid, and its enzymes are encoded by the *atzD*, *atzE* and *atzF* genes. Cyanuric acid is converted by cyanuric acid amidohydrolase AtzD into biuret, which is further transformed by biuret hydrolase AtzE into allophanate. Finally, allophanate is converted by the enzyme allophanate hydrolase AtzF into carbon dioxide and NH_3. An alternative enzyme for the *s*-triazine ring cleavage of cyanuric acid encoded by the *trzD* gene has also been reported (Devers et al., 2007).

The biological degradation of *s*-triazine in soils with a history of *s*-triazine application has been reported (Rousseaux et al., 2003). However, the indigenous microbes are frequently inefficient in the removal of the herbicide. Bioremediation is an interesting technology to accelerate the natural degradation rates of the pollutant and is often more attractive than conventional physicochemical technologies. Bioaugmentation has been used to remove *s*-triazines and to decrease their dispersion to non-agricultural environments (Struthers et al., 1998; Newcombe and Crowley, 1999; Morgante et al., 2010). The selection of the bacterial strain is critical for the success of bioaugmentation. A good candidate for bioaugmentation is the *s*-triazine-degrading bacterium *Pseudomonas* sp. strain MHP41 (Hernández et al., 2008b; Morgante et al., 2010).

The aims of this report were to study the influence of leaching and the role of microorganisms on simazine attenuation in an agricultural soil with a long-term history of simazine application. Undisturbed soil columns as well as microcosm trials were used to study the simazine leaching potential and the simazine removal capability of native soil microorganisms, respectively. The effect of bioaugmentation using the simazine-degrading strain *Pseudomonas* sp. MHP41 in soil that has reduced microbial activity due to *gamma* irradiation was also analyzed.

2. Materials and methods

2.1. Materials

Commercial simazine (Gesatop 90WG, 90% pure) and simazine (99% pure) were purchased from Syngenta (Greensboro, North Carolina, USA) and Atanor S.A. (Buenos Aires, Argentina), respectively. Analytical standard simazine (>99% pure) were purchased from AccuStandard Inc. (New Haven, CT, USA) and Dr. Ehrensdorfer-Schäfers GmbH (Augsburg, Germany).

2.2. Bacterial growth

The *s*-triazine-degrading bacterium *Pseudomonas* sp. strain MHP41 was isolated from an agricultural soil in the Quillota valley, central Chile (Hernández et al., 2008b). MHP41 cells were grown in minimal AM medium (Rousseaux et al., 2001) or MM medium (Morgante et al., 2010) supplemented with simazine (100 mg L^{-1}) as sole nitrogen source. The AM medium (pH 7.0) contained (per L): 1.6 g K_2HPO_4; 0.4 g KH_2PO_4; 0.2 g $MgSO_4$ $7H_2O$; 0.1 g NaCl; 0.02 g $CaCl_2$, 1.0 g sodium citrate as sole carbon source and trace elements (1 mL L^{-1} of a solution containing 2 g L^{-1} boric acid; 1.8 g L^{-1} $MnSO_4 \cdot H_2O$; 0.2 g L^{-1} $ZnSO_4 \cdot 7H_2O$; 0.1 g L^{-1} $CuSO_4 \cdot 5H_2O$; 0.25 g L^{-1} $NaMoO_4 \cdot 2H_2O$); vitamins (1 mL L^{-1} of a solution containing 100 mg L^{-1} of thiamine-HCl and 40 mg L^{-1} of biotin), and $FeSO_4 \cdot 7H_2O$ (1 mL L^{-1} of a 5 g L^{-1} solution). The MM medium (pH 7.0) contained (per L): 0.07 g $Na_2HPO_4 \cdot 2H_2O$, 0.28 g K_2HPO_4, 0.05 g NaCl, 2.95 g sodium succinate as the sole carbon source and 1 mL of trace element stock solution (5 g $MgSO_4 \cdot 7H_2O$, 0.5 g $FeSO_4 \cdot 7H_2O$, 0.25 g $MnSO_4 \cdot H_2O$, 0.32 g $ZnCl_2$, 0.033 g $CaCl_2 \cdot 2H_2O$, 0.018 g $CuSO_4 \cdot 5H_2O$, 0.015 g $CoCl_2 \cdot 6H_2O$, 0.325 g H_3BO_3, 0.5 g EDTA, 7.3 mL HCl 37%). Bacterial growth was monitored by measuring the turbidity at 600 nm ($turbidity_{600nm}$) and by counting colony forming units (CFU) on tryptic soy agar. Bioaugmentation experiments were carried out using calcium alginate-encapsulated MHP41 cells (Morgante et al., 2010). Briefly, MHP41 cells grown in MM medium until $turbidity_{600}$ ~0.8 (1.5×10^8 CFU ml^{-1}), harvested by centrifugation (10,000 g for 20 min at 4 °C) and washed twice in a saline buffer solution were resuspended in 1% w v^{-1} sterile sodium alginate solution (Loba Chemie Ltd., Mumbai, India). The homogeneous mixture was released dropwise into a $CaCl_2$ sterile solution (50 mM) using a sterile syringe. Beads containing the entrapped cells were washed with sterile water before addition to the soil. Alginate beads without cells were used as control.

2.3. Soil sampling

Soil samples were collected from the surface stratum (0–20 cm depth) of an avocado (*Persea americana*) plantation, located in

central Chile (Quillota valley). This soil has been annually treated with simazine (3.5 kg ha^{-1}) for more than 20 years. The soil is a slightly acidic (pH 6.4) and loam soil (10.7% clay, 40.7% silt and 48.6% sand) with an organic matter (OM) content of 8.5%, nitrogen content of 2.0 g kg^{-1} soil and C/N ratio of 12.8 (Morgante et al., 2010). At the collecting time, soil temperature ranged from 16 to 30 °C and residual simazine was not detected in the soil samples.

2.4. Leaching studies on undisturbed soil cores

Undisturbed top soil columns (20 × 15 cm) were obtained from the avocado plantation using a PVC device. Leaching experiments were conducted in freshly collected undisturbed soil columns at three different conditions: i) fresh-applied simazine; ii) two weeks after simazine application, and iii) three weeks after simazine application. Commercial simazine was applied to each core at a concentration of 3.5 kg ha^{-1} (~10 mg kg^{-1}). Soil cores with simazine application were incubated at room temperature (ranged from 13 to 18 °C), and soil moisture was maintained by sprinkling sterile water at 40–50% of the water holding capacity. A chromatographic study has demonstrated that adsorption of simazine to PVC matrix was negligible (data not shown). For the leaching study, an artificial precipitation event of 50 mm during 48 h was simulated using a glass dispensator (Fig. 2). This value of precipitation represents the highest rain event observed directly on field based on the climatic records in the last five years. The artificial precipitation was done with a 0.01 M $CaCl_2$ solution to maintain the soil structure and its percolation capacity during the leaching study (Albarrán et al., 2004). At each sampling time, soil cores and leachate receptor were placed at 4 °C for 12 h, allowing percolation to finish and minimizing further simazine degradation prior chemical analysis. For simazine quantification in soil, soil cores were divided in 0–5 cm, 5–10 cm and 10–15 cm depth layers.

2.5. Microcosm description and bioaugmentation experiments

For the microcosms, the collected soil was sieved sequentially through 5.8 and 2 mm mesh-screens. In order to decrease the native microbiota without disturbing the soil structure, a fraction of the soil was subjected to 3 doses of 25 kGy of *gamma* (γ) radiation (Comisión Chilena de Energía Nuclear, Santiago, Chile). Commercial simazine was applied to soil to obtain a concentration ~14 mg kg^{-1} (Morgante et al., 2010). Each microcosm contained 150 g of simazine-treated soil in a 500 ml sterile flask. Three soil microcosm sets were established: i) control soil; ii) soil perturbed by *gamma* radiation (γ-soil) and iii) γ-soil inoculated with the simazine-degrading bacterium *Pseudomonas* sp. strain MHP41 (γ-soil/MHP41). All treatments were performed in triplicate (under aseptic conditions) and sacrificed at each sampling time to avoid contamination.

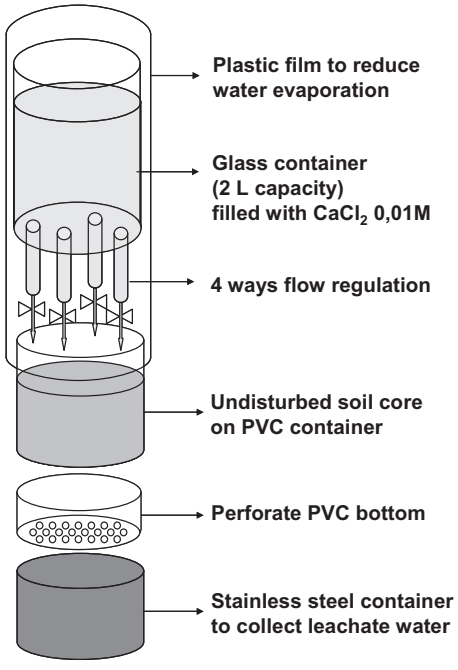

Fig. 2. The system containing the undisturbed soil column. The device was designed and used for simazine leaching studies.

Bioaugmentation was performed as described previously (Morgante et al., 2010). Briefly, alginate-encapsulated cells of strain MHP41 were inoculated into the γ-soil/MHP41 microcosm set (~0.5 g of alginate beads containing cells of strain MHP41 were added to 150 g soil to reach a final concentration of 1 x 10^8 cells g^{-1} of dry soil) every 3–4 days. Sterile alginate beads were added to control and γ-soil microcosms. The alginate beads and the soils were thoroughly mixed with a sterilized spatula. In order to reduce contamination risks due to manipulation, all flasks were covered with polyethylene foil and sampled under aseptic conditions. Microcosms were incubated at room temperature (ranging from 16 to 26 °C). Soil moisture was periodically controlled using infrared absorption mass balance (Sartorius MH30) and maintained by sprinkling sterile water at 40–50% of the water holding capacity. For microbial and chemical analysis, soil samples (50 g) were stored at 4 °C in the dark. Statistical analysis was performed using the two-way ANOVA with treatment and incubation time as factors (Systat 6.1 for Windows). Differences were considered to be significant at $P \leq 0.05$.

2.6. Microbial enumeration in soil

The aerobic cultivable heterotrophic cells from soil samples were enumerated by counting CFU on tryptic soy agar plates. A 10 g soil was placed in a 250-ml Erlenmeyer flask containing 90 ml of sterile sodium chloride solution (0.85% w v^{-1}) and vigorously shaken for 2 h at room temperature. Serial 10 fold dilutions were prepared and aliquots were used to inoculate plates. Plates were incubated at 30 °C for 48 h in the dark.

The enumeration of simazine-degrading microorganisms in the soil was determined using the respiration indicator 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) and a most-probable-number (MPN) method in microtiter plates (Dinamarca et al., 2007). For the MPN enumeration, 1 g of soil sample was added to 9 ml of MM medium (without simazine) and the tubes were vigorously shaken for 2 h at room temperature. Subsequently, ten-fold serial dilutions to extinction were prepared (in triplicate) adding 100 mg L^{-1} of simazine as the sole nitrogen source. One hundred µl aliquots of each dilution were used to inoculate the MPN microplates. Microtiter plates were incubated at 30 °C without shaking for 5 d. After this incubation, TTC (20 µL of a 1% w v^{-1} TTC solution) was added to each well. The positive wells were determined after 4 h incubation at 30 °C by visualization of the TTC formazan production (red colour). The number of cells per gram of dry soil was calculated using an MPN calculator program (Dinamarca et al., 2007).

2.7. Simazine quantification in soil

Simazine was extracted from microcosm soil samples using methanol solution (80% v v^{-1}, pH 2.0) (Morgante et al., 2010). Simazine was quantified using a System Gold HPLC (Beckman, Germany) equipped with a diode array detector and an RP-C18/Lichrospher 5-µm column (Supelco, Bellefonte, USA) (Flores et al.,

2009). Simazine was quantified by comparison with an authentic standard.

Simazine concentration in the soil cores and in the leachate solutions was quantified using GC-ECD (GC-Autosystem-XL, PSS™, Perkin Elmer, USA). Simazine was extracted by adding 35 ml of methanol (80%) to 15 g of air-dried soil (200 rpm during 60 min). This soil suspension was maintained at 4 °C for 2 h for decantation. To minimize methanol concentration, 20 ml aliquot of the supernatant was mixed with 80 ml of water in a glass recipient. This solution was passed through a C_{18} column (C18, 500 mg, AccuBond-Agilent, USA), which subsequently was vacuum-dried during 60 min. Simazine was eluted with ethyl acetate (5 ml), on a conical glass vial. Samples were re-dried under gentle N_2 flow and re-suspended in methanol (3 ml). For simazine quantification on leachate water, whole collected sample was passed through C_{18} column and were processed as described above. One μl of this final fraction was injected on a GC-ECD for simazine identification and quantification. High purity simazine was used as a standard. Simazine detection limit for the whole process was 5.3 ppb. Statistical analysis was performed using simple ANOVA with aged simazine as factor (Statgraphic Plus 5.1). Significant differences were obtained at $P \leq 0.05$.

3. Results and discussion

3.1. Leaching potential of simazine in agricultural soil

The fate of simazine in an agricultural soil was studied. In this study, an agricultural soil with a long-term history of herbicide application was selected. At the collecting time, simazine was not detected in this soil. To study the leaching potential of simazine in the agricultural soil, a special system containing an undisturbed soil column was designed and used in this report (Fig. 2). The soil column represents an appropriate open and intact homogeneous system that resembles natural soil (Burrows and Edwards, 2004). The leaching of simazine in undisturbed soil cores is shown in Fig. 3. After the precipitation event, less than 1% of the herbicide was detected in the leachate solution after 0, 15 and 21 days of simazine application (0.7%, 0.8% and 0.09%, respectively). Residual simazine was only detected on the top 5 cm stratum of the soil column (98%, 36% and 19% after 0, 15 and 21 days, respectively). No simazine was detected at the 5–10 cm depth and 10–15 cm depth soil strata. In agreement, high *s*-triazine retention (>90%) in the top soil stratum (0–10 cm) has been reported (Shaner and Henry, 2007).

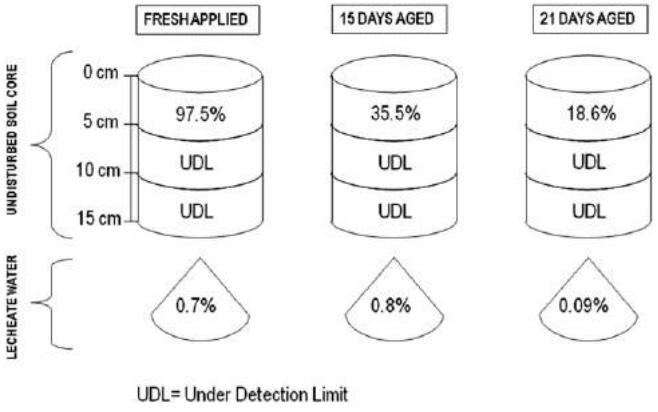

Fig. 3. Effect of simulated precipitation and aging on simazine leaching. Simazine in soil columns was measured by GC-ECD. Simazine quantification under detection limit (UDL) is indicated. Means of simazine recovery on leachate water were significantly different ($P \leq 0.05$).

A simazine adsorption distribution coefficient (K_d) of 9.32 L kg^{-1} for this agricultural soil was reported by Flores et al. (2009). This K_d value indicated a moderate adsorption of simazine to this soil. Adsorption of applied herbicides to soil components is one of the most important processes which determine their leaching potential (Regitano et al., 2006). Both organic matter and clay minerals are involved in the retention of *s*-triazines in soils (Regitano et al., 2006; Flores et al., 2009). Simazine adsorption enthalpy in this soil is exothermic, indicating that simazine adsorption increases at lower temperatures (Flores et al., 2009). Therefore, at high temperatures a higher probability of simazine leaching into the groundwater is expected. Nevertheless, the leaching losses of the applied simazine were very low in the soil indicating that leaching was not a main mechanism of simazine removal. Finally, the negligible leaching observed and the moderate adsorption of simazine to soil components (Flores et al., 2009) suggested that simazine was bioavailable for microorganisms in the top aerobic soil stratum of the agricultural soil.

3.2. Simazine-degrading microorganisms in soil long-term exposed to simazine

In a further analyses, the biological degradation of simazine in the agricultural soil long-term exposed to simazine was investigated. The cultivable heterotrophic bacteria and the simazine-degrading microorganisms were studied in i) control soil); ii) γ-soil and iii) γ-soil inoculated with *Pseudomonas* sp. strain MHP41 (γ-soil/MHP41). The efficient simazine-degrading bacterium *Pseudomonas* sp. strain MHP41 (Hernández et al., 2008b) was selected for bioaugmentation of *gamma* irradiated soil that has reduced microbiota. *Pseudomonas* sp. MHP41 grown in AM broth with simazine (0.5 mM) as the sole nitrogen source reached after 4 d the stationary phase with a $turbidity_{600nm} > 1$ and a maximum of 4.2×10^8 CFU ml^{-1} (Hernández et al., 2008b).

The dynamics of the cultivable heterotrophic bacteria in the soil is shown in Fig. 4. In control soil, the initial heterotrophic bacteria count was 4.5×10^6 CFU g^{-1}. No significant changes were observed in control soil during the incubation (Fig. 4A). On the other hand, *gamma* irradiation decreased the cultivable heterotrophic bacteria count in more than one order of magnitude (2.8×10^5 CFU g^{-1}) at initial time (0 d). During the first two weeks of incubation in γ-soil, an important increase of heterotrophic bacteria was observed, reaching a maximum value of 8.5×10^7 CFU g^{-1} after 9 d. Inoculation of strain MHP41 in γ-soil, increased the heterotrophic bacteria in one order of magnitude after 3 d and more than two orders of magnitude after 9 d.

The simazine-degrading microorganisms of the agricultural soil were studied by the TTC-MPN method. This method is based on the capabilities of microorganisms to use simazine as sole nitrogen source for growth (Dinamarca et al., 2007). The dynamics of the simazine-degrading microorganisms in the different microcosm sets are shown in Fig. 4B. In control soil, the simazine application increased the cultivable simazine-degrading microorganisms in the soil from undetectable (0 d) to 5.2×10^2 cells g^{-1} after 17d (Fig. 4B). This might be an indication that the native degrading microbiota thrives in detectable numbers in the soil with previous treatment history, but probably remains dormant. In γ-soil, negligibly respiratory activity of simazine-degrading microbiota was observed during the first 17 d of incubation. After 23 d, 5.8×10^1 simazine-degrading cells g^{-1} were detected. Bioaugmentation with encapsulated strain MHP41 (γ-soil/MHP41) significantly increased ($p \leq 0.05$) and upheld high stable the simazine-degrading microorganisms, reaching values of 1.4×10^3 cells g^{-1} after 3 d of incubation. These results suggested that the indigenous microbiota could have a major role in the biological degradation of simazine in

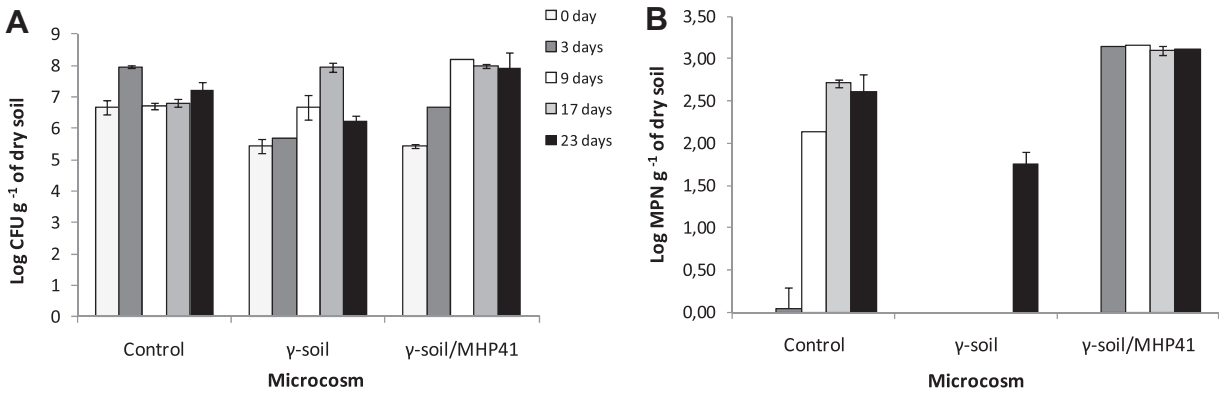

Fig. 4. Heterotrophic bacteria and simazine-degrading microorganisms in agricultural soils subjected to different treatments. Control soil, soil perturbed by *gamma* radiation (γ-soil) and γ-soil inoculated with the simazine-degrading bacterium *Pseudomonas* sp. strain MHP41 (γ-soil/MHP41) were analyzed. The soils were spiked with ~14 mg kg^{-1} simazine. A) Cultivable heterotrophic bacteria enumeration by tryptic soy agar plate counts. B) Simazine-degrading microorganisms estimated by the TTC-MPN method. Values are the average of three independent experiments. Vertical bars indicate standard deviations.

soil long-term exposed to the herbicide. The presence of native simazine-degrading microorganisms in soils long-term exposed to *s*-triazines has been reported (Rousseaux et al., 2003; Rhine et al., 2003; Morgante et al., 2010). The microbial community structure of this soil long-term exposed to simazine has been recently reported (Morgante et al., 2010). Analysis of 16S rRNA gene clone library revealed high bacterial diversity. The most abundant phylotypes thriving in the agricultural soils were identified as *Proteobacteria* (most of them affiliated with *Alphaproteobacteria* and *Betaproteobacteria*), *Acidobacteria*, *Actinobacteria* and *Planctomycetes*. *Bacteriodetes*, *Nitrospirae*, *Verrucomicrobia* and *Gemmatimonadetes* were detected as minor phylogenetic groups in this soil.

The inoculation of *Pseudomonas* sp. strain MHP41 increased the number of simazine-degrading microorganism of soil subjected to *gamma* irradiation. Previous reports established that bioaugmentation enhances the simazine-degrading microorganisms in soils (Struthers et al., 1998; Newcombe and Crowley, 1999; Rousseaux et al., 2003; Morgante et al., 2010). Changes in microbial communities and an increase in *Acidobacteria* and *Planctomycetes* were observed by fluorescence *in situ* hybridization in soil after bioaugmentation with strain MHP41 (Morgante et al., 2010). On the other side, it has been reported that the addition of the herbicide simazine to soil induced changes in the bacterial community structure, increasing *Alphaproteobacteria* (Barra Caracciolo et al., 2005; Morgante et al., 2010) and *Betaproteobacteria* (Barra Caracciolo et al., 2005).

3.3. *Effect of native microbiota and bioaugmentation with Pseudomonas sp. strain MHP41 on simazine removal in soil*

The microbial degradation of simazine in soil was studied by HPLC analysis. The simazine removal in the different microcosm sets is illustrated in Fig. 5. In the control soil, natural attenuation of simazine was observed. After 23 d of treatment, almost complete simazine removal (97%) in soil was observed (Fig. 5). In contrast, the 30% of the initial amount of simazine remained in γ-soil treatment, after 23 d of incubation. Since bioaugmentation with the native strain MHP41 successfully increased simazine-degrading cells in *gamma* irradiated soil, the investigation was further focused on the effect of bioaugmentation on the simazine removal. In the γ-soil, the simazine attenuation was only enhanced after bioaugmentation with strain MHP41 and 87% of the initial amount of simazine was removed after 23 d (Fig. 5). The half-life time of simazine in soil was 4 days in control soil, 17 days in γ-soil and 6 days for the γ-soil inoculated with strain MHP41.

In this study, significant natural attenuation of simazine was observed in the agricultural soil with a long history of herbicide application. However, when the indigenous simazine-degrading microbiota was reduced in more than one order of magnitude by *gamma* irradiation, the simazine removal significantly decreased ($P \leq 0.05$). The longer half-life time of simazine as well as the reduced simazine-degrading activity observed in the γ-soil suggests a central role of microorganisms in the removal of the herbicide. In this study, bioaugmentation with strain MHP41 in soil subjected to *gamma* irradiation increased and restored simazine-degrading microorganisms and increased *s*-triazine removal. The effect of bioaugmentation in γ-soil indicates an essential role of bacteria on simazine removal. The role of bacteria in herbicide removal observed in this study is in agreement with previous reports (Rousseaux et al., 2003; Morgante et al., 2010).

The simazine half-life time values reported in this study are similar with *s*-triazine half-life times in soils with a history of herbicide treatment reported (Struthers et al., 1998; Barra Caracciolo et al., 2005; Shaner and Henry, 2007; Morgante et al., 2010). Simazine and related *s*-triazines may persist in the environment from days up to years depending on physicochemical soil properties, climatic variables and microbial activity (García-Valcárcel and Tadeo, 1999; Gunasekara et al., 2007). Simazine is more persistent at lower temperature and soil moisture (Barra

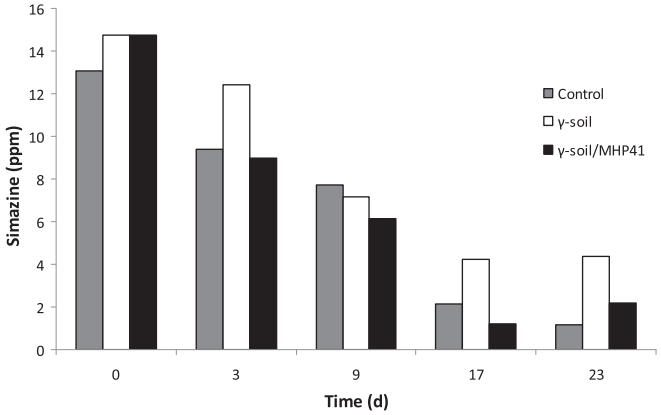

Fig. 5. Role of microorganisms on simazine attenuation in soil. Control soil, soil perturbed by *gamma* radiation (γ-soil) and γ-soil inoculated with the simazine-degrading bacterium *Pseudomonas* sp. strain MHP41 (γ-soil/MHP41) were analyzed. The soils were spiked with ~14 mg kg^{-1} simazine. Simazine in soil was quantified by HPLC. Each value is an average of two independent experiments.

Caracciolo et al., 2005; Shaner and Henry, 2007). Consequently, the removal of the *s*-triazines in this study depends not only on the presence of simazine-degrading microorganisms, but also on incubation conditions that may affect the microbial activity.

4. Conclusions

This study indicates that dissipation of simazine by leaching was negligible whereas the microbial degradation of simazine was crucial for simazine removal in this agricultural soil that has been long-term exposed to this *s*-triazine. *Gamma* irradiation of soils reduced in more than one order of magnitude the cultivable heterotrophic bacteria and reduced the indigenous simazine-degrading microbiota. The simazine half-life time increased from 4 days in control soil to 17 days in γ-soil. Bioaugmentation with strain MHP41 in soil subjected to *gamma* irradiation increased and restored simazine-degrading microorganisms and reduced simazine half-life to 6 days. These results suggest that bioaugmentation is useful for the clean up of herbicide-treated soil with low simazine-degrading microorganisms and that *Pseudomonas* sp. strain MHP41 is an efficient biocatalyst for bioremediation of *s*-triazines. Altogether the results indicate the crucial role of microbes in the removal of *s*-triazine herbicides in soil.

Acknowledgments

MS acknowledges financial support of Millennium Nucleus EMBA P04/007-F, Center of Nanotechnology and Systems Biology, EU ICA4-CT2002-10011, USM 130836, USM 130948 and PBCT Red12 grants. VM and MH gratefully acknowledge MECESUP PUCV-0206, EMBA and CONICYT PhD fellowships.

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Journal of Environmental Management 95 (2012) S306–S318

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journal homepage: www.elsevier.com/locate/jenvman

Review

Enzymes involved in the biodegradation of hexachlorocyclohexane: A mini review

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ARTICLE INFO

Article history:
Received 29 September 2009
Received in revised form
19 June 2011
Accepted 24 June 2011
Available online 10 October 2011

Keywords:
Lindane
γ-Hexachlorocyclohexane
Degradation pathways
Enzymes
Instrumental methods

ABSTRACT

The scope of this paper encompasses the following subjects: (i) aerobic and anaerobic degradation pathways of γ-hexachlorocyclohexane (HCH); (ii) important genes and enzymes involved in the metabolic pathways of γ-HCH degradation; (iii) the instrumental methods for identifying and quantifying intermediate metabolites, such as gas chromatography coupled to mass spectrometry (GC–MS) and other techniques.

It can be concluded that typical anaerobic and aerobic pathways of γ-HCH are well known for a few selected microbial strains, although less is known for anaerobic consortia where the possibility of synergism, antagonism, and mutualism can lead to more particular routes and more effective degradation of γ-HCH. Conversion and removals in the range 39%–100% and 47%–100% have been reported for aerobic and anaerobic cultures, respectively. Most common metabolites reported for aerobic degradation of lindane are γ-pentachlorocyclohexene (γ-PCCH), 2,5-dichlorobenzoquinone (DCBQ), Chlorohydroquinone (CHQ), chlorophenol, and phenol, whereas PCCH, isomers of trichlorobenzene (TCB), chlorobenzene, and benzene are the most typical metabolites found in anaerobic pathways. Enzyme and genetic characterization of the involved molecular mechanisms are in their early infancy; more work is needed to elucidate them in the future.

Advances have been made on identification of enzymes of *Sphingomonas paucimobilis* where the gene LinB codifies for the enzyme haloalkane dehalogenase that acts on 1,3,4,6-tetrachloro 1,4-cyclohexadiene, thus debottlenecking the pathway. Other more common enzymes such as phenol hydroxylase, catechol 1,2-dioxygenase, catechol 2,3-dioxygenase are also involved since they attack intermediate metabolites of lindane such as catechol and less substituted chlorophenols. Chromatography coupled to mass spectrometric detector, especially GC–MS, is the most used technique for resolving for γ-HCH metabolites, although there is an increased participation of HPLC-MS methods. Scintillation methods are very useful to assess final degradation of γ-HCH.

1. Introduction

Halogenated organic substances constitute one of the most important groups of environmental pollutants as a result of their widespread use as herbicides, insecticides, fungicides, solvents, hydraulic and heat transfer fluids, plasticizers, and intermediates for chemical syntheses. Because of their toxicity, bioconcentration, persistence, and ubiquity, the halogenated compounds have raised concern over the possible effects on the quality of life (Fetzner and Lingens, 1994).

The γ-hexachlorocyclohexane (γ-HCH; also called lindane) is a highly halogenated organic insecticide that has been used worldwide, particularly in Mexico, in spite of its banning in first world countries. Lindane has been used for crop protection and prevention of vector-borne diseases for many decades. Negative impacts of lindane on the environment and human health have been reported worldwide (Nagata et al., 1993a,b; Quintero et al., 2005). Theoretically, HCH has eight possible stereoisomers, of which four (α-, β-, γ-, and δ-HCH) predominate in the technical product. γ-HCH is the best known and effective insecticide component of HCH, and only 9–18% of technical HCH (Manickam et al., 2006a,b; Quintero et al., 2007).

2. Aerobic and anaerobic degradation pathways of γ-HCH

Different organisms have been found that are capable of using halogenated compounds as a growth substrate. In

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0301-4797/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jenvman.2011.06.047

particular, several microorganisms are able to degrade γ-HCH such as fungi, cyanobacteria, aerobic (see Table 1) and anaerobic bacteria (Table 2). Yet, only a few of them have been phylogenetically identified (Nagata et al., 2007) and belong to the genera *Sphingomonas, Rhodanobacter* and *Pandoraea* (Mohn et al., 2006).

The key reaction during microbial degradation of halogenated compounds is the removal of the halogen atom, i.e., dehalogenation of the organic halogen. During this step, the halogen atom(s), which is (are) usually responsible for the toxic and xenobiotic character of the compound is most commonly replaced by a hydrogen or a hydroxyl group. Halogen removal reduces both recalcitrance to biodegradation and the risk of forming toxic intermediates during subsequent metabolic steps. For instance, the oxidative conversion of several halogenated organic compounds may lead to the production of acylhalides or 2-haloaldehydes. The latter are very reactive products due to their electrophilicity and may cause cellular damage (Janssen et al., 2001).

2.1. Aerobic degradation pathways of γ-HCH

The aerobic bacterium *Sphingomonas paucimobilis* UT26 can utilize γ-HCH as its sole source of carbon and energy (Imai et al., 1989) (Table 1). Under aerobic conditions, the enzyme γ-hexachlorocyclohexane dechlorinase (LinA) from *Sphingomonas paucimobilis* UT26 catalyzes the elimination of chlorine atoms from the molecule of γ-HCH (Mencía et al., 2006).

The degradation pathway of γ-HCH was extensively analyzed in *S. paucimobilis* UT26. The γ-HCH is transformed to 2,5- dichlorohydroquinone via sequential reactions catalyzed by enzymes LinA, LinB, y LinC. 2,5- dichlorohydroquinone, in turn, is metabolized by enzymes LinD, LinE, LinF, LinGH y LinJ to succinyl-CoA and acetyl-CoA, that are further channeled into and metabolized in the tricarboxylic acid cycle (Fig. 1; Nagata et al., 2007). In this degradation pathway now it is known that several genes are involved, particularly genes that codify for the involved enzymes such as gene LinA for the γ-HCH-dehydrochlorinase that catalyze the reaction of γ-HCH to 1,3,4,6-tetrachloro-1,4-cyclohexadiene (1,4-

Table 1
Summary of aerobic degradation pathways of γ-HCH.

Microorganism	Sources of carbon and energy	Concentrations of pollutant	Matrix	Intermediate metabolites	Removal (%) and removal rate	Ref.
Xanthomonas sp. ICH12	γ–HCH	100 mg/L	Liquid	–γ-PCCH –2,5-DCBQ	• 100% in 8 days • 12.5 mg/L*d	Manickam et al. (2006b)
Pseudomonas aeruginosa ITRC-5	Isomers of HCH	2000 mg/kg	Slurry soil	–γ-PCCH –1,2,4-TCB –CHQ	• 98% in 15 days • 130.6 mg/kg*d	Manickam et al. (2008)
Microbacterium sp. ITCR 1	Isomers of HCH and the intermediates as its sole source of carbon and energy	200 mg/kg	Soil slurry	–2,5DCP	• 96% in 28 days • 6.85 mg/kg*d	Manickam et al. (2006a)
Arthrobacter citreus BI-100	Utilizes γ-HCH as a sole source of carbon and energy	100 mg/L	Liquid	–γ - PCCH -TCCH -TCCD –2–chlorophenol -Phenol -Catechol	• 100% in 8 h • 12.5 mg/L*h	Datta et al. (2000)
Lindane acclimated inocula	γ-HCH	100 mg/L	Soil slurry (agricultural soil with high contents of organic matter and clay)	NR	• 86% in 30 days • 2.86 mg/L*d	Robles-Gonzalez et al. (2008)
Lindane acclimated inocula	Sucrose/γ-HCH Sequential M-A with silicone oil	100 mg/L	Soil slurry (agricultural soil with high contents of organic matter and clay)	–1,4DCB -CB	• 82% in 30 days • 2.73 mg/kg*d	Camacho-Pérez et al. (2010a)
Lindane acclimated inocula	γ-HCH Partially-aerated slurry bioreactor. Triphasic reactor: 20% v/v silicone oil	100 mg/L	Soil slurry (agricultural soil with high contents of organic matter and clay)	NR	•39% in 30 days • 1.3 mg/kg*d	Robles-Gonzalez et al. (2008)
Trametes hirsutus	γ-HCH	0·27 umol/L (0.78 mg/L)	Liquid culture	-TCCH -TCCOL	• 95% in 28 days • 0.026 mg/L*d	Singh and Kuhad (1999)
γ––HCH–degrading microbial consortium	γ–HCH as the sole source of carbon and energy.	10 mg/L	Liquid	95% of Cl^-	•98% in 7 days • 1.4 mg/L*d	Elcey and Kunhi (2010)
Sphingomonas paucimobilis UT26	γ–HCH	4 μg/L	Liquid	γ-PCCH	NR	Imai et al. (1989)
S. paucimobilis UT26	γ–HCH	NA	NA	NA	NA	Mencía et al. (2006)
S. paucimobilis UT26	γ–HCH	NA	NA	NA	NA	Nagata et al. (2007)
Pseudomonas sp. No. 62	γ–HCH			–γ-PCCH –PCB –γ-TCCH	•	Tu, 1975
Pseudomonas putida	Yeast manitol γ–HCH		Liquid	–γ-PCCH –γ-TCCH	•	Matsumura et al., 1976

Notes: γ-HCH: hexachlorocyclohexane; γ-PCCH: γ-pentachlorocyclohexene; 1,2,4-TCB: 1,2,4-trichlorobenzene; 1,4-DCB:1,4-dichlorobenzene; 2,5-DCBQ: 2,5-dichlorobenzoquinone, 2,5-DCP: Dichlorophenol; CB: Clorobenzene; CHQ: Chlorohydroquinone, TCCD : trichlorocyclohexadiene; TCCH: tetrachlorocyclohexene; TCCOL: tetrachlorocyclohexenol; NR: Not reported; NA: Not applicable.

Table 2
Summary of anaerobic degradation pathways of γ-HCH.

Microorganism	External sources of carbon and energy/Electron donors/Electron acceptors	Initial concentration of γ-HCH	Matrix	Experimental conditions	Intermediate metabolites	Removal (%) and removal rate	Ref.
Granular sludge (8 g VSS/L)	Starch (2 g COD/L)	100 mg/kg soil	Soil slurry (Sandy slime soil)	pH 7 Temp. 30 °C 350 RPM V: 4000 mL	PCCH, TCCH, 1,2,3-TCB, 1, 3-DCB, CB	• 100% in 3 days • 33.33 mg/kg*d	Quintero et al. (2006)
Anaerobic granular sludge	–	4.1 mg/kg soil	Soil slurry (sandy clay loam) and 2.3% organic matter	Temp. 30 °C V:30 mL	ND	• 95% in 2 weeks • 0.24 mg/kg*d	Baczynski et al. (2010)
Desulfovibrio gigas and *Desulfococcus multivorans*. (50 mL preculture)	Sulfate (5 mM)	7 mg/L	Liquid	pH 7 Vt: 950 mL 120 RPM	Benzene and CB	• 100% in 19 days • 0.37 mg/L*d	Badea et al. (2009)
Lindane acclimated inocula (500 mg VSS/L)	Sucrose/sulfate	100 mg/kg	Soil slurry (agricultural soil with high contents of organic matter and clay)	pH 7 Vt: 100 mL 120 RPM	PCCH; 1,2,4-TCB; 1,2,3-TCB; CB, benzene	• 88% in 30 days • 2.93 mg/kg*d	Robles-Gonzalez et al. (2008)
Lindane acclimated inocula (500 mg VSS/L)	Sequential M-SR	100 mg/kg	Soil slurry (agricultural soil with high contents of organic matter and clay)	pH 7 Vt: 100 mL 120 RPM	PCCH, 1,2,4-TCB	• 98% in 30 days • 3.26 mg/kg*d	Camacho-Pérez et al. (2010a)
Granular sludge (8 g VSS/L)	Starch (2gCOD/L)	12 mg HCH/L	Liquid	pH 7 Temp. 30 °C 180 RPM Vt: 400 mL	CB, 1,3-DCB, 1,2-DCB, PCCH	• 100% in 4 days • 3 mg/L*d	Quintero et al. (2005)
Lindane acclimated inocula (500 mg VSS/L)	Sucrose/Methanogenic	100 mg/kg	Soil slurry (agricultural soil with high contents of organic matter and clay)	pH 7 Vt: 100 mL 120 RPM	NR	• 47% in 30 days • 1.57 mg/kg*d	Robles-Gonzalez et al. (2008)
Clostridium sp	–	3.7 mg/L	Liquid	NR	75% Chloride ion theoretical lindane degradation	• 99% in 27 h • 0.14 mg/L*h	MacRae et al. (1969)
Anaerobic bacteria from sediments	Short chain fatty acids/Sulfate	7 mg/L	Liquid	Vop: 45 mL T: 30 °C	CB and benzene	• 90% in 20 days • 0.31 mg/L*d	Boyle et al. (1999)
Anaerobic sludge (2000 mL)	Methanol as a supplementary substrate and electron donor (500 mg/L)	100 mg/L of THCH	Liquid	Continuous UASB reactor V: 4000 mL	NR	• 99% in 60 days • 1.65 mg/L*d	Bhat et al., 2008
Clostridium rectum	Alanine, leucine, pyruvate, a leucine–proline mixture, dithiothreitol, and hydrogen gas	0.017 mg/L	Liquid	Vt: 1 L T: 35 °C pH: 7.4	Monochlorobenzene and γ–3,4,5,6–TCCH	• 100% in 3 h • 0.0056 mg/L*h	Ohisa et al. (1980)

Notes: γ-HCH: hexachlorocyclohexane; 1,2,3.TCB:1,2,3-trichorobenzene,1,2,4-TCB: 1,2,4-trichlorobenzene; 1,2-DCB:1,2-dichlorobenzene; 1,3-DCB:1,3-dichlorobenzene; CB: Clorobenzene; COD: chemical oxygen demand; M-SR: methanogenic-sulfate-reducing; ND: Not detected; NR: Not reported; PCCH: Pentachlorocyclohexene; UASB: upflow anaerobic sludge blanket; VSS: Volatile Suspended Solids; TCCH: Tetrachlocyclohexene; THCH: technical grade hexachlorocyclohexane.

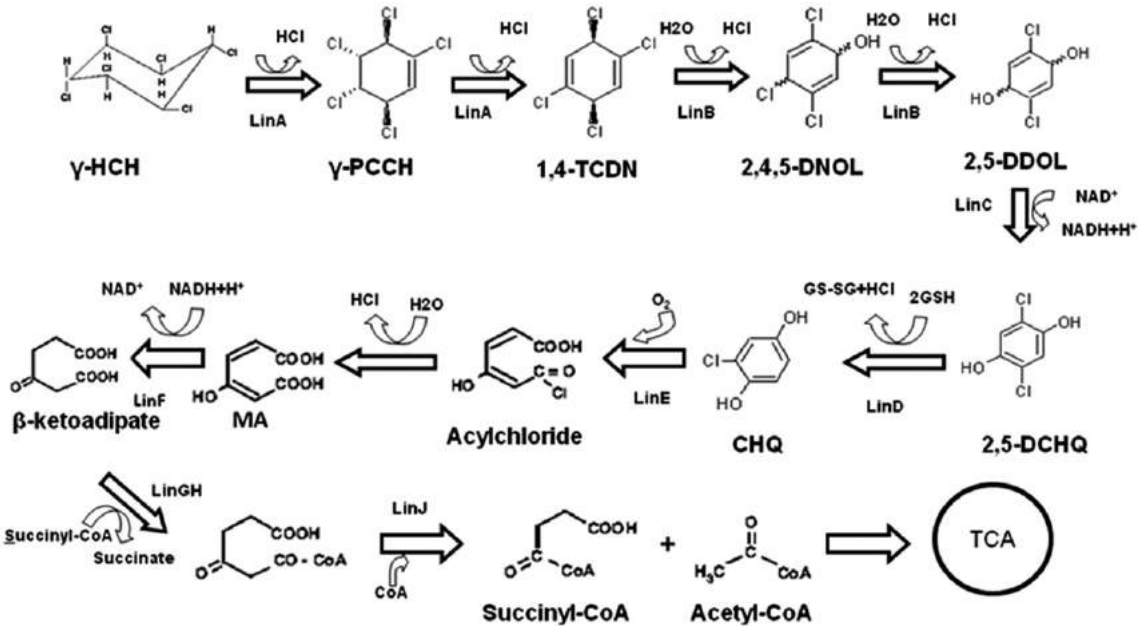

Fig. 1. Proposed degradation pathways of γ-HCH in *S. japonica* UT26 (adapted from Nagata et al., 2007). Keys: γ-PCCH: γ-pentachlorocyclohexene; 1,4-TCDN:1,3,4,6-tetrachloro-1,4-cyclohexadiene; 2,4,5-DNOL: 2,4,5-trichloro-2,5-cyclohexadiene-1-ol; 2,5.DDOL :2,5-dichloro-2,5-cyclohexadiene-1,4-diol; 2,5-DCHQ : 2,5-dichlorohydroquinone; CHQ: Clorohydroquinone; MA: Maleylacetate; TCA:Tricarboxylic acid cycle.

TCDN) via γ-pentachlorocyclohexene (γ-PCCH). Nagata et al. (1993a,b) have proposed that 1,4-TCDN is transformed to 1,2,4-TCB by non-enzymatic reactions due to the fact that 1,4-TCDN contains an instable bond whereas the aromatic ring of 1,2,4-trichlorobenzene (1,2,4-TCB) is more stable. The gene LinB codifies for enzymes of the family of haloalkane dehalogenases that are responsable for catalyzing the reaction of β-HCH to 2,3,4,5,6—pentachlorocyclohexane (PCCH) by hydrolytic dehalogenation. The gene LinC is envolved with dehydrogenases that transforms 2,5-dichloro-2,5-cyclohexadiene-1,4-diol (2,5.DDOL) to 2,5- dichlorohydroquinone (2,5-DCHQ). The latter is further converted to β-cetoadipate by the reductive enzyme dechlorinase LinD (Miyauchi et al., 1998), a ring-cleavage dioxyenase LinE (Miyauchi et al., 1999), and the maleylacetate reductase, LinF (Endo et al., 2005). Other genes have been identified, such as the one for succinyl-CoA:3-oxoadipate CoA transferase (LinGH) and the gene for β-ketoadipyl CoA thiolase (LinJ) where this enzyme is involved in the conversion of β-ketoadioate succinyl-CoA and acetyl-CoA.

Datta et al. (2000) presented data on lindane aerobic degradation by batch cultures of Arthrobacter citreus BI-100 (Table 1); this bacterium was reported earlier to use lindane as carbon source. Six metabolites of γ-HCH of the strain BI-100 were identified, which included γ-PCCH, TCCH, TCCD, 2-chlorophenol, phenol, and catechol produced at different periods of growth. BI-100 appears to degrade γ-HCH via γ-PCCH to TCCH, which is then transformed to other metabolites. However, the TCCH isomer produced by BI-100 could not be ascertained in this study.

According to Datta et al. (2000), aerobic degradation pathways of γ-HCH by *Sphingomonas paucimobilis* (Imai et al., 1989; Mencía et al., 2006), and a strain of *Pseudomonas* sp. No. 62 (Tu, 1975) somewhat differed from the degradation pattern shown for *Achrobacter citreus* BI-100. Yet, γ-PCCH was reported to be the first product of γ-HCH metabolism for the three microorganisms as well as in others (Benezet and Matsumura, 1973; Imai et al., 1989; Matsumura et al., 1976; Sahu et al., 1990; Tu, 1975).

Subsequent to TCCD, 2-chlorophenol appeared as a significant metabolite during the degradation of lindane by Ac BI100. Formation of 2-chlorophenol from TCCD might occur via the formation of transient intermediate(s), which were not detected. Catechol was also identified as one of the metabolites to be produced in the late phase of growth of the Ac BI100; this suggests the possibility of its formation by reductive dechlorination followed by hydroxylation of 2-chlorophenol. The authors suggested that 2-chlorophenol was converted to phenol and then to catechol presumably by reductive dechlorination (DeWeerd and Suflita, 1990), followed by monooxygenase reaction.

Regarding some differences in pathways, Ac BI100 does not present the formation of tetrachloro-cyclohexadiene as a transient product by dehydrochlorination of γ-PCCH that occurs during the metabolism of γ-HCH by Sp. paucimobilis (Nagata et al., 1994). It is interesting to note the formation of TCCD by Ac BI100, in contrast to the production of 1,2,4-trichlorobenzene, as a dead-end product by Sp. paucimobilis during the metabolism of γ-HCH (Nagata et al., 1994). However, no dead-end product was found to accumulate during γ-HCH metabolism by BI-100. Futhermore, Sp. Paucimobilis pathway does not show the appearance of 2-chlorophenol, catechol, and phenol. The study of Datta et al. (2000) concludes with that some γ-HCH metabolites produced by A. citreus BI-100 are quite different from those produced from γ-HCH by any other single microorganism reported in the literature (Matsumura et al., 1976; Nagata et al., 1993a,b; Manickam et al., 2006a,b).

In *Pseudomonas putida* (Matsumura et al., 1976), two patterns of γ-HCH degradation were observed, one being the dehydrochlorination pathway in which γ-PCCH is formed. The second pathway is a NAD-dependent one, that involves the production of large amounts de γ 3,4,5,6-tetrachlorocyclohen-1-ene (γ -BTC). Further degradation of γ -BTC seems to occur by an FAD-dependent reductive dechlorination mechanism. It is known that aromatic ring opening by *P. putida* takes place only when a few chlorines are present in the molecule, i.e., benzene, monochloro- or at most dichlorobenzenes. Therefore, participation of these three different metabolic systems is required to complete the degradation of γ -BTC G-BCH in *P. putida* for the second pathway.

Other researchers have reported lindane removal and degradation by aerobic undefined consortia (Elcey and Kunhi, 2010; Camacho-Pérez, 2010a; Camacho-Pérez et al., 2010b) under

a variety of initial lindane concentrations and cultures (Table 1). For instance, Elcey and Kunhi (2010) have found high lindane removal efficiency (up to 98% at 7-day incubation) in batch liquid cultures with initial 10 mg/L lindane as the sole source of carbon and energy. They did not report metabolites; yet, they observed a 95% of organic chlorine mineralization. On the other hand, Robles-González (2008) tested aerobic batch slurry bioreactors for the remediation of a fine texture, high organic matter soil contaminated with 100 mg/kg lindane. They seeded the slurry bioreactor with an inoculum previously acclimated to low concentrations of lindane in a lab scale suspended growth biomass reactor. An 86% removal at the 30th day was found in the slurry bioreactors; growth of aerobic lindane-clastic bacteria was also observed. Furthermore, Camacho-Pérez et al. (2010a) tested sequential slurry bioreactors operated the first 15 d as methanogenic reactor and the last 15 d as aerobic bioreactor, for the remediation of the same soil. This configuration was coined as sequential methanogenic-aerobic slurry bioreactor. They used acclimated inocula and silicone oil at 20% v/v as a lindane-desorbing agent (leading to a triphasic slurry bioreactor). Lindane removals up to 82% were found when silicone oil was added whereas only 52% were observed for the reactors with no solvent; metabolites of the chlorobenzene family were detected. So, the inclusion of a first anaerobic stage to the aerobic slurry bioreactor operation did not improve lindane removal, compared to that of the full aerobic slurry bioreactor.

2.2. Anaerobic degradation pathways of γ-HCH

Microbial reductive dehalogenation of organochlorine pesticides in marine and estuarine sediments has received increased attention in the last 15 years. MacRae et al. (1969) reported one of the first experiments on the anaerobic degradation of γ-HCH; they observed 75% removal of lindane (Table 2). Quintero et al. (2006) observed total depletion of α and γ-HCH in a polluted soil after 3 days anaerobic incubation; they used an initial lindane concentration of 100 mg kg^{-1} soil, bioaugmentation with methanogenic anaerobic sludge at 8 g VSS L^{-1}, and starch as electron donor. During the degradation, traces of diverse intermediate and end-products compounds were detected, such as pentachlorocyclohexane isomers (PCCH), tetrachlocyclohexane (TCCH), 1,2,3-trichlorobenzene (1,2,3-TCB), 1,3-dichlorobenzene (1,3-DCB), and chlorobenzene (CB). Anaerobic degradation of lindane can also occur via formation of γ-TCCH, as reported for anaerobic bacteria *Clostridium* sp. and *Clostridium rectum* (Ohisa et al., 1980). Boyle et al. (1999) found that anaerobic bacteria from sediments and pure cultures of sulfate-reducing bacteria effected lindane dehalogenation with accumulation of final products such as monochlorobenzene and benzene. Enrichment cultures from marine sediments were supplemented with 7 mg/L lindane and a mixture of short chain fatty acids. It was found that these enrichments consumed lindane and both monochlorobenzene and benzene were detected as transformation products. In further experiments with transfers to fresh media (lactate, citrate as substrates) and using spiking and absence of molybdate as specific inhibitor of sulfate-reducing bacteria, it was shown that sulfate-reducing bacteria were involved in lindane transformation in those cultures. Again, monochlorobenzene and benzene were identified as transformation products.

Quintero et al. (2005) evaluated the anaerobic degradation of γ, α, β, δ-HCH in liquid and slurry cultures. While α- and γ-HCH disappeared after 20–40 days, the most recalcitrant isomers: β-and δ-HCH were only degradaded after 102 days. Intermediate metabolites observed were pentachlorocyclohexane (PCCH), tetrachlorocyclohexane (TCCH), tri, di and mono-chlorobenzenes Fig. 2.

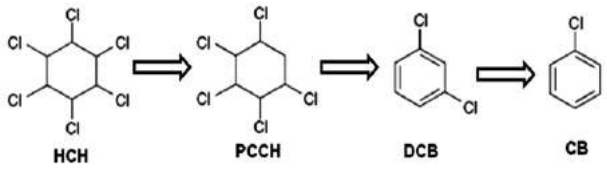

Fig. 2. Proposed degradation pathways of γ-HCH under anaeobic conditions (adapted from Quintero et al., 2005). Keys: PCCH: Pentachlorocyclohexene; DCB: Dichlorobenzene; CB: Clorobenzene.

Camacho-Pérez (2010a,c) evaluated the role of a desorption-aid silicone oil n performance of slurry bioreactors treating a heavy soil polluted with lindane. They reported that the sequential methanogenic-sulfate-reducing slurry bioreactors without silicone oil showed the highest lindane removal efficiency 98%. Yet, units added with silicone oil exhibited a close removal (up to 93%). The second stage of operation (sulfate-reducing) contributed the most to lindane reduction. On average, *ca.* 41% of lindane was removed in the 15-d methanogenic stage whereas the sulfate-reducing stage was responsible for 57% of the consumption with a 93% intrinsic disappearance of the pollutant. After 15 d operation pentachlorocyclohexene was detected in the slurries, whereas 1,2,4-trichlorobenzene was present after 30 d incubation.

On other hand, Robles-Gonzalez (2008) assessed the bioremediation of a heavy soil polluted with 100 mg lindane/kg in full sulfate-reducing slurry bioreactors. Removal was 88% whereas the detected metabolites after 30 d operation were PCCH; 1,2,4-TCB; 1,2,3-TCB; CB, and benzene.

3. Important enzymes and genes involved in the metabolic pathways of γ-HCH degradation

Fig. 3 shows a general scheme of the main enzymes involved in the aerobic degradation pathway of lindane (adapted from Kanehisa Laboratories, http://www.genome.jp/kegg-bin/show_pathway?23816/map00361.args, Nagata et al., 2007, Chang et al., 2009; Braunschweig enzyme database). From this flow diagram we chose to discuss Lin A, Lin B, and selected oxidases. We also discuss a special type of dehalogenases involved in the anaerobic degradation pathways of chlorinated organic compounds that would be relevant in bioaugmentation of bioremediation with specialized bacteria (i.e., dehalorespirers or halorespirers).

3.1. γ- hexachlorocyclohexane dehydrochlorinase (LinA)

Dehydrochlorinases are enzymes that eliminate HCl from a substrate molecule leading to the formation of a double bond (Van Pee and Unversucht, 2003). Three main dehydrochlorinases are recognized so far (Nagata et al., 2001; Trantírek et al., 2001): (i) 3-Chloro-D-alanine dehydrochlorinase from *P. putida* employs the cofactor pyridoxal 59-phosphate during catalysis; (ii) γ-Hexachlorocyclohexane dehydrochlorinase (LinA)1 from the γ-hexachlorocyclohexane-degrading bacterium *Sphingomonas paucimobilis* UT26 catalyzes the conversion of γ-hexachlorocyclohexane (γ-HCH) to 1,2,4-trichlorobenzene (1,2,4-TCB) via γ-1,3,4,5,6-pentachlorocyclohexene (γ-PCCH); and (iii) the DDT-dehydrochlorinase catalyzes dehydrochlorination of 1, 1, 1-trichloro-2,2-bis (4-chlorophenyl) ethane to 1,1-dichloro −2,2-bis (4-chorophenyl ethane) and requires glutathione for its activity.

Yet, LinA is quite different from the other two other dehydrochlorinases metioned above (Trantírek et al., 2001; Nagata et al., 2001). DDT dehydrochlorinase and 3-chloro-D-alanine dehydrochlorinase require glutanione and pyridoxal 5′-phosphate for their

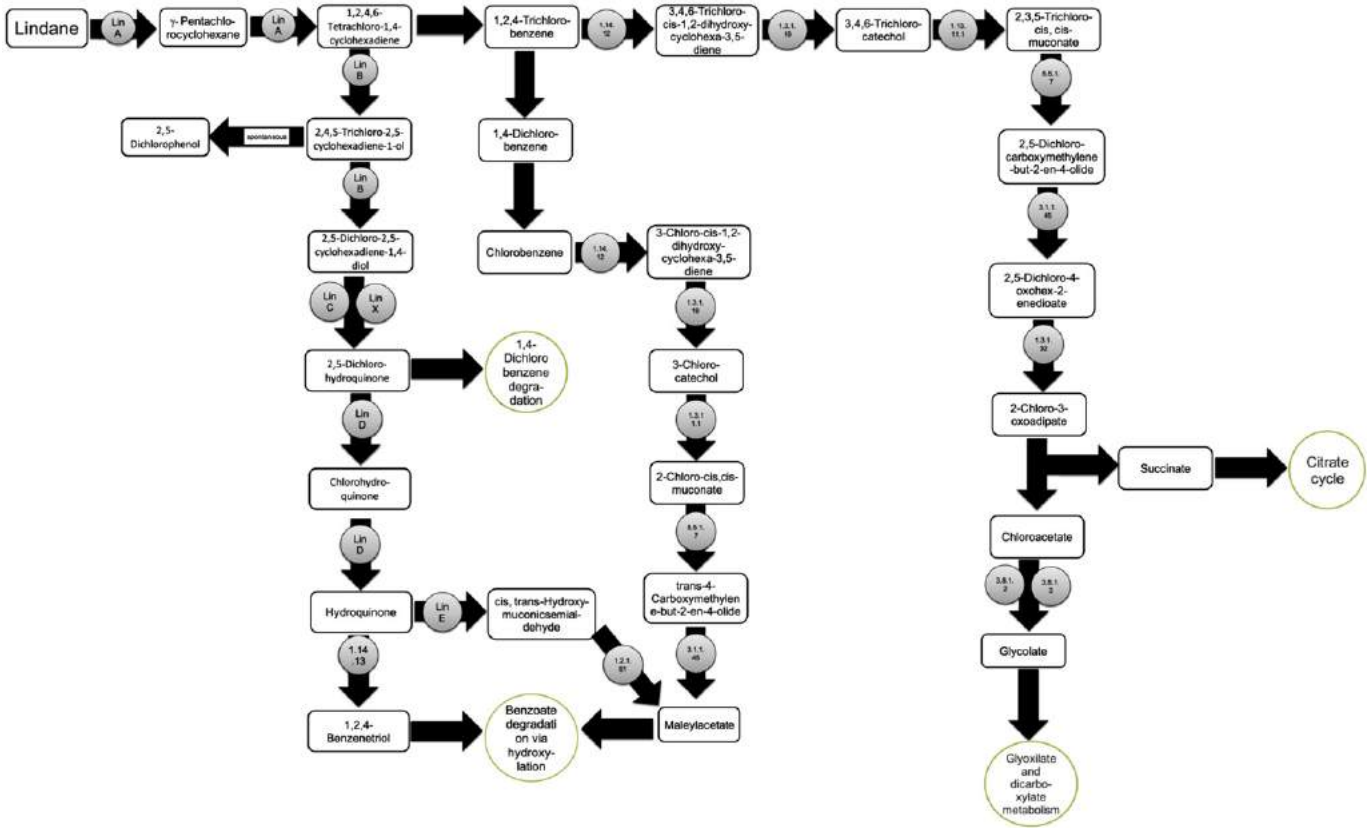

Fig. 3. Enzymes involved in the aerobic degradation of lindane.

activities, respectively. On the other hand, LinA does not require any cofactors (Nagata et al., 2001). Furthermore, the LinA catalyzes two steps of dehydrochlorination from γ-HCH to 1,3,4,6-tetrachloro-1,4-cyclohexadiene (1,4-TCDN) via γ-pentachlorocyclohexane (γ-PCCH).

3.2. Haloalkane dehalogenation LinB

The hydrolysis of haloalkanes to yield alcohols and inorganic halides is catalyzed by special α/β-hydrolases also known as haloalkane dehalogenases. The study of these enzymes is crucial or their development and application to degradation and bioremediation of organohalide-contaminated industrial waste (Oakley et al., 2002).

Haloalkane dehalogenases make up one such important class of enzyme because of their ability to attack polychlorinated aliphatic hydrocarbons, which are produced in several industrial processes (Fetzner and Lingens, 1994). Haloalkane dehalogenases remove halides from organic compounds via a hydrolytic mechanism that results in the production of the corresponding alcohol, inorganic halide and hydrogen ion. One such haloalkane dehalogenase, LinB, is isolated from hexachlorocyclohexane degrading bacterial strain *Sphingomonas paucimobilis* UT26. It is the second enzyme in the biochemical pathway enabling the bacterium to utilize γ-hexachlorocyclohexane as its sole carbon and energy source (Nagata et al., 2007). The first two steps in the degradation of lindane by *Pseudomonas paucimobilis* are catalyzed by a dehydrohalogenase. The elimination of hydrochloric acid catalyzed by this enzyme leads to the formation of a double bond Fig. 4 (Heinz van Pée and Unversucht, 2003).

3.3. Other enzymes

Since transformation of lindane could lead to a varied family of intermediate compounds, we will summarily review the key enzymes that act in further transformation/degradation of most commonly reported metabolites of lindane. Datta et al. (2000) reported phenol, catechol, and a few chlorinated phenols as intermediates of aerobic degradation of lindane by *Achrobacter citreus*. The first step in aerobic metabolism of phenol is phenol hydroxylation to catechol by NADPH-dependant phenol hydroxylase (Viggor et al., 2008). This enzyme consists of a 200–255 kDa dimeric hydroxylase of the form $(\alpha\beta\gamma)_2$, a cofactorless 10–16 kDa regulatory protein that enhances catalytic turnover by 30–150-fold and a FAD- and [2Fe–2S]-containing 38–40 kDa reductase that supplies the hydroxylase with electrons by consuming NADH. It is

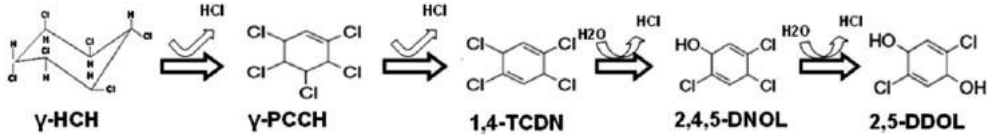

Fig. 4. Degradation of lindane catalyzed by a dehydrohalogenase (adapted from Heinz van Pée and Unversucht, 2003). Keys: γ-PCCH: γ-Pentachlorocyclohexene; 1,4-TCDN:1,3,4,6-tetrachloro-1,4-cyclohexadiene; 2,4,5-DNOL: 2,4,5-trichloro-2,5-cyclohexadiene-1-ol; 2,5.DDOL: 2,5-dichloro-2,5-cyclohexadiene-1,4-diol.

Table 3
Summary of instrumental methods for identifying and quantifying lindane and its intermediate metabolites.

Method and equipment	Intermediate metabolites and retention time (min)	Extraction procedure	Conditions	Remarks	Ref
Lindane: TLC, a 2- to 10-μl sample was spotted on the plates (silica gel 60 20 × 20 cm, 0.20 mm thickness), and the chromatograms were developed in cyclohexane and visualized by spraying the chromogenic reagent, followed by UV exposure. The chlorinated compounds were detected as dark brown spots on TLC plates. GC was performed with a Shimadzu model equipped with Ni^{63} electron capture detector	−2,5−DCP	Extracted twice with an equal volume of hexane and acetone (1:1 v/v) followed once with hexane alone	The carrier gas was nitrogen with a flow rate of 60 ml/min. Temperature for column, injector and detector were 190, 250, and 250 °C, respectively.	Liquid cultures and contaminated soil *Microbacterium* sp	Manickam et al. (2006a)
GC−MS Perkin−Elmer Autosystem XL (Perkin−Elmer, Waltham, MA) gas chromatograph equipped with a PE-Turbomass quadruple mass spectrometer	−γ-PCCH: 8.13 −1,2,4-TCB: 5.57 CHQ: 7.14	Extracted twice with 500 ml of hexane: acetone (1:1 v/v) and once with ethyl acetate. The pooled extracts were passed through anhydrous sodium sulfate to eliminate the water content and were concentrated on a rotary evaporator	Injector temperature 250 °C. Carrier gas was helium at 1 ml/min		Manickam et al. (2006b)
GC−MS autosystem XL-GC, interfaced to a Turbomass mass spectrometer	−γ-PCCH −2,5-DCBQ Mass fragmentation reported: −γ-PCCH: 181 *m/z* −2,5-DCBQ: 176*m/z*	Twice with acetone, hexane and finally once with ethyl acetate. The solvents were removed under vacuum by rotary evaporation, and the residue was redissolved in ethyl acetate prior to analysis	The injector temperature was 250 °C, carrier gas was helium at a flow rate of 1 ml/min		Manickam et al. (2008)
Varian-Saturn GC/MS, equipped with injection port split−splitless, automatic injector and connected to a mass spectrometer with ion traps	−Ethanone: 6.3 −1-benzenecarbobyl chloride: 6.5	3 ml of homogenized medium were immediately transferred to 15 ml volumetric tubes and mixed with 6 ml of a 1:1 hexane−acetone solution, the tubes were hermetically sealed and shaken for 10 min in a vortex in orden to attain the transport metabolites from water or soil to the organic phase, and centrifuged at 2500 rpm for 10 min to split the organic and aqueous phases; an aliquot of the organic phase was taken for GC−MS analysis	Carrier gas was helium at a flow rate of ml/min. The oven temperature was programmed as follows: hold time at 60 °C, 2 min; ramp rate at 20 °C min^{-1} to 180 °C, ramp rate at 5 °C min^{-1} to 200 °C, and finally, a ramp rate at 10 °C min^{-1} to 300 °C. The injection volume 1 μl via a splitless injection at 280 °C.		Quintero et al. (2007)
Applied Biosystem 3200 Q TRAP LC/MS/MS system Triple quadropole with ion trap	−PCCH: 255 *m/z* −DCB: 147 *m/z* −CB: 111 *m/z* −TCB: 181 *m/z* −Benzene: 78 *m/z*	Ibidem	Sample was injected at a rate 10 μl/min through and electrospray injection device		Robles-González (2008)
Lindane: GC with a Hewlett−Packard 5890 (Series II) equipped with an Electron Capture Detector (ECD) and 10 m 0·5 mm OV-17 silicon column. min_1	NA	5 mL ethyl acetate was added to this cell-free culture filtrate. The solution was vortex-mixed to extract lindane and its metabolites from culture filtrate	Carrier gasN_2 (20 mL/min). Injector, detector and column temperatures were 280, 300 and 180 °C, respectively.	Batch liquid cultures; white-rot fungus *Trametes hirsutus*	Singh and Kuhad (1999)
Metabolites: identified by (i) comparing with the GC-ECD peak of standard TCCH, and (ii) GC−MS analysis; JMS DX-303 chromatograph model equipped with an OV- 1 capillary column (0·25 mm × 25 m).	- TCCH: (peak *m/z* 218), fragment ions of *m/z* 183, 147, 122, and 111 - TCCOL: The fragmentation pattern of ions of *m/z* 138, 163, and 199	*ibidem*	Column temperature was increased from 120 to 250 °C at 16 °C/min. Electron impact mass spectrometry was measured at a 70 eV ionization potential, 300 mA trap current and 200 °C ion source temperature.		
Lindane: GC with chromatograph equipped with ECD.	NA (i) γ-PCCH: *m/z* 252;	Culture filtrates at different periods of growth were extracted thrice each time	(i) Column temperature was held at 100 °C for 7 min, then increased to 250 °C at 5 °C/	Batch liquid cultures;	Datta et al. (2000)

Metabolites: (i)A GC–MS of metabolites produced by the strain BI-100 from g-HCH was performed by using a Shimadzu QP 2000 equipped with an ULBON HR-1 equivalent to an OV-1 fused silica capillary column (0.25 mm_50 m; 0.25 mm film thickness) (ii) HPLC equipped with Nova-Pak C18 reverse phase column (3.9_150 mm; particle size 5 mm) AND UV detector (Waters 486) at 254 nm for 2-chlorophenol or phenol, Minimum detection limit is 1 μg.	TCCH: *m*/*z* 218; TCCD; *m*/*z* 183; 2-chlorophenol: *m*/*z* 128; phenol: *m*/*z* 94; catechol: *m*/*z* 110. (ii) 2-chlorophenol (3.2); phenol (2.0); catechol (1.5).	with an equal volume of ethyl acetate. *Ibidem*	min with He as the carrier gas at 2 ml/min. The MS was operated at an electron ionization energy of 70 eV. A 0.5 ml solution containing 10 mg of sample was injected in each case. (ii) A 10 mL solution containing 10–20 mg of sample was injected in each case. Water: methanol (60 : 40) and water: acetic acid :methanol (59.9 : 0.1 : 40) were used as the mobile phase at 1 ml/min.	*Arthrobacter citreus* strain BI-100	
Lindane: GCHewlett-Packard model 5890 with an ECD set to 350 °C, a 30-m DB-5 capillary column (0.25- μm film thickness, 0.25-mm inner diameter).	NA	2.0 ml slurry from the cultures was sampled and extracted with 2.0 mL hexane (supplemented with hexachlorobenzene as an internal standard) for 24 h with gentle shaking.	150 °C for 1 min, 20 °C min 31–235 °C. 2 ml extract was introduced using a split injector (30:1 split ratio) which operated at 280^{3}C.	Enrichment cultures from marine sediments were supplemented with 7 mg/L lindane and a mixture of short chain fatty acids	Boyle et al. (1999)
Metabolites: (i) GC HP 5890 equipped with a 30 m DB-Wax capillary column (0.25 μm thickness × 0.32 mm id) (ii) GC–MS was performed on an HP model 5890 equipped with capillary column DB-5 (0.25-μm × 0.25-mm id × 30 m), coupled to a 5971A mass spectrometer. The gas chromatography	(i)benzene and monochlorobenzene recovered represents approximately 60% of the added lindane (ii) Benzene: of 78 *m*/*z* ion and fragment ion of 52 *m*/*z* for a peak at 2.9 min. Monochlorobenzene: 112 *m*/*z* with an *m*+2 of 114 *m*/*z* and a fragment ion of 77 *m*/*z* for a peak at 8.0 min	Metabolites were extracted immediately with pentane (1:1) supplemented with fluorobenzene as an internal standard.	(i)Operated isothermally at 35 °C. The injector (15:1 split ratio, 2 μL) and detector (flame ionization) were set at 280 °C. (ii) Column at 35 °C for 5 min, 10 °C min^{-1} to 200 °C. The MS was operated in the scan mode using electron ionization. 2 μL samples were introduced using a split injection (10:1 split ratio) set at 280 °C; mass transfer line (detector temperature) 280 °C.	Culture of *Desulfovibrio. gigas*	
GC–MS. A Hewlett–Packard 6890 gas chromatograph coupled to a 5973 quadrupole mass spectrometer was used for identification and structural characterization. γ-HCH and its metabolites were separated on a BPX-5 capillary column (29.1 m × 0.32 mm × 0.25 μm)	-γ–3,4,5,6TCCH -CB -Benzene	Samples xtracted with 1 mL of DCM containing 100 μM hexachlorobenzene (HCB) internal standard for γ-HCH and 100 μM toluene as the internal standard for the metabolites. Vials were shaken for 24 h at 110 rpm, 12 °C. The organic phase was separated transferred into vials containing anhydrous Na_2SO_4 for water removal. 100 μL of this dry organic phase was analyzed by GC–MS	40 °C initial temperature (5 min), increase at 5 °C/min to 110 °C (0 min), 20 °C/min to 180 °C (0 min), 5 °C/min to 230 °C (0 min), and 20 °C min-1–300 °C (3 min).	*Desulfovibrio gigas* and *Desulfococcus multivorans*	Badea et al. (2009)
GC-IRMS. The carbon isotope composition of γ-HCH and metabolites was analyzed. GC Agilent 6890 Series equipped with a Phenomenex ZB1 column (1 μm × 0.32 mm × 60 m) and coupled with a Conflow III interface (ThermoFinnigan, Bremen, Germany) to a MAT 252 MS.	9.2–24 μM CB was detected after 6–30 days with a carbon isotopic composition of-29.6 and 26‰, respectively	Aliquots of the culture medium were regularly taken with plastic syringes for stable isotope analysis (14 mL). Syringes were always flushed with nitrogen before use to avoid oxygen contamination. The volume removed was always compensated with sterile nitrogen in order to avoid negative pressure inside the bottles. Samples for isotope analysis were conserved with 0.5 mL of concentrated hydrochloric acid.	Helium was the carrier at 2.0 mL/min. The split was adjusted to obtain suitable peak areas in the linear range of the $^{13}CO_2/^{12}CO_2$ mass ratio of the target compounds		
Varian-Saturn GC/MS (CP 3900), equipped with injection port split–splitless, automatic injector (CP-8400) and connected to an MS with ion trap	–CB (9.98 min) –1,3-DCB (19.34 min) –1,2-DCB (20.25 min) –PCCH (40.26 min)	Extraction: 3 ml of sample mixed with 6 ml of 1:1 hexane–acetone solution. Tubes were and shaken for 10 min in a vortex, and centrifuged (2500 rpm for 10 min) for	Oven temperature: 35 °C (6 min); ramp rate at 3 °C/min to 180 °C and ramp rate at 8 °C/min to 270 °C. The temperatures corresponding to the transfer line and the	Batch liquid cultures of anaerobic granular sludge	Quintero et al. (2005)

(*continued on next page*)

Table 3 (*continued*)

Method and equipment	Intermediate metabolites and retention time (min)	Extraction procedure	Conditions	Remarks	Ref
(Varian-Saturn 2100). The capillary column CP-Sil 8 CB Low Bleed/MS fused silica WCOT (0.25 mm ID × 30 m).		separation of the organic and aqueous phases. An aliquot of the organic phase was analyzed by GC/MS.	ion trap were 280 and 220 °C, respectively. Ionization energy 70 eV. 1 μL injection volume via splitless injection at 280 °C. Helium was used as a carrier at a flow rate of 1.0 ml min^{-1}.		
Lindane was analyzed by Headspace-Solid Phase Microextraction-Gas Chromatography- Electron Capture Detector (HS-SPME-GC-ECD).	NA	Slurry samples extracted by SPME with polydimethylsiloxane (PDMS) fiber, 100 μm phase thicknes. Times of equilibrium and extraction were 10 min and desorption time 5 min.	Temperatures of column, injector, and detector were 210, 250 and, 350 °C respectively. Nitrogen at 8 mL/min was the carrier gas	Sequential M-SR slurry bioreactor (agricultural soil with high contents of organic matter and clay)	Camacho-Pérez et al. (2010a), Camacho-Pérez (2010b)
Metabolites were analyzed in a Varian CP -3800 equipped with capillary column used was a Factor Four VF-1MS (0.25 mm ID × 30 m)	-4-DCB (18.76 min) −CB (10.11 min)	Metabolites were extracted as in reference 10.	Oven temperature: was programmed as follows: 35 °C (6 min); ramp rate at 3 °C/ min to 180 °C, ramp rate at 8 °C min/1to 270 °C. The temperatures corresponding to the transfer line and the ion trap were 280 and 220 °C, respectively; 70 eV ionization energy. The injection volume was 1 μl. Carrier gas He at 1.0 mL/min.		
Lindane: GC using a Fisons 8800 with a ^{63}Ni ECD column, 0.25 in. DD and 11 ft in length, packed with 1.5% OV17 plus QF1 on 80−100 mesh chromosorb W;	NA	A sample of culture broth was extracted three times with equal volumes of an | n-hexane/acetone mixture (8:1), mixing for 5 min. The solvent layers were pooled and purified by passing through a Florisil column. Moisture was removed with anhydrous Na_2SO_4 granules, and the dehydrated extract was evaporated to dryness. The residue was redissolved in n-hexane and analyzed.	Carrier gas nitrogen at 40 mL/min; column, injector and detector temperatures 210, 230, and 300 °C, respectively.	A γ-HCH-degrading microbial consortium was isolated by enrichment from agricultural soil dedicated to sugar cane growth; the soil had antecedents of technical grade HCH application	Elcey and Kunhi (2010)
Intermediate metabolites: thin layer chromatography (TLC) as well as GC. The latter same as above.	No metabolites found. Chloride anion determined and detected, ca. 80% of theoretical release of chloride from added lindane	TLC was carried out on a silica gel G plate using either cyclohexane or benzene/ ethanol (19:1) as mobile phase. Spots were visualized by spraying o-tolidine and exposing to sunlight. Developed plates were sprayed either with Folin-Ciocalteu reagent for phenolic compounds or with o-tolidine for chloroaromatics. Chlorophenols, chlorobenzenes, and catechols were used as reference standards.			

Notes: 2,5-DCBQ: 2,5-dichlorobenzoquinone, 2,5-DCP: 2,5-Dichlorophenol, DCM : dichloromethane; ECD : electron capture detector, GC-IRMS: Gas chromatography withisotope ratio mass spectrometry; GC−MS: Gas chromatographic coupled to mass spectrometry; HCB: hexachlorobenzend; HCH: hexachlorocyclohexane; id: internal diameter; NA: not applicable; M-SR : methanogenic-sulfate-reducing; NR: not reported; SPME: Solid Phase Microextraction; TLC: thin layer chromatography UV: ultraviolet.

inhibited by phenol; its cofactor is NADH, with K_i = 4.4 mM (Sazinsky et al., 2006; Viggor et al., 2008).

Thus, catechol is a central intermediate in the degradation pathways of various aromatic and non aromatic parent compounds. Catechol is, in turn, metabolized by different strains via either the ortho- or the meta-fission pathway. For instance, catechol 1,2-dioxygenases catalyzes catechol transformation via an ortho-cleavage pathway to form *cis*, *cis*-muconate acids, catechol 1,2-dioxygenase has been detected both intra- and extracellularly, although extracellular presence of these enzymes has been rarely described (Bastos et al., 2000). The molecular weight is 35 kDa, optimal pH is between pH 7.8 and 8.8, temperature optimum 40 °C (*Sphingomonas xenophaga* QYY, Gou et al., 2009). While catechol 2,3-dioxygenase cleaves catechol via a meta-cleavage pathway to form 2-hydroxymuconic semialdehyde. This enzyme is a tetramer that is composed of four identical subunits, each of which contains a ferrous iron atom (Nozaki et al., 1968). Jiang et al. (2004) studied a catechol 2,3-dioxygenase gene from the strain *Pseudomonas* sp. ND6; they found that consisted of 924 nucleotides and encoded a polypeptide of molecular weight 36 kDa containing 307 amino acid residues. Further enzyme kinetic experiments showed that the affinity coefficient K_m and the specific activity were 11 μM and 11.96 U/mg of protein respectively. It was also reported that a nonheme iron is used as a cofactor for both catechol dioxygenases (Gou et al., 2009).

Another enzyme participating in phenol biodegradation is an inducible enzyme named cis, cis-muconate cyclase that catalyzes the transformation of cis,cis-muconate to (+)-mucono-lactone. This enzyme consists of eight subunits with a molecular weight of ca. 24 kDa and identical chemical composition; the optimal pH ranged between 5.5 and 6.0 and K_m was 0.057 mM (Thatcher and Cain, 1974, 1975).

So, in short, degradation of phenol follows a sequence of (a) hydroxylation to catechol, (b) ring-cleavage via catechol-2,3-dioxygenase to 2-hydroxymuconic semialdehyde for meta-pathway, and via catechol-1,2-dioxygenase to cis, cis-muconate for ortho pathway, (c) 2-hydroxymuconic semialdehyde is either oxidized to 4-oxalocrotonate or hydrolyzed to 2-oxopent-4-enoate in case of meta and cis, cis-muconate gets converted into muconolactone for ortho-cleavage (Alexieva et al., 2004; Banerjee and Ghoshal, 2010).

Less substituted chlorophenols can be aerobically degraded by a variety of microorganisms such as *Flavobacterium* sp., *Sphingobium chlorophenolicum*, *Pseudomonas fluorescens* (Xun et al., 1992; Lange et al., 1996; Shah and Thakur, 2003; Su et al., 2008). Xun and Orser (1991) studied a pentachlorophenol monooxygenase catalyzes the oxygenolytic removal of the first chlorine from pentachlorophenol (PCP); it transforms PCP to 2,3,5,6-tetrachloro-*p*-hydroquinone in the presence of oxygen and NADPH (Lange et al., 1996). The enzyme is a monomer with a molecular weight of 63 kDa. The enzymatic reaction required 2 mol of NADPH per mol of halogenated substrate. Each enzyme molecule contained one flavin adenine dinucleotide molecule. Its isolectric point pI is 4.3, the optimal pH and temperature are 7.5–8.5 and 40 °C respectively. The K_m and V_{max} were 30 mM and 16 mumol/min/mg of protein, respectively.

Laccases (p-diphenol: dioxygen oxidoreductase) belong to the so-called "blue-copper" family of oxidases. They catalyze the oxidation of a variety of organic compounds, such as chlorophenols, methoxyphenols, phenols, o- and p-diphenols, as well as lignin-related molecules. This enzyme contains four copper atoms, and is able to oxidize its substrates by using molecular oxygen as an electron acceptor (Qiu and Huang, 2010; Thurston, 1994). Liu et al. (2009) purified a laccase from the ligninlytic fungus *Pleurotus ostreatus* strain 10,969; they found that it was extracellular, molecular weight ca. 49 kDa, 4.0 optimum pH: 4.0, and optimum temperature 50 °C. K_m and V_{max} were determined with values 0.31 mmol and 303.25 mmol/min respectively. Laccase enzymes could be key participants in further degradation of lindane metabolites in fungal bioremediation of soils polluted by lindane, such as in works of Singh and Kuhad (1999); Rigas et al., 2009, 2007; Quintero et al., 2007. Mycoremediation has become a promising branch of bioremediation of soils and waters (Singh, 2006).

An increasing number of bacteria has been isolated that can couple the reductive dehalogenation of various chlorinated compounds to energy conservation by electron transport- coupled phosphorylation (Neumann et al., 1996; Nijenhuis and Zinder, 2005; Magnuson et al., 1998, 2000; Zaa et al., 2010). This process is referred to as halorespiration, or dehalorespiration. Halorespiration may be a significant pathway of anaerobic degradation of chlorinated organic compounds, where the halogen in the C–Cl bond is used as electron acceptor; energy for growth of the microorganisms is generated from exergonic dehalogenation reactions (Holliger and Schraa, 1994; Holliger and Schumacher, 1994). The exergonic dehalogenation reaction, also known as reductive dehalogenation, is a two-electron transfer reaction that involves the release of the halogen as a halide ion and its replacement by hydrogen (Mohn and Tiedje, 1992; El Fantroussi et al., 1998).

Although their role on lindane dechlorination has not been reported yet to the best of our knowledge, it is likely that they could participate at some stage of the reductive dehalogenation process of HCH. Alternatively, halorespirer bacteria could be used in the future for bioaugmentation of lindane-degrading consortia ias they are now used for bioaugmenting microbial communities that degrade chlorinated aliphatic and chlorophenol compounds.

Several microorganisms capable of halorespiration have been isolated in pure culture; these include *Dehalococcoides ethenogenes*, *Desulfomonile tiedjei* DCB-1, *Dehalobacter restrictus* PER-K23, *Dehalospirillum multivorans*.

Desulfuromonas chloroethenica, *Desulfovibrio* sp. *TBP-1*, *and most members of the genus Desulfitobacterium*. They use a wide range of substrates, such as halogenated aliphatic and aromatic compounds (pentachlorophenol and other chlorophenols, chlorobenzoate-derivative compounds, and tetrachloroethene; El Fantroussi et al., 1998; Smidt et al., 2000; Breitenstein et al., 2001; Suyama et al., 2001).

A typical dehalogenase with a molecular weight 58 kDa from the tetrachloroethene-utilizing anaerobe *Dehalospirillum multivorans* catalyzed the reductive dechlorination of tetrachloroethene (TCE) to trichloroethene and of trichloroethene to cis-1,2-dichloroethene; the enzyme specific activity was 2.6 μkat/mg (Neumann et al., 1996). The K_m values of the enzyme for tetrachloroethene and trichloroethene were 0.20 and 0.24 mM, respectively. Enzyme optimum pH and temperature were 8.0 and 42 °C, respectively. One mol of dehalogenase contained 1.0 mol of corrinoid, 9.8 mol of iron, and 8.0 mol of acid-labile sulfur.

4. Instrumental methods for analysis of lindane and metabolites

As a consequence of the great amounts of pesticides used worldwide, their wide spectrum of applications and their physicochemical and toxicological properties, these compounds and their metabolites are considered a significant problem for environment and human health (Planas et al., 2006). In this regard, the analytical chemistry for determining parent chlorinated compounds as well as their intermediate (and possibly final) metabolites has become an important area of research with a fast evolution of instrumental methods.

Concerning determination of lindane and its metabolites, a summary of the most significant and recent instrumental analysis is shown in Table 3. In general, it can be seen that GC coupled to mass spectrometry (MS) is the currently favored method (Manickam et al., 2006a, 2006b, 2008; Quintero et al., 2007; Camacho-Pérez, 2010a), as well as a combination of chromatographic methods in order to complement determination of lindane and a great variety of its metabolites. Chromatography coupled to mass spectrometry has become a significant tool for elucidating pesticide degradation pathways.

Regarding detection methods used for lindane and its metabolites, we can distinguish the following subgroups in the reported literature:

First group: use of gas chromatography (GC) for lindane determination and thin layer chromatography (TLC) for metabolite analysis, where GC is very often fitted with an electron capture detector (GC-ECD) (Manickam et al., 2006a; Elcey and Kunhi, 2010).

Second group: Use of GC-FID and GC-ECD, Singh and Kuhad (1999).

Third group: Use of only GC–MS for both lindane and metabolite detection (Manickam et al., 2006b, 2008; Quintero, et al. 2005, 2007).

Fourth group: Use of GC-ECD, and selected samples analyzed by GC–MS and/or HPLC-MS for metabolites determination (Singh and Kuhad, 1999; Camacho-Pérez, 2010a,b,c; Datta et al., 2000; Robles-González, 2008).

Fifth Group: Combined use of GC-ECD, GC-FID, GC–MS; Boyle et al. (1999).

Sixth Group: Use of GC–MS and isotopic ratio mass spectrometry (GC-IRMS) (Badea et al., 2009; Planas et al., 2006). An interesting application of GC-isotopic ratio mass spectroscopy (IRMS) was made for determining the carbon isotope composition of γ-HCH and its metabolites in a study on reductive dechlorination of lindane by two strains of sulfate-reducing bacteria (Table 3; Badea et al., 2009). The technique allowed to perform a compound-specific isotope analysis (CSIA) that considerably helps in characterizing *in situ* biodegradation processes, both qualitatively and quantitatively. The method is based on the preferential transformation of lighter isotopes during a degradation reaction, thus leading to an enrichment of heavier isotopes in the residual phase during the course of the degradation process.

Concerning the extraction procedures, available information can be organized in two subgroups: a first subgroup relying on solvent extraction, and the second one based on solid phase microextraction techniques.

Research in the first Group report the use of solvent extraction at ambient temperature and very often vortexed (no Soxhlet):

- Hexane-acetone (Manickam et al., 2006a; Quintero et al., 2005, 2007; Elcey and Kunhi 2010.)
- Hexane-acetone followed by ethyl acetate extraction (Manickam et al., 2006b, 2008.)
- Ethyl acetate (Singh and Kuhad, 1999; Datta et al., 2000.)
- Hexane (Boyle et al., 1999.)
- Use of dichloromethane with internal standards (100 μM hexachlorobenzene (HCB) as the internal standard for γ-HCH and 100 μM toluene as the internal standard for the metabolites) (Badea et al., 2009.) Extracts were dried, resuspended, and injected in GC–MS and GC-IMRS.

Interestingly and unfortunately, recovery extents of the solvent extraction procedures were not reported, except for the work by Quintero et al. (2005) who found that average extraction efficiencies (in %) were 84.6 ± 5.8, 92.3 ± 4.1, 91.8 ± 3.4 and 93.7 ± 4.3 for alfa-, beta-, gamma- and delta-HCH, respectively, for the extraction procedure with a mixture of hexane–acetone.

Second Group: Solid phase fiber extraction (Camacho-Pérez, 2010a,b,c; Planas et al., 2006; López et al., 2001; Pawliszyn, 1997; Robles-González, 2008).

For instance, Camacho-Pérez (2010a,b,c) extracted lindane and its metabolites from slurry samples using a Solid-phase microextraction (SPME) with polydimethylsiloxane fiber, 100 μm phase thickness. Lσpez et al. (2001) present a thorough discussion and review of SPME, with several references to pesticide analysis. They point out to several advantages of SPME, i.e., solvent-free extraction technique (and consequently a lower environmental impact and savings in solvent cost and disposal), simplicity or capability of injecting the whole extracted sample, and significant reduction of sample treatment/manipulation. Several applications of SPME to pesticide determination in biological samples can be found for organochlorine, organophosphorus, and dinitroaniline herbicides. Headspace analysis has been preferred in order to avoid interferences from the sample matrix. Another source of important information on SPME as well as its application to pesticide analysis can be found in the book by Pawliszyn (1997).

5. Conclusion

It can be concluded that typical anaerobic and aerobic pathways of γ-HCH are well known for a few selected microbial strains, although less is known for anaerobic consortia where the possibility of synergism, antagonism, and mutualism can lead to more particular routes and more effective degradation of γ-HCH. Enzyme and genetic characterization of the molecular mechanisms involved are in their early infancy; more work is needed to elucidate them in the future. Advances have been made on the enzymes of *Sphingomonas paucimobilis* where the gene LinB codifies for the enzyme haloalkane dehalogenase that acts on 1,3,4,6-tetrachloro 1,4 cyclo hexadiene, thus debottlenecking the pathway. Studies on microbial degradation of γ-HCH indicates that a few pure strains exhibits the traits of overall degradation or mineralization of this compound. The application of microbial consortia in bioremediation of soils and sediments seems to be a promising alternative, although it poses more difficult challenges to microbial and pathway characterization. Chromatography coupled to mass spectrometric detector, especially GC–MS, is the most used technique for resolving for γ-HCH metabolites, although there is an increased participation of HPLC-MS methods. Scintillation methods are very useful to assess final degradation of γ-HCH.

Acknowledgments

We wish to thank CINVESTAV del IPN for partial support to their research, as well as CONACYT for a graduate scholarship to BC-P. NR-S acknowledges support from COFAA-IPN. We also are very grateful to the Editor, Guest Editor and Referees for their insightful comments and suggestions that allowed for a substantial improvement of our manuscript.

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Notation

1,2,3-TCB: 1,2,3-trichlorobenzene
1,2,4-TCB: 1,2,4-trichlorobenzene
1,2-DCB: 1,2-dichlorobenzene
1,3-DCB: 1,3-dichlorobenzene
1,4-TCDN: 1,3,4,6-tetrachloro-1,4-cyclohexadiene
2,4,5-DNOL: 2,4,5-trichloro-2,5-cyclohexadiene-1-ol
2,5-DDOL: 2,5-dichloro-2,5-cyclohexadiene-1,4-diol
2,5-DCBQ: 2,5-dichlorobenzoquinone
2,5-DCHQ: 2,5-dichlorohydroquinone
2,5-DCP: 2,5-dichlorophenol
2,6-DCHQ: 2,6-dichlorohydroquinone
CB: Chlorobenzene
CHQ: Chlorohydroquinone
CSIA: Compound-specific isotope analysis
γ-HCH: γ-Hexachlorocyclohexane
γ-PCCH: γ-Pentachlorocyclohexene
FAD: Flavin adenine dinucleotide
GC–MS: Gas chromatography coupled to mass spectrometry
GC-IRMS: Gas chromatography isotope ratio mass spectroscopy
GSH: Glutathione (reduced form)
GS-SG: Glutathione (oxidized form)
HCH: Hexachlorocyclohexane
HPLC-MS: High-performance liquid chromatographic-mass spectroscopy
K_m*:* Affinity constant in Michaelis–Menten enzyme kinetics
MA: Maleylacetate
NADPH: Nicotinamide adenine dinucleotide phosphate-oxidase
NADH: Nicotinamide adenine dinucleotide reduced
PCCH: Pentachlorocyclohexene
PCP: Pentachlorophenol
pI: Isolectric point
PLP: Pyridoxal 5′-phosphate
TCA: Tricarboxylicacid cycle
TCCH: Tetrachlocyclohexene
TCCD: Trichlocyclohexadiene
TCE: Tetrachloroethene
V_{max}*:* Maximum or saturation rate constant in enzyme kinetic equation

Journal of Environmental Management 95 (2012) S319–S324

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Arbuscular mycorrhizal fungi on growth, nutrient status, and total antioxidant activity of *Melilotus albus* during phytoremediation of a diesel-contaminated substrate

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ARTICLE INFO

Article history:
Received 3 September 2009
Received in revised form
31 January 2011
Accepted 23 February 2011
Available online 21 March 2011

Keywords:
AMF
Phytotoxicity
Petroleum hydrocarbons
Antioxidants
Fungal alkaline phosphatase
Phytoremediation

ABSTRACT

This research evaluated the effects of arbuscular mycorrhizal fungi (AMF) on growth, nutritional status, total antioxidant activity (AOX), total soluble phenolics content (TPC), and total nitrate reductase activity (NRA) of leaves and roots of *Melilotus albus* Medik planted in diesel-contaminated sand (7500 mg kg^{-1}). Seedlings of *Melilotus* either Non inoculated (Non-AMF) or pre-inoculated plants (AMF) with the AMF-inoculum *Glomus* Zac-19 were transplanted to non-contaminated or contaminated sand. After 60 days, diesel significantly reduced plant growth. AMF- plants had no significant greater (64% and 89%, respectively) shoot and leaf dry weight than Non-AMF plants, but AMF plants had lower specific leaf area. AMF-plants had significantly greater content of microelements than non-AMF plants. Regardless diesel contamination, the total AOX and TPC were significantly higher in leaves when compared to roots; in contrast, NRA was higher in roots than leaves. Diesel increased total AOX of leaves, but AMF-plants had significantly lower AOX than non-AMF plants. In contrast, roots of AMF-plants had significantly higher AOX but lower NRA than non-AMF plants. AMF-colonization in roots detected via the fungal alkaline phosphatase activity was significantly reduced by the presence of diesel. AMF-inoculation alleviated diesel toxicity on *M. albus* by enhancing plant biomass, nutrient content, and AOX activity. In addition, AMF-plants significantly contributed in higher degradation of total petroleum hydrocarbons when compared to non-AMF-plants.

1. Introduction

Contamination of soils with toxic organic compounds is an increasing environmental concern. Thus, the application of new biotechnological approaches to detoxify or remediate contaminated soils is much needed. The accidental spillage of oil and oil derivates significantly contribute to the loss of biodiversity in terrestrial ecosystems (López-Martínez et al., 2005; Shahriari et al., 2007). Typically, physical soil properties are severely affected by petroleum hydrocarbons, thus affecting diffusion of oxygen and water, and impairing plant growth (Plice, 1949; Ko and Day, 2004; Merkl et al., 2005; Pilon-Smits, 2005).

Bioremediation is an environmentally sound alternative to current physical and chemical 'clean up' process. Soil microorganisms and/or plants remediate contaminated soil to through naturally mediated degradation process. Phytoremediation utilizes plants to remove metals or to degrade organic contaminants (Pilon-Smits, 2005; Tlustoš et al., 2006). Some plants are tolerant and adapt to the adverse condition experience in petroleum contaminated soil (Merkl et al., 2005). This may be due to enhanced rhizosphere microbial activity which may participate on the degradation, transformation or mineralization of the contaminants (Ferrera-Cerrato et al., 2006; Li et al., 2007; Alarcón et al., 2008).

Plants exposed to soils contaminated with petroleum hydrocarbons (PH) are subjected to growth limitations, due to low water uptake and reduced nutrient availability (Merkl et al., 2005). In short, plants suffer an osmotic stress similar to that caused by drought, largely because hydrocarbons have hydrophobic and lipophylic properties, which significantly reduce water availability and root gas exchange (Ko and Day, 2004; Merkl et al., 2005; Robertson et al., 2007). Under such conditions plants may

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0301-4797/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jenvman.2011.02.015

experiment oxidative stress due to petroleum toxicity by which they must trigger the activity of enzymatic or non-enzymatic antioxidant systems to cope and/or attenuate such stress (Debiane et al., 2008, 2009).

Arbuscular mycorrhizal fungi (AMF) enhance plant nutrient and water uptake, increase plant tolerance to cultural and environmental stresses, and play an important role at the plant–rhizosphere interface (Smith and Read, 2008). Several studies have demonstrated that AMF alleviate cultural and environmental stresses experienced in soils contaminated with PH (Leyval and Binet, 1998; Caravaca et al., 2005; Alarcón et al., 2006; Cho et al., 2006; Sheng et al., 2008). Mycorrhizal fungi have been demonstrated to enhance plant growth, stimulate specific microbial activity, and induce the synthesis of oxidative enzymes that enhance degradation of PH (Criquet et al., 2000; Joner and Leyval, 2003; Corgié et al., 2006). Moreover, AMF may also contribute on improving the phytoremediation performance of contaminated soils (Corgié et al., 2003; Alarcón et al., 2008). Thus, AMF are considered as an important microbial component in the rhizosphere whose benefits on plants are related to improved nutrient status and water absorption, and increased tolerance and survival under environmental adverse conditions (Caravaca et al., 2005; Alarcón et al., 2006; Smith and Read, 2008). Although the inoculation and benefits of AMF have been successfully tested on several horticultural, ornamental and tree species, some studies have been focused on understanding the role of AMF on plants specially grass species, under petroleum contaminated soils (Binet et al., 2000; Gaspar et al., 2002; Alarcón et al., 2008), and few of them have evaluated the benefits of legume species inoculated with AMF (Criquet et al., 2000; Joner and Leyval, 2003; Chiapusio et al., 2007). Thus, this study evaluated the effects of AMF on the alleviation of diesel-induced toxicity to *Melilotus albus* by measuring its growth, nutrient status, and selected physiological responses.

2. Materials and methods

2.1. Cultural conditions, sand contamination, mycorrhizal inoculation, and transplant

This study was conducted under plant growth chamber conditions (26 °C, 80% RH; 12 h photoperiod). Autoclaved river sand was used as substrate for seed germination of *M. albus* Medik (an herbaceous plant). Two seed beds were inoculated with the arbuscular mycorrhizal fungi inoculum *Glomus* Zac-19 (AMF) conformed by three species: *Glomus claroideum*, *Glomus diaphanum*, and *Glomus albidum* (Chamizo et al., 1998). This mycorrhizal inoculum has been probed to promote the growth of several plant species under different stress conditions (Davies et al., 2002; Estrada-Luna and Davies, 2003; Cartmill et al., 2007; 2008). Seed beds were prepared by placing a 5 cm-layer of sterile sand, on which a 5 cm-layer of the AMF inoculum was placed, and finally, another 5 cm-layer of sterile sand was applied to each bed. Two more seed beds only with sterile sand were also set without AMF-inoculation as controls. Seed beds were watered with distilled water, and weekly fertilized with Long Ashton Nutrient Solution (LANS; Hewitt, 1966) modified to supply 11 μg P mL^{-1}, to avoid interferences of this element with the AMF-establishment.

For contaminated treatments, the autoclaved sand was artificially spiked with diesel at 7500 mg kg^{-1}. The diesel concentration was chosen from preliminary experiments in which seed germination, seedling emergence and root morphological changes were assessed for *M. albus* (Hernandez-Ortega et al., unpublished data). Contaminated sand was maintained at room temperature during five days in order to allow its equilibrium in the substrate. Once the root seedlings were colonized by AMF (approximately 40 days after inoculation), they were transplanted to either contaminated or uncontaminated sand (500 g) placed into polystyrene pots (500 g of capacity) as experimental units. Another set of non-inoculated plants (non-AMF) were also transplanted to either contaminated or uncontaminated sand. Each treatment consisted on 30 experimental units in which three plants were transplanted. Plants were weekly fertilized with 50 mL of LANS as previously described, and watered as needed with distilled water during 60 days of experimentation.

2.2. Experimental design

A 2 × 2 factorial experiment was set with four treatments and 30 experimental units, distributed in a completely randomized design. Factors were as follows: two levels of diesel (0 and 7500 mg kg^{-1}), and two levels of AMF-inoculation (non-AMF and AMF plants). Data were analyzed by using the analysis of variance (ANOVA) and by the mean comparison test (Tukey, $\alpha = 0.05$) (SAS Institute Inc., 2002). For each determination, three experimental units were harvested from which each plant was used as subsample for further analysis.

2.3. Plant growth evaluation

Sixty days after transplanting, the plants from three experimental units were harvested to determine the leaf area (cm^2) and dry weight (DW) of leaves, stems, roots and nodules. Leaf area was measured by using a leaf area meter (Area Meter, model LI-3100). Leaf elemental analysis including total nitrogen, phosphorus, calcium, magnesium, iron, copper, zinc, and manganese was analyzed via inductively coupled plasma mass spectrometry (ICP-AES Liberty Series II, EL97053010). The elemental analysis was performed by standardized protocols at the Laboratory of Plant Nutrition (Colegio de Postgraduados) based on Jones et al. (1991).

2.4. Total antioxidant activity, total soluble phenolics content, total extractable nitrate reductase activity, and mycorrhizal colonization

Total antioxidant activity (AOX) was determined by the 1,1-diphenyl-2-picryldrazyl (DPPH) radical decoloration assay utilizing Trolox as antioxidant compound (Matthäus, 2002). In brief, leaf and root extracts were obtained with 80% methanol. The reaction mixture consisted of mixing the extract with DPPH-solution in 96-well microplates. After 15 min of incubating the microplates (22 °C), absorbance readings at 515 nm were taken with a Synergy 2 spectrophotometer (Biotek® Instruments). Total soluble phenolics content (TPC) was determined by using the Folin–Ciocalteu reagent assay utilizing chlorogenic acid as standard (Singleton and Rossi, 1965; Soong and Barlow, 2004). Aliquots from extracts obtained for AOX determination were reacted with Na_2CO_3 and Folin–Ciocalteau reagent in a 96-well microplate. After 30 min of incubation, absorbance readings were taken at 725 nm (Synergy 2 spectrophotometer, Biotek® Instruments).

Total extractable nitrate reductase activity (NRA) was determined by the procedure described by Foyer et al. (1998). In brief, leaf and root samples were ground with an extraction buffer solution consisting of 50 mM Mops-KOH, pH 7.8, 5 mM NaF, 1 μM Na_2MoO_4, 10 μM FAD (flavin adenine dinucleotide), 1 μM leupeptin, 1 μM microcystin, 0.2 g PVP (polyvinylpyrollidone) g^{-1} fresh weight, 2 mM β-mercaptoethanol, and 5 mM EDTA. An aliquot of 200 μL was taken and then reacted with 200 μL of reaction mixture solution consisting of 50 mM Mops-KOH buffer, pH 7.5, supplemented with 1 mM NaF, 10 mM KNO_3, 0.17 mM NADH, and 5 mM EDTA. The reaction was terminated after 15 min by the addition of 200 μL of sulfanilamide (1% [w/v] in 3 N HCl) and 200 μL of

naphthylethylene-diamine dihydrochloride (0.02% [w/v]) to the reaction mixture. Thus, the absorbance at 540 nm was measured (Hewlett Packard HP 8453).

Mycorrhizal colonization was estimated via the alkaline phosphatase vital stain procedure (Pearse, 1968; Tisserant et al., 1993) at 10 sampling times in which three individual plants (one experimental unit) were analyzed from each AMF treatment. Roots were harvested and immediately incubated for 2 h at room temperature in a digestion solution consisting of 0.05 M Tris/citric acid buffer (pH 9.2), 0.05% sorbitol, 15 units cellulase mL^{-1} and 15 units pectinase mL^{-1}. Roots were exposed to a sodium chloride solution (1% active chlorine) for 5 min. Staining procedure consisted on overnight exposure of roots to reaction medium consisted on 0.05 Tris/citric acid buffer (pH 9.2), added with fast blue RR salt (1 mg mL^{-1}), α-naphtyl acid phosphate (1 mg mL^{-1}), $MgCl_2$ (0.5 mg mL^{-1}), and $MnCl_2 \cdot 4H_2O$ (0.8 mg mL^{-1}). Fractional colonization was estimated microscopically as the intensity of AMF-colonization of the root cortex, expressed as a percentage.

2.5. Total petroleum hydrocarbons analysis

After 60 days, the analysis of total petroleum hydrocarbons (TPH) from diesel-contaminated sand was performed by a modified EPA SW-846 Method 8270B (Louchouarn et al., 2000; USEPA, 1986). Extractions were performed using 100% hexane, and the extracts were used in the quantitative determination of TPH by gas chromatographic mass spectrometry GC–MS (Agilent Technologies, model 6890N, Net Work GC system). Samples from AMF-plants and non-AMF plants were collected and analyzed by comparing their TPH-mass spectra (HP Chemstation-NIST 05 Mass spectral search program, version 2.0d) with that observed for initial samples (time zero).

3. Results

3.1. Plant growth responses

The AMF inoculation as independent factor, resulted in significantly greater DW of nodules, stems and shoots than non-AMF plants (Table 1). Diesel factor significantly ($P \leq 0.05$) impaired all the plant growth parameters (Table 1). The AMF × Diesel interaction had no significant effects on plant growth (Table 1). Total DW was reduced ($P \leq 0.01$) in plants under diesel contamination; but AMF plants had non-significant greater plant DW (>33%) than Non-AMF plants (Table 1). Although non-significant differences were observed among treatments, AMF plants under diesel contamination had greater DW of leaves (89%), stems (>53%), shoots (>64%), and total plant DW (>34%) than non-AMF plants (Table 1).

AMF-inoculation did not have significant effect on leaf area; in contrast, diesel contamination, and the interaction AMF × Diesel had significant effects ($P \leq 0.01$) (Table 1). Results showed a reduction of leaf area of plants exposed to diesel; in which AMF-plants had 100% more leaf area than non-AMF plants (Table 1). In contrast, AMF-plants without diesel had significantly lower leaf area than non-AMF plants (Table 1).

Naturally-occurring *Rhizobium* bacterial nodules were observed in roots for all treatments; thus, the number and the DW of nodules were also determined. Regardless diesel contamination, AMF-plants had greater number of nodules than non-AMF plants (Table 1). In addition, the DW of nodules was significantly reduced in non-AMF plants under diesel contamination (Table 1).

3.2. Total antioxidant activity, total soluble phenolics content, total extractable nitrate reductase activity, and mycorrhizal colonization

Regardless diesel contamination or AMF inoculation, total AOX and TPC were significantly higher in leaves than roots; while, NRA was higher in roots than leaves (Fig. 1). In general, diesel contamination resulted in high total AOX, in which AMF plants had significantly lower AOX than non-AMF plants (Fig. 1A); however, root total AOX was significantly higher in AMF-plants than non-AMF plants (Fig. 1A). In contrast, diesel contamination did not significantly affect TPC of plants, though leaves of AMF-plants had significantly greater TPC than non-AMF plants (Fig. 1B).

AMF-plants had greater total N-content than non-AMF plants (Table 2); in contrast, diesel factor and the AMF × Diesel interaction had non-significant differences. There were no significant effects for any independent factor or for the AMF × Diesel interaction on total P, K, Ca, or Mg content (Table 2). Non-AMF plants treated with diesel had reduced content of macroelements when compared to non-AMF plants without the contaminant (Table 2). In contrast, AMF-plants exposed to diesel had less reduction of N (<10%), and K (<20.9%) content, but greater content of P (>7.1%), Ca (>3.9%) and Mg (>61.5%) when compared to AMF-plants without diesel (Table 2). Overall, under diesel contamination, AMF-plants had no significant better nutritional status of N (>70%), P (>87%), K (>55%), Ca (>89%), and Mg (>82%) than non-AMF plants (Table 2). The total content of Fe, Cu and Zn was significantly ($P \leq 0.05$) enhanced due to AMF-inoculation, in which AMF-plants had greater content of Fe, Cu, Zn, and Mn (>159, 116, 170, and 196%, respectively) than non-AMF plants (Table 2). In contrast, diesel significantly reduced ($P \leq 0.01$) the total content of Fe, Cu, Zn, and Mn (Table 2).

AMF-colonization was significantly higher in plants exposed to diesel during the first four days (Fig. 2). However, after the fifth day, colonization significantly decreased under diesel contamination in comparison to plants without diesel (Fig. 2). Moreover, at day 60 in which the lowest AMF-colonization was observed, there were no significant differences among diesel treatments (Fig. 1). No AMF-colonization was observed at non-AMF plants.

Table 1
Growth responses of *Melilotus albus* inoculated with arbuscular mycorrhizal fungi (AMF) and established at diesel-contaminated sand (7500 mg kg^{-1}), after 60 days.

Mycorrhizal Inoculation	Diesel mg kg^{-1}	Nodules	Root	Leaves	Stems	Shoots	Total	Number of nodules	Leaf area cm^2 $plant^{-1}$
		mg $plant^{-1}$							
Non-AMF	0	4.2 ab[a]	212.1 a	517.6 a	838.5 a	1356.1 a	1572.4 a	64 ab	244.7 a
AMF	0	6.6 a	235.0 a	540.0 a	1103.6 a	1643.5 a	1885.1 a	96 a	164.8 b
Non-AMF	7500	2.5 b	124.0 a	82.4 b	175.5 b	257.9 b	384.4 b	20 b	29.9 c
AMF	7500	3.9 ab	84.2 a	156.1 b	269.0 b	425.1 b	513.1 b	56 ab	60.0 c
	Tukey's HSD	3.2	152.0	148.6	292.3	397.1	453.5	52.6	73.8
	AMF	0.05	NS	NS	0.05	0.05	NS	0.05	NS
	Diesel	0.05	0.01	0.001	0.001	0.001	0.001	0.01	0.01
	AMF × Diesel	NS	NS	NS	NS	NS	NS	NS	0.01

[a] Means with the same letter in each column are not statistically different (Tukey, $\alpha = 0.05$); HSD = Honestly significant difference. NS = non-significant, $n = 3$.

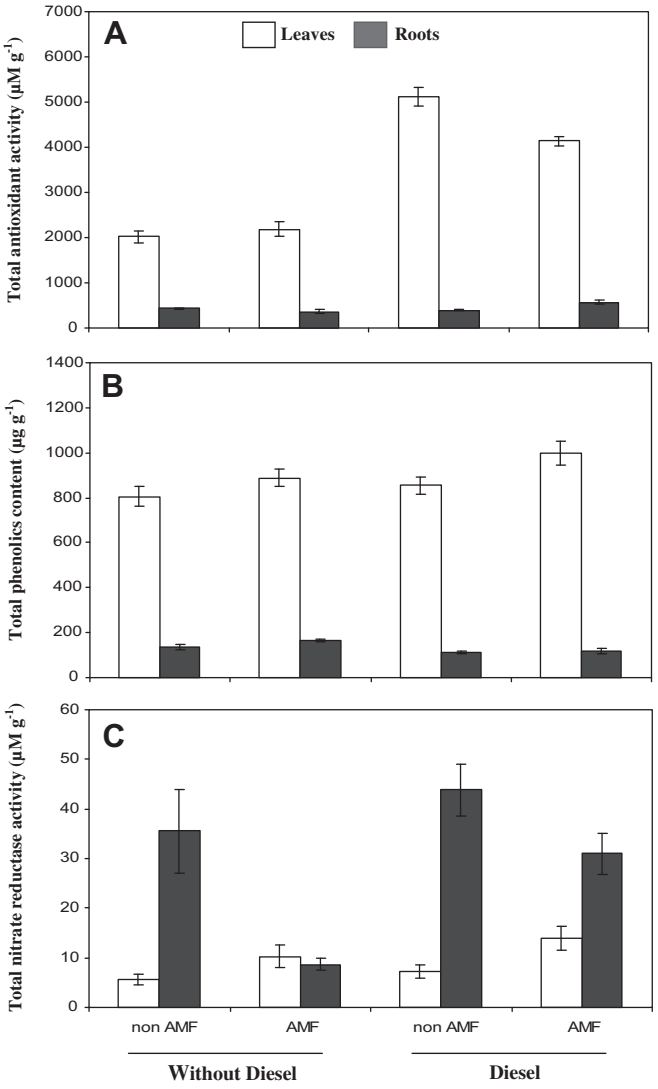

Fig. 1. Effects of arbuscular mycorrhizal fungi (AMF) and diesel contamination (7500 mg kg^{-1}) on the total antioxidant activity (A), total soluble phenolics content (B), and total nitrate reductase activity (C) of leaves and roots of *Melilotus albus*, after 60 days. Means ± Standard error, $n = 3$.

3.3. Total petroleum hydrocarbons analysis

TPH-degradation was significantly ($P \leq 0.05$) enhanced by AMF-inoculation. AMF-plants showed 47.7% of TPH-degradation while non-AMF plants had 29.8% (Fig. 3).

4. Discussion

Diesel contamination resulted in negative effects on the growth of *M. albus*, which may be consequence of limited water and nutrient uptake (Quiñones-Aguilar et al., 2003; Sangabriel et al., 2006). Although non-significant effects were observed in comparison to non-AMF plants, AMF-plants had better growth in presence of diesel. Similar AMF-benefits were described for several plant species at contaminated soils (Leyval and Binet, 1998; Cabello, 1999; Joner and Leyval, 2003; Alarcón et al., 2008). In addition to plant growth, diesel significantly reduced the number of nodules (<69%) which were formed by naturally occurring rhizobia that may be dispersed by seeds (Pérez-Ramirez et al., 1998). Since we did not perform a seed surface disinfection, this may explain in part the proliferation of rhizobia in our experimental system. The legume–rhizobia symbiosis may play a significant role in contaminated soils with hydrocarbons by enhancing the incorporation of N into contaminated systems, and degrading organic contaminants (Keum et al., 2006). Furthermore, AMF significantly enhanced the number of nodules, regardless diesel contamination (Fig. 3), which agrees with findings under uncontaminated systems (Barea et al., 2005; Mortimer et al., 2008; Siviero et al., 2008).

The present study showed some physiological benefits of AMF in *M. albus* exposed to diesel. Total AOX was significantly enhanced due to diesel contamination, indicating that plants were subjected to stress that resulted in impaired growth. This diesel-induced stress may have contributed to increased production of reactive oxygen species (ROS) that have detrimental effects on plant cells (Mittler, 2002). Thus, plants must trigger the synthesis and/or the activity of enzymatic or non-enzymatic antioxidant compounds for ROS scavenging (Mittler, 2002; Lee et al., 2007). Plants at contaminated sand had enhanced total AOX activity that includes TPC, as a way to alleviate the stress induced by diesel toxicity. These physiological responses have been scarcely described for plants established at contaminated soils. In addition, AMF-plants had low AOX activity in leaves, but high AOX activity in roots. The high AOX activity observed in roots of AMF-plants may be in part due to the fact that AMF-roots accumulate ROS (Fester and Hause, 2005), thus, resulting in an enhanced AOX activity. This response reflects beneficial growth effects of AMF to plants under diesel contamination. Beneficial effects of AMF on increasing the activity of specific enzymatic or non-enzymatic antioxidants have been reported for several plants exposed to abiotic stresses (Wu et al., 2006a, 2006b; He et al., 2007; Cartmill et al., 2008). Nevertheless, the physiological benefits of AMF on plants under petroleum hydrocarbons have received little attention (Criquet et al., 2000; Alarcón et al., 2008; Debiane et al., 2009).

Nitrate reductase is an important enzymatic activity in plants exposed to different environmental stresses (Foyer et al., 1998; Sinha and Nicholas, 1981; Taiz and Zeiger, 2002). In this regard,

Table 2
Total elemental content of *Melilotus albus* inoculated with arbuscular mycorrhizal fungi (AMF) and established at diesel-contaminated sand (7500 mg kg^{-1}), after 60 days.

AMF-inoculation	Diesel mg kg^{-1}	N	P	K	Ca	Mg	Fe	Cu	Zn	Mn
		mg $plant^{-1}$					μg $plant^{-1}$			
Non-AMF	0	10.3 a[a]	1.1 a	5.9a	6.8 a	1.6 a	58.5 ab	3.2 b	17.8 b	133.1 ab
AMF	0	15.9 a	1.4 a	6.7 a	10.2 a	2.6 a	121.5 a	5.5 a	30.1 a	191.7 a
Non-AMF	7500	8.4 a	0.8 a	3.4 a	5.6 a	2.3 a	13.4 b	0.6 c	3.1 c	19.9 b
AMF	7500	14.3 a	1.5 a	5.3 a	10.6 a	4.2 a	34.8 b	1.3 bc	8.4 bc	59.0 ab
	Tukey's HSD	9.7	1.32	5.5	9.5	3.2	74.9	2.0	11.8	139.3
	AMF	NS	NS	NS	NS	NS	0.05	0.05	0.01	NS
	Diesel	NS	NS	NS	NS	NS	0.01	0.001	0.001	0.01
	AMF × Diesel	NS	NS	NS	NS	NS	0.01	0.001	0.001	0.05

[a] Means with the same letter in each column are not statistically different (Tukey, $\alpha = 0.05$); HSD = Honestly significant difference. NS = non-significant, $n = 3$.

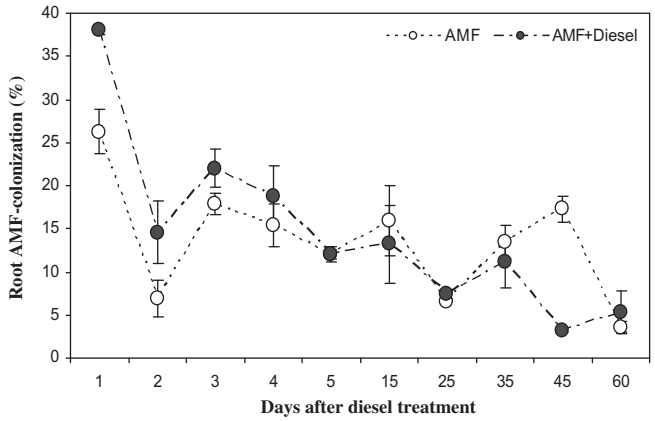

Fig. 2. Effect of diesel contamination (7500 mg kg^{-1}) on the arbuscular mycorrhizal colonization (AMF) measured via the alkaline phosphatase vital stain, in roots of *Melilotus albus*, during 60 days. Means ± Standard error, $n = 3$.

diesel induced greater root NRA, suggesting that nitrogen assimilation is a crucial process for plant establishment at contaminated sand. In addition, AMF-plants had lower root NRA regardless diesel contamination; this may be due to the capability of AMF to transfer inorganic nitrogen ($N{-}NO_3^-$ or $N{-}NH_4^+$) to the host (Bago et al., 2001), thus improving N-uptake (Table 2). In addition, AMF contributed on stimulating rhizobial nodules yielding enhanced N-uptake by plants (Graham and Vance, 2000; Mortimer et al., 2008).

Beneficial effects of AMF on plant nutrition have been documented (Smith and Read, 2008); however, little is known about the effects of organic contaminants on plant nutrition. In the present study, no significant differences were observed among treatments on the content of N, P, K, and Ca, though AMF-plants had greater content of macroelements and microelements than non-AMF plants. There is not much available information about the contribution of AMF on the nutritional status of plants exposed to petroleum hydrocarbons. It is known that some microelements are cofactors for enzymatic antioxidant systems in cells (Lee et al., 2007). This may in part explain how AMF-plants had better physiological mechanisms for reducing the detrimental effects of diesel on growth in the presence of diesel.

Regardless diesel contamination, AMF-colonization diminished along the experimentation. We suggest that this colonization reduction may be a result of the plant flowering observed after 25 days of transplanting. AMF may reduce their colonization status due to the development of reproductive plant organs which are strong sinks of plant-derived carbon, thus affecting its allocation to root cortical cells and its availability to AMF (Bago et al., 2000, 2003). There are some reports indicating the negative or null effects of hydrocarbons on AMF-colonization (Gaspar et al., 2002; Rabie, 2004; Alarcón et al., 2006).

More importantly, AMF contributed on significant degradation/dissipation of diesel in the rhizosphere, which agrees to previous findings (Joner and Leyval, 2003; Volante et al., 2005; Verdin et al., 2006; Alarcón et al., 2008; Cheung et al., 2008; Wu et al., 2009). Our results give more evidence about the influence of AMF for improving the phytoremediation performance of contaminated systems.

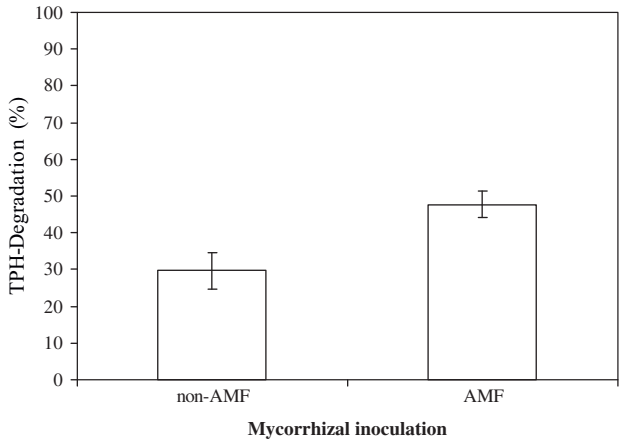

Fig. 3. Effects of the arbuscular mycorrhizal inoculation (AMF) in *Melilotus albus* on the total petroleum hydrocarbons degradation from diesel-contaminated sand (7500 mg kg^{-1}), after 60 days. Means ± Standard error, $n = 3$.

5. Conclusion

Diesel negatively affected both growth and nutritional status of *M. albus*. Although no significant differences were observed under diesel contamination, AMF-plants had better growth responses and content of macroelements than Non-AMF plants. Regardless diesel contamination, AMF significantly enhanced the content of microelements in shoots. Under diesel contamination, roots of AMF plants had high total AOX and NRA, indicating better physiological responses than Non-AMF plants. Furthermore, AMF significantly enhanced the degradation of TPH in the rhizosphere.

Acknowledgments

H.A.H-O thanks the National Council of Science and Technology (CONACYT) Mexico for financial support. Authors also thank the SEP-CONACYT Grant 79456 for financial support. Special thanks to Andrew D. Cartmill and Donita Bryan (Texas A&M University) for invaluable comments/suggestions for improving this manuscript. Sincere thanks to the two anonymous reviewers for invaluable comments and thorough revisions.

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Journal of Environmental Management 95 (2012) S325–S331

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Petroleum-influenced beach sediments of the Campeche Bank, Mexico: Diversity and bacterial community structure assessment

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ARTICLE INFO

Article history:
Received 3 October 2009
Received in revised form
17 June 2011
Accepted 24 June 2011
Available online 28 July 2011

Keywords:
Petroleum
Coastal beaches
PCR-RISA
Campeche Bank
Oil-degrading bacteria
Marinobacter

ABSTRACT

The bacterial diversity and community structure were surveyed in intertidal petroleum-influenced sediments of ~100 km of a beach, in the southern Gulf of Mexico. The beach was divided in twenty sampling sites according to high, moderate and low petroleum influence. Densities of cultured heterotrophic (HAB) and hydrocarbon degrading bacteria (HDB) were highly variable in sediments, with little morphological assortment in colonies. PCR-RISA banding patterns differentiated distinct communities along the beach, and the bacterial diversity changed inversely to the degree of petroleum hydrocarbon influence: the higher TPH concentration, the lower genotype diversity. Seven DNA sequences (Genbank EF191394 – EF191396 and EF191398 – EF191401) were affiliated to uncultured members of *Gemmatimonas*, *Acidobacterium*, *Desulfobacteraceae*, *Rubrobacterales*, *Actinobacterium* and the *Fibrobacteres/Acidobacteria* group; all the above taxa are known for having members with active roles in biogeochemical transformations. The remaining sequences (EF191388 – EF191393 and EF191397) affiliated to *Pseudoalteromonas,* and to oil-degrading genera such as *Pseudomonas*, *Vibrio* and *Marinobacter*, being the last one an obligate oil-degrading bacterium. An exchange of bacteria between the beach and the oil seep environment, and the potential cleaning-up role of bacteria at the southern Gulf of Mexico are discussed.

1. Introduction

The Campeche Bank in the southern Gulf of Mexico is well-known for its intensive production of oil and gas. Geologically, it is divided into Carbonated and Terrigenous regions (geologic provinces), with some unique ecological properties in each one (Yañez-Arancibia and Sánchez-Gil, 1988). The Bank supports the largest Mexican offshore industrial petroleum facility, which supplied 82% of oil and 35% of natural gas (García-Cuéllar et al., 2004), and holds some shallow (≤100 m deep) oil seeps with high hydrocarbon emission potentials (Wilson et al., 1974). Crude petroleum, either spilled or seeped out, has chronically impacted almost 300 km of the beach, from Dos Bocas Harbor (Tabasco State) to Champoton town (Campeche State) (PEMEX, 1987); this area includes a part of the Campeche bank shoreline. Oil as tar balls have been recorded in the intertidal environment in amounts between 9 metric tons to exceptionally 300 metric tons (PEMEX, 1987), and no further oil measurements have been reported since then. Bacteria are the dominant hydrocarbon degraders in the marine environment, and their role in the transformation of petroleum hydrocarbons has been recognized for almost sixty years (ZoBell, 1946; Atlas, 1981; Harayama et al., 2004; Head et al., 2006; Hewson et al., 2007). However, very few studies focused on the microorganisms have been performed in the continental shelf of the southern Gulf of Mexico (e.g., Lizárraga-Partida et al., 1982, 1986), and none in the intertidal sediment environment. Assessment of microbial communities in the beach of the Campeche Bank with emphasis in hydrocarbonoclastic bacterial populations is a necessary task, since a major fate of oil depends on the capacity of microorganisms able to use hydrocarbons as a source of carbon and energy (Leahy and Colwell, 1990). In addition, the finding of any obligate hydrocarbonoclastic bacteria or OHCB (Yakimov et al., 2007) is attractive here, for their natural cleanup potential in chronic and heavy polluted oil marine environments.

The present study surveyed the intertidal bacterial diversity within a stretch (~100 km) of petroleum-impacted beach, using a strategy based on both culture dependent and culture independent methods. One of them, the Ribosomal Intergenic Spacer Analysis (RISA) exploits the variability in length of the intergenic

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doi:10.1016/j.jenvman.2011.06.046

spacer (ITS) between the small (16S) and large (23S) subunit of rDNA (Ranjard et al., 2001). RISA was utilized in this work since it was successfully used to fingerprint complex microbial communities in marine sediments (Hewson et al., 2007), soils (McCaig et al., 1999; Ranjard et al., 2000, 2001), soda lakes (Jan-Roblero et al., 2004) and seawater (Cardinale et al., 2004).

2. Methods

2.1. Description of the sampling area and sampling sites

A beach stretch of ~100 km located between Carmen Island and Sabancuy town, and within the 300 km shoreline between Dos Bocas Harbor to Champoton town was chosen as a case for study (Fig. 1). The target beach is prone to perturbations, mostly by hurricanes and petroleum hydrocarbons discharges from anthropogenic and natural sources (Lizárraga-Partida et al., 1982; Botello et al., 1996; García-Cuéllar et al., 2004). Twenty sampling sites were chosen within the intertidal zone along the 100 km littoral (Fig. 1), based on the total petroleum hydrocarbons (TPH) content and presence of oil seeps. Approximately 250 g of beach sediment samples, from the surface (0–10 cm deep) were collected in sterile containers and kept at 4 °C prior to laboratory processing.

2.2. Measurement of TPH

TPH concentrations were determined in sediments samples with the standard U.S. EPA Method 418.1, which is one of the most widely used methods for the determination of a broad range of hydrocarbons in soil (Weisman, 1998).

2.3. Heterotrophic bacteria (HAB) and petroleum hydrocarbon degrading bacteria (HDB) quantification

To assess the impact of crude petroleum on the environment, one gram of sediment sample from each site was resuspended (1:10 w/v) in artificial seawater, 3.6%, pH 7.5 (Lyman and Fleming, 1940). Then, serial dilutions were plated in triplicate, on marine peptone media to account for HAB (Oppenheimer and ZoBell, 1952), and on mineral agar (MA-oil) supplemented with 0.5% (v/v) of heavy Maya crude oil from the Campeche Bank (API > 30), as the only carbon source (Fernández-Linares et al., 2005); in this medium the HDB were scored. Plates were incubated at 30 °C for 48–72 h and the numbers of colony forming units (CFU) were counted. CFU values were also used to survey the environmental impact degree by crude petroleum (EID), by calculating the ratio HDB/HAB, a surrogate measure suggested by Lizárraga-Partida et al. (1983). Briefly, these authors proposed to scale four degrees of environmental oil pollution based on the percentage of HDB. Plates used for CFU counts with visible morphological differences were maintained at 4 °C and used for further experiments. For genomic DNA extraction, five to twenty different colonies were selected from each site and grown in liquid marine peptone or mineral-oil media. Flasks were incubated aerobically at 30 °C at 180 rpm for 10 days. Bacterial pellets were obtained by centrifugation (2000×*g*, 10 min at 4 °C) and washed several times with artificial seawater solution (3.6%, pH 7.5).

2.4. DNA extraction

Metagenomic DNA was extracted from sediments using the UltraClean Soil DNA Isolation Kit (MoBio, USA). Genomic DNA from the HAB and HDB isolates was extracted from the obtained bacterial pellets using the Wizard kit (Promega Inc., Madison, USA). Obtained DNAs were used as templates for PCR to amplify the 16S-23S ribosomal spacer plus a 500 bp stretch of the 16S rDNA as described by Acinas et al. (1999). Primers employed were B1055-16S and 23SOR (Acinas et al., 1999), which anneal to positions 1055–1074 of the *Escherichia coli* 16S rDNA and 21–38 of the 23S rDNA genes, respectively (Gürtler and Stanisich, 1996).

2.5. PCR-RISA banding patterns, ecological indices and rarefaction analysis

The obtained amplicons were electrophoresed as described by Acinas et al. (1999). Each amplified band of the rDNA intergenic spacer region (between 0.6 and 1.6 kbp) was assumed to represent

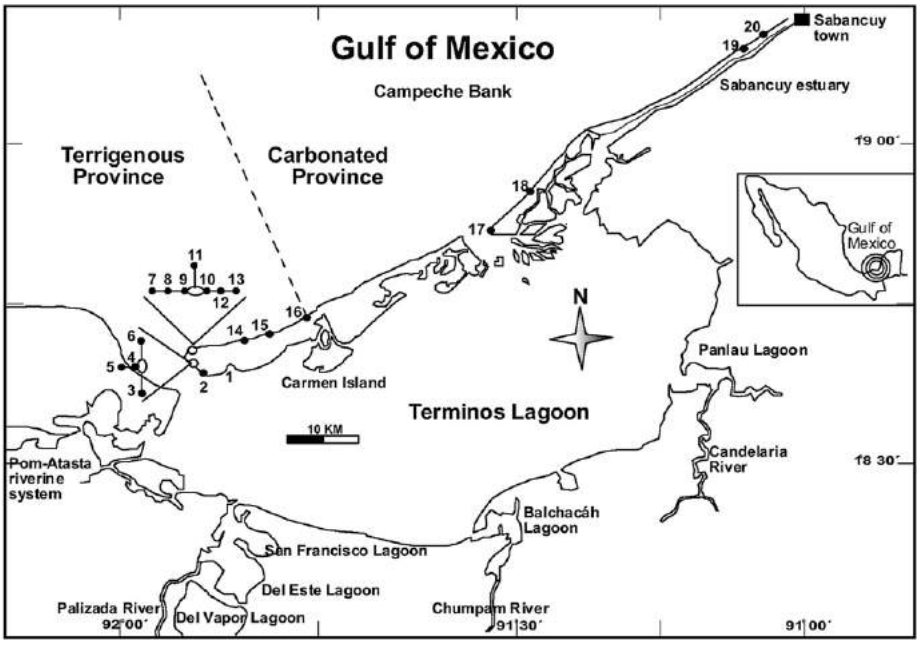


Fig. 1. The coastal beach studied, showing the location of the two sedimentary regions. Numbers 1 to 20 mark the location of the sampling sites. Enlargements containing sampling sites 3 to 6, and 7 to 13, also denote the location of two small oil seepages. Box in the right shows the location of the sampling area in the southern Gulf of Mexico.

a different genotype and treated as an individual operative taxonomic unit (OTU), for calculations of the Shannon–Wiener (H), Pielou (E) and Sorensen indices as indicated elsewhere (Rosano-Hernández et al., 2009). The uncertainty of the Shannon-Weaver index was converted to the True Diversity, or genotype units, as stated by Jost (2006). In addition, both calculations and the statistical standardization of all samples to a common sample size were made by using the rarefaction algorithm (Sanders, 1968) contained in the *Analytical Rarefaction* 1.3 software. Rarefaction provides a good estimate of the expected number of OTUs when specimens are similar and come from related habitats by identical sampling methods (Tipper, 1979).

2.6. rDNA libraries and DNA sequencing

The construction of rDNA libraries from both metagenomic DNA and HAB and HDB isolates was done by cloning the amplified DNA fragments into the pDrive vector and transformed into competent *E. coli* cells included in the kit of One Shot Top10 (Invitrogen, USA). Recombinant colonies were cultured following standard procedures (Sambrook and Russell, 2001). Fourteen purified plasmids were sequenced with the B1055 primer by using the 3100 ABI PRISM sequencer (Perkin–Elmer). GenBank nucleotide sequence accession numbers for the sequenced clones are EF191388 to EF191401.

2.7. Bacterial taxonomic surveillance and phylogenetic analysis

A taxonomic surveillance was made using the BLAST (GenBank, NCBI, USA). Multiple alignments were made with Clustal X using more than fifty partial 16S rDNA sequences retrieved from the GenBank, together with the partial sequences from our clones, identified as Gamma Proteobacteria. The tree topology was inferred by the neighbor-joining method, and distance matrix analyses were performed according to Jukes and Cantor, as implemented in the program MEGA software (Kumar et al., 2008).

3. Results and discussion

3.1. Total petroleum hydrocarbons (TPH) in the beach sediments

Based upon the TPH concentrations and existence of visible petroleum in the sediment, the sampling sites could be classified into three scenarios with high, intermediate and low contaminations, respectively, corresponding to three, eight and nine sites (Table 1). TPH concentrations were highly heterogeneous, showing a patchy distribution of hydrocarbons along the beach. Measured TPH were weathered hydrocarbons in the range of C_6 to C_{40+}, since volatile components were previously lost as effect of weather. Monocyclic aromatic hydrocarbons and *n*-alkanes with a chain length shorter than C_{14} are among the main substances volatilized (Harayama et al., 2004). The remaining exposed hydrocarbon components were likely susceptible to biological transformation, since the toxic, aromatic fraction decrease when the petroleum is exposed to sunlight (Dutta and Harayama, 2000).

3.2. Quantification of HAB and HDB and the environmental impact degree by crude petroleum (EID)

Densities of cultured bacteria were highly variable in the sampled sediments (Table 1). Colonies showed a little morphological assortment in Petri dishes, indicating the dominance of a small number of bacterial groups, and/or the inability of the media to support all the requirements for bacterial growth. The EID showed that the entire beach has been affected by petroleum, but four sampling sites (3–6) were very much affected by crude petroleum, regardless the TPH concentration measured (Table 1). These sites belong to an old half-moon shaped oil seep located in the shoreline of the Carmen Island (Fig. 1), which might be exchanging hydrocarbonoclastic bacteria with the surrounding environment. Nine of the total sampling sites held abundant oil degraders, more than 10% of the total bacterial populations, similar as other petroleum contaminated marine sites (Atlas, 1981).

In general, oil-degrading bacteria from contaminated areas are usually well tolerant to most petroleum hydrocarbons due to their ability to endure the toxic components of oil. Studies on oil seeps indicate that bacteria under oil stress may develop unique metabolic attributes as an adaptive behavior to chronic petroleum inputs (NAS, 2003; LaMontagne et al., 2004). One of them may include the development of a set of genes encoding a solvent efflux pump system as described in *Pseudomonas putida* DOI-TIE (Mosqueda and Ramos, 2000). Moreover, hydrocarbonoclastic bacteria also could thrive by using several components of petroleum as their carbon and energy source, such as the marine group of the obligate hydrocarbonoclastic bacteria, or OHCB (Yakimov et al., 2007).

Intertidal bacterial communities in the studied beach have been exposed chronically to the petroleum-impacted environment for centuries; therefore, they likely hold well-acclimated genes involving in the oil-degrading process, as well as the carbon utilization to biomass conversion, which make them valuable microbial resources of the Campeche Bank.

3.3. PCR-RISA banding patterns, ecological indices and the rarefaction analysis

Analysis of the acquired electrophoretic banding patterns (genotypes) obtained (data not shown), indicated that each of the sampling sites owned a particular bacterial community. Although genotype abundance provided some indication of the bacterial species richness, the DNA amplification by PCR-RISA was focused to those numerically abundant bacterial populations targeted by the primers (Forney et al., 2004). Thus, the total genotype richness here may still remain unknown.

Differences in the community structure of the petroleum-influenced environments were also indicated by some uneven genotype distribution ($E < 0.60$), and the low genotype similarity (Sorensen index < 0.30). The heterogeneous distribution of oil hydrocarbons likely accounted for this patchy pattern; however, in the complex intertidal environment some other unknown environmental factors may also be involved.

In general, local intertidal bacterial populations seem to be very tolerant to persistent inputs of petroleum hydrocarbons. For instance, ecological indices (Shannon and Pielou) showed that local (alpha) genotype diversities were higher in sediments with low and intermediate petroleum levels, than the diversity in sediments with high oil levels, regardless of the little genotype assortment (see Sorensen index, Table 1). Furthermore, the number of different genotypes would be also higher in sediments with a moderate influence condition than the low oil-influenced sediments (Fig. 2).

Interestingly, the TPH was inversely related to the diversity in each location: the highest petroleum concentrations corresponded to the lowest diversity indices (as the number of total and different genotypes, as well as both Shannon index and the True Shannon diversity) (Table 1). Although the beach system appears to support a bacterial community which on the whole seems to be well-acclimated to petroleum hydrocarbons, the inversely relationship between TPH and diversity indices showed that several bacterial populations may be still sensitive to some oil components. Crude petroleum has more than 17 000 distinct chemicals, which make it

Table 1
TPH concentrations, bacterial densities, the environmental impact degree by crude petroleum (EID), genotype numbers and ecological indices in the postulated high, medium and low oil-influenced beach scenarios.

Petroleum-influenced environments										
Scenario	Sampling point	Code banding patterns	TPH (mg kg^{-1})	HAB.gds^{-1} (X106)	HDB.gds^{-1} (X106)	EID	Number of genotypes			
High	4	H	8379	3.7	2.5	4	1	TPH range (mg.kg^{-1})		8379.0 −47645.0
	9	CEHIU	47645	96.6	1.6	2	5	Total points sampled		3
	10	C	32920	1.4	0.5	3	1	Total number of genotypes		7
								Number of expected[a] genotypes		5
								Number of different genotypes		5
								True Shannon diversity		2
								Sorensen index		0.27
								H	H′	E
								0.80	1.95	0.41
Medium	3	CEOS	397	27.2	26.9	4	4	TPH range (mg.kg^{-1})		1223.0 −7490.0
	5	DO	1768	35.1	19.9	4	2	Total points sampled		8
	6	AGHP	467	1.9	1.9	4	4	Total number of genotypes		37
	7	S	1223	<0.00005	0.5	nd	1	Number of expected[a] genotypes		14
	8	GHIKPU	1795	<0.00005	0.4	nd	6	Number of different genotypes		14
	11	CDEGHIOS	7490	2.0	0.9	3	8	True Shannon diversity		7
	12	CDEGHIO	1052	<0.00005	3.8	nd	7	H	H′	E
	13	BEFGK	328	3.4	0.2	3	5	1.95	3.61	0.54
Low	1	CGHOS	328	99.9	1.1	2	5	TPH range (mg.kg^{-1})		97.0−547.0
	2	BEFHIQU	341	<0.00005	281.5	nd	7	Total points sampled		9
	14	CDEFGHKO	nd	nd	nd	nd	8	Total number of genotypes		43
	15	CDHOS	547	nd	nd	nd	5	Number of expected[a] genotypes		13
	16	CEH	342	2.3	0.2	3	3	Number of different genotypes		13
	17	CEHO	97	2.0	0.4	3	4	True Shannon diversity		9
	18	GHOS	130	9.3	0.4	2	4	H	H′	E
	19	GHO	279	2.8	0.4	3	3	2.14	3.76	0.57
	20	FGHO	258	1.2	0.3	3	4			

HDB = Hydrocarbon-Degrading Bacteria; HAB = Heterotrophic Aerobic Bacteria; gds^{-1} = gram of dry sediment; TPH = Total Petroleum Hydrocarbons; H = Shannon Diversity Index; H′ = Theoretical maximum value of the Shannon index. E = Pielou (Evenness) Index. EID in the site: (4) = very affected, if HDB/HAB > 50%; (3) = affected, if HDB/HAB between 6% and 49%; (2) = little affected, if HDB/HAB between 1% and 5%; and (1) = no affected, if HDB/HAB < 1%; thus HAB = 100%, as Lizárraga-Partida et al. (1983).

[a] The number of expected genotypes was obtained through the rarefaction analysis for rarified sample sizes of: 6, 36 and 42 total genotypes, in the high, medium and low petroleum-influenced scenarios, respectively.

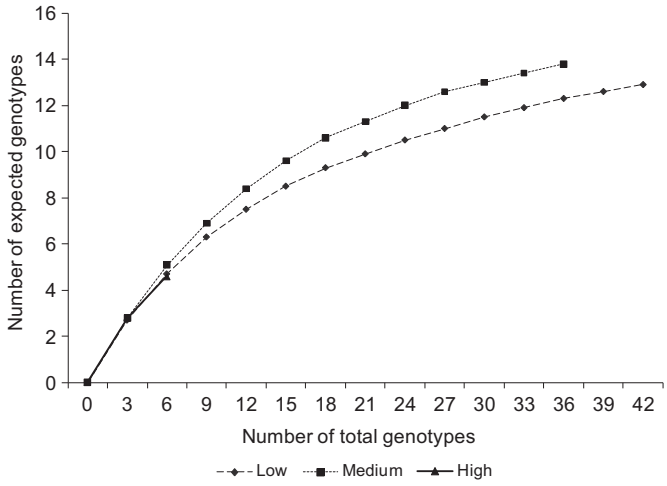


Fig. 2. Expected genotypes in sediments under low, medium and high hydrocarbon-petroleum influences, based on the rarefaction analysis. High and Low curves overlap at the beginning.

the most complex mixture of organic components in the Earth (Van Hamme et al., 2003; Head et al., 2006).

3.4. Bacterial diversity in the Carbonated and Terrigenous regions

The partial 16S rDNA sequences obtained from the sediments sampled in both regions showed high homologies of 97–99% to reported sequences of cultured and uncultured bacteria in which the Gamma-Proteobacteria was the most abundant group (Table 2). Based on the affiliation of sequences recovered, a clear bacterial partition pattern was seen in Terrigenous and Carbonated sediments, similar to other ecological partition models already observed in the Campeche Bank, i.e., heterotrophic bacteria (Lizárraga-Partida et al., 1986), crustaceans, mollusks and fish (Day and Yañez-Arancibia, 1988). Biological partition patterns were associated at that time (Day and Yañez-Arancibia, 1988; Yáñez-Arancibia and Day, 2004) to the development of gradients caused by the huge seasonal exchange of water and sediments, and the heavy organic upload in the area which persist to date.

The bacterial partition pattern revealed that bacteria in the sediments of the Terrigenous estuarine region were more diverse than those from the Carbonated marine region. Uncultured members of *Gemmatimonas*, *Acidobacterium*, *Fibrobacteres/Acidobacteria* group, *Rubrobacterales, Actinobacterium, Desulfobacteraceae*, and *Pseudoalteromonas* —all belonging to the Terrigenous region- fit in the Gemmatimonodates, Fibrobacteres/Acidobacteria, Actinobacteria and Gamma- and Delta proteobacteria classes (Fig. 3).

On the other hand, sequences from the Carbonated region were affiliated to only few genera, *Pseudomonas*, *Marinobacter* and *Vibrio,* which belong to the Gamma-Proteobacteria class (Fig. 3). Based on the characters of *Gemmatimonas aurantiaca*, the first cultured representative of the bacterial phylum Gemmatimonadetes (Zhang et al., 2003), the uncultured *Gemmatimonas* found in the Terrigenous region could be an aerobic, gram negative rod-shaped organism, with functions in nature linked to the accumulation of polyphosphate through the phosphorus removal from the environment (Zhang et al., 2003). The Acidobacteria group has been related to river bacteria (Beier et al., 2008) and to the beach sediments with low pH (Madigan and Martinko, 2006). Acidobacteria, as Gemmatimonas (see above) has been also linked to the removal of phosphorus from the environment (Fu et al., 2008). Interestingly, the discharges of river currents supply great amounts of phosphorus to the Terminos lagoon (Yañez-Arancibia and Day, 1988), which by tidal processes might have been carried to the beach sediments.

Members of the family *Desulfobacteraceae* imply the occurrence of some oxygen-devoid zones in sediments, where sulfate-reduction may take place, even though the beach showed perceptible neither black spots nor H_2S odor. Sulfate reduction, common in coastal sediments (Jørgensen, 1978), relies mostly on the family Desulfobacteraceae which has been associated with the anaerobic biocycling of organic carbon (Leloup et al., 2009). The actinobacterial group, which includes the Rubrobacterales and the Actinobacterium, comprise mostly aerobic gram–positive bacteria as typical inhabitants of soils and marine sediments.

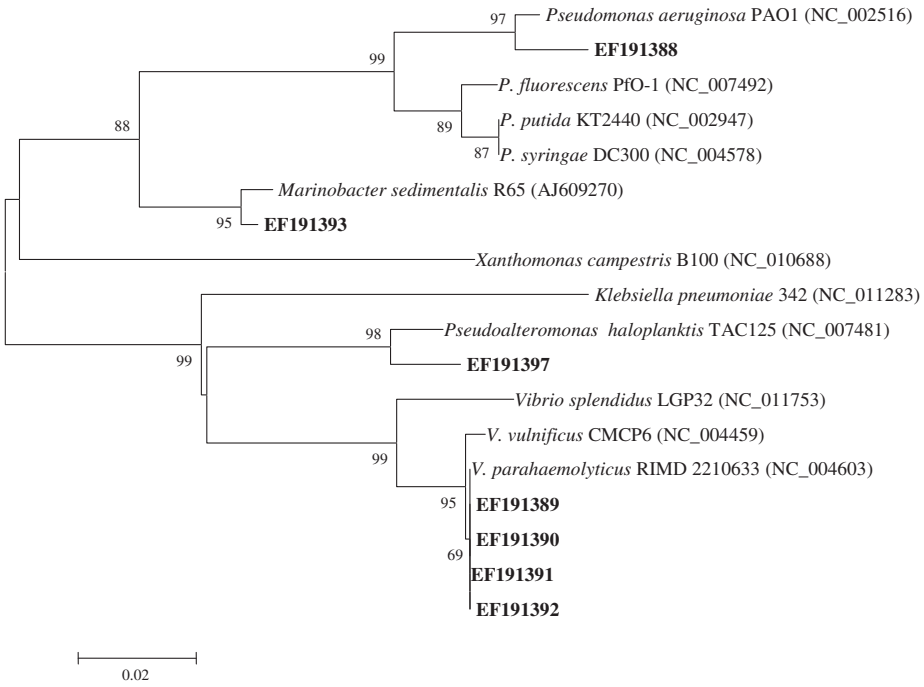
The potential ecological functions of the affiliated bacterial groups mentioned above were in accordance with the environmental properties of each geological region (Table 3), but the genetic composition should not be however extrapolated to any ecosystem function or bacterial activity. Although sediment bacteria play important roles in the biogeochemistry of ocean sediments (Hewson et al., 2007), definitely none of these functions have ever been described before in the beach studied.

Pseudomonas, *Marinobacter* and *Vibrio* were the only genera detected in the carbonated sediments. These genera contain representative species of N_2-fixing (diazothropic), with or without oil degradation activities (Harayama et al., 2004; Madigan and Martinko, 2006). *Pseudomonas* and *Vibrio* are among the most prevalent hydrocarbon degraders with global distribution (Atlas,

Table 2
Recognized bacterial taxa in petroleum-influenced sediments of the Carbonated and Terrigenous regions. Partial DNA sequences came mostly from 16S gene.

Geological province	GenBank accession number	bp	Source	Genbank hit sequence	IsD %[a]	Taxa vs. Lineage
Carbonated	EF191388	660	I,P	FM209186 *Pseudomonas aeruginosa* LESB58	98	Gamma-Proteobacteria
Carbonated	EF191389	530	I,P	BA000031 *Vibrio parahaemolyticus* RIMD 2210633 DNA	99	Gamma-Proteobacteria
Carbonated	EF191390	663	I,P	BA000031 *Vibrio parahaemolyticus* RIMD 2210633 DNA	99	Gamma-Proteobacteria
Carbonated	EF191391	360	I,P	FM 958469 Uncultured Vibrio sp. clone HG136	99	Gamma-Proteobacteria
Carbonated	EF191392	639	I,P	BA000031 *Vibrio parahaemolyticus* RIMD 2210633 DNA	99	Gamma-Proteobacteria
Carbonated	EF191393	783	I,M	CP000514 *Marinobacter aquaeolei* VT8	99	Gamma-Proteobacteria
Terrigenous	EF191394	671	C,P	FJ551968 Uncultured Gemmatimonas sp. clone LTSP_BACT_P1J18	95	Gemmatimonadetes
Terrigenous	EF191395	746	C,P	EU373923 Uncultured Acidobacterium sp. clone HCM3MC91_1G_FL	97	Fibrobacteres/Acidobacteria
Terrigenous	EF191396	705	C,P	EF191396 Uncultured Gemmatimonas sp. clone LTSP_BACT_P4P01	95	Gemmatimonadetes
Terrigenous	EF191397	569	C,P	CR954246 *Pseudoalteromonas haloplanktis* TAC125	99	Gamma-Proteobacteria
Terrigenous	EF191398	749	C,P	FJ516927 Uncultured Desulfobacteraceae bacterium clone TDNP_USbc97_21_4_87	91	Delta-Proteobacteria
Terrigenous	EF191399	757	C,P	AY869377 Uncultured Fibrobacteres/Acidobacteria group bacterium clone I3K-0661	98	Fibrobacteres/Acidobacteria
Terrigenous	EF191400	759	C,P	FJ551414 Uncultured Rubrobacterales bacterium clone LTSP_BACT_P3G01	97	Actinobacteria
Terrigenous	EF191401	726	C,P	AY897259 Uncultured actinobacterium clone Nubeena381	99	Actinobacteria

I = Isolated; C = clone; P = peptonate medium; M = mineral medium plus petroleum.
[a] Comparisons were made with approximately 400 nucleotides from the ′3 16S rRNA gene.



The scale bar represents approximately 0.02 changes per average nucleotide position. Bootstrap confidence values obtained with 1000 re-samplings are given at the branch-points.

Fig. 3. Neighbor-joining tree deduced from partial 16S sequences of clones among the class Proteobacteria.

1981; Van Hamme et al., 2003), and the *Marinobacter* taxa was recently found in sediments of marine oil seeps of the Campeche Bank (Rosano-Hernández et al., 2009). *Marinobacter* is one of the six highly specialized marine genera that obligatory degrade petroleum hydrocarbons (Yakimov et al., 2007).

The finding of *Marinobacter* in beach sediments was indicative of (1) the presence of obligate oil-degrading organisms in the intertidal ecosystem, and (2) the likely occurrence of a bacterial exchange—unknown to date—between the beach and the ancient oil seep sites of the southern Gulf of Mexico. Although the hydrocarbon degradative capacities of the *Marinobacter* found in this work were not further investigated, the taxon might be playing a role in the "natural cleaning-up" of petroleum chronically generated in the Campeche Bank. According to its capacities to exploit spatially and temporally variable resources, and to adapt to various environmental conditions, *Marinobacter* has been considered as one of two potential examples of an opportunitrophic microbe (Singer et al., 2011).

Table 3
Environmental properties of two sedimentary regions in the Gulf of Mexico (modified from Yañez-Arancibia and Sánchez-Gil, 1988).

	Sedimentary provinces	
	Terrigenous	Carbonated
Ecological subsystem or habitat	highly estuarine	typically marine
Sediment type	silt-clay	sand
$CaCO_3$ (%)	0–20	30–100
Organic matter (%)	>10	<10
pH	7.6–8.3	7.7–8.9
Dissolved oxygen (mg l^{-1})	<4	>4
Temperature (°C)	25–28	26–29
Water transparency[a]	low	high

Low = 7–42%; high = 50–99%.
[a] The portion of light that passes through water without distortion or absorption.

4. Conclusions

Differences on the quantity, diversity and distribution of bacterial communities from intertidal beach sediments of the Campeche Bank under different petroleum influences were detected by a protocol based on molecular (PCR-RISA) and cultured-dependent methods. TPH was inversely related to the genotype diversity. Gemmatimonodates, Fibrobacteres/Acidobacteria, Actinobacteria and Gamma- and Delta proteobacteria classes were found in sediments, but their ecological functions in the beach remain unknown. *Marinobacter* was found in the intertidal sediment, which was indicative of OHCB in the beach ecosystem, and a likely exchange of bacteria between the beach and the oil seep sites of the southern Gulf of Mexico. Due to their potential well-acclimated genes involved in the oil-degrading process, bacteria from the Campeche Bank are valuable marine microbial resources. Further research on the genetic diversity of Bacteria and the inclusion of the Archaea and Eucarya domains from other marine environments of the southern Gulf of Mexico is strongly advised.

Acknowledgments

To Drs. H. Macarié and I. Sastre-Conde, as well as three anonymous reviewers for their valuable comments. To Drs. Jesús Caballero-Mellado and Paulina Estrada-de los Santos (Centro de Ciencias Genómicas, UNAM) for the phylogenetic analysis. The IMP provided a grant to María C. Rosano-Hernández. The work was supported by project IMP-D.00023.

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Journal of Environmental Management 95 (2012) S332–S337

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Characterization of rhizosphere bacteria for control of phytopathogenic fungi of tomato

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ARTICLE INFO

Article history:
Received 15 August 2009
Received in revised form
3 March 2011
Accepted 29 March 2011
Available online 20 April 2011

Keywords:
Fluorescent *Pseudomonas* spp.
Antagonistic bacteria
Phytopathogenic fungi
Tomato

ABSTRACT

Fluorescent *Pseudomonas* spp., isolated from rhizosphere soil of tomato and pepper plants, were evaluated *in vitro* as potential antagonists of fungal pathogens. Strains were characterized using the API 20NE biochemical system, and tested against the causal agents of stem canker and leaf blight (*Alternaria alternata* f. sp. *lycopersici*), southern blight (*Sclerotium rolfsii* Sacc.), and root rot (*Fusarium solani*). To this end, dual culture antagonism assays were carried out on 25% Tryptic Soy Agar, King B medium, and Potato Dextrose Agar to determine the effect of the strains on mycelial growth of the pathogens. The effect of two concentrations of $FeCl_3$ on antagonism against *Alternaria alternata* f. sp. *lycopersici* was also tested. In addition, strains were screened for ability to produce exoenzymes and siderophores. Finally, the selected *Pseudomonas* strain, PCI2, was evaluated for effect on tomato seedling development and as a potential candidate for controlling tomato damping-off caused by *Sclerotium rolfsii* Sacc., under growth chamber conditions. All strains significantly inhibited *Alternaria alternata* f. sp. *lycopersici*, particularly in 25% TSA medium. Antagonistic effect on *Sclerotium rolfsii* Sacc. and *Fusarium solani* was greater on King B medium. Protease was produced by 30% of the strains, but no strains produced cellulase or chitinase. Growth chamber studies resulted in significant increases in plant stand as well as in root dry weight. PCI2 was able to establish and survive in tomato plants rhizosphere after 40 days following planting of bacterized seeds.

1. Introduction

Tomato (Lycopersicon esculentum Mill.) is the second leading vegetable crop worldwide, next to potato. World production is $\sim 1 \times 10^6$ tonnes from 3.7×10^6 ha (Food Agricultural Organization, 2010). In Argentina, it is the vegetable occupying the most greenhouse area. The percentage of total production going to industry is 35–40% and the rest is sold as fresh produce domestically. The area dedicated to tomato in field and greenhouse is 1.2×10^4 ha and 3×10^3 ha, respectively; average yield in both cases is ~35–40 tonnes per ha (Nakama and Fernández Lozano, 2006). Due to increasing demand, tomato has a great potential for increased commercialization. More efficient tomato production requires better knowledge of its pathogens and control methods.

The fungus *Alternaria alternata* f. sp. *lycopersici*, frequently isolated from diseased tomato plants, is the cause of stem canker (Gilchrist and Grogan, 1975) and leaf blight (Akhtar et al., 2004). *Sclerotium rolfsii* Sacc. is a soilborne fungus that causes southern blight disease in a wide variety of agricultural and horticultural crops (Flores-Moctezuma et al., 2006). *Fusarium solani* causes root rot in several crops. Penconazole [1-(2,4-dichloro-β-propylphenethyl)-1*H*-1,2,4-triazole], penthachloronitrobenzene (PCNB), and idropione [3-(3,5-dichlorophenyl)-N-(1-methylethyl)2,4-dioxo-1-imidazole-carboxamide] are three chemical fungicides commonly used to control the above pathogenic fungi. Nevertheless, increasing public concern regarding use of chemical pesticides that damage human health or the environment is driving the search for more environmentally "friendly" methods to control plant disease. A realistic alternative, or supplement, to chemical fungicides for management of plant diseases is the use of soilborne, non-

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0301-4797/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jenvman.2011.03.037

pathogenic bacteria that inhibit fungal phytopathogens. Such bacteria are known by several generic names, including "biological control agents" (BCAs) and "plant growth promoting rhizobacteria" (PGPR). Soilborne, fluorescent pseudomonads have received particular attention because of their catabolic versatility, excellent root-colonizing abilities, and production of a wide range of antifungal metabolites (Walsh et al., 2001).

The objectives of this study were (1) to biochemically characterize fluorescent *Pseudomonas* strains, (2) to evaluate their antagonistic activities against phytopathogenic fungi of vegetables *in vitro*, and (3) to determine the effect of a strain, PCI2, on tomato growth as well as to evaluate its potential for controlling tomato damping-off caused by *S. rolfsii* Sacc.

2. Materials and methods

2.1. Isolation and characterization of fluorescent Pseudomonas

Fluorescent *Pseudomonas* spp. were isolated from the rhizosphere of healthy tomato (*L. esculentum* Mill.) and pepper (*Capsicum annuum* L.) plants from four regions of the province of Córdoba, Argentina: Colonia Caroya (20° 36′ N, 102° 13′ W), Embalse (32° 12″ S, 64° 23″ W), Mattaldi (34° 49′ 16″ S, 64° 34′ 22″ W) and Río Cuarto (33° 04′ S, 64° 38′ W). Non rhizosphere soil was removed from the root system of the plants. Roots were then excised and placed into 10 ml of sterile 0.9% NaCl solution and vortexed for 10 min in order to detach the associated rhizosphere soil. Serial dilutions of the resulting root wash were plated on King B medium (KB) (King et al., 1954) supplemented with ampicillin (100 μg ml^{-1}) and cycloheximide (75 μg ml^{-1}) (Simon and Ridge, 1974). Plates were incubated at 28 °C for 24–48 h, at which time the fluorescent colonies were observed under UV light (354 nm). To obtain the most abundant bacteria from each sample, selection of strains showing fluorescence and different colony morphology was performed from the highest dilutions. All bacterial cultures were stored at −20 °C in Tryptic Soy Broth (TSB) supplemented with 20% (v:v) glycerol.

Bacterial characterization was carried out on the basis of colony morphology, Gram stain, oxidase test, production of acids from 1% glucose in Oxidation/Fermentation (OF) basal medium (Hugh and Leifson, 1953), and analysis with the API 20NE biochemical test plus computer software (bioMèrieux S.A., Marcy l'Etoile, France).

2.2. Phytopathogenic fungi and reference bacteria

Fungal phytopathogens used were *Sclerotium rolfsii* Sacc., *Fusarium solani* (from the fungal collection of the Laboratory of Plant–Microbe Interactions, Universidad Nacional de Río Cuarto), and *Alternaria alternata* f. sp. *lycopersici* (kindly supplied by the Laboratory of Mycology, Universidad Nacional de Río Cuarto), all isolated from diseased tomato and pepper plants. Fungi were kept in potato dextrose agar (PDA) plates at room temperature or at 4 °C, and replicated monthly.

Reference bacteria were used in this research. *Pseudomonas fluorescens* CHA0 and *P. aurantiaca* SR1 were grown on KB and 25% Tryptic Soy Agar (TSA). *Serratia marcescens* WF was grown on 25% TSA. *Bradyrhizobium* spp. C 145 and *Sinorhizobium meliloti 3DOh13* were maintained on Yeast Mannitol Agar (Vincent, 1970). All the bacteria were routinely cultured at 28 °C.

2.3. Evaluation of strains for in vitro biological control

2.3.1. Antagonism in dual culture

The fluorescent *Pseudomonas* were tested against *S. rolfsii* Sacc., *A. alternata* and *F. solani* in plate bioassays. *A. alternata* and *F. solani* were cultivated in PDA at 28 °C. Conidia were harvested from the surface of plates by flooding the 10-day-old cultures with 9 ml of sterilized distilled water and gently scraping with a sterilized glass rod; conidial concentration was determined with a Neubauer chamber (Cota et al., 2007). Plates containing the media to be tested (KB, PDA) were prepared. Then, an agar over-layer containing the target fungus, immobilized at a concentration of 10^4–10^5 conidia ml^{-1}, was placed on the medium. The methodology described by Montesinos et al. (1996) was followed in order to prepare the overlay, using 0.7% agar. Four ml of the medium was placed in screw-capped test tubes that, once sterilized, were kept inside of a bath of water at 40 °C. Next, 100 μl of a target conidia suspension was added to each test tube, which were vortexed and the content of each tube was then homogeneously distributed on a plate containing the same culture medium. The bacterial strains tested were sown by gently touching the agar surface with a sterile toothpick, previously inoculated by touching the surface of a single colony. Plates were incubated for 72 h at 28 °C. The degree of inhibition in each medium was determined by measuring the halo around the bacterial strain with no fungal growth. The average of six replicates was considered for the value of the inhibition halo. For screening for potential antagonism against *S. rolfsii* Sacc., mature sclerotia were removed from the surface of 15-day-old cultures with sterile forceps and four were immediately placed around the edges and one in the center of a plate 24 h after the stab-inoculation of four bacterial strains. The experiment was conducted twice.

2.3.2. Mycelial growth inhibition

The bacterial strains were streaked on 1/3 of a Petri plate containing 25% TSA, KB or PDA. A mycelial disc (9 mm diameter) of a 8–15 day-old-culture of an actively growing target fungus was equidistantly placed on the opposite side of the Petri plate 48 h after inoculation of the strain. Plates were incubated for 7 days at 28 °C. The plates with fungal pathogens on one side that were not inoculated with bacterial strains served as controls. For each fungal colony, two diameters, measured at right angles to one another, were averaged to find the mean diameter for that colony. The mean diameter of fungal growth in the presence of each strain was compared to that of the control cultures in order to determine the inhibition percentage. All fungal colony diameters were determined by using three replicates for each strain on each medium. *P. aurantiaca* SR1 (Rosas et al., 2001; Rovera et al., 2008) and *P. fluorescens* CHA0 were used as positive controls.

In addition, each strain was tested on both 25% TSA and KB supplemented with two concentrations of $FeCl_3$ (50 and 100 μM) in order to evaluate the influence of iron on the ability of the strains to control *A. alternata*. Plates were incubated for 7 days at 28 °C. The fungal colony diameter was determined by using three replicates for each strain on each medium. The plates with *A. alternata* on one side that were not inoculated with bacterial strains served as controls. Experiments were conducted twice.

2.3.3. Production of hydrolytic enzymes

Proteolytic activity was detected by inoculating the strains on a medium composed of 1% casein and 2.3% agar dissolved in Castañeda medium (Castañeda-Agulló, 1956). Plates were incubated for 48 h at 28 °C. Casein hydrolysis was detected by the formation of a whitish, opaque halo (coagulated casein) around a translucent area (totally hydrolyzed casein), surrounding the colony. Strains were also tested for its ability to produce extracellular chitinases in a liquid medium; assay medium was prepared with 2% chitin from crab shells (w:v) in tap water (Rojas Avelizapa et al., 1999). *S. marcescens* WF was used as a positive control. Tests were performed twice. To determine cellulolytic activity, carboxymethyl cellulose (CMC) was incorporated at 0.1% into the YEMA–0.2% mannitol agar plates. Colonies were grown for 3 days at 28 °C and

washed off with water. The plates were then flooded with 0.1% (wt:vol) Congo Red in water for 15 min, washed for 10 min with 1 M NaCl, and then washed for 5 min with 5% acetic acid. Degradation of CMC was observed as clearings (reduction of staining) (Zorreguieta et al., 1999). Test was performed twice. *Bradyrhizobium* spp. C 145 was used as the positive control.

2.3.4. Siderophores production

The chrome azurol S (CAS) method described by Alexander and Zuberer (1991) was used for screening strains for siderophore production. Plates were incubated at 28 °C for 5 days, and microorganisms exhibiting an orange halo were considered to be producers of siderophores. *S. meliloti 3DOh13* (Rosas et al., 2006) was used as the positive control.

2.4. Identification and quantification of indole-3-acetic acid (IAA) in culture supernatant of strain PCI2

Strain PCI2 was grown in nutrient broth (NB). Then, 20 ml were taken during the late exponential growth phase (24 h) for identification and quantification of IAA, which were carried out by High Performance Liquid Chromatography HPLC–Mass spectrometry (HPLC–MS). A 100 ng 2H5-IAA (OlChemIm, Czech Republic) deuterated internal standard was included.

2.5. Evaluation of selected strain PCI2 for growth promotion and biological control

2.5.1. Preparation of fungal and bacterial inocula and treatment of seeds

Cultures of *S. rolfsii* Sacc. were maintained on PDA, on which brown sclerotia formed within 8–10 days. Pathogen inoculum added to sterile mixture consisted of 30-day-old sclerotia which were dislodged from the surface of plates and used immediately (Papavizas and Lewis, 1989). Plastic pots (15 cm diameter; 25 cm height) were filled with 600 g of sterile mixture (soil:sand:perlite at 2:1:1 w/w/w), previously sterilized by heating at 180 °C for 2 h on four consecutive days. Each pot was then moistened with sterile distilled water and infested in the mixture surface with 30 mg of sclerotia. Pots were kept for 8 days in a growth chamber under controlled conditions: 16 h light at 28 ± 2 °C, 8 h dark at 16 ± 2 °C (light intensity of 220 μE m^{-2} s^{-1}).

After incubation, tomato seeds (*L. esculentum* Mill.) cv. Platense Italiano (Asociación Cooperativa INTA La Consulta, Mendoza, Argentina) were surface-disinfected for 10 min in 5% sodium hypochlorite solution (60 g l^{-1} of active chlorine), washed ten times in sterilized distilled water, and air dried (Tsahouridou and Thanassoulopoulos, 2002). Then, 10 g of seeds were soaked for 30 min in 2.5 ml of a 10^9 CFU ml^{-1} aqueous cell suspension of strain PCI2. The bacterium was prepared by growing by shaking (80 rpm) in KB broth for 48 h at 28 °C (Jayaraj et al., 2007). Then, eight inoculated seeds were placed into the mixture surface in each pot. The four treatments were: (1) non-infested, non-bacterized healthy control (treated with sterile distilled water), (2) infested with *S. rolfsii* Sacc., non-bacterized control, (3) infested with *S. rolfsii* Sacc. and bacterized with PCI2, and (4) bacterized with PCI2 alone. Pots were incubated in a growth chamber under the conditions described above. Damping-off was determined by counting the total healthy stand after 40 days, compared to non-infested control plants. Shoot and root dry weights (72 h at 70 °C) were recorded from twenty randomly selected plants from each treatment. Pots were arranged in a completely randomized design. The experiment was performed twice, each with six replicates per treatment.

2.5.2. Tomato rhizosphere colonization

Survival of strain PCI2 in the rhizosphere of tomato plants from treatments 1 and 4 was determined according to a modification of the procedure described by Landa et al. (2004) at 10, 25 and 40 days after sowing. Briefly, 1 g of rhizosphere mixture was collected at 10 days from the surroundings of a seedling from each treatment and placed into 9 ml of sterile 0.9% NaCl solution. Also, a seedling from each treatment was carefully removed from a pot at 25 and 40 days and roots were gently shaken to remove all but the tightly adhering potting mixture. One gram of the adhering rhizosphere mixture was collected and placed into 9 ml of sterile 0.9% NaCl solution. Serial dilutions of the suspension were vortexed and plated onto 25% TSA medium. Plates were incubated for 48 h at 28 °C. The developed colonies from each treatment were counted and the number of CFU g^{-1} of mixture was calculated.

2.6. Statistical analyses

The data were analyzed by using analysis of variance (ANOVA). When ANOVA showed treatment effect, the Least Significant Difference (LSD) test was applied to make comparisons between the means at $P < 0.05$. A non parametric Kruskall-Wallis variance analysis was applied to evaluate the differences between the inhibitory capacities of the strains in media supplemented with different concentrations of iron. All data were subjected to statistical analysis using Statgraphics plus software for Windows *V 4.1* (Statistical Graphics Corp., Maryland, USA).

3. Results

Ten bacterial strains were obtained from tomato and pepper roots. All of the strains were Gram-negative rods, oxidase-positive and capable of metabolizing glucose in an oxidative form. The API 20NE test revealed that the strains belong to the species *Pseudomonas fluorescens* (four strains), *P. putida* (four strains), *P. aeruginosa* (one strain) and *Ralstonia pickettii* (one strain).

Recovered bacterial strains were tested for their antagonistic ability against the phytopathogenic fungi *A. alternata*, *S. rolfsii* Sacc. and *F. solani*. As a result, the bacterial antagonistic effect in the dual culture assay depended both on the target pathogen and the culture media used; moreover, the influence of the composition of the medium was observed against all fungi. The strongest *in vitro* antagonism against *A. alternata* was observed on 25% TSA, while the higher inhibitory activity against *F. solani* and *S. rolfsii* Sacc. was observed on KB.

Similarly, the observed *in vitro* inhibition of mycelial growth also varied with the culture medium and the target pathogen. The inhibitory effect on the mycelial growth of *A. alternata* was higher on 25% TSA. All the tested strains resulted in >60% inhibition on 25% TSA, >40% on KB and ≤20% on PDA. As observed for the germination inhibition assay, mycelial growth inhibition of *F. solani* and *S. rolfsii* Sacc. by all the recovered strains was more effective on KB. However, the growth of the fungal pathogens was barely inhibited in the presence of the strains on PDA (Table 1).

Addition of iron to 25% TSA affected the antagonistic activity of the strains against *A. alternata*. A 50 μM $FeCl_3$ concentration significantly decreased the effectiveness of seven strains (P1, P8, Tbr2, TR1, P12, Pbr3 and PCI2). Addition of 100 μM $FeCl_3$ inhibited the antagonistic activity of all of the isolated strains. Moreover, addition of iron to KB caused a stronger decrease in the antagonistic activity of the strains against *A. alternata*. A 50 μM concentration of $FeCl_3$ significantly decreased the effectiveness of strain P1. Addition of 100 μM $FeCl_3$ produced a significant decrease in the antagonistic activity of four strains (P1, TR1, P12 and PCI2) (Table 2). Three

Table 1
Inhibition percentage of mycelial growth of *S. rolfsii* Sacc., *A. alternata* f. sp. *lycopersici* and *F. solani* on three different media.

Bacterial strain	Inhibition percentage of mycelial growth								
	TSA			KB			PDA		
	S. r.	*A. a.*	*F. s.*	*S. r.*	*A. a.*	*F. s.*	*S. r.*	*A. a.*	*F. s.*
P. putida P1	12.39b	66.65b	2.62de	9.01cd	41.43ns	12.55bcde	7.43bc	20.31b	3.60d
P. fluorescens P8	17.19b	77.48ab	3.05cde	8.19cd	55.82ns	11.05cdef	5.78bc	13.54bc	5.40d
P. putida TBR2	4.39c	73.86ab	4.79c	2.45d	47.98ns	10.55def	2.88cd	9.89c	8.10c
P. fluorescens TEI1	1.99c	72.84ab	4.36cd	11.47cd	50.58ns	12.06cdef	4.95bc	14.57bc	5.40d
P. fluorescens TR1	1.79c	82.11a	4.79c	20.07bc	54.50ns	9.54ef	5.37bc	11.98bc	8.10c
P. aeruginosa P4	1.00c	75.93ab	3.48cde	24.58bc	53.19ns	13.56bcd	7.43bc	16.14bc	7.66c
R. pickettii P6	15.99b	75.93ab	4.79c	18.02cd	41.62ns	15.57b	3.71cd	9.89c	4.95d
P. putida P12	1.79c	75.40ab	1.74f	3.68d	44.05ns	14.06bc	2.47cd	15.10bc	9.01c
P. putida PBR3	1.59c	80.04ab	1.74f	1.63d	41.43ns	9.54ef	6.61bc	7.81c	5.40d
P. fluorescens PCI2	2.39c	71.80ab	2.17ef	25.81bc	50.58ns	9.04f	7.02bc	15.62bc	7.66c
P. aurantiaca SR1	44.40a	78.50ab	12.65b	58.93a	50.58ns	15.57b	19.42a	29.68a	13.51b
P. fluorescens CHA0	51.19a	79.00ab	28.38a	59.00a	57.71ns	43.21a	19.90a	13.54bc	20.27a

S. r.: *S.* rolfsii Sacc.; *A. a.*: *A. alternata* f. sp. *lycopersici*; *F. s.*: *F. solani*.
Percentages with the same letter within the same column are not significantly different according to the LSD ($P < 0.05$) test.
ns: not significantly different.

strains (P4, P6 and P8) showed protease activity, whereas none of them produced cellulase or chitinase. Additionally, all of the strains were able to respond to iron limitation producing siderophores in CAS medium.

Of the ten strains isolated from the root system of tomato and pepper plants, strain PCI2 was selected for further study based on its *in vitro* inhibitory activity in the antagonism in dual culture as well as in the mycelial growth inhibition assays against phytopathogenic fungi of tomato, in particular against *S. rolfsii* Sacc. Thus, PCI2 was evaluated for growth promotion of tomato plants and biological control of *S. rolfsii* Sacc. *in vivo*. In *S. rolfsii* Sacc. infested mixture, inoculating tomato seeds with strain PCI2 improved seedling stand by 29% and increased shoot and root dry weight of plants over the untreated pathogen controls by 84.7 mg and 59.9 mg, respectively (Fig. 1). No evident differences between bacterized seeds and control seeds were observed in non-infested potting mixture when recording plant stand; however, inoculation of seeds with PCI2 increased ($P < 0.05$) root dry weight by 71.8 mg. The increase in root dry weight may be due to phytohormone-like substances, since strain PCI2 produces indole-3-acetic acid (IAA) at 4.71 μg ml^{-1} (without addition of tryptophan to culture medium) after 24 h of incubation. Although inoculation with strain PCI2 increased shoot dry weight by 33.6 mg, when compared to healthy controls, differences were not significant (Fig. 1). Fluorescent *Pseudomonas* morphologically similar to PCI2 reached a population density of 10^7–10^8 and 10^6–10^7 CFU g^{-1} mixture after ten and forty days of experimentation, respectively, in the bacterized with PCI2 alone treatment (Fig. 2). Colony counts performed from non-infested, non-bacterized control plants revealed absence of colonies morphologically similar to PCI2.

4. Discussion

The aim of this study was the isolation, characterization, and selection of *Pseudomonas* spp. with antagonistic activity against phytopathogenic fungi, but harmless to vegetable crops. The strains used were initially isolated from rhizosphere of healthy tomato and pepper plants from four regions of Córdoba province. Williams and Asher (1996) concluded that methods employed to isolate rhizobacteria play an important role in identification of potential biocontrol agents, and that the strains should be from the rhizosphere of the target crop.

Antagonistic properties of strains tested *in vitro* were influenced by culture medium composition, the fungal pathogen, and its growth stages. These results were consistent with those of Borowicz and Saad Omer (2000), who proposed that differences between media could result in alterations of metabolites produced, or their relative concentrations. Also, the type of medium used to grow both bacteria and fungi in studies of biological control affects the interaction of the organisms (Benko and Highley, 1990).

Enzymatic degradation of the cell wall of fungal pathogens by biocontrol agents has been reported (Bar-Shimon et al., 2004; Compant et al., 2005). In this work, protease, cellulase and chitinase production were assayed. Protease production proved to be the

Table 2
Inhibition percentage of *A. alternata* f. sp. *lycopersici* mycelial growth in media supplemented with different iron concentrations.

Bacterial strain	Inhibition percentage of*A. alternata* f. sp. *lycopersici* mycelial growth					
	25% TSA			King's B medium		
	No $FeCl_3$	$FeCl_3$ 50 μM	$FeCl_3$ 100 μM	No $FeCl_3$	$FeCl_3$ 50 μM	$FeCl_3$ 100 μM
P. putida P1	66.66a	48.14b	43.95b	40.94a	13.63b	12.69b
P. fluorescens P8	66.66a	56.07b	54.39b	35.42a	37.27a	42.85a
P. putida TBR2	65.98a	54.91b	51.65b	29.91a	20.09a	17.45ab
P. fluorescens TEI1	58.50a	56.07ab	46.85b	30.57a	21.83b	18.26b
P. fluorescens TR1	61.90a	42.20b	42.85b	44.87a	33.65a	13.50b
P. aeruginosa P4	68.02a	56.07ab	50.55b	46.35a	29.91b	30.16b
R. pickettii P6	65.98a	54.91ab	47.25b	29.12ab	30.92a	40.47a
P. putida P12	58.50a	39.30b	48.35b	35.42a	24.54a	7.00b
P. putida PBR3	67.34a	52.60b	53.14b	26.76a	25.47a	12.69b
P. fluorescens PCI2	64.62a	37.57b	48.90b	31.49a	26.54a	9.52b
P. aurantiaca SR1	70.74a	60.69b	60.44b	73.54a	72.72a	49.21b
P. fluorescens CHA0	72.78a	71.67a	75.37a	69.71a	71.81a	75.39a

Percentages with the same letter within the same row (in each medium) are not significantly different according to the non parametric Kruskall-Wallis variance analysis.

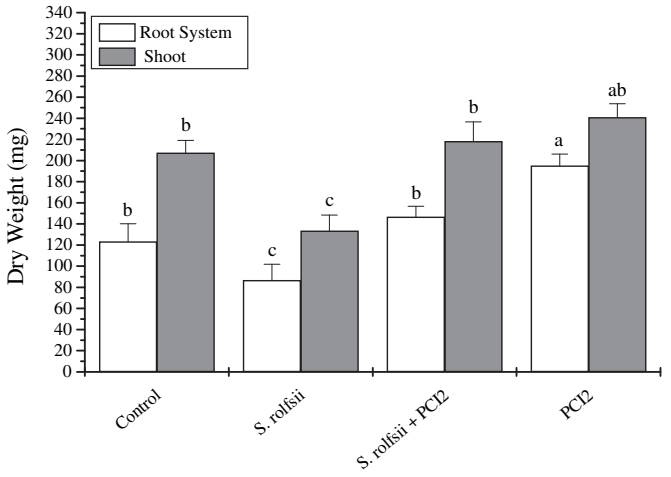

Fig. 1. Biocontrol activity of strain PCI2 against *S. rolfsii* Sacc. Tomato root and shoot dry weights were measured after 40 days of experimentation. Data represent the average of two experiments ± standard deviation. Bars for each plant fraction with different letters are significantly different according to the LSD test ($P < 0.05$).

only exoenzymatic activity detected in the strains. Antagonistic activity of the strains against *A. alternata* declined as iron concentration in both 25% TSA and KB increased, suggesting involvement of siderophores in this system. Based on significant *in vitro* antagonistic effect against leaf pathogen *A. alternata* and root pathogen *S. rolfsii* Sacc., a potential biocontrol agent, strain PCI2, was selected for a future evaluation of ability to suppress fungal pathogens *in vivo*.

A growth chamber assay was performed to evaluate tomato plants response to strain PCI2. Walsh et al. (2001) emphasized the need to investigate *in situ* colonization in the rhizosphere to determine the potential of a *Pseudomonas* strain as an effective BCA. Forty days after sowing of inoculated seeds, PCI2 reached a population density of 10^6–10^7 CFU g^{-1} mixture under growth chamber conditions. In this system, strain PCI2 did not appear to negatively affect development of tomato plants, but it also enhanced growth of the root system. Several reports have indicated that IAA synthesis is related to plant growth stimulation by microorganisms, including *P. putida* (Patten and Glick, 2002). IAA is the most common natural auxin found in plants and its positive effect on root growth and morphology is believed to increase the access to more nutrients in

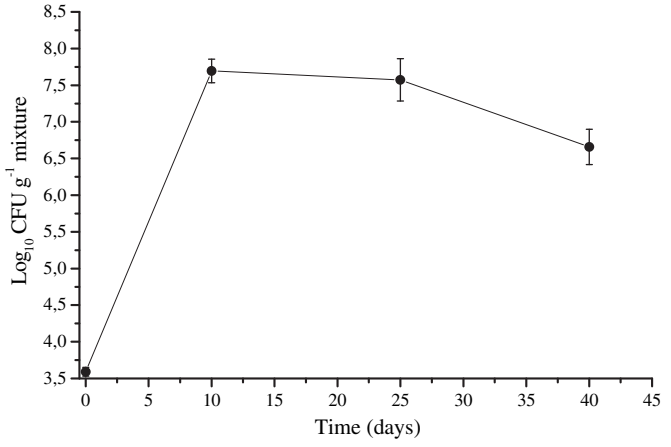

Fig. 2. Persistence of strain PCI2 in the rhizosphere of tomato plants. Data represent the average of two experiments ± standard deviation. For determining colony count at 1 h (zero time), 1 g of rhizosphere mixture was collected from the surroundings of a seed and placed into 9 ml of sterile 0.9% NaCl solution. Serial dilutions of the suspension were vortexed and plated onto 25% TSA medium. Plates were incubated for 48 h at 28 °C; the developed colonies were counted and the number of CFU g^{-1} of mixture was calculated.

the soil (Vessey, 2003). Thus, production of IAA is a characteristic that may enhance PCI2 use as an effective biological control agent to contribute to the control of tomato damping-off caused by *S. rolfsii* Sacc.

5. Conclusions and future perspectives

The use of BCA and/or PGPR fluorescent *Pseudomonas* as bioformulations for sustainable horticulture requires a thorough understanding of their functioning in the complex rhizosphere environment as well as of the response of vegetable crops to introduced microorganisms.

Strain PCI2 showed *in vitro* inhibition of three fungal phytopathogens, it enhanced growth of tomato root system and it showed promise to control tomato damping-off caused by *S. rolfsii* Sacc. by increasing plant stand by 29%. Further work is underway in order to elucidate the specific factors involved in both growth stimulation and protection of tomato plants by PCI2.

To conclude, the potential biocontrol activity of strain PCI2 must be confirmed in long-term greenhouse assays before its development into a commercial formulation for control of vegetables diseases.

Acknowledgments

This work was supported by grants from Secretaría de Ciencia y Técnica of Universidad Nacional de Río Cuarto (Córdoba, Argentina) and Agencia Nacional de Promoción Científica y Tecnológica (Secretaría de Ciencia y Técnica de la Nación).

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Journal of Environmental Management 95 (2012) S338–S342

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Use of several waste substrates for carotenoid-rich yeast biomass production

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ARTICLE INFO

Article history:
Received 3 September 2009
Received in revised form
1 April 2011
Accepted 8 June 2011
Available online 8 July 2011

Keywords:
Carotenoids
Waste substrates
Yeast biomass
Rhodotorula sp.
Sporobolomyces sp.

ABSTRACT

Carotenoids are industrially significant pigments produced in many bacteria, fungi, and plants. Carotenoid biosynthesis in yeasts is involved in stress response mechanisms. Thus, controlled physiological and nutrition stress can be used for enhanced pigment production. Huge commercial demand for natural carotenoids has focused attention on developing of suitable biotechnological techniques including use of liquid waste substrates as carbon and/or nitrogen source. In this work several red yeast strains (*Sporobolomyces roseus*, *Rhodotorula glutinis*, *Rhodotorula mucilaginosa*) were enrolled into a comparative screening study. To increase the yield of these pigments at improved biomass production, several types of exogenous as well as nutrition stress were tested. Each strain was cultivated at optimal growth conditions and in medium with modified carbon and nitrogen sources. Synthetic media with addition of complex substrates (e.g. yeast extract) and vitamin mixtures as well as some waste materials (whey, potato extract) were used as nutrient sources. Peroxide and salt stress were applied too. The production of carotene enriched biomass was carried out in flasks as well as in laboratory fermentor. The best production of biomass was obtained in inorganic medium with yeast extract. In optimal conditions tested strains differ only slightly in biomass production. All strains were able to use most of waste substrates. Biomass and pigment production was more different according to substrate type. In laboratory fermentor better producers of enriched biomass were both *Rhodotorula* strains. The highest yields were obtained in *R. glutinis* CCY 20-2-26 cells cultivated on whey medium (cca 45 g per liter of biomass enriched by 46 mg/L of beta-carotene) and in *R. mucilaginosa* CCY 20-7-31 grown on potato medium and 5% salt (cca 30 g per liter of biomass enriched by 56 mg/L of beta-carotene). Such dried carotenoid-enriched red yeast biomass could be directly used in feed industry as nutrition supplement.

1. Introduction

Carotenoids are naturally occurring lipid-soluble pigments, the majority being C_{40} terpenoids. They act as membrane-protective antioxidants that efficiently scavenge $^1O_2$ and peroxyl radicals; the antioxidant efficiency is apparently related to their structure. Carotenoid pigments occur universally in photosynthetic systems of higher plants, algae and phototrophic bacteria. In non-photosynthetic organisms, carotenoids are important in protecting against photo-oxidative damage. Thus, many non-phototrophic bacteria and fungi rely on carotenoids for protection when growing in light and air (Britton et al., 1998).

Commercially, carotenoids are used as food colorants and nutritional supplements, with an estimated global market of some $935 million by 2005 (Fraser and Bramley, 2004). There is an increased interest in carotenoids as natural antioxidants and free radical scavengers for their ability to reduce and alleviate chronic diseases, various pathological stages and aging. However, the application of chemical synthetic methods to prepare carotenoid compounds as food additives has been strictly regulated in recent years. Therefore attention is paid on the finding of suitable natural sources including biotechnological processes.

The number of red yeasts species *Rhodotorula*, *Rhodosporidium*, *Sporidiobolus*, *Cystofilobasidium* and *Phaffia* are known as producers of carotene pigments. Among yeasts, *Rhodotorula* species is one of main carotenoid-forming microorganisms with predominant synthesis of β-carotene, torulene and torularhodin (Davoli et al., 2004). Nevertheless, although there are many strategies for stimulation of carotene biosynthetic machinery in yeasts, attention is still focused on unexplored yeast's habitats for selection of hyper-

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doi:10.1016/j.jenvman.2011.06.018

producing strains what is the important step toward the design and optimization of biotechnological process for pigment formation (Libkind and van Broock, 2006; Maldonade et al., 2008).

In order to improve the yield of carotenoid pigments and subsequently decrease the cost of this biotechnological process, diverse studies have been performed by optimizing the culture conditions including nutritional and physical factors. Factors such as nature and concentration of carbon and nitrogen sources, minerals, vitamins, pH, aeration, temperature, light and stress have a major influence on cell growth and yield of carotenoids. Numerous substrates have been considered as potential carbon sources for biotechnological production of carotenoids (Bhosale and Gadre, 2001; Lukacs et al., 2005, 2006). Work of Tinoi et al. (2005) demonstrates the effectiveness of using a widely available agro-industrial waste product as substrate and the importance of the sequential simplex optimization method in obtaining high carotenoid yields.

Because overall yield of carotenoids is directly related to the total biomass yield, to keep both high growth rates and high flow carbon efficiency to carotenoids by optimal cultivation conditions is essential in order to achieve the maximal pigment productivity. During growth, different types of environmental and physiological stress conditions constantly challenge all organisms. In microorganisms, the environment of which is highly variable, stress responses are of particular importance. Under stress, different classes of substances are overproduced. Carotenoids are also involved in stress responses of microorganisms (Marova et al., 2004, 2010). Study of the molecular basis of these effects could make it possible to realize regulated overproduction of selected metabolites above all in such industrial applications, when it is not acceptable to use genetically modified strains. Moreover, biotechnological use of specific yeasts strains takes advantage of the utilization of the whole biomass, efficiently enriched for particular metabolites.

The aim of the presented work is to study the influence of several types of waste substrates and stress factors on the production of carotenoids by yeast strains *Rhodotorula glutinis*, *Rhodotorula mucilaginosa* and *Sporobolomyces roseus*.

2. Experimental

2.1. Strains

In the study following red yeast strains were tested: *R. glutinis* CCY 20-2-26, *S. roseus* CCY 19-4-8 and *R. mucilaginosa* CCY 20-7-31. These strains were obtained from the Culture Collection of Yeasts (CCY), Bratislava, Slovakia. The strain was conserved at malt-agar in darkness at 4 °C.

2.2. Cultivation of microorganisms

Red yeasts were cultivated in a simple glucose medium aerobically at 28 °C. Physiological stress was induced by nutrition components (C and N source) and by addition of 5 mM peroxide and 2% and/or 5% NaCl.

Three series of cultivations were realized with each strain. Two-step inoculation was done. All strains were firstly inoculated into a medium containing yeast extract (7 g), $(NH_4)_2SO_4$ (5 g), glucose (40 g), KH_2PO_4 (5 g), $MgSO_4$ (0.34 g) per liter (INO I) and cultivated at 28 °C for 24 h at permanent shaking and lighting. Second inoculum (INO II) was prepared similarly, in 1st series was used the same medium as INO I, in 2nd series lyophilized whey was added (7 g/L) and in 3rd series potato extract (7 g/L) was added into INO II. Cultivation in INO II undergo at 28 °C for 24 h at permanent shaking and lighting. Production media contained $(NH_4)_2SO_4$ (5 g), glucose (40 g), KH_2PO_4 (5 g), $MgSO_4$ (0.34 g) per liter. Several waste substrates were added and cultivation was done for 80 h at 28 °C under permanent lighting and shaking. Production media were prepared according to following scheme:

a) 1st series: INO I—INO II—production: 1 – control, 2 – 5 mM peroxide, 3 – 2% NaCl, 4 – 5% NaCl, 5 – lyophillized whey non-processed (7 g/L), 6 – lyophillized whey processed by deproteination agent (7 g/L), 7 – potato extract (Hi Media; 7 g/L)
b) 2nd series: INO I—INO II (+whey, 7 g/L)—production: 1 – control, 2–5 mM peroxide, 3–2% NaCl, 4–5% NaCl, 5 – lyophilized whey non-processed (7 g/L), 6 – lyophillized whey processed by deproteination agent (7 g/L)
c) 3rd series: INO I—INO II (+potato extract 7 g/L)—production: 1 – control, 2 – 5 mM peroxide, 3 – 2% NaCl, 4 – 5% NaCl, 5 – potato extract (7 g/L).

Whey waste substrate was obtained from dairy industry (Pribina Ltd., Pribyslav, Czech Republic) and its composition was as followed: water (94%), dry weight 60 g/L; ash 31 g/L; lactose 40 g/L; glucose 0.4 g/L; phosphorus 63 mg/L; soluble proteins 2 g/L; total nitrogen 0.12%. Whey substrate was either lyophilized without processing or processed by deproteination. Whey was acidified by 0.1 mol/L H_2SO_4 to pH 4.6, proteins were precipitated by boiling for 20 min and removed by centrifugation (5000 rpm; 10 min). Before cultivation was pH adjusted to neutral by 1 mol/L NaOH.

Potato extract was obtained as microbial food supplement (HiMedia). Other chemicals were of analytical grade purity and obtained from local distributors.

2.3. Cultivation in a laboratory fermentor

Pilot experiments with *R. glutinis* CCY 20-2-26, *R. mucilaginosa* CCY 20-7-31 and *S. roseus* CCY 19-4-8 were carried out in a 2-l laboratory fermentor Biostat B (B. Braun Biotech International, SRN). Two-step inoculation was performed in Erlenmeyer flasks in optimal inoculation medium (see above). The first inoculum (50 ml) was cultivated for 24 h at 28 °C under permanent lighting and shaking. INO I was transferred into 240 ml of fresh inoculation medium (INO II) with or without waste substrate. INO II was grown at the same conditions as INO I. After 24 h, INO II was transferred into a laboratory fermentor containing sterile production medium. Cultivation in a fermentor was carried out at 28 °C under permanent lighting, shaking (150–200 min^{-1}) and aeration (6 L of air/min). Ten types of test stress experiments in 2 L fermentor were performed according to following scheme: 1) INO II (flask) – control; production medium (fermentor) – control glucose medium, 2) INO II (flask) – control; production medium (fermentor) – whey processed by protein precipitation 7 g/L, 3) INO II (flask) – control medium; production medium (fermentor) – potato extract 7 g/L, 4) INO II (flask) – whey processed; production medium (fermentor) – 5% NaCl, 5) INO II (flask) – whey processed; production medium (fermentor) – processed whey, 7 g/L, 6) INO II (flask) – potato extract; production medium (fermentor) – 5% NaCl, 7) INO II (flask) – potato extract; production medium (fermentor) – potato extract.

2.4. Extraction and analysis of carotenoids and other metabolites

Cells were collected by centrifugation (3000 rpm; 30 min). For the subsequent isolation of carotenoids, the whole biomass obtained from 250 ml of medium was used. Yeast cells were disintegrated using a mechanical disruption by shaking with glass beads (70–100 U.S sieve). A mixture of pigments, sterols and other organic compounds was extracted from the cell homogenate using

Table 1
Production of biomass and pigments by red yeasts in Erlenmeyer flasks by *Rhodotorula glutinis* CCY 20-2-26.

Medium composition			Biomass (g/L)	Beta-carotene (μg/g d.w.)
INO I	INO II	Production		
Control	Control	Control	9.28 ± 2.35	425.50 ± 134.90
Control	Control	Peroxide 5 mmol/L	8.12 ± 1.92	210.00 ± 45.60
Control	Control	Salt 5%	7.78 ± 1.65	455.60 ± 104.67
Control	Control	Whey lyophilized	5.91 ± 2.58	1068.20 ± 196.74
Control	Control	Whey deprotein.	9.16 ± 2.02	1268.50 ± 269.15
Control	Control	Potato extract	7.60 ± 2.36	1055.30 ± 225.97
Control	Whey deprotein.	Control	6.10 ± 1.35	365.20 ± 45.69
Control	Whey deprotein.	Peroxide 5 mmol/L	8.25 ± 1.96	241.30 ± 61.76
Control	Whey deprotein.	Salt 5%	9.32 ± 2.58	289.60 ± 45.15
Control	Whey deprotein.	Whey lyophilized	8.91 ± 1.96	1042.50 ± 176.97
Control	Whey deprotein.	Whey deprotein.	9.06 ± 1.58	1012.50 ± 194.33
Control	Potato extract.	Control	7.83 ± 2.14	425.60 ± 85.25
Control	Potato extract	Peroxide 5 mmol/L	8.07 ± 1.98	175.20 ± 39.55
Control	Potato extract	Salt 5%	5.93 ± 1.85	911.50 ± 125.20
Control	Potato extract	Potato extract	5.70 ± 1.59	1085.50 ± 194.33

Table 2
Production of biomass and pigments by red yeasts in Erlenmeyer flasks by *Sporobolomyces roseus* CCY 19-4-8.

Medium composition			Biomass (g/L)	Beta-carotene (μg/g d.w.)
INO I	INO II	Production		
Control	Control	Control	5.82 ± 0.93	65.60 ± 23.27
Control	Control	Peroxide 5 mmol/L	4.84 ± 1.36	440.80 ± 120.00
Control	Control	Salt 5%	4.26 ± 1.05	1500.60 ± 123.40
Control	Control	Whey lyophilized	5.20 ± 0.80	2520.30 ± 225.36
Control	Control	Whey deprotein.	5.25 ± 1.15	2780.50 ± 331.12
Control	Control	Potato extract	5.04 ± 0.74	1745.90 ± 289.00
Control	Whey deprotein.	Control	5.37 ± 0.89	625.50 ± 147.20
Control	Whey deprotein.	Peroxide 5 mmol/L	4.92 ± 0.66	930.00 ± 220.10
Control	Whey deprotein.	Salt 5%	3.86 ± 0.65	1350.80 ± 369.82
Control	Whey deprotein	Whey lyophilized	4.71 ± 0.59	2580.00 ± 258.80
Control	Whey deprotein	Whey deprotein.	5.35 ± 1.11	1710.20 ± 450.10
Control	Potato extract.	Control	3.34 ± 0.98	532.60 ± 162.14
Control	Potato extract	Peroxide 5 mmol/L	4.77 ± 1.31	1300.70 ± 364.40
Control	Potato extract	Salt 5%	3.14 ± 0.45	981.60 ± 230.80
Control	Potato extract	Potato extract	4.61 ± 0.88	594.42 ± 123.12

50 ml of acetone. After saponification of the extract by ethanolic KOH, carotenoids were extracted twice with 50 ml of diethyl ether. The diethyl ether extracts were collected and dried under vacuum. After evaporation, the residue was dissolved by 1–2 ml of methanol (gradient grade) and used for HPLC chromatographic analysis.

Carotenoid pigments extracted from yeast cells were individually identified and quantified by RP-HPLC using a chromatographic system described previously (Marova et al., 2004, 2010). Samples (10 μL valve) were filtered through PTFE filters and injected onto Zorbax EclipsePlus C18 column (150 × 4.6 mm, 5 μm; Agilent Technologies) that had been equilibrated with a mobile phase (methanol/water; 95:5). Isocratic elution was carried out at 45 °C by a flow rate of 1.0 ml/min. Detection of carotenoids was achieved at 450 nm. Data processing of analyses was assessed using Clarity software (DataApex, CZ). Individual carotenoids were verified by on-line LC/MS/ESI analysis (Mass spectrometer LCQ Advantage Max, Thermo Finnigan).

2.5. Statistical analysis

In flask experiments three parallel cultivations were carried out with each strain and each substrate combination. In fermentor experiments three cultivations were performed in control experiments as well as in all cultivations with both *Rhodotorula* strains. Average values and standard deviations were evaluated. Biomass yields and carotenoid concentrations in individual cultivations were compared using Student *t*-test ($p < 0.05$).

In *S. roseus* two parallel experiments with each substrate were carried out.

3. Results and discussion

3.1. Growth and production of metabolites by red yeasts in optimal conditions

The growth curve of *R. glutinis* CCY 20-2-26 as well as other studied red yeast strains (data not shown) exhibited similarly typical two-phase character with prolonged stationary phase probably due to the ability of the yeast cells to utilize lipid storages formed during growth as additional energy source (Marova et al., 2010). The production of carotenoids during growth fluctuated and some local maxima and minima were observed. The maximum of beta-carotene production was obtained in all strains in stationary phase after about 80 h of cultivation.

3.2. Cultivation and production of biomass and pigments in modified conditions

In this work the growth of some red yeast strains on selected waste substrates and subsequent effect of these substrates on beta-carotene production was studied. It was observed that addition of non-processed or processed whey or potato extract to media can increase beta-carotene production, while biomass production changed relatively slightly.

In *R. glutinis* addition of whey substrate into production medium led to 3.5-times increased production of beta-carotene without substantial changes in biomass. Non-processed whey or potato extract added to production media led to about 3-times increase of beta-carotene production accompanied by biomass loss. The highest yield was reached after addition of lyophillized non-processed whey to INO II as well as to production media. Liquid whey exhibited in all strains negative effect. Also potato extract added into INO II led to increased beta-carotene production while biomass yield was lower (Table 1).

S. roseus (Table 2) exhibited significant changes in biomass:carotene ratio dependent on whey substrate addition. Substantial biomass decrease in presence of lyophilized whey in INO II (under 5 g/L) was accompanied by very high beta-carotene yields. Also potato extract addition into production medium led

Table 3
Production of biomass and pigments by red yeasts in Erlenmeyer flasks by *Rhodotorula mucilaginosa* CCY 20-7-31.

Medium composition			Biomass (g/L)	Beta-carotene (μg/g d.w.)
INO I	INO II	Production		
Control	Control	Control	8.12 ± 1.85	62.50 ± 28.20
Control	Control	Peroxide 5 mmol/L	8.18 ± 1.14	51.70 ± 14.20
Control	Control	Salt 5%	5.64 ± 1.58	432.60 ± 56.65
Control	Control	Whey lyophilized	6.48 ± 2.01	200.20 ± 20.11
Control	Control	Whey deprotein.	6.93 ± 1.55	228.60 ± 42.10
Control	Control	Potato extract	7.90 ± 1.82	384.80 ± 63.82
Control	Whey deprotein.	Control	7.24 ± 0.98	45.20 ± 11.20
Control	Whey deprotein.	Peroxide 5 mmol/L	7.46 ± 1.44	51.20 ± 9.36
Control	Whey deprotein.	Salt 5%	4.51 ± 1.12	422.30 ± 14.88
Control	Whey deprotein	Whey lyophilized	4.72 ± 1.54	384.40 ± 48.00
Control	Whey deprotein	Whey deprotein.	6.78 ± 1.88	140.50 ± 29.14
Control	Potato extract.	Control	8.02 ± 2.14	42.60 ± 10.10
Control	Potato extract	Peroxide 5 mmol/L	8.45 ± 1.38	115.70 ± 8.15
Control	Potato extract	Salt 5%	8.11 ± 2.30	1535.60 ± 156.52
Control	Potato extract	Potato extract	7.70 ± 1.58	415.20 ± 25.36

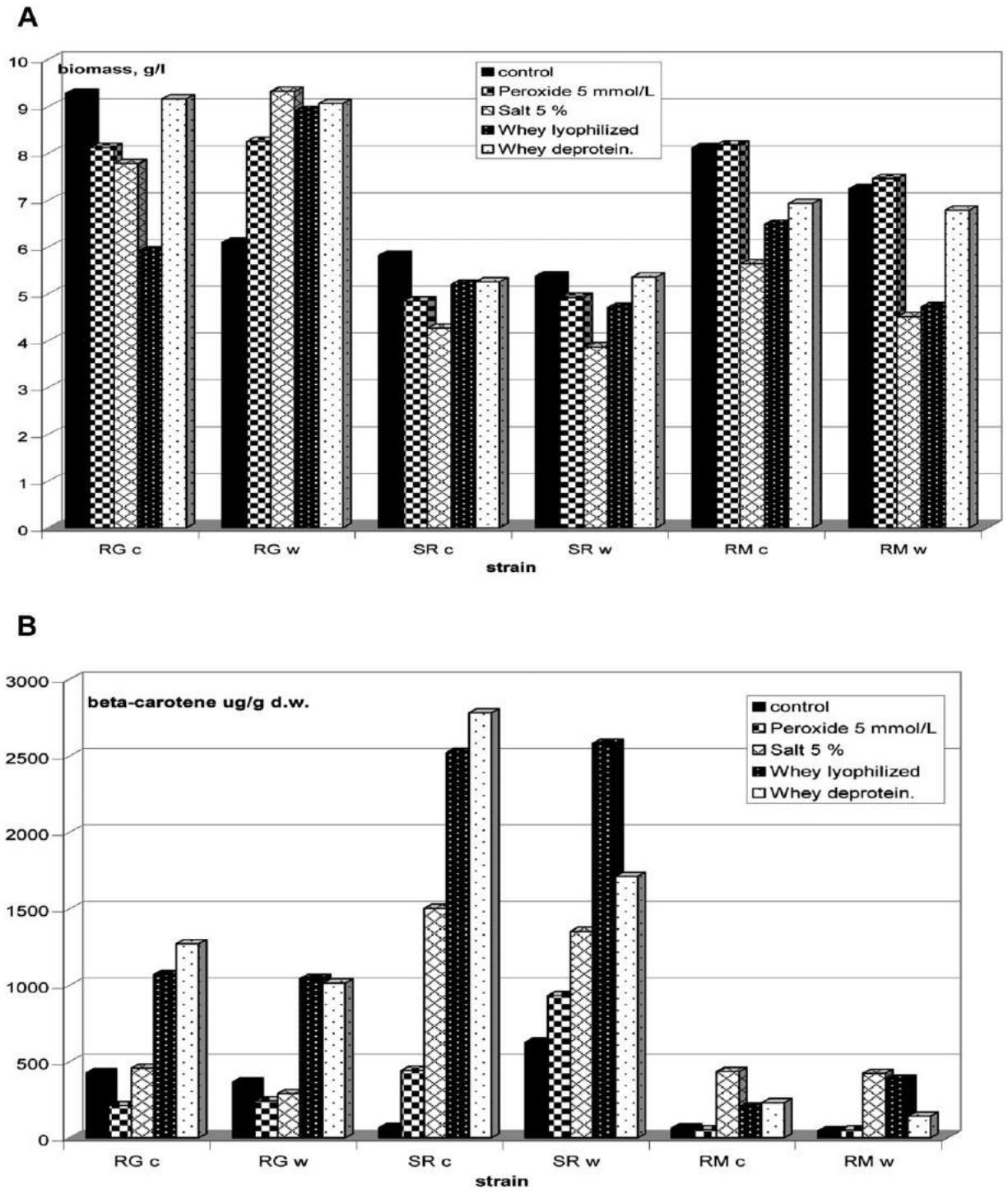

Figure 1. Production of biomass (A) and beta-carotene (B) by studied red yeasts in whey and control media A. Notes: RG c – *Rhodotorula glutinis*, control cultivation; RG w – *R. glutinis*, INO II with deproteined whey; SR c – *Sporobolomyces roseus*, control cultivation; SR w – *S. roseus*, INO II with deproteined whey; RM c – *Rhodotorula mucilaginosa*, control cultivation; RM w – *R. mucilaginosa*, INO II with deproteined whey.

to about 11-fold increase of β-carotene production, while production of biomass was lower than in control. Preincubation of *S. roseus* cells with potato extract and following cultivation in production medium with 5% hydrogen peroxide led to about 20-times higher β-carotene production as in control, in this cultivation conditions biomass decreased only slightly. In general, total production of biomass by *S. roseus* was about 2-times lower as in *R. glutinis.* So, this is the reason why *S. roseus* CCY 19-4-8 strain is less suitable to enriched biomass production.

In optimal conditions *R. mucilaginosa* CCY 20-7-31 seems to be relatively poor producer of carotenoids when compared with the other two strains (Table 3). Production of biomass in this strain was more similar to *R. glutinis* (about 8 g/L). However, cultivation in presence of some complex waste substrate either in INO II or in production medium led to substantial increase of pigment production. Addition of potato extract into INO II combined with salt stress in production medium enabled to reach the highest biomass as well as β-carotene production observed in this strain yet

Table 4
Production of beta-carotene enriched biomass in 2 L laboratory fermentor.

Substrate/stress factor	Biomass			*S. roseus*		*R. mucilaginosa*			
	R.g. (g/L)	S.r. (g/L)	R.m. (g/L)	β-carotene (μg/g d.w.)	β-carotene (μg/g d.w.)	β-carotene (μg/g d.w.)	β-carotene (mg/L)	β-carotene (μg/g d.w.)	β-carotene (mg/L)
Control 0/0	37.14	17.00	26.55	482.76	17.93	191.00	3.25	162.26	4.31
0/whey deprot[a]	44.56	9.59	27.06	1025.12	45.68	2436.00	23.36	325.16	8.80
0/potato	28.12	10.80	38.50	905.10	25.45	1825.06	17.50	680.22	26.18
Whey[a]/salt	40.86	8.16	18.35	685.15	28.00	1744.00	14.23	589.06	10.81
Whey[a]/whey	34.60	10.15	29.82	1480.22	51.22	2896.16	29.40	380.00	11.33
Potato/salt	26.10	7.14	30.12	852.10	22.23	1058.00	7.55	1856.40	55.91
Potato/potato	18.56	6.28	28.48	1211.10	22.48	967.11	6.13	956.22	27.23

[a] In all fermentor experiments whey processed by deproteinization was used.

(1536 μg/g d.w.). It seems that this strain needs for optimal pigment/biomass production some additional nutrition factors which are no present in simple (but cheap) inorganic medium, but can be obtained from different waste substrates (also cheap). Further test experiments with this strain are needed before cultivation on fermentor to find optimal cultivation conditions and production potential.

Comparison of biomass and β-carotene yields produced by all three strains in whey media is demonstrated in Fig. 1.

3.3. Results of cultivation in laboratory fermentor

In next series of preliminary experiments, an orientation batch cultivation of *R. glutinis* CCY 20-2-26, *S. roseus* CCY 19-4-8 and *R. mucilaginosa* CCY 20-7-31 cells was carried out in a 2-L laboratory fermentor. In these conditions several combinations of stress factors and waste substrates were tested.

In experiments with *R. glutinis* the production of yeast biomass in a laboratory fermentor was in most types of cultivation more than 30 g per liter (about 3× higher yield than in Erlenmeyer flasks; Table 4). The balance of cultivation in a fermentor in optimum conditions is as follows: we obtained about 37.1 g/L of biomass containing 17.19 mg per liter of β-carotene (see Table 4). The production of β-carotene was induced in most types of media combinations. High total yield of β-carotene was obtained in whey production medium (44.56 g/L of biomass; 45.68 mg of β-carotene per liter of culture). The highest total yield of β-carotene was obtained using combined whey/whey medium (51.22 mg/L); this cultivation was accompanied also with relatively high biomass production (34.60 mg/L).

In experiments with *S. roseus* CCY 19-4-8 substantially higher production of biomass was obtained in fermentor when compared with cultivation in flasks. Mainly in whey medium about 3-times biomass increase (about 12 g/L) was reached and production of β-carotene was mostly higher than in *R. glutinis*. Because of low biomass production, total yields were in *S. roseus* mostly lower than in *R. glutinis* cells. The best β-carotene production (29.4 g/L) was obtained on whey medium (see Table 4).

Yeast strain *R. mucilaginosa* CCY 20-7-31 exhibited in most cases similar biomass production characteristics as *R. glutninis*, while pigment production was substantially lower (see Table 4). As the only substrate suitable for β-carotene production was found potato extract in INO II combined with 5% salt in production medium. Under these conditions 55.91 mg/L of β-carotene was produced in 30.12 g of cells per liter of medium.

The aim of all preliminary experiments carried out in laboratory fermentor was to obtain basic information about potential biotechnological use of the tested strains to the industrial production of β-carotene enriched biomass. The results of both *Rhodotorula* strains are very promising. The yield of *R. glutinis* CCY 20-2-26 biomass (37–44.5 g/L) produced in minimal cultivation medium was similar to the maximal biomass yield obtained in fed-batch cultivation of *Phaffia rhodozyma* (36 g/L), which is widely used as an industrial producer of astaxanthin (Lukacs et al., 2006). The maximal production of total carotenoids by used *P. rhodozyma* mutant strain was 40 mg/L, which is also similar to the yields obtained in *R. glutinis* CCY 20-2-26 cells grown in whey medium.

4. Conclusions

Changes in medium composition can lead to substantial changes in biomass as well as carotenoid production. Waste substrates can be used as medium component, which can in particular strains and conditions induce carotenoid as well as biomass production. Thus, cheap waste substrates could be used industrially for carotenoid-rich biomass production.

From tested red yeast strains predominantly *R. glutinis* CCY 20-2-26 can be used for industrial production of carotenoid-rich biomass using processed waste substrates and/or mild physiological stress.

Acknowledgments

This work has been supported by project "Centre for Materials Research at FCH BUT" No. CZ.1.05/2.1.00/01.0012 from ERDF and project MSM0021630501 of the Czech Ministry of Education.

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Journal of Environmental Management 95 (2012) S343–S348

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Sludge valorization from wastewater treatment plant to its application on the ceramic industry

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ARTICLE INFO

Article history:
Received 3 September 2009
Received in revised form
27 March 2011
Accepted 8 June 2011
Available online 30 June 2011

Keywords:
Sludge valorization
Wastewater treatment plant
Recycling
Ceramic products

ABSTRACT

The main aim of this study is to assess the effect of incorporating waste sludge on the properties and microstructure of clay used for bricks manufacturing. Wastewater treatment plants produce annually a great volume of sludge. Replacing clay in a ceramic body with different proportions of sludge can reduce the cost due to the utilization of waste and, at the same time, it can help to solve an environmental problem. Compositions were prepared with additions of 1%, 2.5%, 5%, 7.5%, 10% and 15% wt% waste sludge in body clay. In order to determine the technological properties, such as bulk density, linear shrinkage, water suction, water absorption and compressive strength, press-moulded bodies were fired at 950 °C for coherently bonding particles in order to enhance the strength and the other engineering properties of the compacted particles. Thermal heating destroys organic remainder and stabilizes inorganic materials and metals by incorporating oxides from the elemental constituent into a ceramic-like material. Results have shown that incorporating up to 5 wt% of sludge is beneficial for clay bricks. By contrast, the incorporation of sludge amounts over 5 wt% causes deterioration on the mechanical properties, therefore producing low-quality bricks.

1. Introduction

The considerable growth of the waste generated and the global environmental situation in which we find ourselves make it possible for projects on waste reuse to be implemented, provided they are profitable (Andreola et al., 2005; Elías, 2008).

By purifying wastewater, large quantities of sludge are generated. In case this final sludge is not disposed correctly, it can considerably contribute to environmental contamination (Cusidó and Cremades, 2005). Usually, sludge from wastewater treatment plants has been placed in landfills, but many problems, such as acceptance landfill sites, capacity limitations. Incineration may be an alternative solution to reduce its volume, but substantial amounts of ash are produced. Sludge is usually a heterogeneous solid material.

The construction industry is the most indicated technological activity sector to absorb solid wastes, due to the large quantity of raw materials and final products used.

The prospective benefits of using sludge as the brick or tile additive in the fired matrix, oxidizing organic matter and destroying any pathogens during the firing process have been studied by others authors (Alleman et al., 1990; Tay and Show, 1992; Weng et al., 2003; Jordán et al., 2005; Espejel et al., 2006; Merino et al., 2007; Monteiro et al., 2008). Jordán et al. (2005) studied the substitution of clay for sewage sludge in different proportions (0–10 wt%) in a ceramic body. Ceramic bodies were prepared by uniaxial pressing and fired. The authors concluded that the incorporation of sludge gives rise to a decrease of the bending strength, therefore the selection of a adequate percentage of sludge (4–5 wt %) to be added to the body clay to meet the standards. Monteiro et al. (2008) studied the influence of firing temperature on the technological properties of red ceramics prepared with incorporation of 0, 3, 5 and 10 wt% of sludge into the clayey body. The authors concluded the incorporation of sludge must be done in low percentage (3, 5 wt%) to avoid the damage the ceramic processing and the quality of the ceramic.

Ceramic products, bricks and tiles, also have a heterogeneous composition, being formed by clay raw materials with a very wide range composition (Couto et al., 2003). For this reason, this industry sector is suitable for valuation and use of different wastes, among which we can find sludge coming from wastewater treatment plant (Dondi et al., 1997a,b).

Considering that in the province of Jaen (South Spain), the ceramic industry has a great economic importance, the possible application of this recycling technique can be same benefits as

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doi:10.1016/j.jenvman.2011.06.016

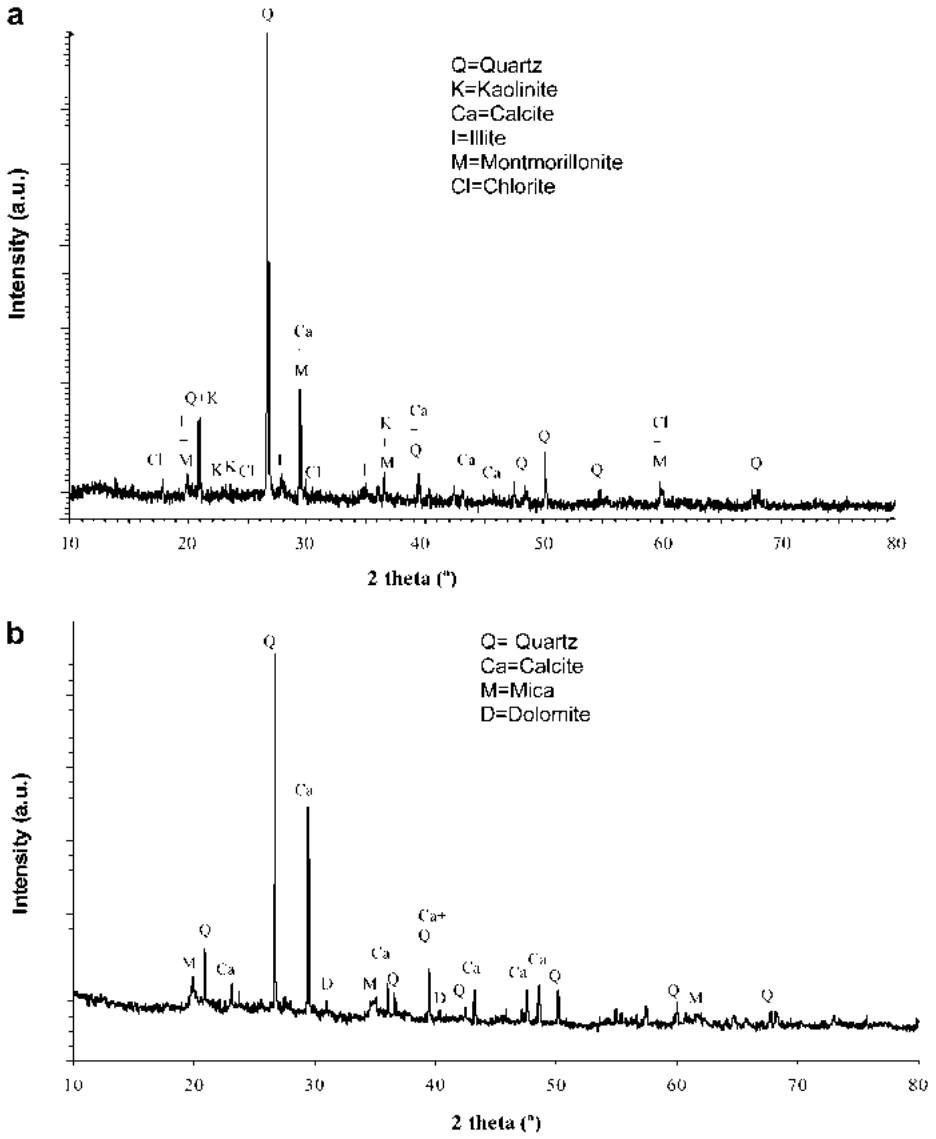

Fig. 1. DRX patterns for powdered (a) clay and (b) sludge.

(i) saving a resource, raw material and energy; (ii) positive effects on the brick-making processes and (iii) reducing the cost of final product and environmental problem due to using waste additive in the process.

This work assesses the use of sludge wastewater treatment plant, normally is placed in landfill or used for agricultural purposes (Directive 86/278/EEC, 1986), to manufacture structural ceramics as an alternative means of sludge disposal. The raw materials were characterized by physical–chemical methods and final ceramics products physical and mechanical properties were investigated, taking into account the composition.

2. Materials and methods

2.1. Materials characterization

Materials used in the present study are clay and sludge from Jaen (South Spain). In order to get a uniform particle size, both sludge and clay were crushed and grounded until a powder with a particle size suitable to pass through a 150 μm sieve was obtained.

Table 1
Chemical composition of the clay and the sludge ash and metal content of the sludge.

Oxide content (%)	Clay	Sludge	Metal content (%)	Sludge
SiO_2	55.82	46.37	Na	1.580
Al_2O_3	12.13	20.33	Mg	0.990
Fe_2O_3	4.83	8.55	Al	2.870
MnO	0.03	0.28	K	0.170
MgO	1.49	2.19	Ca	3.550
CaO	9.21	11.15	Mn	0.160
Na_2O	0.49	0.36	Fe	2.110
K_2O	2.78	3.25	Zn	0.512
TiO_2	0.83	0.85	Si	0.027
P_2O_5	0.12	5.89	Cr	0.090
Zr (ppm)	279.3	161.7	Ni	0.023
LOI	10.55	0.05	Cu	0.048
			Sn	0.015

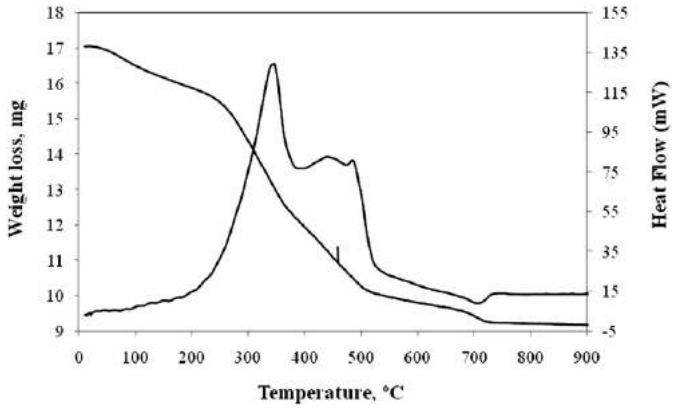

Fig. 2. TGA/DTA curves of sludge.

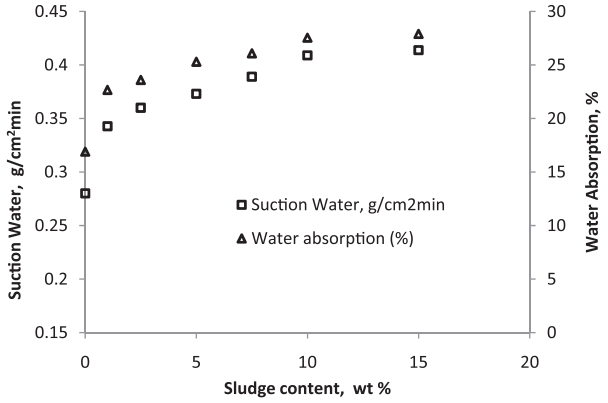

Fig. 3. Effect of sludge content on the water suction and water absorption as a function the amount of sludge addition.

Sludge was previously dried with an electric heater during 48 h at 90 °C in order to remove moisture.

2.2. Sample preparation

The sludge content in the clay mixture varied from 1% to 15% of dry weight. In order to obtain comparative results, series of ten samples were prepared for the tests. Mixtures were moulded under 10 MPa of pressure using a uniaxial laboratory-type pressing Mega KCK-30 A. The necessary amount of water (10 wt%) for mixing was added to obtain adequate plasticity and absence of defects at the compression stage. Solid bricks with 30 × 10 mm cross sections and a length of 60 mm were obtained. The shaped samples were dried during 48 h at 110 °C in an oven to reduce moisture content. Dried samples were fired in a laboratory-type electrically heated furnace at a rate of 10 °C/min up to 950 °C for 24 h. The samples conformed are appointed like *C* for the brick of clay and *M* for the mixtures of clay and sludge in different proportions from 1 to 15 wt% (samples, M1, M2.5, M5, M7.5, M10 and M15).

2.3. Raw materials and conformed materials characterization

Qualitative determination of major crystalline mineralogical phases present in the clay and sludge was achieved by using a Philips X'Pert Pro automatic diffractometer equipped with a Ge (111) primary monochromator, which provides a strictly monochromatic radiation CuK1. Chemical composition was determined by X-ray fluorescence (XRF) in a Philips Magix Pro (PW-2440) equipment. The metal content of the sludge was determined by ICP-ms Agilant series 7500 with internal patron. Thermal behaviour was determined by thermogravimetric analysis (TGA) and differential thermal analysis (DTA). These analyses were conducted simultaneously using a Mettler Toledo 851^e equipment under oxygen. The content of carbonates in clay and sludge were determined by calcimetry.

Weight loss on ignition was obtained by measuring the weight after drying stage at 110 °C and after firing stage at 950 °C. Linear shrinkage was obtained by measuring the length of the samples before and after the firing stage, using a calliper with a precision of ±0.01°mm. Bulk density was obtained according to standard procedure (UNE-EN-772-13). Water absorption was determined according to standard procedure (UNE 67-027). Test on determining water suction was implemented according to standard procedure (UNE 67-031). Compressive strength was measured for fired samples according to standard procedure (UNE 67-026) in a Suzpecar CME 200 SDC laboratory Testing Machine. The effect of freezing was measured according to standards procedure (UNE 67-028). In order to determine the risk of leaching in samples, a lixiviation test was performed following procedure (DIN 38414-S2, 1995). Samples' microstructures were observed with scanning electron micrographs (SEM) by using a JEOL model JSM-5800.

Table 2
Technological properties of construction bricks made from sludge.

Sample	Sludge content (wt%)	Weight loss on ignition (%)	Linear shrinkage (%)	Bulk density (g/cm^3)
Clay	0.0	16.90 ± 0.13	0.33 ± 0.08	1.615 ± 0.090
M1	1.0	14.75 ± 0.34	0.48 ± 0.04	1.600 ± 0.121
M2.5	2.5	15.11 ± 0.75	0.47 ± 0.09	1.546 ± 0.854
M5	5.0	15.68 ± 0.64	1.40 ± 0.07	1.423 ± 0.098
M7.5	7.5	16.25 ± 0.42	0.90 ± 0.04	1.410 ± 0.096
M10	10.0	17.55 ± 1.12	1.88 ± 0.06	1.409 ± 0.103
M15	15.0	18.06 ± 0.96	1.16 ± 0.05	1.340 ± 0.965

3. Results and discussion

The mineralogical composition of raw material and sludge used to design body compositions has been determined by X-rays diffraction (XRD). The crystalline components of the clay are: quartz (SiO_2), kaolinite (aluminium silicate), calcite ($CaCO_3$), illite (potassium aluminium silicate), montmorillonite (aluminium magnesium

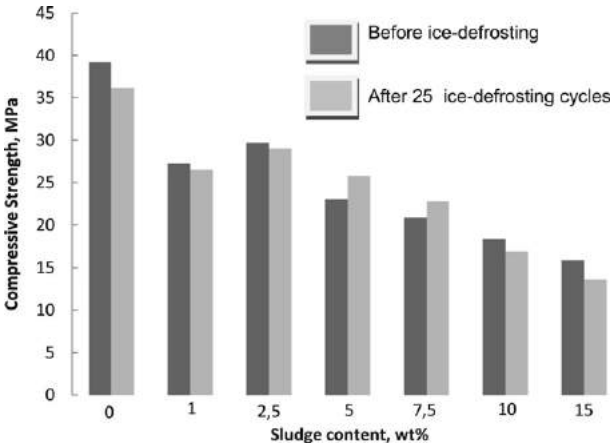

Fig. 4. Compressive strength of the clay as a function the amount of sludge addition before and after freezing.

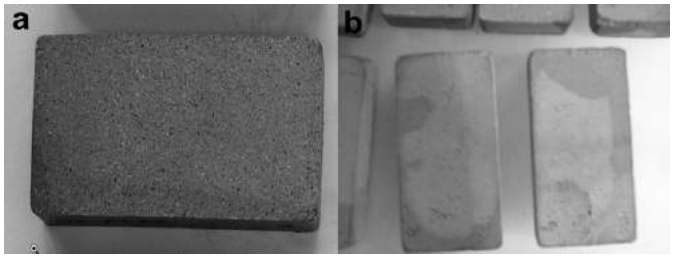

Fig. 5. Effect of freezing on the compressive strength. (a) before ice-defrosting; (b) after 25 ice-defrosting cycles.

silicate) and chlorite (Fig. 1a). From the corresponding XRD graphs of the sludge (Fig. 1b), the presence of quartz (SiO_2), calcite ($CaCO_3$), and phyllosilicates as mica (K–Mg–Fe–Al–Si–O–H_2O) and dolomite ($CaMg(CO_3)_2$) can be concluded as main mineral phases. XRF analysis indicates that after the firing process, the sludge ash is basically composed of high amounts of silica, alumina and iron oxide, mainly due to the presence of phyllosilicates and calcium oxide from the decomposition to calcite phase (Table 1). The inorganic fraction of the sludge showed high contents of iron and aluminium partly due to the flocculating reagent added during the wastewater processing. The present of calcium, magnesium and sodium was likely caused by the sediments from the urban sewage system (Table 1). The sludge contained materials similar to the clays which would indicate the possibility to replace one raw material with another.

Thermal behaviour of sludge was analysed by thermogravimetric (TGA) and thermodifferential (DTA) analysis, as shown in Fig. 2. The heated from room temperature to 200 °C with a weight loss of 4.8%, produces the release of physically adsorbed water, an endothermic peak has been found at about 100 °C. The decomposition of organic matter occurs between 200 and 550 °C, with a weight loss of 36.5%. The first exothermic peak between 200 and 400 °C was associated with biodegradable materials, undigested organics and dead bacteria, together with the emissions of semivolatile compounds (Calvo et al., 2004; Conesa, 2000; Font et al., 2001). The second exothermic peak between 400 and 550 °C was associated to the oxidation of other oxidizable material in the sample. Finally, the last endothermic peak, at 700 °C could be due to the decomposition of calcium carbonate (calcite) with release of CO_2.

After assessing and discussing analytical data, some experimental tests have been carried out with mixtures of different proportions of sludge in order to study their technological properties. The quality of the bricks can be further guaranteed according to the degree of firing linear shrinkage. Good quality bricks usually show shrinkage below 8%. Shrinkage percentage grew up with increasing sludge additions (Table 2). On the contrary, samples with 7.5 and 15 wt% showed lower linear shrinkage. Since levels of swellability and organic content of sludge are higher than those of clay, the addition of sludge to the mixture should enlarge the degree of firing linear shrinkage. As a result, the quality of the bricks is downgraded. The no clear tendency in the results appeared in others works, which confirmed that there was no relation between this technological property and the percentage of applied sludge (Jordán et al., 2005; Montero et al., 2009).

When sintering the brick, a loss of variable weight, according to the percentage of sludge added, is observed, probably due to the combustion of organic matter as well as to the loss of humidity. In the case of a normal clay brick the loss of weight after firing at 950 °C is 16.9%, which could be mainly attributed to the calcium carbonate (11.8 wt%) and organic matter content in clay (Table 2). It assumed that, as temperature was increased, carbonate in clay decomposed into CO_2. The results were superior to those reported in other studies (Tay and Show, 1992; Chiou et al., 2006), this is due to high carbonate content of the clay used. This calcareous clay contributed to a greater lightening of the materials obtained. However, upon the addition of sludge to the mixture, the loss of weight increased, but only mixtures containing higher sludge additions showed higher weight loss on ignition than clay. Weight loss should increase due to the high contribution of organic mass from sludge. These results could indicate, furthermore, the brick weight loss on ignition also depended on the inorganic substances in both clay and sludge being burnt off during the firing process.

The bulk density of the clay bricks was 1.615 g/cm^3. The addition of increasing amounts of sludge causes a decrease in bulk density (Table 2). The main reason for this tendency is the combustion of the matter organic of residue during the sintering period, which forms open and closed pores in the body clay. According to these observations, high sludge waste addition to the clay body improved the thermal properties of the material but also had negative effects on the mechanical resistance of materials and could give rise to products with low compressive strength.

The experimental data of water suction of green and firing samples show an increase in water suction after sintering at 950 °C. Results were expected and, consequently, when the pieces were acted upon by high temperatures, the superficial interconnected porosity was developed. A light increase of this parameter could be observed with increasing sludge additions (Fig. 3). Water suction was 0.34 g/cm^2 min for the test tubes with a 1 wt% of sludge and 0.41 g/cm^2 min when the content of sludge increased up to 15 wt%. Suction water affects quality and durability of the final material. High contents of sludge could cause defects in bricks, a clear tendency on water suction and, therefore, lower durability.

The sludge addition caused an increment in water absorption of the clay body (Fig. 3). For example, increases in the amount of sludge varied from 1 to 15 wt%, the absorption water changed from 22.67% to 27.90%. The results were similar to those obtained by others authors (Montero et al., 2009; Monteiro et al., 2008) using

Table 3
Results lixiviation test.

Sample element ppb	M1	M2.5	M5	M7.5	M10	M15	ppb max. Test TCLP
Cr	924.10 ± 2.34	882.9 ± 2.13	706.6 ± 2.61	809.5 ± 2.44	478.8 ± 2.53	341.3 ± 2.22	5000
Ni	2.17 ± 0.32	10.74 ± 1.11	4.03 ± 0.68	2.11 ± 0.65	25 ± 2.44	1.65 ± 0.34	2000–400
Cu	17.67 ± 1.21	29.75 ± 1.54	18.8 ± 1.12	23.8 ± 1.11	39.27 ± 3.07	29.32 ± 1.33	10,000–2000
Zn	1.19 ± 0.08	5.05 ± 0.64	86.15 ± 2.03	1.17 ± 0.55	84.59 ± 2.22	15.92 ± 1.43	10,000–2000
As	26.22 ± 1.08	33.48 ± 1.81	78.62 ± 2.01	32.23 ± 1.98	137.4 ± 3.76	5.47 ± 0.71	1000–200
Se	1.72 ± 0.06	9.47 ± 0.88	1.46 ± 0.77	1.52 ± 0.62	14.44 ± 2.10	4.71 ± 0.99	1000
Ag	0.81 ± 0.02	5.22 ± 0.93	0.54 ± 0.09	0.66 ± 0.07	4.84 ± 0.69	2.4 ± 0.11	5000
Cd	0.53 ± 0.01	6.42 ± 0.66	0.07 ± 0.02	0.08 ± 0.02	6.78 ± 0.99	0.15 ± 0.03	500–100
Pb	1.87 ± 0.23	11.16 ± 0.94	0.66 ± 0.01	0.33 ± 0.01	13.12 ± 1.01	0.66 ± 0.06	2000–400

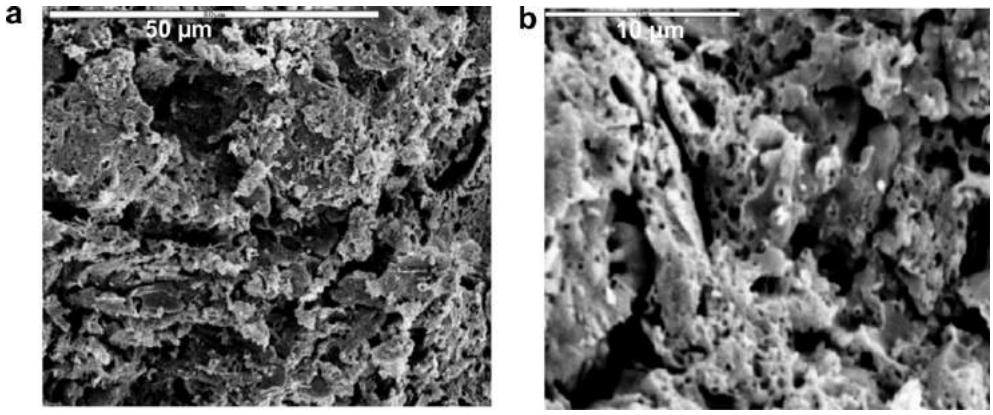

Fig. 6. (a) SEM micrographs of the clay and (b) clay containing 5 wt% of sludge.

the same sludge proportions. They observed that the incorporation of sludge was limited due to the increase of the water absorption and decrease of the mechanical strength. The results of suction and absorption water indicate that an increment in the sludge content produced a more porous material with lower mechanical resistance. Therefore, it has also been noticed that sludge contents above 5 wt% in the clay body could produce some negative effects on technological properties of the bricks obtained.

The sludge content had a significant influence on the mechanical strength of the compositions (Fig. 4). Sludge residue addition reduced the compressive strength of clay samples. In all the samples studied, compressive strength of clay (58.2 MPa) is decreased with sludge additions. Samples with 2.5 wt% residue addition had the highest compressive strength. However, incorporating sludge additions of up to 5 wt% decreased the compressive strength of pure clay by 40%. The overall decrease in the compressive strength over sludge content addition could be attributed to the bulk density reduction (Table 2) along with the increase in water absorption, both connected with the presence of a high porosity level in bodies. Such high porosity level was produced by the combustion of waste organic matter, what is known to have a marked detrimental influence on mechanical strength of ceramic (Carty and Senapati, 1998). The results of samples with 5 wt% of residue were better than those obtained using other wastewater sludge (Tay and Show, 1992; Weng et al., 2003) with values of compressive strength lower than 30 MPa for the same sludge content.

The freezing resistance is defined by the decrease of samples compressive strength before and after undergoing 25 ice-defrosting cycles. After the 25 ice-defrosting cycles, we proceeded to the eyepiece inspection of the probes. During the test no cleavage, fissure or scalping were encountered in samples with sludge content lower than 15 wt%. Superficial deterioration may be clearly observed in the case of samples with higher sludge content (Fig. 5). Then, compression comparative test on samples was conducted again. Samples with sludge content of up to 10 wt% showed a slight decrease in the compression resistance. The highest decrease was observed in sample with 15 wt% of sludge content, so it shows scalping after 25 ice-defrosting cycles (Fig. 4).

Results obtained from the lixiviation test indicated that all the samples within the range of compositions of sludge subject to study would not classify as dangerous and met the current legislation. None of the concentrations of the specific elements override the ones indicated by the standard (Table 3). These results indicated that the degree of metal immovilization achieved by the brick manufacturing processes was high. Therefore, no environmental problems due to heavy metals are expected for the unrestricted use of the sewage-clay bricks.

Morphological study of samples containing clay, as well as that of sample containing 5 wt% of sludge was obtained by means of SEM (Fig. 6). The micrograph of 5 wt% sludge sample showed clearly that waste agglomerates were distributed in the microstructure of clay. Also, the presence of sludge increased clay porosity according to data regarding bulk density (Table 2) and water absorption (Fig. 3).

4. Conclusions

This work showed that the ceramic sector could be a receptor in different types of wastes as sludge wastewater treatment plant. The proportion of sludge in the mixture has been proven as a key factor in altering bricks quality, affecting technological properties of the final ceramics products. Increasing proportions of sludge have been shown to clearly increase water suction and water absorption. On the other hand, sludge addition entails a reduction in compressive strength due to increased porosity caused by the decrease of bulk density. For this reason, selecting the appropriate percentage of sludge to be added to the clay body will be controlled. Therefore, this type of waste should be incorporated in low percentages in order to produce good quality ceramic bricks. In all, the recommended proportion of sludge in brick is up to 5 wt%.

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Journal of Environmental Management 95 (2012) S349–S354

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Recycling of ash from biomass incinerator in clay matrix to produce ceramic bricks

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ARTICLE INFO

Article history:
Received 3 September 2009
Received in revised form
27 September 2010
Accepted 7 October 2010
Available online 10 November 2010

Keywords:
Biomass incineraton ash
Recycling residues
Ceramics
Construction materials

ABSTRACT

The objective of this study is to investigate the effect of incorporation of ash from biomass incinerator as raw material on the production of ceramic bricks for their application in construction. So, for the fabrication of bricks, compositions were prepared with addition of increasing amounts of waste ash (0%, 10%, 20%, 30%, 40% and 50% in wt.) in the clay body. The mixed samples were sintered using conventional powder processing based on powder compaction at 54.5 MPa and firing them at 950 °C without the addition of additives. Effect on apparent density, water absorption and mechanical properties were investigated. The results showed that water absorption increased and apparent density and compressive strength decreased with higher amounts of ash. Bricks with an ash content up to 20% meet the UNE standards compressive strength. As a result, since interesting performances were observed, the potential use of ashes from biomass incinerator up to 20 wt.% in ceramic formulations of industrial interest was confirmed. In this sense, incorporating ashes into clay body reduces environmental problems and total cost of raw material disposition.

1. Introduction

Ceramics are prepared from malleable, earthy materials (such as clay) that are made rigid by high temperatures. Apart from clays, which confer cohesion and plasticity, ceramic pastes also include inert materials that provide structural support that helps to retain shape during drying and firing. Quartz (silica) is the most commonly used inert.

In this sense, a correct environmental solution to the disposal of a wide range of solid wastes is nowadays their incorporation into ceramic (Dondi et al., 1997a, b). The natural variability on the characteristics of clays facilitates the presence of a significant amount of impurities on the final structure of the ceramics.

Biomass incineration reduces the volume of pine bark and provides energy. However, the biomass contains non-flammable materials which are collected in the form of ashes after incineration. Recycling of waste materials occurring at biomass incineration is an increasing problem for the immediate future. Although bottom ash is not classified as a hazardous waste according to the European waste categorization, disposal of such residue may heavily influence the overall cost of incineration.

Currently, only a small percentage of ash is utilized as additive to cement or concrete (Monzó et al., 1999). Ash can be converted into asphaltic paving mixed (Al Sayed et al., 1995) or into permeable paving bricks. Ashes are also useful in agriculture thanks to its composition (Ferreira et al., 2003). However, the remaining is directly discharged into landfill, which is unsatisfactory solution both from ecological and economic points of view. So, there is now increasing incentive to develop economically viable reuse and recycling options, converting waste residues into new marketable materials. The need for an environmentally-correct alternative to recycling ash from biomass incineration is one of the aims of this work.

The production of conventional ceramic can be an important application for ash. The use of fly ash from combustion of sewage sludge for obtaining ceramic materials has recently been suggested by Merino et al., (2005) and Lin, (2006). The production of bricks by mixing ash and clay has been studied by Anderson, (2002) and Hidalgo-Fernández et al., (1999), who suggested the use of limited amounts of ash in mixes. Glass ceramics have been obtained from bottom ashes extracted from municipal solid waste incinerators by Monteiro et al., (2008).

The main aim of this work is to study the effect on the technological properties of ceramic bricks produced by the incorporation of ashes generated by biomass incineration into clay.

2. Materials and methods

2.1. Sample preparation

Ashes from the incineration of biomass were obtained from the Polanco wood plant in the area of Cádiz (Southern Spain) and

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0301-4797/$ – see front matter © 2010 Published by Elsevier Ltd.
doi:10.1016/j.jenvman.2010.10.022

Table 1
Chemical analysis of raw materials.

Materials	SiO_2 (%)	Al_2O_3 (%)	Fe_2O_3 (%)	MnO (%)	MgO (%)	CaO (%)	Na_2O (%)	K_2O (%)	TiO_2 (%)	P_2O_5	Zr (ppm)	LoI (%)
clay	55.82	12.13	4.83	0.03	1.49	9.21	0.49	2.78	0.83	0.12	279.3	10.55
ash	5.01	2.28	2.24	1.83	9.12	43.40	0.25	9.19	0.11	5.43	38.7	18.78

exhibit a rather heterogeneous macroscopic texture and have a very wide particle size range. The clay sample was obtained from a local brick manufacturing plant. In order to get a uniform particle sized, both ashes and clay were crushed and ground until getting a powder with a particle size suitable to pass through a 150 μm sieve. Ashes were dried prior for 4 h at 110 °C to remove moisture in order to obtain representative samples of the starting materials.

The feasibility of using ashes in brick making was investigated. The ash content in the clay mixture was varied from 0 to 50% (by dry weight), without the addition of any additives. In order to obtain comparative results, series of ten samples were prepared for the tests. Mixtures were then homogenized in a blender and molded under 54.6 MPa of pressure using a uniaxial laboratory-type pressing Mega KCK-30 A. The necessary amount of water (10 wt.%) for mixing was added to obtain adequate plasticity and absence of defects in the compression stage. Ash-free mixtures were also made as reference. Solid bricks with 30 × 10 mm cross sections and a length of 60 mm were obtained. The shaped samples were dried during 48 h at 110 °C in an oven to reduce the moisture content. Dried samples were fired in a laboratory-type electrically heated furnace at a race of 10 °C/min up to 950 °C for 24 h. This temperature is normally used in the fabrication of clay bricks. Samples were then cooled to room temperature by natural convection inside the furnace after being turned off. The shaped samples will be designated as C for the brick without ash and C-*x*A for the mixtures where *x* denote the ashes weight percentages (A) in the matrix clay (C).

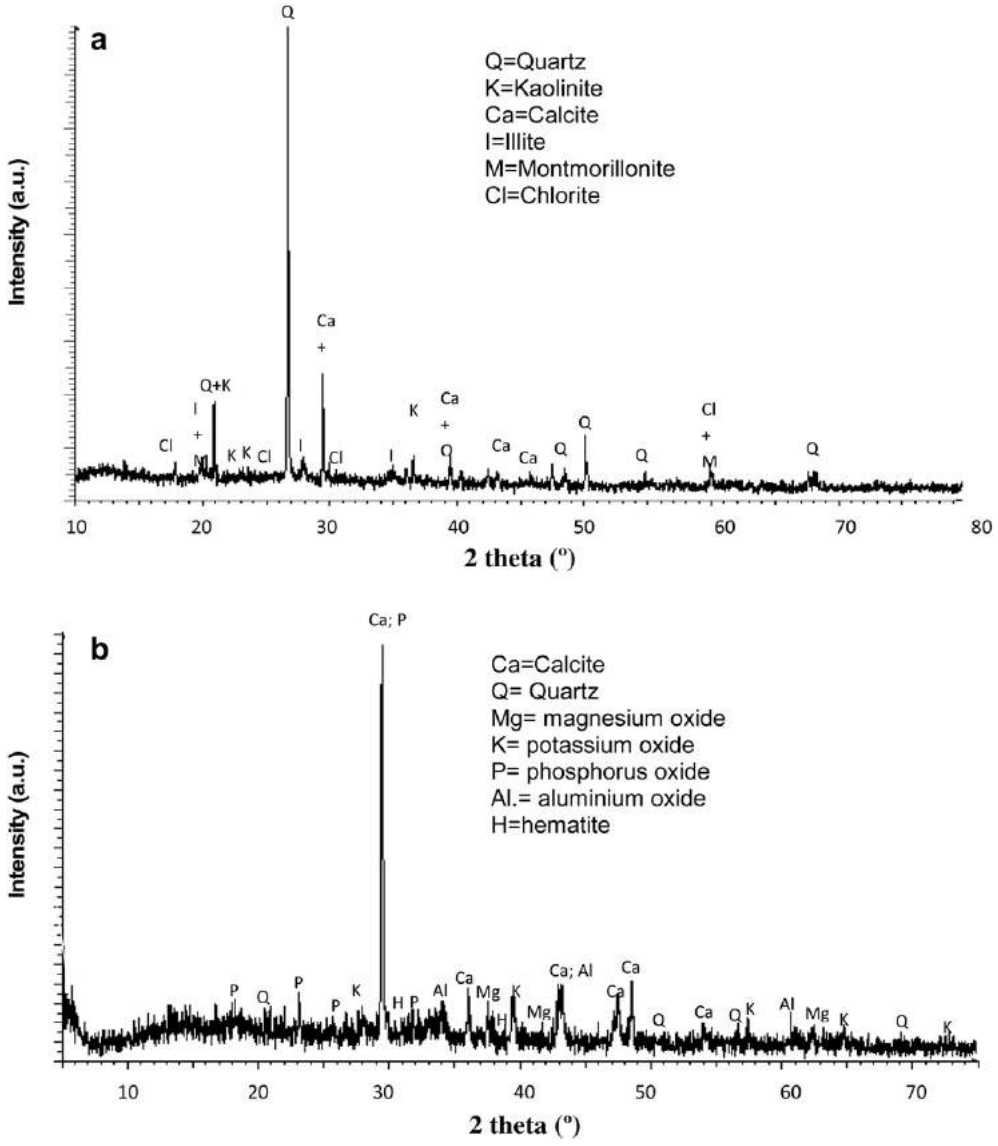

Fig. 1. DRX patterns for powdered (a) clay and (b) ash.

2.2. Raw materials and conformed materials characterization

Qualitative determination of major crystalline mineralogical phases present in the clay and ashes were achieved by using an X-ray diffractometer Siemens D5000 provided with a graphite monochromator and using Cu Kα radiation. The chemical composition was determined by X-ray fluorescence (XRF) in a Philips Magix Pro (PW-2440) equipment.

Thermal behaviour was determined by Thermogravimetric analysis (TGA) using a Mettler Toledo 851^e equipment under oxygen. Samples of about 20–25 mg, placed in a platinum crucible, and heated at a rate of 10 °C/min within the range between room temperature and 1000 °C. Data are shown as percentages of loss of weight as a function of temperature.

The content of carbonates of clay and ash were determined by Bernard calcimetry.

Scanning electron micrographs (SEM) were obtained by using a JEOL model JSM-5800 scanning microscope, with system OXFORD ISIS 486. Samples were placed over an aluminium drum and covered with a gold film by sputtering SCD 005.

A series of tests and inspections were performed to determine physical properties (weight loss on ignition, linear shrinkage, apparent density, water absorption test and water suction test) and mechanical properties (compressive strength) of bricks, for them to be in accordance with the specifications regulated by the UNE standards.

Apparent density was calculated as the ratio of dry mass of the conformed brick to its standard volume. Weight loss on ignition was obtaining by measuring the weigh after drying stage at 110 °C and after firing stage at 950 °C. Linear shrinkage was obtained by measuring the length of the samples before and after the firing stage using a calliper with a precision of ±0.01 mm.

Water absorption was determined according to standard procedure, UNE 22-182. Shaped samples were dried at 110 °C to constant weight. The samples were weighed at dry state (Gs) then introduced in water for 24 h, dried and weighed a second time in water. The samples were introduced again in water for 24 h to constant weight (Ge). The water absorption is the difference between weights (Ge-Gs). The water absorption of samples was calculated according to the following equation:

$$A = ((Ge - Gs)/Gs)*100 \quad (1)$$

The test to determine water suction was implemented according to standard procedure UNE 67-031. Shaped samples were dried at 110 °C to constant weight, Pi. The surface of the face introduced into water, which will cover approximately 3 mm during 1 min and then the samples were weighed (Q_i). The water suction of samples was calculated according to the following equation:

$$S = Qi * Pi / A(g/cm^2 * min) \quad (2)$$

where A is the surface of the shaped bricks.

Compressive strength was measured for fired samples according to standard procedure UNE 67-026 in a Suzpecar CME 200 SDC laboratory Testing Machine. All shaped samples were tested by applying the load centred in the upper face of the brick with speed lower 20 MPa/s until fracture.

3. Discussion

Table 1 shows the chemical composition and the loss on ignition (LoI) of the raw materials, and according to the XRD pattern of raw clay (Fig. 1a) mainly contained quartz (SiO_2) with the presence of calcite ($CaCO_3$) and phyllosilicates as possibly kaolinite (aluminium silicate), illite (potassium aluminium silicate), montmorillonite (aluminium magnesium silicate) and chlorite (a laminar silicate containing magnesium hydroxide and micaceous layers).

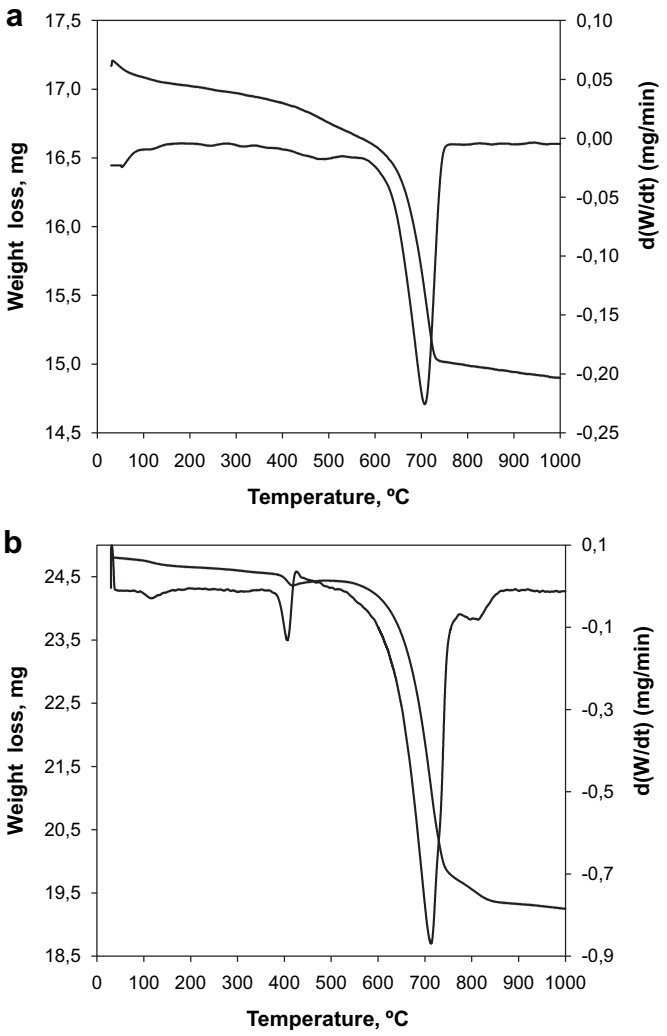

Fig. 2. TGA-DTG curves of (a) clay and (b) ash.

Ashes are basically composed by calcium oxide (CaO) that is mainly due to the calcite phase. The second major chemical constituent of the ashes are potassium oxide (K_2O), magnesium oxide (MgO), quartz (SiO_2) and phosphorus pentoxide (P_2O_5) and in minor quantity aluminium oxide (Al_2O_3) and hematite (Fe_2O_3) according to the XRD pattern of ash (Fig. 1b). The high percentage of LoI indicates an elevated fraction of clay minerals.

Results of thermogravimetric analysis (TGA) and differential thermogravimetric analisis (DTG) of clay and ashes up to 1000 °C are shown in Fig. 2. In the case of clay from room temperature to

Table 2
Characteristics of construction bricks made from ashes.

Sample series name	Ash content (wt.%)	Weight loss on ignition (%)	Linear shrinkage (%)	Suction water (g/cm^2 min)	Apparent Density (g/cm^3)
C	0	16.9	0.33	0.27	1.730
C-10A	10	12.9	0.54	0.29	1.717
C-20A	20	14.5	1.46	0.33	1.656
C-30A	30	16.3	3.78	0.40	1.464
C-40A	40	16.7	3.61	0.43	1.456
C-50A	50	17.9	3.57	0.46	1.463

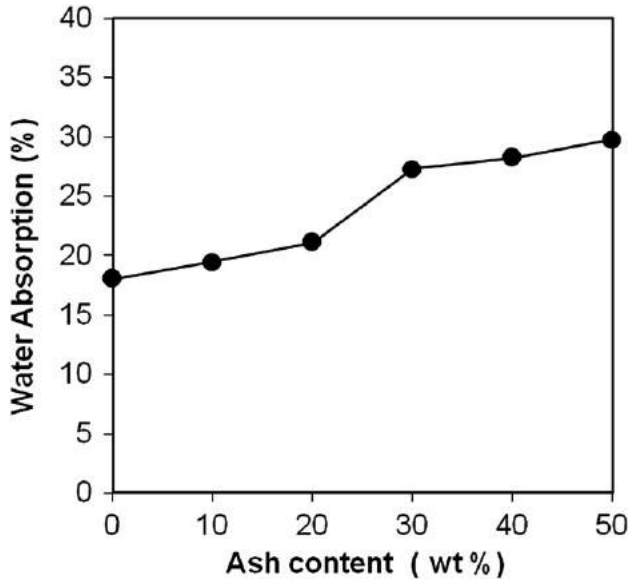

Fig. 3. Effect of ash concentration on water absorption.

160 °C with a weight loss of 0.8%, the heating produces the release of physically adsorbed water. From 160 °C to 580 °C, a weight loss of 2.5% can be assigned to combustion reaction of organic matter and dehydroxylation of clay minerals as, the decomposition of kaolinite at 470 °C and the structural water of illite between 400 °C and 550 °C. Finally, in the interval from 580 °C to 760 °C a weight loss of 9.6% is probably due to the decomposition of $CaCO_3$.

For ashes from room temperature to about 160 °C, heating causes mainly the elimination of hydration water. From 330 °C to about 420 °C the weight loss of 1.1% is probably due to the elimination of structural water (formed from OH-ions). From 420 °C to 570 °C the elimination of the organic matter and charcoal by combustion occurs. From the 570 °C to about 850 °C the weight loss continues in a more intense way (20.5%). The losses must be attributed to the elimination of carbon dioxide from carbonates. In the interval from 850 °C to 1000 °C, neither loss of weight seems to occur.

During the thermal treatment, besides the changes of mass and dimensions, the probes had appreciable colour variations. It was observed that the probes became darker when heated due to iron content of raw materials (Fernández-Abajo, 2000). Weight loss on ignition after sintering is related to the development of the porosity and the densification, and can have an effect on the compressive strength of the thermally treated samples (Lin et al., 2005). As a result of firing the clay has a loss on ignition of 16.9% due to elimination of the organic matter by combustion and water by dehydroxylation reactions of clay minerals. It is assumed that as the temperature was increased, the carbonate (11.8 wt.%) in clay become deformed into CO_2. Weight loss on ignition in mixed probes increased from 13% to 18% when the amount of ash is higher, as shown in Table 2. This was due to elimination of the organic matter content in the mixed samples by combustion and water and mainly to the decomposition of the high content in carbonate (54.7 wt.%) for ashes. Only the sample with major content of ashes (50 wt.%) has a higher weight loss of ignition than clay, due to the carbonates in ash and also to the organic content in the sample. Furthermore, the brick weight loss on ignition also depends on the inorganic substances in both clay and ash being burnt off during the firing process. The incorporation of ash to clay can reduce the sintering temperature, as Ca, K and Mg can act as flux agents. Linear shrinkage of samples (Table 2) increases from 0.33% for clay bricks to a maximum of 3.8% for *C-30A* bricks, so one should notice that the content ashes have a strong influence on linear shrinkage. The observed increase could be due to porosity resulting from carbonate decomposition into CO_2 and CaO during firing, since to the organic content of ash is much lower than that of clay. Crystallization of new calcium phases as anortite, wollastonite and gehlenite (Gonzalez, 2001; Dondi and Fabbri, 2001) can happen due to high content of calcium carbonate in the probes. Crystallization of these phases prevents the significant formation of amorphous phase.

Water absorption is a key factor affecting the durability of bricks and is a measure of open porosity. A low water absorption implies a high durability and resistance to the natural environment. Fig. 3 shows the results of water absorption test for different proportions of ash in the mixture. In this case, there is a clear tendency that the increase in the ash content in the clay body gives rise to an increase in the water absorption. This fact is expected since the calcium carbonate contained in the ashes is eliminated during the thermal process and it leads to an increase of the open porosity of the ceramics bodies. An ash content over 20 wt.% brick samples demonstrated a poor integrity, due to an excessive evolution of gaseous species upon firing.

The results of suction water test are shown in Table 2. Increases in the amount of ash varied from 0 to 50 wt.%, the suction water changed from 0.27 to 0.46 g/cm^2 * min. It is indicated that addition

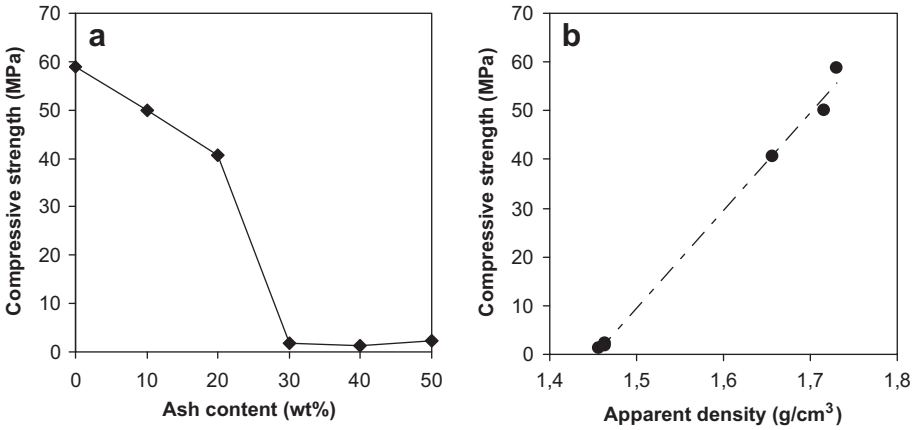

Fig. 4. (a) Compressive strength of the bricks as a function of the amount of the ash addition and (b) Relationship between mechanical resistance and apparent density in the probes studied.

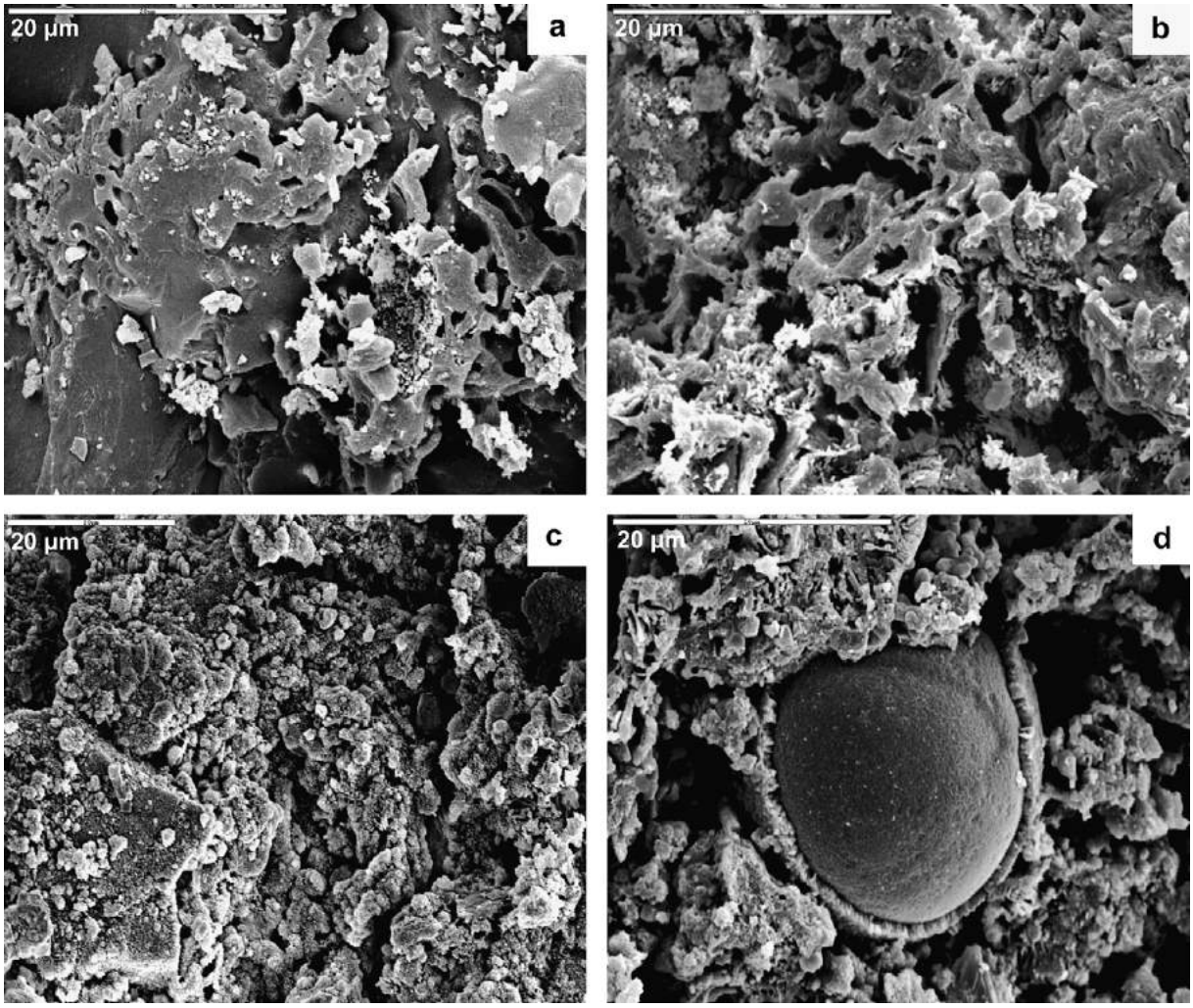

Fig. 5. SEM micrographs of samples (a) clay; (b) C-20A; (c) C-50 A (d) ash.

of ash increases the superficial interconnected porosity in accordance with the absorption date. However, this increase is more pronounced when the amount of ash is higher than 20 wt.%.

During sintering, open and closed pores are usually formed. When the mixture absorbs more water, the brick exhibits a larger pore size, resulting in a low density. The low density and high water absorption values are the indicatives of porous materials. Bricks made with clay have an apparent density of 1.73 g/cm^3 after firing. As shown in Table 2, the apparent density is inversely proportional to the quantity of ash added in the mixture. Addition of 20 wt.% residue to clay body (*C-20A*) caused 4% decrease in apparent density. However, a slight increase of ash, up to 30 wt.%, determined a very significant decrease of apparent density, up to 16%. According to these observations, 20 wt.% ash residue addition to clay body seems to be the maximum addition ratio because porosity decreases the mechanical properties of the materials.

The compressive test is the most important test for assuring the engineering quality of a building material. The results (Fig. 4a) indicate that the strength is greatly dependent on the amount of ash in the brick. Ash residue addition reduces slightly the compressive strength of the clay samples up to 20 wt.% residue addition ascribed to the enhancement of porosity. However, when a 30 wt.% of ash is added to the brick, the achieved brick strength lies from 58.2 MPa, in the case of clay bricks, to 2.0 MPa. This is also consequence of the increase in porosity, which is known to have a marked influence on the mechanical strength of the ceramic (Carty and Senapati, 1998). According to UNE standards, compressive strength of bricks must be 10 MPa, therefore only possible to incorporate up to 20% ash to clay.

The relationship between compressive strengths and apparent density is further evaluated in Fig. 4b, where linear dependences were obtained for firing probes.

Fig. 5 shows the microstructure of the pieces with different percentages of added ash.

In the study of the microstructure of the materials, it is observed that clay porosity (Fig. 5a) changes due to the presence of ash. The greater the amount of ash incorporated in body clay (Fig. 5 b–d) the greater the increase in the open porosity causes as a result of the connection of macropores. These results are consistent with the values of water absorption and compressive strength.

4. Conclusions

Ash from biomass incineration was used as a clay replacement raw material to prepare bricks, and the effects of the proportion of ash in the mixture on the physical and mechanical properties of the fired bricks were investigated. The main advantages of the use of ash in the manufacturing of traditional ceramics are: recycling of

a waste whose production increases everyday with the installation of new plants to produce energy, reduction of raw material costs and produce a higher porosity material, decreasing its density, and increasing its insulation ability. The incorporation of ash is limited due to the increase of the water absorption and decrease of the compression strength. In particular, the water absorption and the compressive strength of bricks with the addition of up 20 wt.% ash are very good and sufficiently high to satisfy the UNE standards. The overall decrease of the physical and mechanical properties of clay bricks with high percentages of ash (30–50 wt.%) can be attributed to high apparent density reduction due to the significant increase open porosity generated by the high carbonate content of the ash. The amount of added ash must be controlled in such a way that the product adequately meets the specific standards required for construction materials. In conclusion, the recycling of ash from biomass incinerator in good quality clay bricks is possible up to a maximum amount of 20 wt.%

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Journal of Environmental Management 95 (2012) S355–S359

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Re-fermentation of washed spent solids from batch hydrogenogenic fermentation for additional production of biohydrogen from the organic fraction of municipal solid waste

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ARTICLE INFO

Article history:
Received 27 September 2009
Received in revised form
16 November 2010
Accepted 18 January 2011
Available online 12 February 2011

Keywords:
Biohydrogen
Organic fraction of municipal solid wastes
Re-fermentation
Solid substrate
Spent solids
Washing

ABSTRACT

In the first batch solid substrate anaerobic hydrogenogenic fermentation with intermittent venting (SSAHF-IV) of the organic fraction of municipal solid waste (OFMSW), a cumulative production of 16.6 mmol H_2/reactor was obtained. Releases of hydrogen partial pressure first by intermittent venting and afterward by flushing headspace of reactors with inert gas N_2 allowed for further hydrogen production in a second to fourth incubation cycle, with no new inoculum nor substrate nor inhibitor added. After the fourth cycle, no more H_2 could be harvested. Interestingly, accumulated hydrogen in 4 cycles was 100% higher than that produced in the first cycle alone. At the end of incubation, partial pressure of H_2 was near zero whereas high concentrations of organic acids and solvents remained in the spent solids. So, since approximate mass balances indicated that there was still a moderate amount of biodegradable matter in the spent solids we hypothesized that the organic metabolites imposed some kind of inhibition on further fermentation of digestates. Spent solids were washed to eliminate organic metabolites and they were used in a second SSAHF-IV. Two more cycles of H_2 production were obtained, with a cumulative production of *ca.* 2.4 mmol H_2/mini-reactor. As a conclusion, washing of spent solids of a previous SSAHF-IV allowed for an increase of hydrogen production by 15% in a second run of SSAHF-IV, leading to the validation of our hypothesis.

1. Introduction

In the last 10 years, interest on biohydrogen has resurrected, particularly the research on dark fermentation of solid wastes (Kovács et al., 2006; Muñoz-Páez et al., 2008a). Hydrogen can be considered the best energy alternative because it can be produced by biological means, it has the highest energy density, it is versatile since can be used both as a primary or secondary energy source, and it is environmentally-friendly since water is the main combustion product and no aggressive pollutants are generated (Mizuno et al., 2002).

In bioydrogen production by dark fermentation of organic wastes, several microbial groups that consume H_2 coexist with the H_2-producing microbes (Valdez-Vazquez and Poggi-Varaldo, 2009). So, it is paramount to process feasibility to find techniques to inhibit the H_2-consuming microorganisms, such as the methanogenic archaea to cite one of the most important groups. It has been reported a variety of methanogenesis inhibitors, *inter alia*: acetylene, bromo-ethanesulphonate (BES), heat-shock pretreatment and low pH (Lay et al., 1999; Valdez-Vazquez and Poggi-Varaldo, 2009; Sprott et al., 1982; Sparling et al., 1997). Methanogenesis inhibition by low pH has been less studied in solid substrate hydrogenogenic fermentation, although it seems to be attractive and feasible for practical applications since low pH would be naturally driven by the accumulation of organic acids in the acidogenic fermentation of organic matter (Muñoz-Páez et al., 2008a). Environmental pH is a factor that has a variety of effects on the physiology of microorganisms. For instance, pH impacts on the electrical charge of the cell membranes, which in turn may influence the uptake of substrates and nutrients. Also, pH may have a noticeable effect on enzymatic activities. It is known that pH may determine to a great

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doi:10.1016/j.jenvman.2011.01.017

extent the type and distribution of fermentation products. In biohydrogen fermentation, the pH strongly influences the active bacteria, affecting among others the overall H_2 production, the specific rate of H_2 generation, and type and concentration of organic acids and solvents (Li et al., 2007).

Another important aspect in biohydrogen fermentation is the possible inhibition of further hydrogen production by accumulation of final products. The accumulation of dissolved H_2 in the liquid phase (associated to high partial pressures of hydrogen in the gas phase) could inhibit hydrogen production (Logan et al., 2002; Sparling et al., 1997; Valdez-Vazquez et al., 2005) and along with low pH it may promote the shift to solventogenic fermentation.

Bioreactor headspace release by venting and eventual flushing with inert gas seems to alleviate such an inhibition (Mizuno et al., 2000; Valdez-Vazquez et al., 2006a).

There is little information on inhibition of the biohydrogenogenesis by accumulation of organic acids and other organic metabolites. It is known that undissociated (or unionized) forms of organic acids are more inhibitory to fermentative bacteria than the anionic forms. It seems that unionized organic acids may uncouple the growth of microorganisms (Booth, 1985; Wang and Wang, 1984). It is likely that hydrogen production cessation in batch solid substrate anaerobic hydrogenogenic fermentation with intermittent venting and headspace flushing (SSAHF-IV) of organic fraction of municipal solid wastes (OFMSW) at the end could also be due to accumulation of organic acids and other organic metabolites (Valdez-Vazquez et al., 2005; van Ginkel and Logan, 2005).

Therefore, the objective of this work was to evaluate the hydrogenogenic second fermentation of washed spent solids originated in a first SSAHF-IV. Washing of spent solids was performed between batch fermentations in order to eliminate the presence of organic metabolites.

2. Materials and methods

2.1. Experimental design and bioreactors

2.1.1. First SSAHF-IV of organic fraction of municipal solid wastes

The process chosen was the so-called mesophilic (35 °C), batch, solid substrate anaerobic hydrogen fermentation with intermittent venting and headspace flushing (SSAHF-IV) according to procedures reported by Valdez-Vazquez et al. (2005).

2.1.1.1. Inocula. Mini-reactors were seeded with digestates from methanogenic solid substrate anaerobic digesters degrading a mixture of organic solid wastes. Those digesters were operated at 21 days mass retention time in mesophilic conditions.

2.1.1.2. Substrate. The substrate was a model organic fraction of municipal solid waste (OFMSW) conformed by a mixture of 40% of paper and 60% food waste. It was prepared with paper and food wastes from CINVESTAV's cafeteria. The mixture was vacuum-dried overnight, milled and stored at 58 °C until its use. The substrate moisture content was adjusted to 25% total solids with a buffer containing 31.6 g $NaHCO_3$ and 63.3 g K_2HPO_4 per liter; buffer and substrate were sterilized in order to control the studied microbial consortium producers of H_2. The main characteristics of the feedstock supplied to the mini-reactors are shown in Table 1.

2.1.1.3. Mini-reactors and experimental procedure. Wide mouth glass bottles of 250 mL volume which were tightly stoppered using rubber stoppers and metallic harnesses were used as mini-reactors. First, in an anerobic glove chamber, 20 g of inocula y 80 g of substrate were added to mini-reactors and blended with the inocula. Background controls with only 20 g of inoculum and abiotic controls (tyndalysed 'substrate plus inoculum') were also run. Next, mini-reactors were made anerobic under N_2 using an evacuation-gas replacement method. At time zero, all bioreactor headspaces were injected with acetylene 1% v/v (Sparling et al., 1997). The mini-reactors were incubated in a constant temperature room at 37 °C.

The bioreactors were first subjected to SSAHF-IV of five cycles, with no addition of new inocula and acetylene. During each cycle, bioreactor headspace was intermittently vented at atmospheric pressure. At the end of each cycle, reactor headspace was flushed with N_2 in order to decrease hydrogen concentration down to zero. Once it was determined the absence of hydrogen production in the fifth cycle, the spent solids were washed (1 part of spent solids in 3 parts of tap water), settled, manually squeezed, and dried for future use.

Table 1
Characterization of substrates in both solid substrate anaerobic hydrogenogenic fermentation with intermittent venting and headspace flushing.

Parameter	SSAHF-IV[a]	
	1	2
	Organic fraction of municipal solid wastes	Washed spent solids
TS[b](%)	24.54 ± 0.13	19.62 ± 0.03
VS[c](%)	14.90 ± 0.85	8.91 ± 0.02
TKN (%)[d]	1.93 ± 0.80	ND[e]
Cellulose (%)[d]	26.2 ± 1.5	18.7 ± 0.8
Lignin (%)[d]	19.4 ± 1.1	16.9 ± 1.2
Phosphorus (mg/kg)	17.3 ± 1.1	13.7 ± 0.3
PH	7.34 ± 0.15	7.0 ± 0.20

[a] Notes: batch solid substrate anaerobic hydrogenogenic fermentation with intermittent venting and flushing of reactor headspace.
[b] Total solids.
[c] Volatile solids.
[d] % of total solids.
[e] Not determined.

2.1.2. Second SSAHF-IV of washed, spent solids from the first fermentation

The washed spent solids from the first SSAHF-IV were loaded along with fresh inocula in bioreactors and subjected to a second SSAHF-IV with a similar procedure as described above. This time, inhibition of methanogenesis was effected by setting pH at 6.3 with a buffer solution (Muñoz-Páez et al., 2008b). Bioreactor incubation and headspace handling were similar to procedures outlined in Subsection 2.1.1. All the experiments of the first and second SSAHF-IV were run by duplicate.

The main characteristics and properties of the OFMSW used in the first SSAHF-IV and the spent solids fed to the second SSAHF-IV are exhibited in Table 1.

2.2. Analyses

The main monitoring parameters were hydrogen production, initial and final pH, and solids contents. Hydrogen and methane contents in biogas were determined by gas chromatography (Poggi-Varaldo et al., 1997) in a Gow–Mac chromatograph model 350 fitted with a thermal conductivity detector (TCD) and Molecular Sieve 5A packed column (injector, detector and column temperatures were 25, 100 and 25 °C, respectively). Argon was the carrier gas. Volatile organic acids and solvents were determined in water extracts of the spent solids of mini-reactors: 5 g of homogenized spent solids was thoroughly mixed with 25 mL of distilled water. The suspension was filtered through a glass-membrane filter and an aliquot of the filtrate was injected in a gas chromatography Varian Star 3400 equipped with a FID for metabolite concentration determination.

The injector and detector temperatures were set at 250 °C. Nitrogen was used as a carrier gas with a 20 mL/min flowrate. The oven temperature was programmed as follows: 60 °C for 2 min, increasing to 140 °C at 5 °C/min, and then kept constant at 140 °C for another 6 min. A 50 m 0.32 mm internal diameter fused silica capillary column coated with 0.2 mm CP-Wax 57 CB was used.

The pH was determined in a slurry 1 part spent solids +5 parts distilled water at 5 °C. Total and volatile solids, Kjeldahl nitrogen, total phosphorus, cellulose and lignin contents in the feedstock and digestates were determined as described elsewhere (Poggi-Varaldo et al., 1997; Muñoz-Páez et al., 2008b; Valdez-Vazquez et al., 2006a).

3. Results and discussion

In the first SSAHF-IV of OFMSW, intermittent venting coupled to convenient flushing of reactor headspace with N_2, allowed for four cycles of hydrogen generation with no addition of new substrate or inoculum between cycles (Fig. 1). A fifth cycle was attempted, but no noticeable hydrogen generation was found (data not shown). A total cumulative production of 16.6 mmol H_2/reactor was obtained. Interestingly, accumulated hydrogen in 4 cycles was 100% higher than that produced in the first cycle alone.

Venting and flushing procedures addressed the decrease of H_2 partial pressure in headspace which in turn would decrease dissolved H_2 concentration in the interstitial liquid of solid substrate. In such a way, the negative effect of H_2 accumulation on further H_2 production was minimized or relieved (Mizuno et al., 2000; Valdez-Vazquez et al., 2005). After each N_2 gassing or flushing, the subsequent production of H_2 was lower than the precedent one. This could be ascribed either to a decrease of biodegradable organic matter with time or to another form of inhibition determined by the accumulation fermentation products other than H_2.

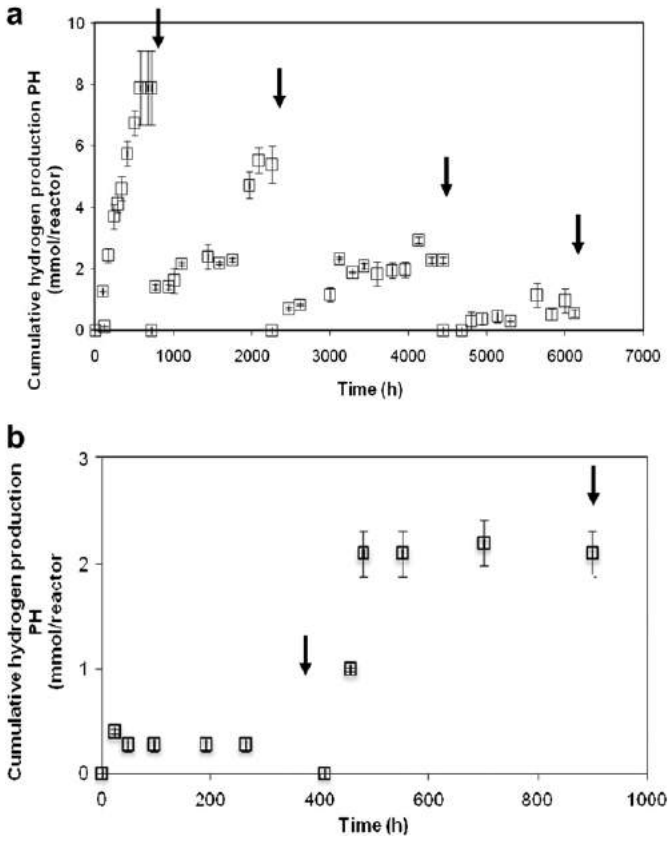

Fig. 1. Time course of H_2 production in batch mini-reactors: (a) first batch fermentation of organic fraction of municipal solid wastes; (b) second batch fermentation of washed, spent solids from the first fermentation. Vertical arrows indicate flushing of bioreactor headspace with N_2.

At the end of incubation, partial pressure of H_2 was low and high concentrations of organic acids and solvents remained in the spent solids. So, since approximate mass balances indicated that there was still a moderate amount of biodegradable matter in the spent solids, we hypothesized that the organic metabolites imposed some kind of inhibition on further hydrogenogenic fermentation.

As it was mentioned above, cessation of hydrogen production after the 4th cycle could be due either to the (i) exhaustion of fermentable organic matter or to an inhibition of the fermentation possibly caused by the accumulation of (ii) solvents or (iii) organic acids.

Exhaustion of fermentable organic matter was not likely to be the cause, since the cellulose-equivalent of biohydrogen harvested in the first fermentation was only a fraction of the initial cellulose (26.2%, Table 1). Furthermore, analysis of washed fermented solids gave a cellulose content of 18.7% (Table 1). On the other hand, solventogenic shift could be ruled out since solvent concentration was lower than that of organic acids (Sum VOA/SumSolvents = 11.3, concentrations in COD-equivalent).

Volatile organic acids may be stimulatory, inhibitory or toxic to fermentative bacteria, depending upon their concentration levels (Stewart, 1975; Ezeji et al., 2004; Wang et al., 2008). Inhibition by VOA is related to the easier transport into bacterial cell of the undissociated forms of organic acids; once inside the cell the organic acids dissociate to hydronium ions plus the corresponding anions, lowering the internal cell pH (Gottschalk, 1986; Jones and Woods, 1986). Proton uptake uncouples the proton motive force; this, in turn, causes a rise in maintenance energy in order to keep the intracellular pH near neutrality. Alternatively, a fraction of maintenance energy should be used to restore the physiological balance in the cell, which can reduce the energy used for bacteria growth and then inhibit the bacteria growth and activity (Jones and Woods, 1986; Fukuzaki et al., 1990; Zoetemeyer et al., 1982). On the other hand, if the dissociated species of these soluble metabolites are present at high concentrations in the fermentation system, then the ionic strength will increase, which may result in the cell lysis of hydrogen-producing bacteria (Niel et al., 2003). The uptake of organic acids may also be linked to a decrease in the available coenzyme A and phosphate pools; this in turn, would decrease the flux of glucose through glycolysis (Gottwald and Gottschalk, 1985).

We thus tend to support that organic acids accumulation was associated to biohydrogen generation cessation in the 5th fermentation cycle in our work. This is also consistent with findings of Wang et al. (2008) who observed that accumulation or spiking with acetate inhibited H_2 production in batch, submerged hydrogenogenic fermentation of glucose. They reported that spiking with acetate was associated to a lower rate of carbohydrate fermentation

Table 2
Metabolite concentrations in solid substrate anaerobic hydrogenogenic fermentation with intermittent venting and headspace flushing.

Metabolite concentration (mg/kg wet basis)	SSAHF-IV [a]	
	1st fermentation	2nd fermentation
	End of 4th cycle	After washing, before 1st cycle
Acetone	36 ± 5	5 ± 0.6
Ethanol	528 ± 47	<DL[b]
Acetate	2406 ± 26	32 ± 4
Propionate	1056 ± 12	14 ± 0.2
Butyrate	4874 ± 101	49 ± 3
Acetone	36 ± 5	5 ± 0.6

[a] Notes: batch solid substrate anaerobic hydrogenogenic fermentation with intermittent venting and flushing of reactor headspace.
[b] Detection level.

Table 3
Hydrogen production in the first and second solid substrate anaerobic hydrogenogenic fermentations with intermittent venting and headspace flushing.

SSAHF-IV[a]							
1st fermentation					2nd fermentation		
Cycle 1	Cycle 2	Cycle 3	Cycle 4	$P_{H\ cum}$[b]	Cycle 1	Cycle 2	$P_{H\ cum}$[b]
7.89 ± 1.21	5.39 ± 0.61	2.28 ± 0.10	1.06 ± 0.22	**16.62**	0.28 ± 0.07	2.1 ± 0.22	**2.38**

[a] Notes: batch solid substrate anaerobic hydrogenogenic fermentation with intermittent venting of reactor headspace.
[b] Cumulative H_2 production (mmolH_2/mini-reactor).

and a significant increase of lag time for hydrogen production onset. In effect, they showed that spiking with ethanol, acetic, propionic and butyric acids could inhibit the ability of mixed cultures to generate biohydrogen from de degradation glucose in submerged batch fermentation tests during the fermentative hydrogen production. They found that the higher their concentrations, the greater their inhibitory effects. When the added concentration of organic acids was 200 mmol/L, the inhibitory effects on the ability of mixed cultures to produce hydrogen were in this order HAc > HPr > HBu.

Also the increased butyric concentration at the end of fermentation would partially explain the lowering of H_2 production in the last cycles of the first SSAHF-IV (Table 2), indeed, the ratio A/B was 0.27, where A is the acetic acid concentration and B is the butyric acid concentration (both in mg COD-equivalent/wet kg). It is known that the butyric pathway of typical Clostridia yields 2 mol H_2/mol hexose whereas the acetic pathway yields 4 mol H_2/mol hexose (Brock and Madigan, 1991). As a reference, it can be shown that when both pathways equally contribute to H_2 production (50%–50%), the ratio A/B is 0.79 (on COD-equivalent concentration). So, the low value of experimental ratio A/B in our work indicates that the first batch fermentation was dominated by the lower H_2-yielding butyric pathway.

Table 2 shows that washing significantly decreased the concentrations of organic metabolites in solids; removals of 86.1, 100.0, 98.7, 98.7 and 99.9% of acetone, ethanol, acetate, propionate and butyrate were achieved.

In the second SSAHF-IV with washed spent solids, two cycles of H_2 production were obtained (Fig. 1, Table 3). In the first cycle, the H_2 production was relatively low; however, in the 2nd cycle, the H_2 generation took off. A 3rd cycle was tried, yet, no hydrogen was obtained (data not shown). Cumulative H_2 production in the 2nd SSAFH-IV amounted to *ca.* 2.4 mmol H_2/mini-reactor that represented nearly a 14.3% of the hydrogen production of the first SSAHF-IV alone.

Even considering that the amount of biodegradable organic matter in spent washed solids was lower than that in the fresh OFMSW, an appreciable amount of hydrogen during the second batch fermentation was observed. This result seems to corroborate our hypothesis that the presence of organic acids and solvents in spent solids were related to the cessation of further H_2 production in the 5th cycle of the first SSAHF-IV. Also, a strategy for maximizing hydrogen yields from OFMSW can be devised, by subjecting washed spent solids to a second hydrogenogenic fermentation or recycling them directly to the first fermentation (mixing OFMSW with washed spent solids).

The extracts produced in the washing are rich in organic acids and some solvents and could be post-treated either by photoheterotrophic non sulfur purple bacteria for more hydrogen generation (Acevedo-Benítez and Poggi-Varaldo, 2008), or in microbial fuel cells for direct bioelectricity production from the soluble organic matter (Poggi-Varaldo et al., 2009), or in methanogenic digesters for methane production (Poggi-Varaldo et al., 2005; Robledo-Narváez et al., 2008). In this way, maximum bioenergy yields could be obtained from the OFMSW.

4. Conclusion

Washing and re-fermentation of spent solids from a first batch solid substrate hydrogenogenic fermentation allowed for the additional generation of biohydrogen, i.e., 14.3% of the biohydrogen obtained in the first fermentation. It seems that washing of solids prevented the inhibition to further H_2 production caused by accumulation of organic acids and other metabolites.

Acknowledgments

The authors wish to thank CINVESTAV del IPN and IMCYTDF for partial support to this research, CONACYT for a graduate scholarship to KMM-P, and COFAA for a fellowship to NR-S. The excellent help of Mr. Javier Pareja and Mr. Rafael Hernández-Vera from the EBRE Group, CINVESTAV del IPN, is gratefully acknowledged. The authors wish to express their gratitude to the Editor, Guest Editors, and Referees, for their kind assistance and valuable comments and suggestions that helped to improve our manuscript.

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Notation

A/B: ratio acetic acid-to-butyric acid, both in COD-equivalent concentrations
BES: bromo-ethanesulphonate
OFMSW: organic fraction of municipal solid wastes
$P_{H\ cum}$*:* cumulative hydrogen production
SSAHF-IV: solid substrate anaerobic hydrogenogenic fermentation with intermittent venting and headspace flushing
TK: total Kjeldahl nitrogen

Journal of Environmental Management 95 (2012) S360–S364

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Improvements in fermentative biological hydrogen production through metabolic engineering

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ARTICLE INFO

Article history:
Received 15 August 2009
Received in revised form
27 June 2010
Accepted 18 July 2010
Available online 8 August 2010

Keywords:
Biofuel
Biohydrogen
Fermentation
Metabolic engineering
Fermentation yields
Anaerobic metabolism

ABSTRACT

Replacement of fossil fuels with alternative energies is increasingly imperative in light of impending climate change and fossil fuel shortages. Biohydrogen has several potential advantages over other biofuels. Dark fermentation as a means of producing biohydrogen is attractive since a variety of readily available waste streams can be used. However, at present its practical application is prevented by the low yields obtained. Here the basic metabolisms leading to hydrogen production are outlined and current research to increase yields, either through modification of existing pathways, or by metabolic engineering to create new, higher yielding, pathways, is discussed. Inactivation of competing reactions and manipulation of culture conditions has lead to higher hydrogen yields, near those predicted by metabolic schemes. However, to be useful, hydrogen production must be increased beyond present limits. Several possibilities for surpassing those limits using metabolic engineering are presented.

1. Introduction

The world is faced with serious environmental problems, many due directly or indirectly to fossil fuel utilization. An estimated 40% of annual deaths are thought to be directly linked to environmental degradation (Pimentel et al., 2007) and poor urban air quality, largely due to fossil fuel combustion, plays a significant role in the estimated 3 million people killed worldwide each year by air pollutants (WHO, 2002), including air-borne particulates emanating from vehicle exhaust, which are estimated to be responsible for 20% of the lung cancer deaths in the USA (Pearce, 2002). Fossil fuel driven climate change already has had an effect on human morbidity with conservatively, 150,000 deaths and over 5 million DALYs (disability adjusted life years) attributable to this factor (Campbell-Lendrum and Woodruff, 2007).

Of course, impending climate change, driven by fossil fuel derived CO_2 emissions, poses a potentially much greater threat to human health and well-being, the magnitude of which is difficult to assess. The anthropogenic climate change already in place, only a small fraction of what is expected to occur, has already had a significant impact. A recent analysis suggests that there are already, conservatively, 150,000 deaths and over 5 million DALYs attributable to this factor (Campbell-Lendrum and Woodruff, 2007).

There is now a widespread awareness that to avert further environmental degradation and the disastrous consequences of climate change, the present almost total reliance on fossil fuels must be replaced by the development of sustainable alternative energy sources and carriers. This has been given even more impetus recently by the realization that the world is entering a phase of ever decreasing fossil fuel reserves and ever increasing fuel prices. A number of options, for both stationary and mobile (i.e. transportation) power generation, are under development. Several biofuels, notably bioethanol and biodiesel, are already being produced on a massive scale, and a number of others have been proposed.

Biohydrogen appears to have a number of potential advantages as a biofuel. For one thing, there is a significant movement to create a hydrogen economy with a great deal of R&D activity in hydrogen storage and utilization. Every major automobile manufacturer is committed to developing and testing hydrogen-powered prototypes (Anonymous, 2010). To power this proposed future hydrogen economy, a green, sustainable means of producing hydrogen must be developed. Biological production of hydrogen through the dark fermentation of wastes (first) and non-food biomass (second) could potentially provide this. As well, although bioethanol and biohydrogen production could take place in similar facilities, energy costs for biohydrogen might be lower since no distillation step would be required. Another advantage is that hydrogen can be converted to power using fuel cells which are inherently much

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0301-4797/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jenvman.2010.07.021

more efficient than the combustion engines used when the biofuel is bioethanol or biodiesel. Using fuel cells also avoids the generation of various pollutants such as NO_x and acetaldehyde (bioethanol). Finally, unlike bioethanol, which emits CO_2 when it is combusted, CO_2 is given off during the production of biohydrogen, thus permitting its centralized capture and sequestration. This could potentially even make biohydrogen production carbon negative.

However, a number of problems must be overcome in order to realize the practical application of dark fermentation for biohydrogen production. Engineering issues, such as bioreactor configuration and operation, do not appear to be particularly technically challenging. It should be noted however that significant technical barriers to the implementation of hydrogen as a fuel do exist in terms of achieving practical means for its purification and storage (Jacoby, 2005; Levin and Chahine, 2010; US DOE Hydrogen Program, 2002, 2006). Likewise, substrate pretreatment problems are either rather easily dealt with, or at worst (cellulosics), are in common with the production of some other biofuels (bioethanol), and are presently the subject of intense investigation. For example, to name only two, $500 million USD has been invested in the formation of the Energy Biosciences Institute (http://www.energybiosciencesinstitute.org/) and $375 million USD in the formation of federal Bioenergy Research centers (http://www.energy.gov/news/archives/5172.htm) both of which will have as a major focus lignocelluloses conversion.

In biohydrogen production through dark fermentation the core issue is the attainable yield, with typical mesophilic fermentations giving at present only about 10–20% of the hydrogen potentially available in the substrate (12 mol H_2/mol hexose) (Hallenbeck, 2005; Hallenbeck and Ghosh, 2009; Hallenbeck et al., 2009; Hawkes et al., 2007; Kraemer and Bagley, 2007). Somewhat higher yields, up to 25% (3 mol H_2/mol hexose) are seen with thermophilic fermentations using either pure cultures or co-cultures at the expense of volumetric productivities (Zeidan and van Niel, 2009; Panagiotopoulos et al., 2010). The key obstacle appears to be constraints imposed by the metabolic pathways involved. Here we discuss recent research that has been carried out in attempts to overcome these restrictions as well as proposals for future research in this direction.

2. Fermentative H_2 producing metabolic pathways

The metabolic pathways implicated in fermentative hydrogen production and the hydrogenase enzymes involved are well known and have been characterized in some detail. A detailed description has been presented previously (Hallenbeck and Benemann, 2002; Hallenbeck, 2005, 2009) and only a brief review will be given here. Two basic metabolic types can be distinguished; facultative anaerobes, such as *Escherchia coli*, and strict anaerobes, like *Clostridia* (Fig. 1). In both cases, the substrate (glucose) is broken down to pyruvate by standard glycolysis (Embden-Meyerhof-Parnas pathway) producing ATP and reducing NAD to NADH. For glycolysis to continue, the NADH must be oxidized. This is achieved by producing a variety of reduced products. In facultative anaerobes this is principally ethanol, although some lactate is made, particularly as the pH becomes acidic, i.e. falls below 6.5 (Stokes, 1949). In strict anaerobes, a variety of products, depending upon the organism and fermentation conditions, of varying states of reduction, are made; i.e. ethanol, butyrate, butanol, acetone (see for example Lee et al., 2008).

The key intermediate is pyruvate whose catabolism differs between the two metabolic types. In general, facultative anaerobes degrade pyruvate to formate and acetyl-CoA through the action of *pfl* and strict anaerobes make acetyl-CoA, CO_2, and reduced ferredoxin using PFOR (pyruvate:ferredoxin oxidoreductase) (Hallenbeck, 2005, 2009). Thus, in one case hydrogen is made from formate by the *fhl* complex which possesses a Ni–Fe hydrogenase, a member of the Ech family of hydrogenases (Vignais and Billoud, 2007; Vignais, 2008) and in the other case, reduced ferredoxin drives hydrogen evolution by a FeFe hydrogenase (Hallenbeck, 2009; Hallenbeck and Ghosh, 2009). Although in principal NADH can be reoxidized by reducing ferredoxin, hence leading to additional hydrogen production, this only occurs at very low hydrogen partial pressures unattainable in practical hydrogen fermentations. It should be noted that some strict anaerobes (i.e. *Clostridia*) appear to have an Ech hydrogenase, although it is unclear if this works to conserve energy through hydrogen consumption, or participates in hydrogen evolution. It has been suggested, based on biochemical evidence, that the Ech of *Thermoanaerobacter tengcongensis* is partly responsible for hydrogen production by that species (Soboh et al., 2004). On the other hand, genetic analysis of the closely related *Thermoanaerobacterium saccharolyticum* has shown that it is not involved in hydrogen evolution in this species (Shaw et al., 2009). Obviously, the physiological function of the Ech hydrogenases in strict anaerobes remains to be demonstrated.

In both the PFL and PFOR pathways, some of the acetyl-CoA must be used for the necessary production of reduced products, as discussed above. Whatever is left over is used for ATP synthesis. From the discussion above, and as shown in Fig. 1, it is apparent that maximum hydrogen yields from the PFL pathway is 2 mol H_2/mol glucose, and from the PFOR pathway one also obtains 2 mol H_2/mol glucose, up to 4 mol H_2/mol glucose only at $P_{H_2} < 0.1$ kPa. These are the basic metabolic constraints on fermentative H_2 production with existing pathways. In practice, H_2 yields are below these values, and various efforts have been made to increase yields to these levels, as discussed in what follows.

2.1. Maximizing yields of existing pathways

Several approaches have been taken to maximize yields with existing pathways (Table 1). For one, elimination of reactions that compete for reductant, either NADH or pyruvate, should in principle increase the yield of H_2 from the fermentation. A second type of modification that might be effective in increasing hydrogen yields is to eliminate the activity of any so-called uptake hydrogenases that might be present. Often bacteria engaging in hydrogen metabolism possess several different hydrogenases that are metabolically or biochemically poised to operate preferentially in one direction; proton reduction or hydrogen oxidation (Hallenbeck and Benemann, 2002; Vignais and Billoud, 2007). Thus it is logical to inactivate any hydrogen oxidation activity that might be present. Indeed, elimination of Hyd1 and Hyd2 of *E. coli* leads to modest increases in hydrogen yields (Penfold et al., 2003; Maeda et al., 2007; Bisaillon et al., 2006; Turcot et al., 2008). For example, in one case elimination of *hyd*1 and *hyd*2 gave a 37% increase in H_2 yield compared to the wild type strain (Bisaillon et al., 2006).

There have been several studies in the facultative anaerobe *E. coli* where various mutations have been introduced to inactivate competing pathways. The most obvious mutation to introduce is to inactivate *ldhA*, lactate dehydrogenase, since normally this potentially could drain the pyruvate pool which otherwise could be used to make formate, and in turn H_2 (Fig. 1, Table 1). Thus, inactivation of this gene leads to a modest increase (20–45%) in net hydrogen production (Yoshida et al., 2006; Bisaillon et al., 2006; Turcot et al., 2008; Maeda et al., 2007,). Since *ldhA* expression only increases when the pH becomes acidic, which might happen as fermentation progresses due to acid production, a large effect of *ldhA* inactivation is not to be expected if the culture medium is well buffered.

The action of fumarate reductase (*frdBC*) could in principal also drain the pyruvate pool since it catalyses the synthesis of succinate

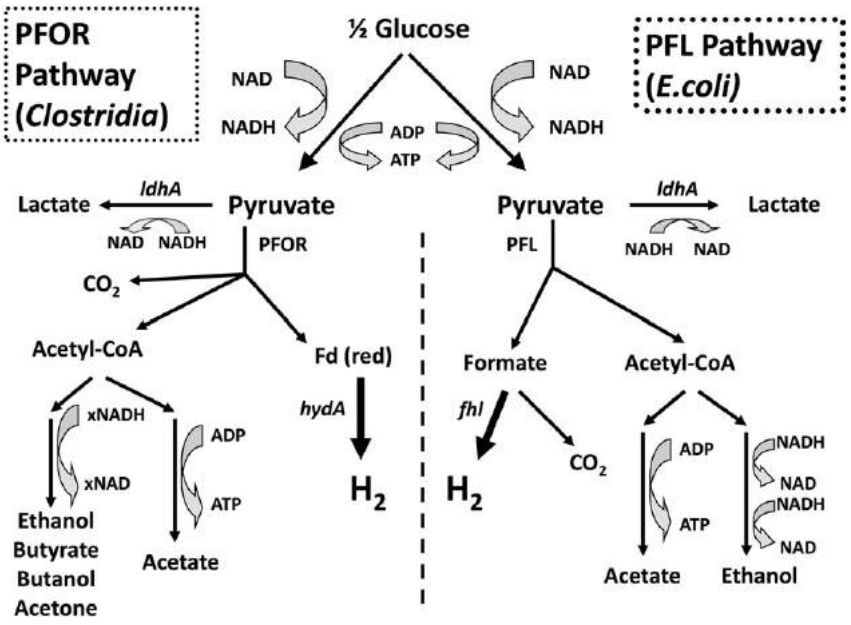

Fig. 1. Metabolic Pathways in H_2 Production. The major pathways in H_2 production are outlined. In all cases, glucose is broken down to pyruvate through the glycolytic pathway. The fate of pyruvate differs depending upon the organism and the specific pyruvate degrading enzymes that are expressed. Basically, two types can be distinguished; the PFL pathway, typical of facultative anaerobes, such as *Escherchia coli*, shown on the right, and the PFOR pathway, typical for strict anaerobes, such as *Clostridia*, shown on the left. Many organisms actually contain both systems with usually only one playing a major role in fermentation (Hallenbeck, 2009). PFOR-pyruvate:ferredoxin oxidoreductase, PFL-pyruvate:formate lyase. *hydA*, [FeFe] type hydrogenase; *fhl*, formate:hydrogen lyase complex, which includes an Ech type hydrogenase (Hallenbeck, 2009). In the PFOR system, additional hydrogen can be derived from electrons from NADH through several different mechanisms. In the PFL system, there is no mechanism for producing hydrogen from the generated NADH. In both systems, ATP is produced when acetate is made from acetyl-CoA, and both types must have means to oxidize NADH to regenerate the NAD necessary for continuing glycolysis. In the PFL system, this is usually through production of ethanol. In the PFOR system (*Clostridia*), a variety of reduced products; ethanol, butanol, butyrate, acetone, are possible, but the individual pathways are not shown here. *ldhA*, lactate dehydrogenase, if present and active, can drain away pyruvate that otherwise could be used for producing hydrogen. As well, minor pathways, e.g. succinate production from phosphoenolpyruvate (*frdBC*) in *E. coli*, are not shown.

from phosphoenolpyruvate, a glycolytic intermediate on the pathway to pyruvate formation. However, little gain is to be expected from inactivation of fumarate reductase since normally there is only a relatively small (10–15%) carbon flux through this pathway (Belaich and Belaich, 1976; Clark, 1989; Alam and Clark, 1989). Indeed, inactivation of the *frd* genes leads to only a modest increase in H_2 yields (Maeda et al., 2007; Yoshida et al., 2006).

Another strategy that has been applied in *E. coli* to increase hydrogen production is to increase expression of the FHL (formate:hydrogen lyase) system. The FHL complex produces H_2 and CO_2 from the formate formed from pyruvate under anaerobic conditions by *pfl*. Two different types of mutations have been introduced. Inactivation of *hycA*, a transcriptional repressor of FhlA, the transcriptional activator of *fhl* synthesis, should lead to greater FHL synthesis (Yoshida et al., 2006; Maeda et al., 2007). Likewise, FhlA-C, an allele (fhlA*) of *fhlA* that carries a deletion of the N-terminal part of FhlA and that is constitutively active has been used (Bisaillon et al., 2006; Turcot et al., 2008).

However, the magnitude of the improvement is variable since the relative amount of undegraded formate available is dependent upon culture conditions and at least in some circumstances can be quite small (7% of the carbon input) (Alam and Clark, 1989). Of course, this points out the evident; increasing the activity of an enzyme in a particular pathway way will increase flux only if the quantity of that enzyme is normally limiting. It is not immediately obvious that it is necessarily the quantity of hydrogenase that is normally limiting production and not flux through the glycolytic pathway, for example. Thus, although expressing the native hydrogenase of *Clostridium paraputrificum* from a plasmid was reported to increase hydrogen yields from N-glucosamine by 1.7 fold (Morimoto et al., 2005), a recent detailed study of hydrogenase overexpression in *Clostridium acetobutylicum* concluded that hydrogenase content was not a limiting factor in hydrogen yields from glucose since no effect of overexpression was found (Klein et al., 2010).

Finally, several attempts to metabolically engineer *Clostridia* to increase hydrogen production have been made. In one example, down regulation of the uptake hydrogenase genes (*hupCBA* operon) in *Clostridium saccharoperbutylacetonicum* strain N1-4 using an antisense RNA strategy, was shown to decrease hydrogen uptake activity significantly causing a 3.1-fold increase in hydrogen production (Nakayama et al., 2008). In another example, decreasing acetate formation through inactivation of *ack* in *Clostridium tyrobutyricum* was reported to give 1.5-fold enhancement in hydrogen production from glucose (2.2 mol of H_2 per mole of glucose versus 1.4 mol of H_2 per mole of glucose) [Liu et al., 2006]. This shows that effects of metabolic perturbation can be different from what might be predicted since acetate formation does not drain the reductant pool and, in fact, eliminating this pathway should theoretically increase acetyl-CoA flux into pathways that would decrease hydrogen production, such as ethanol or butyrate production, since they require reducing power (NADH).

2.2. Metabolic engineering of new pathways

Several approaches have been proposed for introducing new enzymes and/or pathways to overcome thermodynamic/metabolic barriers to increasing hydrogen yields. In one avenue, attempts have been made to express a highly active FeFe hydrogenase (*hydA*) from various sources in *E. coli*. However, *E. coli* does not possess the requisite genes encoding required maturation factors, so successful expression of active hydrogenase require that these, *hydEFG*, be co-expressed (Posewitz et al., 2004; King et al., 2006; Böck et al., 2006). An effective way to do this is to create an artificial operon

Table 1
Targets for modification of existing pathways[a].

Gene	Function	Mode of action	Effect on H_2 yields[b]	Reference
hyd1, hyd2	Uptake hydrogenases	Inactivation increases net H_2 by preventing H_2 oxidation	Modest increase (~35%)[c]	Bisaillon et al., 2006
ldhA	Lactate dehydrogenase	Inactivation increases H_2 by eliminating a drain on pyruvate	Moderate increase (~20–45%)[d]	Bisaillon et al., 2006; Maeda et al., 2007
frdBC	Fumarate reductase	Inactivation increases H_2 by eliminating side reaction in EMP thereby increasing pyruvate	Small increase (~15%)[e]	Maeda et al., 2007
hycA	Inhibitor of *fhlA* expression	Inactivation increases synthesis of FhlA, leading to increase in FHL	No to small increase (20%)[e]	Yoshida et al., 2006; Maeda et al., 2007
fhlA	Activator of expression of Fhl complex	Introduction of constitutive allele (fhlA*), leading to increase in FHL complex	Small (5–10%)[f]	Bisaillon et al., 2006

[a] Shown are targets identified and changed in *E. coli*. The same rationale should apply to other organisms if these targets are present.
[b] Although several studies have been reported on the effects of some of these mutations, in many cases the effects on rates and not yields were described. Here we have included studies that have either reported yields, or have sufficient experimental detail to be able to calculate the yields. Percent increases are given since there were large differences in baseline (wild-type) yields.
[c] Taken from Table 1, Bisaillon et al., 2006.
[d] Based on limiting glucose, Fig. 3 (Bisaillon et al., 2006) and Table 1 (Maeda et al., 2007).
[e] Table 2 (Yoshida et al., 2006), Table 1 (Maeda et al., 2007).
[f] Based on limiting glucose, Fig. 3 (Bisaillon et al., 2006).

carrying *hydA* and *hydEFG* under the control of a single strong *E. coli* promoter (Akhtar and Jones, 2008).

This type of system was used to introduce a ferredoxin-dependent NAD(P)H:H_2 pathway into an *E. coli* strain expressing a [FeFe] hydrogenase along with the requisite maturation enzymes, and ferredoxin (Veit et al., 2008). It is somewhat difficult to judge the degree of success of this approach. Hydrogen production was increased to some extent over background levels. Moreover, the authors were able to demonstrate variation of hydrogen production with putative partial pressures leading them to conclude that the system was impractical due to severe thermodynamic limitations.

However, levels of FeFe hydrogenase activity, even with the artificial operon approach, were quite low making it difficult to draw definitive conclusions. Additionally, H_2 evolution in this system is dependent upon cellular reduced nucleotide levels and it might be possible to use metabolic engineering to increase cellular NAD[P]H production. A theoretical analysis of metabolic fluxes suggested that up to 9 mol H_2/mol glucose could be obtained this way (Oh et al., 2008), but as acknowledged by the author, this figure does not take into account the unfavourable thermodynamics of such a process.

Very recently, a direct artificial pyruvate to hydrogen pathway was constructed using a variation of the approach outlined above but with generation of reduced ferredoxin from pyruvate by overexpression of YdbK, an endogenous "cryptic" PFOR (pyruvate:ferredoxin oxidoreductase). High yields (1.8 mol H_2/mol glucose) with respect to metabolic limitations (2 mol H_2/mol glucose) were achieved in a ΔicsR background with TPP (thiamine pyrophosphate) supplementation (Akhtar and Jones, 2009).

Another approach has previously been suggested where it was proposed to use reverse electron flow to reduce ferredoxin with NADH thus generating enough reducing power to drive hydrogen evolution by hydrogenase (Hallenbeck and Benemann, 2002). Of course, overcoming the thermodynamic barrier would require energy input. It was suggested that a small amount of respiration could be used to generate an electrochemical gradient that would drive ferredoxin reduction. This has not yet been tested and it is not immediately obvious how to construct the appropriate pathways.

However, systems that function in this manner in nature are known, although little understood. One such system is the membrane-bound *rnf* complex (Masepohl and Klipp, 1996). This has been shown to be present in a number of bacteria where it is thought to function to conserve energy under anaerobic conditions by generating an ion gradient when reduced ferredoxin is used to reduce NAD (Seedorf et al., 2008; Biegel et al., 2009). More importantly for the present point, driving hydrogen evolution, *rnf* complexes also thought to function in the reverse direction, using an electrochemical gradient to drive ferredoxin reduction from NADH in supporting nitrogenase activity (Kumagai et al., 1997; Jouanneau et al., 1998). Another example of reversed electron flow is found in some anaerobic bacteria such as *Acidaminococcus fermentans* and *Fusobacterium nucleatum*. In these organisms an electrochemical Na^+ gradient has been proposed to reverse the action of membrane-bound NADH:ferredoxin oxidoreductases thereby reducing ferredoxin with NADH. Reduced ferredoxin in turn drives hydrogen production (Boiangiu et al., 2005). Thus, in the future, it might be possible to adapt this system to driving biohydrogen production by dark fermentation past the present metabolic barrier.

3. Conclusions

Biofuels offer an at least partial solution to some of the problems posed by impending climate change and dwindling fossil fuel reserves. Biohydrogen could be an ideal biofuel, but its production is at present problematic with many technical challenges that must be overcome. Knowledge of existing microbial metabolisms leading to hydrogen production has suggested a number of metabolic modifications that have been shown to increase hydrogen production. However, further increases are necessary and might be possible through the application of various metabolic engineering strategies.

Acknowledgement

This work was supported by the Natural Sciences Research Council of Canada and Foreign Affairs and International Trade Canada (fellowship to D.G.).

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Journal of Environmental Management 95 (2012) S365–S368

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Hydrogen yield from a hydrogenase in *Frankia* R43 at different levels of the carbon source propionate

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ARTICLE INFO

Article history:
Received 31 August 2009
Received in revised form
17 November 2010
Accepted 4 January 2011
Available online 26 January 2011

Keywords:
Bioreactor
Frankia
Hydrogen
Hydrogenase
Propionate

ABSTRACT

Fermentative hydrogen yield was investigated in the *Frankia* strain R43, which was grown in different amounts of the carbon source propionate. In relation to hydrogen yield, the hydrogenase enzyme was characterized by use of Western blot. A bioreactor study revealed a 10-fold increase in growth within 50 h. The study showed that there is an active anaerobic hydrogen production in *Frankia* R43 and that this hydrogenase is immunologically related to the subunit HoxU of *Ralstonia eutropha*.

1. Introduction

A large number of different bacteria are able to ferment carbon sources and produce hydrogen by the aid of hydrogenases. These "dark fermentation" reactions do not require light energy therefore they are capable of forming hydrogen throughout all day and all night. One example of such bacteria is *Frankia* sp., which are filamentous and soil-inhabiting actinomycetous bacteria, with the capability to perform nitrogen fixation, i.e. utilize atmospheric nitrogen and convert it to ammonia. *Frankia* can accumulate in river and lake sediments (Huss-Danell et al., 1997), but the mechanism whereby they survive in these anaerobic environments is poorly understood. One of the mechanisms involved in the survival in sediments might include a hydrogenase (Mohapatra et al., 2004; Vignais and Billoud, 2007). It is known that *Frankia* sp. grow well on propionate, a carbon source found in swine manure (Miller and Varel, 2003).

Hydrogenases (reaction $2H^+ + 2e^- \Leftrightarrow H_2$) are enzymes serving a key role in hydrogen metabolism as catalysts both in hydrogen production and hydrogen oxidation; especially hydrogen production occurs in anaerobic condition. Several prokaryotic and eukaryotic microorganisms possess hydrogenase including *Frankia*. To date, hydrogenase has been classified into 3 major groups based upon biochemical aspects, and they are (i) [Fe]-Hydrogenase, (ii) [NiFe]-Hydrogenase, and (iii) [FeFe]-Hydrogenase (Vignais and Billoud, 2007). The *Frankia* hydrogenase described by Mattsson and Sellstedt (2000) belongs to the cluster of [NiFe] hydrogenase, the largest group of hydrogenase that is made up of NiFe center (Vignais and Billoud, 2007). An additional hydrogen-evolving function of *Frankia* hydrogenase has been presented (Mohapatra et al., 2004). However, the structure of this has yet not been dissected, but recent studies of *Frankia* hydrogenase producing hydrogen, suggests that it shows resemblance to the bidirectional hydrogenase from *Ralstonia eutropha*. This hydrogenase in *R. eutropha* is an enzyme consisting of 6 subunits: HoxFUYHWI, and only partial similarity among the subunits were observed (Grzeszik et al., 1997; Massanz et al., 1998; Burgdorf et al., 2005). The role for the hydrogen producing hydrogenases, also that in *Frankia*, might be to serve a sole function in energy conservation of some microorganisms and by fermenting organic materials releasing carbon dioxide and H_2 (Vignais and Billoud, 2007).

Frankia is a gram-positive filamentous nitrogen-fixing bacterium, living symbiotically as a nitrogen fixer with large spectrum of dicotyledonous plants, so-called actinorhizal plants. Up to date, three major clusters of *Frankia* sp. have been identified based on symbiotic association with plant species (Benson and Dawson, 2007). In the actinomycete *Frankia*, the hydrogen-evolving function of hydrogenase was firstly elaborated in the strain R43 and

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0301-4797/$ – see front matter
doi:10.1016/j.jenvman.2011.01.005

later also proved to occur in additional *Frankia* strains (Mohapatra et al., 2004, 2006; Leul et al., 2005). The hydrogen-evolving enzyme is active both with and without nitrogen added to the growth medium (Mohapatra et al., 2004). This is of importance when using waste streams for hydrogen production, since waste streams are likely to contain nitrogen (Miller and Varel, 2003). A characterisation of the enzyme was demonstrating the facts that the soluble hydrogenase was localised both in vesicles and hyphae (Mohapatra et al., 2006). Protein sequencing of the enzyme suggested a high similarity to bidirectional hydrogenase of cyanobacterial *Anabaena simensis*; however, it lacks of NAD-reduction activity distinguished from cyanobacteria bidirectional hydrogenase (Mohapatra et al., 2004; Leul et al., 2005). However, a correlation between nitrogenase and hydrogenase has been revealed in a condition of nitrogen deprivation (Mattsson and Sellstedt, 2002). Nickel is found to influence the function of hydrogen-evolving enzyme; some strains showed hydrogen production in the media deprived of nitrogen source, and that activity increased upon addition of nickel (Mohapatra et al., 2006). This leads to a speculation whether *Frankia* possesses more than one type of hydrogen-evolving hydrogenases in *Frankia* or a specific regulation of hydrogen producing hydrogenase in those strains of *Frankia* that is unique (Leul et al., 2005). Though there are studies about effects of nickel on the enzyme, the effects of carbon source has never been performed.

The aim of this experiment was to examine effects of propionate as a sole carbon source (i) on hydrogen production by hydrogenase, (ii) presence of the enzyme hydrogenase and (iii) the growth of *Frankia* strain R43 in a bioreactor.

2. Materials and methods

2.1. Frankia strain, culture and growth measurement

The *Frankia* strain R43 (LLR02022) isolated from *Casuarina cunninghamiana* Miq. (Lechevalier, 1986) was investigated regarding the function of hydrogen production by hydrogenase in this experiment. The strain was cultured according to a method of Mohapatra et al. (2004). In brief, the bacterium was firstly grown in approximately 100 ml of PUM medium (Mattsson and Sellstedt, 2000) with nitrogen source supplied with 6 different amounts of sodium propionate (4.6, 2.3, 0.46, 0.23, and 0.13 g L^{-1}) (as later called PUM + N) before incubated on a shaker at 27 °C for 8 days, as shown beneficial for *Frankia* growth and earlier described in Mattsson and Sellstedt (2000). Growth rate of such *Frankia* strain was assessed by mean of total protein concentration and three replicates were used. A total protein was extracted from 8-day *Frankia* grown in PUM + N using protein extraction buffer (PEB) (Agrisera, Sweden). Quantification of the protein was carried with a modified Lowry assay (Bio-Rad DC protein assay, Bio-Rad) using a Beckman spectrophotometer DU 530 Vis (Beckman Ltd., USA,) at absorbance of 750 nm. An equivalent volume of 10 ng μl^{-1} total protein of *Frankia* R43 grown in such concentrations were transferred to PUM media without nitrogen source (designated as PUM − N) medium with corresponding amounts of sodium propionate, put under shaking conditions, and cultured at 27 °C for another 8 days.

2.2. Hydrogen yield

An investigation of H_2-producing enzymatic activities was carried out by using sodium dithionite as electron donor and methyl viologen as election mediator (Plasterk et al., 1981). The reversible reaction: $2\ MV^{+} + 2H^{+} \Leftrightarrow 2MV^{2+} + H_2$ (Merge and Bourdillon, 1985) takes place. The *Frankia* cell suspension was taken after 8 days of growth in the flasks. A total reaction of 2 ml was prepared from a freshly mixture of 1 ml of sodium dithionite and methyl viologen in Tris HCl pH 7.0 and an equal volume of the *Frankia* cell suspension (Mohapatra et al., 2006), in three replicates. Argon was flushed into the vials for 3 min in order to generate anaerobic condition. Hydrogen evolution was recorded at 27 °C, starting at 90 min after incubation using a GC-8AIT gas chromatograph with a TCD detector (Schimadzu Scientific Instruments, Columbia, USA). In the cases of where hydrogen evolution could not be detected after 2 h, re-measurements were made after 24 h.

2.3. PAGE and western blotting

Proteins from the *Frankia* strain R43 were extracted using the protein extraction kit of Agrisera (Agrisera, Sweden). Polyacrylamide gel electrophoresis was carried out with 12–15% Ready gel SDS-PAGE (Bio-Rad, CA) prior to transfer all the proteins into nylon membrane (micron separations inc, Westborough, MA), as described in Leul et al. (2005). The success of protein transfer was assured by staining with Ponceau S before continued with western blotting. Primary antibodies raised against the hydrogenase subunit HoxU of *R. eutropha* (1:250 dilution, 12 h of incubation) were used to survey the presence of hydrogen-evolving enzyme. The secondary antibody rabbit-anti-chicken IgG was then applied at the ratio of 1:10,000 before incubated for 3 h. A horseradish peroxidase chemiluminescence was chosen to report the existence of the enzyme (ECL western blotting system, GE healthcare) with a help of illuminating system by the intelligent dark box LAS3000 (Fujifilm, Japan). Three replicates were used.

2.4. Growth in a bioreactor

To initiate growth, *Frankia* R43 was first grown in PUM in 500 ml bottles, and then the *Frankia* was transferred to a batch bioreactor (27 °C, 150 rpm, 0.46 g L^{-1}) and was grown in 15 L of PUM − N medium. The bacteria were grown in 16 days and three replicate samples were withdrawn for protein determination. However, only 45 h are presented in the graph since stationary phase was obtained after 45 h, and no significant change was observed after this. The growth rate of the bacteria was studied by use of increase in amount of protein, since it is difficult to count cells as *Frankia* have both vesicles and hyphae.

3. Results and discussion

3.1. The effect of carbon source on growth and hydrogen yield

Hydrogen is a widely recognized environmentally friendly energy source. The bacterium *Frankia* has been revealed as a hydrogen producing microorganism (Leul et al., 2005). This study emphasised on the effects of propionate as carbon source on hydrogen production from *Frankia* strain R43. The carbon source propionate is known to be present in swine manure (Miller and Varel, 2003) and it is believed that hydrogen could be produced from waste by the use of fermentative bacteria (Wang et al., 2008). The amount of sodium propionate varied from 4.6 g L^{-1} to 0.13 g L^{-1} in response to examine its effects on hydrogen production. According to the results, the strain responded to the given amount of sodium propionate as such; the growth rate increased most when sodium propionate was 2.3 g L^{-1} (Fig. 1). This suggests a difference in carbon metabolism among *Frankia* strains consenting with the previous finding that three Casuarina-isolated *Frankia* strains were distinctively responding to two different concentrations of sodium propionate (Sellstedt et al., 1994). The dramatic increase in growth rate was observed when the amount of

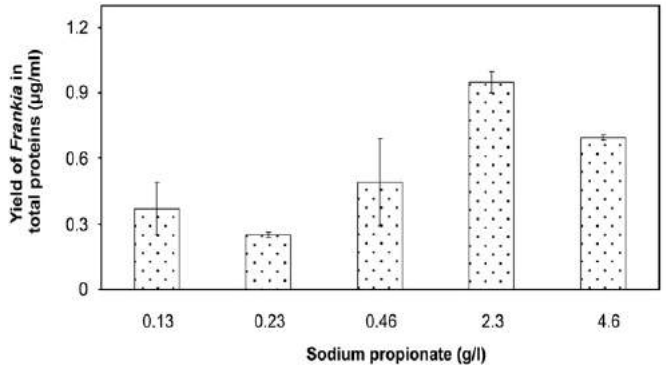

Fig. 1. Growth of *Frankia* strain R43 after 8 days, cultured on different concentrations of sodium propionate. Mean ± standard deviations, with the latter shown as bars.

propionate was raised from 0.23 g L^{-1} to 2.3 g L^{-1}; but the growth rate dropped if propionate was raised from 2.3 to 4.6 g L^{-1}.

3.2. Hydrogen yield

Hydrogen yield was revealed in the strain R43 cultured in one concentration of sodium propionate. The finding accentuates the previous report of the hydrogen evolution in the strain R43 by Mattson (2001), Mohapatra et al. (2004, 2006), and Leul et al. (2005). It was only carbon of 0.46 g L^{-1} that triggered hydrogen production to yield 6.8 nmol H_2 (mg protein)$^{-1}$ h^{-1}, corresponding to 170 ml l^{-1} (Fig. 2). On one hand, propionate is known to be the good carbon source for *Frankia* for it can be rapidly taken up via active transport (Stowers et al., 1986) even though there have not up to now been any concrete evidence demonstrating a correlation between hydrogen production via a hydrogenase and propionate in *Frankia* species. However, it is obvious that *Frankia* houses at least three types of enzymes, namely nitrogenase, uptake hydrogenase and hydrogen-evolving hydrogenase (Mohapatra et al., 2006), that are capable of manufacturing hydrogen in their reactions (Vignais and Billoud, 2007; Leul et al., 2005).

3.3. Protein analysis

Protein gel analysis and immuno-labelling (Fig. 3A and B) was introduced in this experiment to shed more lights into the results derived from gas chromatography. The primary antibodies raised against HoxU of *R. eutropha* were applied and radiated positive signals from two of the tested samples, as shown in Fig. 3B. One dominant band was approximately 30 kDa matching with HoxU of NAD-reducing hydrogenase of *R. eutropha* as reported by Grzeszik et al. (1997), Massanz et al. (1998) and Burgdorf et al. (2005). Interestingly, the distinct band in Fig. 3 is in accordance with the molecular weight of HoxU as shown earlier by Burgdorf et al. (2005). The recognized peptide was present at 0.46 g L^{-1}, while it was absent at 2.3 g L^{-1}. There was also a recognition of a peptide at 4.6 g L^{-1}, but the molecular weight of this peptide was higher, indicating an unprocessed protein, which has earlier been shown to occur in *R. eutropha* (Massanz et al., 1998) and *Frankia* KB5 (Mattsson and Sellstedt, 2000). This result supports the presence of hydrogen-evolving hydrogenase in the strain R43 and is in accordance with the works by Mohapatra et al. (2004, 2006) and Leul et al. (2005). To some extent, the subunits of SH-hydrogenase are believed to be conserved and may have a common origin (Massanz et al., 1998), this might be the reason why *Frankia* R43 hydrogenase with hydrogen production function shares homology with subunits in SH-hydrogenase of *R. eutropha* (Grzeszik et al., 1997; Massanz et al., 1998) and *Rhodococcus opacus* strains (Grzeszik et al., 1997). In addition, the recognition to a peptide as present in Fig. 3B in lane R1, shows presence of hydrogenase, but at a higher molecular weight. And, these physiologically inactive subunits still produce bands in denatured gels (Grzeszik et al., 1997; Massanz et al., 1998). In other words, we speculate that H_2-producing hydrogenase is manufactured even though there is no use of hydrogen as sole energy source. In addition, genomic investigation should be conducted to pave the way for a more detailed exploration of the enzyme.

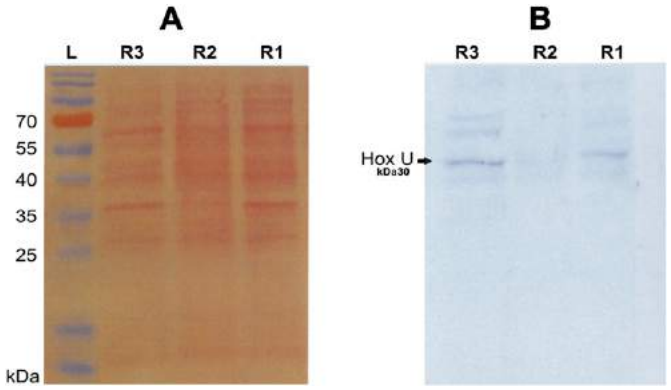

Fig. 3. Western blotting of the *Frankia* strain R43 fed with different levels of the carbon source propionate. A, nitrocellulose membrane after staining with Ponceau S, and B, nitrocellulose membrane after incubation with antibody raised against HoxU of *Ralstonia eutropha*. Lane L represents protein ladder; R1–R3 represent the *Frankia* strain R43 grown in 4.6, 2.3 and 0.46 g L^{-1} of propionate.

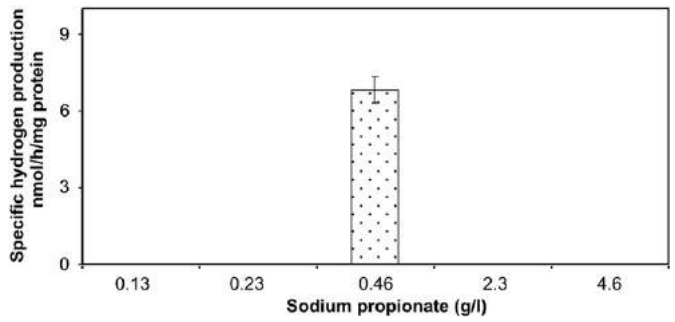

Fig. 2. Specific activity of hydrogen evolution from the *Frankia* strain R43 grown in different concentrations of sodium propionate, as detected by gas chromatography. Mean ± standard deviations, with the latter shown as bars.

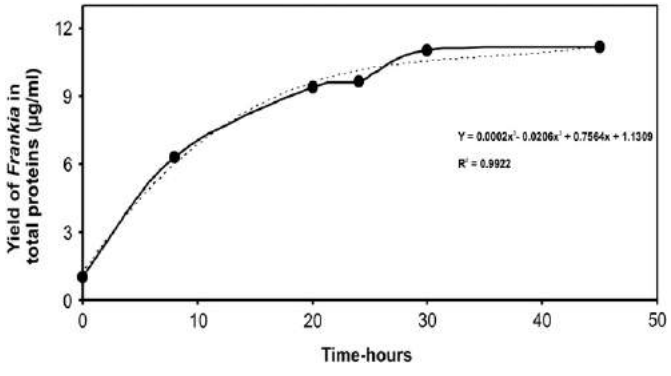

Fig. 4. Growth of Frankia in a bioreactor measured as increase in protein concentration when grown with 0.46 g L^{-1} of propionate. The dotted line represents the polynomal curve adapted to the equation in this figure. Mean ± standard deviations, with the latter being within the symbols.

3.4. Growth in a bioreactor

One μg ml^{-1} was put in the bioreactor and it was shown that there was a 10 times increase in growth after 50 h (Fig. 4). This is promising, since biotechnologically there is a need for fast increase in production of cells that will act as biocatalysts in hydrogen production. Earlier data on growth of *Frankia* in a bioreactor has shown that growth takes days rather than hours (Ringo et al., 1995). These data shows that *Frankia* really can be accounted for as a hydrogen producing bacterium.

4. Conclusions

1. *Frankia* R43 has a very efficient hydrogen producing hydrogenase that can be utilized as an energy producing catalyst.
2. Antibodies against *R. eutropha* HoxU recognized peptides in *Frankia* R43 protein extracts.
3. Level of carbon source had effect on growth and hydrogen evolution in *Frankia* R43, with 2.3 g L^{-1} showing the highest growth and 0.46 g L^{-1} showing the highest hydrogen producing activity.
4. Growth of *Frankia* R43 in a bioreactor increased the growth of *Frankia* 10 times.
5. The advantage of using this bacterium is that it switches to hydrogen production in an anaerobic environment and that it is a naturally occurring non-pathogenic bacterium.

Acknowledgements

Thanks are due to FORMAS for grant to Anita Sellstedt and to C. Tryggers for Rasika Kudahettige.

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