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Enumeration of methanotrophic bacteria in ricefield soils by plating and MPN techniques: a critical approach

S. Escoffier, J. Le Mer and P. A. Roger *

Laboratoire ORSTOM de Microbiologie des Anaérobies
Université de Provence – CESB/ESIL – Case 925,
163, avenue de Luminy, 13288 Marseille Cedex 9, France.

* *Corresponding author.*

E-mail: rogerpa@orstom.esil.univ-mrs.fr

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Abstract

The feasibility of using MPN and plate counts to enumerate methane oxidizing bacteria was tested in 18 rice soils. The NMS Whittenbury medium, considered most suitable for growing methanotrophs, allowed the growth of numerous non methanotrophic bacteria, actinomycetes and fungi on Petri dishes. Despite a satisfactory reproducibility of bacterial counts performed on dishes incubated with or without CH_4 in the incubation atmosphere, methanotroph populations could not usually be estimated from their difference because of (1) the low percentage of methanotrophs in the total population growing on NMS medium and (2) mycelial contaminations developing on dishes. Crystal violet and four antibiotics were found useful to reduce or delay contamination, and thus facilitating methanotroph isolation, but did not allow counting. Graham's staining was found efficient for identifying methanotrophic colonies on plates and did not affect their viability.

The MPN technique, using CH_4 consumption to identify positive tubes, provided stable and reproducible estimates of cultivable methanotrophs after 6-8 weeks of incubation.

Estimates of populations of cultivable methanotrophs ranged from 10^2 to 10^4 in dry soils and from 10^7 to 10^9 in soils preincubated under CH_4, which shows the strong inductive power of CH_4 on these populations and provide an indirect information on their high potential to oxidize CH_4 produced in ricefields.

Keywords: Methanotrophs, enumeration, counts, methods, MPN, plating, ricefield soil.

Étude critique de la numération des bactéries méthanotrophes dans des sols de rizières par étalement sur boîte et MPN.

Résumé

La possibilité de compter les bactéries qui oxydent le méthane par les méthodes du MPN et par étalement sur boîtes a été testée sur 18 sols de rizières. Le milieu NMS de Whittenbury, classiquement utilisé pour la culture des méthanotrophes, permet également la croissance de nombreuses bactéries non méthanotrophes, d'actinomycètes et de champignons filamenteux. Malgré la bonne reproductibilité des comptages bactériens sur les boîtes incubées avec et sans méthane, leur différence ne permet généralement pas d'estimer les populations de méthanotrophes car (1) leur pourcentage par rapport aux populations totales poussant sur milieu NMS est trop faible et (2) les contaminations mycéliennes sur les boîtes sont trop importantes.

L'utilisation de cristal violet ou d'antibiotiques, en réduisant ou ralentissant la croissance des contaminants, facilite le repérage et l'isolement des méthanotrophes mais ne permet pas leur numération. La méthode de coloration de Graham est efficace pour le repérage des colonies méthanotrophes et n'affecte pas leur viabilité.

La méthode du MPN utilisant la consommation de méthane pour identifier les tubes positifs, après 6 ou 8 semaines d'incubation, fournit une estimation stable et reproductible des bactéries méthanotrophes cultivables.

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Les populations de bactéries méthanotrophes cultivables sont comprises entre 10^2 et 10^4 dans les sols secs et entre 10^7 et 10^9 dans les sols préincubés sous méthane. Ces résultats montrent le fort pouvoir inducteur du méthane sur ces populations et donne une information indirecte sur leur fort potentiel pour oxyder le méthane produit par les rizières.

Mots-clés : Bactéries méthanotrophes, comptages, méthodes, MPN, étalements sur boîte, rizière, sol.

INTRODUCTION

Methane emissions from wetland rice fields have been estimated up to 170 Tg yr^{-1} which account for approximately 26% of the global anthropogenic CH_4 budget (Bouwman, 1990). The demand for rice will increase by 65% over the next 30 years (IRRI, 1989) and is most likely to be met by the existing cultivated wetland rice area through intensifying rice production. Global methane emissions from wetland rice agriculture are therefore likely to increase if mitigation techniques are not established.

Methanotrophic bacteria, or methanotrophs, are a subset of the physiological group of methylotrophs, which utilize a variety of one-carbon compounds (Hanson & Hanson, 1996). Methanotrophs are unique in their ability to utilize methane as sole carbon and energy source. They play an important ecological role in reoxidizing part of the CH_4 and reducing the environmental impacts of the release of CH_4. More than 60% of CH_4 produced is reoxidized by methanotrophs (Holzapfel-Pschorn & Seiler, 1986), therefore CH_4 emission from ricefields, as a balance between production and oxidation, is very significantly controlled by methanotrophy. A promising approach to reduce CH_4 emission by ricefields is to enhance methanotrophy by cultural practices such as introducing drainage periods during the cultural cycle (Sass *et al.*, 1992; Watanabe A. *et al.*, 1995).

Methane oxidation occurs in the oxidized environments of the ricefield ecosystem: the submersion water, the water/soil interface and the rhizosphere (Sass *et al.*, 1990). Little is known about the nature and dynamics of methanotrophic populations in ricefield soils. At now, only two identified strains of methanotrophs were isolated from ricefields: *Methylosinus sporium* (Bowman *et al.*, 1993) and *Methylocystis* sp. (Takeda, 1988). Quantitative estimates are also scarce. This is probably due to the difficulties encountered in enumerating and isolating methanotrophs. In the few trials to enumerate methanotrophs in natural environments, the classical MPN technique was used for water samples (Zaiss 1981; Zaiss *et al.*, 1982), and the MPN microtechnique (Rowe *et al.*, 1977) for ricefield soil samples (Bender & Conrad, 1992). The plating method was used by Watanabe I. *et al.* (1995) using the naphtol reaction (Graham *et al.*, 1992) to identify colonies of methanotrophs.

The main limitation for methanotroph enumeration by traditional methods results from the lack of an efficient selective medium. Methanotrophs growing on Petri dishes inoculated with soil suspensions usually represent a small percentage of total growing colonies. Whittenbury *et al.* (1970) reported contamination problems resulting from high humidity which developed in containers used to incubate dishes in an atmosphere enriched with CH_4 (usually 20% CH_4), and the presence of barely visible colonies of actively motile small Gram-negative rods that did not utilize CH_4. Whittenbury *et al.* (1970), Whittenbury & Dalton (1981) and Hanson *et al.* (1992) also often observed a dense growth of mycelial contaminants (actinomycetes and fungi) on dishes. Hanson *et al.* (1992) reported that methanotroph colonies were 10-100 fold less numerous than non-methane utilizing colonies on NMS Petri dishes. In a previous study of a Camargue soil, we found that non-methanotrophic colonies represented at least 65% and often more than 90% of the colonies forming units (CFU) growing on Whittenbury NMS medium (Le Mer *et al.*, 1996). But, contrarily of what was reported by Hanson *et al.* (1992) and Whittenbury & Dalton (1981), we rarely observed amoebae on dishes.

Several authors pointed out that methanotrophs growing on NMS may reflect the conditions employed for growth more than the original population (Whittenbury *et al.*, 1970; Whittenbury & Dalton, 1981; Hanson, 1992) and it is not evident that the methanotrophs in culture are representative of the methanotrophs that are active in situ (King, 1996). Methane concentrations in natural environments are usually expressed in nM: <10 nM in the photic zone of oceans or <2.5 nM in oxic soils, according to King (1996). Therefore, methanotrophs should be in most instances considered as oligotrophic bacteria. The usual method of methanotroph isolation with CH_4 concentrations in the gas phase between 20 and 50% (V/V) – resulting in initial dissolved CH_4 concentrations between 0.28-0.7 mM – might be often inappropriate because (1) it selects copiotrophic methanotrophs that are not active under oligotrophic conditions and (2) it does not allow oligotrophic strains to develop. King (1996) suggested to use low CH_4 concentrations and long term enrichment to isolate oligotrophic methanotrophs. Poindexter (1981) considered as oligotrophic the aquatic systems – and organisms living in them – where carbon flux is lower than 10 $\mu mol\ l^{-1}\ d^{-1}$.

However, in wetland ricefields, these value might be 10 to 100 – fold higher (Denier van der Gon, 1996) and active strains might be considered copiotrophic; therefore, a concentration of 20% CH_4 in the gas phase is most probably appropriate for growing/isolating them (it must be kept in mind that CH_4 concentrations between 5 and 15% in air are explosive).

This paper deals with the feasibility of classical microbiological methods – MPN and plate counts – to enumerate methanotrophs in rice soils. The study of counts by plating on solid medium tested (1) various gelifying agents, (2) additives that could inhibit mycelial and actinomycetal contaminations, and (3) the dynamics of counts. The study of counts by MPN tested the effect of (1) N-source, (2) shaking of the medium, (3) the duration of the incubation, and (4) the method of reading. After determining the most appropriate conditions, both methods were compared for enumerating methanotrophs in a range of rice soils using dry samples and samples enriched in methanotrophs by preincubation under CH_4.

MATERIALS AND METHODS

Ricefield soils

Enumerations were performed on 18 ricefield soils originating from Australia (1), Trinidad (1), California (2), Columbia (1), France (1), and the Philippines (12). The samples from the Philippines were chosen to constitute a representative sample of rice soils encountered in this region.

Media

Media used for counting or isolating methanotrophs were derived from the original salt medium NMS of Whittenbury *et al.* (1970) by using EDTA-Ferric-Sodium salt (Sigma: 15708-41-5) at 0.04 g l^{-1} instead of the Sequestrene iron complex as iron source, as suggested by Henry & Grbic-Galic (1991). The pH of the medium was adjusted to 6.8 before autoclaving at 120°C for 20 min. Two ml of sterile phosphate buffer solution (mixture of Na_2HPO_4 and KH_2PO_4, 15 g in 300 ml distilled water) were added per liter of cooled medium, after autoclaving.

Experiments were conducted with CH_4 as sole carbon source. All incubations were performed under an air/CH_4 (80/20 v/v) mixture.

Enumeration by plating

Plate preparation and incubation

Enumerations were performed on 7 dry soils (Luisiana, Pila, San Dionisio, Amurao, Australia, Maahas, Trinidad) and 4 soils previously incubated for two months at water field capacity under air/CH_4 (80/20 v/v) (Camargue, Australia, Maahas, and Trinidad).

The 10^{-1} soil suspension-dilutions were prepared by using 10 g of dry soil and 90 ml of sterile physiological water stirred for one hour. Successive 10-fold dilutions were then prepared and 0.1 ml of each suspension/dilution was spread on the surface of agar plates using five replicates. Because of the non-selectivity of the NMS medium (Whittenbury *et al.*, 1970), two series of plates were prepared. Both were placed in sealed plastic bags and incubated at 30°C, the first in air and the second in a 80% air/20% CH_4 atmosphere, in order to determine the difference between the number of bacterial colonies growing in the presence of CH_4 (called thereafter Total Bacteria [TB]) and the number of bacterial colonies growing in the absence of CH_4 (called thereafter Bacterial Contaminants [BC]). The difference between TB and BC, expected to correspond to methanotrophs, is called thereafter DTC. We also recorded the percentage of dishes where a dense growth of fungi and actinomycetes refrained counting bacterial colonies.

Counts were performed weekly for 30 days. The first count was done after three days of incubation, because contaminants appear after this time while methanotrophs usually appear only after five days (Hanson *et al.*, 1992).

Gelifying agents

Three gelifying agents were tested: Agar, silicagel, and Gelrite.

- Bacto agar (DIFCO, Detroit, Michigan, U.S.A) was used at 17 g per liter of NMS medium.
- Silicagel was prepared according to a method derived from Mouraret & Baldensperger (1977). An aqueous solution of sodium silicate was prepared from the Prolabo reagent n° 28.084 (d: 1.33, containing 26% silicon dioxide and sodium monoxide) which was diluted in sterile water (1/3.55 v/v) and passed through a strongly acid cation column of Amberlite IRN77 (Prolabo n° 27.362.296). The plates were prepared by (1) mixing (1/1, v/v) the diluted sodium silicate solution with the NMS medium concentrated four times, (2) adjusting pH to 6.8 with KOH 0.1M, and (3) pouring 25 ml of the mixture into sterile glass Petri dishes, which were left over night to allow solidification.
- Gelrite media were prepared according to Lin & Casida (1983) in three steps: (1) dissolution of $CaCl_2.2H_2O$, 0.1% solution in NMS medium with NH_4NO_3 as nitrogen source and of Gelrite in distilled water; (2) autoclaving each solution for 20 min at 120°C; (3) cooling the solutions to 60°C and mixing them before dispensing into plastic Petri dishes at 20 ml per plate.

Selective agents

Because all known methanotrophs are Gram negative, we tested the potential of 13 selective agents

for eliminating eukaryotes, especially fungi, and Gram positive contaminants (actinomycetes and bacteria).

Crystal violet is a staining agent of the murein present in the cell wall of Gram positive microorganisms. It inhibits them by affecting cellular permeability. All known methanotrophs are Gram negative while actinomycetes are Gram positive. Crystal violet was tested at 2, 5, and 10 ppm. Bowman *et al.* (1993) used Crystal violet as a discriminating agent for methanotrophs and observed some inhibition at 10 ppm.

Antibiotics to be tested were selected from a preliminary screening performed by placing 0.5 cm diameter filter paper disks, impregnated with antibiotic solution, on the center of a Petri dish previously inoculated with a contaminant suspension collected from various heavily contaminated dishes.

Bacitracin is known to inhibit a phosphohydrolase involved in the biosynthesis of the murein of the cell wall of Gram positive bacteria. Known methanotrophs are Gram negative bacteria resistant to bacitracin and novobiocin (Green, 1992). Nystatin and cycloheximide are classical inhibitor of fungi (Shearer, 1987).

Concentrations tested were: actidione: 50 ppm; nystatin: 100 U/ml; novobiocin: 10 ppm; bacitracin 30-100 ppm. Antibiotics were also tested in combinations.

Strain isolation from plates

We used the colorimetric assay developed by Graham *et al.* (1992) to detect soluble methane monooxygenase (sMMO) and identify type II methanotrophs on plates. Colonies that developed a purple coloration were counted and individually transferred on NMS agar slopes in Hungate's tubes flushed with air/ CH_4 (80/20 v/v) mixture and incubated at 30°C.

The methanotrophic potential of colonies transferred on NMS agar slopes in Hungate's tubes (collected at random from plates or selected by the sMMO colored reaction) was tested by measuring CH_4 consumption. Gas samples were directly injected in a gas chromatograph (Chrompack CP 9000) equipped with two thermal conductivity detectors and a double column set up allowing to detect CH_4, O_2, CO_2, and N_2 (Le Mer *et al.*, 1996). With regard to the low sensitivity of methane measurement by catharometry, the growth of methanotrophic strains was considered significant when more than 20% of the CH_4 initially present in the tube was oxidized. The arbitrary threshold of 20% insured that CH_4 was significantly consumed in the tube.

MPN counts

Suspension/dilutions of the 18 dry soils were prepared as previously described. Counts were performed in triplicate by preparing three series of dilutions from three soil subsamples from each soil. 0.5 ml of each suspension/dilutions was inoculated in triplicate Hungate's tubes containing 4.5 ml of Whittenbury NMS. Tubes were flushed with a 80% air/20% CH_4 mixture and incubated at 30°C. Enumerations were also performed on four soils (Camargue, Australia, Trinidad, Philippines-Pila) preincubated under CH_4 for at least one month. The whole preincubated soil sample (corresponding to 50 g dry soil) was used to prepare the 10^{-1} dilution. Counts were duplicated using two preincubated soil subsamples for preparing dilutions. Additional experiments were conducted with preincubated Camargue soil to test the effect of shaking on MPN counts. Two sets were incubated without agitation and two were incubated in a gyratory shaker. A tube was considered positive when more than 20% of the CH_4 initially present was oxidized.

We also studied the correlation between CH_4 consumption and the increase in O.D. in MPN tubes after 50 days of incubation, in order to determine if O.D. measurements could replace CH_4 measurements for identifying positive MPN tubes. Both dry and preincubated soils were tested. Optical density (mean of 10 replicates) was measured at 580 nm with a Shimadzu UV 160 Ä spectrophotometer.

RESULTS AND DISCUSSION

Statistical distribution of bacterial counts

The large number of enumerations performed in the course of the experiments allowed to characterize the distribution law of the counts according to the study of the correlation between mean and variance of replicated counts ($m\text{-}s^2$ test) as described by Roger (1996). The correlations between mean and variance of counts of colonies on replicated Petri dishes ($3 < n < 5$) inoculated with soil suspensions and incubated with or without CH_4 were highly significant (*fig.* 1). The slopes (b) of the regression curves on a log-log scale were close to 2, indicating a log-normal distribution of the counts. A log-normal distribution of the data was also observed with the MPN counts ($r^2 = 0.938$, $b = 2.2$). The practical implications for sampling strategies and statistical analysis of microbial counts following such a distribution were discussed by Roger (1996). In particular, statistical analysis and correlation studies should be performed on the logarithms of the data. In addition, the confidence intervals of replicated measurements were dissymetrical and increased with the value of the mean.

Reproducibility and significance of counts

Plating counts

When estimating microbial populations by plating, competition may reduce counts on crowded dishes and refrain the use of counts at two successive dilutions to

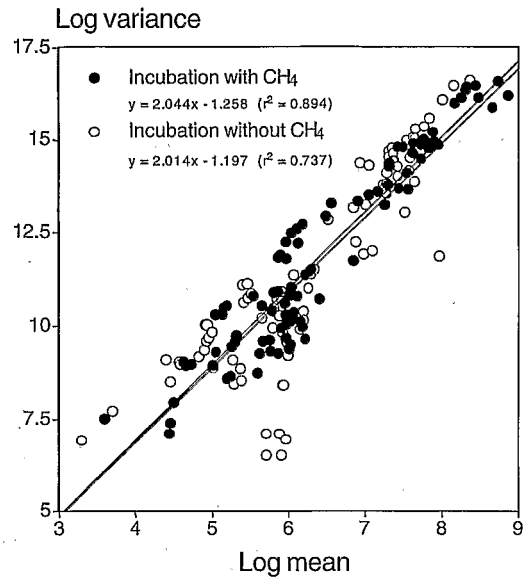

Figure 1. – Correlation between mean and variance of replicated plate counts.

improve the accuracy of the estimates (Roger *et al.*, 1991). *Figure* 2 shows a linear correlation ($r^2 = 0.90$) between the logarithms of counts at two successive dilutions (the number of colonies counted per dish ranged from 10 to a few hundred). The slope of 0.92 indicates an acceptable similarity of estimates obtained at two successive dilutions, which allowed to average counts to improve the accuracy of the estimates.

Replicated counts performed on dishes incubated with (TC) or without (BC) CH_4 showed a satisfactory reproducibility. The coefficients of variations (C.V.) of the logarithms of replicated counts ranged from 3.4% to 5.2% of the mean for dry soils (Pila, $n=46$ and Luisiana, $n=70$) and from 3.0% to 7.7% for wet soils (Trinidad, $n=12$ and Maahas, $n=50$).

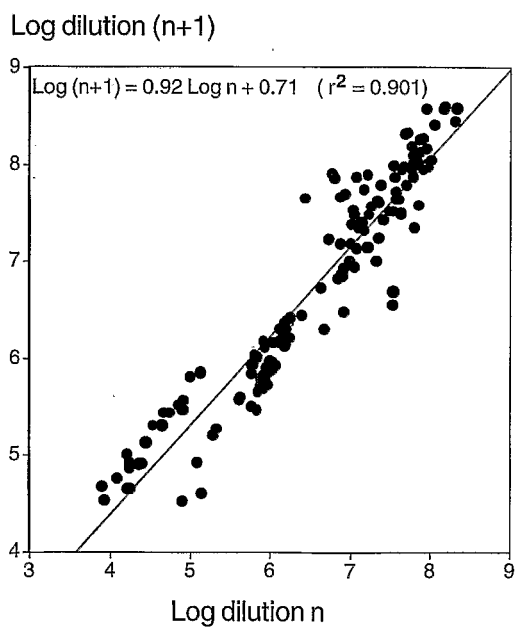

Figure 2. – Correlation between plate counts at two consecutive dilutions.

Estimating methanotrophic populations from the difference in CFU between dishes incubated with and without CH_4 (DTC) requires this difference to be statistically significant. Results indicated that the method was not applicable to dry soils. Among 23 samples corresponding to seven soils, no statistically significant difference was found between counts performed in the presence and the absence of CH_4 (*table* 1*a*). This obviously indicated a very low percentage of putative methanotrophs in the total population of dry soils. When counts were performed on soils preincubated under CH_4 and therefore bearing much higher populations of methanotrophs than dry soils, the level of significance of the difference remained very low with p values ranging from 0.07 to 0.48 (*table* 1*b*).

MPN counts

The average C.V. of MPN counts, estimated from three to seven replicated measurements on 18 soils, was 9.7% and the highest value was 19.3% (*table* 2). Such C.V. values indicate a satisfactory reproducibility of the MPN technique applied to methanotrophs.

Table 1. – Student test of the significance of the difference between bacterial counts on plates incubated in the presence (TB) and in the absence (BC) of CH_4.

1a: Non-preincubated soils.

Soil	Replicates nb*	log TB	log BC	Probability of t
		logN g^{-1} soil dw		
Louisiana	9	5.71	5.77	0.90
Pila	6	5.72	5.61	0.78
Amuroa	2	5.91	5.87	0.70
San Dionisio	2	4.60	4.69	0.73
Australia	2	6.13	6.10	0.72
Maahas	1	5.13	4.94	
Trinidad	1	6.38	6.26	
All soils	23	5.62	5.60	0.89

* number of replicated counts.

1b: Soils preincubated for one month under CH_4.

Soil	nb	log TB	log BC	Probability of t
Australia	2	8.94	8.65	0.07
Maahas	3	9.50	9.11	0.21
Trinidad	3	8.78	8.64	0.24
Camargue	2	9.23	9.06	0.48
All soils	10	9.11	8.87	0.28

Table 2. – Reproducibility of MPN counts performed on 18 dry soils.

Soils	Repetitions (n)	$\log_{10}$ of the count mean	C.V. (%)	Methanotrophs (nb.g^{-1} dry soil)
California 2	3	4.68	6.21	5.5×10^4
Pila	7	4.06	7.74	1.1×10^4
Trinidad	3	4.04	2.83	1.1×10^4
Luisiana	4	3.96	12.98	1.4×10^4
Maahad saline	3	3.71	12.47	6.8×10^3
Maahas + g.m.*	3	3.68	7.90	5.5×10^3
Urdaneta	3	3.68	19.32	1.0×10^4
Bugallon	3	3.57	15.78	6.4×10^3
Maahas	3	3.57	4.13	3.8×10^3
Cali	3	3.54	14.43	5.0×10^3
California 3	3	3.54	14.43	5.0×10^3
Binalonan	3	3.49	7.88	3.5×10^3
Amurao	3	3.48	4.23	3.2×10^3
Camargue	2	3.40	0.00	2.5×10^3
Maligaya	3	3.33	9.57	2.5×10^3
Australia	3	3.01	12.41	1.3×10^3
San Dionisio	3	2.48	5.94	3.2×10^2
Lal-lo	3	2.26	15.80	2.3×10^2

* g.m.: green manure.

Dynamics of the counts

Plating counts

Counts were performed at intervals for 20 to 30 days on Petri dishes inoculated with suspension-dilutions of four dry soils and four soils preincubated under CH_4. *Figure* 3 presents the dynamics of the counts of Total Bacteria (TB) (dishes incubated under CH_4), Bacterial Contaminants (BC) (dishes incubated without CH_4) and their difference (DTC) in Maahas soil.

In Australia, Trinidad, Maahas, and Pila dry soils, TB, BC and DTC stabilized more rapidly than with preincubated soils, not much change in the counts and their difference was observed after 10 to 15 days. However it must be kept in mind that in most cases, the difference between TB and BC was not significant.

With preincubated soils, TB and BC increased for at least 20 days in three of the soils. DTC stabilized within 15 days in three of the soils but kept on increasing till 20 days with Maahas soil. In both Maahas and Trinidad soils, counts at 30 days were refrained by a heavy contamination by fungi and actinomycetes.

Table 3. – Relative DTC (% of TB) expected to correspond to methanotrophs during 30 days of incubation of four preincubated soils*.

Incubation (days)	Soils			
	Australia	Camargue	Maahas	Trinidad
7	59.1	48.1	63.9	23.1
15	48.2	32.4	63.1	19.4
23	49.0	33.8	58.8	17.2

* DTC is calculated from the difference between TB and BC.

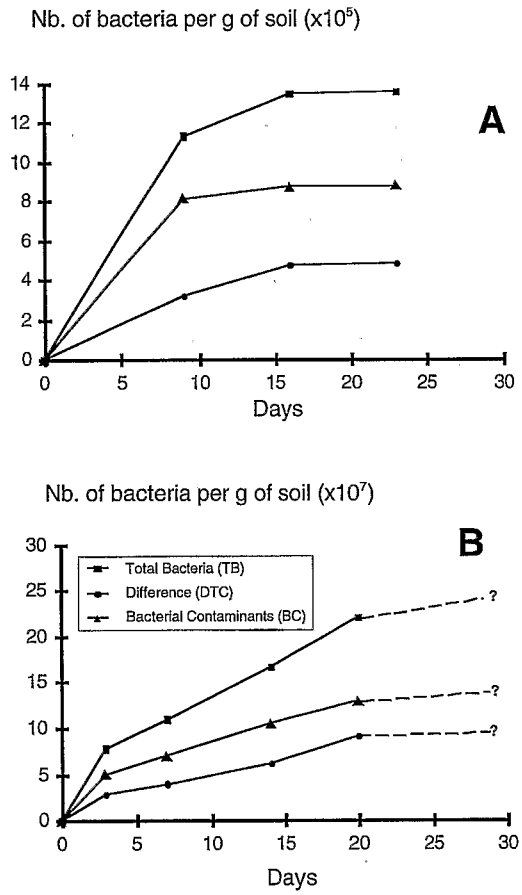

Figure 3. – Dynamics of plate counts on dry soils and preincubated Maahas soil. ? Counts impossible due to mycolial contamination.

In preincubated soils, DTC had a low level of significance ($0.07 < p < 0.2$) in three soils and was not significant in one soil (*table* 1*b*). However, when expressed as a percentage of TB it decreased with time in all soils tested (*table* 3). This tendancy indicates that colony isolation from petri dishes should rather be performed at an early stage of incubation as already observed by Whittenbury *et al.* (1970), Hanson *et al.* (1992), and Graham *et al.* (1992). The correlation between log DTC and log TB was highly significant ($r = +0.738$, $n = 31$, $p < 0.01$) because DTC constituted a significant percentage (48% on the average) of TB. The correlation between DTC and BC was not significant ($r = +0.212$, $n = 31$, $p > 0.10$), which indicated in particular that contaminants had no inhibitory effect on presumed methanotrophs. Therefore, the observed decrease of DTC expressed as a percentage of TB (*table* 3) was not due to an inhibition by the BC but possibly to the delayed growth of bacteria scavenging the metabolic products of methanotrophs (Starostina *et al.*, 1990).

MPN counts

MPN counts performed on dry samples of Luisiana (*fig.* 4), and Pila soils (data not shown) exhibited a

significant lag for about two weeks and reached a plateau after 20-30 days of incubation. In dry ricefield soils, where sporulated type II methanotrophs are probably dominant (Le Mer *et al.*, 1996), a relatively long lag corresponding to spore germination and active cell development was not surprising.

In a soil preincubated under 20% CH_4 for one month, which had reached its maximum potential methanotrophic activity (Le Mer *et al.*, 1996), no lag phase was observed and a plateau was also observed after about 30 days of incubation.

These results indicate that 6-8 weeks incubation are required for ensuring reliable MPN counts of methanotrophs.

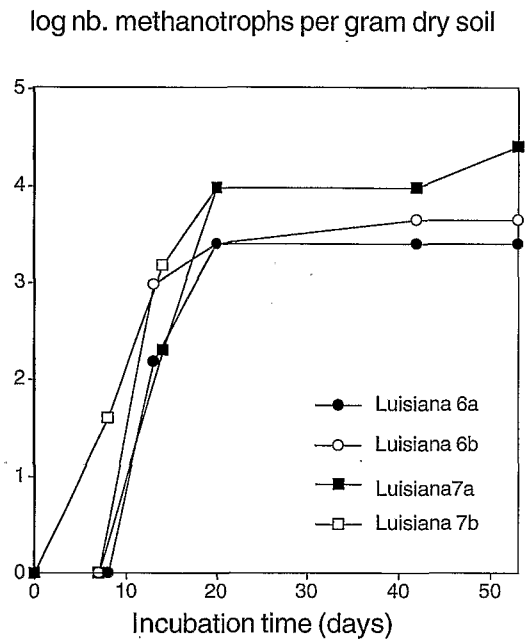

Figure 4. – Dynamics of MPN counts on duplicated enumerations on two samples of Luisiana dry soil.

Plate counts

Effect of various gelifying agents

Silicagel plates required much more time and care for preparation than gelose plates and did not solve the problem of mycelial contamination which was as heavy than that on gelose plates. Moreover, water released from silicagel formed a joint preventing gas diffusion in the Petri plates incubated upside down. No significant difference was observed between the average of 76 paired counts performed on agar and phytagel (data not shown). Phytagel allowed a slightly easier counting because of its clearness, but some lysis by actinomycetes and bacteria was noted. Bacterial contaminants were not affected. Phytagel cost was higher than that of gelose. Therefore, further counts were performed on media solidified with 1.7% gelose.

Mycelial contamination

Both NMS plates incubated with and without CH_4 in the incubation atmosphere were contaminated by actinomycetes and fungi. Similar levels of contamination were observed on plates inoculated with dry or preincubated soils. Actinomycetes were usually recorded after five days of incubation (*fig.* 5, control). Their abundance varied with soils. Sporulation usually started after about 10 days, prohibiting successively step strain isolation and counting bacterial colonies because of the rapid spreading of actinomycetes all over the plates. After one month, most plates were unreadable.

Fungi were usually recorded after about one week of incubation. They sporulated rapidly and invaded plates after two weeks. Contamination by fungi was usually less in terms of number of colonies per plate than by actinomycetes, but was as cumbersome because of the size of the colonies. As with actinomycetes, most plates were unreadable after one month of incubation.

Various antimycelial agents were tested to avoid those contaminants.

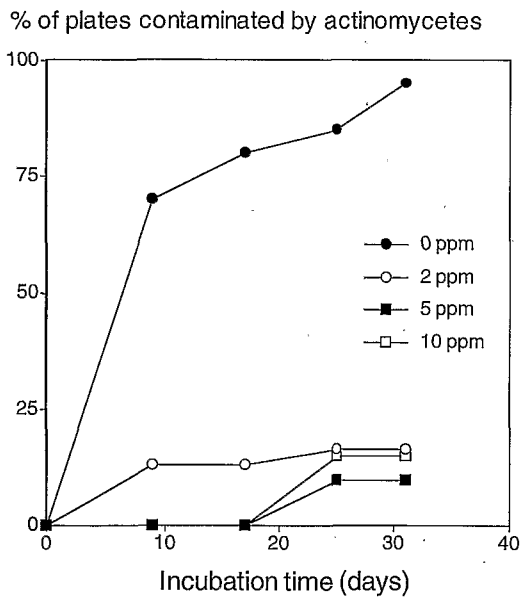

Figure 5. – Effect of crystal violet on plate contamination by actinomycetes (Australia dry soil).

Use of crystal violet and antibiotics to control mycelial contaminants

Inhibition was recorded after two weeks on the test with the filter paper disks. Antimycin A, tetracyclin, vancomycin, D-cycloserin, neomycin, benzalkonium chloride, chloramphenicol, and ofloxacin were inefficient. Actidione, nystatin, novobiocin, and bacitracin exhibited a significant inhibitory effect.

Crystal violet had a clear inhibitory effect on actinomycetes; 2 ppm was enough to markedly reduce their population. Higher concentrations did not fully

eliminate actinomycetes but delayed their growth (*fig.* 5). However, it also significantly ($p=0,05$) decreased (1) TB, BC and DTC and increased the relative abundance of putative methanotrophs (*table* 4). Results indicate that crystal violet at 2 or 5 ppm did not improve counts by plating, but by reducing contamination by actinomycetes and increasing the relative abundance of putative methanotrophs, could be useful for strain isolation.

Table 4. – Effect of crystal violet on bacterial counts on plates [1].

Crystal violet (ppm)	TB[2]		BC[3]		DTC[4]	
	nb g^{-1} dry soil	%	nb g^{-1} dry soil	%	nb g^{-1} dry soil	%
0	7.4×10^5	100	6.4×10^5	100	9.8×10^4	13
2	4.0×10^5	54	2.6×10^5	41	1.6×10^5	34
5	1.2×10^5	16	6.7×10^4	10	5.5×10^4	45
10	2.7×10^4	4	1.0×10^4	2	1.7×10^4	64

[1] Dry soil from Australia. Counts were performed after two weeks of incubation.

[2] TB: Counts on dishes incubated in the presence of CH_4.

[3] BC: Counts on dishes incubated in the absence of CH_4.

[4] DTC: difference between TB and BC, expected to correspond to methanotrophs.

Bacitracin did not markedly affect bacterial counts (TB, BC, DTC) or the relative abundance of putative methanotrophs (%DTC), but significantly reduced contamination by actinomycetes (*table* 5).

Table 5. – Effect of bacitracine on actinomycetes contamination and bacterial counts on plates [1].

Bacitracine (ppm)	TB[2]	BC[3]	DTC[4]	DTC (%)	plates contaminated by actinomycetes (%)
	nb. g^{-1} dry soil				
0	1.9×10^7	2.0×10^7	-1.1×10^6	−6	100
30	1.1×10^7	9.2×10^6	1.8×10^6	16	65
50	1.0×10^7	8.2×10^6	2.0×10^6	19	53
70	9.4×10^6	8.3×10^6	1.1×10^6	12	31
100	9.3×10^6	8.1×10^6	1.3×10^6	14	33

[1] Dry soil from Trinidad. Counts were performed after two weeks of incubation and contamination was estimated after one month [2, 3, 4]. see definitions in *table* 4.

Antibiotic mixture (*table* 6) significantly decreased contamination by actinomycetes. Simultaneously DTC was significantly reduced. Surprisingly, antibiotic mixtures had little effect on contamination by fungi estimated after one month of incubation, however it delayed their growth on the plates by about five days, thus facilitating strain isolation.

Table 6. – Action of combined antibiotics on mycelial contamination and bacterial counts on plates [1].

Treatment	TB[2]	BC[3]	DTC[4]	DTC (%)	% of contaminated plates	
					Actino-mycetes	Fungi
Control	1.1×10^9	5.1×10^8	5.5×10^8	52	53	90
nyst + act	6.3×10^8	5.4×10^8	9.3×10^7	15	15	85
nyst + act + bac + novo	9.6×10^7	7.3×10^7	2.3×10^7	24	0	85

[1] Preincubated soil from Camargue. Counts were performed after two weeks of incubation and contamination was estimated after one month [2, 3, 4]. see definitions in *table* 4.

Colony staining by naphthalene

Naphthalene staining was tested on plates inoculated with three dry soils and one preincubated soil. Percentages of stained colonies averaged 2% with dry soils and 13% with the preincubated soil. Stained colonies were individually streaked on NMS slope agar tubes and incubated under air/CH_4. All colonies exhibited a clear methanotrophic activity. Results demonstrated that naphthalene staining is an efficient method for isolating methanotrophs that possesses a soluble methane- monooxygenase because they apparently keep their viability after the staining allowing their identification. This assay could not be used on plates treated with crystal violet because stained colonies were not clearly differenciated on the violet background.

MPN counts

Nitrogen source

Methanotroph counts by MPN were significantly lower on NH_4NO_3 as compared with NO_3^- (*table* 7). In the tubes containing NH_4NO_3, pH rapidly decreased to about 5, a value inhibitory for methanotrophy. This was obviously due to the well known nitrifying action of methanotrophs (Bedard & Knowles, 1989; King & Schnell, 1994).

The ANMS medium, which contains NH_4NO_3, has been used for isolating methanotrophs from natural environments (Holmes *et al.*, 1995). This medium will

Table 7. – Effect of the nitrogen source on MPN counts (nb. bact.g^{-1} dry soil).

Soils	Nitrogen source	
	KNO_3	NH_4NO_3
Maahas (dry soil)	3.8×10^3	1.1×10^2
Australia (dry soil)	1.3×10^3	2.0×10^2
Australia (preincub. soil)	2.9×10^9	2.1×10^7

underestimate populations if not buffered. It is known that (1) all the methanotrophs can grow on ammonium as nitrogen source, (2) only few strains cannot use nitrate and (3) the growth of nitrate utilizers is better on this substrate than on ammonium (Whittenbury *et al.*, 1970). Therefore, the NMS medium will usually be more suitable than ANMS for methanotroph enumerations.

Shaking

Shaking delayed methanotroph growth and reduced counts by about 10 times (Le Mer *et al.*, 1996). Stable counts were obtained after 20 days for the unshaken control and after 30-40 days for the shacked tubes. Most probably, shaking, which was intended to increase CH_4 availability, simultaneously increased oxygen availability, whereas methanotrophs in general and especially those of type II are microaerophilic (Roslev & King, 1996; Liefke & Onken, 1992). Possibly shaking also dislodged bacterial cells from tube wall where they seem to grow preferentially. However no data were obtained to substantiate these hypothesis.

Relation between optical density and methanotrophy in MPN tubes

In tubes inoculated with preincubated soil, CH_4 consumption (%) and O.D. increase (O.D. after 50 days of incubation minus O.D. at T_0, thereafter called ∂OD) were positively correlated ($p<0,01$) (*fig.* 6). When ∂OD was higher than 0.1, more than 20% of the initial CH_4 was consumed and all tubes could be considered positive (15 data in *figure* 6). When ∂OD was smaller than 0.1 (23 data in *figure* 6) it was not possible to conclude, about 50% of the corresponding CH_4 consumption being lower than 20%. In tubes inoculated with dry soil, there was no correlation between CH_4 consumption and ∂OD (data not shown).

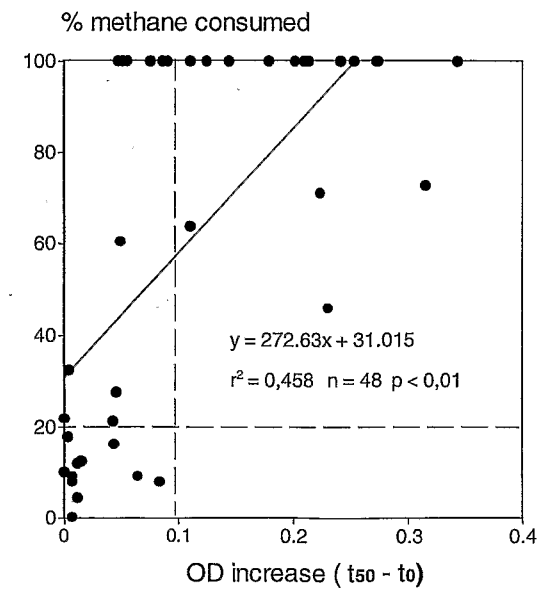

Figure 6. – Correlation between CH_4 consumption and change in optical density in the MPN tubes of Trinidad preincubated soil.

To quantify the implication of the bacterial contaminants in ∂OD, we incubated a set of MPN tubes without CH_4. In tubes inoculated with preincubated soil, we noted a slight growth (∂OD < 0.07). With dry soil, a number of tubes (1/15) exhibited a ∂OD > 0.1 obviously not related to methanotrophy; in such tubes, microbial growth was often filamentous, thus hampering O.D. measurement.

These results indicate that ∂OD measurement was not suitable for estimating methanotrophs by MPN, which should be estimated from CH_4 consumption. In particular methods based on the record of a visible growth in microplates (Rowe *et al.*, 1977) incubated under CH_4 might overestimate populations of cultivable methanotrophs.

Results of counts

Populations of methanotrophs estimated in 18 dry soils ranged from 2×10^2 to 5×10^4. The coefficient of variation of replicated counts ranged from 0 to 20%. Estimates in soils preincubated under 20% CH_4 ranged from 10^7 to 10^9 (data not shown). Data available for comparison are (1) estimates of methanotrophs in a rice soil incubated under CH_4 and oxic conditions: counts increased from 10^4 g^{-1} soil at 0 days to 10^7 g^{-1} soil after 21 days of incubation (Watanabe I. *et al.*, 1995); and (2) estimates in three upland soils and a rice soil by a microtitration method, where the methanotrophic activity of the bacteria was not tested (Rowe *et al.*, 1977): values ranged from 2×10^5 to 4×10^6 for fresh soils and from 6×10^5 to 2×10^7 for soils preincubated under 20% methane (Bender & Conrad, 1992).

CONCLUSIONS

- Estimates of bacterial populations derived from counts on dishes or MPN method followed a log-normal distribution. This was taken into account for statistical analysis of the data.
- No selective medium for methanotrophs is yet available. The NMS Whittenbury medium, currently considered most suitable for growing methanotrophs, allows the growth of numerous non methanotrophic bacteria, actinomycetes and some fungi.
- Non methanotrophic populations developing on plates were Gram positive bacteria – as shown by crystal violet treatment –, fungi and actinomycetes – as shown by microscopic observations –, and probably opportunistic bacteria – as shown by the dynamics of the counts.
- Using silicagel or phytagel to solidify the medium did not solve the problem of mycelial contamination. Crystal violet and selected antibiotics reduced contaminant populations but also the difference

between counts on dishes incubated with or without CH_4.

• Despite a satisfactory reproducibility of bacterial counts performed on dishes incubated with or without CH_4 in the incubation atmosphere, methanotroph populations cannot be estimated from their difference because of (1) the low percentage of putative methanotrophs in the total population growing on NMS medium and (2) mycelial contaminations developing on dishes.

• Plating is however useful for isolating methanotrophs. Crystal violet and four antibiotics were found useful to reduce or delay contamination and thus facilitating methanotroph isolation.

• Graham's staining was found efficient for identifying colonies of methanotrophs possessing a soluble methane-monooxygenase and did not affect their viability, but could not be used together with crystal violet.

• Methanotroph isolations from plates should be easier at early stages of incubation because of (1) the higher relative number of methanotroph colonies in relation with the late growth of opportunistic bacteria (2) the individualization of the colonies which can be identified by Graham's staining and (3) an increasing mycelial contamination spreading on the plates with time.

• Plating and MPN could be compared for few soil samples preincubated under CH_4; counts from plating were about 10 times lower than those by MPN.

• Estimates of populations of cultivable methanotrophs ranged from 10^2 to 10^4 in dry soils and from 10^7 to 10^9 in preincubated soils, which shows the strong inductive power of CH_4 on these populations and provide an indirect information on their high potential capacity to oxidize CH_4 produced in waterlogged ricefields.

The MPN technique using (1) NMS Whittenbury medium, (2) an incubation of 6-8 weeks, (3) an atmosphere of 20% CH_4 in air, (4), no shaking of the standing tubes (5) a temperature of incubation 30°C, and (6) CH_4 consumption measurement to identify positive tubes (>20% CH_4 consumed), provided stable and reproducible estimates of cultivable methanotrophs. Measuring O.D. of the culture could not replace CH_4 consumption measurement to identify positive tubes.

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