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Microbiological aspects of methane emission by a ricefield soil from the Camargue (France): 1. Methanogenesis and related microflora

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Abstract

The major trophic groups of methanogens and the methanogenic potential of a rice soil from Camargue (France) were studied under laboratory conditions, with regard to the effects of soil desiccation and the addition of straw or algal material. The dynamics of cellulose and xylan fermentation by the soil microflora were also established.

Methanogens remained viable for several months in dry oxic soil, indicating that their density at the end of the crop cycle might not be markedly reduced by the dry fallow that usually follows rice harvest. Methanogenic activities were maximum two weeks after soil submersion. The native soil organic matter mineralized after submersion favoured acetotrophic methanogens, while adding straw or algae favoured hydrogenotrophic methanogens. Straw or algae addition increased populations of hydrogenotrophs and formatotrophs similarly, while four times more CH_4 was produced with straw than with algae. Methanogenesis appeared to be more limited by the availability and nature of the substrate than by the density of methanogens. Cellulose fermentation by the soil microflora exhibited a first stage, where hydrogenotrophs were mostly responsible for methanogenesis through interspecies H_2 transfer, and a second stage, where acetotrophs consumed accumulated acetate. Counts, microscopic observations, and strain isolations showed a diversified methanogenic microflora including rods, sarcinae, cocci and spirilla. Four mesophilic, neutrophilic strains were isolated and phenotypically characterized.

Keywords: Methane, rice, soil, acetotrophs, hydrogenotrophs, methylotrophs, formatotrophs, cellulose, xylan, desiccation, straw, algae.

Microbiologie de l'émission du méthane par un sol de rizière de Camargue (France) : (1) méthanogenèse et bactéries méthanogènes.

Résumé

La microflore et le potentiel méthanogène d'un sol de rizière de Camargue (France) ont été étudiés au laboratoire en testant : (1) l'effet de la dessication, (2) l'effet de l'addition de pailles ou d'algues et (3) la dynamique de la fermentation de la cellulose et du xylane par la microflore du sol.

Les bactéries méthanogènes sont restées viables pendant plusieurs mois dans le sol sec oxydé : leur densité en fin de cycle cultural ne serait donc pas significativement diminuée par l'assèchement qui suit habituellement la récolte.

Les activités méthanogènes ont atteint leur maximum deux semaines après la submersion du sol. La matière organique minéralisée après submersion a favorisé les acétotrophes, alors que l'addition de paille ou d'algues a favorisé les hydrogénotrophes. L'addition de pailles ou d'algues a augmenté de façon identique les populations hydrogénotrophes, alors que quatre fois plus de méthane a été produit en présence de pailles. La méthanogenèse apparait être limitée plus par la disponibilité et la nature des substrats que par la densité des bactéries méthanogènes.

L'étude de la fermentation de la cellulose a montré que le méthane était produit dans une première étape par les hydrogénotrophes via un transfert interspécifique d'hydrogène, et dans une seconde par les acétotrophes consommant l'acétate accumulé. Les numérations, les observations microscopiques et la purification de souches ont mis en évidence une microflore méthanogène diversifiée (bâtonnets, sarcines,

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coques et spirilles). Quatre souches mésophiles et neutrophiles ont été isolées et caractérisées d'après leur phénotype.

Mots-clés : Rizière, méthane, méthanogenèse, bactéries méthanogènes, dénombrements.

1. INTRODUCTION

Methane has a high potential for absorbing infrared radiation and is therefore one of the major gases involved in the greenhouse effect. Because of increasing anthropogenic activities, CH_4 concentration in the atmosphere has increased annually by about 1 to 0.8% during the last decades (Blake & Rowland 1988; Steele *et al.*, 1992). The possible effects of this increase on global warming have been widely presented in scientific reviews (Bolle *et al.*, 1986; Zepp, 1994) and popularization articles.

Waterlogged ricefields are considered the first or the second anthropogenic source of atmospheric CH_4 (Minami *et al.*, 1994), however, the estimation of their contribution to global CH_4 budget remains relatively imprecise (20-280 Tg per year) (Sass, 1994). As world rice production will substantially increase during the next decade (IRRI, 1989), it is important to design cultural practices that will improve rice yield while reducing CH_4 emission from rice environments.

Methane emission from ricefields results from (1) production by methanogenic bacteria in reduced soil, (2) consumption by methanotrophic bacteria in the oxic zones of the ecosystem (submersion water, water/soil interface and rice rhizosphere), and (3) transfer processes (diffusion, ebullition) through the soil and the rice plant (Holzapfel-Pschorn *et al.*, 1985; Conrad, 1989). The presence of methanogenic and methanotrophic bacteria in rice soils was indirectly demonstrated by measurements of CH_4 production and oxidation (de Bont *et al.*, 1978; Schütz *et al.*, 1989), but the microflora involved is still very poorly known. Data on population densities are scarce for methanogens and very scarce for methanotrophs (Neue & Roger, 1994). Currently only three genera of methanogens and two genera of methanotrophs have been isolated from rice soils.

This paper is the first of a series that present results of a study aiming at characterizing the methanogenic and methanotrophic populations and estimating the methanogenic and methanotrophic potential of a rice soil in Camargue (France). It reports on methanogens.

Methanogens are strict anaerobic *Archaea.* They constitute the last step in the electron transfer chain generated by the anaerobic degradation of organic matter (Garcia, 1990). They use a restricted range of substrates produced during the anaerobic decomposition of organic matter: H_2/CO_2, acetic acid, formate, methyl compounds like methanol and trimethylamine (TMA), and some secondary alcohols like 2-propanol and 2-butanol (*table* 1). As early as 1970, Takai (1970) reported that acetate and H_2/CO_2 were the two major substrates for methanogenesis in waterlogged ricefields, which is also observed in digestors (Garcia, 1990). Radiotracer experiments confirmed that CH_4 was essentially produced from acetate (Schütz *et al.*, 1989) that originates from (1) rice residues, algae and aquatic plants incorporated into the soil, (2) soil humus, and (3) autolysis products or exudates of the rice roots (Neue and Roger, 1994). About 24±7% was estimated to originate from H_2/CO_2 (Rothfuss & Conrad, 1991).

Twenty genera of CH_4 producing bacteria have currently been described, but only a few studies report the isolation of methanogens from ricefields. Three genera, *Methanobacterium, Methanosarcina,* and *Methanobrevibacter* have been isolated (Raimbault, 1981; Rajagopal *et al.*, 1988; Asakawa *et al.*, 1993; Fetzer *et al.*, 1993). *Methanospirillum, Methanocorpusculum*, which were isolated from freshwater sediments as well as methanogens found as endosymbionts in sapropelic amoeba should at least also be present in wetland rice fields (Neue & Roger, 1994).

This paper presents the results of a study where four major trophic groups of methanogens of a Camargue rice soil were quantified and the dominant strains isolated. We also studied (1) the effect of soil desiccation and organic matter application (rice straw or algae) on methanogenic populations and methanogenesis and (2) the decomposition of two major component of rice straw, cellulose and xylan, by the microflora of this soil.

2. MATERIAL AND METHODS

2.1. Soil

The soil used was collected, as a composite sample, from a wet ricefield in Camargue (France), about two months after the harvest of the rice crop. Part of the soil was kept waterlogged under demineralized water (further referred to as "wet soil"). Part was air-dried (water content: 2.5%) at ambient temperature as large clods and stored at room temperature. Before use, dry soil was crushed and passed through a 5 mm sieve.

Soil properties were as follows: pH: 1/1 H_2O: 7.7; 1/1 0.01 M $CaCl_2$: 7.4; EC (dS/m): 2.43; Organic C (%): 2.06; Nitrogen (%): 0.262; C/N: 7.86; Available P (Olsen, ppm): 45; Exchangeable cations (meq/100g): K:1.35; Na: 0.865; Ca: 90.8; Mg: 4.20; CEC, (meq/100g): 14.0; Avail. N (ppm): 0.10; Clay (%): 37; Silt (%): 56; Sand (%): 7. This soil was a moderately alkaline silt fine clay. As compared with the average

properties of most rice soils (Kawaguchi & Kyuma, 1977) it had an organic matter content above average, but also a high N content, which resulted in a low C/N ratio. Available P content was high. CEC was slightly below average but exchangeable K was very high.

2.2. Microbiological methods

Hungate anaerobic techniques (Hungate, 1969; Macy *et al.*, 1972) were used. Inoculations were done with 10% of culture or soil suspension.

2.2.1. Media for enumeration and culture of methanogens

Basic medium contained (per liter): NH_4Cl: 1 g; KH_2PO_4: 0.3 g; K_2HPO_4: 0.3 g; $MgCl_2.6H_2O$: 0.2 g; $CaCl_2.2H_2O$: 0.1 g; KCl: 0.1 g; CH_3COONa anhydrous: 0.5 g; NaCl: 0.6 g; cystein HCl: 0.5 g; 10 ml of the oligoelement solution of Balch *et al.* (1979); and 1ml of a 0.1% (W/V) rezasurin solution. The pH of the medium was adjusted to 7.0 with 10 M KOH, boiled under a stream of O_2-free N_2 and cooled to room temperature. Portion of medium were distributed into 60 ml serum bottles (20 ml), or into 20 ml Hungate tubes (5 ml), that were closed with butyl rubber stoppers. Hungate tubes and serum bottles were flushed with N_2/CO_2 (80/20 V/V) and sterilized for 45 min at 110°C. After sterilization, 0.01 ml of 2% $Na_2S.9H_2O$ and 0.05 ml of 10% $NaHCO_3$ (sterile, anaerobic solutions) were injected per ml of basic medium into the culture vessels.

For preparing Hungate roll-tubes, 1.6 % agar (Difco) was added to the medium. Depending on the experiments, vitamin [1% of Pfennig et al. (1981) solution], various concentrations of yeast extract (Difco), and Biotrypcase (BioMérieux) were used.

Four morphological and five trophic groups of methanogens can be distinguished (*table* 1). Among trophic groups:

- Strict hydrogenotrophs use exclusively $H_2 + CO_2$,
- Formatotrophs use both formate and $H_2 + CO_2$,
- Acetotrophs can be either stricts or also methylotrophs and hydrogenotrophs
- Stricts methylotrophs use exclusively methanol and methylamines, and
- Alcoholotrophs use alcohols but all also use some of the above energy sources.

In order to quantify the major trophic groups we used four selective media prepared by adding one of the four following substrates to the sterilized basic medium: formate (40 mM), methanol (40 mM), or acetate (20 mM) were added as sterile and anaerobic solution; sterile H_2/CO_2 (80/20 V/V) was injected at a pressure of 2 bars. These substrates are further referred to as "the four selective substrates". Depending on the experiments, other substrates were added.

2.2.2. Enumeration of methanogens.

The populations of major trophic groups of methanogens were estimated on duplicated composite soil samples by the most probable number (MPN) method. Six successive soil dilutions were inoculated in four sets of Hungate tubes containing basic medium supplemented with yeast extract (1 g l^{-1}), Biotrypcase (1 g l^{-1}), and one of the four selective substrates. Trophic groups of methanogens are detected, at least, on one of these substrates. Three tubes were inoculated per dilution. Methanogen growth in test tubes was assayed by measuring CH_4 produced after 60 days of incubation at 37°C. Inoculated tubes containing medium supplemented with yeast extract (1 g l^{-1}) and Biotrypcase (1 g l^{-1}), and no substrate served as control. A tube was considered positive when CH_4 produced was as least twice higher than in the control. Populations were expressed as number per g dry soil.

2.2.3. Isolation procedure

Methanogens were isolated from enrichment cultures obtained by inoculating 10% of a 10^{-1} soil dilution in basic medium supplemented with yeast extract (1 g l^{-1}), Biotrypcase (1 g l^{-1}), and one of the four selective substrates. To reduce contamination by fermenting bacteria, we decreased /suppressed yeast extract and/or Biotrypcase in the medium and replaced them with vitamins when needed, on a trial and error basis. Growth was evidenced by the measurement of CH_4 produced and microscopic examination.

Pure cultures were obtained from enriched cultures by three repeated applications of the agar shake dilution method in anaerobic tubes (Hungate, 1969). Colonies were collected in an anaerobic glove box under a N_2/H_2 atmosphere (95/5 V/V) and used for preparing successive dilutions in liquid medium. Last positive dilution obtained after a maximum of six weeks of incubation was used to prepare cultures that where checked for purity and simultaneously tested for metabolism. We used 11 media prepared from the basic medium enriched with yeast extract and Biotrypcase (1 g l^{-1} each) to which was added one of the following substrates: one of the four selective substrates, 10 mM propanol, 10 mM iso-propanol, 10 mM butanol, 10 mM iso-butanol (to check for metabolism and possible contamination by other methanogens), 20 mM glucose (to check for heterotrophs), 20 mM lactate or H_2/CO_2 at 2 bars + 20 mM sulphate (to check for sulphate reducers).

2.2.4. Strain characterization

Strains were observed with a NIKON Optiphot microscope equipped with a NIKON FX35 camera and a Zeiss, standard 20 microscope equipped with

epifluorescence. Optimum temperature was determined from cultures grown at 15, 20, 25, 30, 35, 37, 40, 42 and 45°C in water baths. Optimum NaCl concentration was determined from cultures grown at 37°C in medium containing 0, 5, 10, 15, 20, 25, 30, 40, 50 and 60 g NaCl l^{-1}. In both cases, media were enriched with yeast extract (1 g l^{-1}) and, when necessary, Biotrypcase (1 g l^{-1}). Bacterial growth was quantified with a Shimadzu UV 160 spectrophotometer by measuring the increase in turbidity at 580 nm in the anaerobic Hungate tubes (homogeneous cultures of rods) or through CH_4 production (aggregated cultures of sarcinae, cultures of cocci with very low O.D.). All experiments were duplicated. Isolated strains were related to genera according to their phenotypic characteristics.

2.3. Chemical analysis

Methane at concentrations lower than 20% of the gas phase was measured with a flame ionisation chromatograph (Girdel serie 30; column Alltech SARL, chromosorb W/A/W, 80/100 mesh, SP 1000, 1% H_3PO_4, 2 m in length, 1/8" in diameter; column temperature: 150°C; detector temperature: 220°C; injector temperature: 220°C; vector gaz: N_2 1.5 bar). Methane at concentrations higher than 20% and H_2 were measured using a thermal conductivity gas chromatograph (Girdel serie 30; two columns in parallel, Alltech SARL, Carbosphere SS 60/80 mesh, 1.8 m in length, 1/8" in diameter; column temperature: 150°C; detector temperature: 180°C; injector temperature: 180°C; vector gaz: N_2 1.2 bar). Gas samples were collected and injected using a syringe connected to a valve to avoid corrections for pressure and volume variations.

Volatile fatty acids in liquid phases were analyzed from 1.5 ml samples centrifuged for 10 min. at 13 000 rpm. One ml of the supernatant, acidified with 0.01 ml of 50% H_3PO_4, was injected in the FID chromatograph.

2.4. Experimental designs

2.4.1. Conservation of methanogens in dried soil

The four trophic groups of methanogens were enumerated on wet soil. Then soil samples of 10 g each were dried at room temperature and enumerations were performed on duplicate samples after 1, 2, and 3 months of desiccation.

2.4.2. Effect of organic matter addition on potential methanogenic activity

Samples of 15 g of dry soil were mixed with 2% (W/W) of dried, ground rice straw, or algal material (algal mat dominated by Sirogyra sp.) and placed with 20 ml of anaerobic distilled water in 120 ml flasks under an atmosphere of N_2. Soil with no organic matter addition was used as control. Methane produced and volatile fatty acids were measured at two weeks intervals for three months of incubation at 37°C. Methanogens were enumerated after 1, 2, and 3 months of incubation. The first dilution was prepared anaerobically directly in the incubation flask.

2.4.3. Anaerobic degradation of cellulose, and xylan by soil microflora

The decomposition by soil microflora of cellulose and xylan, which are major components of rice straw, was studied in order to estimate (1) the relative importance of acetate and fatty volatile acids as products of metabolism from cellulose and hemicellulose, and (2) the significance of H_2 interspecies transfer in this process. Experiments were conducted in 160 ml flasks containing 60 ml of basic medium without acetate, enriched with 0.5 g l^{-1} yeast extract, and pure cellulose (5 g l^{-1}) or xylan (5 g l^{-1}) from oats. Inocula were 6 ml of enrichment cultures prepared from suspensions of wet soil inoculated in similar medium enriched with cellulose or xylan.

3. RESULTS AND DISCUSSION

3.1. Estimation of methanogen populations in wet and dry soil

When interpreting results of MPN counts the following limitations should be kept in mind:

1. The method is known to be biased due to the selectivity of the medium (Rowe *et al.*, 1977). To partially avoid this limitation, (1) we used four specific substrates and (2) counts were performed by considering a tube positive when the quantity of CH_4 produced was as least twice higher than in a control with no specific substrate.

2. Most methanogens grow on two or three of the four selective substrates (*table* 1). Therefore the sum of populations enumerated on each of the four substrates is higher than the total population.

3. Acetoclastic and methanol consuming methanogens are mostly sarcinae (*table* 1), which form aggregates that are difficult to separate into individual cells and thus their populations are underestimated by MPN counts (Fetzer *et al.*, 1993).

4. MPN counts have a low accuracy. When enumerations are not conducted with a number of replicates allowing statistical analysis, differences between results whose ratio is lower than 4 should not be considered significant (Roger *et al.*, 1991).

Populations of the four trophic groups of methanogens did not markedly differ among soil

Table 1. – Morphological and trophic groups of methanogens.

Trophic groups	Strict			Non strict Sarcinae
	Coccus	Rod	Sheath**	
Hydrogenotrophs ($H_2 + CO_2$)	most spp.	most spp.	none	few sp.
Formatotrophs (Formate)	*All formatotrophs are hydrogenotrophs* many sp.	many sp.	none	none
Acetotrophs (Acetate)	none	none	1 genus	all
Methylotrophs (Methanol, methylamine...)	4 genera	none	none	all sp.
Alcoholotrophs (2-butanol, 2-propanol...)	*No strict forms* few	few	none	few

* from a trophic point of view

** *Methanosaeta*

kept under water after collection and soil kept dry as large clods (*table* 2).

When a thin layer of soil kept under water was air dried for 3 months (*table* 3), counts of hydrogenotrophs decreased by four times, counts of acetotrophs and methylotrophs decreased by about 3 times, and counts of formatotrophs did not vary. The observed decreases have probably no significance with regard to the accuracy of MPN counts.

Table 2. – Enumeration of major groups of methanogens in Camargue soil.

	Nb × 10^{-3} of CFU of methanogens on			
	H_2/CO_2	Formate	Acetate	Methanol
Wet soil	18.8	1.3	1.8	6.0
Dry soil	9.6	1.8	2.5	5.0
Dominant morphology	Rod	Rod	Sarcina	Sarcina

CFU: Colony forming unit

Table 3. – Effect of soil desiccation on the major groups of methanogenic bacteria*.

Duration of the desiccation (month)	Nb × 10^{-3} of CFU of methanogens on			
	H_2/CO_2	Formate	Acetate	Methanol
0	18.8	1.3	1.8	6.0
1	3.7	n.d.	1.3	2.1
2	1.8	1.4	1.0	1.5
3	4.7	1.4	0.7	1.9

* Enumarion at 0 time were performed on wet soil.

A number of experimental data indicate that methanogens can survive oxic conditions and desiccation, however, their survival may vary within large limits. Exposing cultures of *Methanosarcina barkeri* to air for 200 min had no effect on its viability but decreased its methanogenic activity. When the same cultures were desiccated under anaerobic conditions, viability was reduced to 10% and methanogenic potential to 0.6%. Desiccation under air markedly reduced viability to 0.5% and methanogenic potential to 0.03% of the control (Fetzer *et al.*, 1993).

The presence of soil increases the resistance of methanogens to aerobic desiccation. Mayer & Conrad (1990) showed that the MPN counts of a culture of *Methanobacterium* sp. mixed to a suspension of sterile soil, and then desiccated and kept under aerobic conditions for two weeks, decreased by about ten times. Rice soils, because of their high content in clay, and possibly in FeS (Fetzer *et al.*, 1993) seems to be efficient in preserving methanogens viability under dry oxic conditions. Populations of methanogens enumerated in a range of dry rice soils from Senegal by Garcia *et al.* (1974) ranged from 10^2 to 10^6. Mayer & Conrad (1990) observed that populations of hydrogenotrophs and acetotrophs were sufficiently abundant (10^4-10^5) in a dry Italian rice soil to allow a significant CH_4 production after 100h of submersion with no increase of the populations. On the other hand, in two upland soils tested for control, the initial populations were much lower and an increase by about 1000 times was needed before methanogenesis appeared, which required between 15 to 30 days.

To our knowledge, no data on the effects of oxic desiccation on the dynamics of soil populations of methanogens is available for comparison with the results we observed with the Camargue soil. However as this soil was collected at the end of the crop cycle and then kept under water, it can be hypothesized that it had already reached a stage where populations were stabilized–because of the lack of easily usable

Table 4. – Effect of straw and algae addition on soil populations of methanogens after 1, 2, and 3 months of incubation (CFU × 10^{-3}).

	Dry soil	Control			Soil + Algae			Soil + Rice straw		
Substrate		1 mo	2 mo	3 mo	1 mo	2 mo	3 mo	1 mo	2 mo	3 mo
H_2/CO_2	9.6	n.d.*	n.d.	5.0	27.8	55.6	125.0	97.2	277.8	347.2
Formate	1.8	1.2	1.2	4.3	15.0	41.7	416.7	125.0	208.3	555.6
Acetate	2.5	n.d.	15.9	27.8	8.3	8.3	12.6	n.d.	4.2	9.7
Methanol	5.0	3.4	20.7	18.8	19.4	27.8	83.3	9.7	15.2	55.6

* n.d.: not determined.
mo: month

substrates–and probably in a quiescent stage more adapted to survive desiccation, which may explain why populations did not markedly decrease.

3.2. Effect of organic matter addition on populations and activities

Upon submersion of dry soil samples with no addition of organic substrate, only populations of acetotrophs exhibited a significant increase by about ten times within three months (*table* 4). This indicates that labile carbon becoming available upon submergence of the dry soil was mostly used by acetogens.

Organic matter addition increased more (12 to 300 times) the counts of hydrogenotrophs and formatotrophs. No significant difference was observed between counts of hydrogenotrophs and formatotrophs, indicating that mostly formatotrophs multiplied. Counts of acetotrophs and methylotrophs increased only by 4 to 16 times after three months. However, MPN counts are not efficient in recording changes in acetotrophic sarcinae populations, which may increase their biomass through the size of their aggregates without affecting much MPN counts, which mostly record the number of aggregates.

Hydrogenotrophs and formatotrophs multiplied faster in soil enriched with straw than in soil enriched with algae. However, after three months, populations of the four trophic groups were quite similar in soil enriched with straw or algal material, while marked differences were observed in terms of potential CH_4 production among the three treatments (*fig.* 1a and b). Maximum methanogenic activity developed after 15 days of submersion for both soil samples, enriched with straw or algae. Mayer & Conrad (1990) already reported that methanogenesis initiation appeared to be limited by the substrate and not by the number of methanogens.

Algal material addition increased CH_4 production by about two times over the control. Straw addition increased it by eight times. The C/N ratios of straw (about 40) and algae (about 12) explain the differences observed. Field experiments showed that incorporation

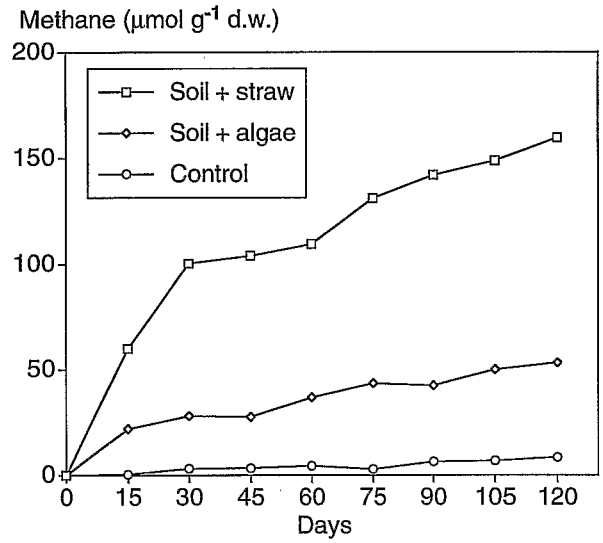

Figure 1a. – Dynamics of CH_4 production by soil samples under continuous incubation (a) cumulated values (b) daily values.

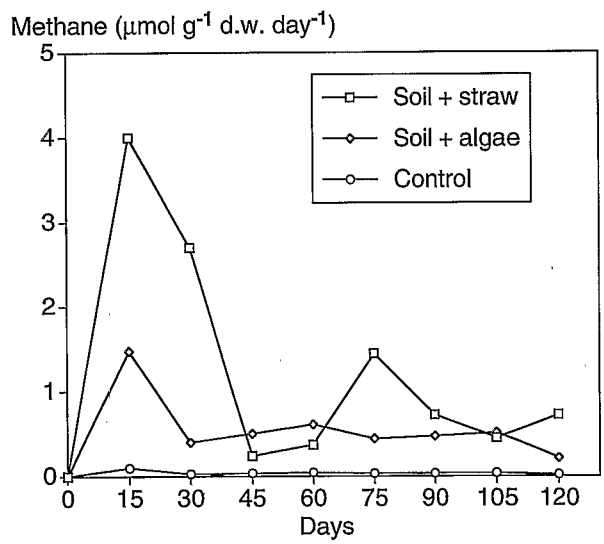

Figure 1b. – Dynamics of CH_4 production by soil samples under continuous incubation (a) cumulated values (b) daily values.

of straw with high C/N ratio favoured CH_4 production (Wang *et al.*, 1992; Watanabe *et al.*, 1994) whereas less CH_4 was produced from green manures with a lower C/N (Lauren *et al.*, 1994).

3.3. Fermentation of cellulose and xylan

In samples enriched with cellulose, CH_4 production started after about 50 h of incubation (*fig.* 2). Hydrogen, which is well known to be produced from cellulose degradation, was not detected, which indicates that it was rapidly consumed by hydrogenotrophs. Acetate was produced after 50 h of incubation. Its maximum concentration (20-25 mM) was maintained between about 100 and 300 h of incubation. Then acetate started to be used by acetotrophs and CH_4 production increased rapidly, leading to a concentration of 60-70 mM after 450 h. No more acetate was detected after 450 h of incubation. Volatile fatty acids (propionate and butyrate) were present at low concentration (< 5 mM) for the whole duration of the experiment.

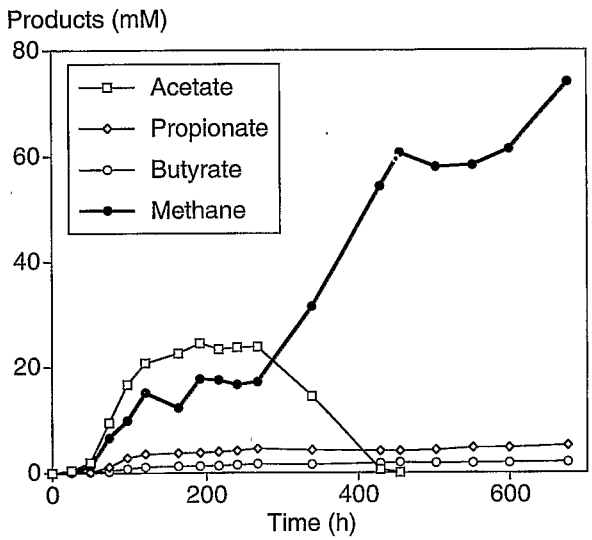

Figure 2. – Dynamics of cellulose fermentation by the Camargue soil microflora.

These results (*fig.* 2) indicate a two step process. In the first stage (0-300 h) hydrogenotrophs were dominant, refraining H_2 accumulation and thus favouring acetate production. In the second stage, acetotrophs, after a lag of about 300 h, started to consumed acetate and CH_4 production increased by 3-4 times.

The fact that H_2 was not detected indicated a synchronism between its release from cellulose degradation and its consumption by hydrogenotrophs, that is an efficient interspecies H_2 transfer which led mainly to the production of acetate. This phenomenon was also observed with mixed defined cultures in mesophilic and thermophilic conditions (Smiti *et al.*, 1986; Laube & Martin, 1981; Latham & Wolin, 1977). The efficiency of interspecies H_2 transfer was confirmed by the low amount of propionate and butyrate accumulating during the fermentation. It is known that H_2 utilization causes a shift in the electron flow, resulting in an increase in acetate production and a decrease in the formation of reduced products such as propionate and butyrate (Stams, 1994).

Rothfuss & Conrad (1991) also reported that, in rice soils, while acetate seems to be the major substrate, H_2 also significantly contributed to methanogenesis through interspecies H_2 transfer within microbial methanogenic associations.

In samples enriched with xylan, the production of acetate and other volatile fatty acids started from the beginning of the incubation (*fig.* 3). A faster accumulation of fatty acids in the medium and higher concentrations were observed, as compared with cellulose. The fast acidification of the medium by acetate and fatty acids delayed the growth of hydrogenotrophs. As a result, H_2 was detected and reached a maximum concentration of about 4 mM. This degradation process was indicative of the absence of interspecies H_2 transfer. Methane started to be produced by hydrogenotrophs only after 200 h of incubation. Acetotrophs were still not active after 300 h and acetate concentration remained stable (17-19 mM) between 70 and 300 h.

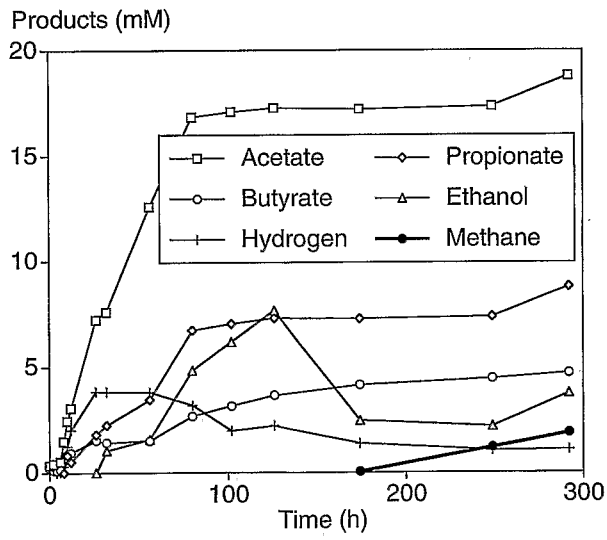

Figure 3. – Dynamics of xylan fermentation by the Camargue soil microflora.

The delay in H_2 and acetate consumption by methanogens probably resulted from (1) a faster growth of fermentative bacteria due to the easier biodegradability of xylan by the fermentative microflora, as compared with cellulose, and (2) the resulting quick release of acidifying products from xylan fermentation. It is known that low pH can

inhibit methanogenesis (Garcia, 1990). Furthermore the non utilization of acetate could also result from the presence of H_2 in the atmosphere of the culture vessel, which is known to inhibit the acetoclastic reaction (Mc Inerney & Bryant, 1981, Smiti *et al.*, 1986).

Hydrogen consumption began after 100 h of incubation and was concomitant with ethanol oxidation. These results are indicative of the establishment of an interspecies H_2 transfer on ethanol which can only be degraded in the presence of sulphate by sulphate-reducing bacteria or by interspecies H_2 transfer (Stams, 1994). We can therefore expect interspecies H_2 transfer to have develop rather on the end-products of xylan fermentation than on xylan oxidation.

The comparison between cellulose and xylan degradation indicates that two fermentation processes developed. They were dependant of the relative growth of fermentative bacteria and methanogens, which govern the establishment of interspecies H_2 transfer. When interpreting these results, it has to be kept in mind that the experiments were conducted with microbial enrichments from soil suspension in a moderately buffered medium and that in situ a more marked buffering effect of the soil might be expected.

These experiments provide no evidence of the existence of syntrophic butyrate or propionate oxidizers in Camargue soil but it its known that the growth of such microorganisms require periods of incubation longer that we used (Boone & Bryant, 1980; McInerney *et al.*, 1981).

3.4. Characterization of the dominant strains

Four morphologically different taxa of methanogens were observed from enumeration and isolation tubes: rods, sarcinae, cocci and spirilla. Four strains were isolated (*table* 5). They were deposited to the "Deutsche Sammlung von Mikroorganismen und Zellkulturen Gmbh" (DSM) and the "Oregon Collection of Methanogens" (OCM).

Strain RiH_2 (=DSM 10113) (=OCM 641) is a non motile rod, strictly hydrogenotroph, and related to the *Methanobacterium* genus. It is mesophilic (range: 25-42 C; optimum around 37°C; no growth observed at 20 and 45°C), neutrophilic (range: 6.0-8.5; optimum around 6.8; no growth observed at pH 5.0 and 9.5), halotolerant (range: 0-25 g/l NaCl).

Strain FCam (=DSM 10111) (=OCM 637) is a non motile rod belonging to *Methanobacterium* genus. It grows on H_2, formate, and some alcohols (propanol, isopropanol). It is mesophilic (range 25-42; optimum around 40°C; no growth observed at 20 and 45°C), neutrophilic (range 6.5-8.5; optimum around 7.0; no growth observed at pH 6.0 and 9.0), and NaCl tolerant (range 0-30g/l NaCl).

Strain CoCam (being deposited) is a motile coccus. It grows on formate and H_2. It is mesophilic (range 20-42°C; optimum around 40°C; no growth observed at 15 and 45°C), neutrophilic (range: 6.5-8.5; optimum around 7.0; no growth observed at pH 5.5 and 9.0) and NaCl tolerant (range: 0-50 g/l).

Strain Sar (=DSM 10131) (=OCM 635) belongs to genus *Methanosarcina.* It grows on methanol, trimethylamine, acetate, and H_2. It is a mesophilic (range 25-42°C; optimum around 35°C; no growth observed at 20 and 45°C), neutrophilic (range: 5.5-7.5; optimum around 6.8; no growth observed at pH 5.0 and 8.0) and not tolerant to NaCl (no growth was observed with more than 1 g/l NaCl).

Spirilla, most probably belonging to the genus *Methanospirillum,* were observed under the microscope at several occasions but could not be isolated.

Table 5. – Major characteristics of the isolated strains.

	Rod 1 (RiH2)	Rod 2 (FCam)	Sarcina (Sar)	Coccus (CoCam)
Growth on methanogenic substate*				
H_2/CO_2	+	+	+	+
Formate	–	+	–	+
Acetate	–	–	+	–
Methanol	–	–	+	–
TMA	–	–	+	–
Propanol	–	+	–	–
Isopropanol	–	+	–	–
Butanol	–	–	–	–
Isobutanol	–	–	–	–
Temperature (°C)				
Range	25-42	25-42	25-42	20-42
Optimum	37	40	35	40
PH				
Range	6.0-8.5	6.5-8.5	5.5-7.5	6.5-8.5
Optimum	6.8	7.0	6.8	7.0
NaCl (g l^{-1})				
Range	0-25	0-30	< 1.0	0-50
Genus	*Methano-bacterium*	*Methano-bacterium*	*Methano sarcia*	n.d.

* No growth was observed on glucose and lactate + sulphate, indicating that strains were axenic.

4. CONCLUSION

Enumerations performed on soil collected at the end of the crop cycle and kept either under water or as dry clods were similar and ranged from 10^3 to 2×10^4. After organic matter addition and incubation under water for three months, populations increased by 4 to 300 times, depending on the trophic group. Results also indicated that the density of the populations occurring at the end of the crop cycle might not be markedly decreased by the dry fallow that usually follows rice harvest.

Maximum methanogenic activities were observed two weeks after the submersion of the dry soil. Values expressed in $\mu mol\ g^{-1}$ dry soil day^{-1} reached 0.01 in the control, 1.5 in the soil enriched with algae and 4.0 in the soil enriched with straw. Extrapolation on the

basis of 1200 tons of dry soil per ha correspond to maximum CH_4 production rates of 0.2 kg ha^{-1} day^{-1} in the control, 29 kg in the soil enriched with algae, and 77 kg in the soil enriched with straw. The native soil organic matter mineralized at the time of resubmersion of the dry soil favoured the growth of acetotrophs, while the addition of new organic matter (straw or algae) favoured hydrogenotrophs and formatotrophs. Straw or algae addition increased populations of hydrogenotrophs and formatotrophs in a similar way, while CH_4 production was about four times higher with straw than with algae. This might confirm that methanogenesis is more influenced by the availability and the nature of the substrate than by the density of populations (Mayer & Conrad, 1990).

The study of cellulose fermentation by the Camargue soil microflora showed a longer lag by acetotrophs than by hydrogenotrophs. This resulted in the establishment of a two stages methanogenic process. In the first stage, hydrogenotrophs were mostly responsible for methanogenesis through interspecies H_2 transfer. In the second stage, acetate accumulated during the first stage was consumed by acetotrophs. When xylan, a substrate more easily degraded than cellulose, was used, a fast acidification of the medium refrained methanogenesis for about 150 h.

Selective counts, microscopic observations, and strain isolation showed that a complex microflora was involved in the production of CH_4 in the Camargue soil. Four morphologically different taxa of methanogens were observed: rods, sarcinae, cocci and spirilla. Four strains were isolated: two rods belonging to the *Methanobacterium* genus (strains RiH_2 and FCam), one coccus (strain CoCam) and one *Methanosarcina* (strain Sar). All strains were mesophilic and neutrophilic. It is the first time that the presence of a *Methanospirillum* in a ricefield soil was recorded.

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