Microbiological aspects of methane emission in a ricefield soil from the Camargue (France): 2. Methanotrophy and related microflora

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Abstract

We studied major factors that influenced the *in vitro* methanotrophic activity of a rice soil from Camargue (France) (soil depth, soil humidity, fertilizer application, and desiccation) to develop a standardized method to estimate the potential methanotrophic activity (PMA) of a soil. Methanotrophs were tentatively enumerated and dominant strains were isolated from the soil incubated for one month under 20% CH_4 in air.

Methanotrophy was a surface phenomenon that was better expressed on an area than on a soil dry weight basis. Maximum methanotrophy (Potential Methanotrophic Activity: PMA) (1.8 mole m^{-2} d^{-1}) was reached by a sample at water holding capacity incubated for one month under 20% CH_4. Methane and O_2 consumption, and CO_2 production were highly correlated. Heterotrophic O_2 consumption was negligible as compared to that by methanotrophs. PMA was not affected by fertilizer application at levels corresponding to field situation and remained reproducible for two years when it was estimated from a soil kept dry.

Plating method was inadequate for enumerating methanotrophs because of the extremely high level of contamination. Populations estimated from MPN measurements were low in dry soil (2.5×10^2 g^{-1}) and increased to 5×10^8 g^{-1} in soils incubated under CH_4 for one month. Isolated strains were morphologically related to type II methanotrophs.

Keywords: Ricefield, methane, methanotrophy, methanotrophic bacteria, enumerations.

Microbiologie de l'émission du méthane par un sol de rizière de Camargue (France) : (2) méthanotrophie et bactéries méthanotrophes.

Résumé

Les principaux facteurs qui influencent l'activité méthanotrophe du sol (épaisseur du sol, humidité, addition d'engrais et dessiccation) ont été étudiés *in vitro* sur un sol de rizière de Camargue (France) afin de standardiser une méthode pour estimer l'activité méthanotrophe potentielle (PMA) d'un sol. La possibilité d'énumérer les méthanotrophes du sol a été étudiée et les souches dominantes du sol incubé pendant un mois sous un mélange de 80 % air/20 % CH_4 ont été isolées.

La méthanotrophie *in vitro* est un phénomène quantifiable par unité de surface plutôt que par unité de poids de sol. L'activité maximale (PMA) (1,8 mole m^{-2} j^{-1}) a été mesurée sur un échantillon à une humidité voisine de la capacité au champ et incubé pendant un mois sous air/CH_4. Les consommations de CH_4 et d'O_2 ainsi que la production de CO_2 sont fortement corrélées. La consommation d'O_2 liée à l'activité hétérotrophe du sol est négligeable comparée à celle due à l'activité méthanotrophe. L'addition d'engrais à des concentrations utilisées en agriculture est sans effet sur la PMA qui est restée stable pendant deux ans dans le sol conservé à l'état sec.

Une très forte contamination rend les étalements sur boîtes de Pétri inexploitables pour dénombrer les bactéries méthanotrophes du sol. Les populations estimées par MPN sont faibles dans le sol sec (2.5×10^2 g^{-1}) et atteignent 5×10^8 g^{-1} dans le sol enrichi. La morphologie des souches isolées indique leur appartenance aux bactéries méthanotrophes de type II.

Mots-clés : Rizière, méthane, méthanotrophie, bactéries méthanotrophes, dénombrements.

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1. INTRODUCTION

Methane emission from ricefields results from production by methanogenic bacteria, consumption by methanotrophic bacteria, and transfer processes through the soil and the rice plant. The presence of methanogenic and methanotrophic microorganisms in rice soils has been indirectly demonstrated by measurements of CH_4 production and oxidation, but the microflora involved is still very poorly characterized. This paper is the second of a series that present a study of the methanogens and methanotrophs and their potential activities in a rice soil from Camargue (France). The first paper reported on methanogens (Joulian *et al.* this issue). This paper reports on methanotrophs.

It is known that ricefield soils are efficient CH_4 producing environments (Neue *et al.*, 1994) where more than 60% of CH_4 produced is reoxidized by methanotrophs in the oxic zones of the ecosystem: submersion water, water/soil interface and rice rhizosphere (Holzapfel-Pschorn *et al.*, 1986; Sass *et al.*, 1990). However, qualitative and quantitative data on methanotrophic populations present in rice soils are very scarce. Only two strains of methanotrophs were isolated from ricefields: *Methylosinus sporium* (Bowman *et al.*, 1993) and *Methylocystis* sp. (Takeda, 1988).

Two types of CH_4 oxidation in soils are known (Bender & Conrad, 1992; Frenzel *et al.*, 1992; Schütz *et al.*, 1989). The first type (high-affinity activity) is observed at atmospheric CH_4 concentration (≤ 12 ppm) and is apparently ubiquitous in soils except those that have been exposed to high NH_4^+ concentrations. Bacterial populations responsible for this type of CH_4 consumption are not clearly identified. The second type of methanotrophy (low-affinity activity) occurs at CH_4 concentrations higher than 40 ppm and is performed by the bacteria called methanotrophs (Jones & Nedwell, 1993; King *et al.*, 1990; Whalen *et al.*, 1990). Methane concentration in the water contained in the two upper centimeters of a rice soil averaged 110 ppm (v/v) (Conrad & Rothfuss, 1991). Methane concentration in the soil atmosphere, when the soil is being drained, is often likely to be higher. These values are significantly higher than the threshold of 11-45 ppm (v/v) CH_4 established by Bender & Conrad (1992) for the methanotrophic activity *sensu stricto*. Therefore methane oxidation in ricefields is mostly of the "low affinity" type.

We used the Camargue soil to study some factors that affect CH_4 oxidation by a rice soil under laboratory conditions, in order to design a standardized method to estimate the potential methanotrophic activity (PMA) of a soil, that is its maximum methane oxidation activity measured *in vitro* under optimized conditions. We also used the classical methods of bacterial enumeration (MPN and plating) to tentatively quantify methanotrophic populations and isolate dominant strains from soil samples enriched in methanotrophs by a preliminary incubation under air mixed with 20% methane.

2. MATERIALS AND METHODS

The properties of the Camargue soil (slightly alkaline silt fine clay) and the preparation methods are described in the previous paper (Joulian *et al.*, this issue).

2.1. Soil incubations

Soil samples were incubated under a CH_4 enriched atmosphere to measure methanotrophic activity and enhance populations of methanotrophs before enumeration and isolation. For estimating PMA, we incubated samples under a mixture of 80% artificial air and 20% CH_4 (4/1; V/V), further referred to as "air/CH_4", which is most frequently used (Bender & Conrad, 1992; Megraw & Knowles, 1987). This concentration (1) is adequate to enhance the "low affinity" methanotrophy, (2) avoid frequent replacement of the gaz phase in the incubation flasks, and (3) avoid the hazard of an explosive mixture of CH_4 in air (between 5 and 15%). It provides an O_2 concentration of 16% at the beginning of the incubation.

Incubations were performed in 600 ml cylindric flaks (bottom area 50 cm^2) bearing a lateral tubing closed with a butyl rubber stopper. The flask atmosphere was replaced daily and analyzed after each replacement. Flasks were incubated in the dark at 30°C, which is optimal for most methanotrophs (Whalen *et al.*, 1990). At 37°C, nine strains of methanotrophs over 23 were inhibited (Green, 1992).

2.2. Gas analysis

Gas were sampled with 1 ml syringes and 0.5 ml was injected in a gas chromatograph (Chrompack CP 9000) equipped with a thermal conductivity detector and a double column set up allowing to bring the gas mixture successively in the contact with the two filaments of the detector and therefore to analyze O_2, N_2, CH_4 and CO_2 with a single injection. The first column (1,5 m × 2 mm) was packed with silicagel 60-80 mesh, the second column (1,5 m × 2 mm) was packed with a molecular sieve 5 Å 60-80 mesh. An empty tubing (1,5 m × 2 mm) inserted between the columns avoided the overlapping of the CH_4 peak detected by the first filament and the O_2 peak detected by the second filament . Carrier gas was helium (15 ml min^{-1}). Temperatures were as follows: injector: 50°C, columns: 50°C, detectors: 150°C.

2.3. Experimental designs for methanotrophy measurement

2.3.1. *Dynamics of methanotrophic activity*

The methanotrophic activity of 50 g dry weight (d.w.) soil samples at approximately field capacity (30% water on d.w. basis) was measured weekly in triplicate for six weeks of incubation under air/CH_4.

2.3.2. *Effect of soil thickness*

The methanotrophic activity of 50 g, 100 g, and 150 g d.w. soil samples at 30% humidity was measured in triplicate after 2 and 3 weeks of incubation under air/CH_4.

2.3.3. *Effect of soil humidity*

The methanotrophic activity of 50 g d.w. soil samples at 20, 25, 30, 35, 40, 50% humidity was measured in triplicate after 2 and 4 weeks of incubation under air/CH_4.

2.3.4. *Effect of fertilizers*

The methanotrophic activity of 50 g d.w. soil samples at 30% humidity was measured after the addition of 12 mg of urea (equivalent to the application of 100 kg N ha^{-1}), or 10 mg of NaH_2PO_4 (50 kg P_2O_5 ha^{-1}), and in a control.

2.3.5. *Preservation of the methanotrophic activity in soil*

In a first experiment, PMA was measured on triplicate soil samples at 30% humidity incubated for three weeks under air/CH_4 with daily replacement of the atmosphere of incubation. Samples were then air dried. After 3 weeks, they were rewetted and used again for PMA measurements. In a second experiment, soil samples were preincubated for one month under air/CH_4. After 1 month, the flask atmosphere was not renewed and became oxygen depleted within about 12 h. Flasks were kept at 30°C and their methanotrophic activity was measured after 1, 2, 3, and 4 weeks of conservation in anaerobiosis.

Gas concentrations were calculated assuming that N_2 was constant in the flask for 1 d. Rates of gases production/consumption were calculated using a linear regression with no constant (in all experiments r^2 [t, CH_4 consumption] was higher than 0,98).

2.4. Methanotrophic bacteria enumerations

2.4.1. *Media*

The basic medium used for counting or isolating methanotrophs was modified from the NMS medium of Whittenbury et al. (1970) by replacing the Sequestrene iron complex with EDTA-Ferric-Na salt (Sigma: 15708-41-5) at 0.04 g l^{-1} as suggested by Henry and Grbic-Galic (1991). The pH was adjusted to 6.8 before autoclaving at 120°C for 20 min. After autoclaving, 2 ml of sterile phosphate buffer solution (mixture of Na_2HPO_4 and KH_2PO_4, 15 g in 300 ml distilled water) was added. Solid media were prepared using 17 g of agar (Bacto agar, DIFCO, Detroit, U.S.A.) in 1 l of liquid medium. Experiments were conducted with CH_4 as sole carbon substrate.

2.4.2. *MPN counts*

The 10^{-1} soil suspension/dilution was prepared from 50 g of soil stirred for 1 h with 450 ml physiological water. This dilution was used to prepare serial 1/10 dilutions, 0.5 ml of which was inoculated in airtight Hungate's tubes containing 4.5 ml of Whittenbury NMS and an air/CH_4 atmosphere. Counts were replicated using two composite soil samples for preparing dilutions. Each dilution was then used to inoculate two sets of triplicate tubes. The four sets were incubated at 30°C. Two sets were incubated without agitation and two sets were incubated in a gyratory shaker. The CH_4 consumption measured weekly for two months was used to identify positive tubes.

2.4.3. *Counts by plating*

0.1 ml of each suspension/dilution was spread on five replicated Whittenbury NMS agar plates. Two series were prepared. They were placed in sealed plastic bags and incubated at 30°C, the first in air and the second in air/CH_4. The apparition of colonies was checked weekly for 30 days.

3. RESULTS AND DISCUSSION

3.1. Dynamics of CH_4 and O_2 consumption, and CO_2 production

The consumption of CH_4 and O_2, and the production of CO_2, remained linear for at least 10 h, indicating that a rate of CH_4 consumption can be defined over several hours and used to estimate PMA (*fig.* 1). The linearity of CH_4 consumption *in vitro* by a landfill cover soil was reported by Whalen *et al.* (1990).

3.2. Effect of the thickness of the soil sample on methanotrophic activity

The minimum quantity of soil sieved at 2 mm ensuring a full coverage of the bottom of the flask (50 cm^2) was 50 g d.w.. When more than 50 g soil

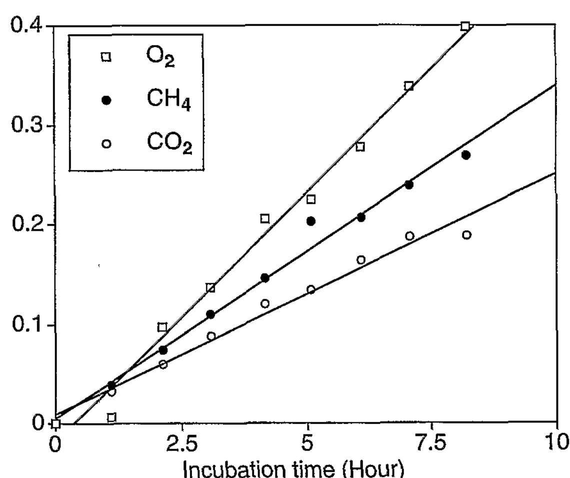


Figure 1. – CH_4 ($r^2=0.992$) and O_2 ($r^2=0.977$) consumption, and CO_2 ($r^2=0.986$) production of the soil after 3 weeks of preincubation under 80% air/20% CH_4.

was used, the thickness of the soil layer increased but the CH_4 consumption per flask and per day measured after three weeks of incubation did not significantly increase (*table* 1). However, the variability among replicated measurements was high, wich refrains definite conclusion. Variability increased with the thickness of the soil layer used, most probably in relation with the heterogeneity of soil samples with regard to aggregation, anaerobiosis in aggregates – leading to methane production – and gas diffusion. It is worth to note that many soil microbial activities and populations exhibit a log-normal distribution leading to a variability of replicated data which increases with the absolute value of the mean (Roger, 1996). In the case of methanotrophy the variability increased with the thickness of the soil samples whereas the average activity did not significantly increase.

Table 1. – Rates of O_2 and CH_4 consumption and CO_2 production after 15 and 21 days of incubation under 20% of methane [a].

Gram of soil per flask	O_2	CH_4	CO_2
	Activities after 15 days (mole m^{-2} d^{-1})		
50	1.37±0.85	0.73±0.44	0.72±0.23
100	2,17±1.26	1.25±0.55	1.03±0.66
150	1.99±2.58	1.04±1.70	1.07±1.21
	Activities after 21 days (mole m^{-2} d^{-1})		
*50	1.83±0.39	1.08±0.22	0.92±0.19
*100	1,76±0.98	1.09±0.62	0.90±0.52
150	2.60±3.66	1.33±1.59	1.36±1.92
200	1.31±1.37	1.01±1.11	0.74±0.75

[a] Each value is the average of 3 or *6 replicates.

These results indicate that (1) *in vitro* methane consumption by a soil sample is a surface phenomenon better estimated on area basis than on dry weight basis and (2) a thin layer of soil provides more reproducible results than a thick layer.

3.3 Effect of soil humidity on methanotrophic activity

Water content appeared to be a key parameter of soil methanotrophic activity . The maximum activity was observed at about 30% humidity (W/W) (*fig.* 2). At 45% water (presence of free water on soil surface) the activity was 15% of the maximum. At higher water concentrations methane consumption was too low to be measured by the catharometric method used.

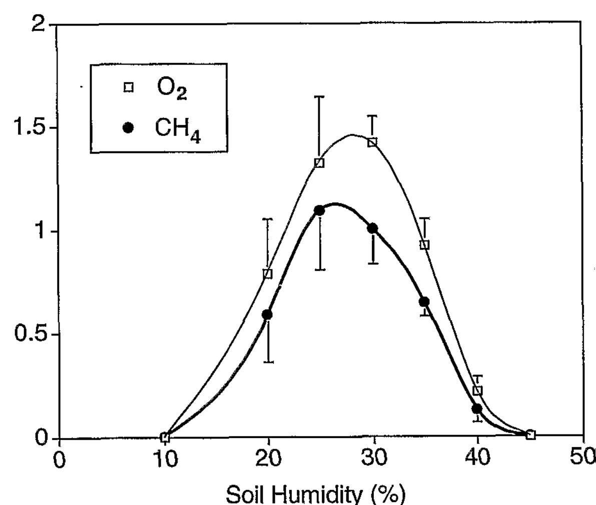


Figure 2. – Rates of O_2 and CH_4 consumption in flasks after 3 wk of incubation under 80% air/20% CH_4. Error bars indicate half of the confidence interval at $p=0.05$.

According to Whalen *et al.* (1990), the influence of water content on soil methanotrophic activity is physical. There is an optimum content which supports rapid gas-phase diffusion of CH_4 to a maximum area of cell surface while preventing desiccation. The decrease in CH_4 oxidation rates when soil moisture increases above the optimum, results from a change from gas phase diffusion to aqueous diffusion (10^4-fold less rapid) for CH_4 transport to cells. Whalen *et al.* (1990) observed that once soils were saturated, no further decrease in CH_4 oxidation rate was observed with increasing moisture content, because CH_4 transport to CH_4 oxidisers was dominated by aqueous molecular diffusion, which is a physical characteristic of a soil. However Nesbit & Breitenbeck (1992) also suggested that the apparent reduction of CH_4 oxidation observed after a moderate increase in soil moisture was

more likely caused by enhanced CH_4 production in anaerobic microsites than by reduced CH_4 oxidation at the soil surface.

In a landfill cover soil, Whalen *et al.* (1990) found an optimum methanotrophic activity at 11% water content. In the Camargue soil optimum was obtained at 30% water content. This obviously resulted from the difference in the water affinity of both soils. The landfill cover soil was a sand mixed with brown and grey clays with a low water affinity, while the Camargue ricefield soil (silt fine clay) obviously had a higher water affinity. Maximum methanotrophic activity observed in the Camargue soil (25 g m^{-2} day^{-1}) was of the same magnitude than that observed by Whalen *et al.* (1990) in a topsoil covering a retired landfill (45 g m^{-2} day^{-1}) which is the highest value currently reported.

3.4. Effect of fertilizer addition

Fertilizers added to soil at concentrations corresponding to those resulting from usual field application had no significant effect on methane oxidation (*fig.* 3).

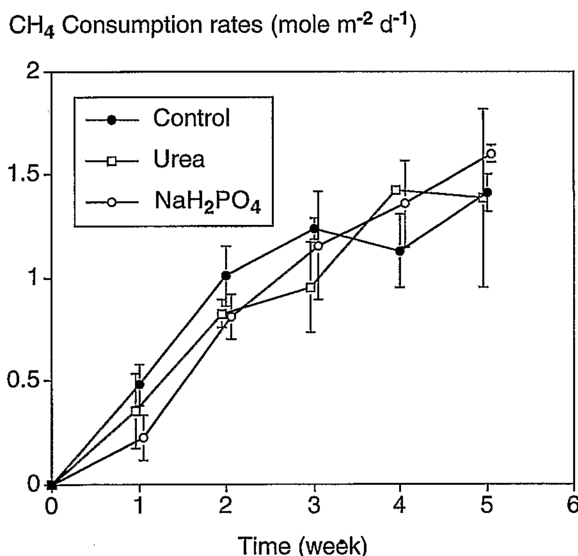


Figure 3. – CH_4 consumption rates after addition of fertilizers at concentrations corresponding to field concentration. Error bars indicate the confidence interval at $p = 0.05$.

A number of reports indicate inhibitory effects of NH_4^+ on methanotrophy (see Dunfield & Knowles, 1995 for references). Inhibition resulting from competition between NH_4^+ and CH_4 was observed at low and high CH_4 concentrations. A direct inhibitory effect of NO_2^- produced by NH_4^+ oxidation was observed only at lower CH_4 concentrations.

In a waterlogged ricefield, though CH_4 concentration was high, Conrad & Rothfuss (1991) showed that NH_4^+ applied to floodwater increased aerobic CH_4 fluxes, due to the inhibition of CH_4 oxidation at the soil surface. This was most probably because of the much higher solubility of NH_4^+ as compared to that of CH_4.

In a humisol, Dunfield & Knowles (1995) observed a competitive inhibition between NH_4^+ and CH_4 and an inhibitory effect of NO_2^-. Inhibition by NO_2^- was substrate-dependent, it was high at CH_4 levels lower than 100 ppm, but decreased at concentrations higher than 100 ppm. This latter observation probably explains that urea had no effect on the PMA, which was measured on wet soil with a high concentration of CH_4 in the gas phase and therefore a high availability of CH_4 to methanotrophs.

To our knowledge, no data are available on the effect of P fertilizer on methanotrophs in soils. As compared with most rice soils (Kawaguchi & Kyuma, 1977), Camargue soil had already a high available P content (45 ppm) and the addition of 45 ppm P as NaH_2PO_4 had no effect on its PMA. A similar result was obtained with three other ricefield soils, one with a very high available P content (58 ppm) and two with low available P content (12 and 5.8 ppm) (data not shown). Inhibition of methanotrophs by phosphate in a culture medium was reported by Whittenbury *et al.* (1970) but the threshold of 0.2% (W/V) was out of the range that can be expected in a ricefield. Phosphorus fertilizer is probably not a limiting factor by excess for methanotrophs in ricefields.

The fact that methane oxidation measured at soil water holding capacity was independent of fertilizer amendments supports the interest of PMA determination in that it is characteristic of a soil, irrespective of factors other than intrinsic soil properties. However, it is clear that PMA measurement should not be extrapolated to field situation.

3.5. Preservation of the methanotrophic activity

3.5.1. In dry soil

The PMA of the dry soil conserved at laboratory temperature in a plastic bag, remained unchanged for at least two years (the coefficient of variation of 50 measurements performed at intervals for two years was 18.4%). On the opposite, it was shown that air-drying the soil suppressed its atmospheric CH_4 oxidation activity when it was rewetted (Nesbit & Breitenbeck, 1992; Bronson & Mosier, 1994). This indicates that soil desiccation does not affect low affinity activity but inhibits high affinity.

3.5.2. In preincubated and dried soil

Measurements on soil preincubated under air/CH_4 for one month, which were performed before and after three weeks of desiccation, showed that PMA

(measured after three weeks of incubation) was not markedly affected by soil drying (*table* 2).

Table 2. – Effect of a three-weeks desiccation on soil methanotrophy [a].

Weeks	O_2 consumed (mole m^{-2} d^{-1})	CH_4 consumed (mole m^{-2} d^{-1})	CO_2 produced (mole m^{-2} d^{-1})
Dry soil remostened at field capacity and incubated under air/CH_4			
1	0.95±0.06	0.48±0.15	0.49±0.05
2	1.45±0.03	0.85±0.08	0.78±0.05
3	1.83±0.06	1.00±0.03	0.94±0.22
Same soil dried for 3 weeks, remostened, and incubated under air/CH_4			
7	0.21±0.04	0.04±0.02	0.15±0.01
8	1.50±0.20	0.78±0.21	0.83±0.09
9	2.21±0.16	1.15±0.18	1.14±0.09

[a] Each value is the average of 3 replicates.

3.5.3. *In preincubated soil kept wet under anaerobiosis*

The methanotrophic activity measured upon exposure to air/CH_4 of preincubated samples that were then kept in anaerobiosis for one, two, three, and four weeks exhibited no lag, did not vary significantly for one month, and decreased after one month (*table* 3).

Table 3. – O_2 and CH_4 consumption and CO_2 production rates (mole m^{-2} d^{-1}) by soil samples preincubated for one month at field capacity in air/CH_4 and then submitted to various periods of anaerobiosis.

Anaerobiosis (days)		0	7	14	21	29	36
Flask 1							
	O_2	0.94	1.16				n.d.
	CH_4	**0.53**	**0.67**				n.d.
	CO_2	0.88	0.69				n.d.
Flask 2							
	O_2	1.02		1.25			0.78
	CH_4	**0.62**		**0.58**			**0.30**
	CO_2	0.61		0.73			0.48
Flask 3							
	O_2	0.97			1.45		0.73
	CH_4	**0.47**			**0.74**		**0.30**
	CO_2	0.51			0.94		0.48
Flask 4							
	O_2	0.96				0.94	0.69
	CH_4	**0.56**				**0.53**	**0.27**
	CO_2	0.56				0.88	0.39

s.e. = 0.086.

n.d. = not determined.

Takeda (1988) observed that *Methylocystis* sp. could survive for two years in the absence of CH_4, under anaerobic conditions, which he explained by the formation of resistance forms (cysts). Roslev & King (1994) studying the oxic and anoxic CH_4 starvation of *Methylosinus trichosporium* OB3b and strain WP 12, showed that survival and recovery were generally highest for cultures starved under anoxic conditions. In their experiments, conducted with pure cultures, the CH_4 oxidation rate decreased and the lag increased during the first month of the experiment. We did not observed such a decrease in Camargue soil for the first month of starvation, but our results confirmed that methanotrophs in wet anaerobic soil maintain their potential for at least several weeks.

The ability of aerobic methanotrophs to withstand anaerobic conditions partly explains the significant potential for CH_4 oxidation of environments not continuously presenting oxic conditions optimum for the growth of methanotrophs. This is the case of ricefields submitted to alternate periods of submersion and desiccation.

Nesbit and Breitenbeck (1992) observed that soils displaying the most vigorous uptake of CH_4 were collected from frequently flooded sites where methanogenic activity was likely to occur in the soil profile. Sass *et al.* (1992) studied CH_4 production and emission in ricefields with (1) permanent flooding and (2) a flooding regime including three intermittent drainage periods of 2-3 days. In both treatments CH_4 emission initially rose with its production. In the continuous flooding treatment, CH_4 production (400 to 1000 mg m^{-2} day^{-1}) and emission (50 to 200 mg m^{-2} day^{-1}) were both significant from the 25th day till the end of the crop cycle. Introducing drainage periods reduced CH_4 production by about 50% and rendered emission negligible till the end of the crop cycle. A week after the first drainage, emission was small (7.9 mg m^{-2} d^{-1}) despite a high production (231 mg m^{-2} d^{-1}) which corresponded to 97% oxidation. Before the third drainage, CH_4 production was 410 mg m^{-2} d^{-1} and emission 6.7 mg m^{-2} d^{-1}, corresponding to 98% oxidation.

Field measurements of methane emission and production (Sass *et al.*, 1992; Watanabe *et al.*, 1995) indicated that methanotrophy (1) better developed in wet soil than in submerged soil, and (2), once established, partly persisted under submerged conditions. Our measurement of methanotrophic populations and activities confirmed these observations. They also showed that methanotrophic populations developed on a wet soil and then kept in anaerobiosis for less than one month rapidly resume a high activity when oxygen becomes available again, which is what happens under alternate periods of drainage and submersion in ricefields.

3.6. Dynamics and stoechiometry of the methanotrophic activity

Methanotrophic activity of the soil during a continuous incubation under CH_4 exhibited a parabolic

dynamic and reached its maximum at 26 days (*fig.* 4). The analysis of pooled data from all the methanotrophic activity measurements performed on the Camargue soil showed that CH_4 and O_2 consumption, and CO_2 production were highly positively correlated (*fig.* 5). In the calculated linear regression equations the constant was almost zero ($<10^{-2}$) indicating that heterotrophic activity was negligible as compared to methanotrophy.

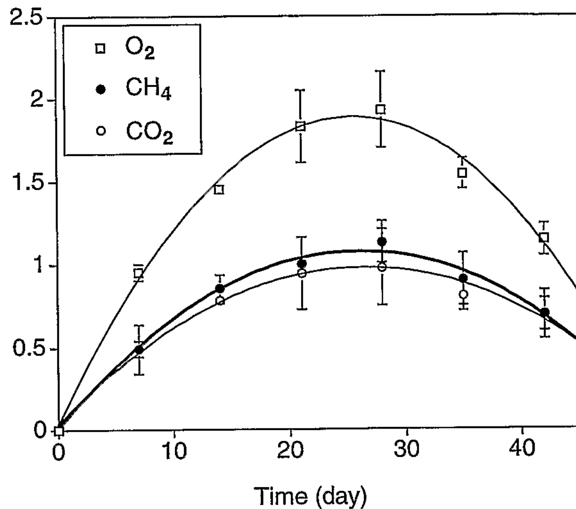
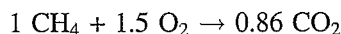


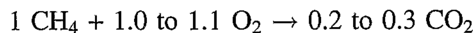
Figure 4. – O_2 and CH_4 consumption and CO_2 production rates during the incubation time under 80% air/20% CH_4. Error bars indicate the confidence interval at $p=0.05$.

The observed gas consumption/production ratios of methanotrophy in Camargue soil was:



$$1\ CH_4 + 1.5\ O_2 \rightarrow 0.86\ CO_2$$

The corresponding equation obtained by Whittenbury *et al.* (1970) using the two most performant strains of his collection grown in a fermentor with optimized gas diffusion was:



$$1\ CH_4 + 1.0 \text{ to } 1.1\ O_2 \rightarrow 0.2 \text{ to } 0.3\ CO_2$$

The comparison of both equations indicates that, in liquid culture, a higher fraction of CH_4 carbon was used for bacterial growth than in soil, thus indicating an activity predominantly of the respiratory type in the soil. This type of activity results from a limiting factor. Our results demonstrated that N or P availability was not limiting. On the other hand, visual observations showed a dense bacterial growth forming patches of vein-like material coating soil aggegates and filling soil porosity, thus refraining gas penetration and limiting to some extent the availability of gazeous substrate in the deeper soil layer.

Despite the low percentage of carbon assimilated, microbial biomass produced was high, because CH_4 consumption was also high. A calculation based on CH_4 consumption and CO_2 emission during the incubation indicated that the organic C content of the soil increased by at least 10% during a one-month incubation.

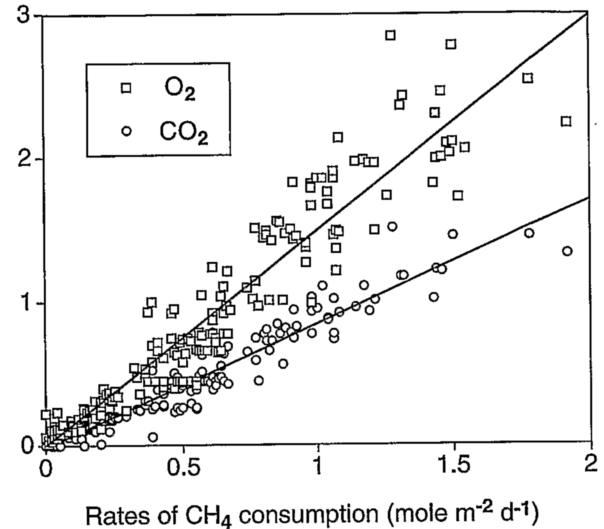


Figure 5. – O_2 consumption and CO_2 production rates in relation with CH_4 consumption rates.
$O_2 = 1.5027\ CH_4 - 3.4618 \times 10^{-3}$ $r^2 = 0.909$.
$CO_2 = 0.85910\ CH_4 - 1.4582 \times 10^{-2}$ $r^2 = 0.931$.

3.7. Density and composition of methanotrophic populations

3.7.1. Estimated density of methanotrophic populations

The methanotroph population of dry soil, estimated by the MPN technique, was low (2.5×10^2 methanotrophs g^{-1} d.w.) and remained stable for more than two years (5 enumerations). Preincubating the soil under air/CH_4 for 30 days considerably enhanced its methanotrophic population, which reached 5×10^8 methanotrophs g^{-1} of dry soil.

The results of MPN counts were not improved by shaking the tubes during their incubation. Maximum counts were obtained after 30-40 days of MPN tubes incubation (*fig.* 6).

Plate counts were refrained by the dense growth of contaminants. About 95% of the plates were contaminated with actinomycetes and nystatin resistant fungi. Counts on readable NMS plates incubated with and without CH_4 were in the same range of magnitude, indicating a low occurrence of methanotrophs on the plates. Estimating methanotroph populations from the difference in colony forming units (CFU) counted

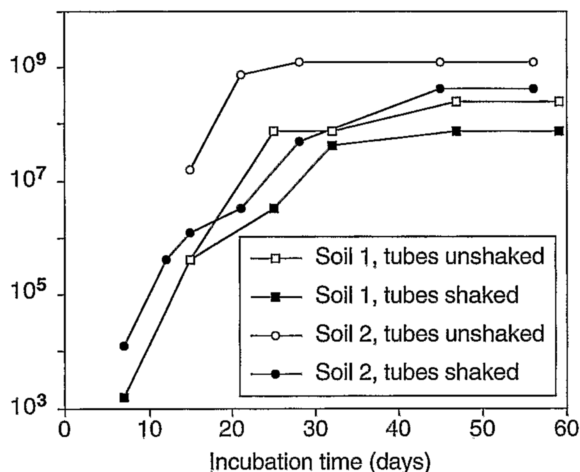


Figure 6. – Enumeration of methanotrophs by MPN counts on soils incubated under 80% air/20% CH_4 for one month.

on plates incubated with and without CH_4 yielded values that were more than 100 times lower than the estimates by MPN on the same sample. The low growth of methanotrophs observed on agar medium, as compared with MPN, was also observed when silicagel or gelrite were used as solidifying agent. Therefore, the difference in numbers of CFU on plates incubated with and without CH_4 was considered as inappropriate for enumerating soil methanotrophs.

3.7.2. Composition of methanotrophic populations

Colonies grown on plates incubated in air/ CH_4 and most likely to be methanotrophs according to Whittenbury *et al.* (1970) (colonies appearing after one week of incubation and keeping on growing for a few weeks) were spread onto slants in Hungate's tubes filled with air/CH_4. Results showed that only 0.5 to 1% of the colonies growing on dishes could grow and oxidise CH_4 on slants.

We isolated 13 methanotrophic cultures. Morphological observations showed the prevalence of type II methanotrophs belonging to the spore-forming genus *Methylosinus*. Further investigations in a range of soils are needed to test whether type II methanotrophs, which possess a soluble methane monooxygenase (sMMO), are dominants in ricefields. The current knowledge on competitive inhibition between CH_4 and NH_4^+ in methanotrophy let hypothesize such a predominance. In ricefields, which are generally fertilized with urea or ammonium sulphate, NH_4^+ is present at concentrations that may reach several ppm (Roger, 1996). In the presence of both NH_4^+ and CH_4, the particulate MMO, which is (1) present in all methanotrophs, (2) constitutive, and (3) able to act as ammonium monooxygenase (Bedard & Knowles, 1989), would cause the accumulation of highly toxic intracellular NO_2^-. On the other hand, at CH_4 concentrations higher than 100 ppm – likely to occur in ricefields –, the synthesis of the sMMO was induced in type II methanotrophs and NH_4^+ was oxidized in NO_2^- outside the cell (King & Schnell, 1994). In waterlogged ricefield soils, this should avoid NO_2^- toxicity because NO_2^- is immediately transformed into NO_3^- (Garcia & Tiedje, 1982). Therefore, it can be hypothesized that in ricefields, type II methanotrophs should be predominant because they are the only form producing sMMO and possess more efficient resistance forms than type I.

4. CONCLUSION

Our results showed that *in vitro* CH_4 consumption by a soil sample remained linear for at least 10 h and was a surface phenomenon better quantified on area basis than on dry weight basis, using a thin layer of soil. Maximum activity measured *in vitro* (PMA) was attained at a soil humidity around field capacity, after 3-4 weeks of incubation under air/CH_4. It remained unchanged for at least two years when estimated in samples of the soil kept dry and was not affected by N or P fertilizer additions corresponding to usual field application. Methane and O_2 consumption, and CO_2 production were highly positively correlated. The stoechiometry of the reaction indicated that in vitro methanotrophy by the soil was mostly of the respiratory type.

Experimental conditions that allowed maximum methanotrophic activity of the soil (PMA) were somewhat artificial for a wetland rice soil because it required a soil at 30% humidity and 20% of CH_4 in the incubation atmosphere. However, it showed a very satisfactory reproducibility (1) for replicated measurements (coefficient of variation ≤ 12.4%) and (2) in time for soil kept in dry state. This type of measurement can probably be used to characterize/classify rice soils with regard to methanotrophy.

Maximum methanotrophic activity measured after three weeks was equivalent to 1.56 mole CH_4 m^{-2} day^{-1} oxidized. After one week, it was already equal to the maximum daily CH_4 production measured after two weeks on the same soil enriched with 2% powdered straw (0.5 mole CH_4 m^{-2} day^{-1}) (Joulian *et al.* this issue). This indicated that indigenous microflora of the Camargue soil has the potential to oxidize all CH_4 produced in the soil.

Methanotrophic population estimated by the MPN technique was 2.5×10^2 g^{-1} d.w. in dry soil and 5×10^8 g^{-1} d.w. in wet soil incubated for 3 weeks under air/CH_4. Plating was found to be inappropriate for enumerating soil methanotrophs because of the

extremely high level of contamination by non-methanotrophic microorganisms, but it allowed the isolation of 13 methanotrophic cultures.

Morphological observations showed the prevalence of type II methanotrophs belonging to the spore-forming genus *Methylosinus.* It was hypothesized that in ricefields, type II methanotrophs must predominate because they are the only form producing soluble MMO (which avoids intracellular accumulation of toxic NO_2^-) and possess more efficient resistance forms than type I.

Field measurements of CH_4 production and emission showed that introducing few short drainage periods during the crop cycle of rice strongly reduced CH_4 emission both by decreasing its production and enhancing its consumption, without reducing rice yield (Sass *et al.*, 1992; Watanabe *et al.*, 1995). Our *in vitro* observations substantiate such field observations by directly showing that (1) methanotrophy developed at a much higher level on wet soil than in submerged conditions, and (2) methanotrophic populations established on a wet soil and kept in anaerobiosis for less than one month, rapidly resumed a high activity upon exposure to O_2 and CH_4. This confirms the observation by Sass *et al.* (1992) that several drainage periods, at about 25 days intervals, have to be introduced during the rice crop cycle to ensure a continuous efficient inhibition of CH_4 emission.

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