APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1997, p. 1199–1207 Vol. 63, No. 4
0099-2240/97/$04.00+0

Activity and Distribution of Methane-Oxidizing Bacteria in Flooded Rice Soil Microcosms and in Rice Plants (*Oryza sativa*)

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Received 21 June 1996/Accepted 17 January 1997

The activity and distribution of CH_4-oxidizing bacteria (MOB) in flooded rice (*Oryza sativa*) soil microcosms was investigated. CH_4 oxidation was shown to occur in undisturbed microcosms by using $^{14}CH_4$, and model calculations indicated that almost 90% of the oxidation measured had taken place at a depth where only roots could provide the O_2 necessary. Slurry from soil planted with rice had an apparent K_m for CH_4 of 4 μM and a V_{max} of 0.1 μmol g (dry weight)$^{-1}$ h^{-1}. At a depth of 1 to 2 cm, there was no significant difference ($P > 0.05$) in numbers of MOB between soil from planted and nonplanted microcosms (mean, 7.7×10^5 g [fresh weight]$^{-1}$). Thus, the densely rooted soil at 1 to 2 cm deep did not represent rhizospheric soil with respect to the number of MOB. A significantly increased number of MOB was found only in soil immediately around the roots (1.2×10^6 g [fresh weight]$^{-1}$), corresponding to a layer of 0.1 to 0.2 mm. Plant-associated CH_4 oxidation was shown in a double chamber with carefully washed intact rice plants. Up to 90% of the CH_4 supplied to the root compartment was oxidized in the plants. CH_4 oxidation on isolated roots was higher and had a larger variability than that in soil slurries. Roots had an apparent K_m for CH_4 of 6 μM and a V_{max} of 5 μmol g (dry weight)$^{-1}$ h^{-1}. The average number of MOB in homogenized roots was larger than on the rhizoplane and increased with plant age. MOB also were found in surface-sterilized roots and basal culms, indicating the ability of these bacteria to colonize the interior of roots and culms.

CH_4 is an important greenhouse gas, and its atmospheric concentration has been increasing for many decades (14, 30, 47). Flooded rice fields are one of the major sources of CH_4 emissions to the atmosphere (36, 49). These emissions are the net result of two processes: CH_4 production by methanogenic bacteria in anoxic environments, and CH_4 oxidation by CH_4-oxidizing bacteria in oxic environments. Anoxic CH_4 oxidation is not important in freshwater habitats (59). In flooded rice fields, most of the bulk soil is anoxic, and CH_4 oxidation is restricted to the soil surface, the roots, and the soil immediately around the roots, i.e., the rhizosphere. Wetland plants, including rice, have a large aerenchyma (air-filled spaces in stem and roots), which facilitates the movement of O_2 to the roots (1). In this way, plants may provide O_2 for CH_4 oxidation, thus reducing CH_4 emission. However, they also provide a pathway for easy ventilation of the soil, thus increasing emissions. In addition, they may enhance emissions by increasing the concentration of labile organic C in the rhizosphere, leading to an increase in CH_4 production. Because of the large surface area of root systems, plants could be very important in determining the overall CH_4 flux to the atmosphere. For natural wetlands as well as for rice fields, it has been shown that plant-mediated transport can account for much of the total observed CH_4 emission (7, 15, 46, 52, 53).

More knowledge of CH_4 oxidation on roots and in the rhizosphere can help us to understand CH_4 emissions from rice and other wetland plants. However, compared to studies concerning CH_4 emissions from rice fields, few studies have dealt with CH_4 oxidation or CH_4-oxidizing bacteria (MOB) in the rhizosphere of rice. CH_4 oxidation has been shown to take place in flooded rice soil by using flux measurements under oxic and anoxic conditions (24, 27) and in the surface layer of a rice field by using slurry measurements (10). CH_4 oxidation also has been shown to take place on the roots of many wetland species (8, 23, 32, 33). The presence of methylotrophic bacteria on the roots of various wetland species has been shown by use of 16S rRNA probes (32). Finally, it has been shown that MOB occur in the rhizosphere of rice plants (18) and on the rice root surface (24). The rhizosphere appears to be a very heterogeneous habitat for MOB. Both CH_4 concentrations (24) and O_2 release by roots (1) are highly variable. In addition, microbe-microbe interactions or plant-microbe interactions could play a role. For example, the presence of heterotrophic bacteria on the roots should lead to competition for O_2, and plants could excrete antimicrobial substances (38).

The aim of this study was to describe both the activity and the distribution of MOB in microcosms which were used as a model for flooded rice fields. A nondestructive method (the use of $^{14}CH_4$ as a tracer) was used to show that CH_4 oxidation took place in flooded microcosms, and a double chamber was used to show that CH_4 oxidation took place on the roots of intact plants. Then the process was characterized and localized by looking at CH_4 oxidation activity in soil slurries and isolated plant parts. Finally, we studied the distribution of MOB in these different compartments. Specifically, we were interested in organisms on the roots and culm, the size of the rhizosphere, and changes associated with plant age.

MATERIALS AND METHODS

Field site and microcosms. Field-grown plants and gas samples were taken in Vercelli, Italy, from a rice field of the Istituto Sperimentale della Risicoltura when the rice was about 80 days old. Soil was taken from the same field in the spring before flooding. Field management and soil type at this site have been described previously (28). The soil was air dried and stored at 25°C. Before use, it was sieved to a diameter of less than 2 mm. Microcosms were prepared by filling waterlogged soil (2 parts of soil and 1 part of deionized water [grams, dry weight, per gram]) in 20-cm-long Plexiglas cores with an inner diameter of 6 cm. They were wrapped in aluminum foil so that light could reach only the soil surface and incubated under 2 cm of floodwater at 25°C under a 12-h light, 12-h dark cycle. The light intensity 30 cm above the soil surface was approximately 70 microeinsteins m^{-2} s^{-1}. One week after being flooded, some microcosms were

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planted with four rice seedlings each (*Oryza sativa*, var. Roma, type japonica), while others remained nonplanted as a control. The vegetation period was 140 days. Weeds (plants other than rice) were removed weekly.

A soil density profile was measured in the microcosms. From this, the following values were taken for calculations: 1.28 g (fresh weight) ml^{-1} at 0 to 0.5 cm deep and 1.58 g (fresh weight) ml^{-1} at 1 to 2 cm deep and in rhizospheric soil. The roots had a density of 0.69 g (fresh weight) ml^{-1}. The average water content of soil was 0.28 g of H_2O g (fresh weight) of $soil^{-1}$, and that of the roots 0.90 g of H_2O g (fresh weight) of $root^{-1}$. For flux calculations according to Fick's first law, the equation $J_D = -D_s \cdot \Phi \cdot \Delta c \cdot \Delta z^{-1}$ was used, with $D_s = D \cdot \Phi^2$, $D = 1.73 \times 10^{-5}$ cm^2 s^{-1} for CH_4 at 25°C (interpolated from reference 34), and $\Phi = 0.84$ for the top 10 mm of soil (41). Δc (concentration gradient) and Δz (distance) are given in the text.

Gas analyses. CH_4 was analyzed by gas chromatography with a flame ionization detector, using either a Carlo Erba gas chromatograph (GC 6000-2) with N_2 as the carrier gas (columns, Porapak Q 80/100 mesh or Mol-Sieve 5A 80/100 mesh at 60°C; detector temperature, 140°C) or a Shimadzu gas chromatograph (GC-8A) with synthetic air as the carrier gas (column, Porapak Q 80/100 mesh at 60°C; detector temperature, 120°C). Detection limits were between 0.2 and 1 part per million (volume) (ppmv) CH_4 in a 1 ml sample, depending on the column. CO_2 was analyzed after conversion to CH_4 in a methanizer (Ni catalyst at 330°C; Chrompak, Frankfurt am Main, Germany). In the ^{14}C experiment, radioactive and nonradioactive CH_4 and CO_2 were analyzed in a gas chromatograph (Shimadzu GC-8A; H_2 as carrier gas; column, Porapak QS 80/100 mesh at 50°C) equipped with a methanizer, flame ionization detector (110°C), and RAGA radioactivity gas proportional counter (Raytest, Straubenhardt, Germany) (13). The detection limit for radioactive gases was 25 Bq ml^{-1}. CH_3F was analyzed with the Carlo Erba gas chromatograph and the Porapak Q column described above (20). O_2 was analyzed on a Shimadzu GC 8A gas chromatograph with a thermal conductivity detector and with N_2 as the carrier gas (column, Mol-Sieve 5A 80/100 mesh at 80°C; detector temperature, 80°C).

$^{14}CH_4$ oxidation in flooded rice microcosms. $^{14}CH_4$ was prepared biologically from $NaH^{14}CO_3$ by using *Methanobacterium thermoautotrophicum* Marburg, DSM 2133 (16, 58). *M. thermoautotrophicum* was grown under a gas atmosphere of 88% H_2 and 12% CO_2 at 65°C in medium containing 0.1 g of $CaCl_2 \cdot 2H_2O$ per liter, 0.1 g of NH_4Cl per liter, and 0.2 g of KH_2PO_4 per liter (modified by Bak from reference 56), 1 ml of vitamin solution per liter (57), 2 ml of trace element solution per liter (55), 30 mM $NaHCO_3$, and 1 mM Na_2S. The medium was prepared as described previously (56) and adjusted to a pH of 7.2 to 7.4. A small amount of dithionite ($Na_2S_2O_4$) was added just before inoculation. A growing culture was concentrated by centrifugation to an optical density at 600 nm of 0.73 in 100 mM phosphate buffer (16). The culture was supplied with H_2 and CO_2 for 24 h. To deplete it of C, it was then supplied with H_2 for another 48 h and bubbled with H_2 twice during this time. Then 500 μl of $NaH^{14}CO_3$ was added. Within 4 days, all of the $NaH^{14}CO_3$ was converted to $^{14}CH_4$. It had a specific radioactivity of 1.25×10^6 Bq μmol^{-1}. Some methanogenic bacteria can produce small amounts of CO, C_2H_2, C_2H_4, C_2H_6, or C_3H_8 (3, 11). We could detect none of these gases in the $^{14}CH_4$ produced, which means that none of those substances can have been present at more than approximately 1% of the $^{14}CH_4$, assuming one labeled C atom per molecule.

Small cores (2.6 by 10 cm) were filled with waterlogged soil, planted with two rice seedlings each, and incubated as described above. When the plants were 46 days old, Plexiglas chambers were mounted gastight on top of the microcosms. Each chamber had a septum for taking gas samples. The soil surface was covered with 2 cm of water. The experiment was run in duplicate and started by adding 1.7×10^6 (core 1) and 2.8×10^6 (core 2) Bq of $^{14}CH_4$ to the gas phase. During the experiment, the plants received light at 120 microeinsteins m^{-2} day^{-1} in a 12-h light, 12-h dark cycle. The concentrations of $^{14}CH_4$, CH_4, $^{14}CO_2$, and CO_2 in the gas phase were monitored over time by taking 0.2-ml gas samples. The sample volume was replaced with air. After 16 (core 1) and 23 (core 2) days, the experiment was stopped. Floodwater, above-ground plant parts, and soil were separated, and the soil was further divided into a 0- to 5-cm and a 5- to 10-cm layer. Dissolved CO_2, CH_4, $^{14}CO_2$, and $^{14}CH_4$ levels were measured in the floodwater and the soil pore water by shaking the samples to extract the gas (22). The roots were then picked from the soil, and organic CO_2 and $^{14}CO_2$ levels were measured in the soil, roots and above-ground plant parts by a modified version of the wet-combustion procedure described in reference 48. Soil and plant parts were dried at 60°C. Then 7 ml of 18 M H_2SO_4 and 5 ml of 2 N $K_2Cr_2O_7$ in 10% (vol/vol) sulfuric acid were added to 0.02 g of plant material or 0.4 g of soil and heated to 130°C for 1 h. Emitted CO_2 was cooled and trapped in 15 ml of 2 N NaOH. This solution was acidified, and CO_2 was extracted into the gas phase (22). The Bunsen coefficient of CO_2 in the acidified solution was determined empirically (12) as 0.62 ± 0.02 (standard error [SE]; $n = 3$) and used to correct for the CO_2 remaining in solution.

CH_4 concentrations in culms of rice plants. Samples were taken by inserting a 50-μl syringe directly into the aerenchyma and/or the medullar cavity of the culm (26) and extracting 10 to 50 μl of gas per sample. A vertical profile of CH_4 concentrations was measured in plants from microcosms (112 and 180 days old) by taking samples in 1- to 5-cm steps between 1 and 30 cm above the soil. A total of 47 samples was measured with $n = 2$ to 11 for each height.

CH_4 oxidation associated with intact plants. We used intact plants in a double chamber to disturb transport processes between culm and roots as little as possible. Plants were grown in soil so that they could develop the typical microbial flora. The double chamber is shown in Fig. 1. The cores (inner diameter, 6 cm; length of the lower part, 20 cm; length of the upper part, 40 cm) and the connecting pieces were made from Plexiglas (modified from reference 17). The lower chamber was filled with 530 ml of He-saturated nutrient solution and 30 ml of CH_4-saturated nutrient solution. Helium was added as an inert control. The nutrient solution was modified from reference 29 with 1.3 mg of Fe^{3+}-citrate per liter and 10 mg of KNO_3 per liter but no C sources. A rice plant was washed carefully to remove all adhering soil, placed into the double chamber as shown in Fig. 1, and fixed in place with modeling clay. Gas bubbles were removed before the upper chamber was set in place. All connections were sealed with O-rings, and the sampling ports were equipped with butyl septa. The double chamber was incubated at the same temperature and light intensity as described for the large microcosms. The concentrations of CH_4 and He were monitored over time in both chambers. The lower chamber was stirred before sampling by moving a magnetic stir bar up and down. The sample volume was 2 ml, and the samples were shaken to extract the gas. The upper chamber was mixed permanently with a fan, and the sample volume was 1 ml. Samples were taken in duplicate from each chamber, and the sample volumes were replaced by nutrient solution (lower chamber) or air (upper chamber). Two experiments were run with this chamber, one with a microcosm plant and one with a field plant (the plant age was 80 days in both cases). In the first experiment, CH_4 and He were dissolved in the nutrient solution in the lower chamber. After 4 days, the nutrient solution and the gas atmosphere were replaced and this time CH_4, He (to the nutrient solution in the lower chamber), and CH_3F (to the gas phase in the upper chamber) were added. CH_3F is an inhibitor of CH_4 oxidation (20, 37). No CH_3F was used in the second experiment.

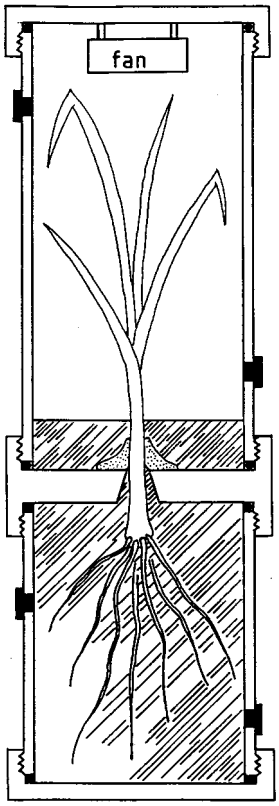

FIG. 1. Schematic drawing of the double chamber used to measure plant-associated CH_4 oxidation.

CH_4 oxidation and CH_4 production in soil slurry and on isolated roots. To measure CH_4 oxidation, soil slurries were prepared by mixing 1 part of microcosm soil with 1 part of water (grams [fresh weight] per gram). Of this slurry, 2 ml was placed into pressure tubes, and the tubes were closed with butyl stoppers. If dilution ratios or volumes other than these were used, it is indicated in the text. CH_4 was added to give different concentrations between 0.13 and 30 μM (100 to 23,000 ppm by volume at 25°C). The tubes were incubated horizontally on a roller at 25°C and the CH_4 concentrations were monitored over time. When the CH_4 concentration reached a plateau for at least 10 days, it was called the threshold concentration. O_2 was added if its concentration dropped below ca. 10% O_2 in air.

Roots were cut from plants and rinsed repeatedly in tap water until the water

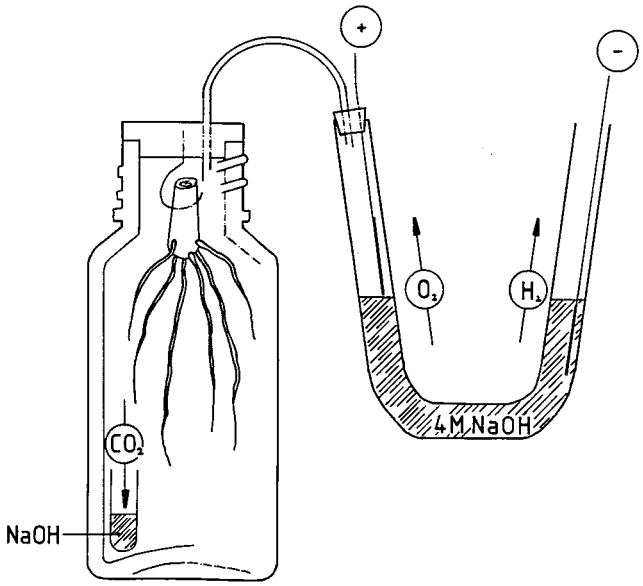

FIG. 2. Schematic drawing of the O_2 supply system used in CH_4 oxidation measurements.

remained clear. Roots which sank to the bottom were considered dead and were discarded. The roots were then blotted and put into pressure tubes. They were incubated standing upright but otherwise treated as the soil samples. The kinetics of CH_4 oxidation were measured on roots still attached to basal culm parts. They were incubated in 150-ml flasks equipped with a system to replace the O_2 consumed. It is based on electrolytic O_2 production (50) (Fig. 2). A small amount of NaOH was added to the flask to adsorb any CO_2 produced. When O_2 was consumed in the flask, the pressure dropped and the electrolyte in the U-shaped glass tube was drawn up. When it reached the platinum wire, electrolytic O_2 production took place (30-V source) until the now increasing pressure lowered the electrolyte level again.

To measure CH_4 production, a 5-ml sample of the 0- to 1-cm soil layer was mixed with 10 ml of anoxic water. The roots were picked out, rinsed carefully in another 10 ml of anoxic water, taken out, and incubated under anoxic conditions in a 150-ml flask for at least 28 h at 25°C in the dark. The two soil slurries from this preparation were incubated in the same way. CH_4 production was monitored over time by taking gas samples.

O_2 consumption in soil slurry. To determine the O_2 consumption in soil, O_2 was analyzed with a commercial O_2 electrode (WTW, Weilheim, Germany) with a detection limit of 3.125 μM O_2. Soil (10 g [dry weight]) from 1 to 2 cm deep was placed into 250-ml flasks and stirred for 15 min. Then the bottles were filled with water. An O_2 electrode with a cone fitting into the flask was inserted without trapping gas bubbles. O_2 consumption in the stirred slurry was monitored over time from 153 to 125 μM O_2 on a recorder.

Counting of bacteria. MOB were counted by the most-probable-number (MPN) method (44). The 1:2 dilution was done in microtiter plates with 100 μl per well and eight parallel determinations. An ammonium mineral medium (modified from reference 25) which contained 0.35 g of $NaH_2PO_4 \cdot 2\ H_2O$ per liter, 0.34 g of KH_2PO_4 per liter, 0.54 g of NH_4Cl per liter 0.39 g of $MgSO_4 \cdot 7\ H_2O$ per liter, 0.5 ml of 0.2 M $CaCl_2$ solution per liter, and 1 ml of trace element solution per liter (55) was used and was adjusted to a pH of 6.8. The incubation temperature was 25°C. After 3 and 4 weeks, the microtiter plates were checked for growth visually. Wells with growth had a cloudy appearance. In addition, some plates were checked with a binocular microscope. The results were the same. All serial dilutions were done in duplicate, and one of the microtiter plates was incubated under a gas atmosphere of 20% CH_4 and 80% synthetic air (21% O_2 in N_2), while the other was incubated under synthetic air as a control. The MPN calculated in the controls was always less than 3% of the MPN under 20% CH_4. Calculation of asymmetric confidence limits and tests for differences between numbers were done by the method in reference 9.

Soil samples were diluted in medium and shaken for 24 h at 4°C (100 rpm). To study the soil surface, soil from 0 to 0.5 cm deep was chosen; to study the effect of roots, soil from 1 to 2 cm deep was used. From this, samples were taken for the serial dilution. In another preparation, soil around the roots was gently removed by hand until only a thin layer of soil remained. Then the roots were rinsed in medium to remove this remaining soil, which was called rhizospheric soil. Samples were taken from the rinsing bath for the serial dilution.

To prepare plants for MPN counts, soil was carefully removed from the plants. Then the roots were cut off and washed as described above. To count rhizoplane (root surface) MOB, roots were mixed with medium and glass beads in a ratio of 1:10:10 (grams [fresh weight] per gram per gram). The mixture was incubated on a rotary shaker for 30 min at 25°C to remove the bacteria from the rhizoplane. Samples of the solution were taken for the serial dilution. To count the total root population of MOB (referred to as total root MOB), the roots were cut into 1- to 2-cm pieces, put into medium, and homogenized for 3 min with a mixer. From this, samples were taken for the serial dilution. Above-ground plant parts were prepared similarly. The plants were rinsed in an upright position under flowing water so as not to contaminate the above-ground parts with microorganisms from roots, and culms or leaves were cut into 2-cm pieces before further handling.

For surface sterilization, a protocol for seeds (39) was adapted. Roots (15 g [fresh weight]) were sterilized for 10 s in 80 ml of 5% NaOCl and then rinsed in sterile water baths (80 ml each) for 1, 2, 3 and finally 10 min. Then, about two-thirds of the material was homogenized to count endorhizospheric MOB, while rhizoplane MOB were counted as a control in the remaining one-third. MOB were found in none of the controls (detection limit, 100 cells g [fresh weight]$^{-1}$); i.e., the surface had been completely sterilized in all experiments. For comparison, MOB were counted in samples of the same material without sterilization. The procedure for surface sterilization of culm parts was the same. Sterilization experiments were done with plants from the field and from microcosms.

RESULTS

CH_4 oxidation in flooded rice microcosms. $^{14}CH_4$ was used to show the oxidation of CH_4 in flooded rice microcosms. Of the $^{14}CH_4$ added, 31.2 and 31.4% were oxidized in the two cores, respectively. Almost all of the oxidized $^{14}CH_4$ was found as $^{14}CO_2$-$H^{14}CO_3^-$ in the soil pore water and as organic ^{14}C when soil and plants were analyzed by wet combustion. No $^{14}CO_2$ was found in the gas phase except on the last day in core 2. On the other hand, the $^{14}CH_4$ which was not oxidized was found almost exclusively in the gas phase. Only 0.5 and 1.2% were found in the pore water of the two cores. CH_4 emitted from the soil and the specific radioactivity of $^{14}CH_4$ in the gas phase decreased exponentially (Fig. 3). The overall recovery of radioactivity was 86 and 72% in the two cores, respectively.

CH_4 concentrations in culms of rice plants. Mean CH_4 concentrations decreased exponentially from 7,025 ppmv (±1,815 [SE]; 9.2 μM at 25°C; $n = 11$) at 1 cm high to 1,032 ppmv (±206; $n = 5$) at 10 cm high and 36 ppmv (34 and 38 ppmv; $n = 2$) at 30 cm high.

CH_4 oxidation in soil slurry. CH_4 oxidation rates were measured in soil slurries from planted and nonplanted microcosms. Different amounts of soil were tested to check for gas phase transfer limitation. In planted microcosms, rates were constant at high CH_4 concentrations (9 to 13 μM, or 7,000 to 10,000 ppmv at 25°C) between 0.5 and 0.9 g (dry weight) of soil. If more soil was used, rates decreased, indicating gas phase transfer limitation. Thus, no more than 0.9 g (dry weight) was used.

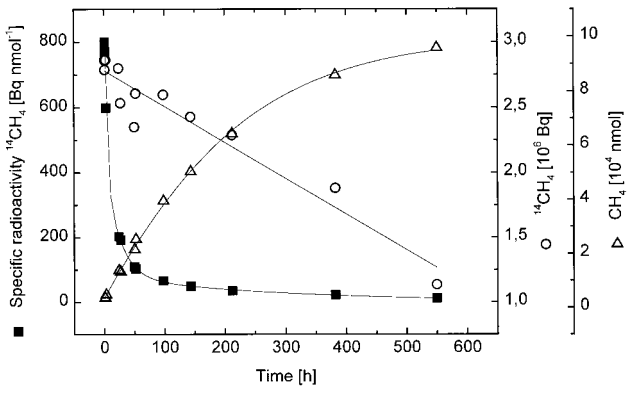

FIG. 3. $^{14}CH_4$, CH_4, and specific radioactivity in the gas phase of a sealed microcosm (core 2) with rice plants after addition of $^{14}CH_4$ to the atmosphere at $t = 0$ h.

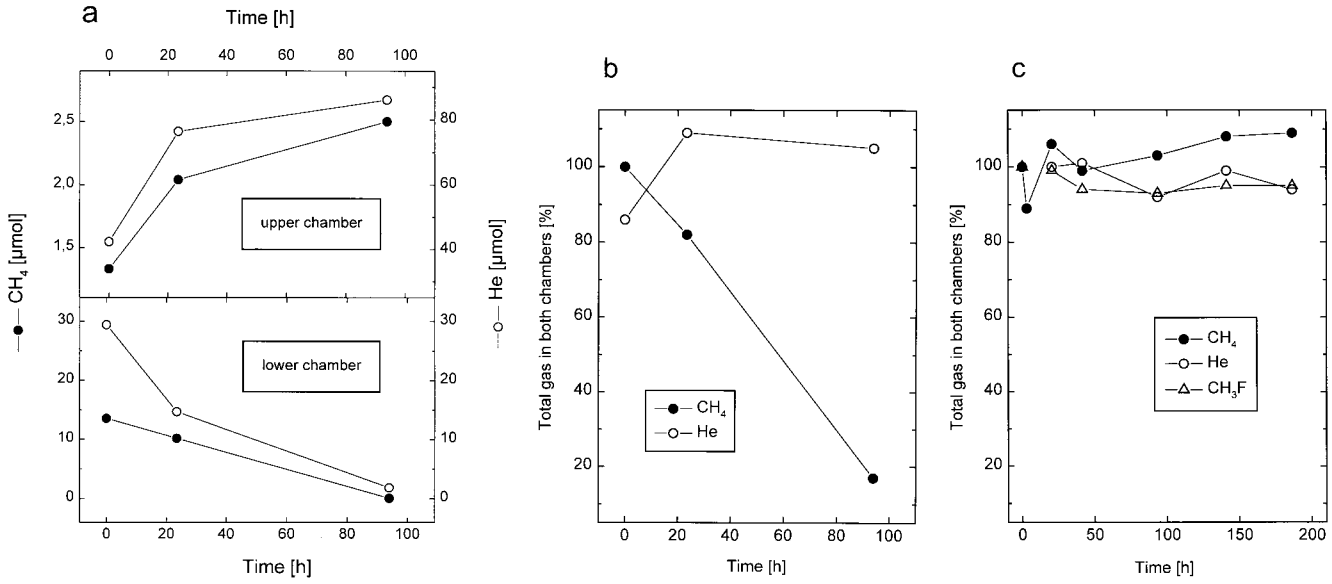

FIG. 4. Results of the double-chamber experiment with a rice plant from a microcosm. (a) CH_4 and He in the two chambers during the first part of the experiment (oxidation). (b) Total percent amounts of CH_4 and He in both chambers during that part. (c) Total percent amounts of CH_4, He, and CH_3F in both chambers during the second part of the experiment (inhibition).

However, rates were near the detection limit, and for low CH_4 concentrations (0.06 μM, or 50 ppmv at 25°C), gas phase transfer limitation could not be entirely ruled out. CH_4 oxidation rates in soil slurries from planted microcosms (0- to 5-cm soil layer) were measured as initial rates at various CH_4 concentrations. A direct Michaelis-Menten fit gave an apparent K_m of 3.91 ± 0.53 μM and a V_{max} of 0.12 ± 0.01 μmol g (dry weight)$^{-1}$ h^{-1} (n = 16). CH_4 was oxidized down to a mixing ratio of 1.2 ppmv, i.e., below atmospheric concentrations. CH_4 oxidation rates also were measured in rhizospheric soil (soil immediately around roots), in soil from the top layer (0 to 3 mm), and in soil from nonplanted microcosms. They were measured at 30 μM CH_4, a typical CH_4 concentration in these microcosms. In each case, the CH_4 oxidation rates were below the detection limit, which was 0.7 μmol g (dry weight)$^{-1}$ h^{-1} for the rhizospheric soil, 0.5 μmol g (dry weight)$^{-1}$ h^{-1} for the top soil, and 0.1 μmol g (dry weight)$^{-1}$ h^{-1} for the nonplanted soil. The detection limit depended on the amount of soil and the incubation time before CH_4 oxidation rates increased sharply. This happened if soil slurries were incubated with >0.6 μM (500 ppmv at 25°C) CH_4. The increase in the CH_4 oxidation rates was observed after 25 to 100 h, usually to values between 0.2 and 2 μmol g (dry weight)$^{-1}$ h^{-1}.

CH_4 oxidation associated with intact plants. The amounts of CH_4 and He in two chambers connected through a rice plant are shown in Fig. 4. Both gases diffused along their concentration gradient from the lower to the upper chamber (Fig. 4a). The total amount of He was roughly constant, showing that the chambers had no leaks. In contrast, the total amount of CH_4 decreased, indicating CH_4 oxidation (Fig. 4b). A plant taken from the field gave similar results. If the rate at which CH_4 disappeared from the lower chamber is termed the gross transportation rate (r_g) and the rate at which it appeared in the upper chamber is termed the net transportation rate (r_n), the percent oxidation rate can be calculated as $(1 - r_n/r_g) \times 100$. From this, it was calculated that up to 90% of the CH_4 was oxidized while diffusing through the plant. The results are summarized in Table 1. In the second part of the experiment shown in Fig. 4, CH_3F was added as an inhibitor (Fig. 4c). This time, the total amount of each gas remained at 100%, indicating that CH_4 oxidation had been completely inhibited by CH_3F.

CH_4 oxidation and CH_4 production on isolated roots. If washed roots were incubated under oxic conditions at 30 μM CH_4, they showed highly variable CH_4 oxidation rates ranging from 2 to 86 μmol g (dry weight)$^{-1}$ h^{-1} (mean, 13.7 ± 2.0; n = 48). Mean rates increased with decreasing root weight (0.24 to 0.003 g [dry weight]), indicating gas phase transfer limitation. However, the variance of the running mean of the rate also increased with increasing rates and thus with decreasing root weight. This means that instead of gas phase transfer limitation, a small-scale variability of the rates might have been the reason for the high rates at low root weight. Rates (r) did not depend on plant age (a) if the effect of root weight (w) was controlled for by partial correlation ($r_{ra.w.} = -0.13$; $P > 0.05$; n = 48). The basal culms also oxidized CH_4. At 2.6 μM CH_4, they showed rates of 0.21 ± 0.06 μmol g (dry weight)$^{-1}$ h^{-1} (n = 3); roots showed rates of 0.31 ± 0.07 μmol g (dry weight)$^{-1}$ h^{-1} (n = 5); and roots still attached to basal culms showed rates of 0.26 and 0.27 μmol g (dry weight)$^{-1}$ h^{-1} (n = 2). Roots still attached to the basal culms showed no gas phase transfer limitation between 0.27 and 0.45 g (dry weight). A direct Michaelis-Menten fit for CH_4 oxidation with roots still attached to the basal culms gave an apparent K_m of 6.10 ± 1.34 μM and a V_{max} of 5.15 ± 0.44 μmol g (dry weight)$^{-1}$ h^{-1} (n =

TABLE 1. CH_4 oxidation by washed[a] intact rice plants (80 days) in a double chamber

Source	Mean oxidation rate			CH_4 concn range (μM) in lower chamber
	μmol g (dry wt) of root^{-1} h^{-1}	mmol m^{-2} day^{-1}[b]	% of gross transportation rate	
Microcosm	1.19	10.0	91	24–0.1
Field	1.44	12.2	57	74–0.3

[a] Soil adhering to roots was carefully removed before the experiment.
[b] To calculate per-area values, 350 g (dry weight) of root per m^2 was used. This value was determined from microcosms of this age.

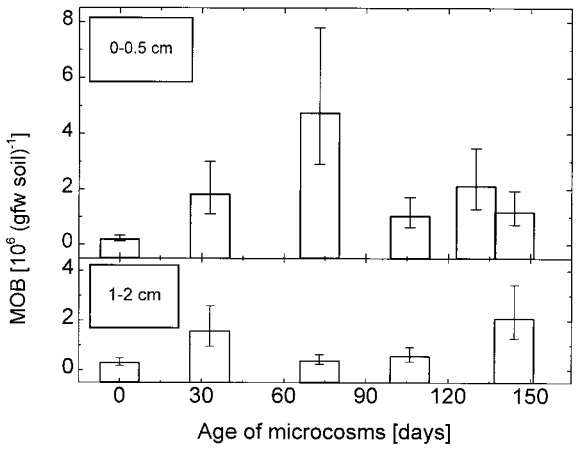

FIG. 5. Number of MOB at different soil depths in nonplanted microcosms during an incubation period of 144 days. Each bar represents 1 MPN ($n = 8$, 95% confidence limits).

10). Roots from plants near flowering (56 and 80 days old) oxidized CH_4 down to a concentration of 2 to 20 ppmv, and roots from younger plants (24 days old) oxidized CH_4 down to a concentration of 100 to 350 ppmv. CH_4 was thus not oxidized to levels below atmospheric concentrations.

With small root samples (<0.24 g [dry weight]), CH_4 oxidation showed a lag phase of 25 to 75 h in about 40% of all experiments. In these cases, the rates were calculated from the decrease in CH_4 concentrations after the lag phase. However, the existence of a lag had no effect on rates (U test; $P < 0.05$, $n_{lag} = 20$, $n_{no\ lag} = 28$). The lag phase was not correlated with plant age and was never observed if large amounts of roots still attached to the basal culm were used (>0.27 g [dry weight]).

In addition to this, we tried to measure CH_4 oxidation on roots by (i) using roots in water, (ii) homogenizing the roots and measuring oxidation directly in the homogenate, and (iii) homogenizing the roots and spreading the homogenate onto substrates (sterilized dry rice soil and quartz sand). However, either the results were not reproducible or the rates were at least 1 order of magnitude lower than those measured by the standard procedures described above.

If isolated roots were incubated anoxically, they produced CH_4 without lag at rates between 1 and 70 nmol g (dry weight)$^{-1}$ h^{-1} (31 ± 15; $n = 4$; plant age, 94 to 144 days). CH_4 production increased from the flowering stage (94 days) to ripening stage (144 days). The CH_4 production on roots was about 8% of the total production of soil and roots in the 0- to 1-cm layer (8.2 ± 2.4; $n = 4$).

MOB in nonrhizospheric soil. MOB were counted in soil from planted and nonplanted microcosms at 0 to 0.5 and 1 to 2 cm deep several times during the vegetation period of rice. Results for soil from nonplanted microcosms are shown in Fig. 5. The numbers varied with time and showed different patterns at the different soil depths, but we have no explanation for the different patterns. The numbers in soil from planted microcosms were very similar. When planted and nonplanted soils were compared at each sampling date (same depth and same day), the numbers were not statistically different in 11 of 12 cases ($P > 0.05$). Table 2 shows the average number of MOB at the different depths. While the 0- to 0.5-cm layers were statistically different ($P \leq 0.05$), the 1- to 2-cm layers, i.e., the root zone, were not ($P > 0.05$).

MOB in rhizospheric soil. Precleaned roots were rinsed repeatedly in medium, and the MOB in the medium were counted. Numbers expressed per gram (fresh weight) of root decreased from one rinsing to the next (Fig. 6). The mean number of MOB rinsed off was 1.2×10^6 MOB g (fresh weight) of soil^{-1} ($n = 32$). This was significantly ($P \leq 0.05$) larger than the number in the rooted soil at 1 to 2 cm deep (Table 2).

This soil with an increased number of MOB can be thought of as a layer around the roots, and the thickness of this soil layer was calculated. First, the volumes of roots and soil were calculated by using the respective densities. Using a root diameter of 0.3 mm (32-day-old plants) or 0.6 mm (70-day-old plants), we then calculated the length of the root, the total volume of root plus soil, and finally the total radius of root plus soil. The thickness of the soil layer is the difference between the total radius and the root radius and was calculated to be 0.06 and 0.11 mm for the two diameters, respectively.

MOB in plants. Figure 7 shows the number of MOB in roots. While the number of rhizoplane MOB was more or less constant with the age of the plants, the number of total root MOB (i.e., MOB after homogenization) increased. The mean number of rhizoplane MOB was 3.3×10^6 MOB g (fresh weight) of root^{-1} ($n = 96$), and the mean number of total root MOB was 6.4×10^6 MOB g (fresh weight) of root^{-1} ($n = 76$). In the basal culm (0 to 2 cm high), there were 3.4×10^6 MOB g (fresh weight) of culm^{-1} ($n = 16$), and in the upper culm (>2 cm), there were 1.1×10^4 MOB g (fresh weight) of culm^{-1} ($n = 8$). In the leaves, numbers were below the detection limit of 100 cells g (fresh weight) of leaf^{-1} ($n = 8$).

Results of the surface sterilization are shown in Fig. 8. MOB were found in roots and in the basal culm of both microcosm and field plants. The average number of endorhizospheric MOB was 2.1×10^4 MOB g (fresh weight) of root^{-1}, or 1% of the mean total root MOB in those experiments, and was in all cases severalfold above the detection limit. For the culm, the respective numbers were 4.3×10^3 MOB g (fresh weight) of culm^{-1}, or 2% of total culm MOB. Rhizoplane and culm surface MOB (i.e., without sterilization) were 27 and 17% of total root and total culm MOB, respectively.

DISCUSSION

We studied the activity and distribution of MOB in flooded rice microcosms to improve our understanding of one of the processes determining CH_4 emissions from rice fields. CH_4

TABLE 2. Numbers of methanotrophic bacteria in different soil depths in flooded rice soil microcosms

Soil depth	Mean no. (10^5 g [fresh wt]$^{-1}$) and 95% confidence interval for:		P^b
	Planted soil	Nonplanted soil	
Top layer (0–0.5 cm)	9.56 (7.84–11.66)	12.87 (10.55–15.70)	0.05
Rooted layer (1–2 cm)	7.98 (6.38–9.98) (NS)[b]	7.34 (5.87–9.18) ($P < 0.001$)[b]	NS

[a] Numbers are averaged from several measurements throughout the vegetation period, with six samples ($n = 48$) at 0 to 0.5 cm deep and five samples ($n = 40$) at 1 to 2 cm deep.

[b] NS, not significantly different ($P > 0.05$). *P* values in parentheses refer to the soil depths; *P* values in the right-hand column refer to the different planting conditions.

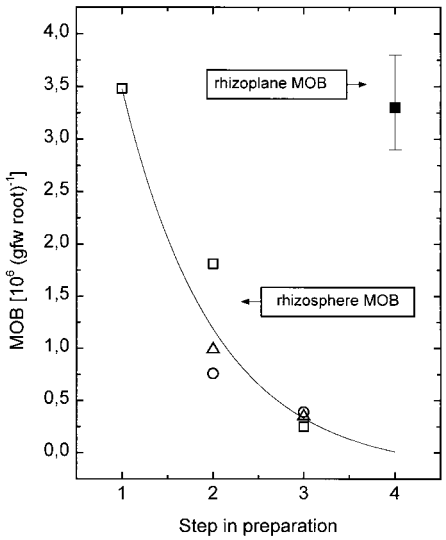

FIG. 6. Number of MOB in soil immediately around rice roots from microcosms. Shown is the number of rhizosphere MOB in consecutive baths removing soil from pre-cleaned roots of different ages: 24 (□), 70 (△), and 130 (○) days. The results of three independent experiments were combined. In each experiment, the roots of all the plants of one microcosm were pooled before the cleaning. Each datum point represents 1 MPN (n = 8). ■, mean number of rhizoplane MOB ± 95% confidence limit (all MPN, n = 96). All values are expressed per gram (fresh weight) of root.

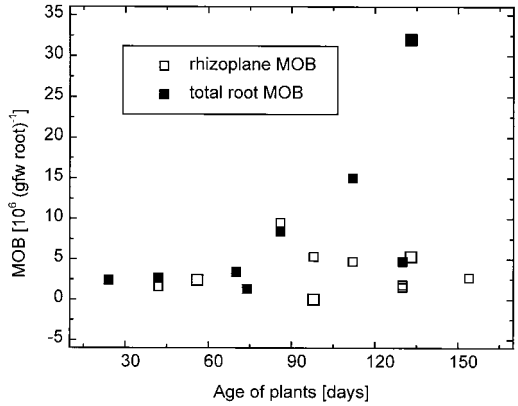

FIG. 7. Number of MOB in rice roots from microcosms during the vegetation period. Each datum point represents the result from one batch of roots (small symbols represent MPN with n = 8; large symbols represent MPN with n = 16 or 32). For one batch, the roots from all the plants of one or a few microcosms were pooled.

oxidation was studied on several levels, from intact microcosms to intact plants to isolated soil and isolated plant parts.

It was shown that $^{14}CH_4$ added to the gas phase around rice plants in closed microcosms was oxidized. In this system, where CH_4 may be produced and oxidized at the same time, oxidation could not be shown unequivocally except by labeling the CH_4. Total CH_4 oxidation was estimated in two ways. Direct estimates from the decrease in specific radioactivity of $^{14}CH_4$ in the gas phase gave 2.4 and 5.4 mmol m^{-2} day^{-1} for the two cores. Indirect estimates from flux measurements gave 3.1 and 3.9 mmol m^{-2} day^{-1}. For the latter, we used the measured CH_4 flux in the ^{14}C microcosms and a mean oxidation rate calculated from a series of oxidation experiments in similar microcosms (5).

To differentiate between soil oxidation and rhizospheric oxidation, we calculated how much CH_4 could reach the soil surface and be oxidized there by using Fick's first law. Assuming a stagnant water layer, with Δc = (mean floodwater CH_4 concentration calculated from mean gas phase concentration) − (minimum pore water CH_4 concentration = 0 μM), and Δz = (water layer) = 2 cm, and with the mean specific radioactivity, a value of 1.9×10^4 Bq was calculated for core 1. This is equal to 3.5% of the 5.3×10^5 Bq which were oxidized in total. For core 2, the value was 5%. Similarly, in case the water was not completely stagnant and the pore water at the soil surface was in equilibrium with the gas phase, we calculated how much $^{14}CH_4$ could then diffuse through the oxic layer. Using Δc = (mean floodwater activity calculated from mean gas phase activity) − (minimum pore water activity = 0 Bq $liter^{-1}$) and Δz = (oxic soil layer) = 3 mm (19, 21), 9 and 12% of the oxidized $^{14}CH_4$ might have been oxidized in the oxic layer. Thus, it seems likely that only a limited amount of $^{14}CH_4$ from the gas phase could be oxidized at the soil surface and that the rhizosphere was the main site of $^{14}CH_4$ oxidation. Total CH_4 oxidation may even be underestimated if a cycling between $^{14}CH_4$ and $^{14}CO_2$ by mineralization of ^{14}C exudates occurred. Soil oxidation would become more important relative to rhizospheric oxidation only if $^{14}CH_4$ were transported belowground through the aerenchyma, passed the rhizosphere without oxidation, and diffused back to the surface through the pore water. Since physical limitations to diffusion in pore water remain, that route is not very likely.

Double-chamber experiments showed that CH_4 oxidation rates per gram (dry weight) were similar between the two plants from the laboratory and the field. Per area, the CH_4 oxidation rate was ca. 11 mmol m^{-2} day^{-1}. A total CH_4 oxidation rate of 13 mmol m^{-2} day^{-1} was calculated for planted microcosms of the same age from the difference between oxic and anoxic fluxes (5). The range of CH_4 concentrations in the double-chamber experiment was comparable to what has been found in microcosms. This means that plant-associated CH_4 oxidation could account for a considerable part of the oxidation observed in microcosms.

Studies with isolated material showed that initial CH_4 oxidation potentials measured at 30 μM CH_4 were much lower in soil than in roots (0.1 and 14 μmol g [dry weight]$^{-1}$ h^{-1}, respectively). To compare CH_4 oxidation potentials for an imaginary field, it was assumed that the upper 3 cm of soil (main root zone) was active. This gives a CH_4 oxidation po-

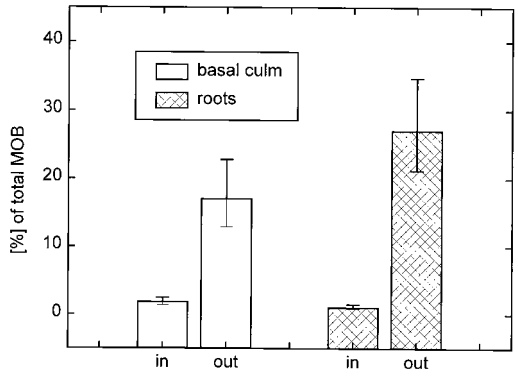

FIG. 8. Number of MOB in rice plants from microcosms and from a rice field. "in" denotes bacteria counted in homogenized roots after surface sterilization; "out" denotes rhizoplane bacteria. Each column represents the mean of several MPN counts (n = 24 for basal culms, n = 32 for roots, 95% confidence limit). The plant age was 80 days for field plants and 130 days for microcosm plants.

tential in the bulk soil of 86 mmol m^{-2} day^{-1}. A CH_4 oxidation potential on roots of 115 mmol m^{-2} day^{-1} was estimated by using the root biomass in 70-day-old microcosms. Thus, on an area basis, the CH_4 oxidation potentials in the bulk soil and on the roots are in the same range. The CH_4 oxidation potential in the oxic surface layer (3 mm [19, 21]) would be less than 34 mmol m^{-2} day^{-1} (calculated from <0.5 μmol g [dry weight]$^{-1}$ h^{-1}). However, from flux calculations obtained with Fick's first law, a maximum flux of 12 mmol CH_4 m^{-2} day^{-1} can be calculated for that layer, with Δc = (highest pore water concentration measured) − (minimum pore water concentration at the surface) = 400 μM − 0 μM = 400 μM (24) and Δz = (oxic soil layer) = 3 mm (19, 21). This means that the CH_4 oxidation potential in the surface layer probably is lower than in a densely rooted bulk soil. However, in situ, the two soil compartments may be active to very different degrees.

The CH_4 oxidation potential increased in the presence of plants. The V_{max} value in the bulk soil was similar to that in the surface layer of this soil (10), while the V_{pot} values on roots were high compared to those given in the literature (23, 32). The apparent K_ms found in soil (4 μM CH_4) and on roots (6 μM CH_4) were similar to those that have been published for freshwater (45), lake sediments (35, 40), and roots of various wetland plants (32, 33). In situ CH_4 concentrations in the soil pore water were mostly between <20 and about 125 μM CH_4 (24). CH_4 concentrations in the aerenchyma just above the soil level were taken as minimum values for the CH_4 concentrations in the roots, since the CH_4 diffuses from the roots to the atmosphere, giving a concentration of ≥ 9.2 μM CH_4 for the roots of older plants. Comparing the apparent K_m values with these CH_4 concentrations shows that in the rhizosphere and in the roots of older plants, CH_4 concentrations should not have been limiting.

CH_4 oxidation on roots, especially young roots, had a higher threshold than in soil. This means that the populations might differ during different stages of plant development and between soil and roots. The spatial heterogeneity of the roots was indicated by the higher variability of the oxidation rates shown by smaller root samples compared to larger ones. Similarly, lag phases were observed only in small root samples. However, lag phases had no effect on CH_4 oxidation rates even if the roots had been incubated at high CH_4 concentrations. This means that a high proportion of the methanotrophic population on roots probably was active in situ. On the other hand, we never observed immediate CH_4 oxidation rates in soil as high as those reached after incubation at high CH_4 concentrations. Since CH_4 concentrations were higher in the soil than in the roots while a lower proportion of the population seemed to be active, the limiting factor must have been O_2 rather than CH_4. This does not mean that O_2 was in excess in the roots. For example, CH_4 oxidation rates were higher on isolated roots (O_2 coming from outside and inside) than in the double chamber (O_2 coming from inside). A "more active" population can indicate adaptations at the enzymatic level, a higher percentage of active than inactive states, or larger cell numbers because of population growth. The latter was in fact observed for both roots and soil upon prolonged incubation at 30 μM CH_4 (data not shown). However, the usually very sudden onset of high CH_4 oxidation rates in soil makes it unlikely that this was the only factor involved.

If roots were incubated anoxically, they showed instantaneous CH_4 production, as has been shown previously (20). Rice roots, except during germination (4), generally have an aerobic metabolism (51), but these observations show that there were anoxic sites. This has also been found for the roots of other wetland species (32) and shows the heterogeneity of these environments. CH_4 originating from roots, about 8% of the total CH_4 production in the layer studied, may not be an important part of the budget quantitatively. However, it could be enough to influence isotopic signals, as suggested (32), since methanogenic bacteria directly on the roots might use C sources different from those used at some distance from the roots.

A large percentage (70%) of the root mass is in the upper 3 cm of the soil. Therefore, a 1- to 2-cm depth was chosen to study the effects of roots on the number of MOB. However, their number was not larger there than at the same depth in nonplanted microcosms. In another study (24), the number of MOB was higher in planted microcosms, possibly due to differences in the sampling procedure. However, we did find an increased number of MOB in a thin layer of about 0.1 mm around the roots. Similarly, if plants were grown in nylon bags so that a dense root mat developed, an increased number of MOB was found in the soil within the bags compared to outside of them (24). The difference was, however, far more pronounced in these mats than in our microcosms. The oxidized zone around roots was calculated in an earlier study by using, among other things, O_2 release by the root and O_2 uptake by the soil as parameters (2). We calculated the thickness of the oxidized zone for our system, using this model for roots with diameters of 0.6 mm and with the mean value of O_2 uptake determined for our soil (2.8 ± 0.35 μmol of O_2 g [dry weight]$^{-1}$ h^{-1}; $n = 9$). A thickness of 0.5 mm was calculated for the oxidized zone compared to 2.9 mm calculated by Armstrong for his system (2). This is higher than the value derived from the MPNs by a factor of 5; however, we did not determine the O_2 release for our system. Thus, there may be large differences depending on growth conditions, rice varieties, and soil, but apparently the rhizosphere around rice roots can be an extremely thin layer.

MOB also were counted in the 0- to 0.5-cm layer. The larger number of MOB in the 0- to 0.5-cm layer of nonplanted compared to planted soil may have been due to differences in light availability resulting in differences in algal growth and O_2 penetration depth (31).

The presence of methylotrophic bacteria on roots of various wetland plants (32) and the presence of MOB on rice roots (24) have been shown previously. We found that the number of total root MOB after homogenization increased much more with plant age than did the rhizoplane population, indicating that growth conditions were better in the roots as a whole than on their surface. We could also show clearly that there are MOB inside sterilized roots from both microcosms and the field. Apart from rhizobia, various groups of bacteria are present in the endorhizosphere (43). However, adding up the endorhizospheric (1% of the total root MOB) and the rhizoplane populations (27%) does not give the total number of MOB. Most probably, this is due to the sterilization time, which, for technical reasons, could not be reduced further. We therefore assume that part of the endorhizospheric population was killed too. We also found MOB on the surface and inside the basal culm (0 to 2 cm). For the reasons given above, the population inside was probably also underestimated. Instantaneous CH_4 oxidation rates measured on basal culms were similar to those on roots, and CH_4 is present there. However, in addition, the conditions are likely to be more constant with time there. Differences caused by different root age or microscale differences in the soil should in part have evened out in the culm. There are few MOB in the upper culm (>2 cm) and even fewer in the leaves, probably because the average CH_4 concentrations in the culm were below the apparent K_m determined here for plant-associated CH_4 oxidation.

Most of the known methanotrophic bacteria can grow on an ammonium-based mineral medium such as was used in this study (54). However, there may be some that do not, and there are probably many others that have not been cultivated at all (see, e.g., references 6 and 42). Therefore, the numbers given here are likely to underestimate the population size, but comparisons between compartments should be possible. To compare numbers of bacteria on roots with numbers in soil, numbers are best expressed per volume substrate because of the differences in density and water content. In planted microcosms, the mean number of total root MOB is 4.4×10^6 ml^{-1}, that of soil at a depth of 0 to 0.5 cm is 1.2×10^6 ml^{-1}, that of soil at a depth of 1 to 2 cm is 1.3×10^6 ml^{-1}, and that of rhizospheric soil is 1.9×10^6 ml^{-1}. Numbers on roots increased over time (for roots younger than 100 days, the mean is only 2.1×10^6 ml^{-1}), while those in soil showed no clear trend. This suggests that roots may be the "better" habitat.

To summarize, rhizospheric and/or plant-associated CH_4 oxidation can be very effective and may be more important than CH_4 oxidation at the soil surface. Rhizospheric CH_4 oxidation seems to be restricted to a very thin layer, and O_2 rather than CH_4 may be the limiting factor. However, not only the rhizosphere but also the interior of the rice plant itself is colonized by MOB. In this context, future studies will focus on the interaction of MOB with rice plants and the controls of CH_4 oxidation.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (FR 1054/1-1, 1-2) and the EC (contract EV5V-CT94-0499).

We thank R. Conrad, P. Janssen, and two anonymous reviewers for helpful discussions, and we thank S. Russo, who supported our work at the rice fields in Vercelli, Italy.

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