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Effects of ammonium-based fertilisation on microbial processes involved in methane emission from soils planted with rice

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Abstract. The emission of the greenhouse gas CH_4 from rice paddies is strongly influenced by management practices such as the input of ammonium-based fertilisers. We assessed the impact of different levels (200 and 400 kgN.ha^{-1}) of urea and $(NH_4)_2HPO_4$ on the microbial processes involved in production and consumption of CH_4 in rice field soil. We used compartmented microcosms which received fertiliser twice weekly. Potential CH_4 production rates were substantially higher in the rice rhizosphere than in unrooted soil, but were not affected by fertilisation. However, CH_4 emission was reduced by the addition of fertiliser and was negatively correlated with pore water NH_4^+ concentration, probably as the consequence of elevated CH_4 oxidation due to fertilisation. CH_4 oxidation as well as numbers of methanotrophs was distinctly stimulated by the addition of fertiliser and by the presence of the rice plant. Without fertiliser addition, nitrogen-limitation of the methanotrophs will restrict the consumption of CH_4. This may have a major impact on the global CH_4 budget, as nitrogen-limiting conditions will be the normal situation in the rice rhizosphere. Elevated potential nitrifying activities and numbers were only detected in microcosms fertilised with urea. However, a substantial part of the nitrification potential in the rhizosphere of rice was attributed to the activity of methanotrophs, as was demonstrated using the inhibitors CH_3F and C_2H_2.

Introduction

Rice paddies are among the most prominent sources in the global CH_4 budget. About 10% of the global annual emission of this greenhouse gas originates from rice cultivation areas (Neue 1997). Rice production has to increase 60% in the next decades to meet the required food demand from population growth (Cassman et al. 1998). This may result in increased CH_4 emission. Hence, knowledge about the sources and sinks of CH_4 in rice paddies, and

of the factors influencing them, is essential for designing possible mitigation strategies.

The input of fertiliser N may become an important controlling factor in methane emission from rice agriculture. The already high nitrogen applications to rice will have to increase, since this is the limiting factor in rice productivity (Cassman et al. 1998). This may affect important processes involved in the methane budget of rice paddies i.e., methanogenesis, methane oxidation, nitrification and also plant growth. Studies investigating fertiliser effects on these processes have yielded contradictory results. After fertilisation with urea or $(NH_4)_2SO_4$, lower (e.g., Schütz et al. 1989; Lindau et al. 1990; Cai et al. 1997) methane emissions were observed and attributed to direct inhibition of methanogenesis. However, higher CH_4 emissions were also detected from rice paddies after fertilisation with urea, $(NH_4)_2HPO_4$ and $(NH_4)_2SO_4$ (Sing et al. 1996; Banik et al. 1996; Cicerone & Shetter 1981).

The consumption of CH_4 by methanotrophs is generally thought to be inhibited by ammonium-based fertilisation, by as yet unraveled mechanisms reviewed by Gulledge et al. (1997). However, results often are contradictory for inhibition (e.g. Steudler 1989; Hütsch et al. 1994; Bosse et al. 1993), as well as no effects (e.g. Dunfield et al. 1995; Delgado & Mosier 1996) have been observed. The obligate aerobic methane oxidisers proliferate in the vicinity of oxygen-releasing roots of rice and other wetland plants (Gilbert & Frenzel 1998; Bosse & Frenzel 1997; Calhoun & King 1998) and may oxidise up to 10–65% of the potentially emitted methane (Denier van der Gon & Neue 1996; Schipper & Reddy 1996; Bosse & Frenzel 1997; King 1996). There is hardly any information about the effects of fertiliser regime on methanotrophic bacteria in these environments and of the impact on methane emission.

Input of fertiliser ammonium may stimulate nitrifying bacteria (Arth et al. 1998) which convert it to nitrite and nitrate. Elevated concentrations of these compounds due to high N input may inhibit methane production (Klüber & Conrad 1998) as well as methane oxidation (King & Schnell 1994). Besides this, ammonia oxidisers are capable of oxidising methane (Jones & Morita 1983; Ward 1987) and methanotrophs may convert ammonium to nitrite (O'Neill & Wilkinson 1977; Yoshinari 1984). The effects of fertilisation on nitrification and the relative contributions of ammonia- and methane-oxidisers to methane and ammonia oxidation in rice paddies have not been amply investigated.

The present study aimed at assessing the effect of ammonium-based fertilisation on microbial processes involved in methane emission from rice soil. We therefore studied the dynamics of methane production, methane oxidation and nitrification in compartmented microcosms fertilised with urea or

di-ammoniumphosphate. In addition, we assessed the effect of fertilisation on the contribution of methane- and ammonia-oxidising bacteria to methane- and ammonia-oxidation in the rice rhizosphere.

Materials and methods

Soil and fieldsite

The soil used in all experiments was sampled from a rice field of the Istituto Sperimentale per la Cerealicoltura in Vercelli (Italy) in the spring of 1997 and was stored at ambient temperature (15–20 °C) after 2 weeks of air-drying. Soil type and rice field management practice have been described earlier (Schütz et al. 1989). Immediately prior to use the soil was crushed with a jaw-crusher and sieved (mesh size 2 mm).

Operation of the model system and growth conditions

As a model system, compartmented microcosms were used as described in detail by Bodelier et al. (1997) and Bodelier and Frenzel (1999). In the center of these cylindrical stainless steel microcosms (12×9 cm; height × diameter), a perforated steel cylinder (12×4 cm, height × diameter) covered on the inside with nylon gauze (mesh size 30 μm), served as physical separation between root and nonroot compartment. Soil from the root compartment was regarded as rhizosphere soil. The microcosms were filled with 700 grams of dry rice soil and subsequently flooded with demineralised water and incubated for 1 week in a growth chamber (Conviron CMS 3244, Controlled Environments Limited, Winnipeg, Canada) at 25 °C and 70% relative humidity (RH) in the dark. After 1 week, 1 rice seedling (*Oryza sativa* var. Roma, type japonica) which had been germinated on wet filter paper at 25 °C in the greenhouse was planted in the root compartment of each microcosm. The planted microcosms were incubated in the growth chamber for 12 weeks at 70% RH and in a light/dark cycle of 12/12 hours at a photosynthetically active radiation (PAR) of 450 μEinstein·m^{-2}·s^{-1} (99 W.m^{-2}) and a temperature regime of night/day of 20 °C/25 °C. The surface of the soil was always covered with 2 cm of demineralised water and shaded with aluminium foil to reduce warming of the microcosms due to illumination.

Design and fertiliser application

The experiment was designed with fertilisation, fertiliser type, fertiliser level and the presence of roots as independent variables. In total, 24 microcosms

Table 1. Schematic presentation of the experimental design (A) and parameters measured prior and after harvesting of the microcosms (B).

A

Treatments	Unplanted	Planted
Unfertilised	4	4
Urea 200 kgN.ha^{-1}		4
Urea 400 kgN.ha^{-1}		4
$(NH_4)_2HPO_4$ 200 kgN.ha^{-1}		4
$(NH_4)_2HPO_4$ 400 kgN.ha^{-1}		4

B

	Plant parameters	Process-related parameters: activities and substrate availability			Organisms
Pre-harvest analyses[1]	Total leaf length Total plant height	CH_4 flux Porewater CH_4	Porewater NH_4^+	Porewater NO_3^-, fatty acids, pH	
Post-harvest analyses	Root biomass	Potential CH_4 production of soil and roots	Potential NH_4^+ oxidation of soil and roots		MPN counts of CH_4 and NH_4^+ oxidising bacteria
	Shoot biomass	Potential CH_4 oxidation of soil and roots	CEA and DRA[2]		

[1] All measurements were done once per week in all microcosms.
[2] Competitive Exclusion Assay (CEA) and Differential Recovery Assay (DRA) were performed to estimate the contribution of methane- and ammonia-oxidising bacteria to NH_4^+ oxidation.

were used for 6 different treatments, with 4 replicate microcosms per treatment as presented schematically in Table 1A. The fertiliser was applied weekly by injecting 10-ml solutions of urea or $(NH_4)_2HPO_4$, containing 67.5 mM N and 135 mM N for the 200 and 400 kg.ha^{-1} treatments, respectively. The solutions were injected both in the root and nonroot compartments by injecting 2ml sub-portions using a 10-ml syringe. The syringe was pulled out of the soil while emptying to achieve an even distribution of fertiliser. During weeks #0–#3 application was made once a week while in week #4–#12 fertiliser was applied twice a week.

In order to assess fertiliser effects on methane emission and microbial processes involved a great number of analysis were performed before plants and soil was harvested. Table 1B gives an overview of the parameters analysed. A detailed description is given below.

Weekly measurements

(i) Plant parameters
Once every week total plant height and leaf length were determined. Plant height was defined as the distance from the soil surface to outmost part of the canopy. Total leaf length was the defined as the distance from the leaf sheath/leaf blade interface to the tip of the leaf.

(ii) CH_4 flux
CH_4 flux was determined once a week by covering each microcosm with an airtight glass cover (see Bodelier et al. 1998) with a volume of 2 liters. The atmosphere within the glass cover was mixed by means of a built-in fan. For a period of 30 minutes, headspace samples were taken every 5 minutes via a septum. The samples were immediately analysed for CH_4 as described below. The methane flux was calculated from linear regressions of methane mixing ratios vs time (adjusted $R^2 > 0.90$).

(iii) Sampling of porewater
In order to monitor CH_4, NH_4^+, NO_3^-, NO_2^-, fatty acids and pH, weekly porewater samples were taken from the root and the nonroot compartments of the microcosms into Venoject blood collecting tubes by means of Rhizon soil solution samplers (Eijkelkamp, Giesbeek, The Netherlands) as described by Bodelier et al. (1997) and Bodelier and Frenzel (1999). 1 ml of porewater was centrifuged (13 800 $\times$ g, 4 °C, 15 minutes) and the supernatant stored at -20 °C for further analyses.

Harvest of rice plants and preparation of soil slurries

After 12 weeks the microcosms were harvested and the soils from the root and the nonroot compartments were treated as follows. Prior to processing the soil, the upper 2–3 cm of both compartments were removed and discarded from further analyses because this soil layer may receive oxygen from the overlaying water and not only from the rice root. The complete root compartment was transferred to a beaker containing 240 ml of demineralised water, and the soil was washed of the roots. The resulting suspension of rhizospheric soil contained approximately 0.25 g of dry soil per ml. The soil from the nonroot compartment was completely transferred to a glass beaker and mixed. 160 grams of this soil was suspended in 240 ml of demineralised water. From both of these slurries 150 ml was diluted with 150 ml of demineralised water and subsequently used for measurement of potential CH_4 oxidation, potential CH_4 production and MPN counts of methanotrophs, ammonia oxidisers and nitrite oxidisers. 150 ml of the original slurries was also diluted with 150 ml

of an $(NH_4)_2SO_4$ solution containing 6.23 mM NH_4^+. This NH_4^+ concentration was chosen to reach a desired concentration of 2 mM in the slurry, accounting for the endogenous ammonium already present and for the adsorption of ammonium to the soil particles (35% of added ammonium) which was determined in a previous experiment with soil from identical microcosms (data not shown). The slurries with added ammonium were subsequently used for the potential ammonia oxidation (PAO), the competitive exclusion assay (CEA) and differential recovery assay (DRA) (see below).

Post-harvest analysis

(i) Plant biomass
Shoots and roots were dried at 60 °C for 48 hours. Dry weight was determined gravimetrically.

(ii) Potential CH_4 oxidation in soil slurries and associated to rice roots
The potential CH_4 oxidation activity of soil from the root and nonroot compartments of 4 replicate microcosms was determined as described by Bodelier and Frenzel (1999). 70 ml of water-diluted slurry was transferred to 500-ml flasks which were supplemented with 10 000 ppm_v (parts per million volume) of CH_4 (99.995%, Messer Griesheim, Siegen, Germany) and incubated at 25 °C on a gyratory shaker (120 rpm). The CH_4 mixing ratio in the headspace was monitored for 3–4 days by GC analysis as described below. Potential CH_4 oxidation activity associated with rice roots was determined using 5 g of fresh root material incubated in 150-ml flasks closed with rubber stoppers. After flushing with synthetic air (21% O_2 in N_2) for 15 min, 1.5 ml of CH_4 (final mixing ratio 10 000 ppm_v) was added. The flasks were incubated unshaken at 25 °C in the dark. The CH_4 mixing ratio was monitored as described below.

(iii) Potential NH_4^+ oxidation in soil slurries and associated to rice roots
For the measurement of potential NH_4^+ oxidation, 70 ml of slurry (supplemented with $(NH_4)_2SO_4$ as described above) was incubated immediately after preparation under the same conditions as described for the CH_4 oxidation assay. Nitrate and nitrite production was monitored in these slurries as described by Bodelier and Frenzel (1999). Potential NH_4^+ oxidation activity associated with rice roots was determined by incubating 5 g of fresh root material together with 50 ml of assay medium (Bodelier & Frenzel 1999) in a 250-ml Erlenmeyer flask. Flasks were incubated on a gyratory shaker (100 rpm) at 25 °C in the dark. Measurement of nitrite and nitrate production during the incubation, and calculation of nitrification rates, were done according to Bodelier and Frenzel (1999).

(iv) Potential CH_4 production in soil slurries and associated to rice roots
Potential CH_4 production was determined by transferring 40 ml of the water-diluted slurries to 150-ml flasks. Residual CH_4 and O_2 was removed by evacuation and subsequent flushing with N_2. The flasks were incubated statically at 25 °C in the dark. CH_4 accumulation in the headspace was monitored for 3 days by GC analysis as described below. Potential methane production was calculated from linear regressions of methane concentration vs time (adjusted $R^2 > 0.90$). The potential CH_4 production associated with rice roots was estimated in the same way, using 5 gram of fresh root material cut into 1-cm pieces.

(v) Competitive exclusion assay using CH_3F (CEA)
This assay was designed by Bodelier and Frenzel (1999) to discriminate between ammonia oxidation by nitrifiers and by methanotrophs. For this assay the slurries diluted with $(NH_4)_2SO_4$ solution were used. From 2 replicate microcosms, 70 ml slurry from the root and nonroot compartments were transferred to 500-ml assay flasks and treated as follows: (1) no CH_4 addition; (2) + CH_4 (10 000 ppm_v); (3) + CH_4 (10 000 ppm_v) + CH_3F (300 ppm_v) (>98%, Fluorochem, Old Glossop, Derbyshire, UK). The latter treatment gives rise to a dissolved molar ratio of CH_4:CH_3F:NH_4^+ = 0.1:1:18. At this concentration of substrate and inhibitor, only methanotrophs but not the ammonia oxidisers are inhibited. In all assay bottles CH_4, NH_4^+, NO_2^- and NO_3^- were monitored as already described. The contribution of methanotrophs to NH_4^+ oxidation in the absence of CH_4 is calculated as [1-(NH_4^+ oxidation rate in the presence of CH_4 and CH_3F/NH_4^+ oxidation rate without CH_4 or CH_3F)]∗100. In the presence of CH_4 this contribution is calculated as [1-(NH_4^+ oxidation rate in the presence of CH_4 and CH_3F/NH_4^+ oxidation rate with only CH_4)]∗100.

(vi) Differential recovery assay using C_2H_2 (DRA)
This assay was designed by Bodelier and Frenzel (1999) to discriminate between methane and ammonia oxidation by nitrifiers and by methanotrophs. 70-ml amounts of the NH_4^+-supplemented slurries from the root and non-root compartments of 2 replicate microcosms were transferred to 500-ml assay flasks, which were supplemented with CH_4 (10 000 ppm_v). The flasks were incubated as already described and CH_4 and NH_4^+ oxidation was monitored for 24 hours, after which C_2H_2 (1 000 ppm_v) (99.6%, Messer Griesheim) was added. After 24 hours the C_2H_2 was removed as described above, and the flasks were supplemented again with CH_4. During the following 2 days the recovery of CH_4 and NH_4^+ oxidation was monitored. Since methanotrophs recover faster than ammonia oxidisers after removal of C_2H_2, the contribution of methanotrophs to NH_4^+ oxidation is calculated by (NH_4^+

oxidation rate prior to inhibition with C_2H_2/NH_4^+ oxidation rate after removal of C_2H_2)$*$100.

(vii) Numbers of methanotrophs

The numbers of methanotrophs in soil from the root and non-root compartments as well as associated with the rice roots were determined by the most probable number (MPN) method according to Gilbert and Frenzel (1995). Soil slurries and root suspensions were serially diluted in microtiter plates containing ammonium-mineral-salts medium. The plates were incubated for 4 weeks at 25 °C in gastight jars containing 20% CH_4 in air. Inoculated plates without CH_4 served as controls. Wells that were turbid were considered positive.

(viii) Numbers of ammonia- and nitrite-oxidisers

Ammonia- and nitrite-oxidising bacteria were enumerated by means of the MPN method. 1ml of slurry was diluted 1:10 with ammonia oxidiser (Watson 1971) or nitrite oxidiser (Laanbroek & Schotman 1991) growth medium and shaken for 2 hours (200 rpm) to extract the bacteria. Subsamples of 0.8 ml of these suspensions were serially diluted in 18 dilution steps of 1:3.5 (vol:vol) in sterile microtiter plates (Nunc^{TM} Brand Products, Denmark) containing growth medium for ammonia- and nitrite-oxidising bacteria, respectively. After an incubation period of 2 months in the dark at 25 °C, ammonium oxidation was scored in each well by checking for the presence of nitrite or nitrate by adding Griess Ilosvay reagents (Spektroquant test, Merck, Darmstadt, Germany) and, subsequently Zinc powder. Nitrite oxidation was detected by addition of the Griess-Ilosvay reagents to check for the disappearance of the added nitrite. Most probable numbers were obtained from statistical tables (Rowe et al. 1975).

Analytical

(i) Gas analyses

CH_4 in flux measurements was analysed using a Shimadzu GC-8A gas chromatograph equipped with a HayesepD column. Nitrogen was used as the carrier gas, synthetic air and H_2 as burning gasses. The oven temperature was 80 °C. Calibration was performed using a standard gas mixture (50.2 ppm_v CH_4 in N_2, Messer Griesheim, Düsseldorf, Germany). CH_3F and C_2H_2 were monitored using a SRI GC (SRI Instruments, Torrance, CA, USA) equipped with an FID and a Porapak N column (length 2 m, mesh 80/400). N_2 was used as the carrier (20 ml·min^{-1}) and synthetic air (222 ml·min^{-1}) and H_2 (20 ml·min^{-1}) as burning gases. The oven temperature was 60 °C. Calibration was performed at each sampling event by triplicate injection of 1000 ppm_v

CH_4 in N_2 (Messer-Griesheim, Siegen, Germany). C_2H_2 and CH_3F standards were prepared by adding defined amounts to serum bottles of known volumes.

(ii) Slurry and porewater analyses
The concentrations of NH_4^+, NO_2^- and NO_3^- in the slurry samples were analysed colorimetrically using a Technicon Traacs 800 autoanalyzer (Technicon Instr. Corp., Tarrytown, USA). NH_4^+ in the weekly porewater samples was analysed by ion chromatography using an HPLC equipped with an LCA A14 column (Sykam, Germany) and using a mixture of ascorbic acid and oxalic acid as eluent. NO_2^- and NO_3^- were also analysed by ion chromatography on an HPLC equipped with an LCA KSP column (Sykam, Gilphing, Germany) and using Na_2CO_3 as the eluent. For fatty acid analysis 300 μl porewater was spiked with 100 μl of 0.4M H_2SO_4 and analysed using an HPLC (Sykam, Gilphing, Germany) equipped with a refractory index and a uv detector.

Statistics

All statistical test were performed using SPSS Base 7.5 for Windows (SPSS inc., Chicago, USA). Treatment effects of independent variables on measured variables were tested with the Kruskall-Wallis test that allows for inhomogeneity of variances. However, this design does not allow for testing of interactive effects of independent variables. Comparison of the means of the potential CH_4 oxidation, potential NH_4^+ oxidation and potential CH_4 production rates of the various treatments were performed using Tukey's test in case of equal variances and using Dunnet's T3 test with unequal variances. Correlations between measured variables were analysed using the Spearman Rank correlation test.

Results

Plant growth

The dry weight of the rice plants after 12 weeks incubation was between 1.5–3.2 gram for roots and between 5.1–10.8 grams for shoots (data not shown). Fertiliser addition led to significantly higher biomass (Table 2) while fertiliser level and type had no effect. Total plant height and leaf lengths were not affected by fertiliser addition (Table 2). According to these parameters the plants grew exponentially between 15 and 35 days after transplanting, then grew at a constant rate (data not shown). Six plants, 3 unfertilised and 3 from fertilised microcosms, started flowering 70 days after transplanting while the other 14 did not initiate panicles during the experimental period.

Table 2. Effects of the presence of rice plants, the presence of rice roots, application of fertiliser, fertiliser level and fertiliser type applied on the measured variables in compartmented microcosms planted with rice. The microcosms either received no fertiliser, urea (200 or 400 kg N·ha^{-1}) or $(NH_4)_2HPO_4$ (200 or 400 kg N·ha^{-1}). (+) and (−) indicate a promoting or suppressing effect of the respective independent variable on the measured dependent variable. The data were analysed using the Kuskall-Wallis test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Values in the table depict the *p*-value of the Kruskall-Wallis test.

Treatment	+/− Plant	+/− Roots	+/− Fertiliser	Fertiliser level	Fertiliser type
Dependent variables	Data of both compartments of planted microcosm compared to unplanted	Data of root compartments compared nonroot compartments of all microcosms	Data of both compartments of fertilised microcosms compared to the unfertilised ones	Data of both compartments of 200 kg.ha^{-1} treated microcosms compared to 400	Data of both compartments of urea compared to $(NH_4)_2HPO_4$-treated systems
Total dry weight			0.014* (+)	0.401	0.248
Root dry weight			0.006*** (+)	0.753	0.495
Shoot dry weight			0.033* (+)	0.401	0.172
Total leaf length			0.253	0.312	0.443
Plant height			0.785	0.065	0.363
Potential CH_4 oxidation (soil)	0.312	0.007** (+)	0.000*** (+)	0.872	0.780
Potential NH_4^+ oxidation (soil)	0.121	0.185	0.028* (+)	0.910	0.000***
Potential CH_4 oxidation (roots)			0.703	0.002**	0.224
Potential NH_4^+ oxidation (roots)			0.007*** (+)	0.156	0.270
Potential CH_4 production (soil)	0.079	0.000*** (+)	0.602	0.797	0.813
Potential CH_4 production (roots)			0.069	1.000	0.396
Most probable numbers of methanotrophs	0.143	0.000*** (+)	0.026* (+)	0.653	0.001***
Most probable numbers of ammonia oxidisers	0.180	0.899	0.002** (+)	0.843	0.251
Most probable numbers of nitrite oxidisers	0.754	0.509	0.019* (+)	0.052	0.000***
CH_4 emission[1]	0.000*** (+)		0.032* (−)	0.000***	0.000***
Porewater NH_4^{+}[1]	0.038* (−)	0.000*** (−)	0.000*** (+)	0.000***	0.268
Porewater CH_4[1]	0.000*** (−)	0.000*** (−)	0.775	0.127	0.062
Porewater pH[1]	0.334	0.147	0.039* (−)	0.075	0.000***

[1] Analysis was done with the compiled data of 12 weeks.

Weekly measured parameters

(i) CH_4 emission

The emission of methane from the microcosms did not increase until 30 days after transplanting and reached levels 50–250 mg $C.m^{-2}.d^{-1}$ (Figure 1). Emissions increased from day 30 on to levels of 1000 mg $C.m^{-2}.d^{-1}$, except for the unplanted and urea 400 treatments that stayed at the same level. Highest emissions were reached with $(NH_4)_2HPO_4$ 200 treatment, which was 19% higher (average over entire growth period) compared to the unfertilised microcosms. However, in general fertilisation led to lower emissions (Table 2). Fertiliser level and type significantly affected emission of methane (Table 2). Fertilisation with urea, as well as with a dose of 400 kg $N.ha^{-1}$ of $(NH_4)_2HPO_4$ led to reduced emissions as compared to the unfertilised situation. Reduction of the average emissions over the entire growth period were 15% (urea 200), 57% (urea 400), 26% ($(NH_4)_2HPO_4$ 200) and 74% (unplanted). Furthermore, methane emission was positively correlated with plant height and total leaf length while negative correlations were found with porewater NH_4^+ and with CH_4 (Table 3).

(ii) Porewater CH_4 and fatty acids

Porewater CH_4 concentrations were between 50–300 μM (Figure 2(A–C)). In general the porewater CH_4 dynamics were similar for all treatments. In the root compartments of all microcosms, both unfertilised (Figure 2(A)) and fertilised (Figure 2(B, C)), CH_4 was lower than in the nonroot compartment in the period between 15–40 days after transplanting. During the rest of the experiment root and nonroot compartment both had CH_4 concentrations of 100–150 μM. Statistical analysis over the entire growth period revealed significantly lower porewater CH_4 concentrations when comparing root compartments with nonroot compartments and planted microcosms with unplanted ones (Table 2). Porewater CH_4 was also negatively correlated with total leaf length and plant height (Table 3). Fertilisation had no effect on porewater CH_4.

Two weeks after flooding (1 week after transplanting), only acetate (140–300 μM) and proprionate (100–170 μM) were detectable in the porewater (data not shown). Two weeks after transplanting, proprionate was not longer detectable ($<$10 μM) while acetate levels decreased to values between 25–40 μM. Three weeks after transplanting, lactate was also detectable (10–15 μM) while acetate levels still decreased ($<$20 μM). During weeks 4–6 only acetate could be detected (20–60 μM). Although statistically not different, acetate concentrations tended to be higher in the root compartments of the unfertilised and the urea – 200 treated microcosms when compared to the

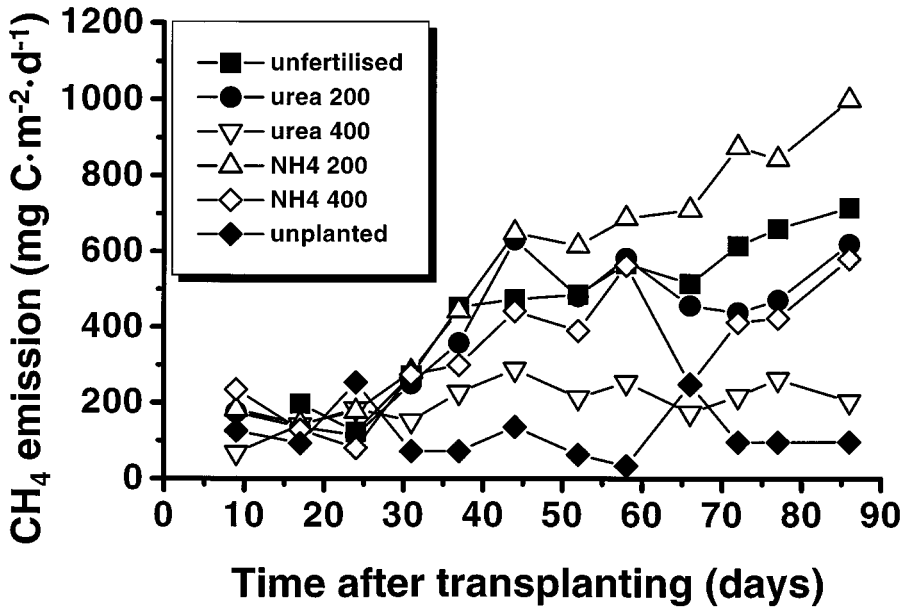

Figure 1. Methane emission from compartmented microcosms which were either unfertilised, unplanted or supplemented with either urea (200 or 400 kgN.ha^{-1}) or $(NH_4)_2HPO_4$ (200 or 400 kgN.ha^{-1}). All values are arithmetic means of 4 replicate microcosms. The coefficients of variation of the average emission during the entire growth period were 41% (unfertilised), 47% (urea 200), 30% (urea 400), 54% ($(NH_4)_2HPO_4$ 200), 46% ($(NH_4)_2HPO_4$ 400) and 59% (unplanted).

Table 3. Spearman rank correlations of variables analysed weekly during a 90-day growing period of rice in compartmented microcosms receiving different fertiliser treatments. The values displayed are the correlation coefficients with the number of observations in brackets. The significance levels are indicated by $*$ $p < 0.05$; $**$ $p < 0.01$.

Variable	CH_4 flux	Plant height	Total leaf length	Porewater pH	Porewater CH_4
Plant height	0.648** (234)				
Total leaf length	0.591** (234)	0.955** (238)			
Porewater pH of root compartment	−0.005 (205)	−0.260 (184)	0.004 (184)		
Porewater CH_4 of root compartment	−0.144* (257)	−0.144* (224)	−0.181* (224)	0.143** (446)	
Porewater NH_4^+ of root compartment	−0.668** (246)	−0.551** (219)	−0.494**(219)	0.158** (438)	0.336** (544)

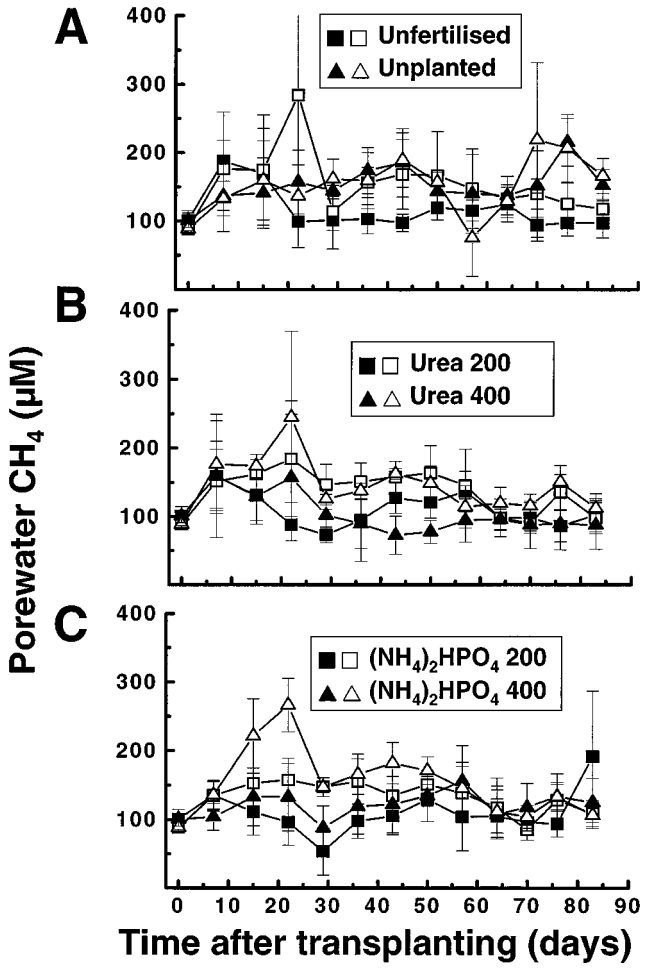

Figure 2. Porewater CH_4 concentrations in the root compartment (closed symbols) and non-root compartment (open symbols) of microcosms which were either unfertilised, unplanted (A) or supplemented with either urea (200 or 400 kgN.ha^{-1}) (B) or $(NH_4)_2HPO_4$ (200 or 400 kgN.ha^{-1}) (C). All values represent are arithmetic means of 4 replicate microcosms. Error bars indicate standard deviation.

respective non-root compartments during this period. During weeks 7–12 all fatty acids analysed were below the detection limits (10 μM).

(iii) Porewater NH_4^+, NO_2^-, NO_3^- and pH

Ammonium concentration in the porewater of the unplanted microcosms was constant at 1.5–2 mM during the entire incubation period (Figure 3(A)). In the root compartment of the unfertilised microcosms NH_4^+ was depleted

after 35 days, while after 70 days NH_4^+ also became undetectable in the non-root compartment (Figure 3(A)). Despite the frequent fertilisation, low levels of NH_4^+ were also observed after day 35 in the root compartment of the 200 kgN.ha^{-1} treatment of the urea (Figure 3(B)) and $(NH_4)_2HPO_4$ (Figure 3C) fertilised microcosms. However, NH_4^+ was detectable throughout the incubation period. With the 400 kgN.ha^{-1} fertiliser dose, porewater NH_4^+ was always in excess with 1.5–5 mM and 0.5–4 mM in the root- and non-root compartments, respectively. Fertiliser addition, fertiliser level and the presence of plants and roots (Table 2) significantly affected porewater NH_4^+ availability. There was no effect of fertiliser type. Uptake of NH_4^+ by the plants was also indicated by the highly negative correlations of porewater NH_4^+ with plant parameters (Table 3).

The NO_3^- and NO_2^- concentrations in the porewater did not exceed the detection limit (5 μM) during the entire experimental period.

The pH of the porewater in the unplanted and unfertilised microcosms was relatively stable over the entire period with values of 7 and 6.8, respectively (data not shown). Fertilisation with urea led to a pH increase as compared to the unfertilised situation, while fertilisation with $(NH_4)_2HPO_4$ decreased the pH, even to below 6 in the root compartment of the 400 kgN.ha^{-1} treatment (data not shown). Statistical analysis showed significant effects of fertilisation and fertiliser type on porewater pH (Table 2).

Post-harvest analysis

(i) CH_4 and NH_4^+ oxidation of rhizosphere and nonrooted soil

CH_4 oxidation in soil slurries from the root compartments of all fertilised microcosms started immediately without a lag phase but at a low initial rate (Figure 4(A)). In the slurries from the unfertilised microcosms CH_4 oxidation started after a lag phase of 50 hours. The lag phase was 30 hours for the unplanted microcosms. When adding ammonium to slurries from unfertilised microcosms, like it was done to perform the CEA assay (Figure 7(A)), methane consumption starts right away which is not the case for slurries from the unplanted microcosms. After the lag phase, the CH_4 oxidation rate stayed constant in the unfertilised soil while the fertilised and unplanted soil displayed a sort of 'induced rate' (Figure 4(A)). The same held true for the soil from the nonroot compartment (Figure 4(B)) except that all treatments showed a lag phase of 30 hours before the onset of CH_4 oxidation. The calculated initial and induced rates are summarised in Table 4. Initial rates could only be detected in the fertilised root compartments and tended to be higher in the urea-fertilised microcosms. The induced rate in the root compartment of the unfertilised microcosms was lower compared to the urea-treated microcosms. In the nonroot compartments, induced rates of

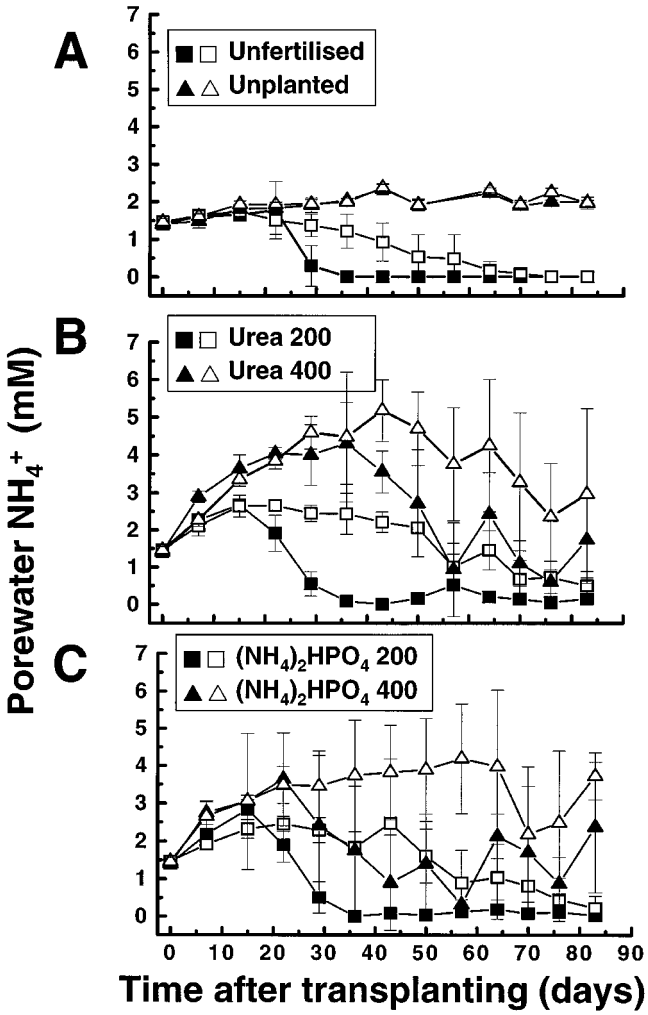

Figure 3. Porewater NH_4^+ concentrations in the root compartment (closed symbols) and nonroot compartment (open symbols) of microcosms which were either unfertilised (A), unplanted (A) or supplemented with either urea (200 or 400 kgN.ha^{-1})(B) or $(NH_4)_2HPO_4$ (200 or 400 kgN.ha^{-1}) (C). All values are arithmetic means of 4 replicate microcosms. Error bars indicate standard deviation.

unfertilised microcosms were lower than in all other treatments, even than to the unplanted systems. The Kruskall-Wallis analysis revealed distinct positive effects of the presence of roots and of the addition of fertiliser on the oxidation of CH_4 (Table 2). Fertiliser dose and type did not influence CH_4 oxidation.

Table 4. Potential CH_4 and NH_4^+ oxidation and CH_4 production rates associated with soil and roots from compartmented microcosms. Values are the arithmetric means (±sd) of 4 replicate microcosms. Different letters indicate significant differences ($p < 0.05$, [1] Dunnet T3 for unequal variances; [2] Tukey's test for equal variances) between the means of the different treatments for root and nonroot samples, respectively.

	CH_4 oxidation rates of soil slurries (μmol·CH_4·g^{-1}dw·h^{-1})				NH_4^+ oxidation rates (nmol NO_3^- + NO_2^- ·g^{-1}dw·h^{-1})		CH_4 production rates (nmol CH_4·g^{-1}dw·h^{-1})	
Treatment	Initial root	Initial nonroot	Induced root[1]	Induced nonroot[2]	Root[1]	nonroot[1]	Root[2]	Nonroot[2]
Unfertilised	nd	nd	0.43 ± 0.18a	0.18 ± 0.03a	2.48 ± 0.38a	2.29 ± 0.16a	24.95 ± 1.99a	6.08 ± 0.61a
Urea 200	0.42 ± 0.12	nd	1.23 ± 0.13b	0.76 ± 0.09b	7.66 ± 0.38b	3.82 ± 0.82ab	44.76 ± 4.85ab	5.36 ± 0.48a
Urea 400	0.16 ± 0.05	nd	1.51 ± 0.13b	0.87 ± 0.11b	22.58 ± 10.34ab	8.38 ± 8.90ab	36.23 ± 3.04ab	4.72 ± 0.71a
$(NH_4)_2HPO_4$ 200	0.12 ± 0.01	nd	1.17 ± 0.26ab	0.85 ± 0.09b	3.38 ± 1.53ab	2.50 ± 0.81a	66.42 ± 6.48b	4.42 ± 0.94a
$(NH_4)_2HPO_4$ 400	0.06 ± 0.02	nd	1.16 ± 0.45ab	0.77 ± 0.23b	2.11 ± 0.46a	2.63 ± 1.13ab	30.86 ± 2.14ab	5.68 ± 0.94a
Unplanted		nd		0.72 ± 0.16b		5.25 ± 0.50b		4.56 ± 0.53a

Treatment	CH_4 oxidation associated with rice roots (μmol·CH_4·g dw $root^{-1}$·h^{-1})[2]	NH_4^+ oxidation rates associated with rice roots (nmol NO_3^- + NO_2^-·g dw $root^{-1}$·h^{-1})[1]	CH_4 production rates associated with rice roots (nmol CH_4·g^{-1}dw·h^{-1})[2]
Unfertilised	6.04 ± 3.79ab	nd	176.16 ± 28.82a
Urea 200	3.55 ± 0.78ab	56.49 ± 51.98a	301.50 ± 60.02a
Urea 400	0.41 ± 0.82a	196.97 ± 171.15a	n.a.
$(NH_4)_2HPO_4$ 200	4.45 ± 0.96b	11.48 ± 4.22a	251.31 ± 52.05a
$(NH_4)_2HPO_4$ 400	2.14 ± 0.96ab	156.79 ± 218.48a	266.65 ± 36.00a

nd = not detectable
na = not analysed

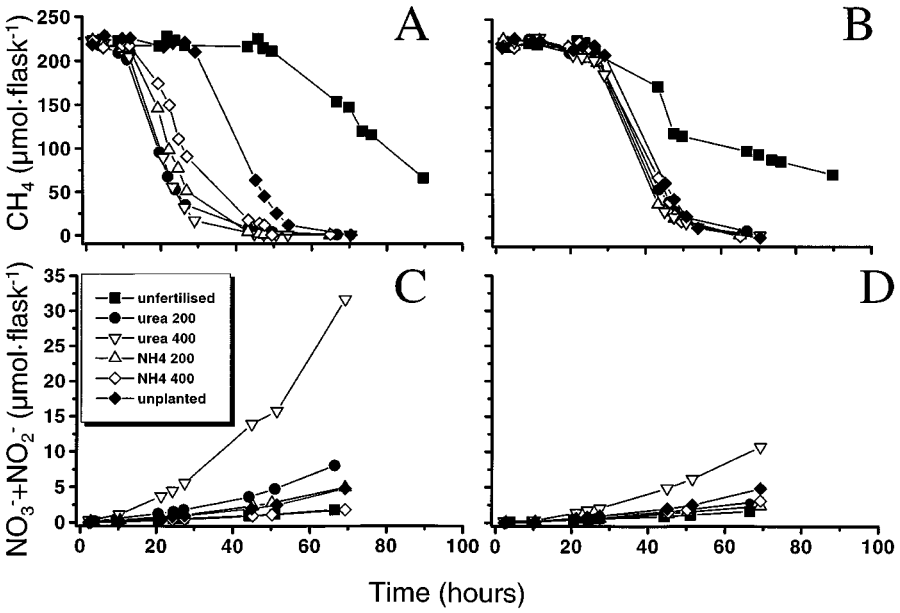

Figure 4. CH_4 (A, B) and NH_4^+ (C, D) oxidation in slurries from the root (A, C) and nonroot (B, D) compartments of microcosms which were either unfertilised, unplanted or supplemented with either urea (200 or 400 kgN.ha^{-1}) or $(NH_4)_2HPO_4$ (200 or 400 kgN.ha^{-1}). All values are arithmetic means of 4 replicate microcosms.

Figures 4(C) and (D) display the NH_4^+ oxidation in root and nonroot compartments, respectively. The production of NO_2^- and NO_3^- was distinctly higher in the root compartments of the urea-treated microcosms (Figure 4(C)). The lowest nitrification rate was detected in the root compartments of unfertilised and $(NH_4)_2HPO_4$400 treatments. Nitrate and nitrite production in the nonroot compartments was similar for all treatments except for the urea 400 microcosms (Figure 4(D)). The potential nitrification rates, calculated from the first 24 hours of incubation, revealed higher rates for the urea-treated microcosms (Table 4). However, due to the high variation only the root compartment of the urea 200 treatment had significantly higher NH_4^+ oxidation rates than the unfertilised situation (Table 4). The potential activities in the unfertilised microcosms were even lower than in the unplanted systems. In general, fertilisation with urea significantly stimulated nitrification (Table 2) while the presence of the plant or roots had no effect.

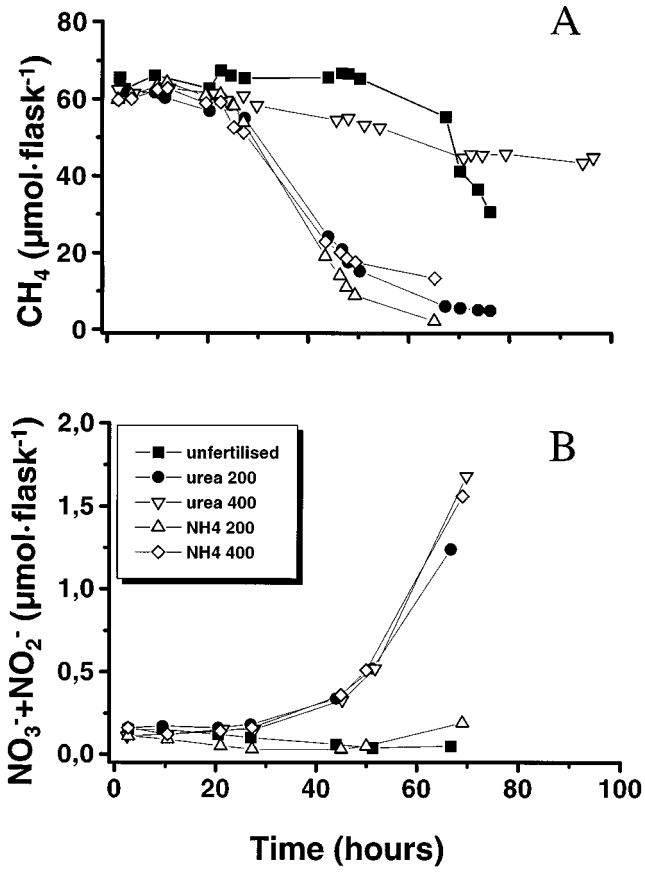

Figure 5. CH_4 (A) and NH_4^+ (B) oxidation associated with rice roots grown in compartmented microcosms which were either unfertilised or supplemented with either urea (200 or 400 kgN.ha^{-1}) or $(NH_4)_2HPO_4$ (200 or 400 kgN.ha^{-1}). All values are arithmetic means of 4 replicate microcosms.

(ii) CH_4 and NH_4^+ oxidation associated with rice roots

CH_4 oxidation associated with rice roots started only after a lag phase of approximately 30 hours (Figure 5(A)). In unfertilised roots it even took 50 hours before CH_4 depletion started. CH_4 oxidation associated to the roots from the urea 400 treatments was surprisingly slow. The calculated induced rates per gram of root did not differ much. A significant difference was found between the urea 400 and $(NH_4)_2HPO_4$ 200 treated roots (Table 4). Kruskall-Wallis analysis revealed an effect of the fertiliser level on CH_4 oxidation by roots (Table 2). A higher fertiliser dose led to lower oxidation rates.

Nitrification associated with rice roots was detectable only in fertilised systems (Figure 5(B)). High production of NO_2^- and NO_3^- was observed on roots from the urea and the $(NH_4)_2HPO_4$ 400 treatments (Table 4). The rates were highly variable between the replicate microcosms and therefore statistically insignificant. However, Kruskall-Wallis analysis indicated a clear stimulation of nitrification associated with rice roots due to fertiliser addition (Table 2).

(iii) CH_4 production in rhizosphere, nonrooted soil and associated with rice roots

The CH_4 production rates of slurries from the rhizosphere of rice plants were 4–15 times the rates detected in the nonroot compartment and in unplanted microcosms (Table 4). In general there was no effect of fertilisation, fertiliser type and level (Table 2). Only the production rates in the root compartment of the $(NH_4)_2HPO_4$ 200 treatment were significantly higher than the unfertilised situation. The presence of roots heavily stimulated CH_4 production in the microcosms (Table 2). The CH_4 production associated with the rice roots was also not affected by fertilisation (Tables 2 and 4).

(iv) Most probable numbers of methanotrophs and nitrifiers

Numbers of methanotrophs were in the range of 10^5–10^7 per gram dry soil and were always higher in the root compartment (Figure 6(A)) as supported by Kruskall-Wallis test (Table 2). Fertilisation led to significantly higher numbers in the root compartments (Figure 6(A) and Table 2). Fertiliser type also affected numbers of methanotrophs (Table 2). The highest numbers were found in the both the root and nonroot compartments of the microcosms fertilised with $(NH_4)_2HPO_4$.

Numbers of ammonia oxidisers were in the range of 10^4–10^6 (Figure 6(B)) and were generally higher in the fertilised microcosms (Table 2). Highest numbers were found in the urea-treated microcosms (Figure 6(B)) while an effect of roots was not observed (Table 2). Moreover, lowest numbers were detected in the root compartment of the unfertilised microcosms.

Nitrite oxidisers reached numbers in the same range as the methanotrophs (Figure 6(C)). No differences were found between root and nonroot compartments. Fertilisation with $(NH_4)_2HPO_4$ 400 led to significantly higher numbers in both root and nonroot compartments.

(v) Correlations between post-harvest analyses

Table 5 shows the Spearman-Rank correlation between the variables analysed after harvesting of the microcosms. The only variables that correlated positively with plant dry matter components were the numbers of methanotrophs and the CH_4 production rate, which correlated with root dry matter. The

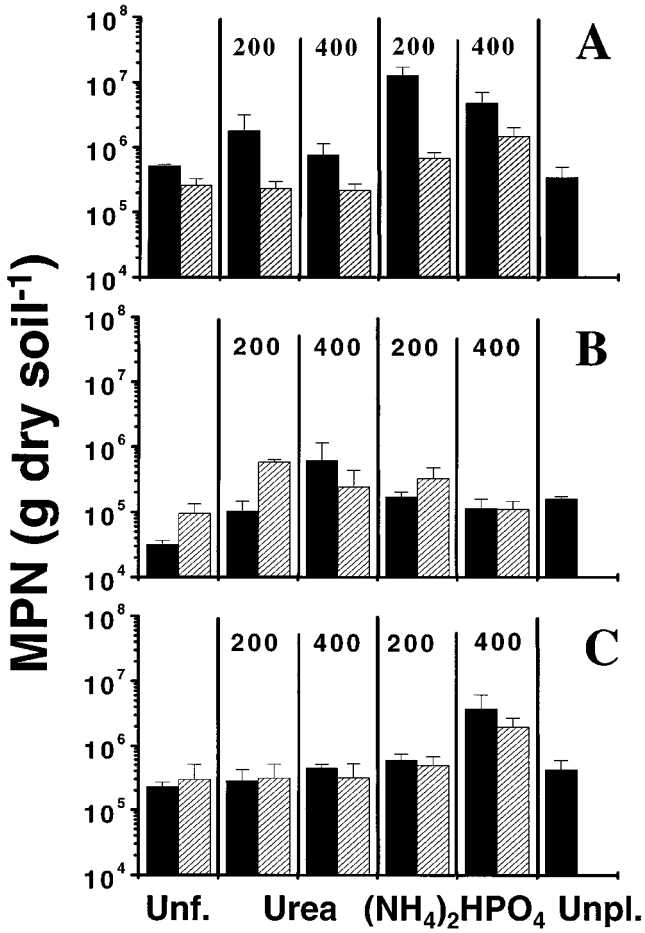

Figure 6. Most probable numbers of CH_4 (A), NH_4^+ (B) and NO_2^- oxidisers in the root (hatched bars) and nonroot compartments (black bars) of microcosms which were either unfertilised, unplanted or supplemented with either urea (200 or 400 kgN.ha^{-1}) or $(NH_4)_2HPO_4$ (200 or 400 kgN.ha^{-1}). All values are arithmetic means of 4 replicate microcosms. Error bars indicate the standard deviation.

CH_4 production of the soil also correlated positively with the numbers of methanotrophs as well as with potential CH_4 oxidation of soil. CH_4 oxidation potential of soil was correlated with NH_4^+ oxidation and with the numbers of methanotrophs and ammonia- and nitrite-oxidisers.

(vi) Contribution of methanotrophs to nitrification in the rice rhizosphere
Figure 7 shows the results of the competitive exclusion assay. NH_4^+ oxidation in the first 24 hours of incubation was stimulated by the addition of CH_4

Table 5. Spearman rank correlation of variables analysed after harvesting of compartmented microcosms receiving different fertiliser treatments. Data are the correlation coefficients with the number of observations in brackets. In the analysis, the CH_4 and NH_4^+ oxidation activities as well as the CH_4 production of soil include the data of both root and nonroot compartments. This also holds true for the numbers of CH_4, NH_4^+ and NO_2^- oxidisers. The CH_4 oxidation activity of soil refers to the induced rates. The significance levels are indicated by $* \; p < 0.05$; $** \; p > 0.01$.

Variable	CH_4 oxidation soil	CH_4 oxidation roots	NH_4^+ oxidation soil	NH_4^+ oxidation roots	CH_4 production soil	CH_4 production roots	MPN CH_4 oxidisers	MPN NH_4^+ oxidisers	MPN NO_2^- oxidisers	Root dry matter	Shoot dry matter
CH_4 oxidation roots	0.205 (19)										
NH_4^+ oxidation soil	0.515** (40)	−0.200 (20)									
NH_4^+ oxidation roots	0.496* (19)	−0.262 (20)	0.382 (20)								
CH_4 production soil	0.514** (39)	0.350 (20)	0.170 (43)	0.069 (20)							
CH_4 production roots	0.368 (15)	−0.031 (16)	0.174 (16)	0.484 (16)	0.021 (16)						
MPN CH_4 oxidisers	0.485** (39)	0.539* (20)	−0.115 (43)	0.169 (20)	0.633** (42)	0.150 (16)					
MPN NH_4^+ oxidisers	0.401* (39)	−0.163 (20)	0.281 (43)	0.441 (20)	−0.050 (42)	0.298 (16)	0.138 (43)				
MPN NO_2^- oxidisers	0.317* (39)	−0.038 (20)	−0.301 (43)	0.349 (20)	0.122 (42)	0.274 (16)	0.446** (43)	0.248 (39)			
Root dry matter	0.403 (19)	0.299 (20)	0.121 (20)	0.413 (20)	0.523* (20)	0.090 (16)	0.622** (20)	0.317 (20)	0.442 (20)		
Shoot dry matter	0.135 (19)	0.411 (20)	−0.172 (20)	0.115 (20)	0.425 (20)	0.181 (16)	0.673** (20)	0.060 (20)	0.344 (20)	0.850** (20)	
Total dry matter	0.212 (19)	0.361 (20)	−0.101 (20)	0.228 (20)	0.436 (20)	0.153 (16)	0.698** (20)	0.162 (20)	0.394 (20)	0.915** (20)	0.985** (20)

(Figure 7(B)), except in the urea 400 treatment. The addition of CH_3F inhibited methanotrophic activity (Figure 7(C)), which enables the calculation of the contribution of methanotrophs to nitrification (Table 6). Methanotrophs potentially accounted for up to 43% of the observed NH_4^+ oxidation in the absence of CH_4. When CH_4 was present the methanotrophic contribution to nitrification was higher, except in the urea 400 treatment and the unplanted microcosms.

The results of the differential recovery assay are displayed in Figure 8. After removal of C_2H_2, CH_4 oxidation recovers within 24 hours (Figure 8(A)). The production of NO_3^- and NO_2^- recovered at the same time (Figure 8(B)) and mirrored the CH_4 oxidation curves. From the NH_4^+ oxidation rates before inhibition and after recovery, the contribution of methanotrophs to nitrification was calculated (Table 6). Except for the urea 400 treatment, the contributions were all-higher than calculated with the CEA assay, and accounted for all of the nitrification activity.

Discussion

Factors controlling CH_4 emission from rice paddies have been intensively studied and reviewed (e.g. Khalil et al. 1998; Neue 1997). Like in other studies (e.g. Huang et al. 1997; Dannenberg & Conrad 1999), the importance of the rice plant in CH_4 emission is clearly demonstrated in our study. However, beside the investigations of Sass et al. (1990) this is the first study demonstrating a clearly higher CH_4 production potential in rhizosperic soil. This is in contrast to the study of Frenzel et al. (1999), which demonstrated lower rates in rhizospheric soil compared to the bulk soil of a nearly identical experimental design. However, the light intensity in our experiments (99 $W.m^{-2}$) was nearly 4 times higher than in the study of Frenzel et al. (1999) (25 $W.m^{-2}$) probably leading to higher availability of exudates which can be converted to methanogenic substrates (Minoda & Kimura 1996; Dannenberg & Conrad 1999). Moreover, higher availability of methanogenic substrates will relieve competition for these compounds between methanogens and sulfate- and iron-reducing bacteria.

Next to the plant, the form, amount and mode of application of mineral fertiliser are major factors affecting CH_4 emission (Minami 1995). However, the microbial processes involved, especially in the consumption of CH_4, have not been well investigated. Hence, explanations for contradictory results of fertiliser additions in various studies are still missing. As depicted in Table 7, ammonium-based fertilisers lead in some instances to higher, and in others to reduced CH_4 fluxes from soils planted with rice. The higher fluxes were often explained by higher plant biomass and consequently higher carbon

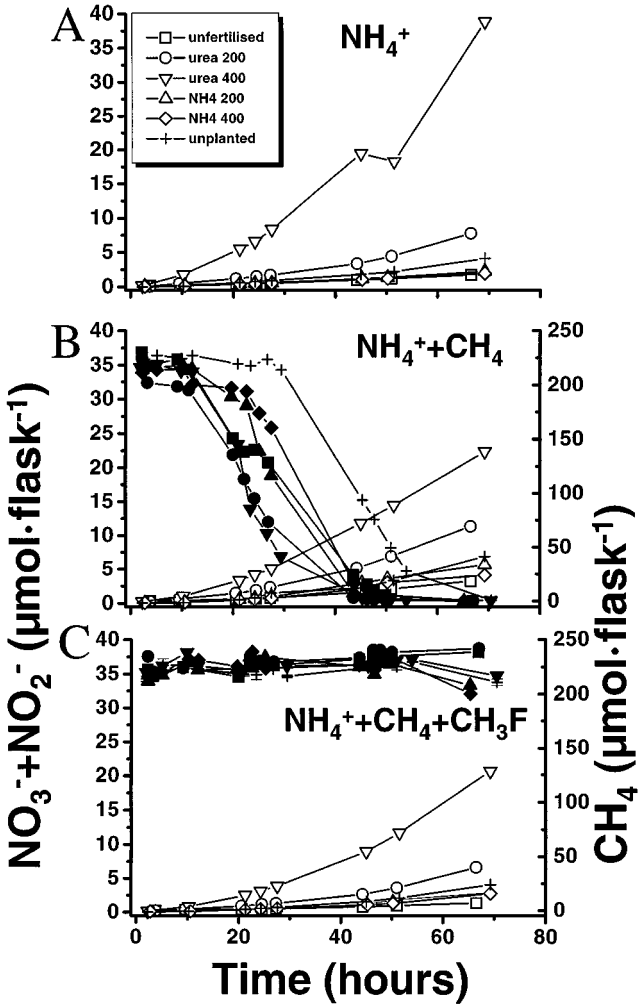

Figure 7. Competitive exclusion assay using CH_3F applied to NH_4^+ (2 mM)-supplemented slurries from the root compartments microcosms which were either unfertilised, unplanted or supplemented with either urea (200 or 400 kgN.ha^{-1}) or $(NH_4)_2HPO_4$ (200 or 400 kgN.ha^{-1}). Panel A depicts NH_4^+ oxidation in the absence of CH_4 and CH_3F, putatively caused by both methanotrophs and nitrifiers. Panel B shows CH_4 (closed symbols) oxidation and NH_4^+ (open symbols) oxidation in the presence of CH_4 (10 000 ppm_v) while panel C displays the same but in the presence of CH_3F (300 ppm_v). Panel C putatively shows NH_4^+ oxidation by only nitrifiers. All values in panels A–C are arithmetic means of 2 replicate microcosms.

Table 6. The contribution of methanotrophs to NH_4^+ oxidation in the root compartment of microcosms receiving different fertiliser treatments. The calculations were based on the data from the CEA and DRA assays of 2 replicate microcosms. All values in the table are the arithmetic means of the respective parameter of 2 replicate microcosms.

Treatment	NH_4^+ oxidation rates CEA assay (nmol NO_3^- + NO_2^-·gdw^{-1}·h^{-1})					NH_4^+ oxidation rates DRA assay (nmol NO_3^- + NO_2^-·gdw^{-1}·h^{-1})		
	$-CH_4$	$+CH_4$	$+CH_4$ + CH_3F	% $-$ CH_4[1]	% $+CH_4$[2]	Prior to C_2H_2 addition	After recovery	% contribution of MOB[3]
Unfertilised	2.26	2.48	1.46	33.76	40.82	2.55	na	na
Urea 200 kg N·ha^{-1}	7.52	9.33	5.15	31.41	42.68	7.82	6.97	89.02
Urea 400 kg N·ha^{-1}	26.60	19.40	17.45	41.26	17.85	19.27	6.51	38.94
$(NH_4)_2HPO_4$ 200 kg N·ha^{-1}	2.13	3.83	2.22	–4.35	38.88	3.43	5.38	152.56
$(NH_4)_2HPO_4$ 400 kg N·ha^{-1}	2.40	2.90	1.99	17.72	31.86	2.83	2.27	80.27
Unplanted	4.83	5.35	3.16	34.15	32.50	3.10	1.76	56.51

[1] $1 - (NH_4^+$ oxidation rate with CH_4 and CH_3F present (only nitrifiers))/(NH_4^+ oxidation rate in the absence of CH_4 and CH_3F (nitrifiers and methanotrophs))$*100$

[2] $1 - (NH_4^+$ oxidation rate with CH_4 and CH_3F present (only nitrifiers))/NH_4^+ oxidation rate in the presence of CH_4 and the absence CH_3F (nitrifiers and methanotrophs))$*100$

[3] (NH_4^+ oxidation rate prior to inhibition with C_2H_2/NH_4^+ oxidation rate after removal of C_2H_2)$*100$

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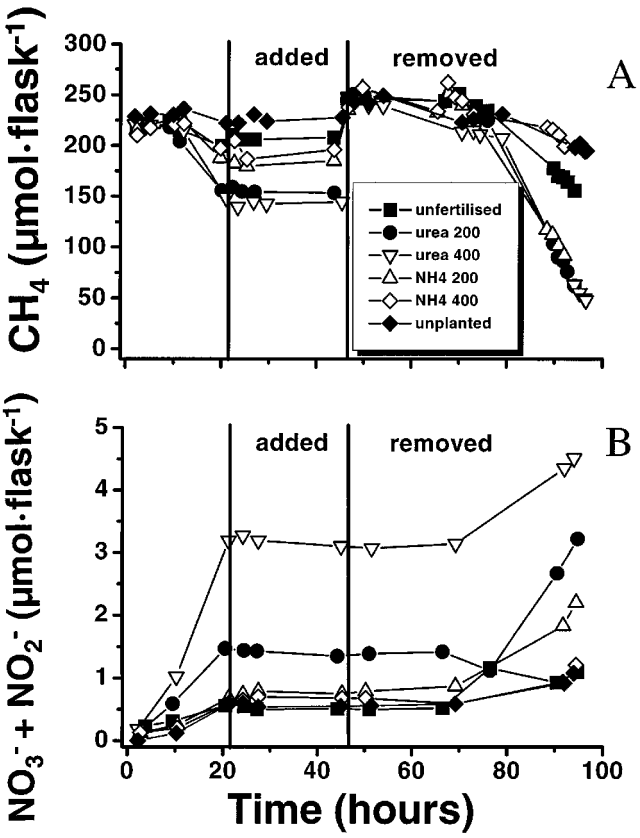

Figure 8. Differential recovery assay using C_2H_2 applied to NH_4^+ (2 mM)-supplemented slurries form the root compartments of microcosms which were either unfertilised, unplanted or supplemented with either urea (200 or 400 kgN.ha^{-1}) or $(NH_4)_2HPO_4$ (200 or 400 kgN.ha^{-1}). Panel A shows CH_4 oxidation before application, in the presence of and after removal of C_2H_2 (1 000 ppm_v). Panel B displays the NH_4^+ in the same assay bottles. All values represent the arithmetic means of 2 replicate microcosms. CH_4- as well as NH_4^+-oxidation within 48 hours after removal of C_2H_2 can be subscribed to methanotrophs.

availability for methanogens. The reduction of emission due to application of $(NH_4)_2SO_4$ is speculated to result from competition between methanogens and sulfate reducers. However, this is not true for urea. Reduced CH_4 emission after urea application is argued to result from the higher concentrations of nitrification products that inhibit methanogenesis (Klüber & Conrad 1998). However, no NO_2^- or NO_3^- was detected in the porewater of our microcosms. Next to this in our study as well as those of Banik et al. (1996) and Bronson et al. (1997), even high levels of urea did not affect potential CH_4 production. In our study the application of 200 and 400 kgN.ha^{-1} urea reduced the

average CH_4 emission over the 12 weeks incubation period by 15 and 57%, respectively, while the application of 400 kgN.ha^{-1} $(NH_4)_2HPO_4$ reduced the emission by 26% compared to the unfertilised microcosms. Bronson et al. (1997) speculated that the reduced emission after fertilisation with urea could only be caused by elevated CH_4 oxidation activity. This speculation is in accordance with the experimental evidence presented in our paper.

Fertilisation, distinctly stimulated both growth and activity of methanotrophs both in rhizospheric soil and associated with the rice roots. Up to 10–30% of total CH_4 production (Denier van der Gon & Neue 1996; Bosse & Frenzel 1997) is oxidised in the rhizosphere of rice. Hence, the "absence" of methane oxidation under N-limiting conditions, and the higher rates with a surplus of N may very well explain the changes of the CH_4 flux in the studies shown in Table 7. The high negative correlation between NH_4^+ in the porewater of the root compartments and CH_4 flux (see Table 3) in this study also suggests that elevated methanotrophic activity is responsible for the reduced emission of CH_4 after fertilisation. In the case of the $(NH_4)_2HPO_4$ 400 treatment this correlation was even as high as $r = -0.97$ ($p < 0.0001$). The reduction of CH_4 emission and the stimulation of methane oxidation by fertiliser addition have been confirmed in greenhouse studies and in field experiments in Vercelli, Italy (Krüger and Frenzel, unpublished data). These authors combine potential rate measurements with *in situ* oxidation rates and used stable isotopes to assess methane oxidation activity under natural conditions. Significant methane oxidation was detected only after fertilisation events, demonstrating the basic importance of ammonium availability to methanotrophs and thus for the global CH_4 budget.

This dramatic effect of N-limitation on methanotrophic bacteria has been neglected until now due to the fact that NH_4^+ is dogmatically thought to have an inhibiting effect on CH_4 oxidation as demonstrated in numerous studies (e.g. Steudler et al. 1989; Nesbit & Breitenbeck 1992; King & Schnell 1994; Hütsch et al. 1994; Gulledge & Schimel 1998). Despite the intense investigation the inhibitory mechanisms are still a matter of debate. Competitive inhibition of MMO by NH_4^+, toxicity of nitrite produced through nitrification by methanotrophs, and osmotic effects due to salt addition are the proposed inhibitory mechanisms. The latter two options will not be important in the rice rhizosphere because added salt or produced nitrite will rapidly be taken up by the plant or be denitrified. Competitive inhibition by NH_4^+ also should not occur in our systems. In littoral sediments (Bosse et al. 1993) and in planted intertidal marshes (Van der Nat et al. 1997) it has been demonstrated that only at NH_4^+:CH_4 ratios higher than 190 and 30, respectively, inhibition of CH_4 oxidation takes place. In our microcosms the highest NH_4^+:CH_4 ratio was 34 while in the assay flasks a ratio as high as 200 did not give any inhibition.

Table 7. Summary of studies performed on the effect of ammonium-based fertilisation on CH_4 emission from rice fields.

Effect of fertiliser on CH_4 emission	Fertiliser	Range of CH_4 emission (mg $C.m^{-2}.d^{-1}$)	Mode of application	Habitat	Explanation by authors of the effect of fertilisation.	References
Higher: 16–44% compared to unfertilised	Urea, 120 $kgN.ha^{-1}$	50–149	50% basal dressing, 50% as 2 split applications	Irrigated rice field in India	• NH_4^+ as N source for methanogens • Inhibition of CH_4 oxidation	Singh et al. 1996
Higher: 156–186% compared to unfertilised	Urea, 406 $kgN.ha^{-1}$ $(NH_4)_2HPO_4$ and $(NH_4)_2SO_4$ 185 $kgN.ha^{-1}$	72–432	Basal dressing	Pot experiment with rice and soil from West Bengal, India	• Higher production and emission due to higher plant biomass	Banik et al. 1996
Higher: 21% compared to unfertilised	$(NH_4)_2SO_4$, 120 $kgN.ha^{-1}$	31–150	Not described	California rice paddies	• Higher C due to higher plant biomass	Cicerone & Shetter 1981
Lower: 6–62% reduction of flux	Urea and $(NH_4)_2SO_4$200 $kgN.ha^{-1}$	90–285	Application before flooding; surface applied; raked into soil; incorporated	Rice paddy, Vercelli, Italy	• Inhibition of methanogens due to competition with sulphate reducers • No explanation for urea	Schütz et al. 1989
Lower: 34–81% compared to unfertilised	Urea and $(NH_4)_2SO_4$ 120 $kgN.ha^{-1}$	15–754	Broadcasted in floodwater. Only 21 day growth period.	Californian rice paddy	• High redox potentials due presence of nitrite or nitrate • Soil gas entrapment	Lindau et al. 1990
Lower: 7–14% reduction with urea; 42–60% with $(NH_4)_2SO_4$	Urea and $(NH_4)_2SO_4$ 100 and 300 $kgN.ha^{-1}$	12–336	20% basal, rest 40,20 and 20% topdressed	Rice paddy, Jiangsu, China		Cai et al. 1997
Lower: 60–75% with urea	Urea and $(NH_4)_2SO_4$120 and 200 $kgN.ha^{-1}$	5–100	4 equal applications at different growth stages	Rice field at the Phillipines (IRRI)	No effect on CH_4 production potential; suggested explanation is elevated CH_4 oxidation	Bronson et al. 1997

Hence, in the rhizosphere of rice and other wetland plants with high CH_4 concentration and low NH_4^+ availability due to plant uptake, inhibition of CH_4 oxidation, by fertiliser nitrogen is not very likely. In contrast, this study demonstrates that in the absence of NH_4^+, methanotrophs were in a dormant or inactive state. Addition of NH_4^+, to slurries from unfertilised microcosms immediately led to methanotrophic activity, which showed a lag phase of 50 hours without the amendment of NH_4^+. This immediate activation suggests that next to ammonium as an N-source an additional effect of ammonium on the methane oxidising enzyme system may be the case. Induction of enzymes may be triggered by the assimilatory utilisation of ammonium.

Another option explaining the discrepancies between our finding and the inhibition found in other studies may be that yet unknown methanotrophs were active in the fertilised systems. However, analyses of the methanotrophic communities of our microcosms revealed the presence of conventional methanotrophs (Bodelier et al. 2000). By using a combination of radioactive fingerprinting and molecular biology techniques the active and present methanotrophic species were detected. Fertilisation lead to higher incorporation of radiolabelled methane ($^{14}CH_4$) into fatty acid ester-linked phospholids (PLFA). The total amount of PLFAs specific for methanotrophs increased upon fertilisation, with the highest increase for type I methanotrophs related to the genus *Methylococcus* (Bodelier et al. 2000).

A positive effect of ammonium-based fertilisation on CH_4 oxidation has already been noted by other authors, although they failed to discuss the phenomenon. Bender and Conrad (1995) showed that the CH_4 oxidation potential of soils with low NH_4^+ contents was distinctly stimulated by addition of 5 mM NH_4^+, while soils with high ammonium contents did not respond to addition of NH_4^+. The authors propose N-limitation to be a possible explanation. Also, atmospheric CH_4 oxidation in upland soils has been demonstrated to be stimulated by ammonium-based fertilisation (Kruse & Iversen 1995; Goldman et al. 1995; Priemé et al. 1997). Positive correlation's between atmospheric CH_4 uptake and NH_4^+ content of heathland (Kruse & Iversen 1995), forest (Goldman et al. 1995) and agricultural (Priemé et al. 1997) soils have been observed. These findings might be explained N-limitation of methanotrophs. When sufficient NH_4^+ is present in soil, the methanotrophs may grow during periods of high CH_4 supply, for instance when the soil becomes partly anoxic after heavy rainfall. After drying of the soil, the numbers of methanotrophs present will be higher, resulting in higher atmospheric CH_4 consumption. This will not be the case in soils with limiting amounts of NH_4^+ prior to anoxic periods

In a previous study we demonstrated that methanotrophs have the potential to contribute substantially to nitrification in the rice rhizosphere (Bodelier &

Frenzel 1999). This was also the case in this study. However, irrespective of the fertiliser regimen and the potential activities of ammonia and methane oxidisers, the contribution of methanotrophs to nitrification was about 40%. This may indicate that a fixed amount of NH_4^+ is cooxidised with CH_4. Hence, there seems to be a connection between the two oxidising pathways, as supported by the negative correlation we found between porewater NH_4^+ and CH_4 emission, and by the positive correlation's between NH_4^+ content of different soils and CH_4 oxidation (Kruse & Iversen 1995; Goldman et al. 1995; Priemé et al. 1997). Bender and Conrad (1995) even found an optimum NH_4^+ concentration (5 mM) for CH_4 oxidation in paddy and cultivated cambisol soil. Growth of methanotrophs due to oxidation of NH_4^+ has not yet been observed, but a stimulatory effect of NH_4^+ on CH_4 metabolism may partially, next to NH_4^+ as an N-source, explain the strong stimulation by fertilisation in our study. This is supported by batch culture studies with *Methylosinus trichosporium* OB3b (Park et al. 1992) where depletion of the N-source (nitrate) in the medium immediately reduced the pMMO activity, while activity was restored again following nitrate addition.

It is evident from this study that ammonia oxidisers play a minor role in the nitrogen cycle in the rice rhizosphere. Only in the case of the urea 400 treatment, when sufficient ammonium was present during the complete experimental period, did numbers and activity rise above the level of the unplanted microcosms. When NH_4^+ was low or absent in the rhizosphere, numbers of ammonia oxidisers were even lower than in the nonrooted soil. The presence of CH_4 as an alternative substrate may prevent loss of viability of nitrifiers during NH_4^+ limitation (Stein & Arp 1998). However, in our microcosms where sufficient $CH_4$was present this was obviously not the case. Apparently, ammonium is required to maintain a viable nitrifier population. A surprising fact was that a surplus of NH_4^+ in the $(NH_4)_2HPO_4$ 400 treatment stimulated neither numbers nor activity of ammonia oxidisers. This may have been caused by the low rhizosphere pH, which dropped below pH 6 12 weeks after transplanting while at that moment, the pH in the rhizosphere of the urea-treated microcosms was 6.8–7.0.

Data on numbers or activity of nitrite oxidisers in the rice rhizosphere are not available. The numbers detected in this study, however, are comparable to those found associated to the emergent macrophyte *Glyceria maxima* (Both et al. 1992; Bodelier et al. 1996). However, it is puzzling that the highest numbers were found in the $(NH_4)_2HPO_4$ 400 treatment where the oxidation rate of NH_4^+ to NO_2^- apparently was low. Moreover, the numbers of nitrite oxisiders were positively correlated with numbers and activity of the methanotrophs. A potential explanation for this phenomenon is that the methanotrophs produce nitrite that can subsequently be used by nitrite oxidisers.

The potential of the methanotrophs to nitrify in our microcosms has been demonstrated in this study.

Effects of fertilisation on the contribution of ammonia oxidisers to CH_4 oxidation can be excluded. A significant contribution of ammonia oxidisers to CH_4 oxidation would be indicated by a lower CH_4 oxidation rate after the removal of C_2H_2 in the DRA assay. During the first 48 hours after removal of C_2H_2 only methanotrophs were active. Hence, a lower rate compared to the rate prior to inhibition would indicate CH_4 oxidation by ammonia oxidisers. However, this was never the case as already demonstrated in a previous paper (Bodelier & Frenzel 1999). Taking into account the very low NH_4^+ oxidation rates and the fact that a substantial percentage of the NH_4^+ oxidation may be carried out by methanotrophs, we can exclude a significant role of ammonia oxidisers in influencing the CH_4 emission from rice paddies.

Conclusions

This study clearly demonstrates that in microcosms planted with rice CH_4 emission is primarly controlled by the presence of the plant when no ammonium-based fertilisers were applied. The plant stimulated the CH_4 production potential by supplying the methanogens with substrates. When ammonium-based fertilisers were applied, methanotrophic activity became the major factor controlling CH_4 emission next to the plant. Increasing the supply of ammonium clearly stimulated numbers and activity of methane-oxidising bacteria, leading to a substantial reduction of CH_4 emission. The stimulating effect may be caused by a relief of N-source limitation or a direct stimulation of CH_4 oxidation by NH_4^+ by an as yet unknown mechanism. The ammonium effect on CH_4 oxidation due to fertiliser addition has to be considered in any CH_4 mitigation strategy involving fertiliser management.

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