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Relationship between major soil properties and culturable microorganisms affecting CH_4 and N_2O dynamics in rice soils

(Beziehungen zwischen wesentlichen Bodeneigenschaften und kultivierbaren, die CH_4- und N_2O-Dynamik beeinflussenden Mikroorganismen)

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Abstract
Microorganisms associated with methane (CH_4) and nitrous oxide (N_2O) dynamics were studied in eight rice (*Oryza sativa* L.) soils by culture-dependent most probable number (MPN) methods. Enumeration of methanogens showed that acetoclastic methanogens (10^4 to 10^9 cells g^{-1}) outnumbered lithotrophic methanogens (10^3 cells g^{-1}). Serological analysis indicated that type II methanotrophs belonging to *Methylosinus* and *Methylocystis* genera are probably the dominant CH_4-oxidizing communities. For chemolithotrophic nitrifiers, nitrite oxidizers outnumbered ammonium oxidizers in most of the studied soils. The populations of both the acetoclastic methanogens ($p = 0.05$) and methanotrophs ($p = 0.06$) exponentially increased with the soil pH. Soil denitrifier populations (in a range of 10^3 to 10^6 cells g^{-1}) exponentially decreased as soil C content increased ($p = 0.03$). Nitrifier and denitrifier populations generally showed a similar trend of variation with soil C/N ratio. The largest nitrifier populations were found in the Texas soil (C/N = 13.9), and largest denitrifier population in the Arkansas soil (C/N = 13.1). The relationships between the studied microbial groups and major soil properties help to understand the dynamics of CH_4 and N_2O production and consumption in soils.

Keywords: *Methane, nitrous oxide, most probable number, C/N ratio, rice soils*

Introduction

Methane (CH_4) and nitrous oxide (N_2O) are two important atmospheric trace gases contributing to global greenhouse effect associated with global climate change. Soil microbial

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processes are a major contributor for the production and consumption of these two gases. Methanogenesis (CH_4 production) is driven by two groups of microorganism, acetoclastic methanogens (A-methanogens, using acetate to produce CH_4) and lithotrophic methanogens (L-methanogens, using H_2 and CO_2 to produce CH_4), mainly under strictly reducing conditions (Zeikus 1977). Methanotrophy (CH_4 oxidation) occurs mainly under aerobic conditions driven by methanotrophs that have two major oxidation pathways (Hanson & Hanson 1996), high CH_4 affinity type-I, and low affinity type II that is generally found in CH_4-rich environments. Nitrous oxide can be produced in both nitrification process under aerobic conditions (by nitrifiers) and denitrification process under moderately reducing conditions (by denitrifiers), and the later process is also responsible for N_2O reduction under more reducing conditions (Conrad 1995). Numerous studies have been conducted and found that nutrient availability, soil pH, organic matter content, and moisture conditions (as indicated by soil redox potential, Eh) are the key factors controlling production and consumption of these two gases (Bronson et al. 1997; Cai et al. 1997; Yu et al. 2004). However, there are few attempts to characterize the inherent soil microbial community structure that ultimately affects CH_4 and N_2O dynamics.

Rice soils experience periodically anaerobic and aerobic conditions because of irrigation and drainage practice in rice fields (Bronson et al. 1997; Yu et al. 2004). Thus, rice soils provide a unique environment for all above-mentioned microbial processes to function at a certain time. A soil microcosm study was previously conducted to focus on productions of three major greenhouse gases (CO_2, CH_4, N_2O) over an entire Eh range that could be maintained (Yu & Patrick 2004). The microbial communities that are responsible for the gas production have not been investigated. The objective of this study using the same rice soils is to characterize the microbial communities that directly affect CH_4 and N_2O dynamics and their relationships to major soil properties.

Materials and methods

Studied soils

Samples (surface 20 cm) from eight rice-growing regions were collected in spring time for this study. The soils were air-dried, sieved (1 mm), thoroughly mixed and stored at room temperature (20°C) before the experiment. Soil pH, total carbon (C) and total nitrogen (N) contents are included in Table I. Additional information on the soil and analysis methods is available in Yu and Patrick (2004).

Incubation of culturable microorganisms

Population densities of four microbial groups associated with dynamics of CH_4 (methanogens and methanotrophs) and N_2O (nitrifiers and denitrifiers) were evaluated by most probable number (MPN) technique. For each of the eight rice soils, soil slurry was prepared in a 500-ml serum bottle capped with a butyl rubber stopper. Each soil slurry consisted of 80 g of air-dried sample soil, 0.8 g of ground rice straw, 50 mg KNO_3–N kg^{-1} soil, and 320 ml of DI water. The eight soil slurries were incubated for 1 month at 25°C. After decanting the surface water, 2.5 g of wet soil (equivalent to 1 g air-dried soil) were transferred from the soil slurry to two 500-ml serum bottles, respectively. One bottle received 100 ml of Na_2S containing (to keep anoxic) mineral solution for identification of methanogens and denitrifiers, and the other one received oxic mineral solutions (no Na_2S) for identification of methanotrophs and nitrifiers. Bottles (16) were placed on a shaker for 2 h at 50 rpm for extracting

Table I. Selected properties of the studied soils.

Soil	Latitude	Longitude	Sand %	Loam %	Clay %	pH in water (1:1)	Total C %	Total N %	C/N ratio	Total S mg Kg^{-1}
Arkansas	35° 04′ N	092° 51′ W	4.1	80.9	15	6.0	0.85	0.07	12.1	12.7
California	33° 17′ N	115° 21′ W	3.3	36.6	60.1	6.7	2.73	0.16	14.8	45.2
Louisiana	30° 17′ N	092° 19′ W	14.3	73.1	12.6	7.3	0.97	0.07	13.9	10.7
Mississippi	31° 19′ N	089° 06′ W	12.6	29.2	58.2	7.7	1.47	0.10	14.7	12.4
Texas	29° 59′ N	096° 44′ W	7.5	28.4	64.1	5.1	1.48	0.11	13.4	38.4
China	41° 32′ N	122° 23′ E	3.1	63.5	33.4	5.6	2.70	0.27	10.0	66.0
Indonesia	07° 58′ S	114° 02′ E	12.2	47	40.8	5.3	1.38	0.10	13.8	65.6
Thailand	13° 37′ N	101° 40′ E	1	29.7	69.3	4.7	1.50	0.12	12.5	190.6

Soil total Mn and Fe are also available in Yu and Patrick (2004) where detail analytical methods for the soil properties were included. Soil total C % is calculated by dividing OM % with 1.72. Regression analysis of soil C and N content shows a significant relationship (C % = 9.2 × N % + 0.4, $R^2 = 0.88$, $p = 0.0004$), indicating most of the soil N is in organic form.

microorganisms. Anaerobic operation for identification of methanogens and denitrifiers was conducted in a glove box filled with nitrogen gas, and aerobic operation for methanotrophs and nitrifiers in room air. Single tenfold serial dilution of the soils was prepared in sterile mineral solution down to 10^{-9} g^{-1} soil. For each serial dilution, three replicates were inoculated with 1 ml aliquot of the solution. The prerequisite for the MPN method is that microorganisms that are to be enumerated selectively must be able to generate some characteristic metabolites or products that can be detected easily by a specific reagent. Details about media ingredients, incubation conditions, and detection methods for the two groups of methanogens can be found in Belyaev et al. (1983), for the methanotrophs in Galchenko (1995), for the two groups of nitrifiers in Alef and Nannipieri (1998), and for the denitrifiers in Malek et al. (1974). Anoxic media for methanogens were specially prepared using the following modified Hungate technique. The medium was boiled, cooled under argon, dispensed into 15 ml Hungate tubes, and autoclaved. A Lumam U2 photomicroscope was used for phase contrast observation and examination of epifluorescence of methanogens due to presence of deazoflavin under UV light (Mink & Dugan 1977). The identification of methanotrophs belonging to 12 species was done by the serological technique as described by Vecherskaya et al. (1993).

Enumeration and statistical analysis

The positive tests were recorded at the end of incubation, and tabulated results were referred to probability tables (Alef & Nannipieri 1998) in order to determine the MPN for each group of microorganism. Linear regression analysis ($\alpha = 0.05$) of soil microbial populations and the major soil properties was conducted using REG procedure of the SAS software (version 8.02, 1999–2001, SAS Institute, Cary, NC).

Results and discussion

Enumeration results of microbial population

Table II summarizes the results of soil microbial MPN into two groups of microorganisms associated with CH_4 and N_2O dynamics, respectively. Enumeration of methanogens revealed

Table II. Population densities of microorganisms affecting CH_4 and N_2O dynamics.

	CH_4 dynamics		
Soil	Acetoclastic methanogens	Lithotrophic methanogens	Methanotrophs
Arkansas	$2.3 \pm 0.5 \times 10^7$	$7.5 \pm 1.7 \times 10^3$	$9.3 \pm 1.8 \times 10^3$
California	$2.3 \pm 0.5 \times 10^9$	$2.3 \pm 0.5 \times 10^3$	$2.7 \pm 0.9 \times 10^5$
Louisiana	$2.3 \pm 0.5 \times 10^7$	$2.1 \pm 0.3 \times 10^4$	$9.3 \pm 1.8 \times 10^6$
Mississippi	$7.5 \pm 1.7 \times 10^8$	$4.3 \pm 0.9 \times 10^3$	$2.3 \pm 0.5 \times 10^6$
Texas	$2.3 \pm 0.5 \times 10^7$	$7.5 \pm 1.7 \times 10^3$	$2.3 \pm 0.5 \times 10^4$
China	$9.3 \pm 1.8 \times 10^6$	$2.3 \pm 0.5 \times 10^5$	$1.5 \pm 0.3 \times 10^6$
Indonesia	$2.3 \pm 0.5 \times 10^4$	$4.3 \pm 0.9 \times 10^3$	$4.3 \pm 0.9 \times 10^5$
Thailand	$2.7 \pm 0.9 \times 10^5$	$9.3 \pm 1.8 \times 10^3$	$9.3 \pm 1.8 \times 10^3$
	N_2O dynamics		
Soil	Ammonium oxidizers	Nitrite oxidizers	Denitrifiers
Arkansas	$7.5 \pm 1.7 \times 10^3$	$2.3 \pm 0.5 \times 10^6$	$4.3 \pm 0.9 \times 10^6$
California	$1.5 \pm 0.3 \times 10^4$	$2.3 \pm 0.5 \times 10^6$	$2.3 \pm 0.5 \times 10^3$
Louisiana	$3.8 \pm 0.9 \times 10^3$	$7.5 \pm 1.7 \times 10^3$	$4.3 \pm 0.9 \times 10^4$
Mississippi	$2.3 \pm 0.5 \times 10^4$	$4.3 \pm 0.9 \times 10^6$	$9.3 \pm 1.8 \times 10^3$
Texas	$4.3 \pm 0.9 \times 10^5$	$2.3 \pm 0.5 \times 10^7$	$2.7 \pm 0.9 \times 10^5$
China	0	$1.5 \pm 0.4 \times 10^2$	$4.3 \pm 0.9 \times 10^3$
Indonesia	$4.3 \pm 1.8 \times 10^3$	0	$2.3 \pm 0.5 \times 10^4$
Thailand	$9.3 \pm 0.9 \times 10^3$	$0.9 \pm 0.2 \times 10^2$	$2.3 \pm 0.5 \times 10^4$

Data represent mean MPN (cell g^{-1} soil) $\pm$ SD ($n = 3$).

that A-methanogens outnumbered L-methanogens by 4–6 orders in the five US soils and by about 2 orders of magnitude in the other three soils. Population densities of A-methanogens varied between 10^7 to 10^9 cells g^{-1} in the five US soils, and between 10^4 to 10^6 cells g^{-1} in other three soils. Soil pH may play a significant role in the A-methanogen populations among different soils. In contrast, populations of L-methanogens were in the order of 10^3 cells g^{-1} in all studied soils with an exception of the China soil (10^5 cells g^{-1}). The difference in population of the two groups of methanogens supports the widely accepted idea that most CH_4 is produced through a mechanism using acetate as substrate (Conrad 1995).

Population of methanotrophs varied between 10^3 to 10^6 cells g^{-1} in the studied soils (Table II), which is in good agreement with methanotroph numbers reported in other rice soils (Bodelier & Frenzel 1999). The members of type II methanotrophs belonging to *Methylosinus* and *Methylocystis* genera were identified positive in the highest dilution steps by serological analysis, and were likely to be the dominant CH_4-oxidizing communities in the studied soils. It may be true in general that type II methanotrophs prevail in rice fields, as found by other studies (Henckel et al. 1999; Macalady et al. 2002). The dominance of type II methanotrophs may be caused by their better survival under dry conditions, and fixation of molecular nitrogen and utilization of lower concentrations of oxygen than type I methanotrophs (Hanson & Hanson 1996; Frenzel 2000).

The autotrophic nitrifiers comprise two physiologically different groups of microorganisms that function together to oxidize ammonium to nitrate. The group, so called nitroso-bacteria, can produce N_2O during oxidation of ammonium to nitrite. Oxygen (O_2) partial pressure is the most important factor regulating the oxidation rate, and more N_2O production tends to occur at low O_2 conditions (Anderson et al. 1993). Autotrophic ammonium oxidation can be

carried out by both ammonium-oxidizing chemolithotrophic nitrifiers (ammonium oxidizers) and methanotrophs (Carini et al. 2003). Most methanotrophs show some affinity for ammonia, which can partially explain the lower abundance of ammonium oxidizers (Table II). The lower abundance of ammonium oxidizers than methanotrophs is in agreement with other results (Bodelier & Frenzel 1999). The results also showed that in most cases nitrite-oxidizing chemolithotrophic nitrifiers (nitrite oxidizers) outnumbered ammonium oxidizers (with two exceptions of Indonesia and Thailand soils), which might suggest a stronger activity of nitrite oxidation than that of ammonium oxidation in soils. This supports the general observation that soils usually have a low content of nitrite that is toxic to most soil biological processes. In addition to biological pathways, nitrite removal can also occur via chemical means (Anderson et al. 1993; Hu et al. 2001).

Denitrifiers are defined as microorganisms that can reduce nitrate to gaseous products (mainly N_2 and N_2O), and can couple this reduction process to generate energy. They are not a phylogenetically homogenous group. Some of them can metabolize a complete process from nitrate to N_2, but most of them can only conduct part of the process. Special interest goes to the group that can reduce N_2O to N_2, determining the N_2O consumption potential in soils. This process is the only known biological mechanism to consume N_2O. However, the current MPN technique cannot distinguish between different groups of denitrifiers. The results show that most denitrifiers were found in the Arkansas and Texas soil ($10^6 - 10^5$ cells g^{-1}), and the least (10^3 cells g^{-1}) were found in the California and China soils (Table II). Soil C content may be an important factor in controlling the soil denitrifier populations (see discussion in next section).

Soil properties and microbial populations affecting CH_4 dynamics

The studied soils were collected from a wide range of rice-producing regions. We found that soil A-methanogen populations exponentially increased with soil pH with statistical significance ($p = 0.05$). Soil L-methanogen populations, on the other hand, were in a lower range and not affected by the soil pH (Figure 1). Higher methanogenesis activities are generally found at near neutral soil pH (Conrad 1995). Low CH_4 production rates observed under acidic conditions can be interpreted by multiple reasons: (i) generally low biological activities under acidic conditions, (ii) small numbers of methanogens, and (iii) inhibition by sulfate (if the low soil pH is associated with high sulfate content as it was the case in the Thailand soil). The numbers of soil methanotrophs also exponentially increased with soil pH, but the correlation ($p = 0.06$) was slightly out of the criteria for statistical significance (Figure 1). The numbers of methanotrophs were consistently less than those of the A-methanogens (with exception of the Indonesia soil for unknown reason). This probably represents a microbial mutual control that soil methanotrophs are potentially able to match up the CH_4 produced by the surrounding methanogens to acquire energy. The result of this methanogens-methanotrophs interrelation provides a negative feedback mechanism of controlling CH_4 productions in rice soils. To minimize CH_4 emission from rice fields, field managements should facilitate less CH_4 production and more CH_4 oxidation. The microbial structure found in this study provides encouraging evidence that such an outcome is achievable.

Soil properties and microbial populations affecting N_2O dynamics

Denitrification is the major source of N_2O in soils. We found that soil denitrifier populations exponentially decreased with increasing soil C content with statistical significance ($p = 0.03$)

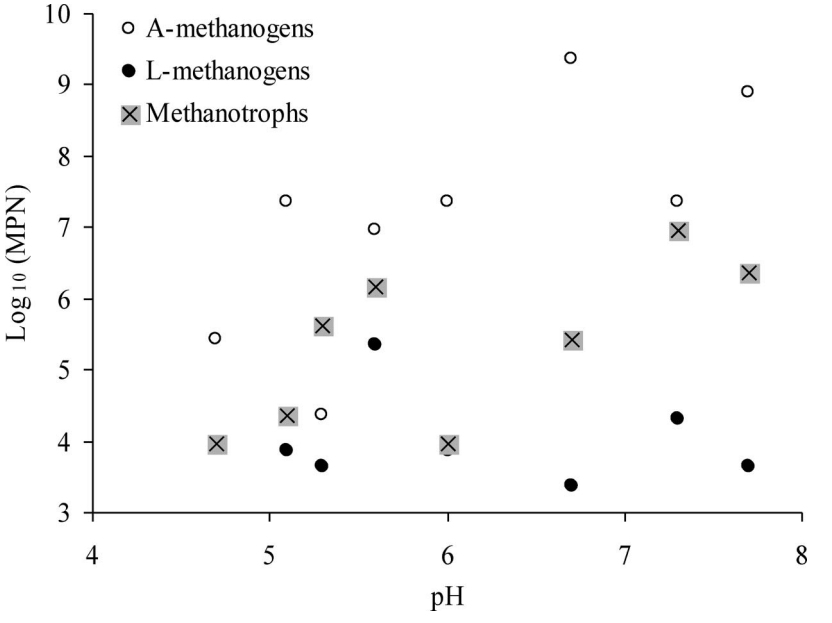

Figure 1. Relationship between population densities of microorganisms affecting CH_4 dynamics and soil pH. Soils, in order of pH from low to high, are Thailand, Texas, Indonesia, China, Arkansas, California, Louisiana, and Mississippi. Regression analysis ($n = 8$): Log_{10} (MPN-A-methanogens) $= 1.08 \times pH + 0.62$, $R^2 = 0.51$, $p = 0.05$, Log_{10} (MPN-L-methanogens) $= -0.10 \times pH + 4.6$, $R^2 = 0.03$, $p = 0.69$, and Log_{10} (MPN-methanotrophs) $= 0.74 \times pH + 0.90$, $R^2 = 0.48$, $p = 0.06$.

(Figure 2). Besides soil denitrifier populations, soil denitrification rate is also a function of soil nitrate (as substrate) and C content (as electron donor). In the studied soils with low C content, electrons may be limited to maintain denitrification activities, but larger denitrifier populations were found in these soils to compensate this limitation in order to maintain the denitrification at a certain rate. In the opposite case of the soils with high C content, we found substantially less denitrifiers, but appropriate denitrification rates were expected to be maintained by the stimulating effect of C on denitrification. Further analysis indicates that the soil nitrifiers and denitrifiers tended to reach a larger number when the soil C/N ratio was medium (Figure 3). The largest nitrifier population was found in the Texas soil with a C/N ratio of 13.9, and the largest denitrifier population in the Arkansas soil with a C/N ratio of 13.1. Globally, C/N ratio for soil organic matter is about 15 (Schlesinger 1991). Soils probably tend to reach equilibrium when the C/N ratio is in the range of 13 – 14. Soil profile analysis in a natural forest with a hydrological gradient showed that, despite of large variation in the soil C and N content at different soil depths and locations, the soil C/N ratio was quite uniform with an average ($\pm$ SD) of 13.3 ± 0.9 for the ridge (dry), 13.6 ± 1.8 for the transition (intermediate), and 13.0 ± 1.4 for the swamp (wet), respectively (Yu et al. 2006). Both the China and California soils had higher C contents (Table I) and smaller populations of denitrifiers (Table II and Figure 2). However, there was a large difference in their C/N ratios (Table I). The smaller denitrifier population in the California soil may be mainly due to abundant amount of electron donors (high C/N ratio), while in the China soil this may be mainly due to the higher availability of N (low C/N ratio). In general, nitrifier and denitrifier populations showed a similar trend of variation with soil C/N ratio (Figure 3), which indicates a close physiological linkage of these two groups of microorganisms associated with

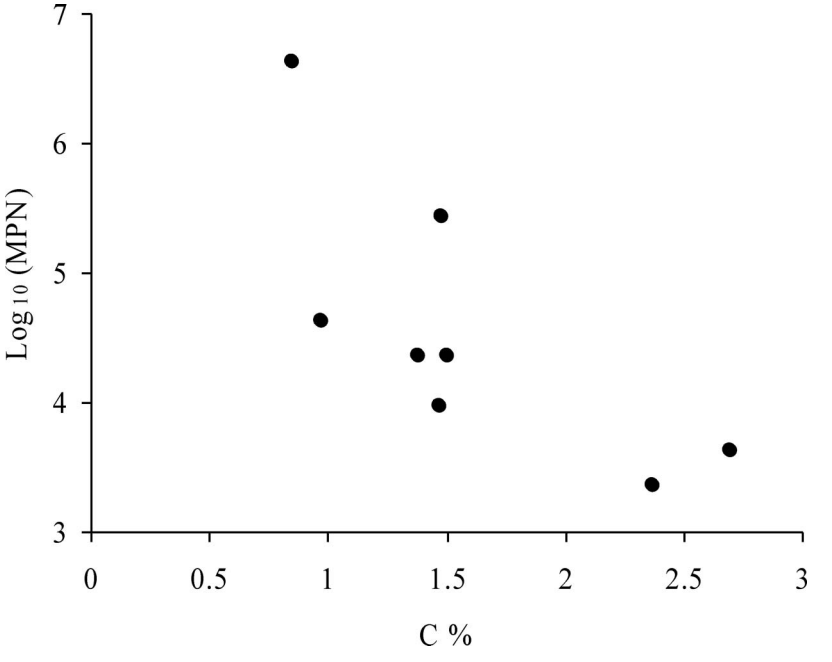

Figure 2. Relationship between population densities of denitrifiers and soil C content. Soils, in order of C % from low to high, are Arkansas, Louisiana, Indonesia, Mississippi, Texas, Thailand, California, and China. Regression analysis ($n = 8$): Log_{10} (MPN-denitrifiers) $= -1.23 \times$ C % $+ 6.5$, $R^2 = 0.56$, $p = 0.03$.

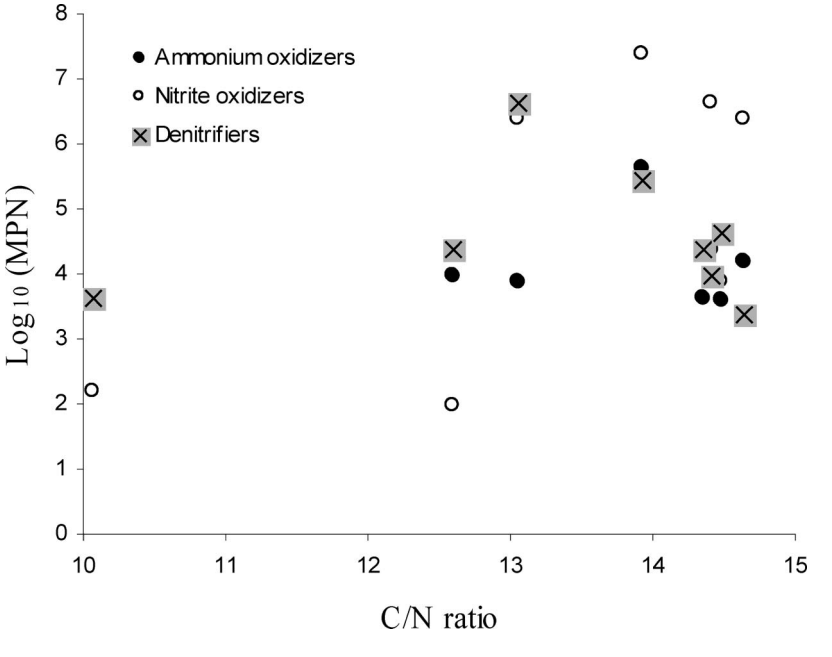

Figure 3. Relationship between population densities of nitrifiers and denitrifiers and soil C/N ratio. Soils, in order of C/N ratio from low to high, are China, Thailand, Arkansas, Texas, Indonesia, Mississippi, Louisiana, and California.

nitrification and denitrification activities in soils. A field study showed that applying different organic matter and mineral N fertilizer showed significant impact on soil C content and microbial biomass (Rühlmann & Ruppel 2005), which suggested that changing soil C/N ratio would likely affect the soil microbial community structure, such as nitrifier and denitrifier populations as shown in this study.

Conclusion

The MPN method used in this study allows enumerating the culturable microorganisms with specific functions. The culturable group of microorganisms probably represents the most active microbial communities that can function rapidly whenever environment becomes favorable. This method is widely used, despite of some deficiencies, because it can provide an approximate estimate of functioning microbial populations. Microorganisms in rice soils tend to adapt the periodical oxic/anoxic cycle, which probably makes the estimates of microbial population in this study more acceptable. Studies showed that the methanogenic population in rice paddies stays constant during dry fallow periods (Peters & Conrad 1995). Culturable methanotrophs were found to remain constant for up to 5 years, and methanotrophic activity was not affected by keeping a rice soil dry for 2 years (Frenzel 2000). Due to changes in environment during sampling and incubation, the observed CH_4 and N_2O production activities in the previous microcosm study (Yu & Patrick 2004) and the microbial communities found in this study can not reflect the actual field conditions. However, population density of the studied microbial groups and their relationships with key edaphic soil properties provides valuable insights to understand and interpret the variations in production and consumption of CH_4 and N_2O. Recent advancement in techniques may better link the microbial populations and their potential activities, such as the molecular approach based on the target genes that code the enzymes responsible for CH_4 and N_2O metabolisms (Rotthauwe et al. 1997; Henckel et al. 1999; Braker et al. 2000). The continuous effort in this direction will help to develop a process-based model in the future to understand the mechanisms of CH_4 and N_2O dynamics in rice soils in order to minimize their emissions to the atmosphere.

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