Abundance and community composition of methanotrophs in a Chinese paddy soil under long-term fertilization practices

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Abstract

Background, aim, and scope As the second most important greenhouse gas, methane (CH_4) is produced from many sources such as paddy fields. Methane-oxidizing bacteria (methanotrophs) consume CH_4 in paddy soil and, therefore, reduce CH_4 emission to the atmosphere. In order to estimate the contribution of paddy fields as a source of CH_4 , it is important to monitor the effects of fertilizer applications on the shifts of soil methanotrophs, which are targets in strategies to combat global climate change. In this study, real-time polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) based on 16S rRNA and *pmoA* genes, respectively, were used to analyze the soil methanotrophic abundance and community diversity under four fertilization treatments: urea (N), urea and potassium chloride (NK), urea, superphosphate, and potassium chloride (NPK), and urea, superphosphate, potassium chloride, and crop residues (NPK+C), compared to an untreated control (CON). The objective of this study was to examine whether soil methanotrophs responded to the long-term, different fertilizer regimes by using a

combination of quantitative and qualitative molecular approaches.

Materials and methods Soil samples were collected from the Taoyuan Experimental Station of Agro-ecosystem Observation at Changde (28°55' N, 111°26' E), central Hunan Province of China, in July 2006. Soil DNAs were extracted from the samples, then the 16S rRNA genes were quantified by real-time PCR and the *pmoA* genes were amplified via general PCR followed by DGGE, cloning, sequencing, and phylogenetic analysis. The community diversity indices were assessed through the DGGE profile.

Results Except for NPK, other treatments of N, NK, and NPK+C showed significantly higher copy numbers of type I methanotrophs ($7.0\text{--}9.6 \times 10^7$) than CON (5.1×10^7). The copy numbers of type II methanotrophs were significantly higher in NPK+C (2.8×10^8) and NK (2.5×10^8) treatments than in CON (1.4×10^8). Moreover, the ratio of type II to type I methanotrophic copy numbers ranged from 1.88 to 3.32, indicating that the type II methanotrophs dominated in all treatments. Cluster analyses based on the DGGE profile showed that the methanotrophic community in NPK+C might respond more sensitively to the environmental variation. Phylogenetic analysis showed that 81% of the obtained *pmoA* sequences were classified as type I methanotrophs. Furthermore, the type I-affiliated sequences were related to *Methylobacter*, *Methylobacterium*, *Methylomonas*, and some uncultured methanotrophic clones, and those type II-like sequences were affiliated with *Methylocystis* and *Methylosinus* genera.

Discussion There was an inhibitory effect on the methanotrophic abundance in the N and a stimulating effect in the NK and NPK+C treatments, respectively. During the rice-growing season, the type II methanotrophs might be more profited from such a coexistence of low O_2 and high CH_4 concentration environment than the type I methanotrophs.

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However, type I methanotrophs seemed to be more frequently detected. The relatively complex diversity pattern in the NPK+C treatment might result from the strong CH₄ production.

Conclusions Long-term fertilization regimes can both affect the abundance and the composition of the type I and type II methanotrophs. The inhibited effects on methanotrophic abundance were found in the N treatment, compared to the stimulated effects from the NK and NPK+C treatments. The fertilizers of nitrogen, potassium, and the crop residues could be important factors controlling the abundance and community composition of the methanotrophs in the paddy soil.

Recommendations and perspectives Methanotrophs are a fascinating group of microorganisms playing an important role in the biogeochemical carbon cycle and in the control of global climate change. However, it is still a challenge for the cultivation of the methanotrophs, although three isolates were obtained in the extreme environments very recently. Therefore, future studies will be undoubtedly conducted via molecular techniques just like the applications in this study.

Keywords 16S rRNA gene · DGGE · Long-term fertilization · Methanotrophs · Paddy soil · *pmoA* gene · Real-time polymerase chain reaction (PCR)

1 Background, aim, and scope

Methane (CH₄) is the second most abundant carbon-containing gas in the atmosphere and contributes approximately 18% to the global warming (IPCC 2007). The atmospheric methane concentration is determined by the balance of methane production and methane oxidation. Rice cultivation is one of the major sources of methane, which annually emits 60 Tg CH₄ into the atmosphere (Lowe 2006). Rice is one of the most important food crops to feed the growing population, especially in Asia (Krüger and Frenzel 2003; Conrad et al. 2006). In order to maximize rice grain yields, rice cultivation needs to be intensified by strengthening and improving the agronomic practices, such as fertilizer applications.

Before releasing into the atmosphere, the produced CH₄ is subject to oxidation by methane-oxidizing bacteria (methanotrophs) in the surface soil layer and the rhizosphere. Methanotrophs, gram-negative bacteria that utilize methane as their sole source of carbon and energy, play an important role in regulating CH₄ emission from the paddy field. Methanotrophs are obligate aerobes and classified into two main groups (type I and type II) differing in phylogeny, physiology, morphology, and biochemistry (Hanson and Hanson 1996). Type I methanotrophs belong to the gamma proteobacteria and assimilate the intermediate formaldehyde via the ribulose monophosphate pathway.

Type II methanotrophs, however, belong to the alpha proteobacteria and utilize the serine pathway for assimilating formaldehyde.

Many methanotrophs are difficult to cultivate. Culture-independent molecular approaches have been widely used to assess the diversity and activity of methanotrophs present in the natural environment. These approaches use phylogenetic and functional gene probes to detect and analyze methanotrophs directly from environmental samples without cultivation (Murrell et al. 1998). For discriminating the type I and type II methanotrophs, different polymerase chain reaction (PCR) primers were designed to amplify 16S rRNA gene fragments of these two distinct groups in earlier studies (Henckel et al. 1999; Wise et al. 1999). Since then, the molecular analysis on the basis of specific 16S rRNA gene probes that target either type I or type II methanotrophs were successfully applied in detecting these two methane oxidizers. For example, type I methanotrophs were found more sensitive to the presence of the rice plants than type II methanotrophs (Bodelier et al. 2000). Soil methanotrophic community structure could be altered after a long-term herbicide application (Seghers et al. 2003).

In addition to the 16S rRNA gene, functional genes of methanotrophs were also used to detect the presence and abundance of the methane oxidizers (Fjellbirkeland et al. 2001; Horz et al. 2001). These functional genes include *pmoA*, *mmoX*, and *mxoF*, which encode subunits of particulate methane monooxygenase, soluble methane monooxygenase, and methanol dehydrogenase, respectively. Among these functional genes, *pmoA* is present in all known methanotrophs with the only exception of *Methylocella* spp. Furthermore, sequence-based *pmoA* phylogeny is consistent with the 16S rRNA-based phylogeny. Thus, the *pmoA* gene is a favorable functional gene and widely used to detect methanotrophs in soils (Holmes et al. 1999; Mohanty et al. 2007).

Our previous investigations showed that long-term fertilization practices could result in significant shifts in the diversity of soil bacteria and fungi (Ge et al. 2008; He et al. 2008) and in the abundance and community composition of some soil functional microorganisms such as ammonia oxidizers (He et al. 2007; Shen et al. 2008). As an important player in carbon cycling in terrestrial ecosystems, soil methanotrophs should not be ignored. In this study, we proposed the following hypotheses to be tested: (1) long-term fertilizer applications result in changes in methanotrophic abundance and community composition in a paddy field and (2) type I and type II methanotrophs respond differently to the fertilization treatments. We thus collected paddy field soil samples from a long-term fertilization experimental site, and the abundance and community composition of the soil methanotrophs were investigated using real-time PCR and denaturing gradient

gel electrophoresis (DGGE) based on both 16S rRNA and *pmoA* genes, respectively.

2 Materials and methods

2.1 Site description and soil sampling

Soil samples were collected from the Taoyuan Experimental Station of Agro-ecosystem Observation, the Chinese Academy of Sciences at Changde (28°55' N, 111°26' E), central Hunan Province of China. This region has a central–north subtropical monsoon moist climate with a mean annual precipitation of 1,447.9 mm and a mean annual temperature of 16.5°C. The paddy soil in this station was derived from quaternary red clay and classified as a waterloggogenic paddy soil. The land utilization was early rice plus late rice plus green manure annually since 1990. Five treatments were selected in this study: (1) treatment 'CON', a control without fertilizers; (2) treatment 'N' in which only nitrogen (N) fertilizer as urea was applied; (3) treatment 'NK', which was treatment N plus K as potassium chloride; (4) treatment 'NPK', which was treatment NK plus P as superphosphate; and (5) treatment 'NPK+C', which was treatment NPK plus recycled crop residues. Each treatment had three replicates which were arranged in fully randomized blocks. The annual amount of N, P, K, and C were shown in Table 1. The soil samples from three individual plots (each plot measured $4.1 \times 8.1 \text{ m}^2$) per treatment were taken randomly from every replicate at horizon A (0–20 cm in depth) in July 2006. The samples were stored at -20°C for DNA extraction and 4°C for other analyses. Basic characteristics of the soil samples are listed in Table 2.

2.2 Extraction of soil DNA

Soil DNA was extracted from 0.5 g (fresh weight) soil samples using MoBio UltraClean™ soil DNA isolation kits (San Diego, CA, USA) according to the manufacturer's

protocol with previously described modifications (He et al. 2007). For a more efficient elution of DNA from spin filter, a volume of 80 μL of solution S5 was used.

2.3 Quantitative analysis of the methanotrophs by real-time PCR

Real-time PCR was performed on an iCycler iQ5 thermocycler (Bio-Rad). Amplification was performed in 25- μL reaction mixtures by using SYBR® Premix Ex Taq™ as described by the suppliers (Takara Bio, Otsu, Shiga, Japan). Two forward primers of MB10 γ (5'-AAGCGGGGATCTTCGGACC-3') and MB9 α (5'-GTTCCGAATAACTCAGGG-3') and their common reverse primer 533r (5'-TTACCGCGGCTGCTGGCAC-3') were used to determine the 16S rRNA gene copy numbers of the type I and type II methanotrophs, respectively (Henckel et al. 1999). The DNA extracts were tenfold diluted and used as template with a final content of 1–10 ng in each reaction mixture. Real-time PCR assay was carried out with the protocol for both target groups as follows: 95°C for 2 min followed by 36 cycles of 1 min at 94°C , 1 min at 60°C , plate read at 83°C . In each real-time PCR amplification, following the three temperature steps, a melting curve analysis was performed to confirm PCR product specificity by measuring fluorescence continuously as the temperature increased from 55°C to 95°C . Data analysis was carried out with the iCycler software (version 1.0.1384.0 CR). The parameter C_t (threshold cycle) was determined as the cycle number at which a statistically significant increase in the reporter fluorescence was detected.

2.4 Standard curve for real-time PCR

The primer pairs MB10 γ /533r and MB9 α /533r were applied to amplify type I and type II methanotroph-specific 16S rRNA gene fragments from the environmental DNA extract and *Methylosinus sporium* (NCIMB 11126), respectively. The PCR products were gel-purified with the Agarose Gel DNA Purification Kit (TaKaRa Biotechnology, Dalian, China) and ligated into the pGEM-T Easy Vector (Promega,

Table 1 The annual amount of the chemical and organic residue cycling fertilizers applied to the examined paddy field since 1990

Treatments	Amount of fertilizer (kg ha^{-1})			Amount of organic residues (kg ha^{-1}) ^a		
	N	P	K	N	P	K
CON	0	0	0	0	0	0
N	262.5/182.3 ^b	0	0	0	0	0
NK	262.5/182.3	0	137.0/197.2	0	0	0
NPK	262.5/182.3	39.3	137.0/197.2	0	0	0
NPK+C	262.5/182.3	39.3	137.0/197.2	175.9	39.9	253.2

^a The amount of organic residue cycling is the average from 1990 to 2001

^b The former is the N and K fertilizer amount of 1990 to 1996 and the latter is the amount after 1996

Table 2 The basic characteristics of the examined paddy soil under different fertilization treatments for 16 years

Treatments	pH (H ₂ O)	O.M. (g kg ⁻¹)	Total N (g kg ⁻¹)	Available P (mg kg ⁻¹)	Available K (mg kg ⁻¹)
CON	4.94±0.01b	29.2±1.1b	3.53±0.31b	19.1±3.06c	47.1±6.02b
N	4.99±0.03b	30.2±5.1b	3.73±0.47b	9.83±1.74d	41.2±7.50b
NK	5.15±0.07a	30.5±5.0b	3.71±0.49b	20.9±4.69c	148±29.3a
NPK	4.80±0.09c	33.1±7.5b	3.96±0.75b	89.2±2.86b	56.1±5.03b
NPK+C	4.92±0.04b	47.7±2.0a	5.39±0.14a	155±6.13a	152±20.9a

The letters (a–d) indicate significant differences between treatments at $P<0.05$

Madison, WI, USA), the ligation products were then used to transform *Escherichia coli* JM109 competent cells following the instructions of the manufacturer. After reamplification with the vector-specific primers T7/SP6 (an additional sequencing identification for type I methanotrophic clone was carried out), the positive clones were selected to extract plasmid DNA with a MiniBEST Plasmid Purification Kit (TaKaRa). The plasmid DNA concentrations were determined using a Nanodrop® ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies) and the copy numbers of 16S rRNA gene were calculated directly from the concentrations of the extracted plasmid DNA. Tenfold serial dilutions of a known copy number of the plasmid DNA were subjected to real-time PCR assay in triplicate to generate an external standard curve.

2.5 PCR–DGGE analysis of the methanotrophic community structure

In order to detect the methanotrophic diversity in the different fertilization treatments, a PCR–DGGE experiment was conducted to amplify methanotrophic *pmoA* gene fragment. The sequences of the forward and reverse primers were A189 (Holmes et al. 1999) and mb661 (Kolb et al. 2003). Notably, a GC clamp (CCC CCC CCC CCC CGC CCC CCG CCC CCC GCC CCC GCC GCC C) was attached to the 5′ end of A189. The PCR condition was described as follows. Briefly, 20 pmol of each primer, 10 nmol of deoxyribonucleoside triphosphate, 75 nmol of MgCl₂, 5 μL of 10× PCR buffer, 2.5 U of *Taq* DNA polymerase (TaKaRa), and approximately 10 ng of template DNA were combined with superpurified H₂O to a total volume of 50 μL in a 0.5-mL tube. A touchdown thermal cycle strategy involved an initial preheating step of 4 min at 94°C for melting double-stranded DNA. Then, a touchdown procedure (consisting of 1 min at 94°C, annealing for 1 min at temperatures decreasing from 60°C to 52°C during the first 16 cycles, and ending with an extension step at 72°C for 1 min) followed, additional 20 cycles (consisting of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C) and a final extension of 10 min at 72°C were performed.

The obtained PCR products were loaded on to 6% (vol/vol) acrylamide/bisacrylamide (37.5:1) gel, which was

made with a denaturing gradient ranging from 40% to 60%. The electrophoresis was run in a DCode Universal Detection System Instrument (Bio-Rad Laboratories, Hercules, CA, USA) at a constant temperature of 60°C for 10 min at 150 V followed by 6 h at 120 V. After running, the gel was stained with 1:10,000 SYBR Gold Nucleic Acid Gel Stain (Invitrogen Molecular Probes, Eugene, USA) for 30 min according to the manufacturer's instructions and then photographed with UV transillumination. Subsequently, a matrix of similarities between the densitometric curves of the band patterns was calculated based on the Dice coefficient with Quantity One 4.5.2 software (Bio-Rad). Finally, the DGGE patterns were clustered based on the unweighted pair-group arithmetic average (UPGAMA) clustering algorithm.

2.6 Cloning, sequencing, and phylogenetic tree construction

The DGGE gel strips of some distinguished bands among all treatments were excised and reamplified following previous PCR conditions. These PCR products were cloned by using the pGEM-T Easy Vector (Promega). Clones that contained correctly inserted fragments were selected and sequenced by using an ABI PRISM 3730 DNA analyzer (Sangon Biotechnology, Shanghai, China). The obtained sequences were manually proofread and corrected if necessary, edited and aligned using BioEdit version 4.8.5. These partial sequences of approximately 508 bp were aligned to *pmoA* gene sequences obtained from the National Center for Biotechnology Information (NCBI) database using the BLAST version 2.2.16 searching program. The most similar and representative GenBank sequences to the clones were extracted from the GenBank. Phylogenetic analyses were conducted using MEGA version 3.1 and the neighbor-joining trees were constructed using *p*-distance with 1,000 replicates to produce bootstrap values.

2.7 Diversity indices

The diversity indices, including richness index and Shannon index, were quantified and calculated based on DGGE data, respectively. Each detected band was defined as a specific phylotypes. The pixel intensity for each band was detected by Quantity One software and considered as the

abundance of a specific phylotype for diversity estimations. Briefly, the Shannon index (H) was calculated based on the following equations in which H is the Shannon index, N_i is the abundance of the i th phylotype, N is the total abundance of all phylotypes in the sample (lane of the DGGE gel), and S is the number of phylotypes:

$$H = - \sum_{i=1}^S p_i \ln p_i = - \sum_{i=1}^S (N_i/N) \ln(N_i/N).$$

2.8 Nucleotide sequence accession numbers

The obtained sequences were submitted to the GenBank database under accession numbers EU193266–EU193301.

3 Results

3.1 Methanotrophic abundance based on 16S rRNA gene

The 16S rRNA gene copy numbers of type I and type II methanotrophs are shown in Fig. 1. For the type I methanotrophs, the fertilization treatments of N, NK, and NPK+C showed significantly higher copy numbers (7.0×10^7 , 9.6×10^7 , and 8.3×10^7 , respectively) than CON (5.1×10^7). There was no significant difference between the NPK and the CON. For the type II methanotrophs, the quantitative results showed that the copy numbers were signifi-

cantly higher in the NPK+C (2.8×10^8) and NK (2.5×10^8) treatments than in CON (1.4×10^8). There was no significant difference among NPK (1.7×10^8), N (1.3×10^8), and CON. Interestingly, the ratio of type II to type I methanotrophic copy numbers ranged from 1.88 to 3.32, indicating that the type II accounted for a significantly higher ratio than type I of the total methanotrophs in all the fertilization treatments.

3.2 Methanotrophic community structure

The DGGE profile of the methanotrophic community of the soil samples showed some variations among the treatments (Fig. 2a). It is clear that fewer bands and lower methanotrophic diversity were detected in the NK treatment (lanes 7–9) than the other two fertilization treatments of N and NPK+C (Table 3). Cluster analyses (Fig. 2b) based on the DGGE profile showed that the N and CON treatments had a relatively similar banding pattern and clustered together initially, while the NK clustered together in another subcluster and clearly distinguished itself from the NPK+C treatment. This could also reflect that the soil methanotrophic community in the NPK+C treatment might be responding more sensitively to the environmental variation here.

3.3 Phylogenetic analysis of *pmoA* gene clones among the treatments

A total of 34 methanotrophic bands (numbering from 1 to 34) in the DGGE gel was excised for further cloning analysis (Fig. 2a). After the subsequent sequencing, we obtained 36 sequences, which were all identified as *pmoA* gene sequences. By aligning with the GenBank database, all retrieved sequences were classified into type I and type II methanotrophs (Fig. 3). Phylogenetic analysis of the methanotrophic *pmoA* gene sequences showed that 29 out of 36 sequences (up to 81%) were classified as type I methanotrophs and the remaining seven sequences were related to type II methanotrophs. Especially in Fig. 3, band 3 and band 7 were both found to contain two sequences, which were further named as bands 3-1, 3-2, 7-1, and 7-2, respectively. However, bands 7-1 and 7-2 were not very closely related, although they were both classified into the *Methylocystis* genera. It was interesting to note that a significantly higher ratio (80%) of sequences appeared in the upper part of the DGGE gel that were affiliated with *Methylobacter*, *Methylochromium*, *Methylomonas*, and some uncultured methanotrophic clones, and all these methanotrophic genera and clones were classified as type I methanotrophs (marked by shade in Fig. 3). Only 20% (three sequences including EU193271, EU193273, and EU193274) was classified into type II methanotrophs. Phylogenetic analysis of these type II-like sequences was affiliated with *Methylocystis* and *Methylosinus* genera.

Fig. 1 Quantification of 16S rRNA gene copy numbers from type I (white bars) and type II methanotrophs (gray bars) in a Chinese paddy soil under different fertilization treatments. Ratios of type II to type I methanotrophic copy numbers are shown above the chart. The different letters above the bars indicate significant differences among treatments at $P < 0.05$

Fig. 2 **a** DGGE profile of methanotrophic *pmoA* gene fragments of soil samples under different fertilization treatments and **b** cluster analysis of left DGGE banding patterns. The small black triangles and numbers on each lane indicate the bands excised for further analysis

4 Discussion

4.1 Effects of fertilizer applications on soil methanotrophic abundance

Quantitative analysis on the basis of group-specific 16S rRNA gene revealed that the size of total soil methanotrophs was subjected to the long-term fertilizer treatments. Compared with the fertilization treatments of NK and NPK+C, long-term N fertilization seemed to inhibit the soil methanotrophs, although higher type I methanotrophic 16S rRNA gene copy numbers were found in the N than in the CON. As N fertilizer was applied in this study, urea is a NH_4^+ -producing compound, and its inhibitory effect on methanotrophs was also demonstrated (Dubey and Singh 2000). In an earlier study, He et al. (2007) found that the lowest ammonia oxidizers population sizes were found in the N-treated upland red soil samples. The negative effects of N fertilizer on methane oxidizers and ammonia oxidizers were similarly found in paddy soil and upland soil samples, which were both collected from central Hunan Province of China. Additionally, Ge et al. (2008) previously found that N fertilizer was a key factor in controlling soil bacterial

community. In contrast to the N application, the application of NK fertilizers seemed to stimulate the growth of methanotrophs and led to an increase in the methanotrophic abundance. This result is in agreement with another field experiment where the amendment of potassium (K) was suggested to be inhibiting methanogenic bacteria, but stimulating the methanotrophic bacterial population (Babu et al. 2006). Interestingly, in Table 2, a significantly higher content of available K was found in NK than in CON and N, indicating that the K fertilizer may play an important role in upkeeping methanotrophic life processes. The NPK fertilizer had given relatively little effects on the soil methanotrophs. However, the treatment of NPK+C fertilizers seemed to stimulate the soil methanotrophs, indicating that the application of recycled crop residues (rice straw) seemed to have a positive influence, both on the soil fertility (see Table 2) and on the soil methanotrophic population size. Dissimilarly, wheat straw was found to have no effect on methanotrophy (Hütsch 1998). We interpreted that this discrepancy may result from the different characteristics of two straws (rice versus wheat) and/or of two tested soils.

Furthermore, our results showed that there is a higher abundance of type II than that of type I methanotrophs in all fertilization treatments. The methanotrophic metabolic differences and the environmental factors might result in this quantification discrepancy. Type I methanotrophs seemed to grow preferentially at high O_2 and low CH_4 concentrations, contrasting with type II methanotrophs which grow preferentially at low O_2 and high CH_4 concentrations (Amaral and Knowles 1995; Amaral et al. 1995). Since the soil samples were collected in the rice-growing season, more CH_4 was produced as well as more O_2 was transferred from the atmosphere into the rice rhizosphere, thus maybe the type II methanotrophs profited

Table 3 Diversity properties of paddy soil methanotrophs using DGGE bands pattern data associated with long-term different fertilization

Treatments	Richness	Shannon (<i>H</i>)
CON	19±2b	2.92±0.08c
N	27±2a	3.24±0.08a
NK	19±2b	2.87±0.06c
NPK+C	23±2a	3.08±0.02b

The letters (a–c) indicate significant differences between treatments at $P < 0.05$

Fig. 3 Evolutionary distance tree of the derived amino acid sequence of *pmoA* genes amplified from excised bands from methanotrophic DGGE gel of four long-term fertilization treatments. The shaded part indicates the sequences representing the bands which were excised from the upper part of the DGGE gel and were classified into type I

methanotrophs. Designation of the clones in **bold** includes the following information: accession number in the GenBank with the fertilization treatments in which the bands were excised in the *parentheses*. Bootstrap values (>50%) are indicated at branch points. The scale bar represents 5% estimated sequence divergence

more in this competition by pursuing a low O_2 and high CH_4 environment and thus revealed a higher abundance than type I methanotrophs. In addition, such a phenomenon could also be explained that perhaps, apart from the fertilization, other factors like plant cultivation, water content, and temperature were responsible for the methane oxidation and propagation of the methanotrophs in the soil (Einola et al. 2007). A previous study similarly demonstrated that only type II populations were strongly correlated with soil porewater methane concentrations and rice growth (Macalady et al. 2002). Therefore, we conclude that the

dominance of the type II methanotrophs might be caused by physiological and environmental factors.

4.2 Effects of fertilizer applications on soil methanotrophic community composition

Considering the minor effects of the NPK treatment on the methanotrophic abundance, only four other treatments (CON, N, NK, and NPK+C) were selected to analyze the methanotrophic community structure using DGGE based on the amplification of the *pmoA* gene. Some distinct

differences in the quantity of bands and diversity indices were found between the N and NK treatments, indicating that the soil methanotrophic community was clearly changed through the K-containing fertilization. Also, a recent study on a Chinese paddy soil demonstrated that K application had no effect on soil microbial parameters, the same as for P and N (Zhong and Cai 2007). It could be interpreted that the numbers of methanotrophs are typically in the order of <1% of total bacteria (Kolb et al. 2003); thus, the clear distinction of a methanotrophic community would be most likely to be neglected in the total bacterial community analysis. A clearly complex diversity pattern was revealed in the NPK+C treatment which might be a result of the strong CH₄ production. Besides the chemical NPK, the addition of recycled crop residues was suggested to be vital to methanogenic archaeal and the methanogenesis in paddy soils (Conrad and Klose 2006). It is well-known that the soil CH₄ oxidation rate can be enhanced by higher CH₄ concentrations, thus promoting the activity, growth, and eventually changing the community structure of methanotrophs (Bender and Conrad 1995). In summary, based on the DGGE profile, it was revealed that long-term application of fertilizers could change the soil methanotrophic community structure.

In this study, a visible and interesting discovery is the methanotrophic community composition of the upper part of the DGGE profile and the subsequent sequence analysis. The type I methanotrophs seemed to be more frequently detected. Many environmental factors such as soil water content and temperature changed continuously along with the growth of rice plants. A lot of work had been carried out to demonstrate the effects of these environmental factors on methane oxidation (King 1997; Smith et al. 2003; Einola et al. 2007). Our results indicated that type I methanotrophs relating to the genera of *Methylobacter*, *Methylomicrobium*, and *Methylomonas* seemed to be the most common methane oxidizers in the paddy soil receiving different fertilizations long-term. Similarly, the *Methylobacter*/*Methylomonas*-related methanotrophs were the active species in the paddy soils and differed from those in the forest soils (Mohanty et al. 2006). Also, our results were in agreement with an earlier work (Fjellbirkeland et al. 2001) in which type I sequences clustering with members of the genera *Methylobacter*, *Methylomicrobium*, and *Methylomonas* were most frequently detected in an agricultural soil.

5 Conclusions

Long-term fertilization regimes can differentially affect the abundance and composition of the methanotrophs. The inhibited effects on methanotrophic abundance were found in the N treatment, compared to the stimulated effects from

the NK and NPK+C treatments. On the other hand, the methanotrophic community structure also responded clearly to the different fertilization. The methanotrophic diversity of the NPK+C treatment was observed clearly distinguished from the other treatments. Furthermore, a clear shift was found in methanotrophic community composition based on the sequences and phylogenetic analysis. A significantly higher ratio of type I methanotrophic sequences clustering with the genera of *Methylobacter*, *Methylomicrobium*, and *Methylomonas* were detected in all treatments. In conclusion, the applications of nitrogen and potassium fertilizers could be important factors controlling the abundance, and some environmental factors such as temperature regulating the community composition, of the methanotrophs. Understanding of the major factors influencing the methanotrophic abundance and composition is vital for linking this functional community to soil ecosystem processes and sustainable management of rice cultivation.

6 Recommendations and perspectives

Methanotrophs are a fascinating group of microorganisms playing an important role in the biogeochemical carbon cycle and in the control of global climate change. However, it is still a challenge for the cultivation of the methanotrophs, although three isolates were very recently obtained in the extreme environments (Dunfield et al. 2007; Pol et al. 2007; Islam et al. 2008). Hence, future studies will undoubtedly be conducted via molecular techniques just like the applications in this study.

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