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Diversity and composition of methanotrophs in paddy soil as affected by different long-term fertilizer management from double-cropping paddy fields in Southern China

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Methane (CH_4) is the most important greenhouse gas, which was produced from paddy fields. The CH_4 production and emission were affected by methane–oxidizing bacteria (methanotrophs). Therefore, it is significant to investigate the effects of fertilizer applications on the change of soil methanotrophs, which affected CH_4 emission. The objective of this paper was to describe changes of CH_4 and diversity and composition of methanotrophs in paddy soil in relation to the application of crop residues, mineral fertilizer, and manure based on a long-term field experiment. In this study, static chamber-gas chromatography technique, real-time polymerase chain reaction (PCR) and Illumina high-throughput sequencing of the 16S rRNA gene, respectively, were used to analyze the CH_4 emissions from paddy fields, soil methanotrophs abundance and community diversity from May to October 2014 under five fertilization treatments: mineral fertilizer (MF), rice residue and mineral fertilizer (RF), low manure rate and mineral fertilizer (LOM), and high manure rate and mineral fertilizer (HOM), as compared to without fertilizer input (CK). The results indicated that CH_4 from fertilization treatments displayed different emission patterns during early and late rice growth period. HOM treatment had the highest CH_4 emissions during early and late rice growth period with 5.074 and 6.099 g m^{-2}, respectively. Some methanotrophs genera (*Methylosinus, Crenothrix, Methylocaldum, Methylomicrobium* and *Methylomonas*) were identified at the early and late rice main growth stages. The abundance and composition of soil methanotrophs were affected by long-term fertilization managements. The methanotrophs abundance was inhibited under MF treatment, while they were stimulated under RF, LOM and HOM treatments. The abundance and community composition of methanotrophs in paddy soil were affected by fertilizers of mineral, crop residues, and manure. It was concluded that application with organic and crop residues enhance the abundance and community composition of methanotrophs in double-cropping paddy fields in Southern China through a long-term fertilizer experiment.

Keywords: CH_4, long-term fertilization, methanotrophs diversity, methanotrophs composition, paddy field.

INTRODUCTION

Methane (CH_4) is the most greenhouse gas in the atmosphere and contributes approximately 18% to global

warming (IPCC, 2007). The atmospheric CH_4 concentration was affected by CH_4 production and oxidation. Paddy field is one of the major sources of CH_4, which annually emits 60 Tg CH_4 into the atmosphere (Lowe, 2006). Rice is the major food crops to feed people, especially in Asia (Krüger and Frenzel, 2003; Conrad et al., 2006). Therefore, some agronomic practices need to be strengthened and improved to obtain higher grain yield, such as fertilizer applications.

The CH_4 emission is affected by methanotrophs in the surface soil layer and rhizosphere, then it releases into the atmosphere. That is, CH_4 emission from paddy field was influenced by methanotrophs, which were gram-negative bacteria that utilize CH_4 as their sole source of carbon and energy (Lowe, 2006). According to the physiology, phylogeny, morphology and biochemistry, methanotrophs were classified into three main groups (type I, type II, and type X) (Hanson and Hanson, 1996). The growth and activity of methanotrophs was influenced by many factors, such as soil conditions, fertilizer application and vegetation cover (Hanson and Hanson, 1996; Zheng et al., 2008). And the fertilizer applications and rice plant are the important factors that affect growth and activity of methanotrophs.

At present, molecular approaches is widely used to assess the diversity and activity of methanotrophs, which used phylogenetic and functional gene probes to detect and analyze methanotrophs from samples (Murrell et al., 1998). In addition to the 16S rRNA gene, the presence and abundance of CH_4 oxidizers were also shown by functional genes of methanotrophs (Fjellbirkeland et al., 2001; Horz et al., 2001). For discriminating the methanotrophs, in earlier studies, different polymerase chain reaction (PCR) primers were designed to amplify 16S rRNA gene fragments of different groups CH_4 oxidizers (Henckel et al., 1999). High-throughput sequencing of the 16S rRNA gene was used to determine that the composition and diversity of microbial in response to soil tillage, different crop rotation and fertilizer applications. Soil methanotrophs community structure was changed after long-term fertilizer applications (Zheng et al., 2008). The relative data was used to measure abundance of operational taxonomic units (OTUs) and to calculate indices of richness and shannon of soil samples.

Our studies indicated that long-term fertilization managements could lead to significant changes in diversity of some soil microbe, enzyme activities, such as aerobic bacterial, actinomycete, fungus and β-glucosidase (Tang et al., 2014). Soil methanotrophs play an important role in carbon cycling in terrestrial ecosystems. The fertilizer application is the important factor that affects growth and activity of methanotrophs. A viable option was applied with manure and crop residue to   changing CH_4 emissions, methanotrophs abundance and community composition in paddy field? Therefore, the objectives of this research were to: (1) CH_4 emissions from paddy fields respond to the long-term fertilization managements, and (2) long-term fertilizer managements lead to changes in methanotrophs abundance and community composition in paddy field. Therefore, the gas and soil samples were collected from a long-term fertilization experimental field and the CH_4 emissions from paddy fields, the methanotrophs abundance and community composition were studied using static chamber-gas chromatography technique, real-time PCR and Illumina high-throughput sequencing based on both 16S rRNA gene, respectively.

MATERIALS AND METHODS

Sites and cropping system

The experiment was established in 1986. It was located in Ning Xiang County (28°07' N, 112°18' E, and altitude 36 m) of Hunan Province, China. Under a continent monsoon climate, the annual mean precipitation was 1553 mm and potential evapotranspiration of 1354 mm. The monthly mean temperature was 17.2°C. Soil texture of the plough layer (0 to 20 cm) was silt clay loam with 13.71% sand and 57.73% silt. At the beginning of the study, the surface soil characteristics (0 to 20 cm) were as follows: soil organic carbon (SOC) 29.4 g kg^{-1}, total nitrogen 2.0 g kg^{-1}, available N 144.1 mg kg^{-1}, total phosphorous 0.59 g kg^{-1}, available P 12.87 mg kg^{-1}, total potassium 20.6 g kg^{-1}, and available potassium 33.0 mg kg^{-1}. There were three crops in a year, barley (*Hordeum vulgare* L.), early rice, and late rice (*Oryza sativa* L.). Barley was sown in the middle of November and harvested in early May of the following year. Early rice was then transplanted and harvested in the middle of July. The growth period of late rice lasted from late July to the end of October.

Experiment design

The experiment had five treatments: control (without fertilizer input, CK), mineral fertilizer (MF), rice residue and mineral fertilizer (RF), low manure rate and mineral fertilizer (LOM), and high manure rate and mineral fertilizer (HOM). The design ensured all fertilized treatments received equal N amount (the amount of N in mineral fertilizer plus that from rice residue or manure). The mineral fertilizers included urea, ordinary superphosphate, and potassium chloride. Details about the fertilizer management are listed in Table 1. Before rice transplanting seedling, manure and air-dried rice residue were incorporated into soil surface. The cultivation depth was about 20 cm. For early rice, and late rice, 70 and 60% of N was applied at seeding, and the remaining N was applied at top dressing stages. All the P_2O_5 and K_2O were applied at seeding stages. There were three replications and each plot size was 66.7 m^2. We referred to the data for the individual cropping periods from May to October, 2014.

1) For the RF treatment, rice straw return rate (air dry) was 2780 and 3600 kg ha^{-1} for early and late rice. (2) For the LOM treatment, manure application rate (decomposed) was 2625.0 and   2670.0 kg

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Table 1. Nutrient supply from rice straw, chicken manure and mineral fertilizer under different fertilizer treatments (kg ha^{-1}).

Treatments	Early rice			Late rice			Total		
	N	P_2O_5	K_2O	N	P_2O_5	K_2O	N	P_2O_5	K_2O
CK	0+0[*]	0+0	0+0	0+0	0+0	0+0	0	0	0
MF	142.5+0	54.0+0	63.0+0	157.5+0	43.2+0	81.0+0	300.0	97.2	144.0
RF	124.4+18.1	50.4+3.6	38.3+24.7	133.0+24.5	37.8+5.4	48.2+32.8	300.0	97.2	144.0
LOM	96.0+46.5	33.0+21.0	33.6+29.4	110.2+47.3	21.8+21.4	51.1+29.9	300.0	97.2	144.0
HOM	49.6+92.9	12.0+42.0	4.2+58.8	63.0+94.5	0.5+42.7	21.2+59.8	300.0	97.2	144.0

*Input from mineral fertilizer + input from organic fertilizer. The treatments are without fertilizer (CK), mineral fertilizer (MF), crop residue and mineral fertilizer (RF), low manure rate and mineral fertilizer (LOM), and high manure rate and mineral fertilizer (HOM).

ha[-1] for early and late rice. (3) For the HOM treatment, manure application rate (decomposed) was 5250.0 and 5340.0 kg ha^{-1} for early and late rice. (4) The N, P_2O_5, and K_2O content of air-dry early rice straw was 0.65, 0.13, and 0.89%; N, P_2O_5, and K_2O content of air-dry late rice straw was 0.68, 0.15, and 0.91%, respectively; and N, P_2O_5, and K_2O content of decomposed chicken manure was 1.77, 0.80, and 1.12%, respectively.

Sample collection

Samples were collected in 2014. In each plot, soil samples in the rhizosphere soil were collected from the rice plants root at different rice growth stages. Three samples were collected from each plot. The samples were immediately frozen until further analyses could be performed.

CH_4 emissions from paddy fields were investigated using the static chamber-GC technique at 9:00 to 11:00 in the morning during rice growth period. From the second day after transplanting of early or late rice, gases sample were collected weekly.

Measurement of CH_4

The quantities of CH_4 emission were survey with a gas chromatograph (Agilent 7890A) equipped with flame ionization detector (FID). CH_4 was separated using 2 m stainless-steel column with an inner diameter of 2 mm 13×MS column (60/80 mesh), with FID at 200°C.

CH_4 fluxes were calculated with the following equation (Liebig et al., 2010):

$$F = \rho h \left[273 / (273+T) \right] dC / dt$$

where F is the CH_4 emission (mg m^{-2} h^{-1}); T is the air temperature (°C) inside the chamber; ρ is the CH_4 density at standard state (0.714 kg m^{-3} for CH_4); h is the headspace height of the chamber (m); and dC/dt is the slope of the curve of gas concentration variation with time.

The total CH_4 emissions (g $CH_4 \cdot$m^{-2}) were sequentially computed from the emissions between every 2 adjacent intervals of the measurements, based on a non-linear, least-squares method of analysis (Singh et al., 1996).

DNA Extraction and PCR

For each sample, DNA was isolated from 0.5 g of soil using the MoBio PowerSoil™ DNA Isolation Kit (Carlsbad, CA, USA). Extractions were executed according to the manufacturer's protocol. All genomic DNA concentration and purity were determined by NanoDrop spectrophotometry (Thermo Scientific, Wilmington, DE, USA). PCR was performed at an initial denaturation temperature of 94°C for 3 min, followed by 20 cycles of 94°C for 45 s, 53°C for 30 s, and 65°C for 90 s. A final elongation step at 65°C was run for 10 min. PCR products were purified using the Qiagen™ PCR purification kit following the manufacturer's protocol with the exception of eluting in sterile water (Qiagen, Valencia, CA, USA) and quantified in Qubit 2.0 Fluorometer (Invitrogen, NY, USA).

Illumina high-throughput sequencing of 16S rRNA genes

Primers 515F and 806R (Caporaso et al., 2010) were used to target the V3–V4 region of the 16S rRNA gene with the addition of a barcoded sequence and the required Illumina adapters. Sequencing was performed on an Illumina (Illumina, Miseq–OE Biotech Company; Shanghai, China) with two paired-end read cycles of 300 bases each. Sequence analysis and OTUs identification was according to the methods of Giongo et al. (2010) and Fagen et al. (2012). Reads were trimmed to remove low quality bases and to remove the first 11 bases corresponding to the primer region by a script based on Trim2 (Huang et al., 2003), and then the reads were separated by barcode. Paired reads were assembled using FLASH to the reference greengenes (Reyon et al., 2012) 16S SSU rRNA database.

IFA analysis of methanotrophs bacteria

The methanotrophs genera were selected using the indirect immunofluorescence (IFA) method (Svetlana et al., 2007). Eight polyclonal antibodies specific for 8 methanotrophs genera, namely, *Methylosinus*, *Crenothrix*, *Methylobacter*, *Methylocaldum*, *Methylococcus*, *Methylomicrobium*, *Methylomonas*, and *Methylosarcina* were applied. The cross-reactivity and specificity of the antibodies were tested previously and found to be species-specific (Svetlana et al., 2007). The total number of methanotrophs was calculated as the sum of 8 pre-selected methanotrophs genera.

Diversity indices

To estimate bacterial diversity of each sample, the number of OTUs, richness and shannon index were calculated using Mothur (Shannon, 1963). The phylogenetic distribution of OTUs was constructed by QIIME software.

Data analysis

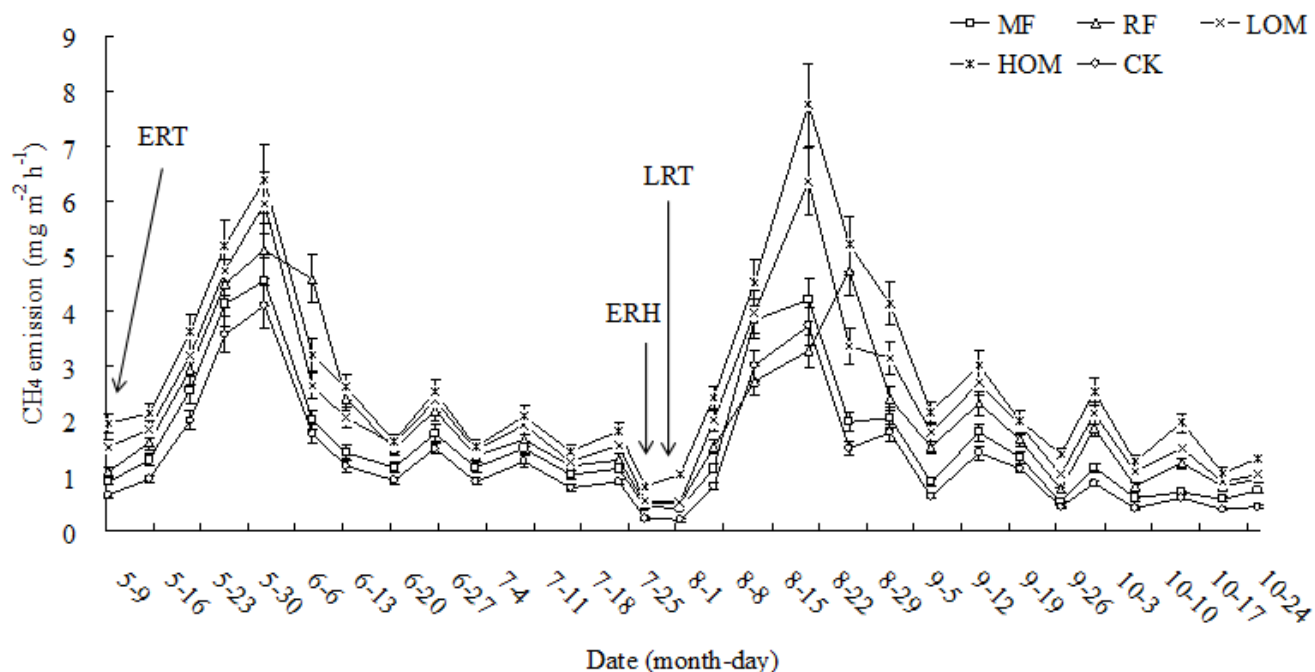The data were analyzed as a randomized complete block, using the

Figure 1. CH$_4$ emission from paddy fields affected by long–term fertilizer managements during early and late rice growth period. MF: Mineral fertilizer; RF: crop residue and mineral fertilizer; LOM: low manure rate and mineral fertilizer; HOM: high manure rate and mineral fertilizer; CK: without fertilizer. ERT: early rice transplanting; ERH: early rice harvesting; LRT: late rice transplanting. CH$_4$ emission rate is the mean of values measured within each treatment (n=3). Bars indicate standard deviation.

PROC ANOVA procedure of SAS (SAS Institute, 2003). Mean values were compared using the least significant difference (LSD) test and a probability value of 0.05 was considered to indicate statistical significance.

RESULTS

CH$_4$ emission

During early rice growing season, the CH$_4$ emission was low after early rice transplanting, but increased quickly until the peak appeared at 23 days after transplanting, and then declined to a low and stable level (Figure 1). The CH$_4$ emission were significantly different among treatments with the sequence of HOM>LOM>RF>MF>CK ($P<0.05$) during early rice growing season (Figure 1).

During late rice growing season, CH$_4$ emission mainly focused at tillering stage and the peak value of CH$_4$ emission was observed at 24 days after transplanting. Then, the emission rate decreased to a low level with relative stability. The sequence of treatments in CH$_4$ emission was HOM>LOM>RF>MF>CK (Figure 1).

During early rice growing season, the accumulate CH$_4$ emission of CK was significantly lower than MF, RF, LOM, and HOM ($P<0.05$) and the sequence with different treatments was HOM>LOM>RF>MF>CK (Table 2). The total CH$_4$ emissions from paddy fields during late rice growing season were 3.212 g m^{-2} in MF, 3.961 g m^{-2} in RF, 4.881 g m^{-2} in LOM, 6.099 g m^{-2} in HOM, and 2.548 g m^{-2}

in CK. The order of treatments in total CH$_4$ emission was HOM>LOM>RF>MF>CK (Table 2). Meanwhile, HOM had larger total CH$_4$ emissions than other treatments during early and late rice growing season.

Operational taxonomic units of methanotrophs bacteria

At the early rice main growth stages, the root methanotrophs bacteria of rice plants from the LOM treatment had the highest number of OTUs (Figure 2). And the highest values of the OTUs in LOM with 76 at seedling stage (SS), the highest values of the OTUs in MF, RF, CK with 23, 41, and 47 at tillering stage (TS) among the different treatments. At the early rice main growth stages, the OTUs values were significantly different among treatments with the sequence of LOW>CK>RF>HOM>MF (Figure 2). At the late rice main growth stages, the highest values of the OTUs at SS in LOM and HOM with 53 and 40, and then dramatically declined to a low level. And the highest values of the OTUs were observed at TS in MF and RF with 39 and 62 after transplanting. Then, the OTUs values dramatically decreased to a low level.

Genetic diversity indices of methanotrophs

At early and late rice main growth stages, the root

Table 2. Effects of long–term fertilizer managements on CH_4 emission from paddy fields during whole growth period of early and late rice (g $CH_4 \cdot m^{-2}$).

Treatments	Early rice	Late rice	Total
MF	3.470±0.147[c]	3.212±0.176[d]	6.682±0.323[d]
RF	4.418±0.131[b]	3.961±0.141[c]	8.379±0.271[c]
LOM	4.521±0.128[b]	4.881±0.114[b]	9.402±0.242[b]
HOM	5.074±0.100[a]	6.099±0.093[a]	11.173±0.193[a]
CK	2.886±0.083[d]	2.548±0.074[e]	5.434±0.157[e]

MF: Mineral fertilizer; RF: crop residue and mineral fertilizer; LOM: low manure rate and mineral fertilizer; HOM: high manure rate and mineral fertilizer; CK: without fertilizer. Values are presented as mean ± SE (n = 3). Means in each column with different letters are significantly different at the $P < 0.05$ level.
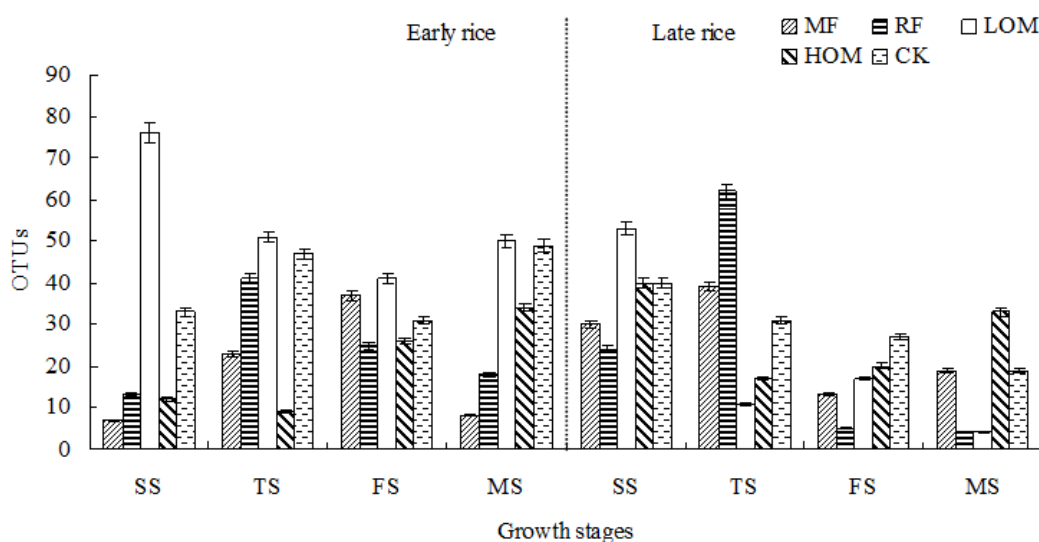

Figure 2. Operational taxonomic units of methanotrophs bacteria with different fertilizer treatments at rice main growth stages. SS: Seedling stage; TS: tillering stage; FS: full heading stage; MS: maturity stage. MF: mineral fertilizer; RF: crop residue and mineral fertilizer; LOM: low manure rate and mineral fertilizer; HOM: high manure rate and mineral fertilizer; CK: without fertilizer. Values are presented as mean ± SE (n = 3).

methanotrophs of rice plants from the paddy fields had the highest values of the richness indexes at TS and SS, respectively, and had the lowest values of the richness indexes at maturity stage (MS). Meanwhile, at early and late rice main growth stages, the values of the richness indexes were higher in HOM than in MF, RF, LOM and CK. The order of treatments in the values of the richness indexes was HOM>LOM>RF>CK>MF (Table 3).

At early and late rice main growth stages, the root methanotrophs of rice plants from the paddy fields had the highest values of the shannon indexes at TS and SS, respectively and had the lowest values of the shannon indexes at MS. Meanwhile, at the early and late rice main growth stages, the values of the shannon indexes were higher in HOM and LOM than in MF, RF and CK. The order of treatments in the values of the shannon indexes was HOM>LOM>RF>CK>MF (Table 3).

Structure of the methanotrophs bacteria community

It was indicated that some methanotrophs genera (*Methylosinus, Crenothrix, Methylobacter, Methylocaldum, Methylococcus, Methylomicrobium, Methylomonas* and *Methylosarcina*) were identified at main growth stages of early rice and late rice. It was shown that some methanotrophs genera were detected at some growth stages of early rice (Table 4). *Methylosinus, Crenothrix, Methylocaldum, Methylomicrobium* and *Methylomonas* were detected more often in all treatments at early rice main growth stages. *Methylobacter* were detected more often in LOM at the SS and MS. *Methylococcus* were detected more often in CK at main growth stages of early rice. *Methylosarcina* were detected more often in HOM and CK at early rice main growth stages.

Methylosinus, Crenothrix, Methylomicrobium,

Table 3. Genetic diversity indices of methanotrophs with different fertilizer treatments during rice main growth stages.

Rice	Treatment	Items							
		Richness index				Shannon index			
		SS	TS	FS	MS	SS	TS	FS	MS
Early rice	MF	22.4±0.65[b]	25.4±0.73[b]	19.7±0.57[bc]	16.8±0.49[b]	2.75±0.08[c]	3.17±0.09[b]	2.68±0.08[b]	2.24±0.06[b]
	RF	23.8±0.69[ab]	26.8±0.77[ab]	21.5±0.62[ab]	17.8±0.51[ab]	2.97±0.09[ab]	3.45±0.10[ab]	2.84±0.08[ab]	2.42±0.07[ab]
	LOM	24.3±0.70[ab]	27.2±0.79[ab]	21.9±0.63[ab]	18.2±0.53[ab]	3.06±0.09[a]	3.57±0.10[a]	2.92±0.08[ab]	2.51±0.07[ab]
	HOM	24.7±0.71[a]	28.3±0.82[a]	22.3±0.64[a]	18.5±0.53[a]	3.18±0.09[a]	3.63±0.10[a]	3.07±0.09[a]	2.63±0.08[a]
	CK	23.0±0.66[ab]	26.0±0.75[ab]	20.3±0.59[c]	17.4±0.50[ab]	2.85±0.08[bc]	3.34±0.10[ab]	2.75±0.08[b]	2.38±0.07[b]
Late rice	MF	23.4±0.68[a]	20.8±0.60[b]	18.7±0.54[b]	15.1±0.44[b]	2.82±0.08[c]	2.73±0.08[c]	2.67±0.08[c]	2.21±0.06[c]
	RF	24.7±0.71[a]	22.3±0.64[ab]	19.7±0.57[ab]	15.8±0.46[ab]	3.02±0.09[ab]	2.97±0.09[ab]	2.95±0.09[ab]	2.47±0.07[ab]
	LOM	25.2±0.73[a]	22.8±0.66[ab]	20.4±0.59[ab]	16.2±0.47[ab]	3.14±0.09[a]	3.04±0.09[a]	3.02±0.09[ab]	2.58±0.07[a]
	HOM	25.6±0.74[a]	23.4±0.68[a]	20.8±0.60[a]	16.6±0.48[a]	3.26±0.09[a]	3.18±0.09[a]	3.14±0.09[a]	2.66±0.08[a]
	CK	24.2±0.70[a]	21.5±0.62[ab]	19.4±0.56[ab]	15.6±0.45[ab]	2.95±0.09[bc]	2.86±0.08[bc]	2.84±0.08[bc]	2.35±0.07[bc]

SS: Seedling stage; TS: tillering stage; FS: full heading stage; MS: maturity stage. MF: mineral fertilizer; RF: crop residue and mineral fertilizer; LOM: low manure rate and mineral fertilizer; HOM: high manure rate and mineral fertilizer; CK: without fertilizer. Values are presented as mean ± SE (n = 3). Means in each column with different letters are significantly different at the $P < 0.05$ level.

Methylomonas and *Methylosarcina* were detected more often in all treatments at late rice main growth stages. *Methylocaldum* were detected more often in MF, RF, HOM and CK at main growth stages of late rice. *Methylococcus* were not detected in LOM and HOM at late rice main growth stages (Table 5).

Heatmap in the different fertilizer treatments during rice growth period

The 8 most abundant genera for five treatments at early rice main growth stages were analyzed and the differences were shown in the heatmap (Figure 3). The abundances of *Methylomonas* and *Crenothrix* were significantly higher in different treatments at main growth stages of early rice. *Methylosinus* were relatively more abundant in different treatments at SS. *Methylocaldum* were relatively more abundant in different treatments at SS and MS. On the other hand, *Methylobacter* and *Methylococcus* were not detected in different treatments at main growth stages of early rice. *Methylomicrobium* and *Methylosarcina* were not detected in different treatments at full heading stage (FS).

At the late rice main growth stages, the relative abundances of *Methylomonas* were significantly higher in different treatments. *Methylosinus* were relatively more abundant in different treatments at SS. *Methylomicrobium* were relatively more abundant in different treatments at FS and MS. *Methylosarcina* were relatively more abundant in different treatments at MS. On the other hand, *Methylococcus* and *Methylocaldum* were not detected in different treatments at main growth stages of late rice. *Methylomicrobium* were less abundant in different treatments at SS and TS. *Crenothrix* were less abundant

in different treatments at MS (Figure 4).

Phylogenetic tree analysis of methanotrophs gene clones among different fertilizer treatments

At the early rice main growth stages, clustering analysis allowed the identifcation of OTUs responsible for the community shifts in different fertilization at genera level. The results showed that OTUs *Methylomonas* and *Crenothrix* were especially abundant in the soil samples. When phylogenetic trees were constructed using abundance belonging to each OTUs, *Methylocaldum* and *Methylosarcina* were grouped into a tight and distinct cluster, *Methylomonas* and *Crenothrix* were also grouped into a tight and distinct cluster. One-third of the clones in OTUs were grouped into a cluster including *Methylocaldum* (Figure 5).

At the late rice main growth stages, the results showed that OTUs *Methylomonas* and *Methylocaldum* were especially abundant in the soil samples. When phylogenetic trees were constructed using abundance belonging to each OTUs, *Crenothrix* and *Methylocaldum* were grouped into a tight and distinct cluster, *Methylomonas* and *Methylosarcina* were also grouped into a tight and distinct cluster. Quarter of the clones in OTUs was grouped into a cluster including *Methylocaldum* (Figure 5).

DISCUSSION

Effects of fertilizer applications on CH_4 emission

CH_4 emission is complex processes including production

Table 4. Genera of selected methanotrophs in different treatments at main growth stages of early rice.

Stage	Treatment	Methanotrophs							
		Methylosinus	*Crenothrix*	*Methylobacter*	*Methylocaldum*	*Methylococcus*	*Methylomicrobium*	*Methylomonas*	*Methylosarcina*
SS	MF	+	+	-	+	-	-	+	+
	RF	+	+	-	+	-	+	+	-
	LOM	+	+	+	+	-	+	+	-
	HOM	+	-	-	+	-	-	-	+
	CK	+	+	-	+	+	+	+	+
TS	MF	+	+	-	+	+	-	+	+
	RF	+	+	-	+	-	+	+	+
	LOM	+	+	-	+	-	+	+	+
	HOM	-	-	-	+	-	+	+	+
	CK	+	+	-	+	+	+	+	+
FS	MF	+	+	-	+	+	+	+	-
	RF	+	+	-	+	+	+	+	-
	LOM	+	+	-	-	-	+	+	-
	HOM	+	+	-	+	+	+	+	+
	CK	+	+	-	+	+	+	+	+
MS	MF	+	-	+	+	-	+	-	+
	RF	+	+	-	+	-	+	+	-
	LOM	+	+	+	+	+	+	+	+
	HOM	+	+	-	-	-	+	+	+
	CK	+	+	-	+	+	+	+	+

+ is present, it exist; - is absent, did not exist.

Table 5. Genera of selected methanotrophs in different treatments at main growth stages of late rice.

Stage	Treatment	Methanotrophs						
		Methylosinus	*Crenothrix*	*Methylocaldum*	*Methylococcus*	*Methylomicrobium*	*Methylomonas*	*Methylosarcina*
SS	MF	+	-	+	+	+	+	+
	RF	+	+	+	+	+	+	+
	LOM	+	+	-	-	+	+	+
	HOM	+	+	+	-	+	+	+
	CK	+	+	+	+	+	+	+

Table 5. Contd.

Stage	Treatment	Methanotrophs						
		Methylosinus	*Crenothrix*	*Methylocaldum*	*Methylococcus*	*Methylomicrobium*	*Methylomonas*	*Methylosarcina*
TS	RF	+	+	+	-	+	+	+
	LOM	+	+	-	-	+	+	-
	HOM	+	+	+	-	+	-	+
	CK	+	+	+	-	+	+	+
FS	MF	+	+	-	-	+	+	-
	RF	-	+	-	+	+	-	+
	LOM	+	+	+	-	+	+	+
	HOM	+	+	+	-	+	+	+
	CK	+	+	+	+	+	+	+
MS	MF	-	+	+	-	+	+	+
	RF	+	-	-	-	+	-	+
	LOM	+	-	-	-	-	+	+
	HOM	+	+	+	-	+	+	+
	CK	-	+	+	+	+	+	+

+ is present, it exist; - is absent, did not exist.

and oxidation. CH_4 production was regulated by vegetation type, fertilizer managements, soil temperature, soil moisture, root activity and many other factors (Wassmann et al., 2004; Kallenbach et al., 2010; Ma et al., 2008). In this study, the total CH_4 emission from paddy fields during rice growth period were much higher in HOM, LOM and RF as compared to CK (Figure 1 and Table 2), which was similar to the result by Wang et al. (2013). The reasons was (1) microbial activities were improved after returning rice residues, manure into the soil for that supplements of carbon source and energy for microbial activities to accelerate consumption of soil oxygen and decrease of soil redox potential (Eh); (2) methanogens became active for the large quantities of carbon source, which provided reactive substrate for CH_4 emission from paddy fields. Meanwhile, compared with the RF, LOM

and HOM, we also observed that MF decreased the CH_4 emission and resulted in a positive CH_4 emission from paddy fields. CH_4 emission was probably reduced because of excessive soil inorganic N level. Reduced root growth due to lower soil inorganic N level as a result of absence of N fertilization also probably reduced the methanotrophs activities, thereby resulting in lower CH_4 emission in MF. Several researchers (Bronson and Mosier, 1994; Powlson et al., 1997) have reported that N fertilization reduced CH_4 emission compared to no N fertilization. However, CH_4 emissions at early and late rice growth periods were decreased to a large extent after field drying, the reason may be (1) the methanogens activities were limited when the soil aeration was increased after field drying; and (2) the ability for transportation and emission of CH_4 was limiting,

which the rice plant physiological activities was decreased.

Effects of fertilizer applications on soil methanotrophs diversity

Organic and crop residues fertilization resulted in increasing methanotrophs bacteria diversity (Figure 2), showing that application of organic, crop residues significantly influenced methanotrophs bacteria composition, and mineral fertilization influenced methanotrophs bacteria composition to a lesser degree.

The response of methanotrophs bacteria community structures to agricultural managements was also represented by Nicol et al. (2003). In this study, it was shown that higher number of OTUs
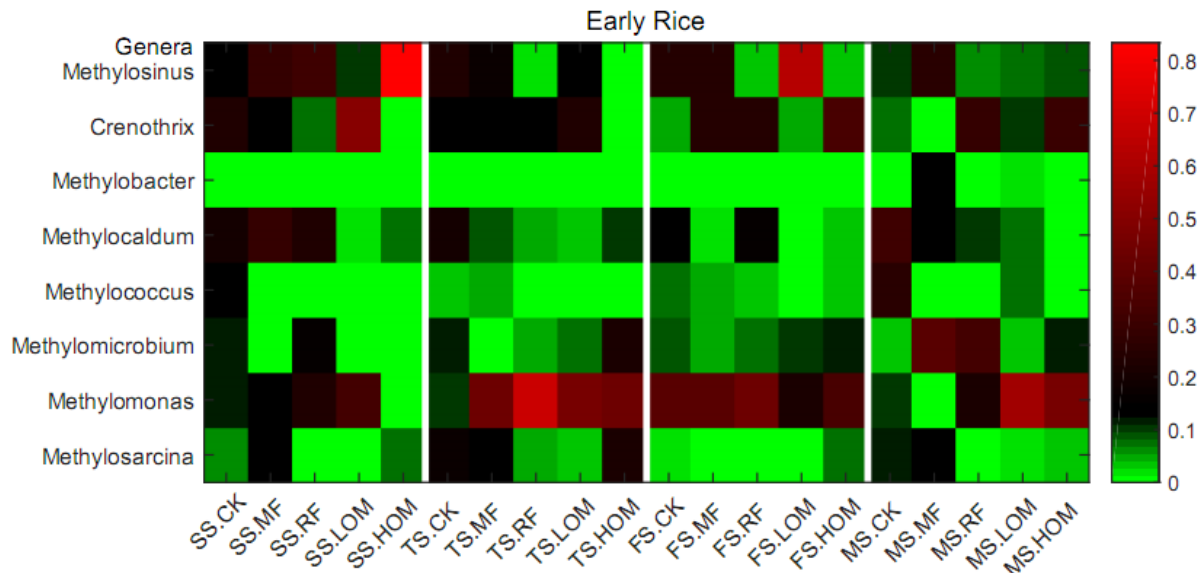
Figure 3. Heatmap illustrating the 8 most abundant methanotrophs genera in the different fertilizer treatments at early rice main growth stages. SS: seedling stage; TS: tillering stage; FS: full heading stage; MS: maturity stage. MF: mineral fertilizer; RF: crop residue and mineral fertilizer; LOM: low manure rate and mineral fertilizer; HOM: high manure rate and mineral fertilizer; CK: without fertilizer.
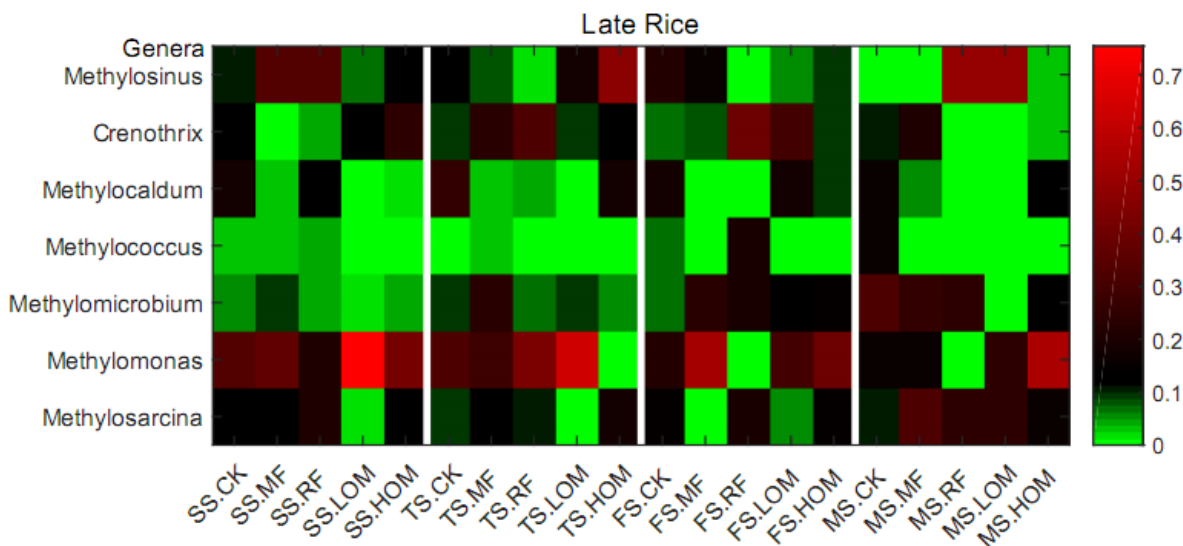

Figure 4. Heatmap illustrating the 7 most abundant methanotrophs genera in the different fertilizer treatments at late rice main growth stages. SS: seedling stage; TS: tillering stage; FS: full heading stage; MS: maturity stage. MF: mineral fertilizer; RF: crop residue and mineral fertilizer; LOM: low manure rate and mineral fertilizer; HOM: high manure rate and mineral fertilizer; CK: without fertilizer.

with organic, crop residues, than that of the mineral fertilizer. The relationship of methanotrophs bacteria and organic, crop residues, and mineral fertilizer in the experiment soil, proposes that organic crop residues is an important nutrient for the found taxa, which is relative to methanotrophs bacteria growth processes.

The methanotrophs microbial diversity was shown by the richness index and shannon index which was larger in HOM, LOM, and RF treatments, which agree with Zheng et al. (2008), who found that soils under NPK and recycled crop residues had higher levels of methanotrophs diversity compared with the conventional fertilization. Meanwhile, shannon index and richness index was significantly higher when application with organic and crop
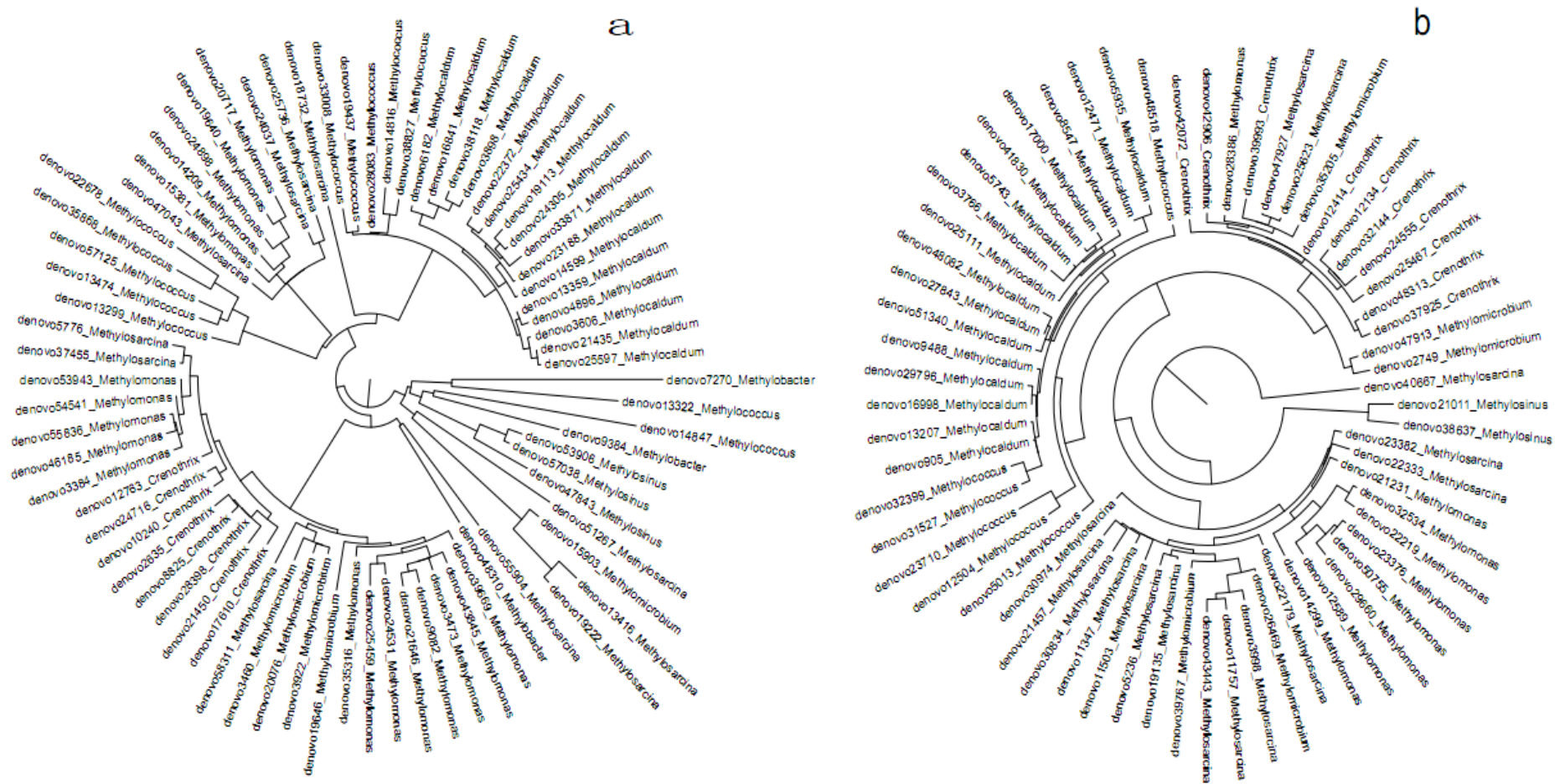
Figure 5. Phylogenetic distribution of OTUs of the clone libraries of 16S ribosomal RNA genes for soil samples under different treatments at early rice (a) and late rice (b) main growth stages.

residues, indicating that the application of organic and crop residues based on application of NPK, with higher rate of C:N, stimulates methanotrophs diversity on the soil. That is, rate of C:N in rice and organic straw substrates is higher than that of other mineral fertilizer (Garcia and Rice, 1994), which provides more substrate to microbial explaining higher shannon index and richness index found in this work on plots where rice was the growing season. Meanwhile, it was key factors that root exudates influenced methanotrophs communities, for that they provide the carbon source to soil microbial (Badri and Vivanco, 2009). Some studies indicated that roots may regulate the soil methanotrophs community in their rhizosphere, change the soil physical and chemical properties, and control the growth of competing plant species (Nardi et al., 2000), which may also affect the

methanotrophs diversity in rhizosphere.

Effects of fertilizer applications on soil methanotrophs community

According to the effects of different fertilizer treatments on methanotrophs abundance, five treatments (MF, RF, LOM, HOM and CK) were chosen to analyze the methanotrophs community structure. Some distinct differences in the diversity indices were found between the mineral fertilizer and application of organic; crop residues fertilizer treatments indicated that the soil methanotrophs community was changed with different fertilization managements. And the diversity pattern was shown in HOM and LOM treatments. In addition to the chemical NPK, the recycled application of crop residues and organic proposal were important to maintain methanogenic bacteria in paddy soils (Conrad and Klose, 2006). It is well-known that the soil CH_4 oxidation rate can be changed by different CH_4 concentrations, thus the growth, activities and community structure of methanotrophs were changed (Bender and Conrad, 1995). In conclusion, according to sequences and phylogenetic analysis, it was shown that soil methanotrophs community structure was changed by different fertilizer treatments.

Some study indicated that the genera of methanotrophs were different between paddy soils and forest soils (Mohanty et al., 2006). In this study, the 8 most abundant genera for five treatments at early rice main growth stages were shown in the heatmap (Figure 3), and the 7 most abundant genera for five treatments at late rice main growth stages were showed in the heatmap (Figure 4), indicated that composition significantly varied among the five estimated treatments (MF, RF, LOM, HOM and CK). Our results showed that methanotrophs were related to the genera of *Methylosinus, Crenothrix, Methylocaldum, Methylomicrobium* and *Methylomonas* with the most usual methane oxidizers in paddy soil with different fertilizer treatments, which were in agreement with an earlier study (Fjellbirkeland et al., 2001).

Conclusions

The results indicated that with the same nitrogen application rate, different organic-inorganic mixed fertilizer application, such as RF, LOM, and HOM, caused substantial CH_4 emissions during early and late rice growth period compared with those from the conventional MF treatment. Meanwhile, the abundance and composition of methanotrophs was affected by long-term fertilization managements. In the MF treatment, methanotrophs abundance was inhibited, compared to the RF, LOM and HOM treatments. The methanotrophs diversity of the HOM and LOM treatments was distinguished from RF, MF and CK. Furthermore, methanotrophs community composition was changed with

different treatments based on the sequences and phylogenetic analysis. And the higher ratio genera of *Methylosinus, Crenothrix, Methylomicrobium, Methylocaldum* and *Methylomonas* were found in the five treatments. In summary, the application of mineral fertilizer was an important factor that affected the abundance of methanotrophs and application of organic; crop residues enhance the abundance of methanotrophs in double-cropping paddy fields in Southern China. It is important to understand that the main effective factors for abundance and composition of methanotrophs, was linked to soil ecosystem processes and sustainable developing management of rice cultivation.

Conflict of Interests

The authors have not declared any conflict of interest.

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