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Diversity and Composition of Methanotrophs in Chinese Paddy Soil as Affected by Different Long–term Fertilizer Managements

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Methane–oxidizing bacteria (methanotrophs) biologically consume and consequently affect the concentration of atmospheric methane (CH_4), the second most prominent greenhouse gas, and therefore play critical roles in the mitigation of global warming effect. Long–term fertilization often affects the methanotrophs community and CH_4 oxidation in various soils. The objective of this study was to characterize for a Chinese paddy soil the changes in CH_4 emissions and the diversity and composition of methanotrophs, that resulted from mineral fertilizer, crop residue, and manure applications. In this long-term (28 years) field study, use was made of static chambers and gas chromatography, and Illumina high-throughput sequencing of the 165rRNA gene, to analyse the CH_4 emissions from a paddy field soil, and the soil methanotroph abundance and community diversity under five fertilizer treatments. These treatments were: mineral fertilizer alone (MF), rice residue plus mineral fertilizer (RF), low manure rate plus mineral fertilizer (LOM), and high manure rate plus mineral fertilizer (HOM), compared to without fertilizer input (CK). The results indicated that CH_4 from fertilization treatments displayed different emission patterns during the barley growing season. The HOM plot had the highest CH_4 emissions (5.074 g m^{-2}) and global warming potentials (GWPs) (1614.77 kg CO_2–eq ha^{-1}), during the barley growing season. It was clear that some methanotrophs species (*Methylosinus*, *Methylomicrobium*, *Methylomonas* and *Methylosarcina*) were identified in the main growth stages of barley. Long–term fertilization managements can both affect the abundance and the composition of the soil methanotrophs. Methanotroph abundance was inhibited by the MF treatment, but enhanced by the RF, LOM and HOM treatments. The mineral fertilizers, crop residues, and manure could be important factors controlling the abundance and community composition of the methanotrophs in the paddy soil. We concluded that the application of organic (chicken manure), and the crop residues enhance the abundance and community composition of methanotrophs in double–cropping paddy fields in southern China through a long–term fertilizer experiment.

Key words: CH_4; Long–term fertilization; Methanotrophs diversity; Methanotrophs composition; Paddy field.

Greenhouse gas (GHG) emissions from paddy soils and the impacts on global climate change have been increasingly concerned. Methane (CH_4) and nitrous oxide (N_2O) are two greenhouse gases with a long–term global warming potential 25 and 310 times that of carbon dioxide (CO_2), respectively. CH_4 is the second most abundant carbon containing gas in the atmosphere and contributes approximately 18% to the global warming [1]. Food crops cultivation is one of the major sources of methane, which annually emits 60 Tg CH_4 into the atmosphere [2]. Barley and rice are the most important food crops to feed the growing population, especially in Asia[3, 4]. In order to maximize barley and rice grain yields, barley and rice cultivation needs to be intensified by strengthening and improving the agronomic practices, such as fertilizer applications [5]. However, the use of fertilizers in agricultural systems often negatively affects the potential of soils to act as a

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CH_4 sink, which leads to elevated CH_4 concentrations in the atmosphere [6].

Before releasing into the atmosphere, the produced CH_4 is subject to oxidation by methane–oxidizing bacteria (methanotrophs) in the surface soil layer and the rhizosphere. Methanotrophs are a group of CH_4–oxidizing bacteria that use CH_4 as sources of carbon and energy. The oxidation of CH_4 by methanotrophs in the soil provides a major sink for CH_4 in the atmosphere [7, 8]. Methanotrophs are obligate aerobes and classified into two main groups (type I and type II) differing in phylogeny, physiology, morphology, and biochemistry [7]. 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. The growth and activity of methanotrophs may be affected by a number of factors, including soil conditions, fertilizer applications, and type of vegetation cover[7, 9, 10].

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 [11]. In addition to the 16S rRNA gene, functional genes of methanotrophs were also used to detect the presence and abundance of the methane oxidizers[12, 13]. For discriminating the methanotrophs, different polymerase chain reaction (PCR) primers were designed to amplify 16S rRNA gene fragments of these different groups methane oxidizers in earlier studies [14, 15]. High–throughput sequencing of the 16S rRNA gene was used to determine how microbial diversity and composition changes in response to tillage, crop rotation, and fertilizer applications. The sequencing data was used to measure the relative abundance of operational taxonomic units (OTUs) and to calculate the richness index and shannon index of soil samples. Soil methanotrophs community structure could be altered after a long–term fertilizer applications [9].

Therefore, the objectives of this research were: (1) to quantify CH_4 emissions from a paddy field and barley grain yield in relation to long–term application of crop residue, manure and mineral fertilizer in the barley growing season, (2) long–term fertilizer applications result in changes in methanotrophs abundance and community composition in a paddy field. We thus collected paddy field gas and soil samples from a long–term fertilization experimental site, and the CH_4 emissions from paddy field, the abundance and community composition of the soil methanotrophs were investigated 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 conducted 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!. Soil texture of the plough layer (0–20 cm) was silt clay loam with 13.7% sand and 57.7% silt. At the beginning of the study (1986), the surface soil characteristics (0–20 cm) were as follows: soil organic carbon (SOC) 29.4 g kg^{-1}, total nitrogen (N) 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. 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 growing season 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 only (MF), rice residue plus mineral fertilizer (RF), low manure rate plus mineral fertilizer (LOM), and high manure rate plus mineral fertilizer (HOM). The application of mineral fertilizer and manure is the common agricultural practice in Hunan Province. The design ensured all fertilized treatments received equal N rate (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 barley sowing, manure and air–dried rice residue was spread onto soil surface manually and was incorporated into soil. The cultivation depth was about 20 cm. For barley, 30% of mineral N fertilizer was applied at seeding, and the remaining N fertilizer was applied by top dressing in the growth periods. All the phosphorus and potassium fertilizers were applied at seeding. The seeding rate application was 250.0 kg hm^{-2} with each treatment. There were three replications and each plot size was 66.7 m^2, the different fertilizer treatments were carried out in a randomized plot design. We referred to the data for the individual cropping periods as 2013–2014.

Sample collection

Samples were collected in 2013–2014. In each plot, the soil samples in the rhizosphere soil were collected from the barley plants root at different barley growth periods. Three subsamples were collected from each plot. The samples were immediately frozen until further analyzes could be performed.

CH_4 emitted from paddy field were collected using the static chamber–GC technique at 9:00–11:00 in the morning during the barley growing season. From the second day after sowing of barley, gases were sampled weekly.

Barley grain yield was determined from 1 m^2 sampling area at harvest and was expressed as rough (unhulled) barley at 14% moisture content. Above ground straw yield was determined after drying the plant materials at 80! for two days.

Measurement of CH_4

The quantities of CH_4 emission were measured with a gas chromatograph (Agilent 7890A) equipped with flame ionization detector (FID). Methane was separated using 2 m stainless–steel column with an inner diameter of 2 mm 13XMS column (60/80 mesh), with FID at 200!.

CH_4 fluxes were calculated with the following equation[16]:

$$F = \tilde{n}h\,[273/(273+T)]\,dC/dt$$

Where, F is the CH_4 flux (mg m^{-2} h^{-1}); T is the air temperature (!) inside the chamber; $\tilde{n}$ 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 were sequentially computed from the emissions between every 2 adjacent intervals of the measurements, based on a non–linear, least–squares method of analysis [17].

GWPs is defined as the cumulative radiative forcing both direct and indirect effects integrated over a period of time from the emission of a unit mass of gas relative to some reference gas. Carbon dioxide was chosen as the reference gas. The GWPs conversion parameters of CH_4 (over 100 years) were adopted with 25 kg ha^{-1} CO_2–equivalent [18].

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 performed according to the manufacturer's protocol. All genomic DNA concentration and purity was determined by NanoDrop spectrophotometry (Thermo Scientific, Wilmington, DE, USA). PCR was performed at an initial denaturation temperature of 94! for 3 min, followed by 20 cycles of 94! for 45 s, 53! for 30 s, and 65! for 90 s. A final elongation step at 65! 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 [19] 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 based on the methods of Giongo et al. (2010) [20] and Fagen et al. (2012) [21]. 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 [22], and then the reads were separated by barcode. Paired reads were assembled using FLASH to the reference greengenes [23, 24] 16S SSU rRNA database. Matches were filtered at 80%

length fraction and OTUs were classified at 97% identity level. The total number of pairs matching 16S rRNA sequences in the database at each level of similarity created an OTUs abundance matrix for each level of taxonomy across samples. Pairs that did not match to the same sequence in the Greengenes database were annotated according to their Last Common Ancestor (LCA), and pairs that did not have an LCA, or any match in the Greengenes database, were considered to be unclassified. To normalize for varying sequencing depths, the OTUs abundance matrices for each sample were divided by the total number of pairs after trimming.

IFA analysis of methanotrophs bacteria

Identification and enumeration of selected methanotrophs species was performed using the indirect immunofluorescence (IFA) method [25]. Seven polyclonal antibodies specific for 7 methanotrophs species, namely, *Methylosinus, Crenothrix, 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 [25]. Firstly, the bacteria retained on the polycarbonate filter were coated with a mixture (1/1) of a species–specific rabbit antiserum (ã–globulins) and rodamine–labelled bovine albumin. Secondly, to visualise the formed immune–complexes, the filters were coated with FITC–labelled donkey antiserum against rabbit ã–globulins, placed in a moist chamber for 20 min; then, filters were washed with a 0.85% NaCl solution. Finally, the filters were examined and counted using a luminescence microscope (LUMAM I–2, LOMO, Russia). As control for the specific fluorescence, we have used filters with pure cultures of the selected methanotrophs species, treated at the same condition. The total number of methanotrophs bacteria was calculated as the sum of 7 pre–selected methanotrophs species.

Diversity indices

To estimate bacterial diversity of each sample, three indices–number of OTUs, richness index, and shannon index were calculated using Mothur [26]. The phylogenetic distribution of OTUs was constructed by QIIME software.

Data analysis

All data were expressed as mean ± standard error. Signiûcance test was done using SPSS 11.0 for windows (LEAD Technologies, Inc, USA). 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

CH_4 emissions from all the treatments were higher in the spring season than those in the winter (Fig. 1). CH_4 fluxes from the control were negative during the early period of crop growth, while positive fluxes were observed after March 2014, and the peak value of CH_4 flux was observed at March 16 after sowing in all treatments in 2014. The cumulative CH_4 flux from the control was 0.656 mg m^{-2} during the whole experimental period (Table 2). The organic–inorganic mixed fertilizer managements resulted in positive CH_4 emissions from the soil (Fig. 1, Table 2). Within barley growth season, the CH_4 flux values were significantly different among treatments. In the early growth period, the order of treatments in CH_4 emission was MF>HOM>LOM>RF>CK. In the late growth period, the order of treatments in CH_4 emission was HOM>LOM>RF>MF>CK.

The production and emission of CH_4 were closely related to farming system, soil type, climate, and field management practices. HOM and LOM had larger total CH_4 emissions than CK in the barley growth period. The total CH_4 emissions from paddy fields during barley whole growth period were 4.165 g m^{-2} in MF, 4.561 g m^{-2} in RF, 5.408 g m^{-2} in LOM, 6.450 g m^{-2} in HOM, 0.656 g m^{-2} in CK, respectively. The sequence of treatments in total CH_4 emission was HOM>LOM>RF>MF>CK (Table 2).

GWPs is an indicator to reflect the relative radioactivity effects of a greenhouse gas, and the GWPs of CO_2 is defined as 1. In this study, the GWPs of CH_4 from barely paddy fields varied with different fertilizer managements, and the trend was shown as HOM>LOM>RF>MF>CK. During the barley whole growth period, HOM had the largest GWPs (1614.77 kg CO_2–eq ha^{-1}) of total CH_4 from paddy fields, followed by LOM (1353.90 kg CO_2eq ha^{-1}), RF (1141.96 kg CO_2–eq ha^{-1}), and CK had the lowest GWPs of total CH_4 (164.33 kg CO_2–eq ha^{-1}) (Table 2).

Barley grain yield of HOM and LOM was the highest, while CK was the lowest (Table 2). Concurrently, we estimated per yield GWPs which was calculated as GWPs divided by barley grain yield. As is shown in Table 2, per yield GWPs of CK was significantly higher than RF, MF, HOM and LOM ($P<0.05$), and the lowest was HOM.

Operational taxonomic units of methanotrophs

In the barley growing season, the root methanotrophs of barley plants from the RF fields had the highest number of OTUs (Fig. 2). And the highest values of the OTUs were observed at tillering stage among the different treatments after transplanting, and then the OTUs values dramatically declined to a low level. In the barley growing season, the OTUs values were significantly different among treatments with the order of RF>HOM>LOW>MF>CK (Fig. 2).

Genetic diversity indices of methanotrophs

In the barley growing season, the root methanotrophs of barley plants from the paddy fields had the highest values of the richness diversity indexes at tillering stage, and had the lowest values of the richness diversity indexes at maturity stage. Meanwhile, during the barley growing season, the values of the richness diversity indexes was higher in HOM, LOM than in MF, RF and CK. The order of treatments in the values of the richness diversity indexes was HOM>LOM>RF>CK>MF (Table 3).

Structure of the methanotrophs community

The results of the detection of 7 targeted methanotrophs by indirect immunefluorescent analysis (IFA) are presented in Table 4. It is important to mention that some methanotrophs species (*Methylosinus*, *Crenothrix*, *Methylo-*

Table 1. Nutrient supply from rice straw, chicken manure and mineral fertilizer under different fertilizer treatments

Treatment	Barley			Total		
	N	P_2O_5	K_2O	N	P_2O_5	K_2O
MF	157.5+0*	43.2+0	81.0+0	157.5	43.2	81.0
RF	133.0+24.5	37.8+5.4	48.2+32.8	157.5	43.2	81.0
LOM	110.2+47.3	21.8+21.4	51.1+29.9	157.5	43.2	81.0
HOM	63.0+94.5	0.5+42.7	21.2+59.8	157.5	43.2	81.0
CK	0+0	0+0	0+0	0	0	0

* Input from mineral fertilizer + input from organic fertilizer. The numbers are in kg hm^{-2}.
Note: The treatments are without fertilizer (CK), mineral fertilizer alone (MF), crop residue plus mineral fertilizer (RF), low manure rate plus mineral fertilizer (LOM), and high manure rate plus mineral fertilizer (HOM).
1) For the RF treatment, rice straw return rate (air dry) was 3600 kg hm^{-2}. 2) For the LOM treatment, manure application rate (decomposed) was 2670.0 kg hm^{-2}. 3) For the HOM treatment, manure application rate (decomposed) was 5340.0 kg hm^{-2}. 4) The N, P, and K content of air-dry rice straw was 0.68%, 0.15%, and 0.91%, respectively, and N, P, and K content of decomposed chicken manure was 1.77%, 0.80%, and 1.12%, respectively.

Table 2. Barley grain yield, global warming potentials (GWPs) of CH_4 and per yield GWPs from barley fields under long–term fertilizer managements

Treatment	CH_4 emission ($g\ m^{-2}$)	GWPs of CH_4 ($kg\ CO_2\ ha^{-1}$)	Barley grain yield ($kg\ ha^{-1}$)	Per yield GWPs CO_2 ($kg\ kg^{-1}$)
MF	4.165+0.186c	1042.60+30.10c	1246.10+35.97b	1.20+0.03bc
RF	4.561+0.132c	1141.96+32.97c	1571.50+45.37a	1.38+0.04b
LOM	5.408+0.120b	1353.90+39.08b	1609.65+46.47a	1.19+0.03bc
HOM	6.450+0.127a	1614.77+46.61a	1670.55+48.22a	1.03+0.03c
CK	0.656+0.019d	164.33+4.74d	888.50+25.65c	5.41+0.02a

MF: mineral fertilizer alone; RF: crop residue plus mineral fertilizer; M1+F: low manure rate plus mineral fertilizer; M2+F: high manure rate plus 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

Table 3. Genetic diversity indices of methanotrophs with different fertilizer treatments during barley growing season

Treatment	Item							
	Richness index				Shannon index			
	SS	TS	FS	MS	SS	TS	FS	MS
MF	18.3±0.54b	20.6±0.61b	15.5±0.50c	12.2±0.36b	1.74±0.05c	2.15±0.07c	1.64±0.05c	1.29±0.04c
RF	19.6±0.58ab	21.9±0.56ab	17.2±0.51ab	12.9±0.38ab	1.95±0.06ab	2.42±0.07ab	1.79±0.05bc	1.41±0.04bc
LOM	20.2±0.57a	22.7±0.63a	17.8±0.50a	13.3±0.37ab	2.04±0.05a	2.53±0.07a	1.89±0.05ab	1.45±0.04ab
HOM	20.4±0.53a	23.4±0.59a	17.9±0.45a	13.7±0.35a	2.11±0.05a	2.58±0.06a	2.03±0.05a	1.50±0.04a
CK	18.8±0.59ab	21.2±0.68b	15.9±0.52bc	12.5±0.40b	1.84±0.06bc	2.31±0.07bc	1.72±0.06c	1.37±0.04c

SS: seedling stage; TS: tillering stage; FS: full heading stage; MS: maturity stage.
MF: mineral fertilizer alone; RF: crop residue plus mineral fertilizer; LOM: low manure rate plus mineral fertilizer; HOM: high manure rate plus 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.

caldum, *Methylococcus*, *Methylomicrobium*, *Methylomonas* and *Methylosarcina*) were identified in all main growth stages of barley. It was clear that some methanotrophs species could only be detected at some growth stages of barley (Table 4). *Methylosinus*, *Methylomicrobium*, *Methylomonas* and *Methylosarcina* were detected more often in all treatments at main growth stages of barley. *Methylocaldum* were detected more often in all treatments at the seedling and maturity stage. *Crenothrix* were not detected in all treatments at the full heading stage. *Methylococcus* were not detected in all treatments at the maturity stage.

Heatmap in the different fertilizer treatments during barley growing season

The 7 most abundant genera for both treatments during barley growing season were analyzed and the differences were represented in a heatmap (Fig. 3). The relative abundances of *Methylosinus* were significantly higher in different treatments at main growth stages of barley. *Methylomicrobium* were relatively more abundant in different treatments at tillering stage. *Methylomonas* were relatively more abundant in LOM and HOM at seedling stage, tillering stage and maturity stage. *Methylosarcina* were relatively more abundant in MF and CK at tillering stage and full heading stage. On the other hand, *Crenothrix* and *Methylococcus* were not detected in different treatments at main growth stages of barley. *Methylocaldum* were not detected in MF, RF at seedling stage, tillering stage and full heading stage. *Methylosarcina* were not detected in different treatments at maturity stage.

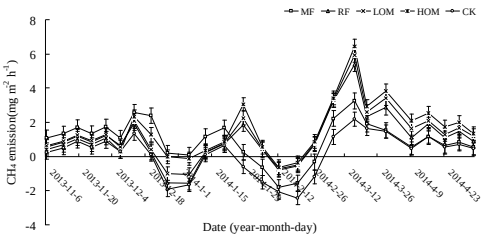
Phylogenetic tree analysis of methanotrophs gene clones among different fertilizer treatments

In the barley growing season, clustering analysis allowed the identifcation of OTUs responsible for the community shifts in different fertilization at species level. The results showed that OTUs *Methylomicrobium* & *Methylosarcina* were especially abundant in the soil samples. When phylogenetic trees were constructed using abundance belonging to each OTUs, *Methylomicrobium* and *Methylocaldum* were grouped into a tight and distinct cluster, *Methylomicrobium and Methylosarcina* were also grouped into a tight and distinct cluster. Quarter of the clones in OTUs were grouped into a cluster including *Methylosarcina* (Fig. 4).

Table 4. Species of selected methanotrophs in the different treatments at the main growth stages of barley

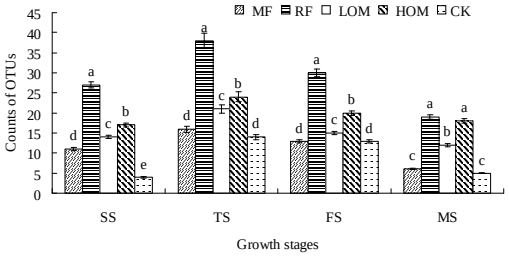
Stage	Treatment	Methanotrophs						
		Methylosinus	Crenothrix	Methylocaldum	Methylococcus	Methylomicrobium	Methylomonas	Methylosarcina
SS	MF	+	—	+	—	+	—	+
	RF	+	+	+	+	+	+	+
	LOM	—	+	+	—	—	+	+
	HOM	+	—	+	—	+	+	+
	CK	+	—	+	+	+	+	—
TS	MF	+	—	—	—	+	—	+
	RF	+	+	+	—	+	+	+
	LOM	+	+	—	—	+	+	+
	HOM	+	—	+	—	+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

SS: seedling stage; TS: tillering stage; FS: full heading stage; MS: maturity stage.
MF: mineral fertilizer alone; RF: crop residue plus mineral fertilizer; LOM: low manure rate plus mineral fertilizer; HOM: high manure rate plus mineral fertilizer; CK: without fertilizer.



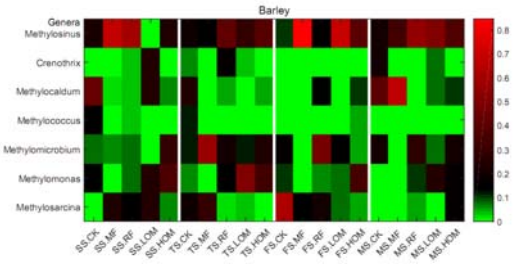
MF: mineral fertilizer alone; RF: crop residue plus mineral fertilizer; LOM: low manure rate plus mineral fertilizer; HOM: high manure rate plus mineral fertilizer; CK: without fertilizer.

Fig. 1. CH_4 fluxes from the soils affected by long–term fertilizer managements in barley field plots. Vertical bars are SE



SS: seedling stage; TS: tillering stage; FS: full heading stage; MS: maturity stage.
MF: mineral fertilizer alone; RF: crop residue plus mineral fertilizer; LOM: low manure rate plus mineral fertilizer; HOM: high manure rate plus mineral fertilizer; CK: without fertilizer. Values are presented as mean ± SE (n = 3).
Error bars represent the standard error of mean. Different letters indicate significance at $P < 0.05$, according to the least significant difference test.

Fig. 2. Operational taxonomic units of methanotrophs with different fertilizer treatments during barley growing season



SS: seedling stage; TS: tillering stage; FS: full heading stage; MS: maturity stage.
MF: mineral fertilizer alone; RF: crop residue plus mineral fertilizer; LOM: low manure rate plus mineral fertilizer; HOM: high manure rate plus mineral fertilizer; CK: without fertilizer.

Fig. 3. Heatmap illustrating the 7 most abundant methanotrophs genera in the different fertilizer treatments during barley growing season

Fig. 4. Phylogenetic distribution of OTUs of the clone libraries of 16S ribosomal RNA genes for soil samples under different treatments during barley growing season

DISCUSSION

Effects of fertilizer applications on CH_4 emission

Methane emission is complex processes including production, oxidation, and emission. CH_4 production was regulated by vegetation type, fertilizer managements, soil temperature, soil moisture, root activity and many other factors [27-30]. In this study, higher CH_4 emission in RF with 4.561 g m^{-2}, LOM with 5.408 g m^{-2}, and HOM with 6.450 g m^{-2}, suggests higher root growth due to increased N supply by mineral fertilizer, manure and crop residues. Manure and crop residues probably stimulated the activity of methanogen that produce CH_4. The reasons for above observations may be: first, microbial activities would have improved after returning manure and crop residues into the soil due to the carbon (C) supplements source and energy for microbial activities to accelerate consumption of soil oxygen and decrease of soil redox potential (Eh); second, methanogens became active due to the large quantities of C source, which provided reactive substrate for CH_4 emission from paddy fields. When N was supplied by mineral fertilizer, such as in MF with 4.165 g m^{-2}, compared with the RF, LOM and HOM, 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 activity, thereby resulting in lower CH_4 emission in MF. Several researchers [31, 32] have reported that N fertilization reduced soil CH_4 emission compared to no N fertilization while others did not observe effects of N fertilization on the emission [33, 34].

Global warming potential can be used as an index to estimate the potential effects of different greenhouse gases on the global climate system. Chu *et al*. (2007) [35] found that the N fertilization increased soil–derived GWPs significantly in the barley season. For a comprehensive consideration, we introduced the GWPs of CH_4 and per yield GWPs into this study for global warming calculations. In this study, it is certain that the CH_4 GWPs for the HOM and LOM were higher than RF and MF ($P<0.05$), due to their greater CH_4 emissions. Manure and crop residues addition increased barley grain production compared to the control. Therefore, the control without fertilizer may mitigate GHG emissions but may not sustain barley yields. Barley production was significantly higher in the HOM, LOM and RF than in the control. But the per yield GWPs of HOM, LOM was significantly lower than the control and MF ($P<0.05$). Therefore, we recommend application of manure and crop residues pattern in double cropping rice areas in the Middle and Lower reaches of Yangtze River in China, which correspond to HOM, LOM and RF as a management option under intensive cropping system. Further studies are underway to examine if HOM, LOM and RF with reduced N fertilization rate might mitigate GHG emissions and sustain crop yields. However, for global warming potential evaluating of management systems, it would be mandarory to consider the soil C dynamics associated with crop production inputs and machinery use.

Effects of fertilizer applications on soil methanotrophs diversity

Archaeal 16S rRNA gene revealed that long–term fertilization selected archaeal communities according to the fertilization treatments of the soil. Organic and crop residues fertilization resulted in increasing methanotrophs diversity (Fig. 2), showing that the application of organic, crop residues significantly influenced methanotrophs composition, mineral fertilization influenced methanotrophs composition to a lesser degree. The response of methanotrophs communities to agricultural practices was also shown by Nicol et al. (2003) [36] who determined different methanotrophs community structures in improved (N–fertilized) and unimproved grassland soils. In this work, we found higher number of OTUs correlated whit organic, crop residues, than OTUs correlated with mineral fertilizer. And the relationship of microorganisms and organic, crop residues, mineral fertilizer in the studied soil, suggests that organic, crop residues is an essential nutrient for the found taxa, whose may be involved on methanotrophs growth processes.

The methanotrophs diversity, measured by the shannon index and richness index, was significantly higher in samples from HOM, LOM, RF plots in five different fertilizer treatments, which agree with Zheng et al. (2008) [9], who found that soils under NPK plus recycled crop residues had the highest levels of methanotrophs diversity compared to the conventional fertilization. Also, at all taxonomic levels, shannon index and richness index was significantly higher when application of organic and crop residues, indicating that the application of organic and crop residues based on application of NPK, with higher C:N ratio, stimulates methanotrophs diversity on the soil. C:N ratio is higher in organic and crop straw substrates [37, 38], which provides more substrate to microorganisms explaining the highest shannon index and richness index found in this work on plots during barley growing season. Besides crops and C:N ratio, root exudates are also key factors that influence methanotrophs communities, as they provide a carbon source to soil microorganisms [39, 40]. Through the exudation of a wide variety of compounds, roots may regulate the soil methanotrophs community in their immediate vicinity, stimulate beneficial symbioses, change the chemical and physical properties of the soil, and inhibit the growth of competing plant species [41], which may also determine the methanotrophs diversity around rhizosphere.

Effects of fertilizer applications on soil methanotrophs community composition

The soil methanotroph community

structure for five treatments (MF, RF, LOM, HOM and CK) were analysed to assess the effects of the different treatments on methanotroph abundance. In this study, some distinct differences in the diversity indices were found between the mineral fertilizer and application of organic, crop residues fertilizer treatments, indicating that the soil methanotrophs community was clearly changed through the different fertilization managements. A clearly complex diversity pattern was revealed in the HOM and LOM treatments which might be a result of the strong CH_4 production [6]. Besides the chemical NPK, the addition of recycled crop residues and application of organic was suggested to be vital to methanogenic archaeal and the methanogenesis in paddy soils [42]. It is well–known that the soil CH_4 oxidation rate can be enhanced by higher CH_4 concentrations, thus promoting the activity, growth, and eventually changing the community structure of methanotrophs [43]. In summary, based on the sequences and phylogenetic analysis, it was revealed that long–term application of fertilizers could change the soil methanotrophs community structure.

In this study, the methanotrophs community compositions of the part of the subsequent sequence were analysis. The 7 most abundant genera from all treatments during the barley growing season, respectively, represented by the heatmap (Fig. 3), demonstrated that composition varied significantly among the different treatments. Many environmental factors such as soil water content and temperature changed continuously along with the growth of barley plants. A lot of work had been carried out to demonstrate the effects of these environmental factors on methane oxidation [44, 45, 46]. Our results indicated that methanotrophs relating to the genera of *Methylosinus*, *Methylomicrobium*, *Methylomonas* and *Methylosarcina* seemed to be the most common in the paddy soil receiving different fertilizations long–term. Similarly, the *Methylomonas*–related methanotrophs were the active species in the paddy soils and differed from those in the forest soils [47]. Also, our results were in agreement with an earlier work [12] in which type I sequences clustering with members of the genera *Methylobacter*, *Methylomicrobium*, and *Methylomonas* were most frequently detected in an agricultural soil.

CONCLUSIONS

GHG emissions from large paddy fields and excessive fertilizer application can significantly contribute to global warming. The results indicated that with the same N application rate, different organic–inorganic mixed fertilizer application, such as RF, LOM, and HOM, caused substantial CH_4 emissions and GWPs of CH_4 during the barley growing season compared with those from the conventional MF treatment. However, HOM and LOM resulted in a significantly lower yield–scaled GWPs compared to the control treatment. Meanwhile, long–term fertilization managements can differentially affect the abundance and composition of the methanotrophs. The inhibited effects on methanotrophs abundance were found in the MF treatment, compared to the stimulated effects from the RF, LOM and HOM treatments. On the other hand, the methanotrophs community structure also responded clearly to the different fertilization managements. The methanotrophs diversity of the HOM and LOM treatments was observed clearly distinguished from the other treatments. Furthermore, a clear shift was found in methanotrophs community composition based on the sequences and phylogenetic analysis. A significantly higher ratio of type methanotrophs sequences clustering with the genera of *Methylosinus*, *Methylomicrobium*, *Methylomonas* and *Methylosarcina* were detected in all treatments at main growth stages of barley. In conclusion, the applications of mineral fertilizer could be important factors controlling the abundance, and the application of organic, crop residues enhance the abundance of methanotrophs in double–cropping paddy fields in southern China. Understanding of the major factors influencing the methanotrophs abundance and composition is vital for linking this functional community to soil ecosystem processes and sustainable management of barley cultivation.

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