

Responses of soil methanogens, methanotrophs, and methane fluxes to land-use conversion and fertilization in a hilly red soil region of southern China

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Abstract Changes in land-uses and fertilization are important factors regulating methane (CH₄) emissions from paddy soils. However, the responses of soil CH₄ emissions to these factors and the underlying mechanisms remain unclear. The objective of this study was to explore the effects of land-use conversion from paddies to orchards and fertilization on soil CH₄ fluxes, and the abundance and community compositions of methanogens and methanotrophs. Soil CH₄ fluxes were quantified by static chamber and gas chromatography technology. Abundance and community structures of methanogens and methanotrophs (based on *mcrA* and *pmoA* genes, respectively) were determined by quantitative real-time PCR (qPCR), and terminal restriction fragment length polymorphism (TRFLP), cloning and sequence analysis, respectively. Results showed that land-use conversion from paddies to orchards dramatically decreased soil CH₄ fluxes, whereas fertilization did not distinctly affect soil CH₄ fluxes. Furthermore, abundance of methanogens and methanotrophs were decreased after converting paddies to orchards. Fertilization decreased the

abundance of these microorganisms, but the values were not statistically significant. Moreover, land-use conversion had fatal effects on some members of the methanogenic archaea (*Methanoregula* and *Methanosaeta*), increased type II methanotrophs (*Methylocystis* and *Methylosinus*), and decreased type I methanotrophs (*Methylobacter* and *Methylococcus*). However, fertilization could only significantly affect type I methanotrophs in the orchard plots. In addition, CH₄ fluxes from paddy soils were positively correlated with soil dissolved organic carbon contents and methanogens abundance, whereas CH₄ fluxes in orchard plots were negatively related to methanotroph abundance. Therefore, our results suggested that land-use conversion from paddies to orchards could change the abundance and community compositions of methanogens and methanotrophs, and ultimately alter the soil CH₄ fluxes. Overall, our study shed insight on the underlying mechanisms of how land-use conversion from paddies to orchards decreased CH₄ emissions.

Keywords Land-use conversion · CH₄ fluxes · Methanogens · Methanotrophs · Fertilization

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Introduction

Methane (CH₄), the second largest contributor to radiative forcing after carbon dioxide (CO₂), causes approximately 15–20% of the observed global warming (IPCC 2013). Agriculture methane emissions are crucial to the global methane estimates, and these emissions magnitude are highly dependent on land-uses and fertilizer application (Scheer et al. 2008; Liu et al. 2016). Paddy cultivations have long been recognized as the main contributors to anthropogenic CH₄ emissions, ranging from 30 to 50 Mt y⁻¹ (Sass et al. 1994), whereas aerobic upland soils

are the second greatest global CH₄ sinks, accounting for 6–10% of the total atmospheric CH₄ uptake or about 30 Tg y⁻¹ CH₄ (IPCC 2013). Therefore, the expected land-use conversion from paddies to uplands could possibly mitigate the global warming potentials by reducing CH₄ emissions.

It is urgently demanded to understand the extent to which land-use conversion from paddy fields to upland plots contribute to greenhouse gases budgets, but CH₄ fluxes record from continuous measurements of paddy fields and upland plots are limited. In the recent decades, results from field measurements have demonstrated that converting from paddies to uplands would change soil from emission source to sink of atmospheric CH₄ (Nishimura et al. 2008; Liu et al. 2016). The dynamics of CH₄ in paddy fields significantly differ from those in upland plots in which aerobic decomposition process is dominant (Nishimura et al. 2008; Scheer et al. 2008). However, there have been far fewer field investigations on the underlying mechanisms of decreases in CH₄ fluxes induced by converting paddies to uplands. Meanwhile, fertilization is the management practice most frequently used for paddy fields and upland plots (Wang et al. 2015; Fan et al. 2016). While it has been reported that fertilization had controversial effects on soil CH₄ fluxes in different land-uses, ranging from positive (Wang et al. 2015), negative (Liu et al. 2016; Zhou et al. 2016), and no significant effects (Fan et al. 2016). Thus, adequate estimates of soil CH₄ fluxes, coupling with the changes in land-uses and fertilization, would be more helpful for the global greenhouse gases emissions budget.

Generally, CH₄ fluxes from soils are determined by the counterbalance of methane production and subsequent oxidation by methanogenic and methanotrophic microorganisms, respectively (Hanson and Hanson 1996; Watanabe et al. 2007). The *mcrA* gene commonly encodes the K-subunit of methyl coenzyme M reductase (MCR), which is the key catabolic enzyme of methanogens (Freitag and Prosser 2009). The *pmoA* gene, commonly recognized as a phylogenetic marker of methanotrophs in ecological studies, encoding the membrane-bound subunit of the particulate methane monooxygenase (MMO) (Henckel et al. 1999). Moreover, it is widely accepted that the particulate methane monooxygenase (*pmoA*) gene provides a molecular indicator of methane oxidation potential (Scheer et al. 2008; Singh et al. 2009). Therefore, the abundance and community compositions of methanogens and methanotrophs could be respectively characterized by the *mcrA* and *pmoA* genes. Within the last decade, studies on the abundance and community compositions of the methanogens and methanotrophs under different land-uses have become more common (Bao et al. 2016; Wang et al. 2015). Nevertheless, the changes of the methanogens and methanotrophs induced by land-use conversion from paddies to orchards are scarce. In addition, researches on the linkage between the relating microorganisms and in situ soil CH₄ fluxes remains unclear. Therefore, it

is essential to explore the relationships between methanogens, methanotrophs, and soil CH₄ fluxes to clarify the underlying mechanism driving the soil-atmosphere exchange of CH₄.

China is one of the most important rice-producing countries in the world and the annual CH₄ emissions from Chinese rice cultivations are about 7.4 Tg CH₄ Y⁻¹ (Yan et al. 2009). The hilly red soil region is characteristic of typical agricultural soil and covers an area of almost 11.8% of the country land surface, producing 80% of the rice, and supporting 22.5% of China's population (Liu et al. 2016). Over the past few years, this region has undergone great changes in land-uses, in particular the land-use conversion from paddies to uplands for cultivating fruits and economic orchards. These changes in land-uses and fertilization might cause marked variations in the soil CH₄ fluxes owing to the alteration in the microbial activity. However, little information is available on the exhaustive assessments of the responses of soil methanogens, and methanotrophs, as well as in situ CH₄ fluxes to the land-use conversion and fertilization in the hilly red soil region of southern China. Therefore, the present study aimed to (1) evaluate the effects of land-use conversion and fertilization on soil CH₄ fluxes; (2) examine the variations in the abundance and communities of methanogenic archaea and methanotrophic bacteria, with the *mcrA* and *pmoA* genes, respectively; and (3) explore the potential linkages between soil CH₄ fluxes and the abundance and communities of methanogens and methanotrophs.

Materials and methods

Site description and experimental design

The experiment site was conducted at Qianyanzhou Ecological Research Station of Chinese Academy of Science (CAS) (26° 44' 46" N, 115° 04' 05" E) in Taihe city, Jiangxi province, southern China. According to the meteorological data from 1989 to 2010, this area was characteristic of a subtropical monsoon climate, with an average annual air temperature of 18.0 °C and precipitation of 1509.0 mm. The soil is typically red soil found in southern China and classified as Cambosols in the Ultisols soil classification.

In the present experiment, the two most common land-uses were selected, namely rice paddy (*Oryza sativa* L.) and citrus orchard (*Citrus reticulata* L.). The paddy fields had been continuously cultivated for approximately 10 years and a portion of the fields has been converted to orchard plots in June 2012. Meanwhile, in order to evaluate fertilization effects, fertilization and non-fertilization treatments were established under the two land-uses. Therefore, four treatments with four replicates were employed, referred as citrus orchard with fertilization (OF), citrus orchard without fertilization (ONF), rice paddy with fertilization (PF), and rice paddy without fertilization

(PNF). The planting density was 235,000 ha⁻¹ and the spacing was 0.25 × 0.17 m in the paddy plots, whereas the converted orchards were planted at a planting density of 833 plants ha⁻¹ with a spacing of 3 × 4 m. Under the two land-uses, fertilizer was evenly sprinkled over the soil surface. The details about the management in the orchards and paddies were described in our early studies (Liu et al. 2016). These fertilization systems, fertilizer rates, and plant population were chosen according to common experiences from local farmers. The detailed description about the intensive cultivations and fertilization managements during our observing period are given in Table 1.

Field soil CH₄ fluxes measurements

The exchanges of CH₄ fluxes between soil and atmosphere from Aug 2013 to Jul 2014 were measured using static chamber and gas chromatography technology. The chambers were made of stainless steel materials and comprise two separate sections: a cylindrical base frame and a chamber. The base frames (a bottom area of 0.12 m²) without covering the vegetation were installed at depth of 0.05 m in all plots several days before the initial sampling and kept in the places marked. The chambers had a bottom area of 0.12 m² and heights of 0.39 m (used for orchards) and 0.69 m (used for paddies), respectively. During the gas sampling, chambers were temporarily mounted onto the base frames. All gases were sampled in the 09:00–11:00 a.m. The gas samples were collected daily for 4 days after fertilization and once or twice per week for the remainder of the time. The gases were taken with 100 ml plastic syringes at 0, 10, 20, 30, and 40 min after the chamber was closed. Within 24 h after gas

collection, the CH₄ concentrations of the gas samples were measured using gas chromatography (Agilent 7890A, Santa Clara, California, USA). Then, the soil CH₄ fluxes were computed on the basis of the rate of variations of CH₄ concentrations in the chambers, and calculated as the slope of linear or nonlinear regression between concentrations and time. During gas sampling, the temperature of the chamber was recorded using a portable thermometer and then used to calibrate the actual gases concentrations.

Soil sampling and chemical analysis

During Aug 2013 and Jul 2014, soil samples from the top layer (0–10 cm) were sampled once per month or once in every 2 months. In each plot, five soil cores (diameter = 3 cm) were randomly collected and combined together. Then, each bulk soil sample was removed with stones and other materials. After that, the composite soil samples were trisected: the first section was air-dried to analyze soil organic carbon (SOC), total nitrogen (TN), C:N ratio, and pH; the second section was restored at -20 °C for analysis of soil ammonia (NH₄⁺-N) and dissolved carbon (DOC) contents, and the remaining section was freeze-dried and restored at -80 °C for molecular analysis.

Daily air temperature and precipitation were recorded by the climate station located in this region. Soil temperature and moisture were measured using an automatic data recording system via wireless technology. The pH was measured by a pH meter. Soil SOC, TN, and C:N ratio were determined by an element analyzer (Elementar, Hanau, Germany). For the DOC contents, 20 g of soil was extracted by adding 0.1 L distilled water; after that, the admixture was shaken for 1 h, and filtered, and assessed with a TOC-TN analyzer (Elementar,

Table 1 Description of management practices used for the paddy and orchard

Land use	Management	Date
Orchard	Compound fertilizer (67.2 kg N ha ⁻¹) and urea (20.8 kg N ha ⁻¹) were applied to the fertilization treatment	Jul 17, 2013
	Compound fertilizer (67.2 kg N ha ⁻¹) and urea (20.8 kg N ha ⁻¹) were applied to the fertilization treatment	Apr 17, 2014
Paddy	Rice transplanting and compound fertilizer (72 kg N ha ⁻¹) were applied to the fertilization treatment	Jul 30, 2013
	Urea (106 kg N ha ⁻¹) was applied to the fertilization treatment	Aug 10, 2013
	Rice harvest	Nov 14, 2013
	Rice transplanting and compound fertilizer (72 kg N ha ⁻¹) were applied to the fertilization treatment	Apr 19, 2014
	Urea (106 kg N ha ⁻¹) was applied to the fertilization treatment	Apr 29, 2014
	Rice harvest	Jul 22, 2014

Laurel, NJ). Soil NH_4^+ -N contents were analyzed with a flow-injection auto analyzer (Seal AA3, Norderstedt, Germany).

DNA extraction and quantitative real-time PCR

Soil DNA was extracted from freeze-dried bulk soil (0.3 g) using the Mobio Powersoil™ DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA, USA). The concentration and quality of the extraction was checked by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). All the soil DNA extracts were diluted with nuclease-free DI water (DNA:water = 1:5) to reduce potential PCR inhibition and preserved at $-80\text{ }^\circ\text{C}$.

Quantitative real-time PCR (qPCR) was applied to quantified the copy numbers of methanogenic *mcrA* gene (primer set ME1 (GCMATGCARATHGGWATGTC)/ME2 (TCATKGCRTAGTTDGGRTAGT)) (Hales et al. 1996) and methanotrophic *pmoA* gene (primer set A189F (GGN GAC TGG GAC TTC TGG)/mb661 (CCG GMG CAA CGT CYT TAC C)) (McDonald et al. 2008). The copy numbers were determined by qPCR using an iCycler iQ2 Thermocycler (Bio-Rad Laboratories, Hercules, CA, USA). All the DNA samples were run in triplicates in a final volume of 25 μl reaction system containing 12.5 μl of SYBR® Premix Ex Taq™ (TaKaRa Biotechnology, Dalian, China), 0.5 μl of each primer (10 μM), 2 μl of the diluted DNA (1–10 ng) and 9.5 μl of nuclease-free DI water. In order to generate a standard curve, individual clones with accurate inserts were cultured in Luria–Bertani medium and the plasmid DNA was extracted, purified, and quantified. Plasmid DNA was prepared in a 10-fold dilution series to yield a standard curve covering six orders of magnitude (10^2 to 10^8 copies) per assay. The qPCR amplification efficiencies were in the range of 95–100%, and the R^2 was 0.995–0.998. Besides, dissociation curves and gel electrophoresis were employed to confirm whether the amplified DNA fragments were of correct size. The final copies of the methanogenic archaea and methanotrophic bacteria were determined by calculation against the plasmid DNA copies.

Terminal restriction fragment length polymorphism (TRFLP), cloning, and sequence analysis

The methanogen and methanotroph community compositions were determined using soil samples collected in Jul 2014. The PCR amplification was carried out with the same primer pairs as those used for qPCR assays, with the forward primers being labeled with 6-carboxyfluorescein. Then, the PCR products obtained were purified by the PCR purification kit (Promega, San Luis Obispo, USA). Subsequently, the fluorescent-labeled PCR

products were digested using the restriction enzymes *Eco311* and *MspI* (Takara Biotechnology, Dalian, China), respectively, for the *mcrA* and *pmoA* gene products at $37\text{ }^\circ\text{C}$ for 3 h, followed by inactivation at $65\text{ }^\circ\text{C}$ for 20 min. The digests were purified by ethanol precipitation and re-suspended in 20 μl of water. Then, 10 μl of the digest were mixed with 15 μl of HiDiformamide and 0.3 μl of internal DNA fragment length standard (Applied Biosystems). The terminal restriction fragments (T-RFs) were calculated with the GeneMarker software package (version 2.2.0). The relative high abundance T-RFs (>1%), which fell in the range of 0–500 bp, were employed for analysis.

Two clone libraries were acquired using the *mcrA* and *pmoA* gene fragments retrieved from the PNF and ONF treatments, respectively. The primers used for PCR amplification were the same as those employed earlier but not fluorescent labeled. The purified PCR products were ligated with pGEM-T Easy Vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* JM109 cells (Takara Biotechnology, Dalian, China). Subsequently, approximately 50 clones were chosen randomly and sequenced with an ABI 3730 sequencer (Applied Biosystems). Then high-quality sequences were compared to the genes in the NCBI database and examined for chimeras manually, and sequences with alignment splits were removed. The sequences were aligned and two phylogenetic trees were acquired with neighbor-joining tree analysis (1000 bootstrap replicates) by MEGA 6 software (Tamura et al., 2013). To match every clone with a particular T-RF, the sequences acquired were run in REMA software to determine the size of the T-RF (Szubert et al. 2007). The whole sequences acquired in our work were submitted to the GenBank database with the following accession numbers: KX130584–KX130599 for the *mcrA* gene, and KX077180–KX077192 and KX130600 for the *pmoA* gene.

Statistical analyses

Repeated measures ANOVA were applied to examine the differences in soil properties, abundance of *mcrA* and *pmoA* genes, and CH_4 fluxes among the four treatments. Repeated measures ANOVA were also conducted to examine the effects of land-use conversion, fertilization as well as their interactions on soil properties, abundance of *mcrA* and *pmoA* genes, and CH_4 fluxes. Regression and multiple regression analyses were employed to examine the relationships among CH_4 fluxes, abundance of *mcrA* and *pmoA* genes, and soil properties. All the statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL, USA). Redundancy analysis (RDA) of the T-RFLP profiles was performed to summarize the variations in the methanogen and methanotroph communities that could definitely be explained by the soil properties using CANOCO software (Microcomputer Power, Ithaca, NY, USA).

Results

Soil properties

The soil moisture of the paddy plots was greater than that of the orchards, while the reverse patterns were observed for soil temperature (Table 2). Both land-use conversion and fertilization distinctly reduced soil pH ($P < 0.001$), with the lowest pH being detected in the OF treatment (Table 2). Moreover, land-use conversion from paddies to orchards distinctly increased the SOC contents, TN contents, and C:N ratio (Table 2, $P < 0.05$). Fertilization did not affect soil moisture, soil temperature, SOC, and C:N ratio under the orchards and paddies (Table 2, $P > 0.05$). Soil TN contents were affected by fertilization (Table 2, $P < 0.05$). The soil DOC contents were significantly lower in the orchard plots when compared with those in the paddy fields (Table 2). Fertilization significantly affect the soil DOC contents (Table 2, $P < 0.05$). In addition, fertilization significantly increased soil $\text{NH}_4^+\text{-N}$ contents, when compared with unfertilized plots (Table 2, $P < 0.05$).

Environmental conditions and soil CH_4 fluxes

The annual precipitation was 1424.8 mm, which is below than the long-term average (1509.0 mm). Rainfall between March and June accounted for 57% of the annual precipitation, with the largest and smallest monthly rainfall occurring in May and February, respectively (Fig. 1a). The average annual air temperature was 19.3 °C, with maximum and minimum daily air temperature being 31.9 °C (August 2013) and 0.02 °C (February 2014), respectively (Fig. 1a).

The amounts and patterns of CH_4 fluxes were significantly different between the two land-uses (Fig. 1b, c). The CH_4 emission fluxes from the conventional paddy plots exhibited distinct seasonal variations, peaked at almost 20 days after

flooding and declined and became stabilized on the day before harvest (Fig. 1c). In the converted orchard plots, most of the CH_4 exchange fluxes were negative and higher amounts of CH_4 were oxidized in summer when the soil moisture was moderate and temperature was higher (Fig. 1a, b). The average CH_4 fluxes for the paddy fields were significantly greater than those for the orchard plots during the observation period (Table 3, $P < 0.05$). However, the average soil CH_4 flux between unfertilized and fertilized treatment were not significantly different under the orchard plots and paddy field (Table 3, $P > 0.05$).

Abundance of methanogens and methanotrophs

The *mcrA* copy numbers ranged from 2.55×10^6 to 2.53×10^7 and from 2.26×10^6 to 6.40×10^7 copies g^{-1} dry soil, while the *pmoA* copy numbers ranged from 1.42×10^5 to 4.95×10^5 and from 4.81×10^5 to 4.76×10^6 copies g^{-1} dry soil in the orchard and paddy soils, respectively (Fig. 2a, b). As for the paddy fields, the *mcrA* copy numbers were highest in June 2014, whereas maximum *pmoA* copy numbers were observed in November 2013 (Fig. 2a, b). In the orchard plots, the highest *mcrA* and *pmoA* abundance were noted in May 2014 and June 2014, respectively (Fig. 2a, b). Furthermore, the *mcrA* and *pmoA* abundance was statistically different between the land-uses, with lower mean *mcrA* and *pmoA* abundance detected in orchard plots, irrespective of the fertilization treatment (Table 3). However, although the fertilization treatment showed lower *mcrA* and *pmoA* abundance when compared with the non-fertilization treatment in both orchard and paddy plots, the difference between them was not statistically significant (Table 3).

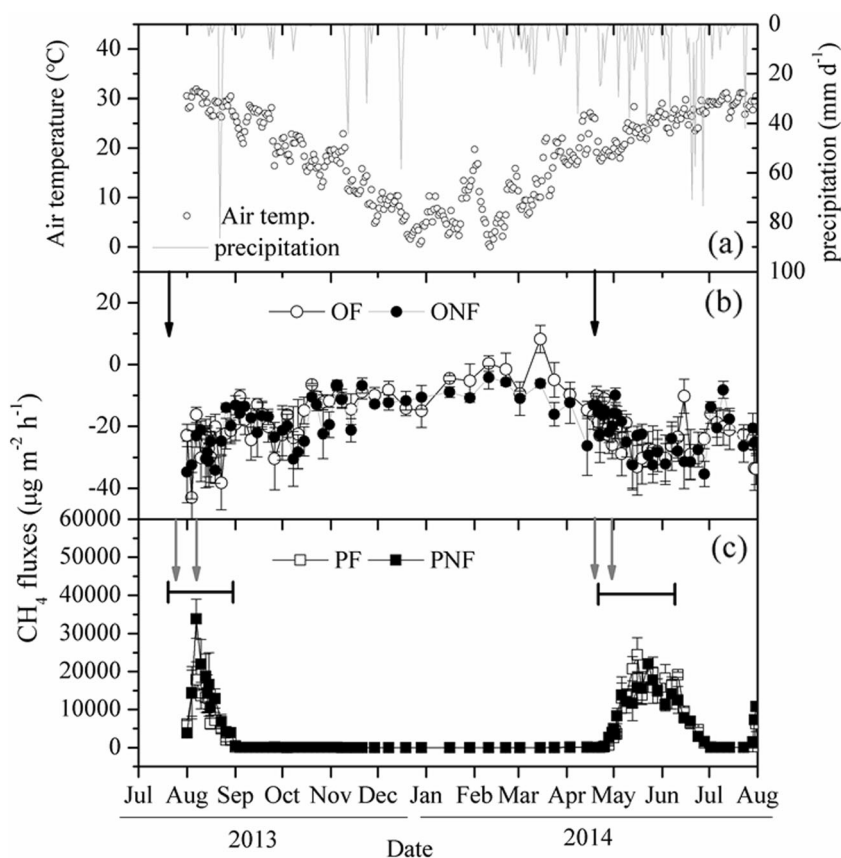
Table 4 summarizes the relationships among soil *mcrA* and *pmoA* abundance and soil properties. For the orchard plots, the coefficients of the best analysis (i.e., CH_4 fluxes and SOC

Table 2 Effects of land-use conversion and fertilization on soil properties

Treatment	Soil moisture (%)	Soil temperature (°C)	pH	SOC (g kg^{-1})	TN (g kg^{-1})	C:N ratio	DOC (mg kg^{-1})	$\text{NH}_4^+\text{-N}$ (mg kg^{-1})
OF	22.74 ± 0.61b	23.98 ± 0.28a	4.76 ± 0.03d	10.20 ± 0.54a	1.16 ± 0.05a	8.81 ± 0.11a	20.78 ± 3.12c	12.65 ± 1.25a
ONF	22.14 ± 0.33b	23.61 ± 0.41a	4.87 ± 0.03c	9.79 ± 0.52ab	1.12 ± 0.06ab	8.72 ± 0.06a	18.25 ± 2.34c	8.33 ± 0.47b
PF	36.97 ± 0.22a	21.46 ± 0.08b	4.95 ± 0.03b	9.02 ± 0.44b	1.08 ± 0.03b	8.44 ± 0.12b	32.44 ± 1.96a	10.89 ± 0.94a
PNF	37.17 ± 0.34a	21.63 ± 0.11b	5.04 ± 0.04a	9.09 ± 0.35b	1.06 ± 0.03b	8.50 ± 0.11b	29.23 ± 2.40b	8.52 ± 1.07b
Significance of								
Land-use conversion	<i>0.000</i>	<i>0.021</i>	<i>0.000</i>	<i>0.013</i>	<i>0.000</i>	<i>0.005</i>	<i>0.000</i>	0.055
Fertilization	0.435	0.235	<i>0.000</i>	0.141	<i>0.033</i>	0.416	<i>0.026</i>	<i>0.000</i>
Land-use conversion × fertilization	0.475	0.465	0.995	0.682	0.165	0.165	0.821	0.019

Values are means with standard errors. Different letters in the same column indicate significant differences ($P < 0.05$). Significant effects ($P < 0.05$) are highlighted in italics. Treatments are OF orchard with fertilization, ONF orchard without fertilization, PF paddy with fertilization, PNF paddy without fertilization

Fig. 1 Seasonal variation of the daily air temperature and precipitation (a), and soil CH₄ fluxes in the orchard plots (b) and paddy fields (c) during the observation period. The values are means with standard errors. *Black arrows* indicate the fertilizer applied to OF; *gray arrows* indicate fertilizer applied to PF. *Horizontal bars* indicated the period of flooding in the paddy plots. Treatments are *OF*, orchard with fertilization; *ONF*, orchard without fertilization; *PF*, paddy with fertilization; *PNF*, paddy without fertilization



contents) were used in multiple regression study against *pmoA* abundance, and an R^2 of 0.57 was obtained. However, no significant correlation between *mcrA* abundance and soil properties was observed. For the paddy plots, the best multiple regression result for the *pmoA* abundance including soil DOC and CH₄ fluxes, with an R^2 of 0.53, whereas the best fit of soil properties against *mcrA* abundance including soil moisture, pH, and soil temperature, with an R^2 of 0.86.

Community structure of methanogens and methanotrophs

In total, six T-RFs were considered to be main peaks in the T-RFLP spectrum of *mcrA* gene (Fig. 3a). The clone sequences were analyzed to associate the T-RFs with the respective methanogens, which suggested that five T-RFs could be specified to a single lineage, i.e., T-RFs of 97, 292, and 139 bp to *Methanosarcina* and T-RFs of 195 and 280 bp to *Methanocella*

Table 3 Effects of land-use conversion and fertilization on mean soil CH₄ fluxes, *mcrA*, and *pmoA* abundance

Treatment	CH ₄ fluxes (µg C m ⁻² h ⁻¹)	<i>mcrA</i> abundance (copies g ⁻¹ dry soil)	<i>pmoA</i> abundance (copies g ⁻¹ dry soil)
OF	-19.12 ± 4.79b	5.80 × 10 ⁶ ± 9.92 × 10 ⁵ b	2.50 × 10 ⁵ ± 5.40 × 10 ⁴ b
ONF	-19.99 ± 4.47b	8.53 × 10 ⁶ ± 2.61 × 10 ⁵ b	3.10 × 10 ⁵ ± 7.08 × 10 ⁴ b
PF	4398.94 ± 904.36a	2.70 × 10 ⁷ ± 5.74 × 10 ⁶ a	1.37 × 10 ⁶ ± 5.74 × 10 ⁵ a
PNF	4855.70 ± 1238.0a	2.90 × 10 ⁷ ± 7.37 × 10 ⁶ a	1.54 × 10 ⁶ ± 6.00 × 10 ⁵ a
Significance of			
Land-use conversion	<i>0.000</i>	<i>0.000</i>	<i>0.000</i>
Fertilization	0.458	0.069	0.431
Land-use conversion × fertilization	0.457	0.422	0.716

Values are means with standard errors. Different letters in the same column indicate significant differences ($P < 0.05$). Significant effects ($P < 0.05$) are highlighted in italics. Treatments are *OF* orchard with fertilization, *ONF* orchard without fertilization, *PF* paddy with fertilization, *PNF* paddy without fertilization

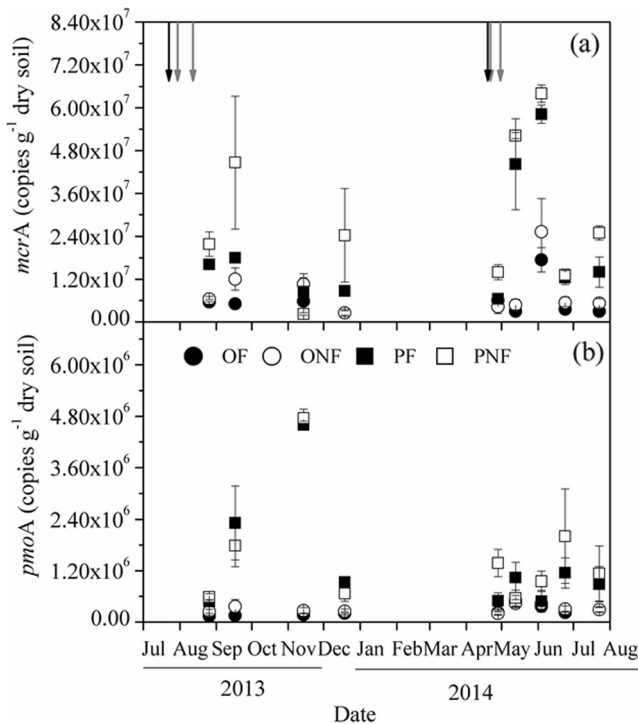


Fig. 2 Seasonal variation of *mcrA* (a), and *pmoA* abundance (b) under the four treatments. Values are means with standard errors. Black arrows indicate fertilizer applied to OF; gray arrows indicate fertilizer applied to PF; the arrows in (a) applied to (b). Treatments are OF, orchard with fertilization; ONF, orchard without fertilization; PF, paddy with fertilization; PNF, paddy without fertilization

(Fig. S1). However, T-RF of 329 bp was characteristic of *Methanosarcina*, *Methanoregula*, and *Methanosaeta* (Fig. S1). In the orchard plots, members of *Methanosarcina* and *Methanocella* were observed (Fig. 3a), whereas in the paddy fields, members of *Methanosarcina*, *Methanocella*, *Methanoregula*, and *Methanosaeta* were detected (Fig. 3a).

Moreover, the community compositions of methanogens were almost similar between fertilization and non-fertilization treatment under the two land-uses (Fig. 3a).

Seven T-RFs were considered to be main peaks in the T-RFLP profiles for the *pmoA* gene (Fig. 3b). Four T-RFs were specified to a single lineage, such as T-RF of 130 bp to *Methylococcus*, T-RF of 13 bp to *Methylobacter*, and T-RFs of 401 and 53 bp to *Methylocystis*, whereas T-RFs of 19 and 295 bp were assigned to type II methanotrophs (*Methylocystis* and *Methylosinus*) (Fig. S2). However, T-RF of 76 bp could not be characteristic of any sequences (Fig. S2). In the four treatments, the T-RF of 295 bp showed the highest relative abundance (Fig. 3b). Furthermore, land-use conversion from paddies to orchards reduced type I methanotrophs (*Methylococcus* and *Methylobacter*), but increased type II methanotrophs (*Methylocystis* and *Methylosinus*) ($P < 0.05$). Moreover, fertilization significantly affected T-RF of 13 bp under the orchard plots ($P < 0.05$).

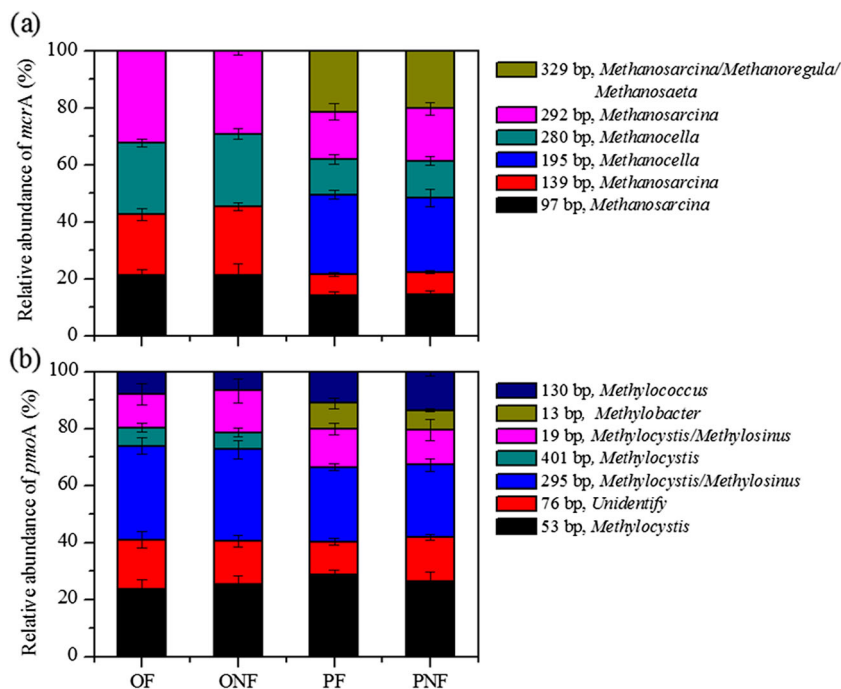
The RDA analysis was conducted by combing compositions of soil methanogen and methanotroph to show the relationships among soil properties and microbial compositions (Fig. 4). The results showed that the first (RD1) and second (RD2) axes explained 89.8 and 2.3% of the total variations of the community structures of methanogens and methanotrophs, respectively (Fig. 4) Furthermore, the results indicated that the orchard plots were best characterized by soil temperature, SOC, TN, C:N ratio, and *Methylocystis* and *Methanosarcina*, whereas the paddy fields were characterized by soil CH₄ fluxes, DOC contents, pH, soil moisture, and *Methanoregula*, *Methanosarcina*, *Methylobacter*, *Methanosaeta*, *Methylosystis*. The different cluster of symbols in Fig. 4 indicates that land-use conversion but not of fertilization significantly induced the variation of microbial communities.

Table 4 Multiple regressions among CH₄ fluxes, copy numbers of *mcrA* gene and *pmoA* gene, and soil properties

Land use	Soil fluxes/gene abundance	Regression equation	R ²	P value
Orchard	CH ₄ fluxes (μg C m ⁻² h ⁻¹)	$y = -10.27 - 4.44 \times 10^{-5} pmoA$	0.41	0.004
	<i>pmoA</i> (copies g ⁻¹ dry soil)	$y = 4.76 \times 10^5 - 9.23 \times 10^4 CH_4 - 4.01 \times 10^4 SOC$	0.57	0.002
	<i>mcrA</i> (copies g ⁻¹ dry soil)	—	—	—
Paddy	CH ₄ fluxes (μg C m ⁻² h ⁻¹)	$y = -8.51 + 5.18 \times 10^{-5} mcrA + 3.76 \times 10^2 DOC$	0.84	0.000
	<i>pmoA</i> (copies g ⁻¹ dry soil)	$y = 4.96 \times 10^6 - 1.43 \times 10^5 DOC + 2.09 \times 10^2 CH_4$	0.53	0.003
	<i>mcrA</i> (copies g ⁻¹ dry soil)	$y = -2.85 \times 10^8 + 1.37 \times 10^6 SM + 4.77 \times 10^7 pH + 8.11 \times 10^5 ST$	0.86	0.000

SOC soil organic carbon contents, DOC soil dissolved carbon contents, SM soil moisture, ST soil temperature

Fig. 3 Community structures of methanogens and methanotrophs based on T-RFLP analysis targeting *mcrA* (a) and *pmoA* (b) genes in the four treatments. Values are means with standard errors. Treatments are *OF*, orchard with fertilization; *ONF*, orchard without fertilization; *PF*, paddy with fertilization; *PNF*, paddy without fertilization



Correlations between CH₄ fluxes and various variables

In the orchard plots, soil CH₄ fluxes could not be fitted to *mcrA* abundance (Fig. 5a), but were negatively correlated with *pmoA* abundance (Fig. 5b). Meanwhile, the relationships between soil CH₄ fluxes and *mcrA*:*pmoA* ratio well fitted the quadratic equations (Fig. 5c). In the paddy plots, the

relationship between CH₄ fluxes and *mcrA* abundance and *mcrA*:*pmoA* ratio fitted the linear equations (Fig. 5d, f). The multiple regression analysis confirmed the correlations between CH₄ uptakes and *pmoA* abundance in the orchards (Table 4). Meanwhile, the DOC contents and *mcrA* abundance were the main factors controlling soil CH₄ fluxes from the paddies (Table 4).

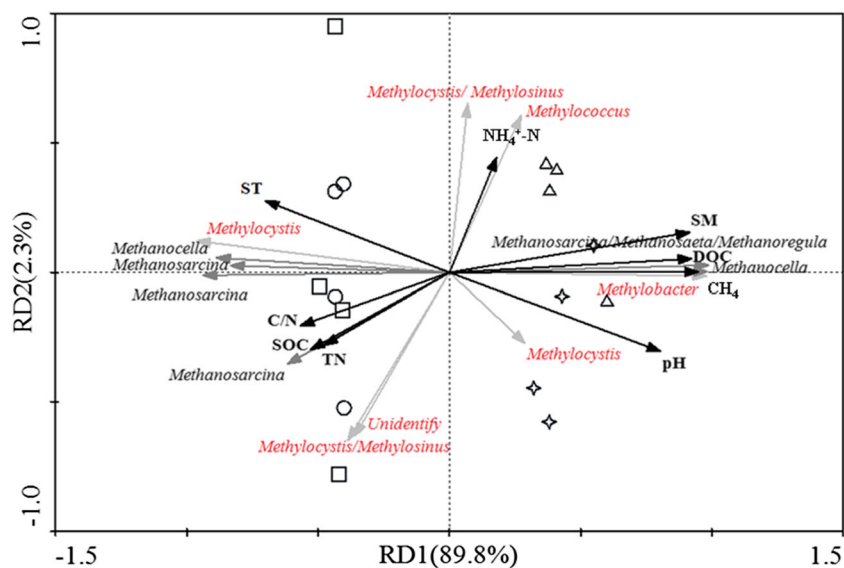
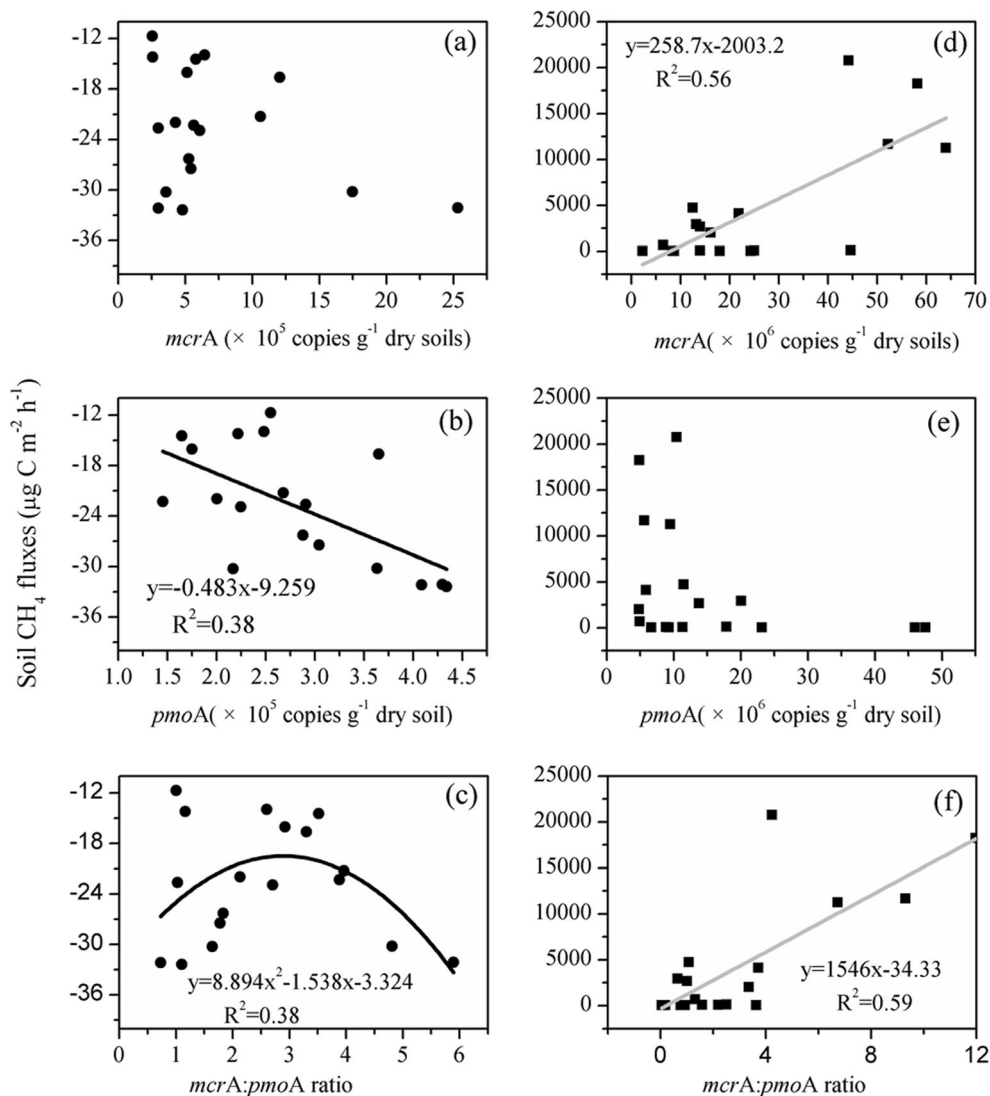


Fig. 4 RDA showing the relationships among land-uses (symbols), soil properties (black vectors), and relative abundance of methanogens (gray vectors) and methanotrophs (light gray vectors). The data are shown for four different land uses, namely, orchard with fertilization (circles); orchard without fertilization (square); paddy with fertilization (triangles); and paddy without fertilization (stars). *SOC* soil organic carbon contents, *DOC* soil dissolved carbon contents, *SM* soil moisture, *ST* soil temperature, *TN* total nitrogen contents, *C/N* C:N ratio

relationship between CH₄ fluxes and *mcrA* abundance and *mcrA*:*pmoA* ratio fitted the linear equations (Fig. 5d, f). The multiple regression analysis confirmed the correlations between CH₄ uptakes and *pmoA* abundance in the orchards (Table 4). Meanwhile, the DOC contents and *mcrA* abundance were the main factors controlling soil CH₄ fluxes from the paddies (Table 4).

Fig. 5 Relationships between soil CH₄ fluxes and *mcrA* abundance, *pmoA* abundance, and *mcrA:pmoA* ratio in orchard plots (a–c) and paddy fields (d–f)



Discussion

Soil CH₄ fluxes

The seasonal variations of soil CH₄ flux in this study exhibited similar patterns to those documented in the paddy fields and upland plots (Eusufzai et al. 2010; Wang et al. 2015). The paddy fields showed higher CH₄ fluxes than those from orchard plots, especially in the flooding period (Fig. 1b, c), which was consistent with the argument that drainage would bring about the disappearance of CH₄ emissions but appearance of CH₄ uptakes (Jäckel et al. 2001; Nishimura et al. 2008). It is noteworthy that several reasons might contribute to the reduced CH₄ emissions induced by such land-use conversion. First, CH₄ emissions could have probably been caused by anaerobic soil conditions induced by flooding depth on soil surface (data are not shown). It has been reported that

longer period of flooding in paddy fields significantly leads to anaerobic soil conditions, in contrast to the aerobic soil condition in upland orchard fields (Inubushi et al. 2003; Nishimura et al. 2008). Second, soil DOC is an important parameter of CH₄ emissions from paddy soils because the available carbon substrates supply energy sources and consequently enhance methanogenic activity (Zou et al. 2005; Liu et al. 2015b). According to the common experiences, some crop residue was typically incorporated in the paddies, which would result in more soil DOC contents (Table 2). Lastly, land-use conversion could markedly influence the abundance and community structures of methanogens and methanotrophs, thus determining the soil CH₄ fluxes (Jäckel et al. 2001; Liu et al. 2015a). From the microbiological point, CH₄ fluxes are largely determined by methanogenic archaea and methanotrophic bacteria. Therefore, changes in the CH₄ fluxes induced by land-use conversion or fertilization would

be inherently caused by the responses of these two functional groups. In the following paragraphs, we will illuminate how these factors affect the soil CH₄ fluxes.

Although fertilization could reduce CH₄ fluxes in some cases (Liu et al. 2016; Zhou et al. 2016), it might increase or fail to affect soil CH₄ fluxes depending on the fertilizer rate (Fan et al. 2016; Reay and Nedwell 2004; Wang et al. 2015). As it is well known that various factors lead to the controversial response of soil CH₄ fluxes induced by fertilization. On the one hand, an increase in denitrification intermediates (N₂O, NO, NO₂⁻) induced by fertilization can inhibit methanogens (Bao et al. 2016). On the other hand, NH₄⁺ could suppress CH₄ oxidation by binding to the particulate methane monooxygenase and reacting with it (Schimel 2000). Nevertheless, nitrogen fertilizers were shown to be advantageous for the growth of methanogens and some methane-oxidizing bacteria (Bodelier et al. 2000; Lu and Conrad 2005). In this study, fertilization did not significantly affect the abundance of *mcrA* and *pmoA* gene (Table 3), which might be explained by that the inhibiting and stimulating effects of fertilization on the methanogens and methanotrophs were almost equal. Similarly, Dan et al. (2001) proposed that fertilization stimulated both CH₄ production and CH₄ oxidation, resulting in no significant effect on CH₄ fluxes.

Abundance and community compositions of methanogens and methanotrophs

In the present study, the abundance of methanogens was significantly lower in the orchard plots than that in the paddy plots (Table 3), which means that land-use conversion from paddies to orchards decreased the abundance of methanogenic archaea. Recently, Liu et al. (2015a) also observed significant decreases in the abundance of methanogens as a result of a relatively lengthy duration of upland conditions in the field study. However, methanogens have been shown to survive well without any distinctive changes in the copy numbers under upland crop cultivation for 7 months (Ma and Lu 2011). These findings together confirmed our previous results that soils were CH₄ emissions sources in the early days after land-use conversion from paddies to orchards, and became CH₄ uptakes in the second conversion stage (Liu et al. 2016). Nevertheless, the results on the existence of methanogenic archaea in upland plots are highly controversial (Angel et al. 2012; Hu et al. 2013; Lammel et al. 2015). In our results, methanogenic archaea were detected in the newly converted orchard plots (1 year). A possible explanation might be that the presence of detoxification enzymes, such as superoxide dismutase and catalase in some members of methanogens, defended them from harmful products derived from oxygen (Brioukhanov et al. 2000). Therefore, a long-term study on the abundance of methanogenic archaea in an age-sequence of

converted orchards should be conducted to clearly show how long they would be survived after land-use conversion from paddies to orchards.

The abundance of methanotrophs in the orchard plots was expected to be higher than that in the paddy fields, as that methanotrophs were considered to be more common in upland fields (Singh et al. 2009; Wang et al. 2015; Lammel et al. 2015). Surprisingly, the abundance of methanotrophs was decreased after land-use conversion from paddies to orchards (Table 3), demonstrating that the abundance of methanotrophs was actively increased in the paddy plots. These results of no significant increases in the methanotrophs abundance in orchard plots were in well agreement with those stated by Jaatinen et al. (2004) who observed higher CH₄ oxidizing potential in a Finnish mire than in a well-drained soil. Similarly, Mohanty et al. (2016) also proved that methanotrophs were generally activated at high CH₄ concentrations in the culture experiment. Moreover, the significant positive correlations between *pmoA* abundance and CH₄ fluxes in paddy plots confirmed the stimulation of *pmoA* by CH₄ concentrations in the paddy soils (Table 4). Furthermore, although the abundance of *mcrA* and *pmoA* differed with time, there was no clear response to fertilization at subsequent “pre-fertilization” date (Fig. 2a, b), because our sampling time was not at each “fertilization” date. These variations across time suggested that temporal dynamics were more important in structuring the microbial processes in the study site.

Our study showed that *Methanosarcina* and *Methanocella* were observed in the orchard plots and paddy fields, whereas *Methanosaeta* and *Methanoregula* were just detected in the paddy fields (Fig. 3a). Those results suggested that land-use conversion from paddies to orchards resulted in a rather poor diversity of methanogens, which were in well agreement with the statement of former results (Liu et al. 2015a; Scavino et al. 2013). Generally, *Methanosarcina* and *Methanocella* were the most abundant methanogens in paddy fields, probably due to their tolerance to oxygen (Angel et al. 2011). However, methanogens belonging to the family *Methanosarcina* were also detected in the orchard plots owing to their ability to utilize a range of different precursors for methanogenesis (Jetten et al. 1990). *Methanocella* were known for their ability to withstand toxic oxygen populations (Erkel et al. 2006), which made them better adapted to both paddy and orchard soils (Fig. 3a). In addition, it has been reported that a relatively shorter period of drainage of 7 months may be less harmful to the methanogen community structure (Ma and Lu 2011). Fertilization were shown to have no significant effects on the community compositions of methanogens, which corresponded well with the results of Ho et al. (2016) who proposed the theory of site history and the “resting” community.

Land-use conversion increased type II methanotrophs (i.e., *Methylocystis* and *Methylosinus*), and decreased type I

methanotrophs (i.e. *Methylobacter* and *Methylococcus*) (Fig. 3b). In a study on three land-use types, the community compositions of methanotrophic bacterial were observed to be strongly affected by land-use changes (Zheng et al. 2010). Former studies on the field study indicated that the methanogen communities did not change in the fallow period of rice paddies (Krüger et al. 2005; Ma and Lu 2011). However, there was no field observation on the methanotrophs community induced by land-use conversion from paddies to orchards, thus more studies are urgently needed. In general, soil properties select particular groups of microorganisms potentially by shaping the ecological niches such as substrates concentration and soil properties of the targeted microorganisms (Fig. 4). In addition, although fertilization was found to decrease the T-RF of 13 bp in the orchard plots, no consistent patterns could be generalized with respect to the effects of fertilization on methanotrophs (Alam and Jia 2012).

Correlations between soil CH₄ fluxes and methanogens and methanotrophs

The conventional explanation for soil CH₄ fluxes induced by land-use conversion included soil condition and physical properties (Inubushi et al. 2003; Nishimura et al. 2008). In the present study, soil CH₄ fluxes were significantly related to the abundance of *mcrA* and soil DOC contents (Table 4), indicating that CH₄ production and carbon substrates were directly related to the CH₄ emissions from paddy soils. Meanwhile, *mcrA* and *pmoA* genes play an obvious mechanistic role in the actual CH₄ fluxes from paddy soils (Lee et al. 2014), as that significant positive relationships between CH₄ fluxes and *mcrA/pmoA* were observed (Fig. 5f). Moreover, it is well known that most of the methanogens could utilize H₂/CO₂ for CH₄ production, while *Methanosaeta* can utilize acetate (Metje and Frenzel 2007). Furthermore, *Methanosaeta* have been described as slow-growing microorganisms and have a much lower acetate threshold (Jetten et al. 1990). In general, CH₄ productions from acetate are much higher (typically 67%) than those from H₂/CO₂ (generally 33%) (Metje and Frenzel 2007). Thus, in the present study, the survival of *Methanosaeta* in the paddy plots might have resulted in more CH₄ emissions, which might be an alternative explanation for more CH₄ fluxes from paddy soils.

Intuitively, the potential CH₄ uptakes in the uplands can be attributed to the stimulation of methanotrophs. Though abundance of methanotrophic bacteria was decreased after land-use conversion (Table 3), the soil CH₄ uptakes of orchard plots were negatively correlated with the *pmoA* abundance (Fig. 5b). The result suggested that *pmoA* abundance could dominate soil CH₄ uptakes in the upland plots, which was in line with former studies (Singh et al. 2009; Wang et al. 2015). Moreover, despite the *pmoA* abundance decreased (Table 3),

the type II methanotrophs increased in the orchard plots (Fig. 3b), which could result in the CH₄ uptakes. Singh and Tate (2007) also reported that samples of forest soil, where type II methanotrophs were predominant, were at least 10 times more efficient in oxidizing CH₄ than those of pasture soil in which type I methanotrophs were predominant. Thus, effective CH₄ oxidation induced by land-use conversion from paddies to orchards could be owing to the significant stimulation of type II methanotrophs. Moreover, it was suggested that type I methanotrophs participated in CH₄ consumption only from 50 ppmv methane upward, whereas type II methanotrophs consumed at lower methane concentrations (Knief et al. 2006). Therefore, we could speculate that the methanotrophs of paddy soils mainly oxidized the soil CH₄ productions, and those in the orchard soils consumed the atmospheric CH₄. In addition, it has been reported that fertilization did not affect CH₄ consumptions of the soil where type I methanotrophs predominant, but inhibits CH₄ uptakes in the soil predominantly comprising type II methanotrophs (Mohanty et al. 2006). Conversely, in the present study, the soil was clearly dominated by type II methanotrophs, the CH₄ consumption was not inhibited by fertilization, as some others reported that a few methanotrophs genera are shown to be able to withstand nitrogen species (Noll et al. 2008).

Conclusion

Taken together, our results suggested that land-use conversion from paddies to orchards change soils from emission sources to sink of atmospheric CH₄, whereas fertilization did not significantly affect soil CH₄ fluxes under the two land-uses. The conversion of paddies to orchards decreased the abundance of methanogens and methanotrophs, resulted in poor diversity of methanogens community, and increased type II methanotrophs. The higher CH₄ emissions from paddy soils could mainly attribute to the increased soil DOC contents, more methanogens, and compositions of methanogenic archaea. The compositions of methanotrophs and soil conditions together drive the ability of oxidizing atmosphere CH₄ of orchard soils. Moreover, no distinct changes in methanogens and methanotrophs could help to explain the stable response of CH₄ fluxes to fertilization. Overall, we provide a certain extent evidence of microbial mechanisms for the soil CH₄ fluxes induced by land-use conversion and fertilization. Moreover, the findings of CH₄ fluxes and dynamics of methanogens and methanotrophs following land-use conversion and fertilization could provide new insights into the soil carbon biogeochemical cycles in red soils.

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