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Microbial mechanisms in the reduction of CH_4 emission from double rice cropping system amended by biochar: A four-year study

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ABSTRACT

Biochar amendment can reduce CH_4 emissions from paddy soils. However, little is known about how the soil microbial communities associated with paddy soil CH_4 emissions respond to biochar aging after biochar amendment. In this study, we examined the effects of biochar on CH_4 emissions, soil properties, and abundance/community composition of methanogens and methanotrophs in a double rice cropping system from 2012 to 2016. Straw-derived biochar was applied once in 2012 at 24 and 48 t ha^{-1}. Biochar application decreased the annual CH_4 emissions by 20–51%. Biochar increased the abundances of both methanogens and methanotrophs, with a larger increase of methanotrophs than methanogens in the first year, mainly caused by the increases in soil dissolved organic carbon, NH_4^+-N, and porosity. Biochar suppressed the abundance of methanogens and had little effect on the methanotrophs in the following three years, probably due to the increased soil porosity. Eventually, the ratios of abundance of methanogens to methanotrophs decreased by 11–31% in each of the four years and were positively correlated to CH_4 emissions. Biochar addition increased the relative abundances of *Methanocella* and *Methanospirillum* and reduced those of *Methanoregula* and *Methanosaeta* for methanogens, while it increased the proportion of the basophilic methanotrophs *Methylomicrobium* and decreased that of *Methylocaldum* in the growing season 2014. Our results demonstrate that biochar aging greatly alters the responses of abundances and community compositions of soil methanogens and methanotrophs to biochar addition. The reduction of CH_4 emissions owing to biochar in the long run was probably mainly due to the lesser suppression of abundance and activity of methanotrophs compared with methanogens.

1. Introduction

Rice paddy fields are major greenhouse gases (GHG) emission sources. CH_4 accounts for > 75% of the CO_2-equivalence of the total GHG emissions from paddy fields (FAO, 2016; Liu et al., 2014; Su et al., 2015; Wu et al., 2018). Annual paddy field CH_4 emissions account for 12% of the global total (IPCC, 2013). Moreover, the CH_4 emissions from rice paddy fields have been increasing at the rate of 1.2 Tg per decade between 1961 and 2016 (1961: 17.4 Tg; 2106: 24.4 Tg) (FAO, 2016). Therefore, decreasing paddy CH_4 emissions could substantially mitigate global GHG emissions.

Biochar is produced from the pyrolysis of organic material (Meyer et al., 2011). It has a high recalcitrant carbon (C) content and is porous and alkaline (Lehmann, 2007). Biochar can store C in soils for long periods of time (Lehmann and Joseph, 2015). Biochar can also improve soil fertility and crop yields (Hussain et al., 2016; Liu et al., 2016; Sui et al., 2016). As reported by Liu et al. (2016), straw-derived biochar amendment increases soil pH, total organic carbon (TOC), and available P and K. In a two-year field trial, biochar enhanced rice grain yield by 8.5–10.7% compared to the unamended control. Numerous studies have shown that biochar consistently lowered CH_4 emissions from paddy fields (Chen et al., 2018; Khan et al., 2013; Qin et al., 2016; Wang et al., 2018). Qin et al. (2016) reported that application of straw-derived biochar produced from pyrolysis at 350–500 °C significantly

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decreased the average seasonal total CH_4 emissions by 18–40% over eight rice cropping seasons. In a pot experiment, Khan et al. (2013) showed that sewage sludge biochar transformed paddy soil into a CH_4 sink, in which the added biochar was produced at pyrolysis temperatures of 550 °C with a residence time of 6 h. Nevertheless, some studies also showed that biochar amendment could not significantly affect CH_4 emissions (Liu et al., 2015), and even increased CH_4 emissions in paddy soils (Koyama et al., 2015; Singla et al., 2014). In these instances, corn straw (Liu et al., 2015), rice husk (Koyama et al., 2015), and biogas digested slurry (Singla et al., 2014) derived biochars were produced by pyrolysis at 400 °C, 350–400 °C, and 370 °C with residence times of 10 h, 15 min, and 7 min, respectively. The different responses of paddy CH_4 emissions to biochar amendment may be correlated to biochar pyrolysis temperature, residence time, and feedstock. In general, the CH_4 emissions from paddy soils with biochar amendment decreased with increasing biochar pyrolysis temperature and residence time, and increased with the proportion of bioavailable C and N from the biochar derived from raw materials (Lehmann and Joseph, 2015).

The microbial mechanisms of CH_4 emission reduction by biochar have been investigated in experiments within the first year after biochar application. For example, the studies of Feng et al. (2012), Liu et al. (2011), and Qin et al. (2016) were conducted in 116, 49, and 365 days after a single biochar amendment, respectively. Methanogens and methanotrophs regulate paddy field CH_4 emissions (Conrad, 2007; Dubey et al., 2014). Organic material decomposition in paddy soil generates copious CH_4 since methanogens use H_2, CO_2, and acetic acid as substrates for CH_4 formation (Conrad, 2002). However, up to 90% of the produced CH_4 can be oxidized by methanotrophs before CH_4 escapes to the atmosphere (Bosse and Frenzel, 1997). Biochar amendment significantly decreases paddy field CH_4 emissions mainly by increasing methanotroph abundance (Feng et al., 2012; Qin et al., 2016). Feng et al. (2012) found that a single biochar addition significantly increased methanogen abundance and decreased methanogen/methanotroph ratios within one rice season. Therefore, biochar significantly decreased CH_4 emissions in paddy soils. Qin et al. (2016) reported that significant decreases in CH_4 emissions in response to biochar treatment could be explained by increases in methanotroph biodiversity and abundance. Nevertheless, biochar amendment had no significant effect on methanogen abundance in a paddy field one year after a single biochar application. Potential explanations of this phenomenon are that biochar addition enhanced labile C content and provided an optimum soil pH (6–8) in acid soils (Hanson and Hanson, 1996; Le Mer and Roger, 2001). Thus, the pH overlaps with the optimum pH range of methanogens and methanotrophs (Le Mer and Roger, 2001; Semrau et al., 2010), but the increase of methanotroph abundance may be greater than that of methanogen abundance because the methanotrophic community was more sensitive to the rise of soil pH than methanogens (Jeffery et al., 2016). Additionally, the high porosity and pH of biochar may increase methanotroph abundance by enhancing their ability to capture CH_4 and by decreasing Al^{3+} toxicity levels for methanotrophs (Tamai et al., 2007). However, denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) data indicated that biochar addition does not significantly affect methanogen or methanotroph community structure (Feng et al., 2012; Liu et al., 2011). Moreover, changes in microbial community structure were not significantly correlated with decreases in CH_4 emissions (Feng et al., 2012; Liu et al., 2011; Qin et al., 2016).

Less is still known on the microbial mechanisms of CH_4 mitigation affected by biochar aging following a single application in soils for longer than two years. The effects of biochar on soil microbial communities may vary with time in soils because biochar ages and its physical properties, aromatic moieties, and labile fractions change over time (Kuzyakov et al., 2014; Quilliam et al., 2013; Zhu et al., 2017). Spokas (2013) found that there were increased N_2O emissions and reduced CH_4 oxidation for the soil amended with three-year aged biochar as compared to the soil amended with fresh biochar. He ascribed these results to the decrease in the presence of microbial chemical inhibitors in the aged biochar. One-year aged biochar had also been reported to increase soil microbial biomass and potential activities of chitinase, cellobiohydrolase, and xylanase under flooding condition as compared with fresh biochar (Wang et al., 2016b). Therefore, we hypothesized that in paddy fields with biochar addition, 1) the changes of abundances and community structures of soil methanogen and methanotroph cause the reduction of CH_4 emissions; 2) the microbial mechanism for the reduced CH_4 emissions varies with time owing to a change in soil properties with biochar aging. To test the hypotheses, the effects of a single biochar application on CH_4 emissions, soil physicochemical properties as well as the abundances and community structures of soil methanogen and methanotroph were investigated in a double rice cropping system over a four-year period (2012–2016).

2. Materials and methods

2.1. Experimental site

The field experiment was conducted over four annual cycles from April 2012 to April 2016 in a typical double rice cropping system (~50 y old) in Jinjing, Changsha County, Hunan Province, China (28°33′04″N, 113°19′52″E, elevation 80 m). Each cycle consisted of early rice, late rice, and fallow seasons. The region has a subtropical monsoon climate with mean annual precipitation and air temperature of 17.5 °C and 1330 mm, respectively. The frost-free period is ~300 d y^{-1}. Meteorological data were recorded by a weather station (Inteliment Advantage; Inteliment Technologies, Philadelphia, PA, USA) near the sampling sites. The paddy soil was classified as a Stannic Anthrosol according to Chinese soil taxonomy (Gong et al., 2007), an Ultisol according to USDA soil taxonomy, and a Hydragric Anthrosol according to the World Reference Base (FAO, 2015) (Table 1).

2.2. Experimental design

The long-term field experiment started in 2012. A single-factor randomized block design was used. It consisted of three treatments and three replicated plots (7 m × 5 m). The three treatments were: (a) no biochar application (control (CK)); (b) low biochar application rate (24 t ha^{-1}) corresponding to 1% of the topsoil (0–20 cm) weight (LB); and (c) high biochar application rate (48 t ha^{-1}) corresponding to 2% of the topsoil weight (HB). The biochar was purchased from Sanli New Energy Ltd. (Shangqiu, China) and produced from wheat straw pyrolyzed at 500 °C (Liu et al., 2014; Shen et al., 2014; Wang et al., 2018). The basic properties of the biochar are listed in Table 1. The biochar was evenly applied over the experimental plots and thoroughly mixed into the plow layer on April 25, 2012. No further biochar applications were made during the experiment. Chemical fertilizers were added at

Table 1
Basic properties of experimental field soil and biochar.

	TOC (g kg^{-1})	Total N (g kg^{-1})	Total P (g kg^{-1})	Total K (g kg^{-1})	pH	CEC (cmol kg^{-1})	Bulk density (g cm^{-3})	Ash content (%)	Sand (%)	Silt (%)	Clay (%)
Soil	17.5	1.62	0.55	28.4	5.1	8.0	1.31	–	42.4	30.4	27.2
Biochar	418.3	5.80	1.20	9.2	9.3	28.6	0.18	37.2	–	–	–

the same rate for all three treatments. Urea (120 kg N ha^{-1} in the early rice season and 150 kg N ha^{-1} in the late rice season) was applied three times each in the early and late rice season. Half of it was used as a basal fertilizer before transplanting, another 30% was applied at early tillering, and the remaining 20% at heading. Calcium superphosphate (40 kg P_2O_5 ha^{-1}) and potassium sulfate (100 kg K_2O ha^{-1}) were applied once as basal fertilizers before transplanting. The irrigation regime followed the local practice: initial flooding, then midseason drainage, then reflooding, and final drainage. Early-season rice (*Oryza sativa* L., cv. 'Xiangzaoxian No. 45') seedlings were transplanted at a hill density of 16.7 cm × 20.0 cm between April 27 and May 4 and harvested between July 12 and July 18. Late-season rice (*Oryza sativa* L., cv. T-you 207) seedlings were transplanted at a 20.0 cm × 20.0 cm hill density between July 19 and July 25 and harvested between October 18 and October 24.

2.3. CH_4 emission measurement

CH_4 flux was determined by the static opaque chamber-gas chromatograph method according to Zheng et al. (2008a). Stainless steel collars (0.64 m × 0.64 m) were inserted into the soil in each plot to a depth of 20 cm. Rice seedlings were transplanted into the collar at the normal density. Stainless steel opaque chambers (0.64 m long × 0.64 m wide × 1.00 m high) were covered with insulating foam, sealed watertight, and temporarily mounted onto the collars for gas flux measurements. The top of each chamber was fitted with a three-way stopcock, a portable digital thermocouple (JM624, Tianjin Optical Precision Instrument Co. Ltd., Tianjin, China) to measure air temperature, and a circulating fan to mix the air. Gases were sampled from 9:00 to 11:00 a.m. weekly and twice a week during the aeration and fertilization periods. Five gas samples were collected per chamber with 60 mL propylene syringes at 0 min, 10 min, 20 min, 30 min, and 40 min after the chamber enclosed the stainless steel collar. The air samples were compressed into pre-evacuated 12-mL glass vials sealed with butyl rubber lids and then analyzed in the laboratory. CH_4 concentrations were determined with a gas chromatograph (Agilent 7890D; Agilent Technologies, Santa Clara, CA, USA) fitted with a hydrogen flame ionization detector (FID) to analyze CH_4 at 250 °C as described by Zheng et al. (2008a). CH_4 flux was calculated by linear regression of the rate of change in CH_4 concentration during the period in which the chamber was sealed. The flux rate was rejected if the regression coefficient was < 0.90. Chamber height, internal air temperature, and external air pressure were also recorded (Zheng et al., 2008a). The annual CH_4 flux rates were sequentially accumulated from the emissions averaged for every two adjacent measurement intervals (Fu et al., 2012; Zou et al., 2005). Average emission and standard error (SE) for each treatment were calculated from three replicates.

2.4. Soil sampling and physicochemical analysis

Five soil sample cores (0–20 cm depth) were collected from each plot with a stainless steel auger (internal diameter of 3 cm). The soil samples were thoroughly mixed to make a composite sample. Visible gravel and plant residue were removed, and each soil sample was divided into two parts. One was stored at 4 °C, and then air dried at room temperature (10–25 °C) for physicochemical analysis. The other was stored at −80 °C until DNA extraction. During the 2012–2016 annual cycles, soil samples were collected approximately once monthly to measure ammonium (NH_4^+-N), nitrate (NO_3^--N), and dissolved organic carbon (DOC) during the cropping seasons. NH_4^+-N, NO_3^--N, and DOC were extracted with 0.5 M K_2SO_4. Soil samples from the tillering and ripening stages were also taken to analyze microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN). MBC and MBN were determined by the chloroform fumigation and extraction method (Wu et al., 1990, 2006). DOC and MBC were measured with a total organic carbon analyzer (TOC-VWP; Shimadzu Corporation, Kyoto, Japan). NH_4^+-N, NO_3^--N, and MBN was measured with a flow-injection auto-analyzer (Tecator FIA Strar 5000 Analyzer; Foss A/S, Hillerød, Denmark). Soil samples were collected to determine pH, total organic carbon (TOC), and total soil nitrogen (TSN) in the ripening stage in each late rice season. For pH determination, the samples were air-dried and milled to pass through a 2-mm sieve. To measure TOC and TSN, they were finely milled to pass through a 0.15-mm sieve. Soil pH, TOC, and TSN were determined according to the methods of Bao (2005). The pH was measured with a pH meter (Metro-pH320; Mettler-Toledo Instruments Shanghai Co. Ltd., Shanghai, China) at a soil:water ratio of 1:2.5. TOC was determined by wet digestion with H_2SO_4–K_2CrO_7. The sample for TSN measurement was first digested with H_2SO_4 using $CuSO_4$, K_2SO_4, and Se as catalysts. After digestion, the N content was measured with a flow-injection auto-analyzer (Tecator FIA Strar 5000 Analyzer; Foss A/S, Hillerød, Denmark). Soil sample collection frequency and parameters are listed in Table S1.

2.5. DNA extraction

The DNA in each composite soil sample collected at tillering (August 22, 2012, August 22, 2013, May 27, 2014, August 20, 2014, May 20, 2015, and August 9, 2015), flowering (June 25, 2014, June 18, 2015, and September 12, 2015), filling (October 5, 2014), ripening (July 19, 2014, October 19, 2014, July 13, 2015, and October 19, 2015), and the fallow season (April 22, 2013 and March 25, 2015) was extracted according to the method of Chen et al. (2010) with minor modifications (Table S1). MP FastPrep®-24 (MP Biomedicals, LLC, Santa Ana, CA, USA) was used in the first step instead of the 1-h water bath treatment. The concentration and quality of extracted DNA were measured with a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

2.6. Real-time PCR

Methanogen and methanotroph abundances were measured in triplicate by qRT-PCR (ABI Prism 7900; Applied Biosystems, Foster City, CA, USA) using the primer sets MLF/MLR (Luton et al., 2002) and A189F/Mb661R (Kolb et al., 2003). These targeted the methyl coenzyme M reductase (*mcrA*) and particulate CH_4 monooxygenase (*pmoA*) genes, respectively. Each reaction was performed in a 10-μL mixture containing 5 μL of 2 × SYBR Green PCR Master Mix (TaKaRa Bio Inc., Shiga, Japan), 0.2 μL Rox Reference Dye II, 0.3 μM each of the forward and reverse primers, and 5 ng DNA template. The amplification conditions for *mcrA* were as follows: 95 °C for 3 min; 5 cycles of 95 °C for 30 s, 48 °C for 45 s, and 72 °C for 30 s; 30 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. The thermal cycling conditions for *pmoA* were as follows: 95 °C for 5 min; 5 cycles of 95 °C for 25 s, 65 °C for 30 s, and 72 °C for 30 s; 35 cycles of 95 °C for 25 s, 55 °C for 30 s, and 72 °C for 30 s. Standard curves were generated using 10 × stepwise dilutions of plasmid DNA with the target genes. Quantitative real-time PCR was performed in triplicate. The reaction efficiencies were 96–108% and R^2 was 0.995–0.998.

2.7. Illumina HiSeq sequencing

In the 2014–2015 annual cycle, the primer pairs 1106F/1378R (Watanabe et al., 2007), 197F/533R, and 142F/533R (Tsien et al., 1990) were used to amplify the 16S rRNA gene fragments of the methanogenic archaea and the type I and II methanotrophs, respectively, in the late rice (tillering and ripening stages) and the fallow seasons. A unique 6-nt barcode was added to the 5′ end of the forward primers to distinguish the PCR products. All PCR amplifications were performed in 30-μL reaction volumes containing 15 μL of 2 × Phusion Master Mix (New England Biolabs, Ipswich, MA, USA), 0.2 μM of both forward and reverse primers, and 10 ng template DNA. The thermal cycling conditions for the methanogenic archaeal 16S rRNA gene fragments were as

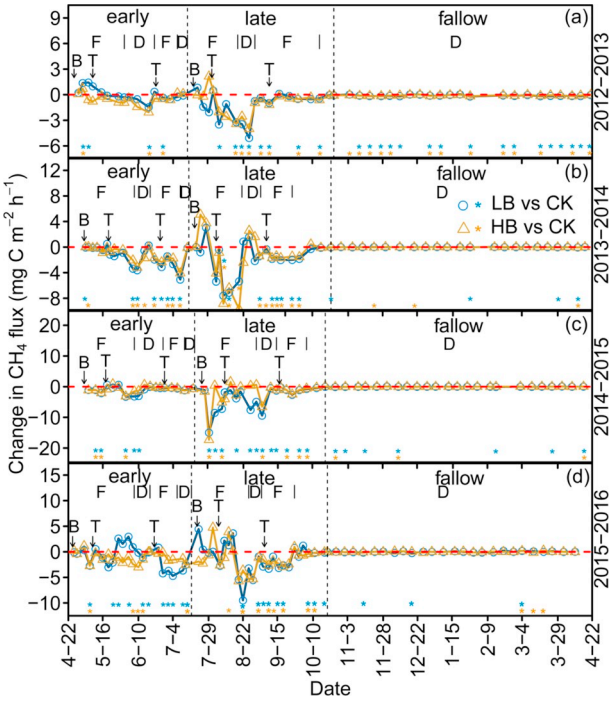

Fig. 1. Dynamic differences in CH_4 flux between biochar and CK treatments from the paddy field in the 2012–2013 (a), 2013–2014 (b), 2014–2015 (c), and 2015–2016 (d) annual cycles, respectively. CK: control. LB: 24 t ha^{-1} biochar. HB: 48 t ha^{-1} biochar. Early, late, and fallow: early rice, late rice, and fallow seasons, respectively. F and D: flooding and drainage periods for the four annual cycles, respectively. B and T: base fertilizer application and urea top-dressing dates, respectively. *: significant differences between biochar and CK treatments in each measurement period according to Duncan's multiple range test ($p < 0.05$).

follows: an initial denaturation at 98 °C for 1 min followed by 30 cycles of 95 °C for 10 s, 50 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min. The thermal cycling conditions for type I and II methanotrophic 16S rRNA gene fragments were as follows: an initial denaturation at 94 °C for 1 min followed by 31 cycles of 95 °C for 10 s, 50 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 5 min. The triplicate PCR products were pooled and mixed with equal amounts of 1 × loading buffer containing SYBR Green. They were separated by electrophoresis in 2% (w/v) agarose gel and purified with a GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA). The purified PCR products were sequenced by the Illumina HiSeq platform at Novogene Bioinformatics Technology Co. Ltd. (Beijing, China).

2.8. Sequence processing

Raw sequences were quality-filtered and demultiplexed with Quantitative Insights Into Microbial Ecology (QIIME) v. 1.91 (Caporaso et al., 2010) using the following criteria: 1) The reads were truncated at any position with > 3 consecutive quality scores ≤ 25.2) Reads with low-quality base calls ≥25% of the total sequence were discarded. 3) All sequences ≤200 bp for methanogenic archaeal 16S rRNA gene, and ≤250 bp for the 16S rRNA genes of type I and II methanotrophs were discarded. 4) The chimeric sequences were removed using a *de novo* algorithm. Operational taxonomic units (OTUs) were clustered with Usearch v. 7.0.1001 at a 97% similarity cutoff (Edgar, 2010); singleton OTUs were removed. To ensure equal sampling depth, the OTU tables were rarefied to 16,809, 1,054, and 1166 sequences per sample for methanogenic archaeal, type I, and type II methanotrophic 16S rRNA genes, respectively. Representative OTU sequences were aligned with PyNAST in the QIIME platform (Caporaso et al., 2010). The representative sequences were compared against the Silva 16S rRNA reference database (QIIME release 123) (Quast et al., 2013) using the rdp method with an 80% confidence threshold in QIIME v. 1.91 (Caporaso et al., 2010; Wang et al., 2007).

The raw sequences of the methanogenic archaeal and type I and II methanotrophic 16S rRNA genes obtained in this study were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the accession number PRJNA476849 and sample accession numbers SAMN09460594-SAMN09460620, SAMN09523800-SAMN09523826, and SAMN09534091-SAMN09534117.

2.9. Statistical analysis

One-way analysis of variance (ANOVA) was performed with Duncan's multiple range test to show significant differences between the treatments in R v. 3.3.1 (ISM, 2010) ($p < 0.05$). Repeated measurement analysis was applied to show the significant effects of biochar amendment, time series, and their interactions on the *mcrA* and *pmoA* copy numbers. OTU richness, Shannon indices, and abundance-based coverage estimators (ACE) were calculated in QIIME (Hill et al., 2003). Principal coordinate analyses (PCoA) and analyses of similarities (ANOSIM) based on the Bray-Curtis distance matrices of communities were performed to compare methanogen and methanotroph community structures between the treatments in R using the “ape” and “vegan” packages, respectively (Clarke, 1993; Oksanen et al., 2008; Paradis et al., 2004; Warton et al., 2012). Pearson correlation and Redundancy analyses (RDA) were performed to test the associations among the CH_4 emission rates, soil properties, and abundances and community compositions of methanogens and methanotrophs. RDA was performed in the “vegan” package, and ANOVA (999 permutations) was used to test the significance of RDA models in R (Oksanen et al., 2008).

3. Results

3.1. CH_4 emissions

During the four annual cycles, CH_4 fluxes were generally lower in the biochar treatments than they were in CK (Fig. 1). Relative to CK, the changes in daily CH_4 fluxes for the LB and HB treatments mainly showed negative values in the cropping seasons (with a mean of −1.36 and −1.18 mg C m^{-2} h^{-1} (corresponding to the change rates of −32% and −28%), respectively), while the changes were negligible during the fallow season (with a mean of −0.03 and −0.02 mg C m^{-2} h^{-1} (corresponding to the change rates of −57% and −45%), respectively). The large decrease of CH_4 emissions in biochar treatments mainly occurred during the tillering and mid-season drainage stages. There were also larger decreases of CH_4 emissions in the late rice season than in the early rice season, which might be due to that CH_4 emissions were much higher during the late rice season than during the early rice season since higher air temperature and the rice cultivar with a higher yield in the late rice season favored CH_4 emissions (Wang et al., 2018). In the fallow season, the paddy soils were drained and the air temperature during the period was also low, which could result in negligible CH_4 fluxes in paddy fields (Wang et al., 2018), and thus the changes in daily CH_4 fluxes for the biochar treatments were also small as compared to CK.

Biochar addition significantly decreased cumulative CH_4 emissions in the 2012–2016 annual cycles ($p < 0.05$) (Fig. 2). Compared to CK, the annual total CH_4 emissions for the LB and HB treatments were 35–40%, 40–34%, 51–30%, and 20–22% lower in the 2012–2013, 2013–2014, 2014–2015, and 2015–2016 annual cycles, respectively ($p < 0.05$) (Fig. 2). The average annual cumulative CH_4 emissions in the LB and HB treatments were 35% and 29% lower than that in the control, respectively ($p < 0.05$) (Fig. 2).

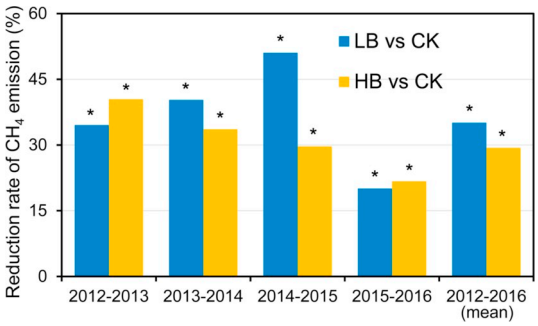

Fig. 2. Reduction rate in cumulative CH_4 emissions between biochar and CK in the 2012–2016 annual cycles. CK: control. LB: 24 t ha^{-1} biochar. HB: 48 t ha^{-1} biochar. *: significant differences between biochar and CK treatments within the same cycle according to Duncan's multiple range test ($p < 0.05$).

3.2. Soil properties related to CH_4 emissions

The measured soil properties all showed short-term or long-term responses which were affected by biochar amendment (Fig. 3). Soil NH_4^+-N, DOC, MBC, and MBN content in the biochar treatments mainly increased in the first year after biochar addition, and soil NH_4^+-N and DOC content also increased with biochar application rate ($p < 0.05$). There were no significant differences for soil NH_4^+-N ($p = 0.21$–0.89), DOC ($p = 0.07$–0.96), MBC ($p = 0.09$–0.74), and MBN content ($p = 0.15$–0.98) between CK and biochar treatments in the following three years, except that NH_4^+-N and DOC content for the biochar treatments significantly decreased in the 2013–2014 and 2015–2016 annual cycles, respectively, compared to CK ($p < 0.05$). Soil NO_3^--N content did not show significant differences across the treatments in the 2012–2015 annual cycles ($p = 0.26$–0.96), except that the biochar treatments had higher soil NO_3^--N content in the 2015–2016 annual cycle, compared to CK ($p < 0.05$). Soil MBC and

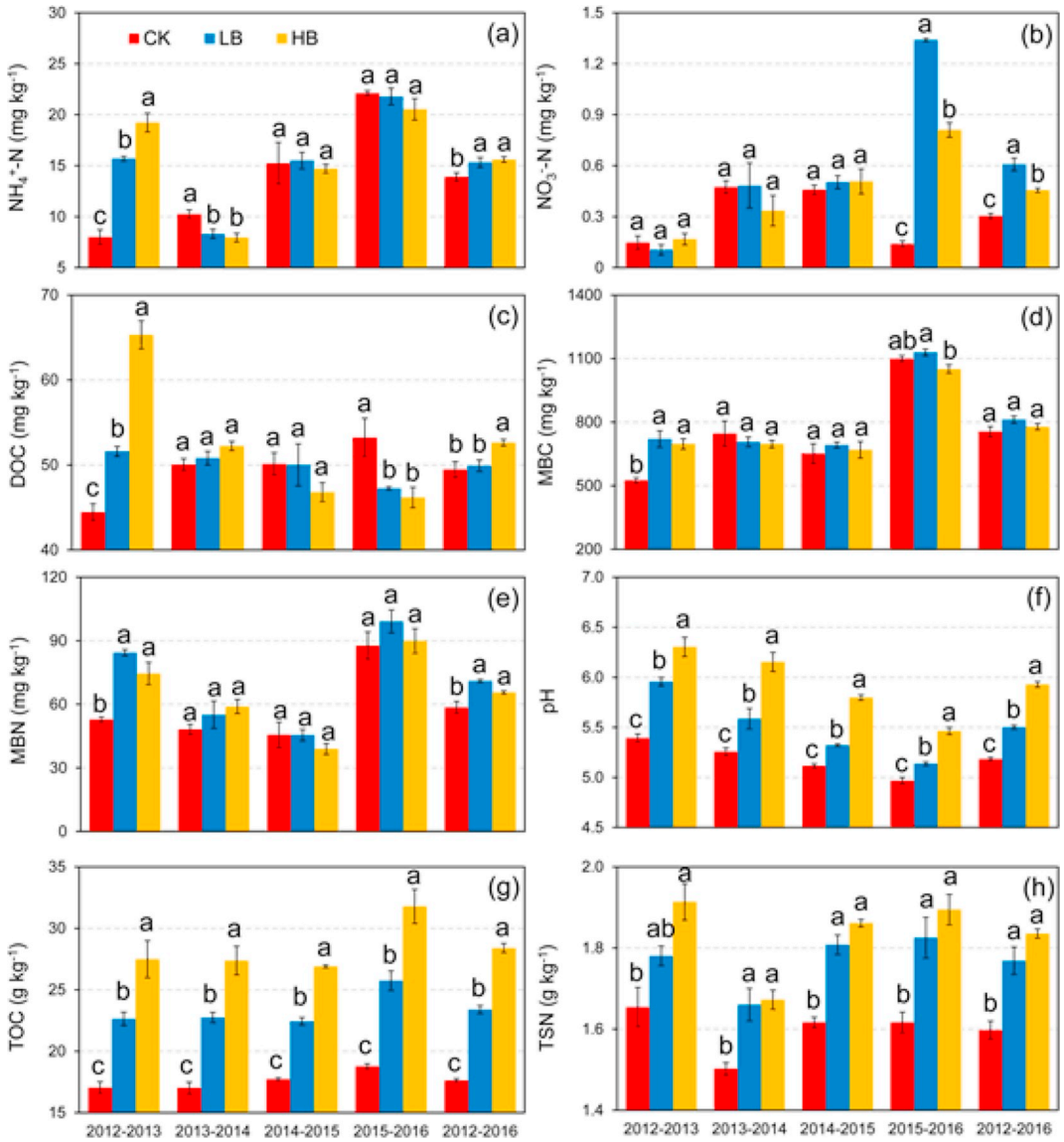

Fig. 3. Mean NH_4^+-N (a), NO_3^--N (b), DOC (c), MBC (d), MBN (e), pH (f), TOC (g), and TSN content (h) for the three treatments in the 2012–2016 annual cycles. CK: control. LB: 24 t ha^{-1} biochar. HB: 48 t ha^{-1} biochar. Different lowercase letters indicate significant differences between treatments within the same cycle at the 5% level according to Duncan's multiple range test. Vertical bars represent standard errors (n = 3).

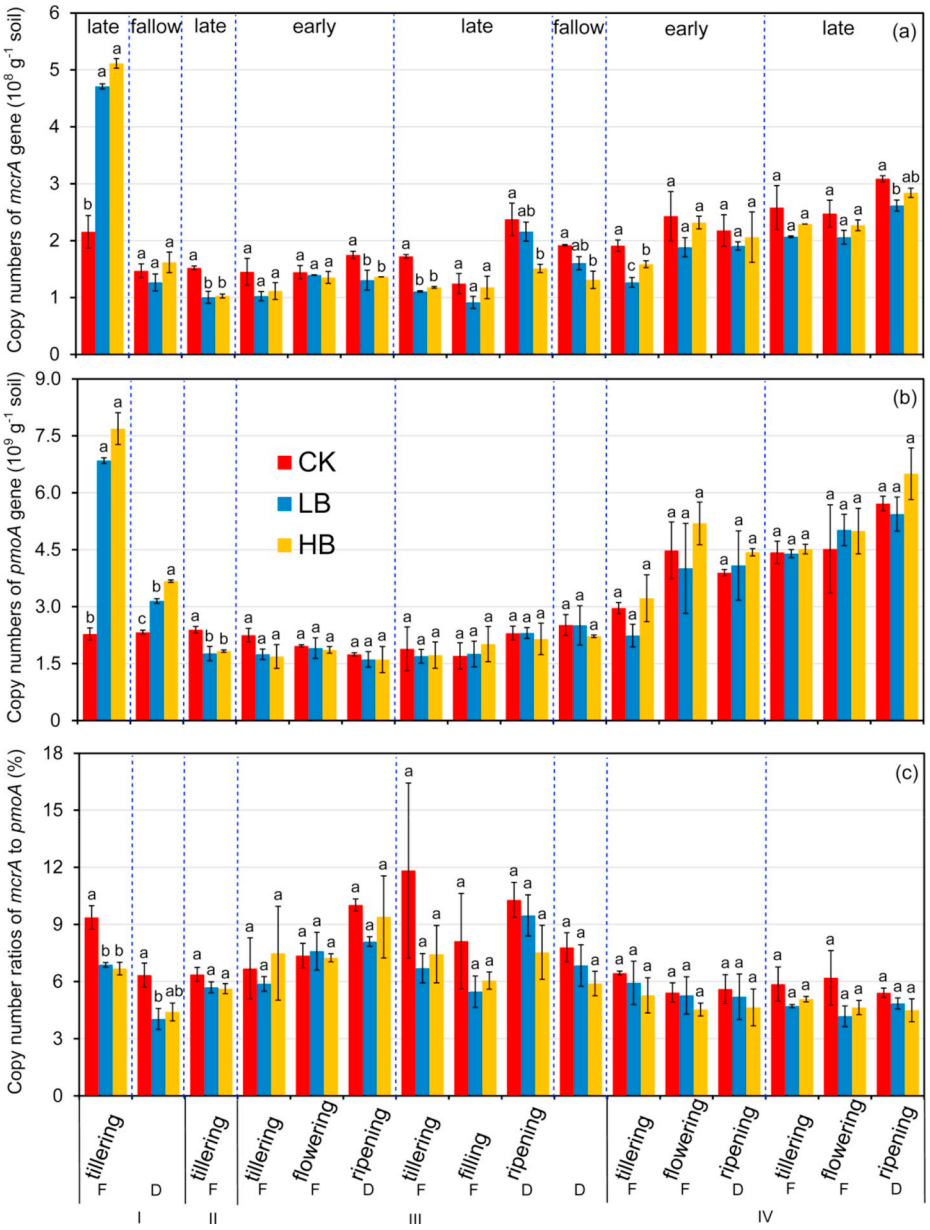

Fig. 4. Effects of biochar application on *mcrA* (a) and *pmoA* (b) copy numbers and *mcrA*: *pmoA* copy number ratios (c) in the 2012–2013 (I), 2013–2014 (II), 2014–2015 (III), and 2015–2016 (IV) annual cycles. CK: control LB: 24 t ha^{-1} biochar. HB: 48 t ha^{-1} biochar. Early, late, and fallow: early rice, late rice, and fallow seasons, respectively. Tillering, flowering, filling, and ripening: tillering, flowering, filling, and ripening stages, respectively. F and D: flooding and drainage periods, respectively. Different lowercase letters indicate significant differences among treatments at the same growth stage according to Duncan's multiple range test ($p < 0.05$). Vertical bars represent standard errors (n = 3).

NO_3^--N content in HB treatment were significantly lower in the 2015–2016 annual cycle, compared to LB treatment ($p < 0.05$). Soil pH, TOC, and TSN content in the biochar treatments all increased significantly as compared with CK in the 2012–2016 annual cycles ($p < 0.05$), and also increased significantly with biochar application rate except for TSN ($p = 0.06$–0.77).

3.3. Soil methanogenic and methanotrophic abundances

Across the four annual cycles and treatments, the temporal patterns of the methanogen and methanotroph abundances and the methanogen/methanotroph ratios significantly changed over time ($p < 0.001$) (Table S2; Fig. 4). The methanogen and methanotroph abundances for CK generally increased over the four annual cycles (Fig. 4a and b). The methanogen and methanotroph abundances of the biochar treatments showed a declining trend from the 2012–2013 annual cycle to the 2013–2014 annual cycle, then increased to maxima in the 2015–2016 annual cycle (Fig. 4a and b). The methanogen/methanotroph ratios for the treatments decreased from the 2012–2013 annual cycle to the 2013–2014 annual cycle, increased to the peaks during the 2014–2015 annual cycle, and then decreased again in the 2015–2016 annual cycle (Fig. 4c).

Compared to CK, biochar application increased methanogen and methanotroph abundances in the 2012–2013 annual cycle and decreased methanogen abundances in the 2013–2016 annual cycles (Fig. 4a and b), while it significantly decreased the methanogen/methanotroph ratios during the 2012–2016 annual cycles ($p < 0.05$) (Fig. 4c). Relative to CK, biochar treatments increased methanogen and methanotroph abundances at tillering by 118.7–137.4% and 200.2–237.0% in the 2012–2013 annual cycle, respectively ($p < 0.05$) (Fig. 4a and b). Methanogen and methanotroph abundances for the biochar treatments increased by 10.1% ($p = 0.51$) and 35.4–57.7% ($p < 0.05$) in the fallow season of the 2012–2013 annual cycle, respectively, but LB treatment decreased methanogen abundance by 13.9% ($p = 0.38$). There was no significant difference in methanogen and methanotroph abundances between LB and HB treatments in the 2012–2013 annual cycle ($p = 0.07$–0.52), except that methanotroph abundance for the HB treatment increased significantly, by 16.5% in the fallow season, compared to LB treatment ($p < 0.05$) (Fig. 4a and b). The methanogen abundances for the biochar treatments decreased

by 11.3–35.7%, 3.5–26.5%, 5.4–36.4%, and 16.4–31.6% at the tillering, flowering, ripening, and fallow stages in the 2013–2016 annual cycles, respectively (Fig. 4a), with an average reduction of 16.7–20.6% ($p < 0.05$) during the three-year cycles. Compared to CK, the biochar treatments decreased the methanotroph abundances by 23.5–26.2% at tillering in the 2013–2014 annual cycle ($p < 0.05$). Nevertheless, there were no significant differences in methanotroph abundance across the treatments during the 2014–2016 annual cycles ($p = 0.13$–0.99) (Fig. 4b). There was no significant difference in methanogen and methanotroph abundances between LB and HB treatments in the 2013–2016 annual cycles ($p = 0.06$–0.98), except that HB significantly increased the methanogen abundance by 25.2% at tillering in the early rice season of the 2015–2016 annual cycle, compared to LB ($p < 0.05$) (Fig. 4a and b). Compared to CK, the average methanogen/methanotroph ratios for the biochar treatments decreased by 29.4–30.5% in the 2012–2013 annual cycle ($p < 0.05$), by 16.9–17.5% in the 2013–2016 annual cycles ($p = 0.11$), and by 18.7–19.1% for all four annual cycles ($p < 0.05$) (Fig. 4c). Relative to LB, the average methanogen/methanotroph ratios in the HB treatment increased by 1.6% in the 2012–2013 annual cycle ($p = 0.89$), while it decreased by 0.7% ($p = 0.95$) and 0.4% ($p = 0.97$) in the 2013–2016 and 2012–2016 annual cycles, respectively (Fig. 4c).

For the 2012–2016 annual cycles, the correlation analysis showed that the methanogen abundances were significantly positively correlated to soil DOC, NH_4^+-N content, and pH ($p < 0.05$), and significantly negatively correlated with soil NO_3^--N content ($p < 0.01$) (Fig. S1a). The methanotroph abundances were significantly positively correlated with the DOC, NH_4^+-N, MBN, and pH ($p < 0.05$), and significantly negatively correlated to the CH_4 emissions ($p < 0.01$). The methanogen/methanotroph ratios were significantly positively correlated with the CH_4 emissions ($p < 0.001$), and significantly negatively correlated with MBC and TOC ($p < 0.05$) (Fig. S1a). Similar results can be found in the RDA (Fig. 5a). The RDA explained approximately 50.4% of the total variation in the abundances of methanogen and methanotroph, methanogen/methanotroph ratios, and CH_4 fluxes, and the first two axes explain 46.8% of the variation (Fig. 5a). The methanogen and methanotroph abundances, methanogen/methanotroph ratios, and CH_4 fluxes for biochar treatments in the 2012–2013 annual cycle were clearly separated from those for all treatments in the 2013–2016 annual cycles (Fig. 5a). Furthermore, the analysis showed that NH_4^+-N, NO_3^--N, DOC, MBC, and MBN content, and pH were significantly correlated with methanogen and methanotroph abundances, methanogen/methanotroph ratios, and CH_4 fluxes, thereby accounting for 5.7%, 9.1%, 7.9%, 4.2%, 11.3%, and 4.9% of the total variation, respectively ($p < 0.05$) (Fig. 5a). The methanogen and methanotroph abundances along axis 1 (36.6% of the total variation) were positively correlated with DOC, NH_4^+-N, and MBN content, and pH. The methanogen/methanotroph ratios and CH_4 fluxes along axis 2 (10.2% of the total variation) were negatively correlated with TOC, MBC, MBN, and NO_3^--N content (Fig. 5a). For the 2012–2013 annual cycle, the RDA explained approximately 30.6% of the total variation in the abundances of methanogen and methanotroph, methanogen/methanotroph ratios, and CH_4 fluxes, and the first two axes explain 30.0% of the variation (Fig. 5b). The methanogen and methanotroph abundances, methanogen/methanotroph ratios, and CH_4 fluxes in biochar treatments were clearly separated from those for the CK treatment (Fig. 5b). The abundances of methanogens and methanotrophs along axis 1 (27.8% of the total variation) were positively correlated with NH_4^+-N, DOC, pH, TOC, and TSN content, and the methanogen/methanotroph ratios and CH_4 fluxes along axis 1 were negatively correlated with NH_4^+-N, DOC, pH, TOC, and TSN content (Fig. 5b). For the 2013–2016 annual cycles, the RDA explained approximately 45.1% of the total variation in the abundances of methanogen and methanotroph, methanogen/methanotroph ratios, and CH_4 fluxes, and the first two axes explain 42.4% of the variation (Fig. 5c). The abundances of methanogens and methanotrophs along axis 1 (30.8% of the total variation) were positively correlated with NH_4^+-N, MBC, and MBN content, and negatively correlated with pH (Fig. 5c). The methanogen/methanotroph ratios and CH_4 emission along axis 2 (11.6% of the total variation) were negatively correlated with TOC and NO_3^--N content (Fig. 5c). Similar results can be found in the correlation analysis (Figs. S1b and c).

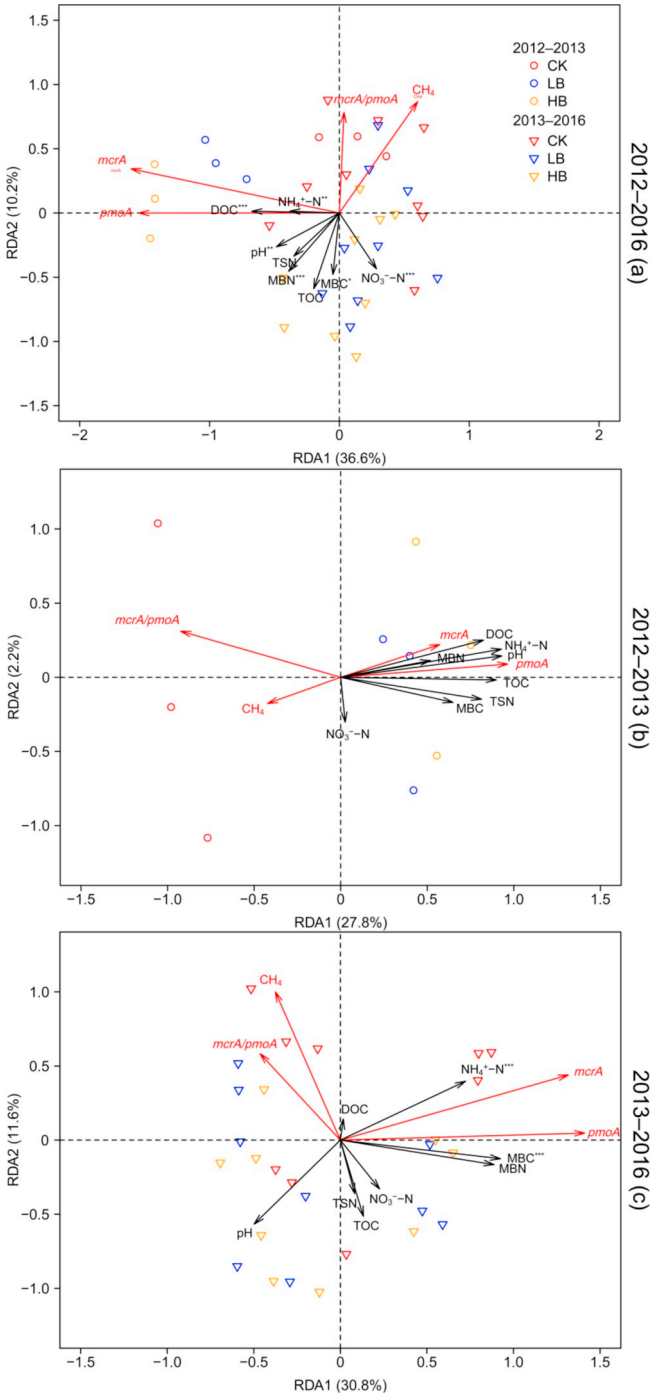

Fig. 5. Redundancy analysis (RDA) tested the associations among the *mcrA* and *pmoA* copy numbers, CH_4 fluxes, and soil properties in the 2012–2016 (a), 2012–2013 (b), and 2013–2016 (c) annual cycles, respectively. CH_4: CH_4 fluxes at the tillering stages (mg C m^{-2} h^{-1}). NH_4^+-N, NO_3^--N, DOC, MBC, and MBN: average soil ammonium, nitrate, dissolved organic carbon, microbial biomass carbon, and microbial biomass nitrogen content, respectively. pH, TOC, and TSN: soil pH value, total organic carbon, and nitrogen content, respectively. *mcrA* and *pmoA*: *mcrA* and *pmoA* copy numbers at the tillering stages, respectively. *mcrA/pmoA*: ratio of the copy numbers of *mcrA* to *pmoA* at the tillering stages. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

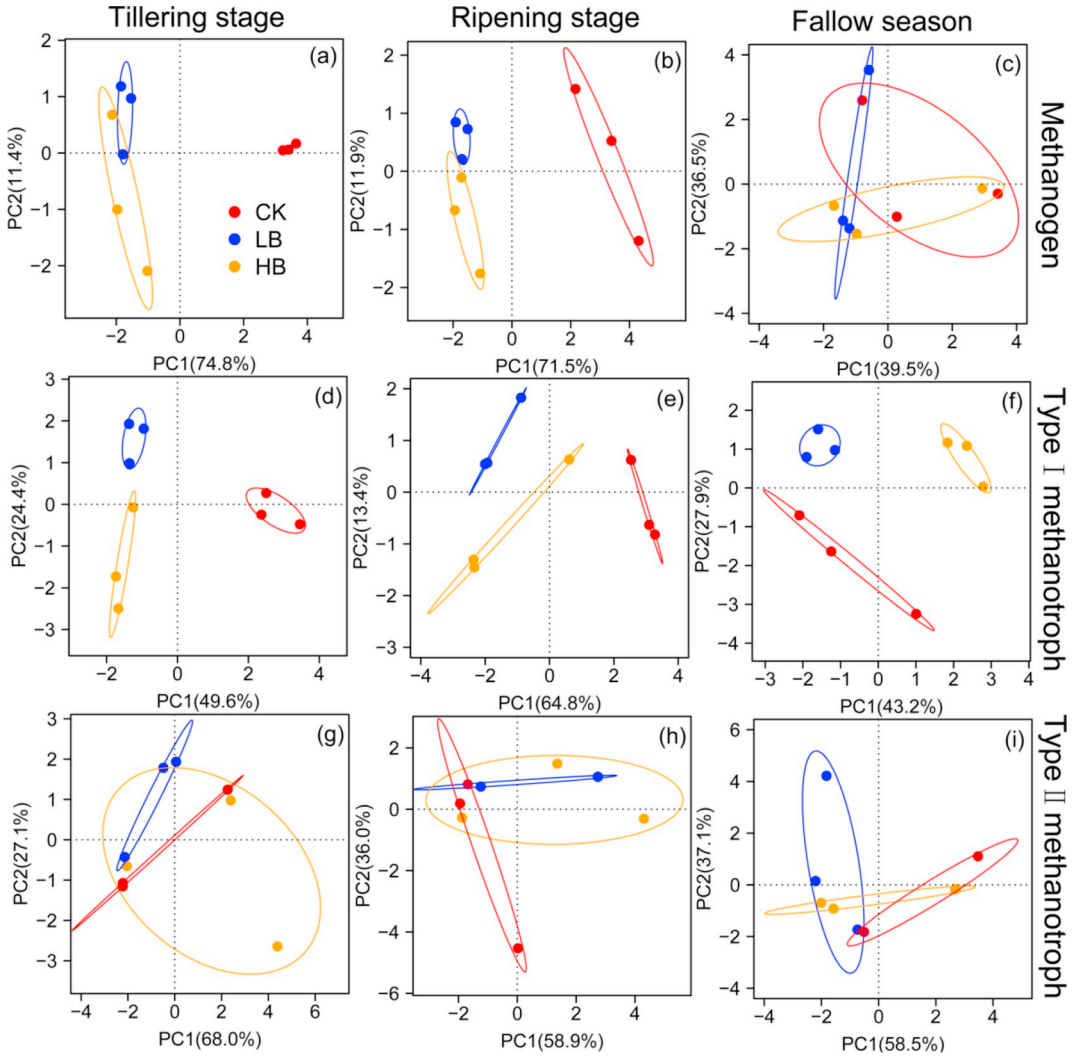

Fig. 6. Principal coordinates analysis (PCoA) of methanogenic (a–c), type I (d–f), and type II (g–i) methanotrophic communities at the tillering (a, d, and g), ripening (b, e, and h), and fallow (c, f, and i) stages of the 2014–2015 annual cycle. Ellipses: 95% confidence areas of the treatments.

3.4. Methanogenic and methanotrophic community structures

The PCoA ordination indicated that biochar addition substantially affected the soil methanogen and type I methanotroph community structures in the 2014–2015 annual cycle (except for the methanogen community structures in the fallow season) ($p < 0.05$) (Fig. 6a–f). There was no significant difference between CK and the biochar treatments in terms of their effects on the type II methanotroph community structures ($p > 0.05$) (Fig. 6g–i). About 76–86%, 71–78%, and 95–96% of the total variability was explained by the first two principle coordinate axes for the methanogen, type I, and type II methanotroph community structures in the 2014–2015 annual cycle, respectively (Fig. 6). Similar results were obtained using ANOSIM (Table S4).

Biochar addition caused changes to soil methanogen community structure, and these changes varied across the rice seasons. The most abundant methanogen genera across the treatments were *Methanocella* (14.8–29.3%), *Methanoregula* (11.1–23.6%), *Methanospirillum* (3.4–10.3%), *Methanosarcina* (6.5–13.3%), *Methanosaeta* (3.8–10.7%), and *Methanobacterium* (4.3–9.5%) (Fig. 7a). In the tillering stage, biochar addition increased the relative abundances of *Methanocella* ($p < 0.05$), *Methanospirillum* ($p = 0.23$, LB; $p < 0.05$, HB), and *Methanosarcina* ($p = 0.07$–0.22), while it reduced the relative abundances of *Methanoregula* ($p = 0.24$–0.44), *Methanosaeta* ($p < 0.001$), and *Methanobacterium* ($p = 0.07$–0.40). In the ripening stage, the relative abundances of *Methanocella* ($p < 0.001$), *Methanospirillum* ($p = 0.08$–0.26), and *Methanobacterium* ($p < 0.05$; $p = 0.12$) increased, while the abundances of *Methanoregula* ($p = 0.07$–0.28), *Methanosarcina* ($p < 0.05$), and *Methanosaeta* ($p < 0.05$; $p = 0.21$) decreased in the biochar treatments as compared to CK. In the fallow season, the relative abundances for *Methanosarcina* ($p < 0.05$; $p = 0.41$) and *Methanobacterium* ($p < 0.05$; $p = 0.06$) increased in the biochar treatments as compared to CK.

Soil methanotroph community composition also changed with biochar amendment and showed difference responses across rice seasons. The dominant genera in the type I methanotrophic community for all treatments were *Methylocaldum* (36.3–78.7%), *Methylomonas* (12.1–33.5%), *Methylomicrobium* (0.3–32.7%), and *Methylococcus* (0.5–4.5%) (Fig. 7b). All type II methanotrophs belonged to the genus *Methylocystis*. In both the tillering and ripening stages, biochar addition increased the relative abundances of *Methylomicrobium*, while it decreased the relative abundances of *Methylocaldum* ($p < 0.05$). In the fallow season, compared to CK, the HB treatment significantly decreased the relative abundance of *Methylomonas* and increased the abundance of *Methylococcus* ($p < 0.05$).

For the 2014–2015 annual cycle, the correlation analysis showed that the DOC content was significantly positively correlated to the relative abundances of *Methylocaldum*, and significantly negatively correlated with the relative abundances of *Methylomicrobium* ($p < 0.05$)

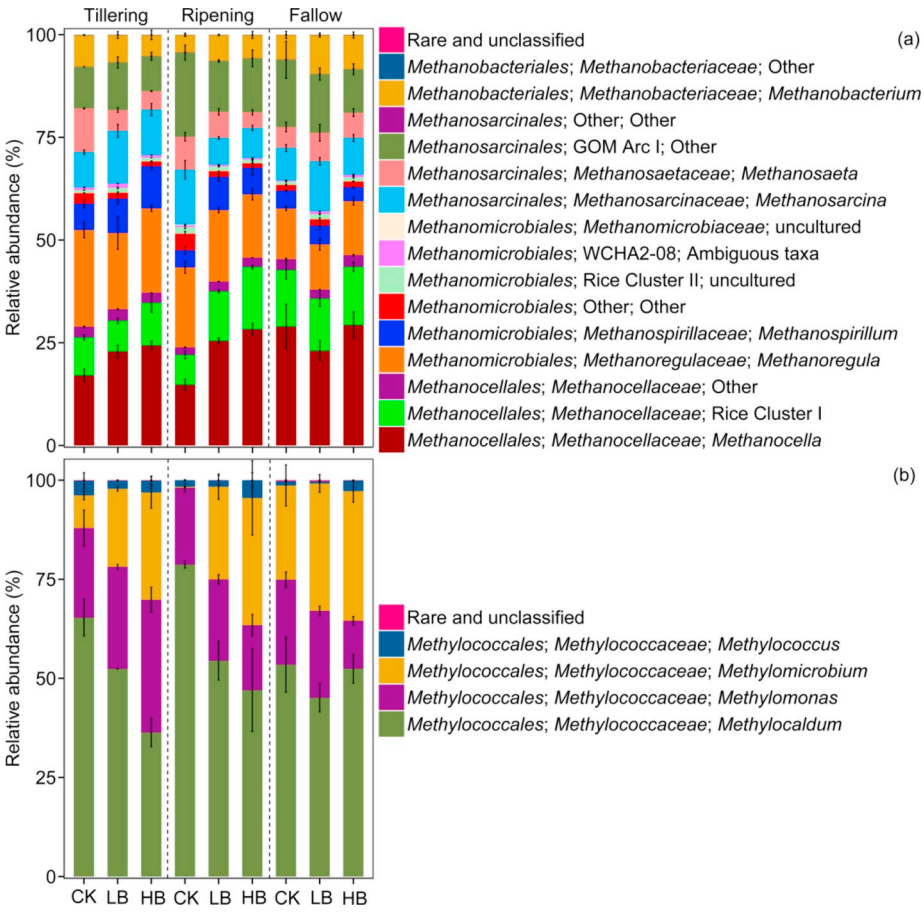

Fig. 7. Taxonomies of methanogenic (a) and type I methanotrophic genera (b) in the late rice season of the 2014–2015 annual cycle. CK: control. LB: 24 t ha^{-1} biochar. HB: 48 t ha^{-1} biochar. Tillering and ripening: tillering and ripening stages, respectively. Fallow: fallow season. Vertical bars represent standard errors (n = 3).

(Fig. S2). The pH and TOC content were significantly positively correlated to the relative abundances of *Methanocella* and *Methylomicrobium*, and significantly negatively correlated with the relative abundances of *Methanosaeta*, aceticlastic methanogen (relative abundance of Methanosarcina and Methanosaeta), and *Methylocaldum* ($p < 0.05$) (Fig. S2). The TSN content was significantly positively correlated with *Methylomicrobium* abundance, and significantly negatively correlated to the relative abundances of *Methylocaldum* and *Methanosaeta* ($p < 0.05$) (Fig. S2). The RDA showed a similar result (Fig. S3). The analysis explained approximately 32.8% of the total variation in the dominant methanogenic and methanotrophic genera, and the first two axes explained 25.6% of the variation (Fig. S3). The dominant methanogen and methanotroph genera for biochar treatments were clearly separated from those for CK treatment (Fig. S3). The *Methanocella* and *Methylomicrobium* abundances along axis 1 (18.8% of the total variation) were positively correlated with pH, TOC, and TSN content, but were negatively correlated with DOC and MBN content (Fig. S3). *Methanosaeta*, aceticlastic methanogen, and *Methylocaldum* abundances along axis 1 were positively correlated with DOC and MBN content, but were negatively correlated with pH, TOC, and TSN content (Fig. S3).

4. Discussion

4.1. Contrasting responses of soil methanogenic and methanotrophic abundances in the four years after biochar amendment

In our four-year study, we found contrasting responses of soil methanogenic and methanotrophic abundances in the first year and the following three years after biochar amendment. To the best of our knowledge, the present study is the first to investigate the effects of biochar application on the annual variations in methanogen and methanotroph abundances for four years after the amendment (Cai et al., 2018; Feng et al., 2012; Han et al., 2016; Qin et al., 2016). As was the case for the previous studies (Cai et al., 2018; Feng et al., 2012; Han et al., 2016), the *mcrA* and *pmoA* copy numbers in this study were higher for the biochar treatments than they were for CK during the first year after the amendment (Fig. 4a and b). Biochar application increased soil NH_4^+-N, DOC, TOC, TSN, pH, and plant residue levels in the first year after biochar addition (Fig. 3a, c, and f-h; Wang et al., 2018). The activities and growth of both methanogens and methanotrophs can be stimulated by ammonium-based fertilizers. Ammonium can stimulate methanogens growth in paddy soil by providing N source as well as more C substrates from rhizodeposit and plant litter due to increased rice biomass (Banik et al., 1996; Banger et al., 2012). The rice biomass in the biochar treatments was only increased by 4–7% as compared with CK (Wang et al., 2018), thus the increased C substrate from rice plant was not likely to be the major contributor for the increased methanogen abundance in the biochar treatments. Ammonium can also stimulate methanotrophs growth in paddy soils when applied at low rates (Bodelier et al., 2000b; Hu and Lu, 2015), but may inhibit methanotrophs growth at high rates due to the competition between CH_4 oxidization and nitrification of NH_4^+ (Bodelier et al., 2000a; Alam and Jia, 2012). Alam and Jia (2012) found that CH_4 oxidation in a paddy soil was inhibited when urea was applied at a rate of 200 mg N kg^{-1} dry soil or above. As the available N input from biochar was not very high (soil ammonium N content was 103 and 154 mg kg^{-1} dry soil for LB and HB treatments after biochar amendment in the tillering stage in early rice season 2012, respectively), methanotrophs growth was not likely suppressed in this study. Bodelier et al. (2000b) found that type I methanotrophs showed a larger increase as compared to type II methanotrophs when ammonium fertilizer was applied. In a DNA-based stable isotope probing experiment, Hu and Lu (2015) also found that soil NH_4^+-N stimulated the incorporation of $^{13}CH_4$ into the type I methanotrophs but not type II. Thus, the increase of methanotrophs

abundance in the biochar treatments might be dominated by the type I group in the first annual cycle in this study.

In paddy soils, methanogens mainly use acetate and CO_2/H_2 as substrates to produce CH_4 (Schütz et al., 1989). These substrates can be formed during soil humus or plant materials degradation. Soil DOC usually contains labile organic C compounds, which can provide substrates for methanogens during their degradation, and thus could stimulate methanogens growth (Fig. 5b and S1b; Mayer and Conrad, 1990; Qin et al., 2018; Zhang et al., 2018). Methanotrophs mainly use CH_4 as carbon source and energy (Hanson and Hanson, 1996). Increased soil DOC could promote CH_4 production, and thus could provide more substrate for methanotrophs and stimulate methanotrophs growth. Besides, facultative methanotrophs can use some kinds of DOC (e.g., acetate, ethanol, and larger organic acids) as the substrates, and thus soil DOC could also enhance methanotrophs growth directly (Im et al., 2011; Semrau et al., 2011).

The soil pH also have important effects on methanogens and methanotrophs, and both of them have an optimum activity under neutrality and slightly alkaline conditions (Le Mer and Roger, 2001). However, it was reported that methanogens were more sensitive to acidic environments (Dunfield et al., 1993; Jeffery et al., 2016), due to that low pH may affect methanogens *per se* or H_2 production in the soils (Dunfield et al., 1993). In this study, though biochar amendment increased soil pH, the soil pH was still acidic. Thus the increase of methanotroph abundance was greater than that of methanogen abundance in the first annual cycle in the biochar treatments (Fig. 5b and S1b).

On the other hand, biochar addition could increase soil porosity, aeration, and redox potential (Eh) (Chen et al., 2018), which could thus reduce the abundance of methanogen. Former studies also showed that soil methanogens were much sensitive to soil Eh (Chen and Lin, 1993; Mayer and Conrad, 1990; Wang et al., 1993). This is consistent with our results that methanotroph abundance increased more than that of methanogens in the 2012–2013 annual cycle (Fig. 4c) and methanogen/methanotroph ratios decreased. Therefore, biochar reduced CH_4 emissions probably by enhancing the abundance of methanotrophs more than that of methanogens in the first year after amendment.

Nevertheless, the *mcrA* and *pmoA* copy numbers decreased by 79–80% and 74–76%, respectively, for the biochar treatments in the late season of the 2013–2014 annual cycle relative to those for the biochar treatments in the late rice season of the 2012–2013 annual cycle ($p < 0.05$) (Fig. 4a and b). The extra NH_4^+-N and DOC from biochar addition were exhausted in the first year (Fig. 3), thus the growth of soil methanogens and methanotrophs may be mainly affected by the variation of soil porosity due to biochar amendment in the following years. Former studies showed that CH_4 production was significantly reduced when soil porosity or soil Eh increase (Schütz et al., 1989; Wang et al., 1993). Biochar addition could increase soil porosity, and it could thus reduce the abundance of methanogen as well as the methanogen/methanotroph ratio. This is also reflected in the negative correlation between methanogen/methanotroph ratio and TOC (Fig. 5c), since biochar is carbon rich and highly porous, and soil amendment with biochar could thus increase both TOC and soil porosity (Chen et al., 2018). No significant change of the abundance of methanotroph in the biochar treatments in the following years may be due to that mathanotrophs were not sensitive to the variation of soil porosity caused by biochar amendment. In their incubation experiments, Cai et al. (2018) found that the straw biochar pyrolyzed at 700 °C decreased methanogen abundance but had no significant influence on methanotroph abundance. Sonoki et al. (2013) observed that wood biochar pyrolyzed at 400–600 °C decreased methanogen abundance. Biochar derived from low-temperature pyrolysis and fresh biochar contain easily decomposable substances. In contrast, old biochar and biochar produced by high-temperature pyrolysis have fewer labile fractions, and are more porous than new biochar and biochar produced by low-temperature pyrolysis (Fig. 3) (Ding et al., 2016; Dong et al., 2017). This indicated that when no additional substrates (e.g. NH_4^+-N, labile components) were supplied after biochar amendment (Fig. 3), decrease of methanogen abundance and methanogen/methanotroph ratio might be the major reason for the decreased CH_4 emissions in paddy fields with biochar amendment. This is consistent with our findings that the methanogen/methanotroph ratios also showed positive correlation with CH_4 fluxes (Fig. 5c and S1c). Considering many biochar products contain additional nutrients or labile components (Cross and Sohi, 2011; Knoblauch et al., 2011), their short-term or long-term effects on soil microorganisms may be different due to biochar aging (e.g. depletion of available nutrient, degradation of labile components), and thus should be examined not only in short-term experiments, but also in long-term experiments.

4.2. Biochar-induced changes in the methanogen and methanotroph communities

As was the case for previous studies (Zhang et al., 2016; Zu et al., 2016), high through-put sequencing indicated that *Methanocella*, *Methanoregula*, *Methanospirillum*, *Methanosarcina*, *Methanosaeta*, and *Methanobacterium* were the dominant methanogen genera in the paddy soil of our study site (Fig. 7a). The high porosity of biochar might have increased soil permeability relative to that for CK (Chen et al., 2018; Lehmann and Joseph, 2015). In this way, biochar lowered the abundance of all methanogens except *Methanocella* and *Methanosarcina* which can survive oxygen stress (Angel et al., 2012; Yuan et al., 2011). Biochar application decreased the abundance of the aceticlastic methanogen *Methanosarcina* at ripening ($p < 0.05$) possibly by improving paddy soil porosity, reducing soil acetate levels (Kusel and Drake, 1995), and stabilizing soil native TOC (Sigren et al., 1997; Wang et al., 2016a; Weng et al., 2017). The relative abundance of *Methanoregula* was decreased by biochar addition in the 2014–2015 annual cycle except for the HB treatment in the fallow season (Fig. 7a). These effects may be explained by the increases in soil pH and aeration achieved by biochar amendment (Chen et al., 2018; Brauer et al., 2006; Jetten et al., 1992) (Fig. 3f). Nevertheless, the soil was drained during the fallow season so these effects of biochar were not evident at that time (Fig. 7a). In general, 65–81% of the CH_4 generated from paddy fields in China is derived from acetate consumption (Conrad et al., 2009; Yao and Conrad, 2000). To the best of our knowledge, only *Methanosarcina* and *Methanosaeta* can utilize acetate as a carbon and energy source (Whitman et al., 2014). In our study, biochar addition decreased aceticlastic methanogen abundance except in the fallow season ($p < 0.05$) (Fig. 7a). Biochar may stabilize native TOC by forming microaggregates (Sigren et al., 1997; Wang et al., 2016a; Weng et al., 2017), and it may also facilitate redox reactions by mediating electron shuttling which lowers paddy soil acetate levels (Briones, 2012; Mochidzuki et al., 2003). Thus, the TOC content was significantly negatively correlated with the relative abundance of aceticlastic methanogen (Figs. S2 and S3). Relative to CK, the biochar treatments may drastically reduce CH_4 production by changing methanogen community structure.

The paddy methanotroph community was dominated by *Methylocaldum*, *Methylomonas*, *Methylomicrobium*, *Methylococcus,* and *Methylocystis.* These findings corroborate those reported from DGGE analyses of the methanotroph community in the paddy fields of China. In those studies, either methanotroph 16S rRNA or *pmoA* was targeted (Li et al., 2014; Zheng et al., 2008b). To the best of our knowledge, few studies have reported the effects of biochar on the paddy methanotroph community especially in the third year after biochar addition. Biochar application significantly changed the type I methanotroph community structure, but did not affect the type II methanotroph community structure as much (Fig. 6d–i). One reason for this is probably because biochar decreased annual cumulative CH_4 emissions by 30–51% in the third year after it was applied ($p < 0.05$) (Fig. 2). The substrate utilization by type II methanotrophs is generally more versatile than that by type I methanotroph; type II methanotrophs can alter their survival strategy when available substrate level fluctuates or is limited (Ho

et al., 2013). Many species of the genus *Methylocystis* contain two enzymes with low and high levels of affinity for adapting to various CH_4 concentrations and can consume ethanol and acetate for growth (Baani and Liesack, 2008; Belova et al., 2011; Im et al., 2011). *Methylocaldum* uses CH_4 as its sole carbon and energy source (Takeuchi, 2016), thus biochar addition decreased the relative abundance of *Methylocaldum* by the 2014–2015 annual cycle (Fig. 7b). Nevertheless, *Methylomonas*, *Methylomicrobium*, and *Methylococcus* can use glucose and methanol instead of CH_4 (Bowman, 2015a, 2015b, 2016; Kalyuzhnaya, 2016). The other reason is probably because biochar treatments increased soil pH, relative to CK (5.3–5.8 vs. 5.1) ($p < 0.05$) (Fig. 3f), which was shifted to the optimal pH range of 6.0–10.0 for *Methylomicrobium* growth (Kalyuzhnaya, 2016); thus the relative abundances of *Methylomicrobium* were increased by the biochar application (Fig. 7b, S2, and S3).

5. Conclusions

Our field study demonstrated that biochar amendment decreased annual CH_4 emissions in a double rice cropping system by 20–51% over a four-year period. The ratios of soil methanogen to methanotroph abundance (*mcrA/pmoA* ratio) in the biochar treatments also decreased by 10.6–30.5% in the four years, which indicates that the CH_4 emission reduction can be explained by the decreases of *mcrA/pmoA* ratios. Biochar increased methanogen and methanotroph abundances with a larger increase of methanotrophs than methanogens in the first year, which is mainly ascribed to increased soil NH_4^+, DOC content, and porosity in the biochar treatments. Biochar suppressed methanogen abundance and had little effect on methanotroph abundance in the following three years due to increased soil porosity. Our study indicated that biochar addition could change the abundances and community structures of soil methanogen and methanotroph, and thus cause the reduction of CH_4 emissions in paddy fields. In view of the variations of soil properties during biochar aging, long-term investigations should be considered in studies looking into microbial mechanisms in the soils with biochar addition. Further studies are still needed to investigate the microbial mechanisms underpinning biochar applications on soil nitrogen and phosphorus cycling.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2019.05.012.

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