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ENVIRONMENTAL BIOTECHNOLOGY

Effect of nitrogen fertilizer and/or rice straw amendment on methanogenic archaeal communities and methane production from a rice paddy soil

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Abstract Nitrogen fertilization and returning straw to paddy soil are important factors that regulate CH_4 production. To evaluate the effect of rice straw and/or nitrate amendment on methanogens, a paddy soil was anaerobically incubated for 40 days. The results indicated that while straw addition increased CH_4 production and the abundances of *mcrA* genes and their transcripts, nitrate amendment showed inhibitory effects on them. The terminal restriction fragment length polymorphism (T-RFLP) analysis based on *mcrA* gene revealed that straw addition obviously changed methanogenic community structure. Based on *mcrA* gene level, straw-alone addition stimulated *Methanosarcinaceaes* at the early stage of incubation (first 11 days), but nitrate showed inhibitory effect. The relative abundance of *Methanobacteriaceae* was also stimulated by straw addition during the first 11 days. Furthermore, *Methanosaetaceae* were enriched by nitrate-alone addition after 11 days, while *Methanocellaceae* were enriched by nitrate addition especially within the first 5 days. The transcriptional methanogenic community indicated more dynamic and complicated responses to straw and/or nitrate addition. Based on *mcrA* transcript level, nitrate addition alone resulted in the increase of *Methanocellaceae* and the shift from *Methanosarcinaceae* to *Methanosaetaceae* during the first 5 days of incubation. Straw treatments increased the relative abundance of *Methanobacteriaceae* after 11 days. These results demonstrate that nitrate addition influences methanogens which are transcriptionally and functionally active and can alleviate CH_4 production associated with straw amendment in paddy soil incubations, presumably through competition for common substrates between nitrate-utilizing organisms and methanogens.

Keywords Rice straw · Nitrate · Methane · Methanogens · Paddy soil

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Introduction

Methane (CH_4) is a key contributor to global warming. Rice fields are a major source of CH_4, accounting for approximately 5–19 % of global CH_4 emissions (IPCC 2007). CH_4 is produced by methanogenic archaea as a final metabolic product of the anaerobic degradation of organic matters (Liesack et al. 2000). All known methanogens belong to tone of seven orders within the phylum *Euryarchaeota*: *Methanomicrobiales*, *Methanobacteriales*, *Methanosarcinales*, *Methanocellales*, *Methanococcales*, *Methanopyrales* (Sakai et al. 2009), and the recently discovered *Methanomassiliicoccales* (Borrel et al. 2014). Methyl coenzyme-M reductase (MCR), the key enzyme of methanogenesis (Ermler et al. 1997), is a specific marker for

methanogens. This enzyme catalyzes the reduction of methyl-coenzyme M, which leads to the release of methane (Ellermann et al. 1988). *mcrA* gene is a functional marker gene encoding the MCR. This gene is highly conserved in all methanogens (Lehmacher and Klenk 1994; Springer et al. 1995), which renders it suitable for application in molecular ecology studies, and has been widely used for investigation of methanogenic communities in rice paddy soils (Ma et al. 2012; Lu et al. 2015).

Returning rice straw to rice field soil is a common practice to maintain the soil fertility in Asian agriculture (Tirol-Padre et al. 2005). However, incorporation of straw also increases CH_4 production and emissions (Denier Van Der Gon and Neue 1995; Zou et al. 2005). Several incubation studies have shown a highly dynamic structure of methanogenic archaeal communities during straw decomposition, with members responding differently to organic residue type, incubation temperature, and duration of incubation (Conrad and Klose 2006; Peng et al. 2008; Conrad et al. 2012; Lu et al. 2015). It is thought that the concentrations of acetate and H_2 are the key factors that control methanogenic community composition during the degradation of rice straw (Conrad 2007).

The intensive use of inorganic nitrogen (N) fertilizer in rice fields has been dramatically increased to meet the increasing food demands of a continuingly growing population (Zhu and Chen 2002). In rice field soil, most N is applied in inorganic form, such as urea. While applied for utilization by plants, the oxidation of ammonia during nitrification in oxic surface soils results in the production of nitrate which may have consequences for methane production, as nitrate in flooded rice fields has been widely observed to have an inhibitory effect on CH_4 production (Klüber and Conrad 1998a, 1998b; Lu et al. 2000; Yuan and Lu 2009). Two main mechanisms have been reported to inhibit CH_4 production from the presence of nitrate. Firstly, denitrification intermediates such as nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O) from nitrate reduction can inhibit methanogenic microorganisms (Clarens et al. 1998; Roy and Conrad 1999). Secondly, CH_4 production can be reduced when nitrate reducers outcompete methanogens for common substrates. During the phase of reduction of NO_3^-, NO_2^-, and N_2O, the partial pressure of H_2 can decrease below concentrations that are required to support CH_4 production (Klüber and Conrad 1998b; Roy and Conrad 1999). The production of organic matter decomposition also serves as an important electron source for iron(III) and sulfate (SO_4^{2-}) reduction in anaerobic respiration processes, and CH_4 production is suppressed by the reduction of these electron acceptors (Yao et al. 1999). It has been shown that nitrate amendment resulted in suppression of CH_4 production in anoxic incubation of excised rice roots (Scheid et al. 2003). However, there are few studies examining the effects of combined rice straw and nitrate amendments on specific methanogenic populations.

Therefore, to determine which populations were influenced under different amendments, rice field soil was anaerobically incubated at a moderate temperature (30 °C) in the presence of rice straw alone, nitrate alone, and both straw and nitrate. CH_4 production, soil physicochemical properties, and the DNA and complementary DNA (cDNA)-based methanogenic communities were analyzed with incubation time. The specific aims were to investigate the influence of rice straw and/or nitrate addition on (i) CH_4 production and (ii) the structure and dynamics of the active methanogenic communities.

Material and methods

Preparation and incubation of soil slurries

Rice field soil samples were taken from the plow layer (0–20 cm) of rice fields (28° 12′ 22.2″ N, 116° 56′ 02.2″ E) in Jiangxi province of China. The soil type was silt loam and pH was 6.42. Other characteristics and pre-treatment of soil were described in a previous study (Bao et al. 2014). Soil sample was air-dried and passed through a 2-mm sieve. Rice straw were dried for 48 h at 60 °C and cut into <1-mm-long pieces. Glass bottles (100 ml) were filled with 15 g of dry soil, 0.15 g of dry straw and 21.5 ml of autoclaved and degassed water and shaken manually to homogenize the soil slurries. Then, the bottles were closed with sterilized butyl stoppers and crimp seals and flushed with nitrogen gas three times. One milliliter of KNO_3 solution (200 mg kg^{-1} N dry soil) was added to generate the final concentration of about 10 mM nitrate in soil slurries (final ratio of water to soil was 1.5:1). All bottles were incubated at 30 °C in the dark for 40 days without shaking. Four treatments were established, i.e., control (no straw or nitrate) (Ctrl), rice straw alone (S), nitrate alone (N), and rice straw and nitrate added together (S + N). For chemical and microbial analysis at each time point, three replicate bottles were established per treatment, and one set of replicates (n = 3) was used to track the partial pressures of CH_4, CO_2, H_2, and N_2O that were produced during the incubation. Over the first 15 days of incubation, measurements were carried out on each day, then measurements were taken every 2 to 7 days.

Chemical analyses

CH_4 and CO_2 were analyzed using a gas chromatograph (Shanghai Precision and Scientific Instrument, China) equipped with a flame ionization detector, a catalytic methanizer for CO_2 (Chrompack, nickat replacement reator) and a 80-cm-long Propack QS 50/100 mesh column operated at 50 °C, with N_2 used as the carrier gas. A reductive gas detector (RGD2) (Trace Analytical, Menlo Park, CA, USA) was used to determinate low H_2 concentrations (<10 Pa) with a molecular sieve column and synthetic air (80 % N_2 and 20 % O_2) as the carrier gas. Gas samples were detected with a

thermal conductivity detector with a molecular sieve column and N_2 as the carrier gas when H_2 partial pressure was >10 Pa. N_2O was analyzed using an Agilent 7980A gas chromatograph (Agilent Technologies) equipped with an ECD. Concentrations of Fe(II), NO_3^-, NO_2^-, SO_4^{2-}, and acetate in soil solutions were determined as described previously (Bao et al. 2014).

Nucleic acid extraction and purification

Soil samples were taken for nucleic acid extraction on days 0, 5, 11, and 23 according to the patterns of CH_4 production in all treatments. Total DNA and RNA were co-extracted and purified using a protocol described previously (Noll et al. 2005; Ma et al. 2012). For the analysis of DNA, no further purification steps were carried out and nucleic acids were stored at −20 °C. For the analysis of rRNA, DNA was removed by DNase treatment before purification using the RNeasy® Mini Kit (Qiagen) as described previously (Xu et al. 2012). The quality of DNA and rRNA extracts was quantified by UV-vis spectrophotometer (ND-1000, NanoDrop, USA) and checked by 1 % agarose gel electrophoresis.

cDNA synthesis

rRNA was transcribed to cDNA using the PrimeScript® first-strand cDNA Synthesis Kit (Takara) according to Xu et al. (2012). To verify the absence of DNA, a control reaction was performed with water instead of reverse transcriptase. cDNA was stored at −20 °C until subsequent analysis.

Terminal restriction fragment length polymorphism analysis

Terminal restriction fragment length polymorphism (T-RFLP) analyses were used to determine the composition of methanogenic communities. *mcrA* genes and transcripts were amplified by PCR using the same primers used for cloning with the forward primer additionally labeled with 6-carboxyfluorescein. Purified PCR products of *mcrA* genes and transcripts were digested at 37 °C for 3 h with *Sau96I* (Fermentas) (Lueders et al. 2001). Digestion products were size separated using a 3730*xl* Genetic Analyzer (Applied Biosystems). The relative abundance of each terminal restriction fragment (T-RF) was calculated as described previously (Xiao et al. 2013).

Quantitative analysis of *mcrA* genes and transcripts

The abundance of *mcrA* genes and their transcripts was determined using the mlas/mcrA-rev primer pair (Steinberg and Regan 2009). Reactions were performed on an iCycler iQ™ Thermocyler (Bio-Rad, USA) using a previously described method (Xu et al. 2012; Ma et al. 2012). For generating standard curves, 2.49×10^2 to 2.49×10^8 copies per reaction were used.

PCR, cloning, sequencing, and phylogenetic analysis

PCR reactions followed the protocols as described previously (Lueders et al. 2001). The primers were MCRf/MCRr. DNA and cDNA were used as PCR templates for *mcrA* gene and transcript analyses, respectively. According to the results from T-RFLP, the community structures of methanogens on day 11 showed great differences among almost all treatments compared with other sampling time points based on both *mcrA* gene and its transcript analysis. Thus, clone libraries were constructed from *mcrA* gene and transcript amplicons derived from N and S + N treatments sampled on day 11 to allow assigning T-RFs to specific sequence groups using a pGEM®-T Easy Vector kit (Promega) according to the manufacturer's instruction.

Fifty-three and 110 randomly selected positive clones were sequenced from *mcrA* gene and transcript, respectively. The clones with correct insert size were sequenced in SinoGenoMax Co., Ltd. (Beijing, China). Clones with >95 % sequences similarity were considered to be the same operational taxonomic unit (OTU). We used the representative sequence from each OTU for phylogenetic analysis. The *mcrA* sequences were aligned with the related sequences using the Clustal X program. Neighbor-joining phylogenetic trees were constructed from the aligned sequences with MEGA 4.1 (Tamura et al. 2007) by using p-distance. Bootstrapping (1000 replicate reconstructions) was used to estimate the reliability of phylogenetic reconstructions.

The nucleotide sequences of *mcrA* gene and *mcrA* transcript fragments determined in this study were submitted in GenBank database under the following accession numbers: *mcrA* gene: KU052990–KU053010 and *mcrA* transcript: KU052963–KU052989.

Statistical analysis

Redundancy analysis (RDA) of T-RFLP profiles was performed to summarize the variations in methanogenic communities that could potentially be explained by the measured variables (treatments, sampling time, and all physicochemical parameters) using CANOCO 4.5 software (Microcomputer Power, Ithaca, NY, USA). RDA was also performed to analyze the variations of CH_4 production and all variables (variations of methanogens (including the relative abundance of T-RFs, the copy numbers of *mcrA* genes or transcripts), treatments, sampling time, and all physicochemical parameters), with significance determined by Monte Carlo permutation test ($P < 0.05$). RDA was chosen according to the results of detrended correspondence analysis (DCA).

Results

Methane production and soil chemistry

Straw addition increased CH_4 production. CH_4 production in the S treatment was higher than in the S + N treatment during incubation. CH_4 production started with longer lag phases in both N and Ctrl treatments, and CH_4 production in the N treatment was slightly lower than in the Ctrl during incubation. The CH_4 partial pressures at the end of the incubation reached 2.54, 17.44, 1.05, and 11.52 kPa in the Ctrl, S, N, and S + N treatments, respectively (Fig. 1a).

CO_2 production occurred from the beginning of the incubation, and straw addition increased CO_2 production. There were no differences of CO_2 production not only between the S and S + N treatments (Fig. 1b), but also between the N and Ctrl treatments. H_2 production increased in the S treatment with two distinct peaks in H_2 concentrations. A lower concentration of H_2 with a lag phase was observed in the S + N treatment. The obvious accumulation of H_2 in the S and S + N treatments occurred within about 10 days. No apparent H_2 production was found in the N treatment. H_2 accumulated greatly in the Ctrl treatment within 1 week of incubation (Fig. 1c). Straw addition significantly increased acetate production. Two peaks of acetate accumulation were observed in the S + N treatment, with obvious consumption taking longer with the S treatment. The obvious consumption of acetate in the N treatment was shown within 3 days of incubation. No acetate accumulated in the Ctrl treatment (Fig. 1d).

NO_3^- increased after 1 day of incubation but decreased immediately in both N and S + N treatments. NO_3^- reduced within 3 days of incubation in the S + N treatment, but another high peak of NO_3^- appeared on the fifth day in the N treatment, and the reduction of them was finished within 6 days of incubation. NO_2^- production in the S + N treatment was greater than in the N treatment. Reduction of NO_2^- was finished within 3 and 4 days of incubation in the S + N and N treatments, respectively. Greater production and longer reduction

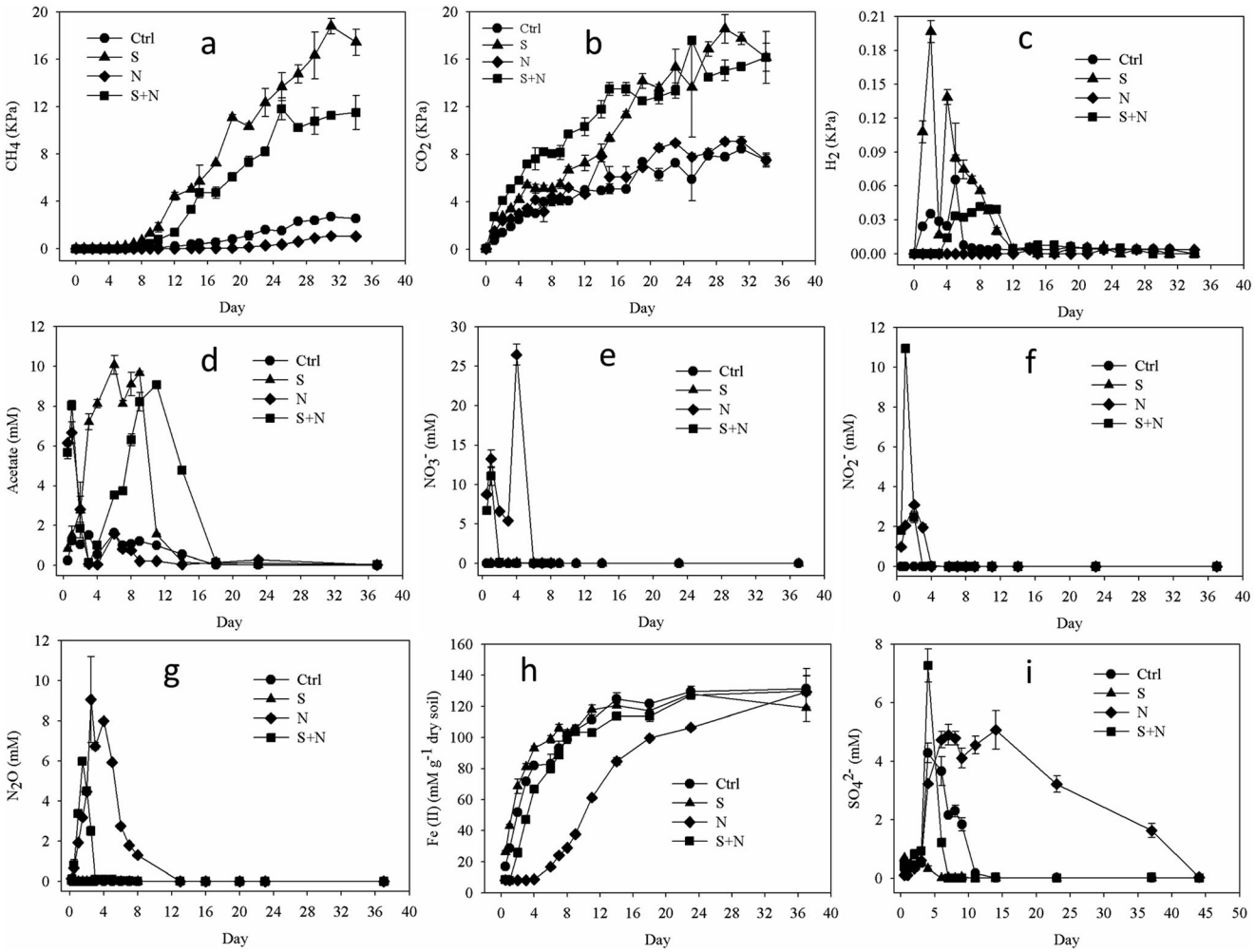

Fig. 1 The time course of CH_4 (**a**), CO_2 (**b**), H_2 (**c**), acetate (**d**), NO_3^- (**e**), NO_2^- (**f**), N_2O (**g**), Fe(II) (**h**), and SO_4^{2-} (**i**) in anaerobic incubation with treatments of Ctrl, S, N, and S + N, respectively. Data are mean ± SE ($n = 3$). Ctrl means control, S means rice straw, N means nitrate, and S + N means straw in combination with nitrate

period of N_2O were observed in the N treatment compared with the S + N treatment. Reduction of N_2O took about 3 and 14 days in the S + N and N treatments, respectively (Fig. 1e–g, respectively).

Fe(III) reduction was greatly inhibited in the N treatment; the reduction of them nearly took 40 days after incubation. The patterns of Fe(III) reduction in other treatments were similar (Fig. 1h). SO_4^{2-} obviously accumulated after 3 days of incubation in all treatments except the S treatment. A very sharp peak of SO_4^{2-} was observed in the S + N treatment but decreased quickly within 4 days. The reduction of SO_4^{2-} in the N treatment took a longer time than that in the Ctrl treatment (Fig. 1i).

Quantification of *mcrA* genes and transcripts

Straw addition increased the abundance of both *mcrA* genes and their transcripts. The abundance of *mcrA* genes and transcripts was higher in the S treatment than in the S + N treatment, with values reaching 1.44×10^{11} genes and 1.15×10^{13} transcripts g^{-1} dry soil in the S treatments, respectively. In S + N treatment, the abundance of *mcrA* genes and transcripts was 1.22×10^{11} genes and 8.97×10^{12} transcripts g^{-1} dry soil, respectively. The *mcrA* gene and transcript abundances remained relatively constant during the entire incubation period in the Ctrl and N treatments. The abundance of *mcrA* genes in the Ctrl and N treatments ranged from 1.54×10^9 to 2.07×10^{10} copies g^{-1} dry soil, and the abundance of *mcrA* transcripts in these two treatments ranged from 1.27×10^{10} to 7.18×10^{11} copies g^{-1} dry soil (Fig. 2a, b). The patterns of variations in *mcrA* genes and transcripts were significantly ($P < 0.01$) correlated with CH_4 production. The abundance of *mcrA* genes and transcripts explained 72 % ($r^2 = 0.72$) and 96 % ($r^2 = 0.96$) of the observed variations in CH_4 production, respectively.

Dynamics of methanogenic archaeal community structure

T-RFLP analyses of the *mcrA* genes and transcripts were performed on different samples taken on days 0, 5, 11, and 23 to determine the population dynamics of methanogenic communities. In total, seven and eight characteristic terminal restriction fragments (T-RFs) were identified as major peaks in T-RFLP profiles of the *mcrA* genes (Fig. 3) and their transcripts (Fig. 4), respectively. The phylogenetic trees showed that methanogenic community mainly comprised *Methanosarcinaceae*, *Methanocellaceae*, *Methanosaetaceae*, and *Methanobacteriaceae*. Clone sequences were also used to relate T-RFs to the respective methanogens, i.e., four T-RFs (390, 422 , 489 , and 268 bp) to *Methanosarcinaceae*, two T-RFs (234 and 402 bp) to *Methanocellaceae*, and T-RFs of 502 and 416 bp to *Methanobacteriaceae* and *Methanosaetaceae*, respectively (Table S1, Figs. S1 and S2).

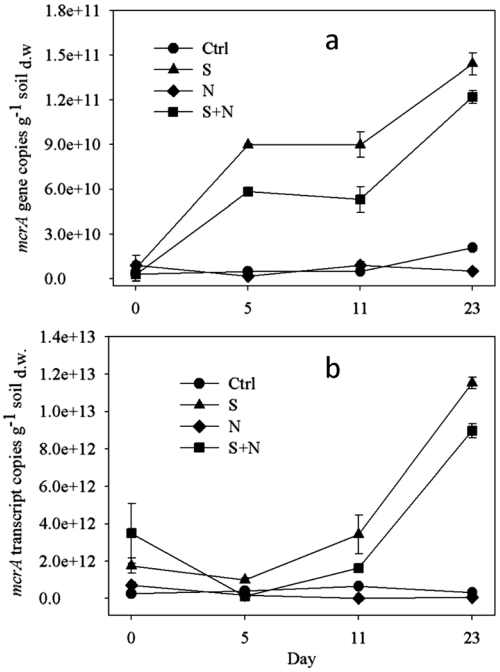

Fig. 2 The copy numbers of *mcrA* genes (**a**) and *mcrA* transcripts (**b**) (mean ± SE, $n = 3$) in all treatments over sampling time based on real-time PCR

The T-RFLP patterns derived from *mcrA* genes revealed that the dynamics of methanogenic community were similar between the S and S + N treatments and between the Ctrl and N treatments. *Methanosarcinaceae* were predominant in all treatments. The relative abundance of *Methanocellaceae* increased in the S and S + N treatments on day 23 but decreased in the S treatment on day 5. *Methanobacteriaceae* decreased in the Ctrl treatment from day 0 to day 23, but only decreased on day 23 in the other three treatments. *Methanosaetaceae* increased in the N treatment but decreased in treatments with straw on both day 11 and day 23 (Fig. 3).

mcrA cDNA-based T-RFLP fingerprints were assumed to represent the community of active methanogens. cDNA-based T-RFLP profiles were distinct from those derived from genes. The dynamics of methanogenic community were different between treatments and showed strong variation across sampling time. *Methanosarcinaceae* increased in treatments without N on day 5 and day 11 but decreased on day 23. In the N and S + N treatments, *Methanosarcinaceae* decreased on day 5 but increased on day 11 and day 23. The relative abundances of *Methanocellaceae*, *Methanobacteriaceae*, and *Methanosaetaceae* showed great fluctuation in all treatments and over sampling time. *Methanocellaceae* increased in the N treatment on day 5. *Methanobacteriaceae* showed increases in the S and S + N treatments on day 11 and day 23. *Methanosaetaceae* showed increases in the N and S + N treatments on day 5 but decreased on days 11 and 23. *Methanosaetaceae* also decreased in the S treatment after 5 days of the incubation (Fig. 4).

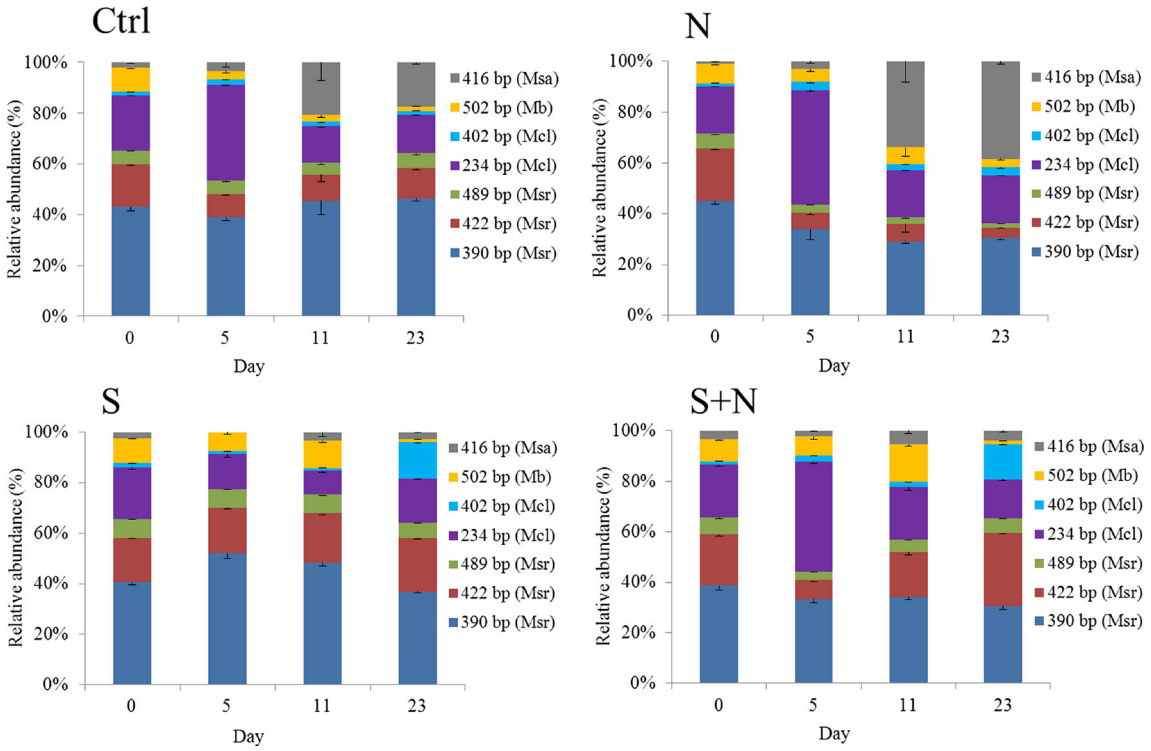

Fig. 3 Community structures of methanogenic community based on T-RFLP analysis targeting *mcrA* genes in anaerobic incubation with treatments of Ctrl, S, N, and S + N and sampling time in rice field soil. Only the relative abundances (mean ± SE, $n = 3$) of major terminal restriction fragments (T-RFs) (relative abundance >1 %) are shown. *Msr Methanosarcinaceae*, *Msa Methanosaetaceae*, *Mcl Methanocellaceae*, *Mb Methanobacteriaceae*

Redundancy analysis (RDA) of T-RFLP profiles was performed to summarize the percentage of total variation in methanogenic communities that could potentially be explained by the measured variables (treatments, sampling time, and all physicochemical parameters) (Table 1). At the gene level, treatments (Ctrl, S, N, and S + N), sampling time (days 0, 5, 11 and 23), and physicochemical parameters (CO_2, H_2, acetate, NO_3^-, NO_2^-, N_2O, SO_4^{2-}, and Fe(II)) as explanatory variables accounted for 81 % of the total variation. The factors with a significant effect were sampling time and SO_4^{2-} and CO_2 concentrations, which contributed to 30, 21, and 12 % of the total variation of methanogenic community, respectively. Based on the *mcrA* transcript level, treatments, sampling time, and physicochemical parameters as explanatory variables accounted for 77 % of the total variation. Sampling time and Fe(II) concentration as significant effect factors contributed to 25 and 18 % of the total variation of methanogenic community, respectively.

Effects of variables on CH_4 production

Table 2 shows the effects of all measured variables (treatments, sampling time, all physicochemical parameters, the relative abundance of T-RFs based on DNA and cDNA level, and the copy numbers of *mcrA* gene and their transcript on CH_4 production by RDA (Table 2)). All factors together explained 89 % of the total variance of CH_4 production. Significant factors included CO_2 (75 %), SO_4^{2-} (4 %), acetate (3 %), the relative abundance of T-RFs of 422 bp (*Methanosarcinaceae*, based on DNA level, 2 %), and *mcrA* gene abundance (1 %).

Discussion

Physicochemical parameters

As reported in previous studies (Peng et al. 2008; Lu et al. 2015), our study showed that straw addition increased CH_4 production. The addition of nitrate with straw reduced CH_4 production compared with the treatment with straw alone. The amounts of CH_4 production were similar between Ctrl and the treatment with nitrate alone. The differences of CH_4 production between treatments most likely related to the production of substrates, such as H_2 and acetate (Conrad 2007). A recent study found that the peak of acetate concentration coincided with the beginning of increased methane production (Wegner and Liesack 2015). H_2 and acetate in microcosms with both straw and nitrate addition were lower than those in microcosms with straw alone, which was possibly due to competition for methanogenic substrates by denitrifying bacteria or the toxic effects of denitrification intermediates (NO_2^-, NO,

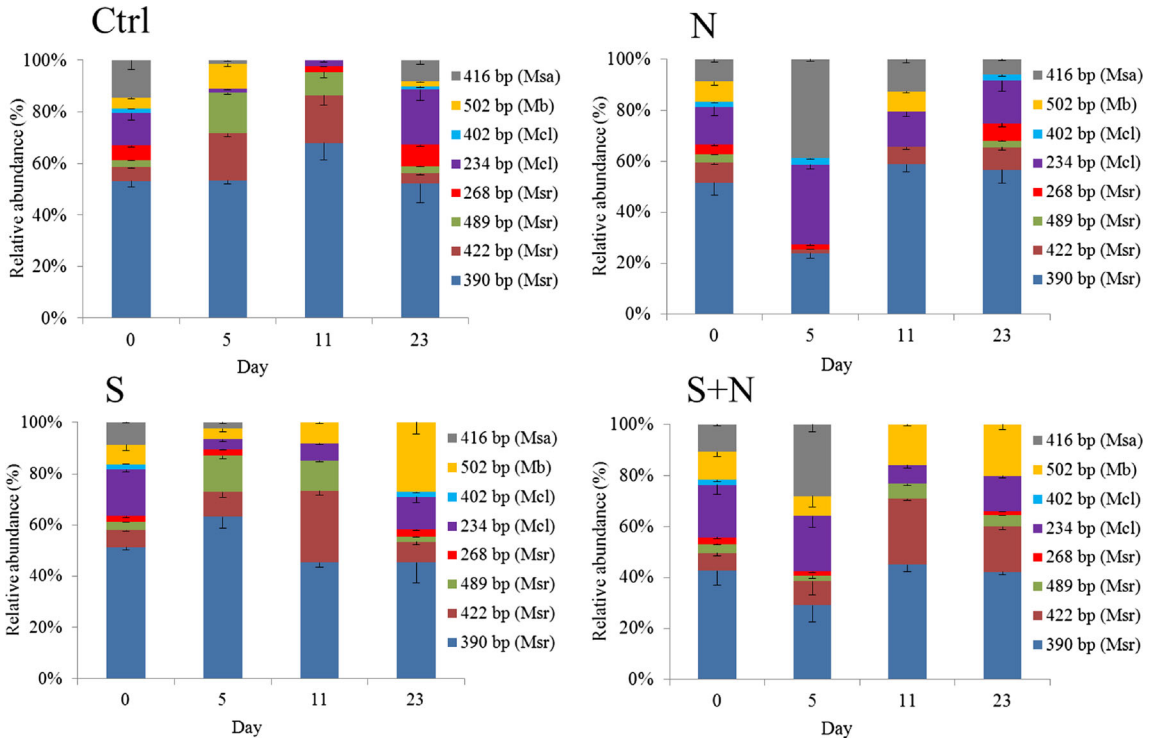

Fig. 4 Community structures of methanogenic community based on T-RFLP analysis targeting *mcrA* transcripts in anaerobic incubation with treatments of Ctrl, S, N, and S + N and sampling time in rice field soil. Only the relative abundances (mean ± SE, n = 3) of major terminal restriction fragments (T-RFs) (relative abundance >1 %) as digested by Sau96I enzyme from PCR products are shown. *Msr Methanosarcinaceae*, *Msa Methanosaetaceae*, *Mcl Methanocellaceae*, *Mb Methanobacteriaceae*

and N_2O) on fermentative bacteria which were involved in acetate or H_2 production (Meyer 1981) or possible toxic effect of products of nitrate reduction (NO, NO_2^-, and N_2O) on methanogens (Chidthaisong and Conrad 2000). Thus, the inhibitory effect of denitrification intermediates on methanogens was a possible contributing factor for lower CH_4 production in treatment with both straw and nitrate. Such toxic effects have been reported in anoxic soil or pure culture studies (Klüber and Conrad 1998a; Roy and Conrad 1999). For example, the suppression of methanogenic acetate utilization by methanogens has prolonged although nitrate reduction was completely finished in methanogenic soil incubation (Klüber and Conrad 1998b). The microbial reduction of accumulated SO_4^{2-} that was released from clays and hydrous aluminum oxides during anoxic incubation in treatments with nitrate addition may decrease electron donors (H_2 and acetate) available to the methanogens (Yao et al. 1999) and thus decreased CH_4 production. The deficiency of elector donors in treatments with nitrate alone possibly resulted in low reduction rates of Fe(III), so further influenced CH_4 production.

Responses of the methanogenic communities to treatments

Straw addition resulted in increases in biomass and activity of methanogens. These growth and activity were most likely supported by substrates (H_2 and acetate) derived from the decomposition of organic matter (Conrad and Klose 2006; Conrad et al. 2012). Nitrate showed inhibitory effect on both *mcrA* gene and transcript abundances in the presence of straw. Ctrl and treatment with nitrate-alone addition showed similar patterns of *mcrA* gene and transcript abundances, with low

Table 1 Redundancy analysis (RDA) of methanogenic composition (based on DNA and cDNA level) observed in all the treatments (Ctrl, S, N, S + N) throughout all sampling times (days 0, 5, 11, 23)

DNA level			cDNA level		
Significant impact factors	Explains %	p value	Significant impact factors	Explains %	p value
Sampling time	30	0.002	Sampling time	25	0.018
SO_4^{2-}	21	0.012	Fe(II)	18	0.018
CO_2	12	0.006			

Input explanatory variables include amendments, sampling time, and physicochemical parameters. The values from the Explains % column represent the percentage of total variation per explanatory variable

Table 2 Redundancy analysis (RDA) for the dependent variables on CH_4 production

CH_4 production Significant impact factors	Explains %	*p* value
CO_2	75	0.002
SO_4^{2-}	4	0.002
Acetate	3	0.026
T-RFs of 422 bp (DNA)	2	0.018
mcrA genes copy numbers	1	0.014

Input variables were physicochemical parameters, *mcrA* genes and their transcript copy numbers, and relative abundances of different T-RFs based on *mcrA* genes and their transcripts. The values from the Explains % column represent the percentage of total variation per explanatory variable

abundances of *mcrA* genes and transcripts during the entire incubation period. These results could be related to differences in the rates of substrate production between treatments and indicated that energy substrates were the limited factors to microbial growth and activity. While both *mcrA* gene and transcript abundances were positively correlated with methane production, transcript abundance was more significant, as observed in other studies (Freitag and Prosser 2009; Ma et al. 2012; Xu et al. 2012), indicating that the variations of *mcrA* transcript abundance were more powerful in predicting CH_4 production.

We analyzed the succession and dynamics of the methanogenic community with the purpose of understanding the effects of straw and nitrate addition. The methanogenic community, as determined by gene-based T-RFLP analysis, changed in response to straw addition but did not alter in nitrate-alone treatment. However, examining the transcriptionally active community revealed a more dynamic and complicated response to straw and/or nitrate addition. This probably resulted from that the transcription level analysis is much more sensitive than the gene level, as activity of cells is directly associated with turnover and synthesis of rRNA (Molin and Givskov 1999).

Based on analysis of *mcrA* genes, *Methanosarcinaceae* were found to be the predominant archaea of the methanogenic community, and straw addition (alone) stimulated their growth in the early stage of incubation, as observed previously (Peng et al. 2008; Conrad et al. 2012). *Methanosarcinaceae* are known as fast-growing and substrate-versatile methanogens and not only can use high concentrations (0.2–1.2 mM) of acetate, but also can utilize H_2-CO_2 or methanol (Jetten et al. 1990; Conrad 2007). In straw-amended treatments, acetate and H_2 accumulated to high concentrations resulting from anaerobic straw degradation in the same incubation stage. Nitrate inhibited the *Methanosarcinaceae* during the early stages of incubation, particularly on day 5 at the transcript level, presumably due to the toxicity of denitrification intermediates directly on *Methanosarcinaceae* or indirectly by inhibiting fermentative bacteria (Fig. 1c, 1d), and subsequent substrate production. Similar observations have been reported before (Scheid et al. 2003; Meyer 1981), where the growth of methanogens was inhibited even after nitrate was completely reduced, indicating the inhibitory effect of nitrate-derived intermediates on methanogen growth.

Methanosaeta spp. have been described as slow-growing methanogens, utilizing acetate over a lower range of concentrations (7–70 μM) (Jetten et al. 1992). *Methanosaetaceae* are usually dominant when acetate concentrations decrease to a low level (Peng et al. 2008). In this study, the relative abundance of *Methanosaetaceae* in gene level obviously increased in the Ctrl and nitrate-alone treatment on days 5 and 11. A clear shift from *Methanosarcinaceae* to *Methanosaetaceae* was observed on day 5 in treatments with nitrate by analysis of transcripts. An expected change of acetate concentrations was observed in these treatments, with accumulation in the early stages of incubation with a subsequent decrease; this was strongly in accordance with earlier reports (Jetten et al. 1992; Peng et al. 2008). Moreover, higher levels of nitrate (NO_3^-), nitrous oxide (N_2O), and sulfate (SO_4^{2-}) in the same period in these treatments probably affected *Methanosarcinaceae* and *Methanosaetaceae*. These results further demonstrated that analysis of *mcrA* transcripts was more sensitive and powerful than analysis of *mcrA* genes.

Methanocellaceae are known as RC-I methanogens and play a key role in CH_4 production in paddy soil (Lu and Conrad 2005). Hydrogenotrophic *Methanocellaceae* were favored in nitrate treatments in early incubation stage (on day 5) in both gene and transcript levels. Wu et al. (2009) have observed the increase of hydrogenotrophic methanogens on the rice roots with the increasing application of N fertilizer in paddy soil. The increase of *Methanocellaceae* (based on both gene and transcript levels) may be stimulated due to application of N fertilizer in this study. Additionally, SO_4^{2-} nearly kept at high level in nitrate-alone treatment during the whole incubation period, which probably stimulated the increase of *Methanocellaceae*. Lyu and Lu (2015) have reported that *Methanocellales* (as *Methanocella arvoryzae* and *Methanocella paludicola*) were most likely to use sulfate as a sulfur source and assimilate sulfate into biomass. High activity of *Methanocellaceae* (based on transcript level) was also observed in later incubation stage (on day 23) in all treatments. This shift could be explained by the change of H_2 concentration in the headspace. Low H_2 concentrations were favored *Methanocellaceae* (Lu and Conrad 2005; Peng et al. 2008). Previous studies found that *Methanocellaceae* predominated methanogenic activity at high temperatures (e.g., 45 °C) (Peng et al. 2008; Lu et al. 2015). *Methanocellaceae* increased at 30 °C in our study, indicating that they can also play an important role in CH_4 production at moderate temperatures.

Methanobacteriaceae using H_2 plus CO_2 have been isolated from rice field soil (Joulian et al. 1998). In this study,

Methanobacteriaceae were favored in the treatments with straw application, which could be explained by variation of H_2 concentration (Lu and Conrad 2005; Peng et al. 2008), indicating that *Methanobacteriaceae* contributed to CH_4 production in straw-applied rice field soil.

In conclusion, nitrate amendment suppressed the straw-induced increase in CH_4 production in laboratory incubations. Methanogenic community structures, as determined by *mcrA* gene analysis, changed in response to straw addition, but at *mcrA* transcript level, methanogenic community structures showed more sensitive, revealing differential response to a combined addition of nitrate and straw, as well as temporal fluctuations. These results suggest that nitrate application could alleviate CH_4 production during anaerobic degradation of rice straw in paddy soils by inhibiting the production of substrates such as H_2 and acetate and thus might influence CH_4 emission from rice fields and even its contribution to global warming. Investigating the CH_4 production and the dynamics of the methanogenic archaeal community upon rice straw and/or nitrate addition in paddy soils could provide insights into C and N biogeochemical cycles in rice fields and even further important suggestions for regulating CH_4 emission from rice field soils via agricultural management practices.

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Compliance with ethical standards

Conflict of interest No conflict of interest exists in this manuscript. No part of this paper has been published or submitted elsewhere.

References

Bao QL, Xiao KQ, Chen Z, Yao HY, Zhu YG (2014) Methane production and methanogenic archaeal communities in two types of paddy soil amended with different amounts of rice straw. FEMS Microbiol Ecol 88:372–385

Borrel G, Parisot N, Harris HMB, Peyretaillade E, Gaci N, Tottey W, Bardot O, Raymann K, Gribaldo S, Peyret P, O'Toole PW, Brugère JF (2014) Comparative genomics highlights the unique biology of *Methanomassiliicoccales*, a thermoplasmatales-related seventh order of methanogenic archaea that encodes pyrrolysine. BMC Genomics 15:679

Chidthaisong A, Conrad R (2000) Turn of glucose and acetate coupled to reduction of nitrate, ferric iron and sulfate and to methanogenesis in anoxic rice field soil. FEMS Microbiol Ecol 31:73–86

Clarens M, Bernet N, Delgenès JP, Moletta R (1998) Effects of nitrogen oxides and denitrification by *Pseudomonas stutzeri* on acetotrophic methanogenesis by *Methanosarcinamazei*. FEMS Microbiol Ecol 25:271–276

Conrad R (2007) Microbial ecology of methanogens and methanotrophs. Advan Agron 96:1–63

Conrad R, Klose M (2006) Dynamics of the methanogenic archaeal community in anoxic rice soil upon addition of straw. Eur J Soil Sci 57: 476–484

Conrad R, Klose M, Lu YH, Chidthaisong A (2012) Methanogenic pathway and archaeal communities in three different anoxic soils amended with rice straw and maize straw. Front Microbiol 3:1–11

Denier Van Der Gon HAC, Neue HU (1995) Influence of organic matter incorporation on the methane emission from a wetland rice field. Glob Biogeochem Cycles 9:11–22

Ellermann J, Hedderich R, Böcher R, Thauer RK (1988) The final step in methane formation-investigations with highly purified methyl-CoM reductase (component-C) from *Methanobacterium thermoautotrophicum* (strain Marburg). Eur J Biochem 172:669–677

Ermler U, Grabarse W, Shima S, Goubeaud M, Thauer RK (1997) Crystal structure of methyl coenzyme M reductase: the key enzyme of biological methane formation. Science 278:1457–1462

Freitag TE, Prosser JI (2009) Correlation of methane production and functional genes transcriptional activity in a peat soil. Appl Environ Microbiol 75:6679–6687

Intergovernmental Panel on Climate Change (IPCC) (2007) Couplings between changes in the climate system and biochemistry. Climate change 2007: the physical science basis (Denman KL, ed.), pp. 541–584. Cambridge University Press, Cambridge

Jetten MSM, Stams AJM, Zehnder AJB (1990) Acetate threshold values and acetate activating enzymes in methanogenic bacteria. FEMS Microbiol Ecol 73:439–344

Jetten MSM, Stams AJM, Zehnder AJB (1992) Methanogenesis from acetate—a comparison of the acetate metabolism in *Methanothrix soehngenii* and *Methanosarcina* spp. FEMS Microbiol Rev 88:181–197

Joulian C, Ollivier B, Patel BKC, Roger PA (1998) Phenotypic and phylogenetic characterization of dominant culturable methanogens isolated from rice field soils. FEMS Microbiol Ecol 25:135–145

Klüber HD, Conrad R (1998a) Inhibitory effects of nitrate, nitrite, NO and N_2O on methanogenesis by *Methanosarcinabarkeri* and *Methanobacteriumbryantii*. FEMS Microbiol L Eco 25:331–339

Klüber HD, Conrad R (1998b) Effects of nitrate, nitrite, NO and N_2O on methanogenesis and other redox processes in anoxic rice field soil. FEMS Microbiol Ecol 25:301–318

Lehmacher A, Klenk HP (1994) Characterization and phylogeny of MCRII, a gene-cluster encoding an isoenzyme of methyl coenzyme-M reductase from hyperthermophilic *Methanothermus fervidus*. Mol Gen Genet 243:198–206

Liesack W, Schnell S, Revsbech NP (2000) Microbiology of flooded rice paddies. FEMS Microbiol Rev 24:625–645

Lu YH, Conrad R (2005) In situ stable isotope probing of methanogenic archaeal in the rice rhizosphere. Science 309:1088–1090

Lu YH, Wassmann R, Neue HU, Huang C (2000) Dissolved organic carbon and methane emissions from a rice paddy fertilized with ammonium and nitrate. J Environ Qual 29:1733–1740

Lu Y, Fu L, Lu YH, Hugenholtz F, Ma K (2015) Effect of temperature on the structure and activity of a methanogenic archaeal community during rice straw decomposition. Soil Biol Biochem 81:17–27

Lueders T, Chin KJ, Conrad R, Friedrich M (2001) Molecular analyses of methyl-coenzyme reductase a-subunit (*mcrA*) genes in rice field soil and enrichment cultures reveal the methanogenic phenotype of a novel archaeal lineage. Environ Microbiol 3:194–204

Lyu Z, Lu YH (2015) Comparative genomics of three *Methanocellales* strains reveal novel taxonomic and metabolic features. Environ Microbiol Rep 7:526–537

Ma K, Conrad R, Lu YH (2012) Responses of methanogen *mcrA* genes and their transcripts to an alternate dry/wet cycle of paddy field soil. Appl Environ Microbiol 78:445–454

Meyer J (1981) Comparison of carbon monoxide, nitric oxide, and nitrite as inhibitors of the nitrogenase from *Clostridium pasteurianum*. Arch Biochem Biophys 210:246–256

Molin S, Givskov M (1999) Application of molecular tools for in situ monitoring of bacterial growth activity. Environ Microbiol 1:383–391

Noll M, Matthies D, Frenzel P, Derakshani M, Liesack W (2005) Succession of bacterial community structure and diversity in a paddy soil oxygen gradient. Environ Microbiol 7:382–395

Peng JJ, Lü Z, Rui JP, Lu YH (2008) Dynamics of the methanogenic archaeal community during plant residue decomposition in an anoxic rice field soil. Appl Environ Microbiol 74:2894–2901

Roy R, Conrad R (1999) Effect of methanogenic precursors (acetate, hydrogen, propionate) on the suppression of methane production by nitrate in anoxic rice field soil. FEMS Microbiol Ecol 28:49–61

Sakai S, Imachi H, Sekiguchi Y, Tseng I-C, Ohashi A, Harada H, Kamagata Y (2009) Cultivation of methanogens under low-hydrogen conditions by using the co-culture method. Appl Environ Microbiol 75:4892–4896

Scheid D, Stubner S, Conrad R (2003) Effects of nitrate- and sulfate-amendment on the methanogenic populations in rice root incubations. FEMS Microbiol Ecol 43:309–315

Springer E, Sachs MS, Woese CR, Boone DR (1995) Partial gene-sequences for the a-subunit of methyl-coenzyme M reductase (MCRI) as a phylogenetic tool for the family *Methanosarcinaceae*. Int J Syst Bacteriol 45:554–559

Steinberg LM, Regan JM (2009) *mcrA*-targeted real time quantitative PCR method to examine methanogen communities. Appl Environ Microbiol 75:4435–4442

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA 4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Tirol-Padre A, Tsuchiya K, Inubushi K, Ladha JK (2005) Enhancing soil quality through residue management in a rice-wheat system in Fukuoka, Japan. J Soil Sci Plant Nutr 51:849–860

Wegner CE, Liesack W (2015) Microbial community dynamics during the early stages of plant polymer breakdown in paddy soil. Environ Microbiol Published Online. doi:10.1111/1462-2920.12815

Wu LQ, Ma K, Li Q, Ke XB, Lu YH (2009) Composition of archaeal community in a paddy field as affected by rice cultivar and N fertilizer. Microb Ecol 58:819–826

Xiao KQ, Bao P, Bao QL, Jia Y, Huang FY, Su JQ, Zhu YG (2013) Quantitative analyses of ribulose-1, 5-bisphosphate carboxylase/oxygenase (RubisCO) large-subunit genes (cbbL) in typical paddy soils. FEMS Microbiol Ecol 38:11–20

Xu Y, Ma K, Huang SW, Liu LM, Lu YH (2012) Diel cycle of methanogen *mcrA* transcripts in rice rhizosphere. Environ Microbiol Rep 4:655–663

Yao H, Conrad R, Wassmann N, Neue HU (1999) Effect of soil characteristics on sequential reduction and methane production in sixteen paddy soils from China, the Philippines, and Italy. Biogeochemistry 47:269–295

Yuan Q, Lu YH (2009) Response of methanogenic archaeal community to nitrate addition in rice field soil. Environ Microbiol Rep 1:362–369

Zhu ZL, Chen DL (2002) Nitrogen fertilizer use in China-contributions to food production, impacts on the environment and best management strategies. Nutr Cycl Agroecosys 63:117–127

Zou JW, Huang JY, Zheng XH, Sass RL (2005) A 3-year field measurement of methane and nitrous oxide emissions from rice paddies in China: effects of water regime, crop residue and fertilizer application. Global Biogeochem Cycles 19:153–174