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Linking phosphorus & potassium deficiency to microbial methane cycling in rice paddies

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Abstract Phosphorus (P) and potassium (K) availability in soil are crucial for the growth and development of resident microorganisms, which in turn modulate local and global carbon emissions from the terrestrial biosphere. Carbon dioxide and methane are key climate active soil emissions, the latter being 25 times more active than CO_2 on a per unit basis and its biological production regulated by the balance of activities of key production (methanogenic) and consumption (methanotrophic) taxa. Here, we assessed whether deficiencies in P and K modulated the activities of methanogens,

Biogeosciences Discuss., doi:10.5194/bg-2016-213, 2016
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Published: 18 July 2016

methanotrophs, or both in long term (20 y) experimental systems undergoing limitation in either one or both nutrients. Biogeochemical measures of methane production, in tandem with *mcrA* (methanogen) and *pmoA* (methanotroph) activity under nutrient limitation demonstrated that P deficiency significantly reduced methane flux rates, whereas K deficiency did not. Under P deficiency, methanotroph transcript copy number significantly increased in tandem with a decrease in methanogen transcript activity, suggesting that P deficiency reduced CH_4 emissions via reduced methane production in tandem with an increased methane consumption potential. Assessments of community structures based upon transcript or gene abundance indicated transcriptional activities were more sensitive to P and K deficiency than DNA copy number, with phosphorus deficiency inducing greater shifts in the active methanotroph community than for potassium deficiency. In contrast, both phosphorus and potassium deficiencies exhibited similar community structures of active methanogens at the mRNA level, suggesting that methane emissions from paddy soils under nutrient limitation are regulated at the transcriptional level and not the methanogen/methanotroph population size.

Keywords: Methane; Methanogen; Methanotroph; Paddy field

1. Introduction

Methane (CH_4) is the second most abundant greenhouse gas, next only to CO_2, in the atmosphere and contributes approximately 20-30% of the global warming effect (IPCC, 2007). The atmospheric

Biogeosciences Discuss., doi:10.5194/bg-2016-213, 2016
Manuscript under review for journal Biogeosciences
Published: 18 July 2016

concentration of CH_4 has increased from a pre-industrial value of 0.715 ppm to 1.824 ppm in 2013 (IPCC, 2007; WMO, 2010), with anthropogenic activities accounting for 60% of the global budget of annual CH_4 emission (Insum and Wett, 2008). One significant global source of methane is rice paddy cultivation, covering a worldwide area of 155 million hectares and contributing 10% of the annual anthropogenic CH_4 emissions (Nazaries et al., 2013).

The net methane flux in rice paddy cultivation is determined by the balance between the activities of biological methane production by a specific group of *Archaea* (methanogens) and the subsequent oxidation of CH_4 at anoxic/oxic interfaces by aerobic methanotrophs (Le Mer and Roger, 2001). The biochemistry and molecular biology of both methanogens and methanotrophs has been extensively reviewed (Thauer et al., 2008; Trotsenko and Murrell, 2008). In addition to 16S rRNA gene based assays, metabolic genes (*mcrA* - encoding the alpha subunit of methyl co-enzyme reductase and the *pmoA* gene, encoding the alpha subunit of membrane bound particulate methane monooxygenase) have been successfully used as biomarkers to target both methanogens and aerobic proteobacterial methanotrophs (MOB), respectively in the environment (Steinberg and Regan, 2008; McDonald et al., 2008).

In previous DNA-based analyses, it was reported that changes in the population size of methanogens and methanotrophs were closely related to the variations in CH_4 production and oxidation potential in paddy soils (Dubey and Singh, 2000; Bao et al., 2014; Mohanty et al., 2014), lake sediments

Biogeosciences Discuss., doi:10.5194/bg-2016-213, 2016
Manuscript under review for journal Biogeosciences
Published: 18 July 2016

and wetland soils (Liu et al., 2014). However, studies have also indicated that the *mcrA* and *pmoA* gene copy numbers were not significantly correlated with the activities of CH_4 production and oxidation, despite the fact that they responded to environmental disturbance (Ahn et al., 2014; Lee et al., 2014; Zheng et al., 2013). Other investigators have suggested that assessment of methanogen community composition, based on *mcrA* gene diversity, remained highly stable in response to environmental changes, showing no significant correlation with the rate of CH_4 production in various soil ecosystems (Ma et al., 2012; Xu et al., 2012; Zhang et al., 2014).

It is reasonable to assume that gene abundance analyses alone may not be adequate to link the methanogenic and/or methanotrophic potential of communities with the methane flux (Zheng et al., 2013; Ma et al., 2012; Yuan et al., 2011). However, the transcriptional analysis (mRNA) of *mcrA* and *pmoA* genes can provide information on the 'active' communities of methanogens and MOB (Ma et al., 2012; Zhang et al., 2014; Freitag and Prosser, 2009; Freitag et al., 2010; Angel et al., 2011). Ma et al. (2012) reported that the abundance of *mcrA* transcripts showed a greater correlation with CH_4 production rates compared to the *mcrA* gene copies. Similarly, Ahn et al. (2014) also suggested that the transcript abundance of *mcrA* and *pmoA* genes could correlate with the CH_4 emission pattern whilst the gene abundance remained relatively stable in rice paddy soil. Interestingly, a study on peat soils indicated that the abundance of *mcrA* or *pmoA* transcripts alone was not correlated with CH_4 flux, instead, the transcript/gene ratios of both *mcrA* and *pmoA* genes actually exhibited a linear correlation

Biogeosciences Discuss., doi:10.5194/bg-2016-213, 2016
Manuscript under review for journal Biogeosciences
Published: 18 July 2016

with CH_4 emission (Freitag et al., 2010).

Important macronutrients, nitrogen (N), phosphorus (P) and potassium (K) influence soil fertility and crop production (Ogden et al., 2002; Pujos and Morard, 1997). Although previous studies have focussed on the effect of N on methane flux, in particular on MOB activity and diversity, little is known on the effects of both P and K on both methanogens and MOB (extensively reviewed in (Veraart et al., 2015)). Specifically, P and K deficiency is present in 50.59% and 12.16% of the total cultivation area in China, respectively. Previous studies indicate that CH_4 emissions in P and K deficient plots are significantly lower than balanced inorganic fertilization plots (Yang et al., 2010; Shang et al., 2011). However, the role of microbial functional diversity (of both methanogens and MOB) underpinning these differences in the methane flux is uncertain.

It is clear from emerging evidence that different conclusions can be drawn from DNA or mRNA based studies, particularly when studying correlation between community composition and net methane flux. Therefore, in this study we have used a multi-level approach i.e. both DNA and mRNA-based analyses in tandem with net methane flux rates in rice paddies. We subsequently used this multi-level approach to resolve the impact of key nutrients upon the community composition and abundance of both resident (DNA based) and active (mRNA-based) methanogens and MOB and its subsequent influence upon overall methane flux. Long term (20 y) nutrient manipulation experiments were selected which have been maintained under P and K deficiencies (Shang et al., 2011; Chen et al., 2010) to

Biogeosciences Discuss., doi:10.5194/bg-2016-213, 2016
Manuscript under review for journal Biogeosciences
Published: 18 July 2016

resolve the modulation of methane flux by P and K.

2. Materials and Methods

2.1. Experimental site and soil sampling

The experimental site is located within the Taoyuan Agro-ecosystem Research Station of the Chinese Academy of Sciences (28°55′ N, 111°26′ E), Hunan province, China. The area is characterized by a subtropical monsoon climate with an annual average air temperature of 16.5℃ and a mean annual precipitation of 1448 mm. Soil samples were collected from a long-term paddy rice field fertilization experiment established in 1990 (Yang et al., 2010; Chen et al., 2010). The paddy soil was derived from quaternary red clay and the cropping regime was a double rice cropping system. The experiment contained ten treatments with three replicates, organised by randomized blocking design, with each plot of 33 m^2. The four treatments selected for this study were as follows: balanced fertilization (NPK, amended with nitrogen, phosphorus and potassium fertilizers), phosphorus deficiency (–P, amended with nitrogen and potassium fertilizers), potassium deficiency (–K, amended with nitrogen and phosphorus fertilizers) and dual deficiency of phosphorus and potassium (–PK, only amended with nitrogen fertilizer). The annual fertilizers input were urea, superphosphate, and potassium chloride at 182.3 kg N ha^{-1} $year^{-1}$, 39.3 kg P ha^{-1} $year^{-1}$, 197.2 kg K ha^{-1} $year^{-1}$, respectively. Soil samples were collected in August and September, 2010, when the rice was at the tillering (flooding) and ripening

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Manuscript under review for journal Biogeosciences
Published: 18 July 2016

(drying) stage, respectively. Five soil columns (0–20 cm depth and 5 cm diameter) were randomly taken from each plot and homogenised. The samples were divided into two aliquots, one immediately frozen in liquid nitrogen and stored at -80 ^{o}C for nucleic acid extraction and the remainder used for analyses of soil properties. Immediately after gas and soil sampling, six randomly chosen rice plants were harvested. After clean washed off adhering soil from roots, the plant samples were oven dried to constant weight at 60 ^{o}C and aboveground and underground biomasses were estimated separately. Data are standardized to 1 m^2 plots.

2.2. Methane emission measurement

In situ methane fluxes from the experimental field plots were sampled using static chambers (Shang et al., 2011) at tillering and ripening stages with a 30 mL syringe and stored in pre-evacuated vials (Labcolimited high Wycombe UK). The sampled CH_4 was analysed using a gas chromatograph equipped with a FID detector (Agilent 7890A, USA).

2.3. Soil microbial DNA and mRNA extractions

Soil microbial DNA was extracted according to Chen et al (2010) with minor modifications. Briefly, after the addition of lysing solution, MP FastPrep-24 (MP Biomedicals, USA) was used instead of a vortex followed by a 15 min water bath treatment at 68 ℃. DNA concentration and quality were

Biogeosciences Discuss., doi:10.5194/bg-2016-213, 2016
Manuscript under review for journal Biogeosciences
Published: 18 July 2016

measured using a NanoDrop NA-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Extraction of total RNA from soils was performed according to the method described by Mettel et al. (2010). The extracted nucleic acid was rendered DNA free by DNase (Promega, USA) digestion according to the manufacturer's instructions. To remove humic acids, the total RNA was reversibly bound to Q-Sepharose and followed by stepwise elution using 1.5 M NaCl, precipitated with isopropanol and resuspended in TE buffer (pH 8.0). In order to remove the 5S rRNA and remaining salts, an RNeasy MinElute Kit (Qiagen, Germany) was used to further purify the total RNA and mRNA associated only with prokaryotes was captured using, the mRNA-ONLY prokaryotic mRNA isolation kit (Epicentre Biotechnologies, United States). Finally, the enriched 700 ng mRNA was reverse-transcribed to cDNA using the Fermentas K1622 RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, USA) and the resulting cDNA was stored at -80^{o}C.

2.4. PCR amplification and T-RFLP detection

Primers mals/mcrA-rev (Steinberg and Regan, 2008) and A189F/Mb661R (Holmes et al., 1995) were used for PCR amplification of the *mcrA* and *pmoA* gene, respectively. The PCR reaction solution (50 μL) consisted of 60 ng of DNA template, 0.3 μM of each primer and 25 μL 2 × Power Taq Master Mix (TIANGEN, China). Reaction conditions for the *mcrA* gene included an initial denaturation step at 95 ℃ for 3 min, followed by five cycles of denaturation at 95 ℃ for 30 s, annealing at 48 ℃ for 45 s, and

Biogeosciences Discuss., doi:10.5194/bg-2016-213, 2016
Manuscript under review for journal Biogeosciences
Published: 18 July 2016

145 extension at 72 ℃ for 30 s, with a ramp rate of 0.1 ℃ s^{-1} from the annealing to the extension temperature. These initial five cycles were followed with 30 cycles of denaturation at 95 ℃ for 30 s, annealing at 55 ℃ for 45 s, and extension at 72 ℃ for 30 s, followed by a final extension step at 72 ℃ for 10 min. The PCR conditions for *pmoA* gene amplification was as follows: after an initial denaturation step at 95℃ for 5 min, followed by 5 cycles of denaturation at 95℃ for 25 s, annealing at 65℃ for 30 s, extension
150 at 72℃ for 30 s. These initial five cycles were followed with 30 cycles of denaturation at 95℃ for 25 s, annealing at 55℃ for 30 s, and extension at 72℃ for 30 s, followed by a final extension step at 72℃ for 10 min.

T-RFLP analysis was performed at Sangni Corporation (Shanghai, China) using an ABI Prism 3100 Genetic Analyzer. T-RFLP profiles for *mcrA* and *pmoA* genes were generated with the
155 endonucleases *HaeIII* (Fermentas, USA) and *HhaI* (Fermentas, USA), respectively. Data analysis of the resultant T-RFLP profiles was performed using PeakScan (version 1.0, Applied Biosystems, Inc.). Fragments with a signal above 1% of the sum of all peak heights were included and peak positions that differed in size by ≤ 2 bp in an individual profile were binned and considered as one fragment. Minimum T-RF size for inclusion within the cluster analysis was set at 50 bp or larger.

160 Local databases of *mcrA* and *pmoA* gene sequences were constructed using over 2,000 downloaded from Functional Gene Pipeline/Repository (FGPR, http://fungene.cme.msu.edu/) and National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). *In silico* digestion was

Biogeosciences Discuss., doi:10.5194/bg-2016-213, 2016
Manuscript under review for journal Biogeosciences
Published: 18 July 2016

performed on these sequences using restriction endonuclease sequences and the T-RFs were assigned to specific methanogen and MOB lineages, which was subsequently used to predict and verify the assignment of individual T-RFs in this study.

2.5. Quantitative PCR

McrA and *pmoA* qPCR were performed using the mals/mcrA-rev (Steinberg and Regan, 2008) and A189F/Mb661R (Kolb et al., 2003) primer pairs, respectively. Real-time PCR assays were performed in a volume of 10 μL containing 5 μL 2 × SYBR Premix Ex Taq TM (Takara, Japan), 150 nmol L^{-1} forward and reverse primers and 5 ng of template DNA. Thermal cycling conditions for the two genes were also the same as described for the T-RFLP analysis. The standard curves for the *mcrA* and *pmoA* genes were created using a 10–fold dilution series of plasmids containing the target gene of interest derived by PCR and cloning from soil.

For determination of absolute quantities of *mcrA* and *pmoA* transcripts, the quantitative PCR was performed using 10 ng of cDNA template. The standard was prepared from *in vitro* transcription of *mcrA* and *pmoA* clones derived from soil using the Riboprobe *in vitro* Transcription System (Promega) according to the manufacturer's instructions. The *in vitro* transcript was purified by phenol-chloroform extraction and quantified using a RiboGreen RNA quantification kit (Invitrogen). The resultant transcripts were reverse transcribed as described above and a dilution series (10 fold) of cDNA was

Biogeosciences Discuss., doi:10.5194/bg-2016-213, 2016
Manuscript under review for journal Biogeosciences
Published: 18 July 2016

used as the standard.

2.6. Statistical analysis

Soil properties such as pH, soil organic carbon and total nitrogen together with gene abundance between the treatments were compared by ANOVA analysis using the Statistical Package for Social Science (SPSS 13, SPSS Inc., Chicago, IL, USA). Significance among means was identified using least significant differences. Pearson correlation analysis between CH_4 flux and population size of resident and active methanogens and methanotrophs was also performed using SPSS.

3. Results

3.1. Influence of P and K deficiency on soil properties and plant biomass

Compared to the fully fertilized NPK treatment, –P and –PK treatments induced significant decreases in soil organic carbon (SOC), total phosphorus (TP) and available P (AP) content, whereas –K only caused significant decline in available potassium (AK) content (Table 1). Significant lower plant biomass were also observed in –P, –K and –PK plots compared to NPK treatment, suggesting that deficit in soil P and K availability had restricted the growth of rice plant (Table 2). Especially, at the rice tillering stage, the –P treatment revealed a reduction of 41% and 28% ($P < 0.01$) in aboveground and belowground plant biomass, respectively.

Biogeosciences Discuss., doi:10.5194/bg-2016-213, 2016
Manuscript under review for journal Biogeosciences
Published: 18 July 2016

3.2. Influence of P and K deficiency on CH_4 flux

The measurements from the field plots and soil incubation showed that CH_4 fluxes from all the treatments mainly occurred at tillering stage while it was at very low levels at ripening stage (Table 1). The methane emission rates at tillering stage exhibited that the NPK treatment possessed the highest rate while the lowest appeared in the –P treatment which was significantly differed from NPK (P < 0.05). The CH_4 flux in –K treatment were not significantly different from NPK (P > 0.05). Meanwhile, the –PK treatment showed significantly higher CH_4 flux than –P (P < 0.05) but less than NPK treatment.

3.3. Influence of P and K deficiency on methanogenic populations

When assessing the abundance of the *mcrA* gene, based upon both DNA- and mRNA-based analyses, we observed significantly higher gene copy numbers at the tillering stage when compared to the ripening stage across all the treatments (Fig. 1a). At the tillering stage, both P and K deficiency did not significantly modulate the abundance of *mcrA* gene copy numbers when compared to the NPK treatment. However, at the transcription level all three-deficiency treatments (–P, –K and –PK) revealed significant decrease in *mcrA* transcript abundance in comparison to the fully fertilized NPK treatment (P < 0.05, Fig. 1b). Although no significant difference in *mcrA* transcript abundance was observed

Biogeosciences Discuss., doi:10.5194/bg-2016-213, 2016
Manuscript under review for journal Biogeosciences
Published: 18 July 2016

between –K and –P treatments ($P > 0.05$), the –PK treatment exhibited lower *mcrA* transcript abundance when compared to both –K and –P treatments (Fig. 1b). Similar to the the lower methane emissions observed at the ripening stage, all the treatments revealed lower *mcrA* transcript copy numbers under both –P and –K treatments, suggesting, as above, that the nutrient deficiency effect was independent of the strength of methane flux or rice cultivation stage (Fig. 1b).

The T-RFLP patterns of resident methanogenic community structures at the rice tillering stage were relatively stable in response to both P and K deficiency (Fig. 2a). However, both –P and –K treatments induced shift in the active community composition of methanogens when assessing community structure by transcript abundance (Fig. 2a). These two treatments severely limited the expression of *mcrA* from less abundant members of methanogens represented by T-RFs 118, 208, 277 and 292 bp, but stimulated the activity of other methanogens represented by T-RFs 95 and 202 bp (Fig. 2a). When both P and K were deficient, the active methanogen community composition was roughly similar with that in–P and –K plots, besides that, the further shifts also happened in this treatment, such as the methanogens represented by T-RF 216 bp, corresponding to uncultivated archaeal methanogens, was relatively less abundant in –PK when compared to –P and –K treatments. At ripening stage, both DNA- and mRNA-based analyses revealed similar community composition as that at the tillering stage, and –P and –K exhibited similar effects on methanogen community compositions (Fig. 2a).

Biogeosciences Discuss., doi:10.5194/bg-2016-213, 2016
Manuscript under review for journal Biogeosciences
Published: 18 July 2016

3.4. Influence of P and K deficiency on MOB populations

For the resident (DNA) MOB abundance, no significant differences were detected between treatments at the tillering stage. Similarly, at the ripening stage, except for the –P treatment, all the treatments revealed no significant differences between observed MOB community structure ($P > 0.05$, Fig. 1c). However, the abundance of the active (mRNA) MOB under nutrient deficient conditions showed a different picture (Fig. 1d). At the tillering stage, represented by high CH_4 flux, P deficiency significantly increased (~85%) *pmoA* transcript abundance while K deficiency led to a significant reduction (~75%) in *pmoA* transcript when compared to the NPK treatment (Fig. 1d). When both P and K were deficient, the abundance of *pmoA* transcripts only accounted for 24% of the NPK treatment and was the lowest between all the treatments. At the ripening stage, a different trend was observed with potassium deficiency resulting in significant increase in *pmoA* transcript abundance when compared to NPK, –P and –PK treatments.

The MOB community compositions, based on both DNA and mRNA analyses, revealed differential responses under the deficiency of both P and K (Fig. 2b). At the tillering stage, both –P and –PK treatments displayed similar T-RFLP patterns but different from the MOB community compositions under potassium deficiency alone, especially at the mRNA level. –P and –PK treatments resulted in community shifts within the active MOB populations, particularly T-RF 150 bp, predicted to represent the type I methanotroph *Methylococcus/Methylocaldum*. Transcript abundances of this

Biogeosciences Discuss., doi:10.5194/bg-2016-213, 2016
Manuscript under review for journal Biogeosciences
Published: 18 July 2016

methanotrophs increased 10 fold, whilst transcripts representing likely members of the genus *Methylococcus* (T-RF 108 bp) significantly reduced in –P and –PK treatment compared to NPK control ($P < 0.05$). Meanwhile, –P and –PK treatments induced a decline in the relative abundance of an unknown type II methanotroph genus or *Methylosinus/Methylocystis* (T-RF 81 bp). For other taxa, T-RF 143 bp was observed in phosphorus deficiency treatments (–P and –PK). Potassium deficiency resulted in a substantial reduction of the relative abundance of T-RF 128 bp (corresponding to several genera, including type I and type II methanotrophs) in resident methanotrophic community (Fig. 2b).

At the ripening stage, –P also induced a significant increase in the relative abundance of T-RF 249 bp within the resident MOB community and a significant reduction of T-RFs 70 bp and 108 bp in the active MOB. Remarkably, –K not only caused significant increase in the relative abundance of T-RF 81 bp in the resident MOB community but also increased the relative abundance of the T-RF 70 bp in the active MOB populations (Fig. 2b). In addition, –PK showed similar T-RFLP pattern to –K.

4. Discussion

Methane emission from rice paddy cultivation is the consequence of the activity of both methanogenic and methanotrophic populations. Phosphorus and potassium availability have been known to influence methane emissions from peat (Aerts and Toet, 1997) and paddy soils (Yang et al., 2010; Shang et al., 2011; Han et al., 2002). In this study, we report that phosphorus deficiency induced

Biogeosciences Discuss., doi:10.5194/bg-2016-213, 2016
Manuscript under review for journal Biogeosciences
Published: 18 July 2016

significant reductions in CH_4 emission, whereas potassium deficiency did not influence net methane flux when compared to balanced fertilization control plots. Further, these data indicated no significant correlation between CH_4 flux and the abundance of methanogens and methanotrophs population (based on DNA), as reported by Zheng et al (2013) that showed the abundance of the *pmoA* gene was not correlated to soil methane oxidation rates in paddy fields. Therefore, as previously hypothesised, it seems that methane flux under rice paddy cultivation cannot be explained only through DNA-based analysis of the methanogenic and MOB communities.

In contrast, mRNA-based assessments of the active community composition of methanogens and MOB have been shown to closely relate to methane flux (Freitag and Prosser, 2009; Freitag et al., 2010; Kumaresan et al., 2011). Here, we demonstrated that the abundance of both active methanogens and MOB, represented by transcript abundance of *mcrA* and *pmoA*, was significantly influenced by different fertilization regimes when compared to DNA-based approaches at the tillering stage when higher CH_4 emission was observed, and the CH_4 flux was closely related with the transcript ratio of *mcrA*/*pmoA* (r^2=0.682; $P < 0.05$). Based upon this observation, we propose that the transcript analyses can be better indicator of the link between methanogen and methanotroph community function and its relation to overall CH_4 flux, particularly in paddy soils.

Deficiencies within the key nutrients of P and K showed differential influence upon the size and composition of methanogens and MOB communities. Both P and K deficiencies resulted in a reduction

Biogeosciences Discuss., doi:10.5194/bg-2016-213, 2016
Manuscript under review for journal Biogeosciences
Published: 18 July 2016

in *mcrA* transcripts abundance with similar transcript composition at the tillering stage. However, the deficiency of these two elements resulted in different consequences for *pmoA*-containing MOB communities at the transcript level. For example, P deficiency caused significant increase in the abundance of *pmoA* transcripts and also influenced the relative abundance of the active MOB populations, while K deficiency induced significant reduction in the abundance of *pmoA* transcripts but did not affect the community composition. Differences in CH_4 flux, influenced by –P and –K treatments could be explained by the response of *pmoA*-containing MOB communities and its influence on methane oxidation rates (Zheng et al., 2008). The distinct responses of methanogens and MOB to the nutrient deficiency are likely to be linked to the difference in their adaptation and response strategies to different environment conditions. Whilst P and K deficiencies did not affect methanogens, active MOB community composition is sensitive to variations in the environmental factors (Le Mer and Roger, 2001; Chowdhury and Dick, 2013). Phosphorus is an essential life element that is a crucial component of nucleotides and energetic material, such as ATP (Rausch and Bucher, 2002). Phosphorus deficiency not only affects plant growth but also the growth of microorganisms, but in this study, the T-RF representing the genus *Methylococcus*/*Methylocaldum* was markedly enriched within the –P treatment (Fig. 2b). Previous studies have reported that *Methylococcus* and *Methylocaldum* sp. were dominant members of methanotrophic communities in low P oligotrophic soil (Chauhan et al., 2012). We speculate that the possible adaptations of this MOB to a P deficient environment might be attributed to one or more

Biogeosciences Discuss., doi:10.5194/bg-2016-213, 2016
Manuscript under review for journal Biogeosciences
Published: 18 July 2016

adaptive strategies. First, the possession of high-affinity P transporters, capable of producing P-liberating enzymes, as has been documented previously (Veraart et al., 2015; Sebastian and Ammerman, 2009). Second, P use minimisation through low P containing membranes using non-phosphorus lipids (Van Mooy et al., 2009) or smaller genomes and lower RNA content, which can minimize their P-requirements may explain their ability to thrive in low P environments (Sterner and Elser, 2002). In contrast, potassium plays an important role in methanotrophic processes via enhancing the redox potential and reducing the contents of active reducing substances in flooding soil (Zheng et al., 2008; Babu et al., 2006), and as a consequence, the copy numbers of *pmoA* transcripts are sharply decreased under –K condition. Concerning the distinct variations in the active methanogen and MOB population size and community composition in –PK treatment from that in –P and –K treatments, for example, –PK treatment displayed similar *pmoA* transcripts abundance with –K treatment, but the T-RFLP pattern of *pmoA* transcripts was similar with that in –P treatment, the relative high CH_4 emission was assumed to be a consequence of combine effects of phosphorus and potassium deficiencies.

5. Conclusions

CH_4 emission was much more affected by phosphorus deficiency compared to potassium deficiency in the paddy rice soil. P deficiency may significantly decrease CH_4 flux rate via reducing the

325 activity of methanogens and enhancing the activity of methanotrophs. Comparatively, the transcript abundance and composition of *mcrA* and *pmoA* to the nutrient deficiency were more sensitive than DNA-based analysis and P deficiency induced more variations within community composition of the active MOB than K deficiency.

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Manuscript under review for journal Biogeosciences
Published: 18 July 2016

Table 1 The basic characteristics of the examined paddy soil under different fertilization treatments and the CH_4 flux from from field plots at rice tillering stage and rice ripening stage.

Treatment[b]	Soil properties[a]							CH_4 flux	
	SOC (g kg^{-1})	**TN (N g kg^{-1})**	**TP (P g kg^{-1})**	**TK (K g kg^{-1})**	**AP (P mg kg^{-1})**	**AK (K mg kg^{-1})**	**pH (1:2.5 H_2O)**	**Tillering**	**Ripening**
NPK	20.53±1.01a[c]	1.91±0.09ab	0.69±0.05a	13.69±0.10a	12.23±1.51b	135.40±33.98a	5.15±0.15a	25.83±4.28a	0.31±0.26a
–K	20.51±0.36a	2.10±0.16a	0.66±0.06a	13.77±0.46a	14.38±1.56a	63.40±5.38b	5.16±0.15a	19.32±4.05ab	0.06±0.46a
–P	18.60±0.06b	1.86±0.03b	0.42±0.06b	14.02±0.31a	4.45±1.03c	138.83±35.93a	5.27±0.23a	15.67±1.44b	0.17±0.02a
–PK	17.94±0.11b	1.96±0.06ab	0.43±0.06b	13.76±0.11a	4.13±0.37c	59.28±3.61b	5.19±0.20a	21.79±1.45a	0.20±0.23a

[a] Soil properties: ***SOC,*** total carbon, ***TN,*** total nitrogen, ***TK,*** total potassium, ***TP,*** total phosphorus, ***AP,*** available phosphorus and ***AK,*** available potassium.

[b]Treatments: ***NPK,*** balance chemical fertilization; ***–K,*** potassium deficiency; ***–P,*** phosphorus deficiency; ***–PK,*** phosphorus and potassium deficiency.

[c] Significant differences ($P < 0.05$) between treatments are shown with letters a, b, or c; mean ± SEM, n=3 for each treatment.

Biogeosciences Discuss., doi:10.5194/bg-2016-213, 2016
Manuscript under review for journal Biogeosciences
Published: 18 July 2016

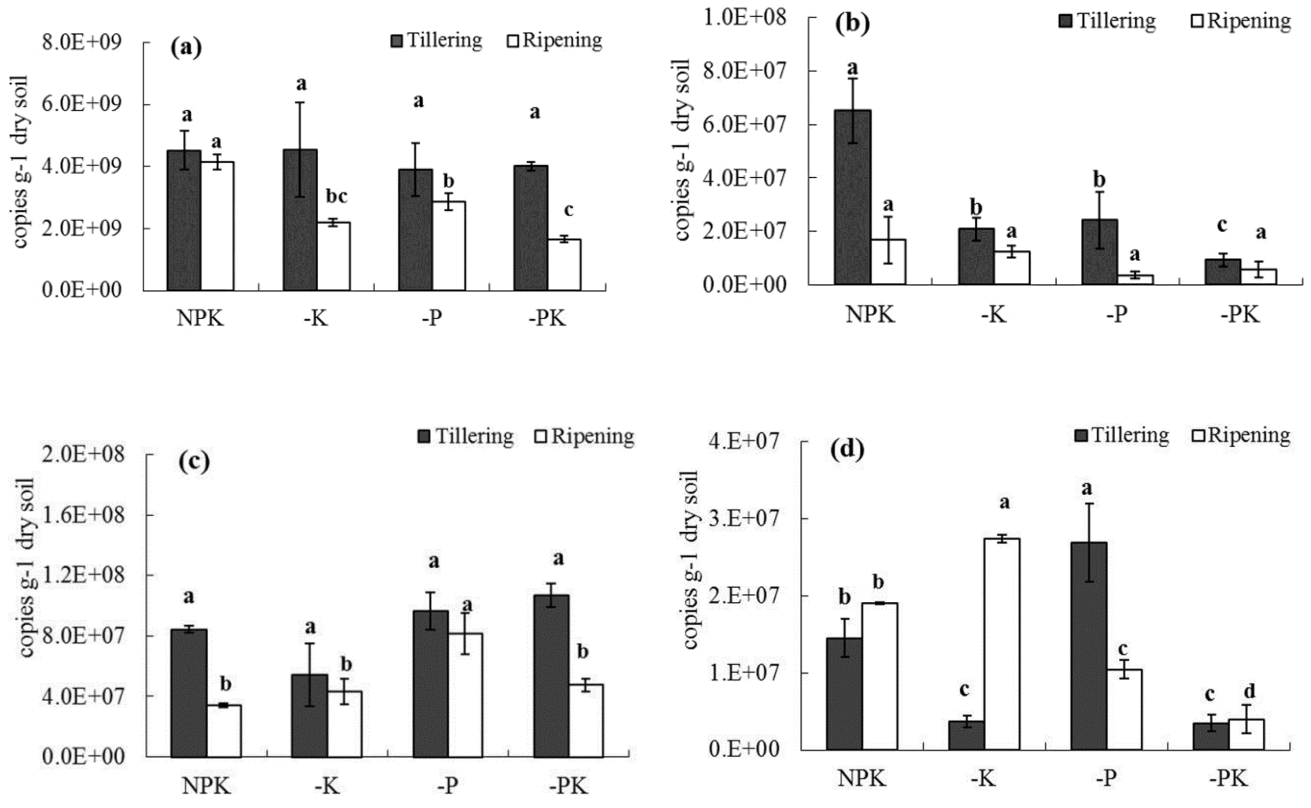
Table 2 The Plant biomass under different fertilization treatments.

Treatment [a]	Plant biomass (g m^{-2})			
	Aboveground		Belowground	
	Tillering	Ripening	Tillering	Ripening
NPK	207.94 ±1.46a [b]	447.11 ±7.53a	68.03 ±3.28a	108.2 ±0.76a
–K	190.51 ±6.45b	371.53 ±6.07b	60.20 ±2.74b	103.56 ±0.60a
–P	121.74 ±7.70c	303.81 ±50.43b	49.03 ±1.57c	93.55 ±10.50a
–PK	131.41 ±12.99c	313.96 ±43.04b	52.10 ±0.30c	90.64 ±5.79a

[a] Treatments: ***NPK,*** balance chemical fertilization; ***–K,*** potassium deficiency; ***–P,*** phosphorus deficiency; ***–PK,*** phosphorus and potassium deficiency.

[b] Significant differences ($P < 0.05$) between treatments are shown with letters a, b, or c; mean ± SEM, n=3 for each treatment.

Biogeosciences Discuss., doi:10.5194/bg-2016-213, 2016
Manuscript under review for journal Biogeosciences
Published: 18 July 2016



465 **Fig. 1** Copy numbers of *mcrA* gene (a) and gene transcripts (b) and *pmoA* gene (c) and gene transcripts (d) in relation to nutrient P and K deficiency. ***NPK***, balance chemical fertilization; ***–K***, potassium deficiency; ***–P***, phosphorus deficiency; ***–PK***, phosphorus and potassium deficiency. Significant differences ($P < 0.05$) between the soils are shown using letters a, b, or c. Statistical analysis between soils from tillering and ripening stage was performed separately. Soils with the same letter
470 at each depth are not significantly different at the $P < 0.05$ level.

Biogeosciences Discuss., doi:10.5194/bg-2016-213, 2016
Manuscript under review for journal Biogeosciences
Published: 18 July 2016

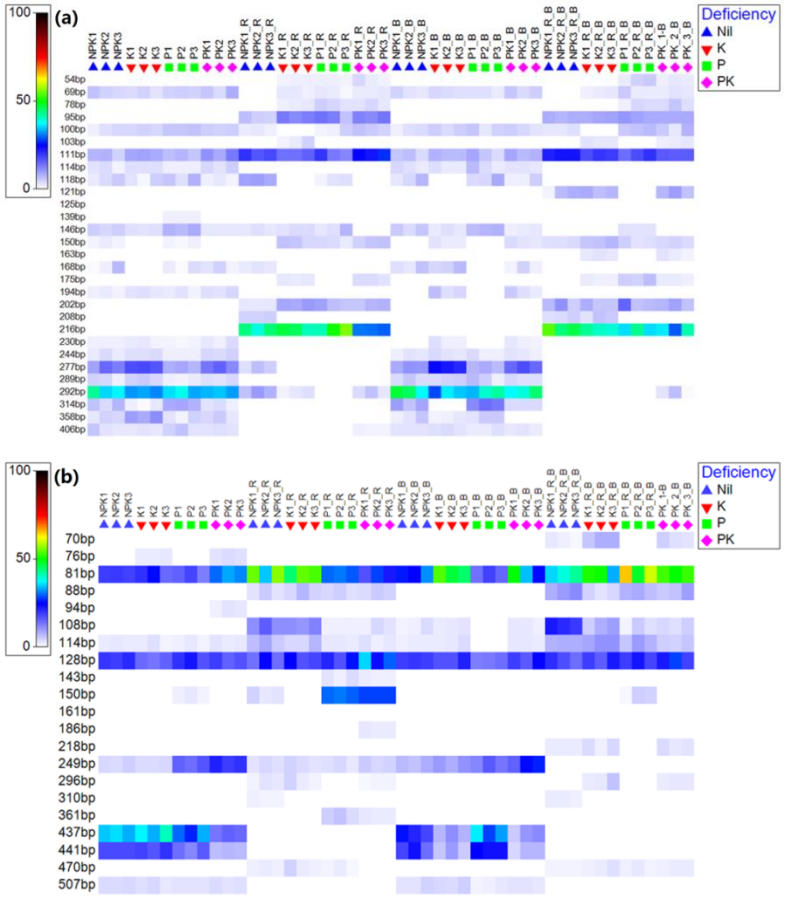


Fig. 2 Heatmap of *mcrA*-(a) and *pmoA*-based (b) T-RFLP profiles showing average relative abundances of *mcrA* T-RFs with endonuclease *HaeIII* and *pmoA* T-RFs with endonuclease *HhaI* in soils. The relative abundance of T-RFs is given as a percentage of the total peak height. Fragment sizes within the graph indicate the sizes (bp) of the experimental T-RFs by T-RFLP. Letters "R" after the treatments indicate samples from mRNA-derived profile, and letters "B" indicate the samples from ripening stage. ***NPK***, balance chemical fertilization; ***K***, potassium deficiency; ***P***, phosphorus deficiency; ***PK***, phosphorus and potassium deficiency.