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Interactive comment on "Linking phosphorus and potassium deficiency to microbial methane cycling in rice paddies" *by* Rong Sheng et al.

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Dear Madam/Sir,

Thank you for your comments and suggestions on our manuscript. We have revised the manuscript accordingly, and highlighted the changes in the revised version. The detailed corrections are listed below point by point:

General comments

The authors performed an interesting study on effects of P and K availability in rice paddy soils on methane emission and methanogen and methanotroph presence and community composition. They find reduced CH4 emission in low P plots, which they attribute to higher methanotroph activity and lower methanogen activity. Effects of

potassium on CH4 emission are less pronounced. Copy numbers of mcrA and pmoA genes are not linked to CH4 emission and hardly show a response to fertilization treatments. Transcripts of pmoA showed differences in the active MOB community between treatments, also dependent on the rice growth stage.

Strong points of the manuscript are the inclusion of mcrA and pmoA transcript analysis in combination with CH4-flux data, and the use of long-term fertilization plots. However, I would like to see improvement on the following issues:

(1) Introduction/Discussion

##Comments: To my understanding, it is quite clear when P or K are limiting plants, but concentrations at which they become limiting to microbes are far less understood. Therefore, I recommend to use terms like 'P/K deficient' with care. For example, your results point at an increase in MOB activity in low P plots, perhaps indicating not so much P-limitation, but an alleviation of excess P? Also, effects may arise from altered (C):N:P:K stoichiometry, rather than concentrations in itself.

Answer: We agree to the comment that it is less understood about the lack of phosphorus and potassium for plants whether also influence microbial activities. That is the major reason for us to conduct this study. In this study, we observed that the soils without applying P fertilizers for more than 20 years contained very low available P and showed severe limitation to rice growth. But the various influences of low P availability on methanogenic and MOB communities were observed. The population sizes of mcrA-containing methanogens were not obviously affected by the low P availability, but low P concentration exhibited significant restrictions to the transcript abundance, which may in turn limit the activities of methanogens. On the contrary, the copy number of both pmoA gene and gene transcripts were not restricted by low P availability. These phenomena suggested that the low P content restricted plant growth may not limit methanogen and MOB population sizes, but whether it influence the transcription activity of functional gene of methanogens or MOB might rely on functional groups and

environmental factors. Such as the transcription of mcrA gene was blocked by the low P availability while the transcription of pmoA gene was not restricted under same P level. From this study, we can hardly conclude which P level is critical for specific functional microbial group and whether the results is caused by N:P:K stoichiometry, to answer these questions, further investigations will be required. We observed a higher transcription activity of pmoA gene in low P plots based on mRNA analysis, which indicated the plots possess more active MOB and suggested possibly higher activities of methane oxidation. Further studies based on protein level may be useful for assessing the in situ activities of MOB.

Discuss how, because different MOB respond in different ways, results may strongly depend on the initial community composition. Different soils may react in different ways.

Answer: We have added related discussion in Line 371 - Line 380 in the manuscript. Details are as follows: "It should be noted that previous studies have documented that soils derived from different parent materials may possess different initial microbial community structure (Ulrich and Becker, 2006; Sheng et al., 2015). Agricultural practices such as crop planting, fertilization and irrigation can modify the initial microbial community structures to some extent based on the initial microbial communities (Fierer et al., 2003). Therefore, different soils may have different methanogenic and methanotrophic community composition structures, their shift patterns in different soils responded to low P availability may vary among different soil types. The variations of the methanogenic and methanotrophic communities in responding to the depleting P and K levels in the paddy soil derived from quaternary red clay may be transferrable to other soils in tendency, but the varying species might be obviously different."

(2) Methods

##Comments: The CH4-flux method is poorly described. Please provide more detail on the method. Where and how were the samples taken? Did the chambers include rice plants? Did you measure time-series? From how many static chambers per plot?

How many replicate gas samples? How many replicates in time?

Answer: We have added more details about the in situ CH4 flux measurement in Line 125 to Line 136 in the "Materials and methods" section. Additionally, we also added the information about CH4 flux measurement using soil incubation in Line 137 –Line 148. The details are as following: "In situ methane fluxes from the experimental field plots were sampled using static chambers (Shang et al., 2011) at tillering and ripening stages. The sampling chamber was made of PVC with a size of $60\times70\times90$ cm, which was equipped with one circulating fan inside to ensure sufficient gas mixing and wrapped with a layer of sponge to minimize air temperature changes inside the chamber during the period of sampling. After rice transplant, a PVC frame was fixed into a random site in each plot. The top edge of the frame had a groove for filling with water to seal the rim of the chamber. Each frame enclosed 6 rice plants and the height aboveground of the frame is only 5 cm to avoid affecting the growth of rice plants. Gas samples were taken from the chamber headspace with a 30 mL syringe and stored in pre-evacuated vials (Labcolimited high Wycombe UK). At each sampling stage, CH4 fluxes were measured in triplicate plots for all treatments once a day for 3 days. Confirmation of a similar variation trend of CH4 fluxes was observed during these 3 days, we only presented the data from the third day when soil samples were collected in this study. After in situ CH4 flux sampling, fresh soil samples (0–20 cm) were immediately taken from the plots to conduct incubation experiment to determine methane emission rates under controlled environment. The incubation was carried out as follows: after 24 h pre-incubation at 30 oC, equal amounts of fresh soil samples from each treatment (three replicates) were homogenised and 30 g soil (dry weight) was placed into a 250 mL plastic box that can be sealed. For tillering stage samples, soil water content was adjusted to field flooding condition by maintaining 2 cm free surface water. For the ripening stage samples, water content in the soils was adjusted to the same level (50% moisture content, w/w) according to the highest water content of the fresh soil samples. Afterwards, the plastic boxes were sealed and incubated at 30 oC. Headspace gas sampling was conducted at 0 and 60 min, respectively, using a 5 mL syringe and

stored in pre-evacuated vials (Labcolimited high Wycombe UK)."

##Comments: How were total and available P and K determined? Do they reflect availability to plants? / to which extent is P or K unavailable to plants available to microbes?

Answer: We have added the information about the measurement of soil properties in Line 116 - Line 123 in the "Materials and methods" section. Briefly, total phosphorus (TP) and potassium (TK) were measured by Inductively Coupled Plasma Spectrometry (Agilen, USA) after fusion in NaOH. Available K (AK) was determined by Atomic Absorption Spectroscopy (Seal, Germany) after extraction with NH4OAc. Available P (AP) was measured using UV-Vis Spectrophotometer (PerkinElmer, USA) following extraction with 0.5 M NaHCO3. In this study, we use the variation in plant biomass to show the P and K availability to plants, but to which extent is P or K unavailable to plants available to microbes is a question worthy to be discussed but remains unclear, because the microbial diversity is very high in soil and the requirements for nutrients for different microbes may vary significantly.

(3) Results

##Comments: The figures can be improved. It would be helpful to show hierarchical clustering of the samples based on their T-RFLP profiles, to show which samples/treatments are most similar (per sample class).

Answer: We have done the cluster analysis and added in the manuscript as Figure 4 (rice tillering stage) and Supplementary Figure 1 (rice ripening stage).

##Comments: Add gene names and DNA vs mRNA copies inside the panels or on the y-axes of the graphs.

Answer: We have revised in the manuscript.

##Comments: Show correlations between CH4-flux and DNA and mRNA copies, and also present the relation between CH4 flux and mcrA/pmoA transcripts here. You refer

to these in the discussion but they are missing in the results section.

Answer: We have added the correlation analysis between methanogenic, methanotrophic populations, soil properties and CH4 flux in Line 297 – Line 302 in the "Results" section. The details are as follows: Correlation analysis indicated that the CH4 fluxes from field and soil incubation were significantly correlated to the transcript ratio of mcrA/pmoA ($P < 0.05$ and $P < 0.01$, respectively, Table 3) at the tillering stage. In addition, CH4 flux from soil incubation was also significantly correlated with the contents of both total and available phosphorus (TP and AP, $P < 0.01$), SOC ($P < 0.05$) and plant biomass ($P < 0.05$). (4) Discussion ##Comments: Please also better explain why one would expect DNA copy numbers to be less indicative of community functioning than transcripts.

Answer: We have added the sentences "Although other studies also reported that the community structures based on DNA analysis could respond to soil environmental changes and they could reflect the existing state of functional groups (Ahn et al., 2014; Lee et al., 2014; Zheng et al., 2013), the analysis based on gene transcripts are increasingly reported to provide more useful information in understanding the activities of functional microbial communities than the DNA analysis, as gene transcripts are indicative for the active groups against a large resident microbial population (Nicolaisen et al., 2008; Nicol et al., 2008; Freitag et al., 2010)." in Line 331 to Line 357 to explain this question.

##Comments: I am missing some discussion on what these results mean in terms of CH4-mitigation potential? Low emissions seem to come at the expense of plant biomass (and possibly nutritional value?).

Answer: We have added related discussion in Line 365 – Line 371 in the "Discussion" section. Details are as follows: "In addition, we focused on the analysis of the possible contributions of methanogens and methanotrophs on methane emission in relation to the soil P and K status, but in fact the plant biomass was also affected. Although we

observed that CH4 emission was significantly related to plant biomass, CH4 emissions did not always rely on the plant biomass. For instance, the crop yield was significantly different but the CH4 emission was similar between NPK and –PK treatments. Similar result was also detected by Shang et al. (2011). So, the mitigation of CH4 emission under very low soil P content might be influenced by both poor P nutrition of methanogens and methanotrophs and low plant biomass."

(5) Specific comments

##Comments: Line 31: I would end this sentence at 'transcriptional level', as the relation between 'population size' and DNA copies is debatable.

Answer: We have deleted the sentence.

##Comments: Line 125, why?

Answer: We modify the method due to the following reasons: 1) The MP FastPrep can improve the cell lysis efficiency compared to vortex; 2) Using MP FastPrep can save time and labor.

##Comments: Line 254 This seems to conflict with the previous sentence, where members of methylococcus increased. Are these T-RFs representing different species, meaning some methylococcus species increase whereas others decrease?

Answer: Yes, Methylococcus is a genus we predicted using in silico digestion, unfortunately, we can not specify which species are these T-RFs affiliated to, hence, under P limited condition, maybe some species of this genus were restricted while others were resistant.

##Comments: Line 280 describe how they were influenced by the fertilizer regime

Answer: We had described the influence of fertilization regime in the following paragraph. Here we focus on the comparative analysis between DNA and mRNA level.

##Comments: Line 286 the 'size' of the resident communities is hardly affected. It

would be interesting to also discuss the effect of the growth stage of rice on CH4 flux and methanogen and MOB communities.

Answer: We thought that the different size of resident communities between two sampling time might also attribute to the differences in soil water condition, soil nutrient availability and some other factors, so we did not discuss the specific effect of the growth stage of rice on CH4 flux and methanogen and MOB communities in this study.

##Comments: Line 303. How can you be sure that they were P deficient?

Answer: The significant lower plant biomass in P deficient treatments can verify that the soils were P deficient. Of course, the word "deficiency" here is specific to rice plant rather than soil microbes.

##Comments: Line 325: Add that effects are species specific, different soils may show different effects

Answer: We have added sentence: "We have observed these effects in our quaternary red soils, but to what extent it is transferrable to other soils remains to be established." in Line 389 – Line 391 in the "Conclusions" section.

Comments: Technical comments Line 78, round to whole numbers

Answer: We have revised in the manuscript.

Comments: Line 87, key nutrients -> phosphorus and potassium

Answer: We have revised in the manuscript.

Comments: Line 113, after washing off

Answer: We have revised in the manuscript.

Comments: Line 204 different

Answer: We have revised in the manuscript.

References

Nicol, G.W., Leininger, S., Schleper, C., Prosser, J.I.: The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. Environ. Microbiol., 10, 2966–2978, 2008. Nicolaisen, M.H., Bælum, J., Jacobsen, C.S., Sørensen, J.: Transcription dynamics of the functional tfdA gene during MCPA herbicide degradation by Cupriavidus necator AEO106 (pRO101) in agricultural soil. Environ. Microbiol., 10, 571–579, 2008.

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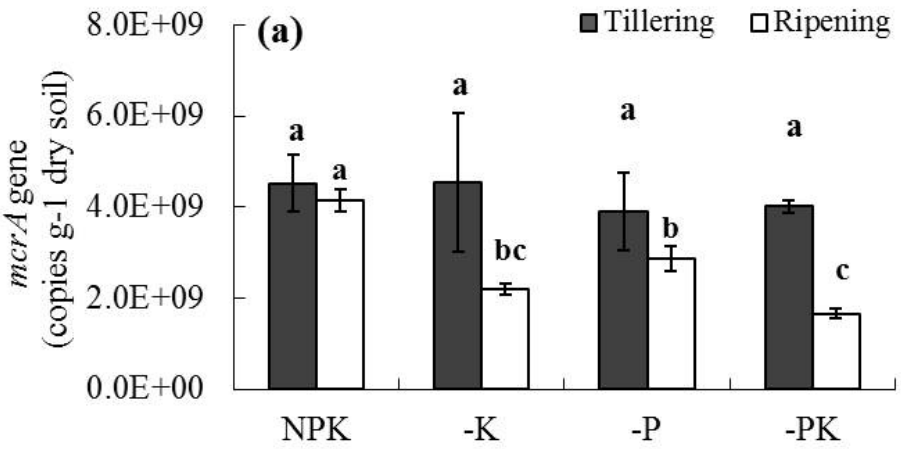

Fig. 1. Revised Fig. 2a

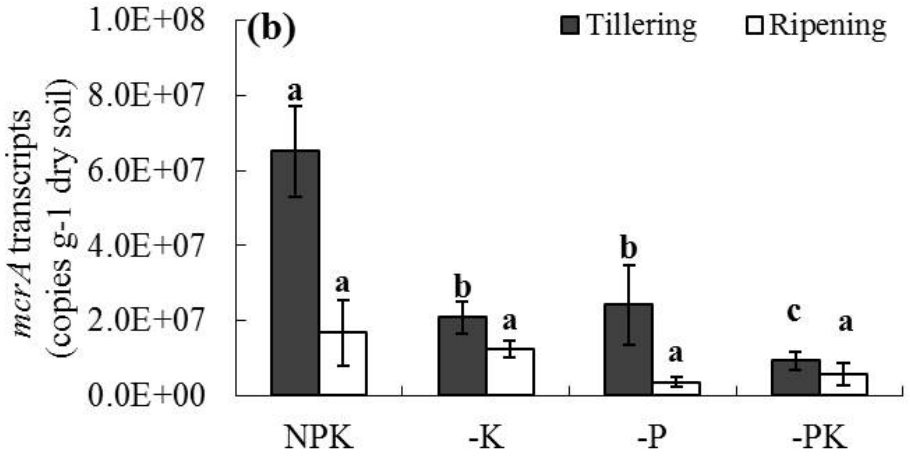

Fig. 2. Revised Fig. 2b

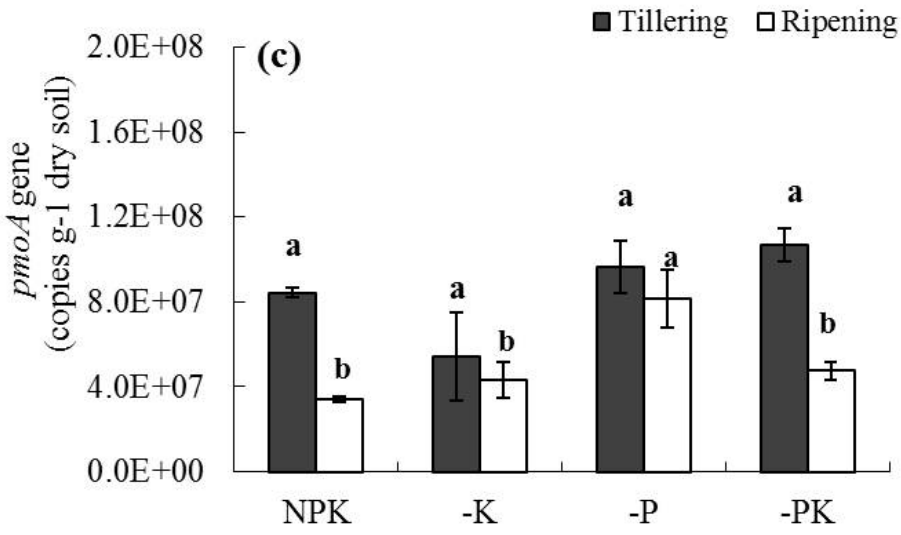

Fig. 3. Revised Fig. 2c

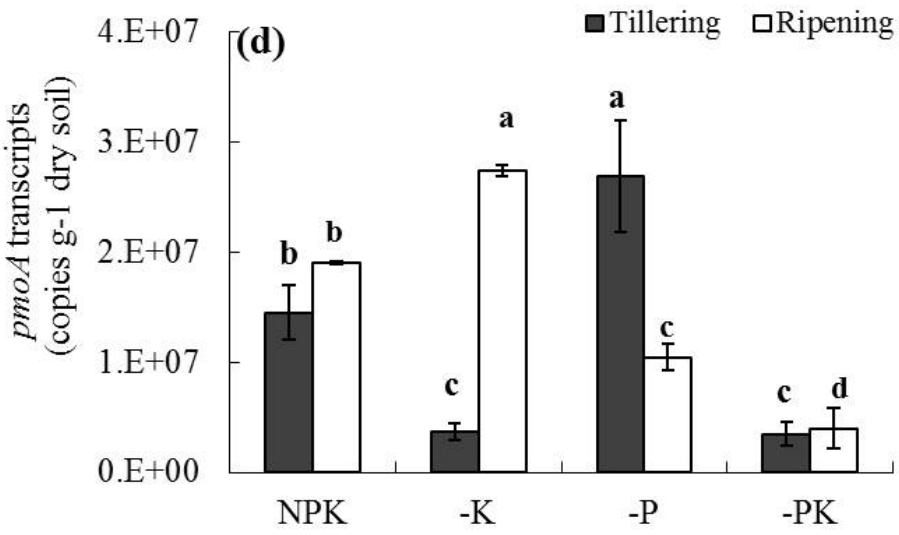

Fig. 4. Revised Fig. 2d

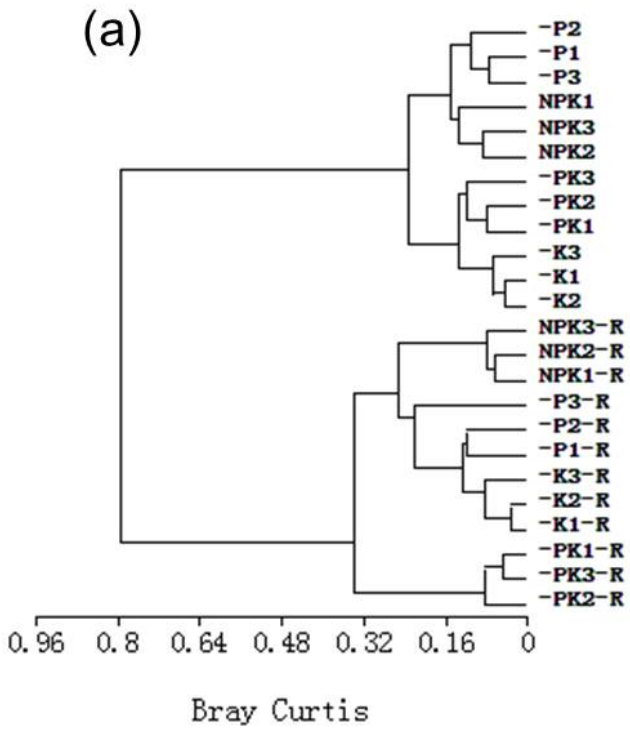

Fig. 5. New added Fig. 4a

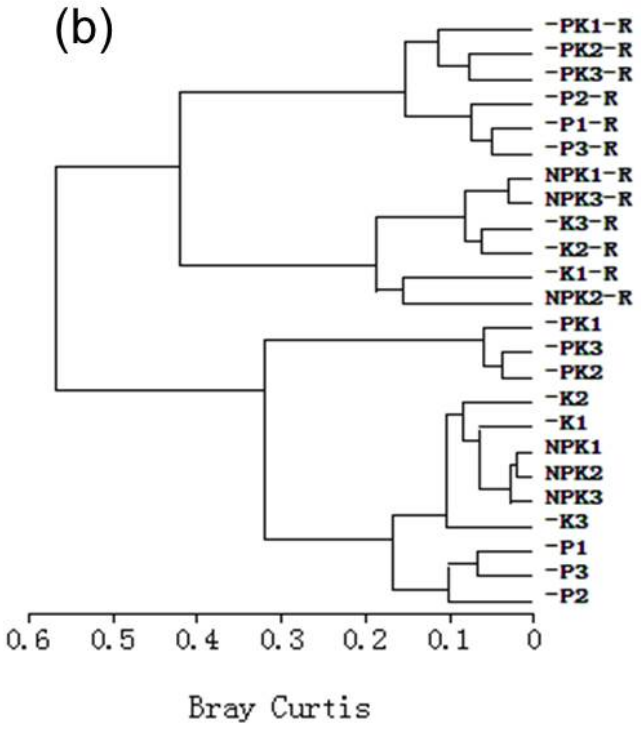

Fig. 6. New added Fig. 4b

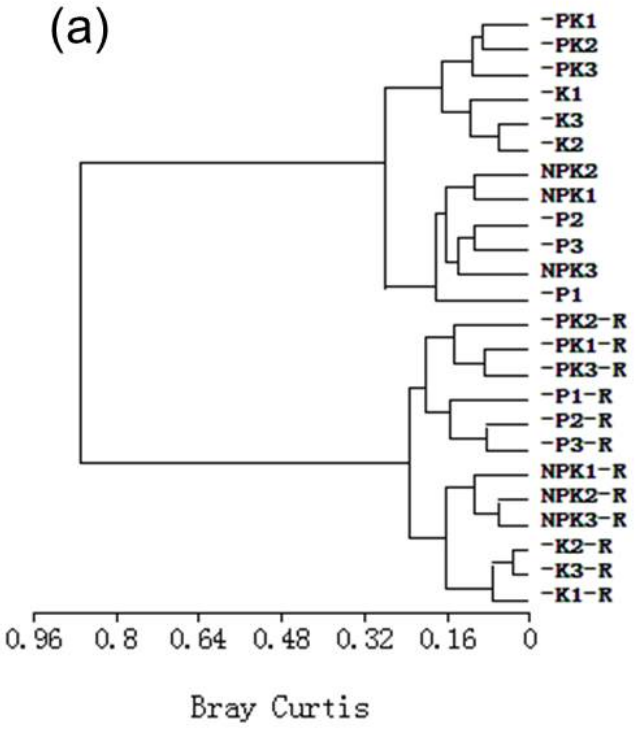

Fig. 7. Summplementary Fig. 1a

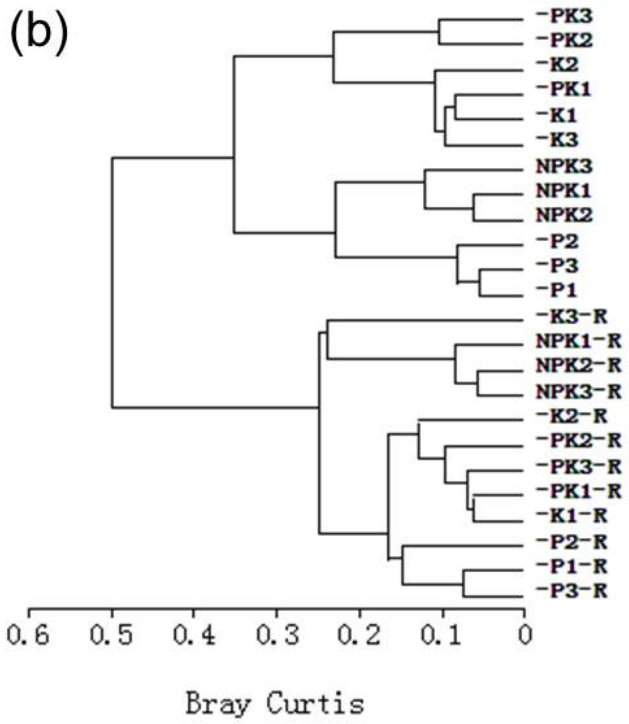

Fig. 8. Summplementary Fig. 1b