Influence of rice straw amendment on mercury methylation and nitrification in paddy soils

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ABSTRACT
Currently, rice straw return in place of burning is becoming more intensive in China than observed previously. However, little is known on the effect of returned rice straw on mercury (Hg) methylation and microbial activity in contaminated paddy fields. Here, we conduct a microcosm experiment to evaluate the effect of rice straw amendment on the Hg methylation and potential nitrification in two paddy soils with distinct Hg levels. Our results show that amended rice straw enhanced Hg methylation for relatively high Hg content soil, but not for low Hg soil, spiking the same additional fresh Hg, methylmercury (MeHg) concentration was significantly correlated to the dissolved organic carbon (DOC) content and relative abundance of dominant microbes associated with Hg methylation. Similarly, amended rice straw was found to only enhance the potential nitrification rate in soil with relatively high Hg content. These findings provide evidence that amended rice straw differentially modulates Hg methylation and nitrification in Hg contaminated soils possibly resulting from different characteristics in the soil microbial community. This highlights that caution should be taken when returning rice straw to contaminated paddy fields, as this practice may increase the risk of more MeHg production.

Main finding: Rice straw amendment enhanced both Hg methylation and nitrification potential in the relatively high, but not low, Hg soil.

1. Introduction
The production of neurotoxic methylmercury (MeHg) by anaerobic microbes in paddy fields has raised widespread concern due to its uptake and accumulation by rice (Liu et al., 2014a; Meng et al., 2014; Rothenberg et al., 2014; Zhang et al., 2010). The recent identification of mercury (Hg) methylating hgcAB genes provides a foundation for evaluating microbial Hg-methylation potential in the environment (Parks et al., 2013; Gilmour et al., 2013). Previous study has showed highly diverse anaerobic microbes in paddy soils that had potential to convert inorganic Hg into methylmercury (MeHg) (Liu et al., 2014a), which were predominantly distributed in Deltaproteobacteria, Firmicutes, Euryarchaeota and several unclassified groups based on analysis of HgcAB orthologs. In natural ecosystems, however, Hg methylation could not be completely predicted by hgcAB carrying microbes, which may be modulated by Hg availability, soil variables such as redox status, SO42− and characteristics of dissolved organic carbon (DOC) (French et al., 2014; Graham et al., 2013, 2012b; Liu et al., 2014b).

Rice straw is commonly incorporated into fields to enhance soil nutrient (Mandal et al., 2004; Yadvinder-Singh et al., 2004), and now this agricultural practice is getting more intensive than before in China due to a new policy that prohibits burning straw as this is considered to be one of the most important sources of air pollution. Rice straw is also used to remediate metal-contaminated soils because of its ability of reducing metal reactivity through interaction with its degradation products (Sud et al., 2008; Zhu et al., 2015). Previous studies have suggested that decomposition of rice straw by anaerobic breakdown was the main source of organic carbon in paddy soils (Liesack et al., 2000; Ye et al., 2015), which may affect Hg uptake and methylation by microbes due to its strong binding with Hg2+ that reduce Hg bioavailability and reactivity (Barkay et al., 1997; Zhu et al., 2015). However, recent studies argued that dissolved organic matter (DOM) enhanced Hg methylation at relatively low DOM content or under low sulfidic conditions (French et al., 2014; Graham et al., 2012a). This is
partially consistent with the recent field study indicating a positive correlation between MeHg and soil organic matter content in paddy fields around Hg mining area (Liu et al., 2014a). However, it is difficult to confirm whether DOM directly enhanced Hg methylation by forming available Hg-DOM complexes or indirectly by increasing activity of Hg methylating microbes in the paddy soils (Driscoll et al., 2012; Miskimin, 1991; Ullrich et al., 2001). Therefore, it is crucial to understand effect of rice straw return on Hg methylation in paddy environments.

In paddy ecosystems, amendment of organic matter may also change microbial community composition, which in turn influences soil functions such as respiration, nutrient cycle and other biogeochemical processes (Ryals et al., 2014). Among them, nitrification has been considered to be one of important indicators reflecting soil microbial process (Broo et al., 2005; Liu et al., 2010), and potential nitrification rate (PNR) has been widely used as an endpoint in evaluating soil quality (Liu et al., 2012; Smolders et al., 2001; Subrahmanyam et al., 2014). Experimental PNR involves ammonia oxidation from to ammonium to nitrite, which is the first and rate limiting step of nitrification (Brochier-Armanet et al., 2008; He et al., 2012). This pivotal process was generally presumed to be performed by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). AOA have been suggested to be sensitive to various soil perturbations (Bissett et al., 2013), while AOA seems to be insensitive to environmental changes due to strongly rigid cell membranes and other cellular macromolecules (Cray et al., 2013). For example, previous studies have indicated the positive effects of organic matter on function and community of ammonia-oxidizers attributing that organic matter provides more available ammonium during degradation (He et al., 2007; Kalvelage et al., 2013). In addition, it has been reported that Hg pollution influenced on AOB activity and community composition based on a short-term microcosm experiment (Liu et al., 2010). Up to date, however, little is known on the responses of ammonia-oxidizers to amended rice straw in Hg contaminated paddy soils.

Here, we conduct a microcosm experiment to evaluate how rice straw amendment will influence Hg methylation and nitrification processes in paddy soils with different Hg background levels. We analyzed bacterial community via illumina high throughput sequencing, which could be also used to identify Hg methylation associated microbes according to reported Hg methylating microbes containing hgeAB homologs (Gilmour et al., 2013). We also correlated Hg methylation to DOC content and the Hg methylators in the paddy soils. Soil nitrification potential and community of ammonia-oxidizers were measured to evaluate effects of rice straw amendment on soil microbial function. We tested the following hypotheses: (1) amended rice straw will have different effects on Hg methylation and nitrification in soil with different Hg background levels; and (2) amended rice straw will affect microbial communities accounting for Hg methylation and nitrification in the different soils.

2. Methods and materials

2.1. Experimental design and soil analysis

Soils with relatively low and high Hg background levels were collected at different paddy sites around Wanshan Hg mining areas in China, with an average Hg content of 0.52 and 5.53 mg kg⁻¹, respectively. The two paddy soils have similar chemical properties in pH (7.31–7.40) and organic matter content (5.20% and 5.14%, respectively). Soil samples were passed through a 4 mm sieve and then stored at 4 °C for microcosm experiment. In order to test the effect of rice straw amendment on potential for Hg methylation by indigenous microbial community, freshly spiked inorganic Hg (concentration of 2 mg Hg per kg soil) was introduced into the two soils. We established four treatments as follows: 1) soil with relatively low Hg background level (L); 2) the low Hg soil with 1% straw amendment (LS); 3) soil with relatively high Hg background level (H) and 4) high Hg soil with 1% straw amendment (HS). Briefly, 200 g of soil was introduced in a conical PVC pot (8 cm diameter and 10 cm height), and then the rice straw (0.2 cm in length, with less than 0.1 μg kg⁻¹ Hg) was added into the pots and mixed homogenously with soil. The inorganic Hg was added through 150 ml deionized water mixed HgCl₂ solution, leading to a paddy condition by flooding the soils with 2.5 cm depth of water. Each treatment was run with four replicates, giving a total of 16 experimental units. All the experimental pots were incubated at room temperature in dark condition for 120 days, and deionized water was added periodically to keep the soils saturated throughout the experiment. At the termination of this experiment, soil samples were collected and passed through a 2.0 mm sieve. One sub-sample was stored at −20 °C for analysis of THg and MeHg and microbial DNA extraction, and another sub-sample was air-dried for general chemical analyses.

2.2. Soil chemical analysis

Ammonium, nitrate and DOC in the soils were extracted with 0.5 M K₂SO₄ in a ratio of 1:5 by shaking at 200 rpm for 1 h and filtered using 0.45-μm MilliPore filter. The ammonium and nitrate contents in the filtered extract was analyzed within 24 h using a Continuous Flow Analyzer (SAN++, Skalar, Holand). DOC content in the extracts was analyzed by TOC analyzer (TOC-L Analyzer, Shimadzu, Japan). Potential nitrification rate (PNR) was assessed using the chloride inhibition soil-slurry method. In brief, 5 g of fresh soil was placed in a 50 ml falcon tube containing 20 ml of 1 mM (NH₄)₂SO₄. Potassium chloride with a final concentration of 50 mg L⁻¹ was added to inhibit nitrite oxidation. After 24 h incubation of the suspension in a dark incubator at 25 °C, NO₂⁻N in the soil was extracted using 5 ml of 2 M KCl, then the NO₂⁻N concentration in extraction was determined spectro-photometrically at 540 nm with N-(1-naphthyl) ethylenediamine dihydrochloride as an indicator. Potential nitrification rate was calculated as the linear increase in nitrite concentrations during incubation.

2.3. Mercury speciation analysis

For Hg analysis, 0.500 g of each soil sample was first digested with concentrated HNO₃+HCl (10 ml, 1:1 v/v) in a teflon tube at 100 °C for 2 h (Zheng et al., 2008). Total Hg concentration was then determined using an atomic fluorescence spectrometer (AFS, AFS-8220 Jitian Analytical Instrument Co., Beijing, China). A standard reference soil GBW-07405 (GSS-5), obtained from Center for National Standard Reference Materials of China, was included in the analytical process for quality assurance/quality control. MeHg was extracted using CuSO₄-methanol/solvent from the soil followed by ethylation and trapping on a Tenax column via N₂-purging based on modifications to the US EPA 1630 method. MeHg in the extract was measured with an automated MeHg analytical system (TEKRAN 2700 GC-CVAFS). Quality controls included measuring analytical blanks and a certified reference material (ERM-CC580) between sample runs. The analytical blanks were <2 pg L⁻¹ for MeHg and the recovery of the ERM ranged from 85.7 to 104.2% MeHg.

2.4. Soil DNA extraction and real-time PCR (qPCR)

The total microbial genomic DNA was extracted from 0.25 g of soil using the MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's
instructions. The quantity and quality of extracted DNA were checked photometrically using a NanoDrop® ND-1000 UV–Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The abundances of amoA gene for (AOA and AOB) were quantified on an iCycler iQ5 thermocycler (Biorad, USA). Primer pairs of CrenamoA23f/CrenamoA616r (Tourna et al., 2008) and amoA-1F/amoA-2R (Rotthauwe et al., 1997) were used for quantifying of amoA genes, respectively. Standard curves were generated using ten-fold serial dilutions of plasmids containing the correct insert of amoA genes. The 25-μl reaction mixture contained 12.5 μl SYBR Premix Ex Taq, 0.5 μl of each primer and 2 μl of 10-fold diluted DNA template (1–10 ng). Thermal-cycling conditions were as follows: 5 min initial denaturation at 95 °C, 40 cycles of 30 s at 95 °C, 30 s at 53 °C for AOA, and 55 °C for AOB, 1 min at 72 °C, followed by a plate read at 83 °C. To prepare the standard curves, amoA gene sequences were amplified from the extracted DNA using the primer pairs above. The PCR amplicons were ligated into a pGEM-T Easy vector (Promega, USA) and transformed into Escherichia coli JM109 cells. Positive clones containing the target gene insert were sequenced, and the most abundant clone was used for the plasmid DNA extraction. After measuring the DNA concentration using a NanoDrop® ND-1000 UV–Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), the purified plasmid DNA was serially diluted 10-fold and subjected to qPCR in triplicate to generate an external standard curve. Melt curve analyses were conducted following each assay to verify the specificity of the amplification products, and the PCR efficiency for different assays ranged between 86% and 99%.

2.5. Illumina sequencing and bioinformatics processing

The V4 region of the bacterial 16S rRNA gene (about 390 nucleotides) was targeted with the primer pairs of 515f and barcoded-907r (Ren et al., 2015). Primer sequences were modified by adding Illumina adaptor A to the 5'-ends of the forward primers, and adaptor B followed by 12 nucleotide barcode sequences to the 3'-ends of the reverse primers. The 50 μl reaction system consists of 20 μl Premix Ex Taq (Takara Biotechnology, Dalian, China), 0.4 μl of each primer (10 μM), 4 μl of five-fold diluted template DNA (1–10 ng) and 25.2 μl sterilized water. Thermal-cycling conditions were an initial denaturation of 3 min at 94 °C; six touch down cycles of 45 s at 94 °C, 60 s from 65 °C to 58 °C, 70 s at 72 °C, followed by 22 cycles of 45 s at 94 °C, 60 s at 58 °C, and 60 s at 72 °C, with a final elongation of 72 °C for 10 min. The PCR products were purified using a Wizard SV Gel and PCR Clean-up system (Promega, USA), and then sequenced on the MiSeq platform (Illumina, San Diego, CA, USA) at Novogene, Beijing, China.

Raw sequences generated through MiSeq paired-end sequencing were merged using fast length adjustment of short reads (FLASH) (Magoc and Salzberg, 2011) with Q30 of clean full-length reads ranging from 95.0 to 95.8%. UPARSE, a chimera filtering approach, was used to pick operational taxonomic units (OTUs) at the 97% similarity (Edgar, 2013) on the Bio-Linux platform. Representative sequences were processed using the QIIME pipeline (Caporaso et al., 2010). PyNAST alignment, and ribosomal database project (RDP) assignment were carried out based on the latest Green genes database (McDonald et al., 2012). Resampling according to the minimum sequence numbers across all samples was performed before the downstream analyses. The community composition provides the classification information at different taxonomic levels. Shifts in the bacterial community compositions were visualized using a principal coordinate analysis (PCoA) of the pairwise Bray–Curtis dissimilarity matrices based on the 97% OTU similarity across the four different treatments.

2.6. Statistical analysis

One-way ANOVA analyses were conducted to evaluate the difference in contents of THg, MeHg, DOC, PNR, ammonium, nitrate and gene abundances from the treatments. We used linear regression analyses to evaluate the correlation between DOC and Hg methylation and relative abundance of Hg methylation associating microbes. The principle coordinates analysis (PCoA) was based on the Bray–Curtis dissimilarity using QIIME 1.7.0. The differences of microbial community were measured by pairwise Bray–Curtis dissimilarities between L, LS, H, and HS using permutation multivariate analysis of variance (PerMANOVA) in R statistical platform (http://www.r-project.org).

3. Results

3.1. Variations in MeHg and THg contents after rice straw amendment

Rice straw amendment significantly increased MeHg content (P < 0.05) in the relatively high Hg paddy soil but did not change MeHg content in the relatively low Hg soil (Fig. 1), although they were spiked the same quantity of inorganic Hg at the beginning of incubation. The MeHg content in the high Hg soil was higher than that in the low Hg soil, and we did not observe a change in total Hg (THg) content after the straw amendment in the two soils. Average methylation (ratio of MeHg to THg at the end of the experiment) in the L, LS, H, and HS treatment was 0.036%, 0.036%, 0.023% and 0.045%, respectively, and was positively correlated to DOC content (Fig. 2).
3.2. Bacterial community and Hg methylation associating microbes

In total, more than 12 microbial phyla were detected from the experimental soils. *Proteobacteria* was most abundant phylum, followed by *Bacteroidetes, Chloroflexi, Acidobacteria, Actinobacteria* and *Firmicutes* (Fig. 3), accounting or greater than 98.1% of the bacterial sequences. Bray–Curtis analysis was performed to illustrate the effect of rice straw on bacterial community variance (beta diversity) in low and high Hg background level soils (Fig. S1). Communities from the same treatment tended to cluster together, while those from different soil and rice straw amendment were distinctly different from each other. Interestingly, we observed that the amended straw decreased ($P < 0.05$) bacterial alpha diversity in both soils (Shannon index, Fig. S2).

We identified the 8 closest genera that may associate with Hg methylation according to the predicted Hg methylators by Gilmour et al. (2013). It is obvious that *Geobacter* spp (belonging to *Deltaproteobacteria*), *Methanocella* spp and *Methanothergula* spp (belonging to *Methanomicrobia*) dominated all the proposed Hg methylators (Table S1), which totally account for 92% of all the predicted methylators. We found that introduced rice straw significantly enhanced the relative abundance of *Geobacter* spp and *Methanocella* spp in both low and high Hg paddy soils.

3.3. Nitrification potential and community structure of ammonia-oxidizers

Rice straw amendment significantly increased PNR ($P < 0.05$) in the high Hg soil but did not change the PNR in the low Hg soil (Fig. 4). Differently, the amended straw significantly increased ammonium content in both soils, whereas no change was observed for nitrate content. We did not observe any negative impact of Hg pollution on soil PNR, nitrate or ammonium contents in the present study. There was no difference of AOB or AOA abundance in the four treatments despite a much higher AOA abundance than AOB (Fig. S3). We noticed interactive community structures of AOA and AOB from the four different treatments (Fig. S4), though neither Hg pollution nor amended rice straw affected alpha diversity of the two ammonia-oxidizers.

4. Discussions

Understanding how rice straw mediates Hg methylation in natural paddy ecosystems is crucial for improving our ability to predict the ecological consequences of ongoing agricultural practice such as returning of rice straw to fields. Despite that rice straw has been commonly used to improve soil nutrient and decrease metal bioavailability, it has been largely unknown whether the amended rice straw will differentially affect fate of metal and soil function in Hg-contaminated paddy soils. Our results provide evidence that the amended rice straw enhanced Hg methylation and potential nitrification in the relatively high Hg (but not for low Hg) paddy soil.

We observed a positive effect of amended rice straw to MeHg production in the high, but not the low, Hg content paddy soil, highlighting differentiate effects of rice straw on the Hg methylation in the two soils with distinct Hg background levels. This may be related to the different properties of the high Hg soil characterized by long-term Hg contamination in contrast to the low Hg soil collected far from the contaminated site (Liu et al., 2014b). For example, the microbial community in the high Hg paddy soil may play more important role in microbial Hg methylation and decomposition of the rice straw than those in low Hg soil. Generally, Hg methylation in environments is mainly regulated by Hg availability to microbes and activity of Hg methylators (Barke et al., 1997; Chiasson-Gould et al., 2014; Hsu-Kim et al., 2013). In the present study, the positive correlation between Hg MeHg content suggests the great importance of DOC on enhancing Hg methylation. The DOC derived from decomposition of rice straw may increase Hg availability to Hg methylating microbes by forming DOM–Hg complexes (Driscoll et al., 2012; Graham et al., 2013). This is consistent with previous studies suggesting an enhanced Hg methylation under low sulfidic (Graham et al., 2012a) or low DOC conditions (French et al., 2014). Thus, the effect of DOM on Hg uptake and methylation by microbes may strongly depend on environmental conditions (Schurup et al., 2015). Here, the reason why the same amount of amended rice straw resulted in a different DOC increase in the two soils may be related to the microbial community that degraded rice straw. It has been suggested that *Bacteroidetes, Acidobacteria, Chloroflexi* and *Firmicutes* are the major microbial groups involved in breakdown of chemical composition of rice straw such as cellulose, hemicellulose and chitin (Wegner and Liesack, 2015). Interestingly, we found *Bacteroidetes*, the second most abundant phylum in this study, strongly correlated to DOC content (Fig. S5), suggesting the important role of *Bacteroidetes* in degradation of the rice straw in these paddy soils. Therefore, higher relative abundance of *Bacteroidetes* (Fig. 3) in the high Hg background soil with straw amendment may explain the observed highest DOC content considered to be a key factor enhancing Hg methylation in the present study.

The different Hg methylation in the two soils may be also related to community characteristics of Hg methylators. For example, increasing DOC in the low Hg soil did not increase soil MeHg content, which may be affected by the microbial community that can methylate Hg. Our results show the dominant microbes in the soils cover all the reported potential Hg methylating phyla such as *Proteobacteria, Firmicutes Chloroflexi* and *Euryarchaeota* (Fig. 3), with different relative abundance in the four treatments, respectively. However, these phyla abundance may not explain higher MeHg production in the high Hg background level soil, since there is different capacity of Hg methylation across various taxa (Gilmour et al., 2013; Yu et al., 2012). We identified Hg methylation associating microbes at genus level according to reported species carrying *hgcAB* genes (Gilmour et al., 2013; Parks et al., 2013), which may partially predict Hg methylation in the paddy soils. Among them, *Geobacter, Methanocella* and *Methanothergula* are the most abundant groups that have Hg methylation potential, accounting for 92% the relative abundance of detected sequences like Hg methylators in this study (Table S1). The positive correlation
between the potential Hg methylating microbes and methylation (Fig. 5) may support our argument that microbial community is another important factor in addition to Hg bioavailability regulating Hg methylation in environments.

Rice straw amendment also enhanced PNR in the soil with relatively high Hg background level, however, this promotion effect was not obvious in the low Hg soil. This different effect of rice straw amendments on the potential nitri

Fig. 3. Relative abundances of the dominant bacterial phyla in the four treatments. 1, 2, 3 and 4, represent four replicates of each treatment, respectively. Relative abundances are based on the proportional frequencies of the DNA sequences that could be classified at the phylum level.

Fig. 4. Variations in potential nitrification rate (PNR), ammonium (left-y axis) and nitrate (blue dot, right-y axis) contents of soils from the four treatments. Different capital letters indicate significant differences between the treatments ($P < 0.05$).

between the potential Hg methylating microbes and methylation (Fig. 5) may support our argument that microbial community is another important factor in addition to Hg bioavailability regulating Hg methylation in environments.

Rice straw amendment also enhanced PNR in the soil with relatively high Hg background level, however, this promotion effect was not obvious in the low Hg soil. This different effect of rice straw amendments on the potential nitrification in the two soils could be attributed to the different substrate (ammonium) content and activity of ammonia-oxidizers (Prosser and Nicol, 2012). In this study, increased ammonium content derived from decomposition of the amended straw may provide more substrate for nitrification by ammonia-oxidizers than that without rice straw treatments. Interestingly, we did not observe a PNR increase in the low Hg soil, though the amended straw also enhanced ammonium content. An alternative mechanism for this may be related to the different degree of ammonium increase after rice straw amendment in the two soils. We found the amended straw enhanced more than 2.7 fold ammonium in the high Hg soil while only a 1.5 fold increase of ammonium in the low Hg soil, and these different enhancement of substrate may lead to a differentiate effect on nitrification potential. Additionally, much higher DOC content in the high Hg soil than that in the low Hg soil after rice straw amendment may support a higher activity of the ammonia-oxidizers, since DOC is considered to play a regulating role in influencing microbial activity (Schnürer et al., 1985). This is also supported by the positive correlation between DOC content and PNR (Fig. S6). As explained above, highest relative abundance of Bacteroidetes in the high Hg soils with rice straw may account for the highest DOC content. These different PNR could be not explained by the amoA gene abundance which was less affected by the amended rice straw in this study, and this agrees with previous studies indicating no correlation between PNR and amoA gene abundance (Li et al., 2014; Liu et al., 2010). However, the shifts of AOA and AOB community structure may be related to the different effects of rice straw on PNR in the two soils, since microbial diversity is one of the important factors regulating microbial functions in ecosystems (Singh et al., 2014).

5. Conclusions

In conclusion, our results provide novel evidence that rice straw amendment enhanced both Hg methylation and nitrification potential in the relatively high, but not low, Hg soil, suggesting the differentiate effect of rice straw return on Hg methylation in
different Hg background level soils. Our findings emphasize the great importance of microbial community on the Hg methylation and nitrification for paddy fields. We also highlight differentiated composition of the rice straw by microbes in the different soils. Overall, our results indicate that rice straw return increased microbial activity in both nitrogen cycle and Hg methylation in paddy fields. These findings challenge traditional view that suggests the return of straw to fields will enhance soil fertility and reduce metal environmental risk. We suggest that rice straw should be prudently returned to paddy fields with Hg contamination, since it may increase risk of more MeHg production.

Acknowledgments
This work was supported by the National Natural Science Foundation of China (41571453, 51221892). We would like to thank Dr. Qiang-Gong Zhang in Institute of Tibetan Plateau Research, Chinese Academy of Sciences, for his assistance in measurement of methylmercury. We are also grateful to Jun-Tao Wang for his assistance in bioinformatics analysis.

Appendix A. Supplementary data
Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2015.11.023.

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