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Characteristics of archaea and bacteria in rice rhizosphere along a mercury gradient

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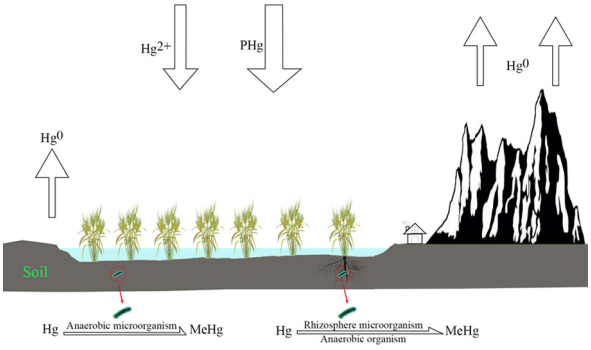
HIGHLIGHTS

- Communities of bacteria and archaea in Hg-polluted rice rhizosphere were studied firstly.
- Bacteria between high and low-Hg sites clustered together respectively, so did archaea.
- *merA* abundance was significant higher in high-Hg sites than low-Hg sites.
- Methanogen Hg-methylators could potentially be affiliated to *Methanosarcina*.

GRAPHICAL ABSTRACT



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ABSTRACT

Several strains of archaea have the ability to methylate or resist mercury (Hg), and the paddy field is regarded to be conducive to Hg methylation. However, our knowledge of Hg-methylating or Hg-resistant archaea in paddy soils is very limited so far. Therefore, the distribution of archaea and bacteria in the rhizosphere (RS) and bulk soil (BS) of the rice growing in Xiushan Hg-mining area of southwest China was investigated. Bacterial and archaeal 16S rRNA gene amplicon sequencing of the rice rhizosphere along the Hg gradient was conducted. THg concentrations in RS were significantly higher than that in BS at site S1 and S2, while MeHg concentrations in RS was always higher than that in BS, except S6. Bacterial species richness estimates were much higher than that in archaea. The bacterial α-diversity in high-Hg sites was significant higher than that in low-Hg sites based on ACE and Shannon indices. At the genus level, *Thiobacillus*, *Xanthomonas*, *Defluviicoccus* and *Candidatus Nitrosoarchaeum* were significantly more abundant in the rhizosphere of high-Hg sites, which meant that strains in these genera might play important roles in response to Hg stress. Hg-methylating archaea in the paddy field could potentially be affiliated to strains in *Methanosarcina*, but further evidence need to be found. The results provide reference to understand archaeal rhizosphere community along an Hg gradient paddy soils.

1. Introduction

The toxicity of mercury (Hg) is closely related to its chemical forms (Clarkson, 1998). Highly neurotoxic methylmercury (CH_3Hg^+, MeHg) can be significantly accumulated in the aquatic and terrestrial food

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webs, especially the fish and rice, which are confirmed to be the main source of exposure for humans worldwide (Meng et al., 2014; Stein et al., 1996). Both inorganic Hg (Hg^{2+}) and elemental Hg (Hg^0) can be converted into MeHg via biotic and abiotic processes in the environment (Hu et al., 2013; Schaefer et al., 2011). Biotic Hg methylation indicates that MeHg is formed in the environment by a variety of anaerobic microorganisms, which is regarded as the predominant agent associated with Hg pollution (Hsu-Kim et al., 2013; Raposo et al., 2008). Sulfate-reducing bacteria (SRB), iron-reducing bacteria (IRB), and methanogens are primarily responsible for microbial MeHg production in nature (Compeau and Bartha, 1985; Fleming et al., 2006; Gilmour et al., 2013; Hamelin et al., 2011; Kerin et al., 2007; Parks et al., 2013; Yu et al., 2013). Parks et al. (2013) found that a two-gene cluster, *hgcAB*, is indispensable for Hg methylation and believed that SRB are the main producers of MeHg, although IRB and methanogens can also be involved. Gilmour et al. (2013) confirmed that Hg can be methylated by SRB, IRB, methanogens, fermentative, acetogenic, and cellulolytic microorganisms, which significantly expands the range of Hg methylators and our knowledge of MeHg producing habitats, especially rice paddies.

Meanwhile, a unique Hg resistant *mer* operon system has evolved over a long period of time in the environment of Hg-poisoning. The microorganisms involved in Hg-resistant process are very extensive, including aerobic, facultative anaerobes and anaerobic microbes that all containing *merB* and *merA* genes (Li et al., 2010; Susana et al., 2011). MerB transporter first interrupts the C—Hg bond of MeHg, and degrades it to Hg^{2+}, then the mercuric reductase enzyme (MerA) reduces Hg^{2+} into a lower toxic and volatile elemental Hg (0), overflowing the cells and volatilizing into the atmosphere (Li et al., 2010; Susana et al., 2011). Mercury methylation and demethylation is supposed to simultaneously exist in the anaerobic environment and form a cycle, which determines the net production of MeHg. Exploring the microorganisms involving in the processes of Hg-methylation and Hg-resistant pathways is essential to understanding the biogeochemical cycle of Hg.

Rice paddy soils could go through anaerobic conditions due to oxygen depletion after flooding (Liu et al., 2014a; Liu et al., 2014b), and the flooded anaerobic soils are typically methanogenic (Chin and Conrad, 1995). Mercury methylation by methanogens provides a potential explanation for the observation of MeHg in rice paddies (Gilmour et al., 2013). Archaea exert crucial roles in Hg transformation, including Hg reduction, methylation, and demethylation (or resistance). To date, the confirmed archaeal Hg methylators includes *Methanolobus tindarius* (DSM 2278), *Methanomethylovorans hollandica* (DSM 15978), *Methanospirillum hungatei* JF1 (DSM 864), *Methanomassiliicoccus luminyensis* B10 (DSM 25270), *Methanocorpusculum bavaricum* (DSM 4179), *Methanofollis liminatans* GKZPZ(DSM 4140), *Methanosphaerula palustris* E1-9c (DSM 19958) and *Methanocella paludicola* SANAE (DSM 17711), all possessing *hgcAB* gene clusters and 7 of them belonging to *Methanomicrobia* (Gilmour et al., 2018; Gilmour et al., 2013; Podar et al., 2015; Yu et al., 2013). The confirmation of Hg methylation by methanogens has potential significance for human health, especially in southwestern China where rice is the main route of human MeHg exposure. (Feng et al., 2008; Li et al., 2015; Meng et al., 2011; Xu et al., 2018; Zhang et al., 2010). Therefore, the biotical methylation and demethylation of MeHg in paddy soils, especially methanogens, is seemed to be a foremost public health concern for human beings.

However, our knowledge of Hg-methylating and Hg-resistant archaea in paddy soils, especially in the rhizosphere (RS) and bulk soil (BS) of the rice, currently is very limited. Researchers had analyzed bacterial abundance and community by pyrosequencing bacterial 16S rRNA gene (*24F*, *454R*) and *hgcA* gene (*hgcA4F*, *hgcA4R*) in paddy soils of Wanshan Hg-mining areas, Guizhou (Liu et al., 2014a; Liu et al., 2014b). However, these research did not find any Hg-methylating bacteria at the genus level, nor did they confirm their role in the formation of MeHg. People cannot obtain the explanation of MeHg production in paddy fields based on bacteria research. Unfortunately, little is known regarding the responses of Hg-methylating archaea to Hg pollution in the paddy soils so far, especially in the rice rhizosphere growing in the Hg-impacted areas. Research on Hg-methylating and Hg-resistant archaea in the rice rhizosphere is crucial to guide research on mitigating MeHg production in the rice fields. Therefore, the primary objective of this study was to investigate the distribution of archaea and bacteria in the rice rhizosphere along a Hg gradient in Xiushan Hg-mining areas so as to analyze the impact of Hg pollution on the bacterial and archaeal communities.

2. Materials and methods

2.1. Study area

This study is conducted in Xiushan Hg-mining area, Yangshikeng (YSK) (E: 108°53′20″-108°54′01″; N: 28°34′5″-28°36′4″), Chongqing, southwestern China (Fig. 1), which locates 32 km northwest Xiushan County and covers approximately 2.5 km^2 (Xu et al., 2018). The study area is karstic and hilly, and elevation ranges from 246 m to 1631 m, with annual mean temperature 16 °C and average annual rainfall approximately 1341 mm. The reasons to choose Xiushan Hg-mining area are as follows. Firstly, the state-owned company began mining at the YSK in 1960s, with annual mining capacity of around 25 tons. Large quantities of mine-waste have been deposited along the Shileixi creek in the past years. Considerable quantities of mine-waste calcines were mainly piled up around the abandoned mining places. Secondly, the main mineral in the study region is cinnabar, whose grade ranges from 0.13% to 0.28% (Xu et al., 2018). As a tributary of the Rongxi River, the Shileixi creek is directly influenced by the mining activities of cinnabar. Thirdly, previous research has found that the total Hg (THg) and MeHg levels in the paddy soils, water, and rice of Xiushan Hg-mining area were significantly higher than those in non-mining areas and Chinese standards, and slightly lower or comparable to other Hg-mining areas worldwide (Feng et al., 2008; Lin et al., 2010; Meng et al., 2014; Qiu et al., 2005; Xu et al., 2018; H. Zhang et al., 2014b; Zhao et al., 2016). Moreover, rice was the staple food of 183,000 native residents living downstream of the YSK area. Therefore, it is essential to research on the impact of Hg pollution on the archaea and bacteria existing in the rice rhizosphere.

2.2. Sampling

Samples were collected in early September of 2015, at the end of flooding period and the rice was mature. There were six sampling sites, from site 1 (S1) to site 6 (S6), with different distances away from the center of Hg-mining area (Fig. 1). There were both three replicates for the rhizosphere (RS) and bulk soil (BS) samples at each site. Therefore, a total of 36 soil samples were collected with a depth of 0–20 cm, dividing into two types, RS and BS. Sampling referred to the shake method by Riley and Barber (Riley and Barber, 1969). A complete root system was dug out of the soil, and the large blocks not containing roots were gently shaken off. This was regarded as non-rhizosphere soil, namely the bulk soil, BS. Then, forcefully shake off the soil attached to the root, and obtain the rhizosphere soil, RS.

Samples for THg and MeHg analyses were encapsulated in polyethylene plastic bags after thorough homogenization to prevent cross contamination, and frozen by liquid nitrogen immediately after collected. The transportation, preservation and the pretreatment of the samples before GC-CVAFS analysis referred to our previous research (Ma et al., 2016, 2017a). Samples for bacterial and archaeal 16S RNA gene amplicon sequencing analysis were collected in sterile air-tight Falcon tubes with clean PE gloves, quickly frozen by liquid nitrogen, put on ice, carried to the laboratory within 24 h, and then stored at −80 °C before DNA extraction (Du et al., 2017; Ma et al., 2017a).

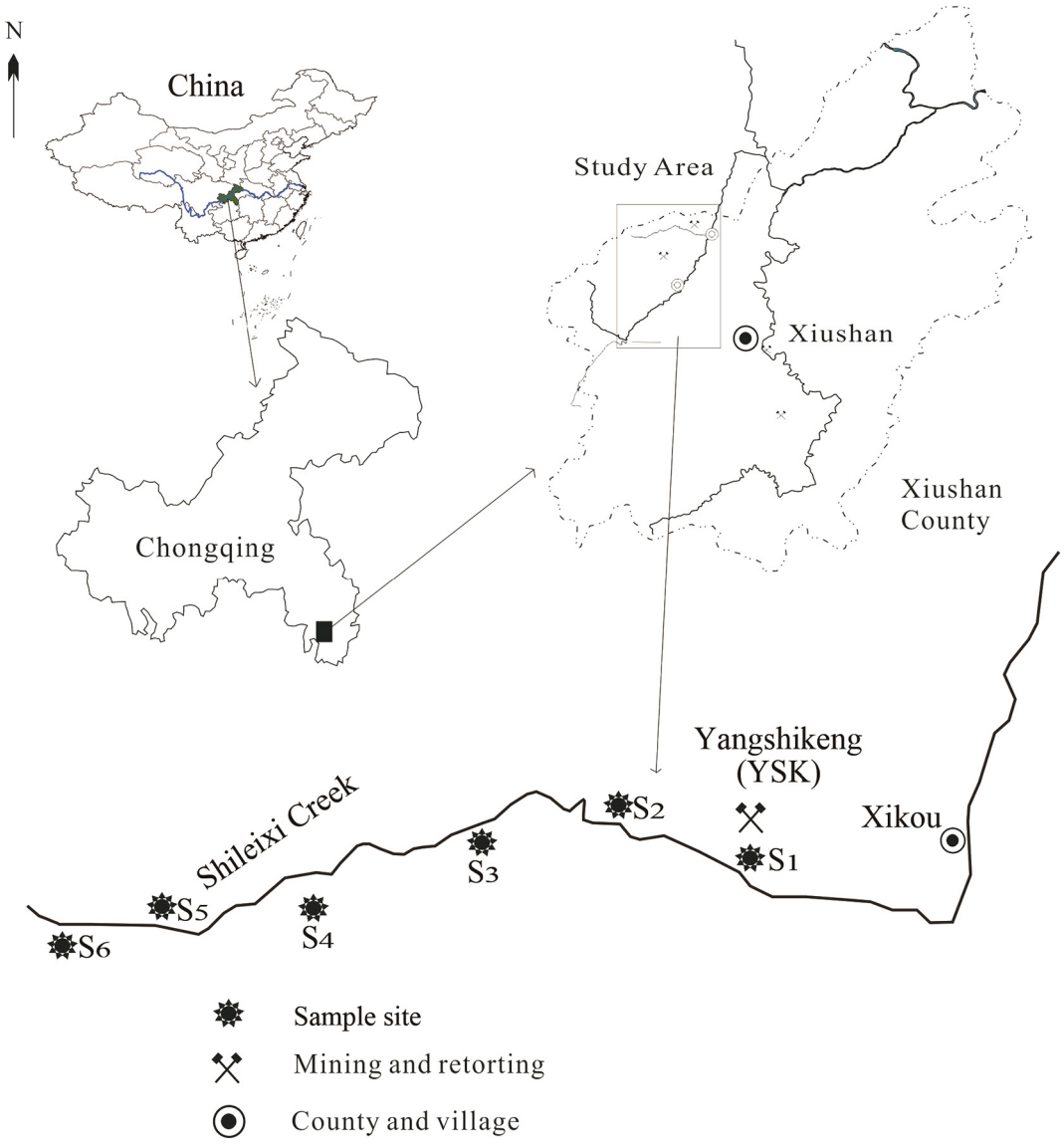

Fig. 1. Schematic diagram of the study site, Xiushan Hg-mining area, Chongqing, southwestern China.

2.3. THg and MeHg analysis and soil DNA extraction

Measurement of soil THg and MeHg concentrations, as well as the extraction, purification, quantification and preservation of genomic DNA from the soil were the same with our previous research (Ma et al., 2017a). Detailed introduction was seen in the Supplementary Information (SI).

2.4. High-throughput bacterial and archaeal 16S rRNA gene amplicon sequencing and analysis

The total DNA was used as templates for high-throughput bacterial and archaeal sequencing of 16S rRNA amplicons by Illumina MiSeq PE300 platform. The isolated DNA was sent to Shanghai Majorbio to perform PCR amplification and sequencing. Primers 338F (5′-ACTCCTACG GGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) targeting both the V3 and V4 regions of bacterial 16S rRNA genes, 524F_10_ext (5′-TGYCAGCCGCCGCGGTAA-3′) and Arch958R_mod (5′-YCCGGCGTTGAVTCCAATT-3′) covering V4 and V5 regions of archaeal 16S rRNA gene were selected (Mori et al., 2014; Pires et al., 2012; Xu et al., 2016). The analysis and data processing were described in SI (Ma et al., 2017a, 2018), including the quantification and normalization of PCR products, removal of PhiX and low quality sequences, merging of the forward and reverse sequences, chimera detection, OTU classification, taxonomic assignment, as well as the corresponded statistical analyses. For each site, the three replicated samples for sequencing were mixed and then used for DNA extraction. Therefore, a total of 12 samples were sequenced, with the number of reads ranging from 30,365 to 44,727, and average length 439.4 bp for bacteria and 448.0 bp for archaea.

2.5. Quantification of dsrB, merA, hgcA and mcrA genes

The absolute quantification of *dsrB* (SRB), *merA* (Hg-resistant microorganisms), *hgcA* (Hg-methylation microorganisms) and *mcrA* (methanogen) gene were conducted by q-PCR with ABi3500 q-PCR system (Thermo Fisher, USA). Primers used for q-PCR assays were introduced in Table S1. The parameters of q-PCR reactions and the construction of standard plasmids were described in SI.

2.6. Quality control and statistical analysis

The Kruskal-Wallis H test was used for the comparison of relative abundances of microbial taxa in RS and BS between high-Hg and low-Hg sites, with significant level of 0.05. Comparisons of the THg and MeHg levels, MeHg/THg ratios, as well as species richness and α-diversities were calculated by the Student's *t*-test, with significant level of 0.05. The gene quantities of the q-PCR results seemed to be statistically significant at $p < 0.05$ based on ANOVA test. All the analyses related with the 16S rRNA gene amplicon sequencing were done by R based on the I-Sanger platform (Shanghai Majorbio), including RDA and the Wilcoxon rank-sum tests. Origin 8.0 was used for drawing the figures of THg and MeHg concentrations. The details of QA and QC of THg and MeHg analyses were described in SI.

3. Results

3.1. The levels of THg and MeHg in the paddy soils and their correspondence with environmental variables

THg and MeHg concentrations in the RS and BS of the rice along an Hg gradient in Xiushan Hg-mining area were shown in Fig. 2. THg concentrations between RS and BS in high-Hg sites (S1, S2 and S3) had highly significant difference (**, $p < 0.01$, *t*-test), and the difference was significant in the low-Hg sites (S4, S5 and S6, *, $p < 0.05$, *t*-test, Fig. 2a). THg concentrations in RS of site S1 and S2 were significantly higher than that in BS (**, $p < 0.01$, *t*-test), whereas with the distance from the Hg pollution source increasing, the concentrations of THg in RS were less than that in BS (Fig. 2a). Methylmercury concentrations in the six sites ranged from 0.06 $ng \cdot g^{-1}$ to 3.88 $ng \cdot g^{-1}$. The concentrations of MeHg in RS was always higher than that in BS, except S6 (Fig. 2b). MeHg concentrations between RS and BS of the rice in site S1 and S2 had highly significant difference (**, $p < 0.01$, *t*-test), and the difference was significant in the other four sites (*, $p < 0.05$, *t*-test, Fig. 2b). At the same time, we found that the ratios of MeHg to THg in high-Hg sites (S1, S2 and S3) were significantly higher than that in low-Hg sites (S4, S5 and S6) (**, $p < 0.01$, *t*-test) (Fig. 2c).

The relative contributions of environmental factors to THg and MeHg concentrations were expressed by redundancy analysis (RDA) plots in Fig. 3. Previous research indicated that the concentrations of organic matter (OM), NH_4^+, SO_4^{2-}, Fe^{2+}, and pH had significant correspondence with THg and MeHg levels in biotic Hg-methylation (Du et al., 2017). Therefore, these factors were measured and analyzed in our research. In addition, elevations of the sampling sites ranged from 427 m (S6) to 1020 (S1), so the elevation was also regarded as one of the variables. The concentrations of NH_4^+, Fe^{2+}, OM and SO_4^{2-}, as well as elevations positively correlated with MeHg levels, while pH had negative relationship with MeHg levels based on RDA plots. The intersection angles between Fe^{2+} (or SO_4^{2-}) and MeHg levels were smaller than that between OM (or NH_4^+) and MeHg levels, which indicated that MeHg levels had higher positive correspondence with Fe^{2+} and SO_4^{2-}. Moreover, the variables of Fe^{2+}, SO_4^{2-}, OM and NH_4^+ mainly affect the levels of MeHg, not THg, which can be seen from the smaller intersection angles between these variables and MeHg but larger angles with THg. RS had higher MeHg levels than BS, which might because that the variables that significantly affect MeHg levels were all positively correlated with RS, while negatively correlated with BS.

3.2. Bacterial and archaeal species richness, evenness and diversity

A total of 422,397 and 459,966 high-quality bacterial and archaeal 16S rRNA sequences were obtained after processing, with an average length of 448.02 bp and 439.39 bp respectively. 23,598 bacteria OTUs were generated after clustering at a 97% similarity level, among which 5080 OTUs were mapped to 280 known genera. While for archaea, 2372 archaea OTUs were produced, and 547 OTUs were mapped to 16 known genera. At the genus level, a relatively small number of

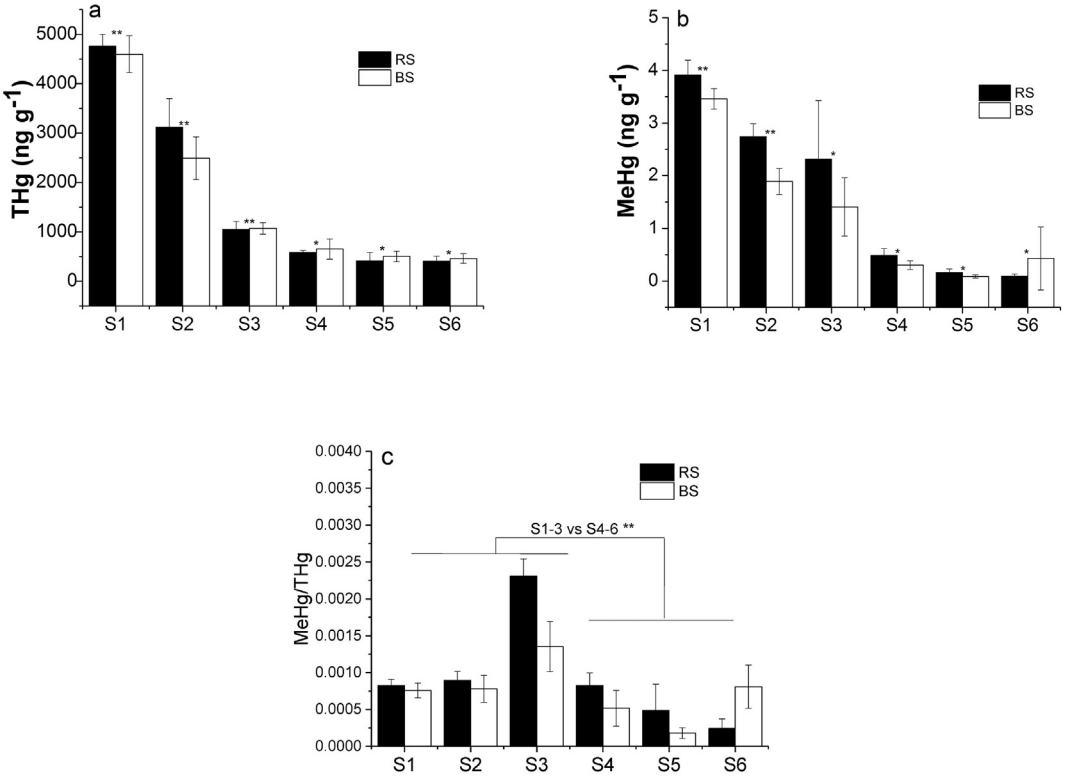

Fig. 2. THg (a) and MeHg (b) levels, and MeHg/THg ratios (c) in the rhizosphere and bulk soil of the rice growing in Xiushan Hg-mining area. *Small bars* show standard errors. The asterisk indicates the difference of Hg levels based on one-sample Student's *t*-test (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; without *: $p > 0.05$).

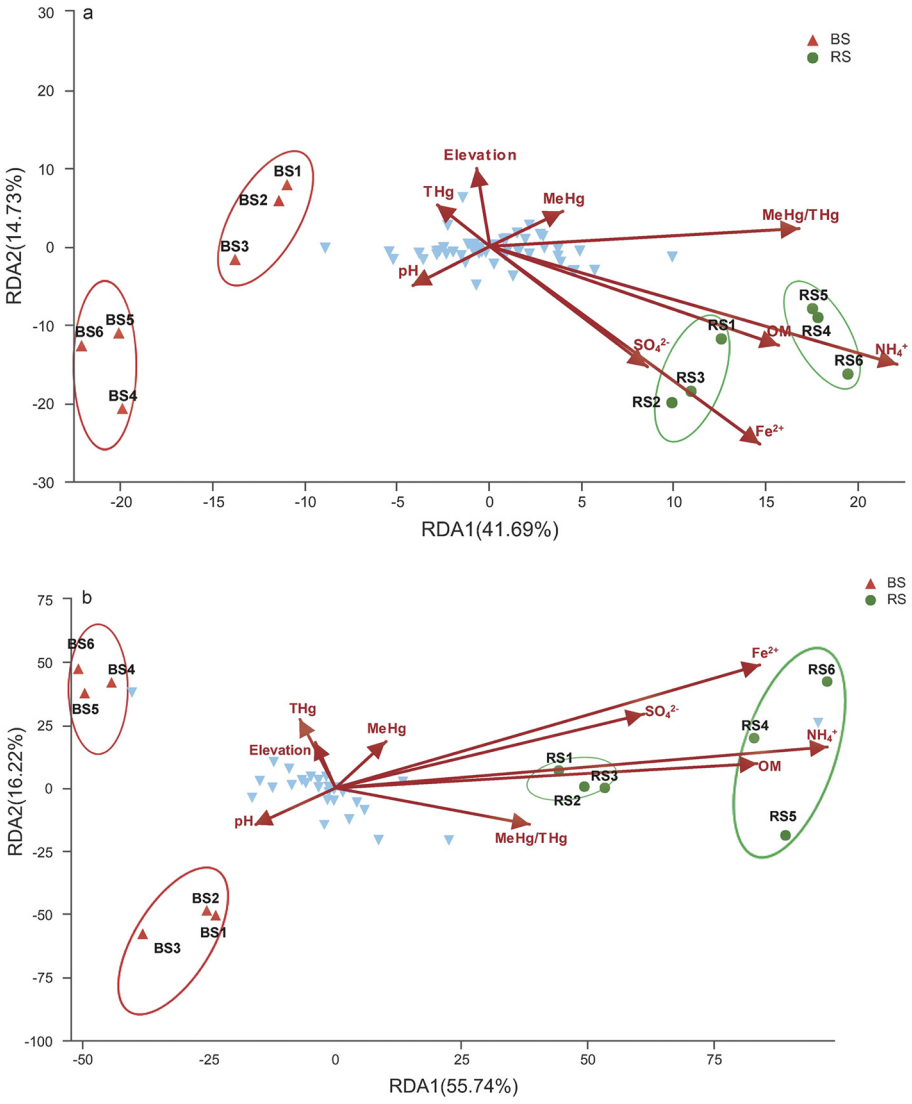

Fig. 3. RDA plots showing the relationship between environmental factors, Hg concentrations and bacterial (a) and archaeal (b) communities.

sequences were not assigned to any known genera (6.41% for bacteria, 3.84% for archaea), indicating nearly few potentially novel bacteria and archaea existing in the sampling site (Table S2). Rarefaction curves of the RS and BS samples based on a 97% phylogenetic cluster similarity reached the near plateau phase, representing good sampling depth (Fig. S1).

The α-diversity of bacterial estimates was 2395.60 and 2120.18, while archaeal estimates were 207.13 and 176.19 respectively for RS and BS based on Chao estimator (Table 1). The α-diversity of bacterial estimates in high-Hg sites was significant higher than that in low-Hg sites (**, $p < 0.01$, student's t-test, Fig. S2a, Table S3), and archaeal estimates in RS were significant higher than that in BS in low-Hg sites (*, $p < 0.05$, student's t-test, Fig. S2b, Table S3) based on ACE index. Along the Hg gradient, the α-diversity of bacterial ACE estimates in the rhizosphere was highest in RS2, and lowest in RS1 (Table S3). While in the bulk soils, the α-diversity of bacterial ACE estimates was highest in BS2 and lowest in BS5. Along the Hg gradient, archaeal ACE estimates in the rhizosphere were maximum in RS2 and minimum in RS1 (Table S3). While in the bulk soils, archaeal ACE estimates were maximum in BS1 and minimum in BS5. Bacterial α-diversity in BS was significant higher than that in RS in the high-Hg sites (*, $p < 0.05$, student's t-test, Fig. S2c, Table S3) based on Shannon index. Archaeal α-diversity between high-Hg sites and low-Hg sites did not have significant difference ($p > 0.05$, student's t-test, Fig. S2d) based on Shannon index. Along the Hg gradient, bacterial diversities in the rhizosphere was highest in RS2 and lowest in RS1, and those in the BS were highest in BS2 and lowest in BS6 (Table S3). Along

Table 1
Bacterial and archaeal community structural parameters in the rhizosphere and bulk soil.

Sample	Bacteria				Archaea			
	OTUs	Chao	ACE	Coverage	OTUs	Chao	ACE	Coverage
RS	3011	2395.60	2366.59	0.9811	272	207.13	206.25	0.9990
BS	2943	2120.18	2081.51	0.9836	282	176.19	177.28	0.9992

Estimates (Chao & ACE) was calculated based on Hellinger-transformed OTU counts data.
RS: Rhizoshpere soil; BS: bulk soil.

the Hg gradient, results of archaeal diversity estimates in the rhizosphere were highest in RS2 and lowest in RS1; while that in the bulk soils were highest in BS3 and lowest in BS6.

3.3. Bacterial and archaeal compositions, community structures and their response to soil physicochemical properties

The bacterial community structures in RS and BS of the high-Hg and low-Hg sites at the phylum and OTU levels were shown in Fig. S3. Within the bacteria domain, *Proteobacteria* were the most abundant phylum (36.17% in relative abundance), followed by *Chloroflexi* (19.37%), *Acidobacteria* (13.94%), *Actinobacteria* (8.06%), and *Nitrospirae* (5.45%), with all the other phyla below 4.00%. Moreover, *Chloroflexi*, *Gemmatimonadetes* and an unclassified phylum were significant different between high-Hg and low-Hg sites (*, $p < 0.05$, Kruskal-Wallis H test, Fig. S3a). While the bacterial community structures didn't have significant difference among the groups at the OTU levels ($p > 0.05$, Kruskal-Wallis H test, Fig. S3b).

The archaeal community structures in RS and BS of the high-Hg and low-Hg sites at the phylum and OTU levels were shown in Fig. S4. Within the archaea domain, the relative abundance of *Thaumarchaeota* (41.58%) was highest, followed by *Miscellaneous_Crenarchaeotic_Group MCG* (35.70%), and *Euryarchaeota* (19.83%), with all the other phyla below 2.00%. At the phylum level, there was not significant difference among the groups ($p > 0.05$, Kruskal-Wallis H test, Fig. S4a). While at the OTU levels, we found that OTU203 and OTU212 were significantly higher in the RS of high-Hg and low-Hg sites than that in the BS (*, $p < 0.05$, Kruskal-Wallis H test, Fig. S4b).

Analysis of the relative abundance of bacterial taxonomic groups at the genus level showed that the ten most abundant genera were *Anaerolinea* (mean relative abundance, RS, 10.61%; BS, 8.92%), *Nitrospira* (4.99%, 5.45%), *Nitrosomonas* (3.63%, 3.40%), *Chloroflexus* (2.93%, 3.04%), *Gemmatimonas* (1.68%, 2.68%), *Defluviicoccus* (2.20%, 0.61%), *Xanthobacter* (1.07%, 1.60%), *Marmoricola* (1.52%, 0.75%), *Thiobacillus* (0.73%, 0.93%), and *Alcaligenes* (1.14%, 0.39%) (Fig. 4a). The abundances of *Thiobacillus*, *Xanthomonas* and *Defluviicoccus* in high-Hg sites decreased comparing with that in low-Hg sites, significantly decreasing by 44.10%, 34.41% and 28.21% respectively. Nevertheless, the abundances of *Roseiflexus*, *Candidatus_Solibacter*, and *Gemmatimonas* in the high-Hg sites increased by 32.10%, 20.53% and 18.24% respectively comparing with low-Hg sites. *Defluviicoccus* and an unclassified genus were significant higher in RS than that in BS no matter in the high-Hg sites with the low-Hg sites (Fig. S5a).

The relative abundance of archaeal taxonomic groups at the genus level revealed that the ten most abundant genera were *Miscellaneous_Crenarchaeotic_Group* (RS, 35.01%; BS, 36.41%), *Soil_Crenarchaeotic_Group_SCG* (16.92%, 22.52%), *Candidatus Nitrosoarchaeum* (21.35%, 0.36%), *Methanosaeta* (6.19%, 5.86%), *Candidatus Nitrosotalea* (0.05%, 12.05%), *Methanoperedens* (2.97%, 2.36%), *Methanobacterium* (2.73%, 1.68%), *Thermoplasmatales* (2.11%, 1.24%), *Methanosarcina* (0.06%, 3.20%), and *Methanomassiliicoccus* (2.04%, 0.97%) (Fig. 4b). The abundances of most archaeal genera in high-Hg sites significantly declined comparing with low-Hg sites, especially *Methanosaeta*, *Miscellaneous_Crenarchaeotic_Group*, *Candidatus_Nitrosoarchaeum* and *Soil_Crenarchaeotic_Group_SCG*, whose abundances dramatically decreased by 74.83%, 70.01%, 69.37% and 68.74% respectively when Hg concentrations significantly decreased. However, the abundances of *Methanoperedens* and an unclassified archaea genus in the high-Hg sites significantly increased by 40.37% and 52.04% comparing with low-Hg sites. In addition, the abundance of *Candidatus_Nitrosoarchaeum* was significant higher in RS than that in BS (Fig. S5b).

The relative contributions of environmental factors and Hg concentrations to the bacterial and archaeal communities were also expressed by RDA plots in Fig. 3. The variance in the relationship between bacterial OTUs and environmental factors had a total of 56.42% contribution (Fig. 3a), while for archaeal communities, the variable contribution was 71.96% (Fig. 3b). The variance in the relationship between bacterial and archaeal OTUs indicated that NH_4^+ was the most significant variable that influenced both bacterial ($r^2 = 0.7361$, $p = 0.003$) and archaeal ($r^2 = 0.5415$, $p = 0.029$) communities, followed by Fe^{2+}, OM, SO_4^{2-} and MeHg/THg ratios. The pH, elevation, THg and MeHg levels had relatively less correspondence with microbial communities, with p-values > 0.05. Both the bacterial and archaeal communities in the RS (or BS) of high-Hg sites clustered together, indicating that they had similar community structures. Moreover, the bacterial and archaeal communities in samples of high-Hg sites were less affected by these variables comparing with those of low-Hg sites. In addition, bacterial and archaeal communities between RS and BS distributed in different quadrants, indicating that the effect of these variables on rhizosphere communities significantly differed with that in BS.

3.4. Phylogenetic analysis of archaea

Due to the important role of archaea in paddy soils as regard to Hg methylation, the phylogenetic analysis of archaea was investigated. Two phylogenetic trees were constructed, including the sequenced archaeal genera of this research and the previously confirmed archaeal Hg methylators (Figs. 5, S6). Results showed that three genera, *Soil_Crenarchaeotic_Group_SCG*, *Candidatus_Nitrosoarchaeum*, and *Candidatus Nitrosotalea* had a close genetic relationship. Among the three genera, *Soil_Crenarchaeotic_Group_SCG* and *Candidatus Nitrosotalea* had relatively higher number of reads both in RS and BS comparing with other genera, whereas the genus of *Candidatus_Nitrosoarchaeum* almost only existed in RS. In the upper branch of the three genera, we can find another genus with the highest reads in RS and BS, namely *Miscellaneous_Crenarchaeotic_Group*. This indicated that the four genera had akin relationship with each other, and they represent the majority of the genera found in the two sample types.

4. Discussion

4.1. The levels of THg and MeHg in the paddy soils and their correspondence with environmental variables

THg and MeHg concentrations along the Hg gradient had similar trends, namely higher concentrations occurred in the sites near the pollution source, and the farther the distance, the lower the concentrations of both THg and MeHg. Soil THg concentrations in the six sites ranged from 405.1 $ng \cdot g^{-1}$ to 4762 $ng \cdot g^{-1}$, which were comparable to or slightly lower than those reported from other Hg-mining areas worldwide (Qiu et al., 2012; Qiu et al., 2005; Tomiyasu et al., 2012; Xu et al., 2018; Zhao et al., 2016). Similarly, MeHg concentrations in the six sites were also comparable to or slightly lower than those reported from other heavily Hg-polluted places (Qiu et al., 2012; Qiu et al., 2005; Tomiyasu et al., 2012; Xu et al., 2018; Zhao et al., 2016). The reasons perhaps were that many studies had confirmed that flooded soils were an important source of MeHg in the wetland ecosystem (Roulet et al., 2001; Wang et al., 2002). Usually in flooded conditions, abundant soluble carbon and humic acid in wetland environment provide ideal living conditions for methylated microorganisms, which lead to enhanced methylation of Hg. Seasonal irrigation in rice growing period can also lead to the formation of anaerobic environment conducive to Hg methylation in the surface layer of paddy soils (Porvari and Verta, 1995). Moreover, Hg methylation in rice paddy soils is impacted by high levels of atmospheric Hg deposition, followed by the uptake of MeHg by the rice. Therefore, MeHg concentrations in Xiushan Hg-mining areas were relatively higher than the background value.

Methylmercury concentrations in RS were remarkably higher than that in BS of the rice, especially in the sites near Hg-pollution source, namely higher Hg-concentration sites. RS is different from BS in that it is near the root surface and it holds higher microbial activities owing

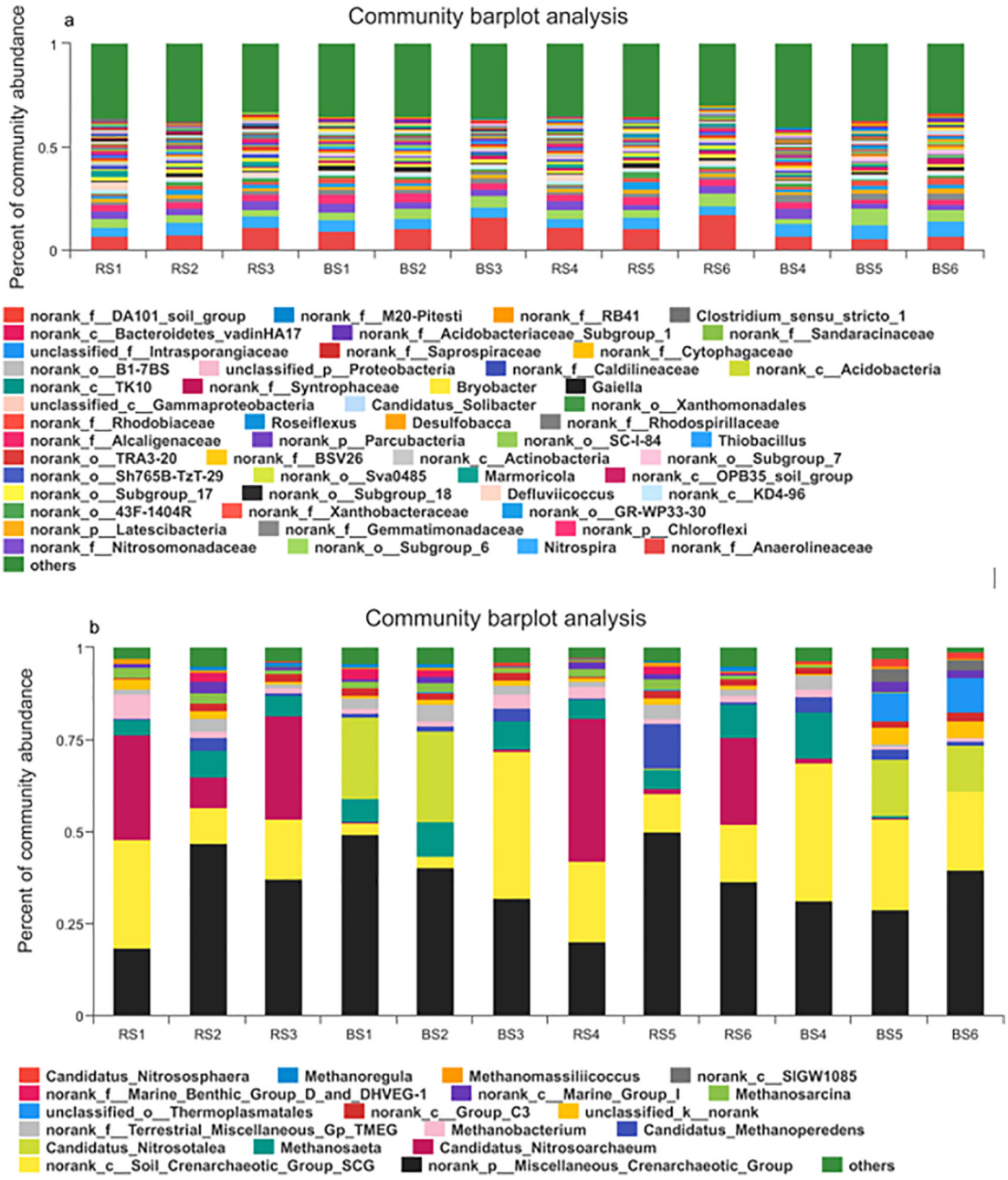

Fig. 4. Bacterial (a) and archaeal (b) community bar plots showing the percent of community abundances on the genus levels.

to the organic materials derived from roots (Vancura and Hovadík, 1965). So the reason for higher MeHg levels in RS perhaps was due to the higher biotical activities of microorganisms among the rhizosphere microbiome. Previous studies found that rhizosphere microorganisms could promote the accumulation of various heavy metals, including Hg (Liao et al., 2006; Sessitsch and Puschenreiter, 2008; Souza et al., 1999; Sun et al., 2011; Xu et al., 2007). Our research confirmed that the plant root and the rhizosphere microorganisms could promote the methylation and accumulation of Hg in rice, which was in agreement with previous studies (Souza et al., 1999; Sun et al., 2011). Thus, it is important to know what kind of microorganisms, especially archaea, plays essential roles in Hg methylation and accumulation in the RS.

The ratios of MeHg to THg in high-Hg sites were significantly higher than that in low-Hg sites, which might indicate that the Hg methylation abilities in high-Hg sites were remarkably stronger than that in low-Hg sites. Interestingly, the ratios of MeHg to THg were especially higher in S3, which was in agreement with the highest *hgcA* gene copies in S3 (Figs. 2c & S7). This implied that the soils at S3 had the maximum Hg-methylating abilities among the sampling sites. We noted that the gene copies of *mcrA* was also maximum in S3, which indicated that methanogens might exert important roles in Hg methylation process. This might be one of the reasons, but need to be further confirmed in the future.

In addition, pH values correlated negatively with MeHg levels in the paddy soils, which differed with our previous results in the fluctuating water-level soil (Ma et al., 2018). We could see that the effect of pH on MeHg levels in different environment could contribute to different results. The concentrations of SO_4^{2-} and Fe^{2+} were the most important factors influencing MeHg levels, which were in accordance with previous studies (Achá et al., 2005; Benoit et al., 2001; Drott et al., 2007; Gilmour et al., 1992; Ma et al., 2017b). It can be seen from the RDA plots that OM positively correlated with RS, while negatively correlated

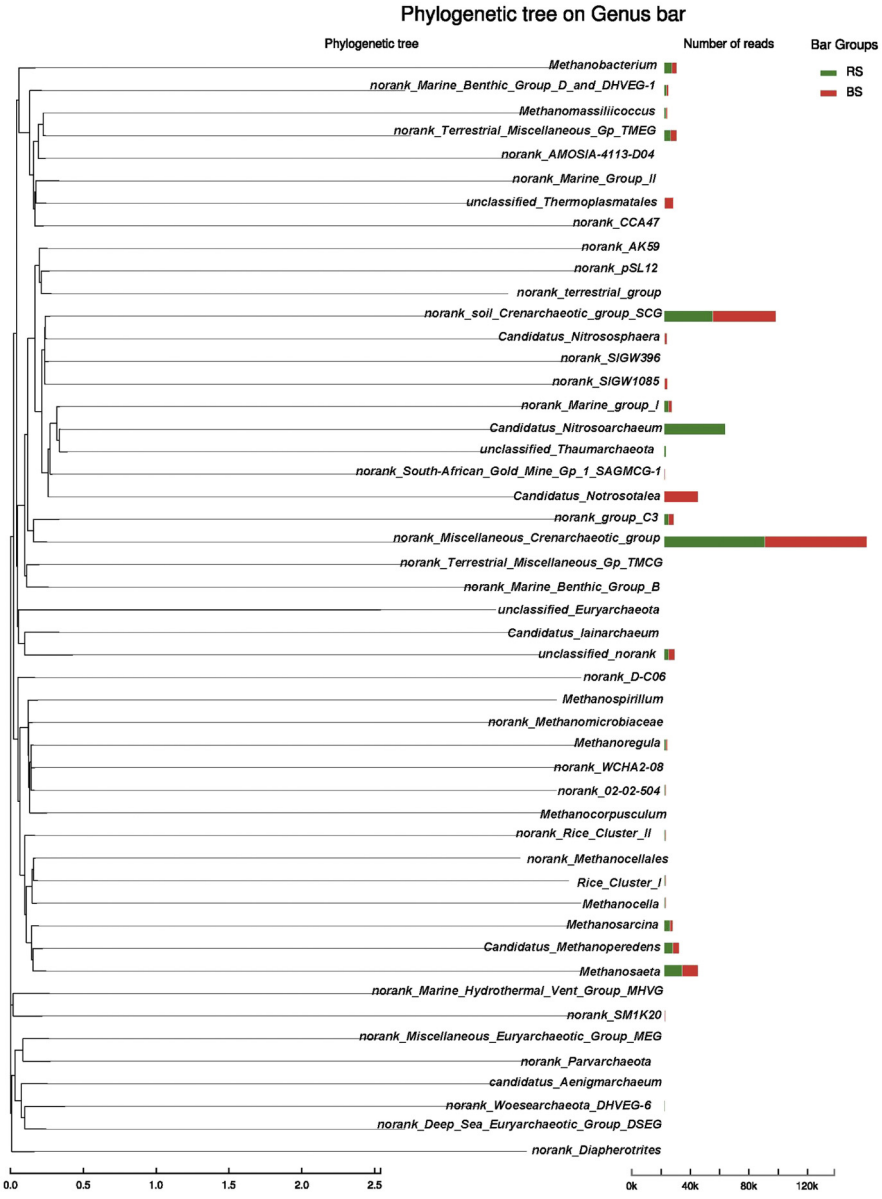

Fig. 5. Approximately-maximum-likelihood phylogenetic tree with the number of reads from the archaea genera.

with BS (Fig. 3). OM had stronger correspondence with MeHg levels in RS than that in BS, so it could be predicted that the higher OM content associated with root might be the reason.

4.2. The diversities of bacteria and archaea in the paddy soils

4.2.1. The α-diversity along Hg gradient

In the Hg contaminated paddy field, bacterial and archaeal species richness and diversity estimates in the rhizosphere were both highest in RS2 and lowest in RS1. Namely RS1 had the lowest α-diversity among the rhizoshpere, while RS2 harbored the highest bacterial and archaeal α-diversities. For the bulk soil, BS2 and BS1 harbored the highest bacterial and arachaeal α-diversities respectively, while the lowest diversity occurred in BS5. However, if we separate the samples into high-Hg sites and low-Hg sites, we found that the α-diversities of bacterial and archaeal estimates between high-Hg sites and low-Hg sites were significant different based on ACE index (Fig. S2). This indicated that the influence of Hg concentrations on rhizosphere microbiome was very complicated, but it seemed that Hg concentrations had significantly affected bacterial and archaeal α-diversities. Several previous research on bacterial diversities in heavy metal contaminated soils demonstrated high microbial diversities (Ellis et al., 2003; Filali et al., 2000; Konstantinidis et al., 2003). However, some other studies also found that significantly negative correlations existed between bacterial/fungal abundance and diversities and the concentrations of many heavy metals (Chen et al., 2014). This contradiction was mainly due to the complex interactions between rhizosphere microbial community and various heavy metals, including Hg. It is known that soil rhizosphere microbiome could interact directly with the heavy metals to reduce their toxicity to the plant, and thus affect their bioavailability (Bhateria and Snehlata, 2013; Muehe et al., 2015). Comparing with uncontaminated paddy soils, the Shannon index of bacteria in soils without Hg-pollution was around 8 (F. Zhang et al., 2014a), which were significantly higher than our research (Table S3, archaea: 2–3; bacteria: 6–7). This result was in accordance with previous research showing that heavy metal pollution decreased bacterial and archaeal abundance and diversity in the paddy soils (Chen et al., 2014). When comparing our results with other Hg-contaminated paddy soils, we found that the Shannon index of archaea in our research was lower than the index of Hg-methylating microorganisms in Wanshan (3.5–4.0) and Xunyang

(2.5–4.0) Hg-mining area, but higher than Chashan Hg-mining area (1.0–3.0), southwest China (Liu, 2017).

4.2.2. The α-diversity between RS and BS

ACE and Chao indices indicated that the species richness estimates were higher in RS than that in BS (Table S3). Shannon and Simpson indices showed that the α-diversities between RS and BS for both the bacteria and archaea did not have any significant difference (Table S3). The reasons perhaps were that the rhizosphere microbiome had undergone a long-term interaction with Hg. A series of Hg tolerant bacteria and archaea gradually dominated among the rhizosphere microbiome. The transportation of heavy metals, including Hg, through bioaccumulation had been reported in many bacterial genera. A large number of heavy metal tolerant bacteria were reported to promote plant growth and affect rhizosphere microbial communities and activities, including Hg tolerant bacteria *Rhizopus arrhizus* (Bhateria and Snehlata, 2013; Leita et al., 1995; Umrania, 2006) and maybe several strains of archaea or even fungi being founded in the future. Mercury, like cadmium and lead, has no known biological and/or physiological functions (Bhateria and Snehlata, 2013). When Hg-tolerant microorganisms are exposed to high concentrations of Hg, the Hg enters the cells, reacts with them and finally forms toxic compounds, like MeHg (Hsu-Kim et al., 2013). These Hg tolerant microbes accustomed with the Hg contaminated environment and gradually dominated among the rhizosphere microbiome. For survival under Hg-stressed environment, these microorganisms were predicted to evolve the mechanism of demethylation to resist the toxicity of Hg (Li et al., 2010; Susana et al., 2011). Therefore, the species richness and α-diversity in the RS were elevated or comparable to the BS.

4.2.3. The α-diversity between bacteria and archaea

Bacterial species richness estimates were much higher than that in archaea, and ACE and Chao indices showed that bacterial species richness between RS and BS was significant different, while archaea not. This indicated that a wider variety of bacteria lived in the RS and BS of the rice growing in the sampling sites. This may because the special and strict living environment for the growing of many archaea, such as thermophiles or halophilic archaea. As we know that most archaea living in extreme environments, such as high salt lake water, extremely hot, acid and absolute anaerobic environment, some in the extremely cold environment. In this study, the paddy field is mainly proper living condition for methanogens. That may also be why the archaeal species richness did not have significant difference even between RS and BS. "Rhizosphere effect" indicates that the number and activity of microorganisms in the rhizosphere of plants are higher than that in non-rhizosphere soil due to rich nutrition and energy from the root tissue. Our research found that average estimators in RS were significantly higher than that in BS, which indicated that the "rhizosphere effect" in the paddy soils of Hg-mining area was also present.

4.3. Bacterial and archaeal compositions, community structures and their responses to soil physicochemical properties

Results showed that *proteobacteria* were predominantly found in the rice rhizosphere, which was in agreement with previous studies about the effect of heavy metals on the diversity of bacterial communities (Bhateria and Snehlata, 2013; Chen et al., 2014; Liu et al., 2014a; Mengoni et al., 2004). The bacterial phylum of *Chloroflexi* and *Gemmatimonadetes* in RS of the high-Hg sites were significantly less than that in RS of the low-Hg sites, which indicated that the quantities or activities of the two phyla decreased under the influence of high concentration of Hg in rice rhizosphere. However, our result differed from Liu et al. (2014a), who showed that the relative abundance of *Nitrospirae* decreased, while that of *Gemmatimonadetes* increased with the increase of Hg concentrations. The rice had not been identified as a hyperaccumulating plant of Hg, so bacteria and archaea in rice rhizosphere did not being confirmed to facilitate Hg uptake by the stem, leaf or root of the rice, and the accumulated and transformed Hg in the soil mainly affected rhizosphere communities. This result was accordance with other research with different heavy metals (Bhateria and Snehlata, 2013; Whiting et al., 2001). Interestingly, *Chloroflexi* was more abundant in BS of the high-Hg sites than that in BS of the low-Hg sites, which indicated that *Chloroflexi* in the BS was less affected by Hg pollution. The abundances of archaeal phyla between RS and BS in the high-Hg and low-Hg sites didn't have significant difference, which indicated that Hg had little impact on archaea at the phylum and OTU levels.

Bacterial community structure analysis at the genus level showed that *Thiobacillus*, *Xanthomonas* and *Defluviicoccus* were significant more abundant in high-Hg sites compared to low-Hg sites, while *Roseiflexus*, *Candidatus_Solibacter* and *Gemmatimonas* was significantly less abundant in high-Hg sites than that in low-Hg sites. This indicated that the quantities or activities of the genera of *Roseiflexus*, *Candidatus_Solibacter* and *Gemmatimonas* decreased under the influence of high concentration of Hg, while *Thiobacillus*, *Xanthomonas* and *Defluviicoccus* might be accustomed to the long-term Hg stress near the Hg-pollution center. *Gemmatimonas* and *Defluviicoccus* were the main difference between RS and BS. *Thiobacillus* (*β-proteobacteria*), *Xanthomonas* (*r-proteobacteria*) and *Defluviicoccus* (*α-proteobacteria*) belongs to different groups, and their Hg methylation characteristics are unknown. Hg-resistant characteristics of *Thiobacillus* and *Xanthomonas* had been researched previously, and *Xanthomonas* was formerly been isolated from an Hg mine and were found to have Hg resistance transposon (Kholodii et al., 2000; Takeuchi et al., 1999). Considering results of the gene copies of the quantified *hgcA*, *dsrB*, *merA* and *mcrA* genes, it can be seen that *merA* quantities in the rhizosphere of high-Hg sites were extremely significant different from that in low-Hg sites (*merA* RS1-3 vs RS4-6***, ANOVA, $p < 0.001$, Fig. S7). The gene quantity results confirmed that higher abundance of Hg-resistant microorganisms existed in the high-Hg sites, which made the *merA* quantities in high-Hg sites be significantly higher than that in the low-Hg sites. Another genus that was more abundant in the high-Hg sites was *Defluviicoccus*, whose Hg-resistant abilities had not been confirmed yet. *Defluviicoccus* contains only a single species, namely *Defluviicoccus vanus*, which is found in the wastewater treatment processes (Maszenan et al., 2005). *Thiobacillus*, *Xanthomonas* and *Defluviicoccus* might play an important role in Hg methylation or demethylation among the rhizosphere bacterial communities, and whose reads in RS1 was significantly higher than that in RS6. Moreover, the Hg-resistant genes, *merA* and *merB*, as well as the Hg-methylating genes, *hgcA* and *hgcB* in strains of the genus of *Defluviicoccus* need to be investigated further.

When comparing the relative abundance of all the archaeal genera between high-Hg and low-Hg sites, we discovered that *Methanosaet, Miscellaneous_Crenarchaeotic_Group*, *Candidatus_Nitrosoarchaeum* and *Soil_Crenarchaeotic_Group_SCG* were significant more abundant in high-Hg sites. This meant that archaeal strains in the four genera might be related with Hg-methylation or demethylation in the paddy soils. The *mcrA* and *hgcA* gene quantities in BS of the high-Hg sites were significant more than that in BS of the low-Hg sites (*mcrA* BS1-3 vs BS4-6**, $p < 0.01$; *hgcA* BS1-3 vs BS4-6*, $p < 0.05$, ANOVA, Fig. S7), which indicated that higher abundance of methanogens with Hg-methylating abilities might exist in BS of the high-Hg sites. In addition, *Candidatus Nitrosoarchaeum*, an ammonia-oxidizing archaea in the phylum of *Thaumarchaeota*, was also significant more abundant in RS compared to BS. *Thaumarchaeota* is initially classified as mesophilic *Crenarchaeota*, and they are ubiquitous in the terrestrial environments and dominant among the soil archaea (Schleper and Nicol, 2010). Moreover, *Candidatus Nitrosoarchaeum* was previously found in the rhizosphere of *Caragana sinica* to resist the stress from heavy metals (Kim et al., 2011). Therefore, it is predicted that methanogens with Hg-methylating abilities mainly distribute in RS of the high-Hg sites, and *Candidatus Nitrosoarchaeum* may interact directly or indirectly with

Hg, participating in the biogeochemical cycle of Hg in the rhizosphere, ultimately survives and plays an important role among the rhizosphere archaeal communities. In addition, the abundance of *Candidatus Nitrosoarchaeum* became smaller or even vanished in BS of the low-Hg sites, which could also prove that *Candidatus Nitrosoarchaeum* might be the result of microbe's combating with heavy metal pressure. However, this was also just our conjecture, and further tests were needed to prove it.

RDA analysis showed that the variable NH_4^+, not SO_4^{2-} or Fe^{2+}, seemed to be the most important factors that influencing the bacterial and archaeal communities, which inferred that microorganisms involving in nitrogen cycle in the paddy soils might play more essential roles in microbial communities. Previous research had confirmed that NH_4^+ played key roles in determining the archaeal distribution in the flooded habitat (Zheng et al., 2013), which accorded with our results. The effect of MeHg to THg ratios on bacterial communities was stronger than that on archaeal communities, which might mean that Hg-pollution had larger influence on bacterial communities. In addition, it should be noted that the RS communities were significant different from the BS communities. The RS communities were positively affected by the variables except pH and elevation, while the effect of variables on BS communities was reverse. This might be the reason for the differences between the communities of RS and BS. It might also be due to the role of rhizosphere secretion on changing microbial activities and community structures. In addition, we could also find that the bacterial and archaeal communities in high-Hg sites were less affected by the variables than that in the low-Hg sites. This might also be one of the reasons for the differences of community structures between high-Hg and low-Hg sites.

4.4. Phylogenetic analysis of archaea

The phylogenetic trees of archaea indicated that previously confirmed Hg-methylators *Methanolobus tindarius* (DSM2278) and *Methanomethylovorans hollandica* (DSM15978) had akin relationship with the dominant genus *Methanosarcina* (Fig. S6), whose reads accounted for 2.04% (RS) and 0.97% (BS) respectively (Fig. 4b). This indicated that strains in the genus *Methanosarcina* might have relationship with Hg methylation in the studied paddy soils. However, several research found that even rare microbes contributing <1% of the total microbial abundance could have profound impacts on biogeochemical cycles (Pester et al., 2010). Therefore, further research need to be done to isolate strains in the genus of *Methanosarcina* and to test their Hg-methylating abilities. Another confirmed archaeal Hg methylators, *Methanospirillum hungatei* JF1 (DSM 864) had akin relationship with *Methanospirillum* (Fig. S6), but the Hg methylating abilities of other strains in this genus were not confirmed so far. Moreover, the number of reads for *Methanospirillum* was quite low in our research, accounting for <0.003% for both RS and BS (Fig. 4b). Therefore, it is predicted that the Hg-methylating archaea in the paddy field maybe strains in the genus *Methanosarcina*. Nevertheless, the dominant archaeal strains in this genus need to be isolated and their Hg-methylating abilities need to be further researched in the future. Results of this research might provide reference to understand archaeal rhizosphere community along an Hg gradient paddy soils, but further research on isolating archaea and confirming their Hg-methylating or Hg-resistant abilities is needed.

5. Conclusion

Thiobacillus, *Xanthomonas*, *Defluviicoccus* and *Candidatus Nitrosoarchaeum* might play an important role in the response to Hg stress, including Hg-methylation or demethylation among the rhizosphere bacterial and archaeal communities. Further research on Hg-resistance abilities of *Defluviicoccus vanus* and *Candidatus Nitrosoarchaeum* need to be conducted. Phylogenetic tree of the archaeal genera showed that two confirmed Hg methyltors, *Methanolobus tindarius* (DSM2278) and *Methanomethylovorans hollandica* (DSM15978) had akin relationship with the dominant genus *Methanosarcina*, whose reads accounted for 2.04% (RS) and 0.97% (BS) respectively in our research. Our conjecture is that the Hg-methylating archaea in the paddy field could potentially be affiliated to the genus of *Methanosarcina*. So, archaeal strains in this genus need to be isolated and their Hg-methylating abilities deserve further investigation. However, we should admit several limitations of this research. For instance, the sequencing sample numbers should be enlarged to cover all the average samples. No further proof is available to rule out bacteria and their roles in Hg methylation in paddy fields. Overall, our findings still demonstrated that *Thiobacillus*, *Xanthomonas*, *Defluviicoccus* and *Candidatus Nitrosoarchaeum* might play roles among the rhizosphere microbiome under the long-term Hg stress to combat the toxicity of Hg. Additionally, the genus of *Methanosarcina* might exert significant role in the methylation process of Hg. These findings are conducive to a better appreciation of the role of archaea in the biogeochemical cycling of Hg.

Notes

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary methods, figures and tables. This material is available free of charge via the Internet at https://www.journals.elsevier.com/science-of-the-total-environment/. Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.07.175.

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