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Mercury methylation in the soils and sediments of Three Gorges Reservoir Region

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Abstract

Purpose Previous studies demonstrated that microorganisms had an important role in the mercury (Hg) methylation process in the water and sediments of Three Gorges Reservoir (TGR). The purpose of this research was to analyze the microbial methylation of Hg in the soils and sediments of the water-level-fluctuating zone (WLFZ) of TGR.

Materials and methods Different types of soils, sediment (≤ 155 m), semi-inundated soil (≥ 155 m), and non-inundated soil (≥ 175 m) of the WLFZ of Shibao (S), Zhenxi (Z), and Tujing (T) were investigated. Real-time PCR, terminal restriction fragment length polymorphism (T-RFLP), cloning, sequencing, and phylogenetic analysis were used to analyze the abundance and diversity of *dsrB*, *hgcA*, and *mcrA* genes, and their relationship with the levels of total Hg (THg), MeHg, and several biogeochemical factors that probably affected microbial methylation reaction.

Results and discussion THg concentrations in different soil types of the WLFZ of TGR did not show significant differences ($p > 0.05$) in site S, while there were significant differences ($p < 0.05$) of MeHg levels in different soil types of the three sites. Phylogenetic analyses found that the dominant groups of microorganisms with *dsrB* in the sediment and non-inundated soil in site S differed remarkably. Microorganisms that probably related with Hg methylation mainly distributed in the sediment, with *δ-proteobacteria* as the dominant class. Real-time PCR found that soil MeHg levels correlated positively with the resident quantities of microorganisms with *dsrB*. The abundance of *dsrB* was much higher than that of *hgcA* which may indicate that only a small part of sulfate-reducing bacteria (SRB) related with Hg methylation.

Conclusions Soil MeHg levels correlated positively with the resident quantities of microorganisms with the *dsrB* gene. The phylogenetic analysis indicated that SRB that probably related with Hg methylation distributed mainly in the sediment, rather than in the non-inundated soil.

Keywords *dsrB*/*hgcA*/*mcrA* · Methylmercury · Soil/sediment · Three Gorges Reservoir · Water-level-fluctuating zone

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1 Introduction

Mercury (Hg) is a pervasive global pollutant released from both natural and anthropogenic sources (Benoit et al. 1998; Lin et al. 2012). Mercury exists in the environment as the elemental Hg (Hg^0), inorganic Hg (Hg^{2+}), and organomercury compounds, such as monomethylmercury (CH_3Hg^+), methylmercury (MeHg), and dimethylmercury (DMHg) (Abelson 1970; Mason et al. 1996). Methylmercury is the form of most concern since it can be biomagnified in the food webs of aquatic systems, reaching high concentrations in fish, thus posing a threat to human health (Abelson 1970; Boening 2000; Morel et al. 2003; Yu 2011). Both Hg^0 and Hg^{2+} can transform into MeHg via biotic and abiotic processes (Barkay et al. 1997; Lin et al.

2012; Hu et al. 2013). However, a predominant view is that MeHg is produced in the environment primarily by microorganisms, especially anaerobic microorganisms that exist in most natural settings (Raposo et al. 2008). Therefore, it is essential to research on regulating mechanisms of Hg methylation by anaerobic microorganisms (Hsu-Kim et al. 2013).

Many studies had indicated that methylation rates in sediments significantly correlated with the quantities of sulfate-reducing bacteria (SRB), iron-reducing bacteria (IRB), and methanogens (Fleming et al. 2006; Ranchou-Peyruse et al. 2009; Hu et al. 2011; Schaefer et al. 2011; Yu et al. 2013). However, it should be noted that not all SRB, IRB, or methanogens were capable of methylating Hg. Fortunately, the genes and proteins involved in Hg methylation reaction had finally been identified (Parks et al. 2013). A two-gene cluster, *hgcA* and *hgcB*, required for Hg methylation was found and confirmed by several novel microorganisms from new environments (Gilmour et al. 2013). A few studies have been done based on *hgcAB* genes (Bae et al. 2014; Liu et al. 2014a, b; Schaefer et al. 2014). Moreover, Parks et al. (2013) confirmed that SRB were still the main producers of MeHg, although IRB, methanogens, and other microorganisms can also be involved. Model strains of SRB and IRB had widely been researched (Gilmour et al. 2011). Many researchers focus on SRB and its methylation effect (Henry 1992; Acha et al. 2005, 2011; Foti et al. 2007; Bridou et al. 2011). The gene encoding a component of dissimilatory (bi)sulfite reductase, *dsrB*, is common among all known SRB. So, the *dsrB* gene has been widely used as a biomarker for determining the SRB diversity in various environments (Geets et al. 2006). Therefore, it is still meaningful to research on the Hg methylation by SRB based on the *dsrB* gene.

The reservoir has been confirmed as an Hg methylation environment. Several studies have shown that reservoirs tend to have elevated MeHg levels compared with natural lakes and rivers, especially in newly created reservoirs (Montgomery et al. 2000; Brigham et al. 2002; Kamman et al. 2005; He et al. 2008; Feng et al. 2009a). Eckley et al. (2015) found that the seasonally inundated sediments had significantly higher methylation activities than the permanently inundated area of an older reservoir. Undoubtedly, the ecological safety of Three Gorges Reservoir (TGR), the biggest reservoir of China, has attracted much more focus. Therefore, TGR has been selected in our research based on the following reasons.

First, previous research has indicated that Hg concentrations in the background soils of the Circum-Pacific Global Mercuriferous Belt in which TGR situated is elevated up to 2–4 orders of magnitude compared with the national background value ($0.038 \ \mu g \ g^{-1}$) (Feng et al. 2005). Second, many studies have been carried out to measure the concentrations of MeHg and total Hg (THg) in the water column, soils, sediments, and fish of TGR and found that it is an Hg-sensitive area (Xiang et al. 2014; Zhao et al. 2014; Li et al. 2015).

Third, the alternation of water levels from 175 to 145 m every year produces a water-level-fluctuating zone (WLFZ) of over $400 \ km^2$ along the Yangtze River. A large number of vegetables and agricultural crops grow luxuriantly in the WLFZs of TGR from March to August and die from September to the next April, which makes TGR quite different from other old reservoirs. Finally, the exchange of water level makes the soils in the WLFZ of TGR experience a cycle of anaerobic and aerobic conditions, leading to different characteristics of Hg methylation reactions. Although the biogeochemistry of MeHg production in reservoirs has been studied well for years, the relationship of community structure of Hg methylators with the levels of THg and MeHg in soils under alternating anaerobic and aerobic conditions remains poorly understood. As of yet, the distribution of SRB in soils and sediments of the WLFZ and its relationship with the levels of THg and MeHg are still unknown. Therefore, the primary objectives of this research were to investigate the distribution, abundance, and diversities of the *dsrB* gene, and its relationship with the levels of THg and MeHg in soils of the WLFZ of TGR.

2 Materials and methods

2.1 Study area and sampling

Chongqing is situated in the upper reaches of Yangtze River in the eastern Sichuan Basin, China. The water level of Yangtze River fluctuates between 145 and 175 m every year, which produces WLFZs of 30 m in altitude, most of which are located in Chongqing. This research was conducted in three WLFZs in the Chongqing region, namely sites Zhenxi (Z), Shibao (S), and Tujing (T), among which two were mainstreams (sites Z and S) and one was the tributary (site T) of Yangtze River (Fig. 1). The three sites all belong to subtropical monsoon climate, with annual average temperature around 18 °C and annual precipitation around 920–1200 mm. The WLFZ of the three sites belongs to the eastern Sichuan fold belt of the third subsidence belt of the Cathaysian tectonic system. Sampling sites selected in this research are inundated by water from October to March and expose to the air from September to the next April (Du et al. 2016). Site Z is located in Shuikou village, Zhenxi County, Fuling District. Site S is located in Xinzheng village, Shibao Town, Zhong County. Site T is in the Pingshan village, Tujing Town, Zhong County. All the three sites are situated in the upstream of TGR. The types of land use in sites Z and S are simple. The soil of 150–175 m from March to August in the two sites is occupied by local farmers to plant corn. For site T, the monitoring sites are mainly wasted land, being surrounded by eucalyptus forest. It is not barren since many kinds of natural plants and weeds grow lushly in the WLFZ of site T. Brief
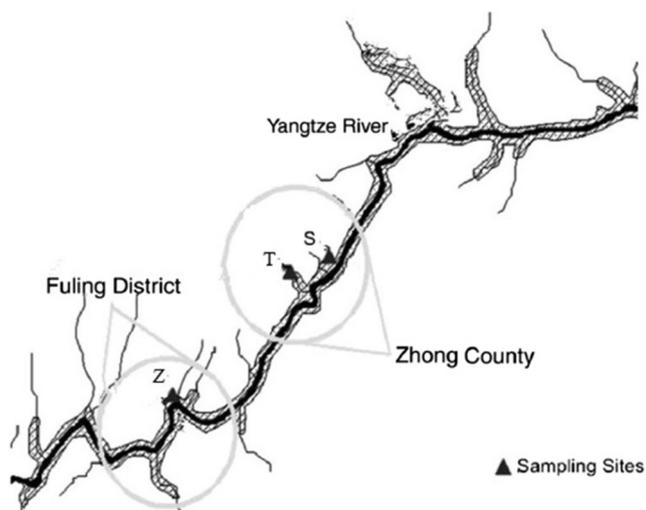
Fig. 1 Schematic diagram of the three study sites, Zhenxi (Z), Shibao (S), and Tujing (T) in Chongqing, China

descriptions and chemical indices of the three sampling sites are listed in Tables 1 and 2.

Sampling was conducted in the end of April 2014 when the water level was around 155 m. Samples in all sites include three types: sediment, semi-inundated soil, and non-inundated soil. The sediment is inundated by water throughout the year, namely inundated soils. Semi-inundated soil means that it is sediment for half a year, becoming soils exposed to the air for another half a year. Non-inundated soils mean the soils at the altitude of 175 m, which is never inundated by water throughout the year. Soil depth of the three sites was all upper soils, 0–10 cm. Therefore, a total of nine soil/sediment samples were taken at the three sites. For site Z, ZI, ZS, and ZN represented sediment (inundated soil), semi-inundated soil, and non-inundated soil collected in Zhenxi, respectively. By the same naming rule, for sites S and T, SI, SS, and SN and TI, TS, and TN represented sediment, semi-inundated, and non-inundated soils collected in Shibao and Tujing, respectively.

Samples for community diversity analysis were collected by clean PE gloves, placed in sterile air-tight Falcon tubes on ice, transported to the laboratory within 24 h, and stored at −80 °C prior to DNA extraction. Samples for THg and MeHg analyses were placed into two sealed envelope pockets after thorough homogenization, frozen by liquid nitrogen immediately after collected, kept in a refrigerated box, transferred to the laboratory within 24 h, and then stored at −20 °C. The soil samples were freeze-dried, homogenized to fine powder (100 meshes), and stored at −20 °C, to be used for determining MeHg levels by GC-cold vapor atomic fluorescence spectrometry (CVAFS) after aqueous-phase ethylation.

2.2 Analysis of environmental factors

Soil MeHg levels were determined using HNO_3 leaching/CH_2Cl_2 extraction, ethylation, trapping on a Tenax trap,

isothermal GC separation, and the CVAFS detection method (Liang et al. 2004). Soil THg levels were determined by acid (1:3 HCl + HNO_3) digestion followed by CVAFS detection (Qiu et al. 2006; Feng et al. 2009b). K_2CrO_7 oxidation titration coupled with a volumetric technique was used to test the concentrations of soil organic matter (OM) (Jiang 2005). Soil pH was detected by a potentiometric method, and soil sulfate was analyzed by barium chromate spectrophotometry (370 nm) (Lu 2000). Ammonium in the soils and sediments was extracted with 2 M KCl and determined by bromophenol blue colorimetry. Dissolved iron (Fe^{2+}) was determined using a modified bathophenanthroline procedure (Nurnberg 1984).

2.3 Soil DNA extraction and quantification of *hgcA*, *dsrB*, and *mcrA* genes

DNA was extracted from 0.25 g soil samples with PowerSoil DNA Isolation Kit (Mo Bio, USA) based on the manufacturer's protocol (Liu et al. 2014a, b). The purity and concentration of total DNA extracted were measured by 1% agarose gel electrophoresis (Bio-Rad, USA) and a NanoDrop 2000 spectrophotometer (Thermo Fisher, USA), respectively, and then the DNA was stored at −20 °C before use.

Real-time PCR (also referred to as quantitative PCR, qPCR) has emerged as a promising tool for studying soil microbial communities nowadays. One advantage for qPCR is that it allows for a relatively rapid yet quantitative assessment of the abundances of specific phylogenetic groups of microorganisms in various environments. So, in order to know the abundance of Hg methylators (*hgcA*), SRB (*dsrB*), and methanogens (*mcrA*), qPCR assay was used to obtain the quantities of different genes in the soils and sediments of TGR. The qPCR was conducted in polypropylene 96-well plates on an ABI 7500 Real-Time PCR system (Life Technologies, USA) with MightyAmp for Real Time (SYBR® Plus) (Takara, Japan). Each 20-μL reaction contained the following (the same for all genes): 10 μL MightyAmp for Real Time (SYBR Plus) (2×), 0.4 μL forward and reverse primers (10 μM), 0.4 μL ROX Reference Dye II (50×), 2 μL temperate DNA, and 6.8 μL ddH₂O. PCR conditions were 2 min at 98 °C, followed by 40 cycles of 98 °C for 10 s, 34 s at the annealing temperature (Table 3), and 68 °C for 1 min. All the primers were synthesized by Shanghai Sangon.

In the absolute quantification test, the plasmid standard containing the target genes was generated as follows. First, the gene sequences were amplified from extracted DNA with primers mentioned above. The amplified products were run on a 1% agarose gel to confirm the specificity of the amplification and then cut and reclaimed by the Wizard SV GEL and PCR Clean-up System (Promega, USA). The purified PCR products were cloned into the pGEM-T Easy Vector (Promega, USA) and then transformed into the *Escherichia coli* JM109 competent cells (Promega, USA). Plasmids were extracted using the EZNA Plasmid Mini Kit I (Omega, USA) with

Table 1 Basic information of research sites

Sampling sites	Longitude/latitude	Annual average temperature (°C)	Soil type	Main stream/ tributary
Fuling Zhenxi (site Z)	E 107° 28′ 4″; N 29° 54′ 7″	17–18	Purple soil	Main stream
Shibao (site S)	E 108° 7′ 41″; N 30° 25′ 40″	18.2	Purple and paddy soil	Main stream
Tujing (site T)	E 108° 7′ 41″; N 30° 25′ 40″	18.2	Purple and paddy soil	Tributary

concentrations determined by a NanoDrop 2000 spectrophotometer (Thermo Fisher, USA). The plasmids used as standards for quantitative analysis were extracted from correct clones of target genes, with confirmation by sequencing (Shanghai Lifei). Copy numbers of the target genes were calculated directly from the concentrations of extracted plasmid DNA. An external standard curves were generated using triplicate tenfold serial dilutions of a known copy number of plasmid DNA.

2.4 Terminal restriction fragment length polymorphism analysis

Terminal restriction fragment length polymorphism (T-RFLP) analysis is one of the most common techniques used to study the diversity of complex microbial communities (Duran et al. 2008). So, T-RFLP was used to analyze the community diversity of SRB in the soils and sediments of the WLFZ of TGR. ABI 3730 sequencer was used to scan the spectrogram with *Hha*I digestion. Primers used for T-RFLP were *DsrB*-F (5′ FAM, ACSCACTGGAAGCACGGYGG) and *DsrB*_R (GTAGCAGTTWCCGCAGWACAT) which produced a 1900-bp product. A 50-μL reaction mixture included 5 μL of 10× Taq PCR buffer (Mg^{2+} free), 4 μL dNTPs (2.5 mM), 3 μL MgCl$_2$ (25 mM), 1.0 μL of each primer, 2 μL template DNA,

0.5 μL Taq (5 units/μL, Takara, Japan), and 33.5 μL sterilized distilled water. The PCR condition used was as follows: 1 min at 98 °C, 30 cycles of 98 °C for 10 s, 30 s at 50 °C, 72 °C for 1 min, and a final extension at 72 °C for 5 min.

FAM-labeled PCR products were electrophoresed on a 1% agarose gel. The target fragment was cut and reclaimed by the Wizard SV GEL and PCR Clean-up System (Promega, USA). The purified PCR product was subjected to restriction enzyme digestion. *Hha*I (Takara, Japan) was used in a final volume of 20 μL at 37 °C for 30 min. Digested amplicons were precipitated with 2 μL NaOAC (3 M) and 40 μL absolute alcohol at − 20 °C for 30 min. DNA pellets were washed by 70% ethanol, dried in a DNA concentrator (miVac, UK) for 4 min, and then dissolved in 10 μL sterilized distilled water. The lengths of terminal restriction fragments (T-RFs) were detected by capillary electrophoresis by Shanghai Sangon. T-RFLP profiles were analyzed by GeneMapper software 5 (Applied Biosystems, USA). The relative abundance of a T-RF was calculated by dividing the peak area of the T-RF by the total peak area of all T-RFs in the profile. Peaks with a height less than 1% of the total peak heights were not used for further analysis. Peak areas of the rest of T-RFs were normalized, and T-RFs with relative abundance of lower than 1% were canceled. For T-RFs whose relative abundance was larger than 5%, they were considered as dominant fragments.

Table 2 Physicochemical features of the soils and sediments of the WLFZ of TGR

Samples	pH	OM (%)	SO$_4^{2-}$ (mg kg^{-1})	NH$_4^+$ (mg kg^{-1})	Fe^{2+} (g kg^{-1})
ZI	7.82	6.36	24.8	4.23	5.86
ZS	7.79	7.63	15.4	4.56	5.72
ZN	7.41	12.6	10.0	4.97	1.34
SI	7.76	8.82	23.6	4.59	4.85
SS	7.45	3.66	15.0	4.87	3.88
SN	5.26	15.4	8.21	5.28	1.05
TI	6.92	7.03	19.3	4.03	4.58
TS	6.71	8.09	10.2	4.34	4.72
TN	6.28	5.52	7.13	4.64	1.02

For the different sampling sites, site Z is Zhenxi, site S is Shibao, and site T is Tujing. For the different soil types, I is for the sediment/inundated soils, S for the semi-inundated soils, and N for the non-inundated soils, namely soils of 175 m

Table 3 PCR conditions for the genes researched

Gene	Primers	5′ → 3′ sequences	Amplicon size (bp)	Annealing T (°C)	References
dsrB	DSRp2060F	CAACATCGTY CAYACCCAGGG	350	55	(Geets et al. 2006; Liu et al. 2014a)
	DSR4R	GTGTAGCAGTTACCGCA			
hgcA	*hgcA_*F	GGNRTYAAYRTNTGGTGYGC	950	50	(Bae et al. 2014)
	*hgcB_*R	CADGCNCCRCAYTCVATRCA			
mcrA	mlas	GGTGGTGTMGGDT TCACMCARTA	470	55	(Hales et al. 1996)
	mcrA-R	CGTTCATBGCGTAGTT VGGRTAGT			

2.5 Cloning, sequencing, and phylogenetic analysis

To characterize the diversity of SRB based on *dsrB*, two clone libraries were constructed. One was from sample SI (the sediment of site S), and the other was from sample SN (non-inundated soil of site S), both of which were from site S. *DsrB* was amplified with the same primers as described in qPCR assay to produce 350-bp PCR products as the target fragment. PCR products were separated, purified, and then ligated into the pGEM-T Easy Vector (Promega, USA). The target PCR products were transformed into *E. coli* JM109 competent cells (Promega, USA) following the manuals of the manufacturer. A total of 118 and 116 *dsrB* gene clones for SN and SI were sequenced by the ABI 3730 sequencer by Shanghai Lifei. Sequences sharing more than 95% gene nucleotide homology were grouped into the same operational taxonomy units (OTUs). Representative sequences of each OTU were aligned and analyzed with DNAman v6.0. Phylogenetic trees were constructed with MEGA v6.0, employing a neighbor-joining method with a bootstrap value of 1000. OTU coverage (C) of each library was calculated with the formula $C = (1 - n / N) \times 100\%$, where N was the number of clones sequenced and n was the number of OTUs.

2.6 Quality control and statistical analysis

Quality control of the analytical processes was performed by using field blanks, system blanks, spike recoveries, and sample duplicates. Field blanks and duplicates were collected regularly throughout each sampling campaign. Detection limits were based on three times the standard deviations of the blank. Detection limits of THg and MeHg in soils were 0.02 and 0.01 ng g^{-1}, respectively. The method blank was lower than the detection limits in all cases. The precision was determined by relative standard deviations for duplicate samples, which were 5.8 and 6.2% for THg and MeHg analyses. Recoveries for matrix spikes ranged from 96 to 113% for THg and from 90 to 112% for MeHg.

One-way analysis of variance (ANOVA) was used to assess the statistical significance of the data, with $p < 0.05$ being considered as significant. MS Paint v6.1, Microsoft Office 2013, and Origin 8.0 were used for drawing. Canoco for Windows 5.0 was used to conduct the principal component analysis (PCA) of microbial community and biogeochemical factors that probably affected Hg methylation.

3 Results and discussion

3.1 The levels of THg and MeHg in the experimental soils

The concentrations of THg and MeHg in the soils and sediments of the three sites are shown in Fig. 2. The concentrations of THg in different soil types of the WLFZ of TGR did not show significant differences ($p > 0.05$) in site S, while for sites Z and T, the difference was significant ($p < 0.01$ and $p < 0.05$, respectively). As for the concentration of MeHg among the same sites, there were significant differences ($p < 0.05$) of MeHg levels in different soil types of the three sites. All the sediment samples in the three sites had higher concentrations of MeHg, SO_4^{2-}, and Fe^{2+}, but lower OM content (Fig. 2B, Table 2). As for the same soil types, THg and MeHg levels in the sediments showed significant differences between sites Z and S ($p < 0.001$), as well as sites S and T ($p < 0.001$), except sites Z and T ($p > 0.05$). There were also significant differences ($p < 0.01$ and $p < 0.001$, respectively) of THg and MeHg levels among semi-inundated or non-inundated soils in the three sites. The concentrations of MeHg reached maximum in the sediment of site S, 1.149 ng g^{-1}, while non-inundated soil in site Z had the minimum MeHg level, 0.071 ng g^{-1}. Inundated and semi-inundated soils in the three sites had the most obvious changes for the production of MeHg compared with non-inundated soil. MeHg levels in the inundated and semi-inundated soils in site S were remarkably higher than those in the other two sites. The reason for this is not known. It may because that site S is located in the main stream of Yangtze River while site T is in the tributary, although the two sites are both in Zhong County. Water flows slowly in site T, and the WLFZ in site T is waste land, with no agricultural activities and no people living around. Moreover, sites Z and S are both in the main
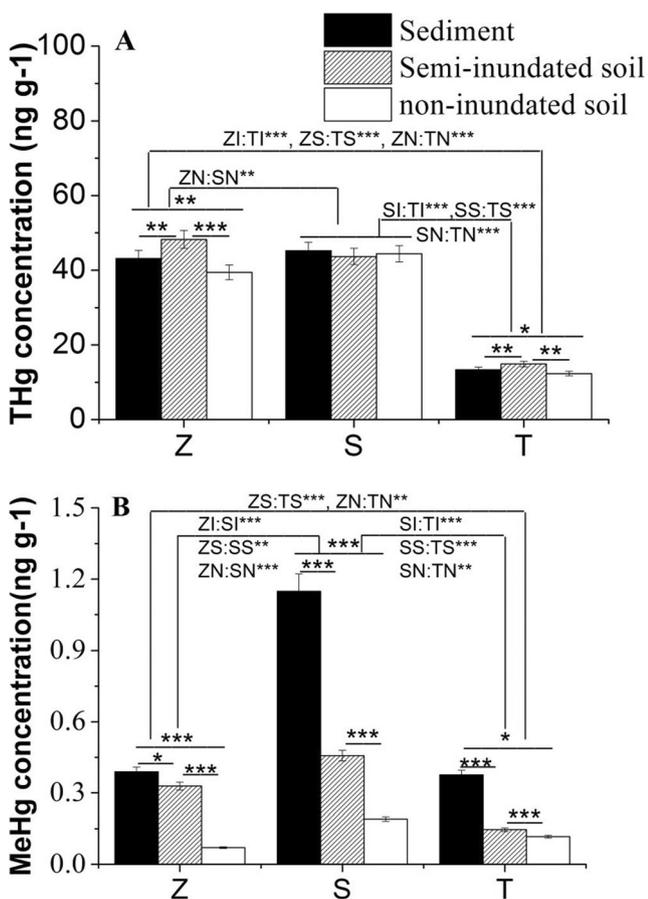
Fig. 2 The levels of THg (**a**) and MeHg (**b**) in different soil types of the WLFZ in the three sites. *Small bars* show standard errors, $N = 3$. The asterisk indicates the difference of THg and MeHg levels based on one-way ANOVA (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; without *$p > 0.05$)
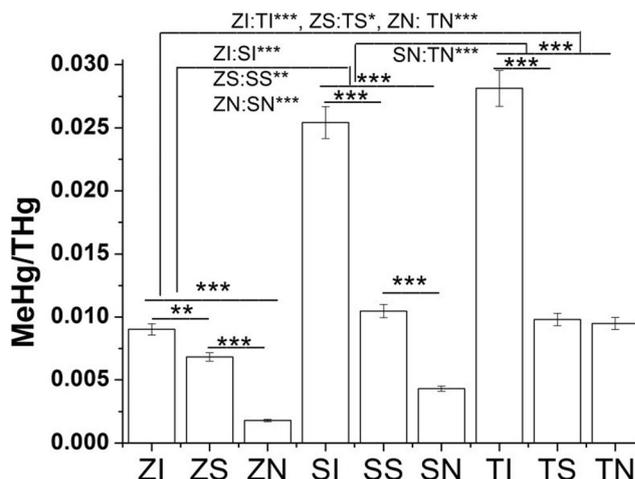

Fig. 3 The ratios of MeHg to THg in different soil types of the WLFZ in the three sites. *Small bars* show standard errors, $N = 3$. The asterisk indicates the difference of MeHg/THg ratios based on one-way ANOVA (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; without *$p > 0.05$)

stream, but site Z has a quite different soil condition, and human activities in site Z are quite fewer.

Since, the ratios of MeHg to THg can be an index of Hg methylation efficiency according to previous research (Sando et al. 2007). In order to show the methylation progress of the researched soils and sediments, the ratios of MeHg to THg were calculated. The ratios of MeHg to THg reached maximum (0.52%) in the sediment of site T (TI), and it was minimum (0.07%) in the non-inundated soil of site Z (ZN) (Fig. 3). It was obvious that MeHg/THg was remarkably higher in the sediments than in the other two soil types in the three sites ($p < 0.001$). For different soil types, the ratio of MeHg to THg was highest in the sediment, followed by the semi-inundated soil and non-inundated soil, which indicated that the sediment contributed more to the production of MeHg. However, MeHg/THg ratios of the sediment in the WLFZ of TGR were a little bit lower compared with other research (Brigham et al. 2002; Sando et al. 2007; Feng et al. 2009a; Yu 2011). Moreover, it should also be noticed that MeHg/THg was only a rough indicator of the Hg methylation process. Methylmercury is more volatile than Hg^{2+}, such that low MeHg/THg ratios may mean rapid rates of

methylation, depending on the characteristics of the soil. Moreover, MeHg tends to stick to the sediments and soils quite well. Therefore, the loss of MeHg may be primarily due to de-methylation. Further research on the characteristic difference of soil types and MeHg demethylation needs to be done.

3.2 Resident abundance of Hg methylators, SRB, and methanogens

The abundance of Hg methylators, SRB, and methanogens based on *hgcA*, *dsrB*, and *mcrA* gene sequences in the tested soils and sediments can be seen in Fig. 4. The levels of MeHg in the soils and sediments correlated positively with the resident quantities of microorganisms with the *dsrB* gene in sites Z, S, and T (Figs. 2B and 4), while MeHg levels in the soils and sediments correlated positively with *hgcA* quantities in sites S and T, except site Z. The abundance of *dsrB* was significantly higher than that of *hgcA* ($p < 0.001$, not shown in Fig. 4), which may indicate that only a small part of SRB related with Hg methylation. Among the same groups, both the *hgcA* and *dsrB* gene copies had an extremely significant difference ($p < 0.001$) between the sediment and non-inundated soil in site T, and *dsrB* gene copies had a significant difference ($p < 0.01$) between the sediment and semi-inundated soil in sites Z and S. The *hgcA* and *dsrB* gene copies were highest in the sediment, followed by the semi-inundated soil and non-inundated soil, which indicated that the sediment had the largest number of *hgcA* and *dsrB* genes compared with the other soil types. The reason perhaps was that microorganisms in the soils inundated in the water were under anaerobic condition for a longer time, which was a necessary condition for Hg methylation reaction. So, the numbers of SRB and other Hg methylators were higher than those in the other soil types.

Fig. 4 Resident abundance of mercury methylators, SRB, and methanogens based on *hgcA*, *dsrB*, and *mcrA* gene sequences. *Small bars* show standard errors, $N = 3$. The asterisk indicates the difference of gene quantities based on one-way ANOVA (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; without *$p > 0.05$). Italic letters on the lines showing significant difference include *h* (*hgcA*), *d* (*dsrB*), and *m* (*mcrA*). Differences between groups (Z, S, and N) are not shown here
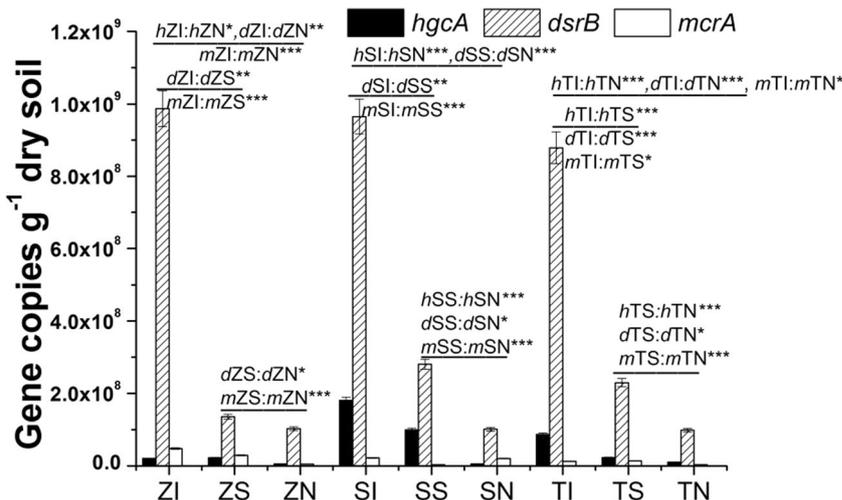
A total of 35 dominant T-RFs were detected based on the *dsrB* gene. The relative abundance of the dominant T-RFs of the *dsrB* gene is shown in Fig. 5. Samples of ZI, ZS, and SS had the fewest T-RFs, while SI had the most T-RFs, followed by TI, TS, TN, ZN, and SN (Fig. 5). Sites Z, S, and T differed greatly in the size of the fragments, indicating that different species were present at different locations. Site T was the only one that located in the tributary of Yangtze River, where all the three types of soils had a higher relative abundance compared with the other two sites. Around the sampling place of site T, it is a waste land surrounded by eucalyptus forest. Many kinds of natural plants and weeds grow lushly in the WLFZ, and it has not been utilized by farmers for growing vegetables, whereas soils collected in sites Z and S were both around the agricultural planting area. That may be one reason for the different results of relative abundance of the *dsrB* gene in different soil types between site T and the other two sites. Comparing site Z with S, the ones both located in the main stream of Yangtze River, T-RFs of 203 bp only occurred in ZI and ZS. For the sediment in the three sites, SI had the most T-RFs, followed by TI, and ZI had the fewest T-RFs. It may

result from the variation of the water levels, which may also affect the diversity of the T-RFs. For non-inundated soils in the three sites, the number of T-RFs was the same. It may result from the least influence of water level changes, since soils at this altitude were exposed to the air in the whole year.

3.3 Relationship of microbial community and the levels of THg, MeHg, and other biogeochemical factors

PCA between community structure and the levels of THg, MeHg, and other biogeochemical factors, such as OM, NH_4^+, SO_4^{2-}, Fe^{2+}, and pH, is shown in Fig. 6. The non-inundated soils in sites Z, S, and T (ZN, SN, and TN) were totally different from other soil types. The levels of THg, MeHg, NH_4^+, SO_4^{2-}, Fe^{2+}, and pH were all the main factors that correlated with the dominant T-RFs (Fig. 6). OM concentrations correlated negatively with soil MeHg levels, while the levels of SO_4^{2-}, Fe^{2+}, and pH correlated positively with soil MeHg levels. These results were in accordance with previous studies demonstrating that SO_4^{2-} and Fe^{2+} concentrations were both important factors dominated in the Hg methylation process (Dyrssen and Wedborg 1991; Gilmour et al. 1992; Benoit et al. 1999, 2001; Acha et al. 2005; Drott et al. 2007). Some researchers also focused on Hg methylation in the presence of polysulfides and found that polysulfides had the same effect with sulfide, both of which could result in the increase of MeHg levels (Jay et al. 2000, 2002).

With *dsrB* as the target gene, for RFs in the first quadrant, 60, 161, 173, 186, and 191 bp corresponded positively with OM. For RFs in the second quadrant, 189 bp corresponded positively with THg and NH_4^+. For RFs in the third quadrant, 175, 200, 203, and 276 bp corresponded positively with SO_4^{2-}, Fe^{2+}, MeHg, and pH. For RFs in the fourth quadrant, 94, 181, 211, and 258 bp corresponded negatively with NH_4^+ and THg. For inundated and semi-inundated soils in sites Z, S, and T, OM, NH_4^+, SO_4^{2-}, Fe^{2+}, and pH corresponded positively with the dominant T-RFs, while THg, NH_4^+, and OM reacted negatively.
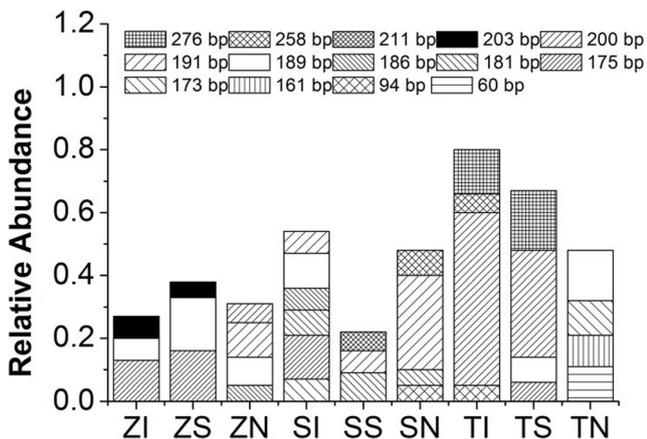

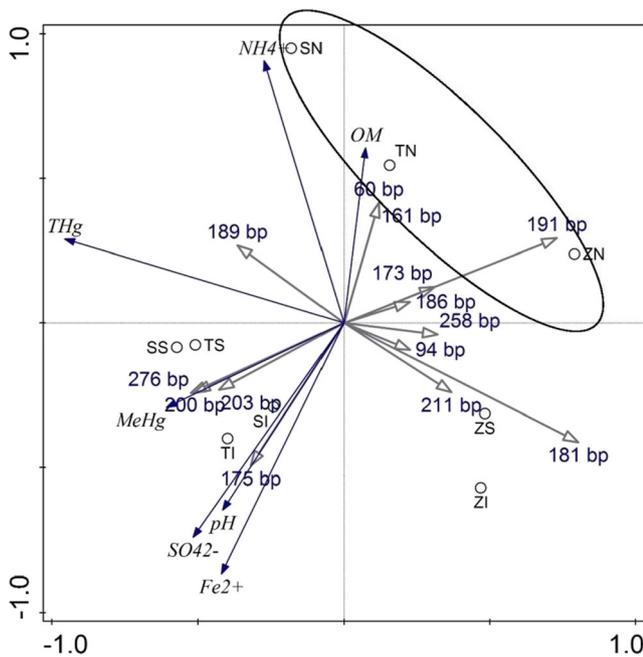**Fig. 5** Relative abundance of dominant T-RFs based on the *dsrB* gene in different soil types of the WLFZ of TGR

Fig. 6 PCA results of the dominant T-RFs and environmental factors based on the *dsrB* gene

Table 4 The OTUs and Shannon-Weaver, Simpson, and Pielou indices of each sample based on the *dsrB* gene

Sampling sites	OTUs	H	D	E
ZI	4	3.843	0.9303	0.8275
ZS	4	3.690	0.9268	0.8276
ZN	4	3.799	0.9282	0.8398
SI	3	4.235	0.9606	0.8631
SS	3	3.619	0.9394	0.8239
SN	4	3.083	0.8826	0.7543
TI	6	1.774	0.6702	0.5914
TS	3	3.249	0.8455	0.8315
TN	4	3.316	0.9285	0.7952

OTU means the numbers of dominant T-RFs based on *dsrB* gene sequences

3.4 Diversity of SRB based on the *dsrB* gene

Shannon-Wiener (*H*), Simpson (*D*), and Pielou (*E*) indices based on α-diversity were used to describe SRB diversities in different soil types of the WLFZ of TGR (Table 4). *H*, *D*, and *E* indices in the mainstream of Yangtze River (sites Z and S) were both highest in the sediment and lowest in the non-inundated soil. As for the tributary of Yangtze River, site T, the indices of *H* and *D* were both highest in the non-inundated soil and lowest in the sediment, while *E* was highest in the semi-inundated soil and lowest in the sediment.

Results of α-diversity indicated that the WLFZ in the mainstream and tributary of Yangtze River had different SRB diversities. The reason perhaps was that there were quite different environmental characteristics in the main stream and tributaries of Yangtze River. Sites Z and S are located in the main stream where water flows fast, local residents live in the bank, and the soil is occupied by agricultural crops. However, site T is located in the tributary where the water is relatively conservative, with no people living in the bank. The sampling place has no agricultural activities for years.

3.5 Phylogenetic analysis of SRB based on *dsrB*

In order to compare the difference of SRB community structures in the sediment and non-inundated soil, two phylogenetic trees of *dsrB* gene sequences from SI and SN were constructed by using the confirmed *dsrB* gene as reference species retrieved from NCBI (Fig. S1 and S2, Electronic Supplementary Material). *DsrB* gene sequences in SI fell into *Desulfosarcina*,

Desulfofaha, *Desulfonema*, *Desulfococcus*, *Desulfotignum*, *Desulfovibrio*, *Desulfomicrobium*, and *Desulfotomaculum* of δ-proteobacteria and some unclassified clusters (Fig. S1, Electronic Supplementary Material), while *dsrB* gene sequences in SN fell mainly into *Desulfomonile* and *Syntrophobacter* of δ-proteobacteria and some unclassified clusters, with much fewer species found in SN compared with SI (Fig. S2, Electronic Supplementary Material). The OTU coverage (*C*) of libraries constructed by SI and SN was 39.4 and 46.7% respectively. Sequences based on the *dsrB* gene found in SI and SN were mostly δ-proteobacteria, with only one exception that *Olavius algarvensis* symbiotic bacteria were only found in SI. The mercury methylation effect of this species was not known until now. Moreover, *Syntrophobacter* was found in SN but not in SI. The reasons for the difference of SRB genus distribution between SI and SN may be that SI is in the anaerobic condition throughout the year while SN is never inundated by water during the whole year. It is known that SRB, IRB, and methanogens in the phylum of δ-proteobacteria are the most important families for Hg methylation, some of which are confirmed as Hg-methylating bacteria such as the model strains *Desulfovibrio desulfuricans* ND132 and *Geobacter sulfurreducens* PCA. However, only SRB and *Syntrophobacter* were found in this research. That may reveal the limit of our sequencing results based only on the *dsrB* gene.

4 Conclusions

Soil MeHg levels correlated positively with the resident quantities of microorganisms with the *dsrB* gene. Both the *hgcA* and *dsrB* gene copies had an extremely significant difference ($p < 0.001$) between the sediment and non-inundated soil in site T. We can see from the phylogenetic analysis that SRB is probably related with Hg methylation distributed mainly in the sediment, rather than in the non-inundated soil. The relative

abundance of the dominant T-RFs of the *dsrB* gene indicated that SI had the most T-RFs, while ZI, ZS, and SS had the fewest T-RFs. The three sites differed greatly in the size of the fragments, indicating that different species were present at different locations. PCA results showed that ZN, SN, and TN were totally different from other two soil types, and THg, MeHg, NH_4^+, SO_4^{2-}, Fe^{2+}, and pH were all the main factors that correlated with the dominant T-RFs. Alpha-diversity results indicated that the WLFZ in the mainstream and tributary of Yangtze River had different SRB diversities. Phylogenetic results showed that *O. algarvensis* symbiotic bacteria and *Syntrophobacter* were the main difference between SI and SN.

In addition, there were still some limitations in this research. Only soils in April were researched, and results of the effect of water level on the distribution of Hg methylation microorganisms were still unknown, which might be the future plan of this research. The phylogenetic groups did not correspond with the T-RFs due to different primers used for T-RFLP and phylogenetic trees, which lead to the uninformative results of T-RFLP. SRB, IRB, methanogens, and some other microorganisms were confirmed to be involved in the Hg methylation process. Therefore, the gene quantities of *dsrB*, *hgcA*, and *McrA* were detected. However, *dsrB* as an indicator in the community analysis is not enough for reflecting Hg methylators other than SRB. As we have mentioned above, not all SRB are capable of methylating Hg. So, further research of community structure based on *hgcAB* genes (and may be *dsrAB* and *hgcAB* together) is needed to offset the limitations based only on the *dsrB* gene. To sum up, more rigorous research need to be conducted in the future. Nevertheless, this research can still give a preliminary picture of quantities and diversities of SRB in the WLFZ of TGR.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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