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Patterns of bacterial diversity along long-term mercury-contaminated gradients in the paddy soils

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Abstract

Mercury (Hg) pollution is usually regarded as an environmental stress in reducing microbial diversity and altering bacterial community structure. However, these results are based on relatively short-term studies, which might obscure the real response of microbial species to Hg contamination. Here, we analysed the bacterial abundance and community composition in paddy soils that have been potentially contaminated by Hg for more than 600 years. Expectedly, the soil Hg pollution significantly influenced the bacterial community structure. However, the bacterial abundance was significantly correlated with the soil organic matter content rather than the total Hg (THg) concentration. The bacterial alpha diversity increased at relatively low levels of THg and methylmercury (MeHg) and subsequently approached a plateau above 4.86 mg kg^{-1} THg or 18.62 ng g^{-1} MeHg, respectively. Contrasting with the general prediction of decreasing diversity along Hg stress, our results seem to be consistent with the intermediate disturbance hypotheses with the peak biological diversity under intermediate disturbance or stress. This result was partly supported by the inconsistent response of bacterial species to Hg stress. For instance, the relative abundance of *Nitrospirae* decreased, while that of *Gemmatimonadetes* increased significantly along the increasing soil THg and MeHg concentrations. In addition, the content of SO_4^{2-}, THg, MeHg and soil depth were the four main factors influencing bacterial community structures based on the Canonical Correspondence Analysis (CCA). Overall, our findings provide novel insight into the distribution patterns of bacterial community along long-term Hg-contaminated gradients in paddy soils.

Keywords: microbial diversity, long-term contamination, environmental disturbance,

methylmercury, paddy soil

Introduction

Mercury (Hg) is a global pollutant that can be transported over long distances [1]. Extensive research has been devoted to exploring the influence of Hg pollution on human health [2, 3]. Recently, the impact of Hg on soil microbial communities in environments also has received great concern [4-7] because soil microorganisms are responsible for many fundamental ecological processes such as C and N biogeochemical cycles [8]. It has also been suggested that Hg contamination could adversely impact soil respiration [9], enzyme activities [10， 11] and the potential nitrification rate [12]. These reductions in ecological function could be associated with shifts in the soil microbial community structure [13, 14]. For instance, Hg contamination altered the bacterial community structure and decreased microbial diversity in soils as expressed in terms of the number and relative abundance of Denaturing Gradient Gel Electrophoresis (DGGE) bands [5]. Nevertheless, effects of Hg on microbial communities were different between tropical and temperate soils [6]. Furthermore, Hg stress could also affect the community structure of some functional groups, such as ammonia-oxidising bacteria [12] and sulphate-reducing bacteria [15], which play key roles in N transformation and sulphate reduction, respectively. However, these studies on the influence of Hg contamination on bacterial communities are mainly conducted over relatively short time periods or on upland soils, and little is known of the effects of long-term Hg contamination on bacterial communities in paddy habitats.

In addition, bacteria also exert crucial roles in Hg transformation, including Hg reduction and methylation. For example, inorganic Hg^{2+} in environments can be reduced to volatile Hg^{0}

by Hg-resistant bacteria [16]. The resistance of bacteria to inorganic Hg^{2+} is associated with the presence of the Hg resistance gene (*merA*) [16， 17], which has been considered widely distributed in the phyla of *Firmicutes*, *Actinobacteria* and *Proteobacteria* [16]. Meanwhile, ionic Hg in anaerobic habitats may also be potentially methylated to highly toxic methylmercury (MeHg) by Hg-methylating microbes [18，19], such as sulphate-reducing bacteria (SRB) [20, 21]， iron-reducing bacteria (FeRB) [22, 23] and methanogens [24, 25]. However, little is known regarding the responses of these Hg methylating bacteria to long-term soil Hg contamination. Therefore, it is likely that understanding the bacterial community structure can help in evaluating both the capacity of ecosystem to resist Hg disturbance and the potential for Hg methylation [26].

Here, we focus on bacterial communities in the paddy soils of the Wanshan district, Guizhou province of China, which have been contaminated by Hg for more than 600 years. The Wanshan district is an important rice-producing region in southwest China where soil Hg pollution is an important stress factor resulting from historic Hg mining. The Wanshan Hg mine is the biggest Hg mine in Asia and has caused serious Hg contamination of the local environment and adjacent ecosystems. Although many studies have been conducted across the Hg mining area on the health risks of Hg exposure [27-29], limited knowledge is available on the effects of long-term Hg contamination on bacterial community composition in the paddy soils. The paddy soil is a typical anaerobic habitat, where microorganisms could play key roles in both Hg reduction and methylation [15, 30]. Knowledge regarding the bacterial community along Hg-contaminated gradients is crucial to predict the responses of microbial diversity and function to global Hg transportation and precipitation.

We covered a THg gradient from 1.11 to 29.07 mg kg^{-1} in the paddy soils around the Hg mining area [16]. Bar-coding pyrosequencing targeting 16S rRNA genes was used to explore the diversity and structure of the bacterial communities in the soils. We correlated the THg, MeHg, pH, organic matter (OM), SO_4^{2-}, NH_4^+ and depth to the bacterial community, revealing the potential underlying factors influencing the patterns of diversity. In addition, the richness of species was correlated to the concentrations of THg and MeHg in all of the examined soils because the species composition may strongly affect the ecosystem functions [31].

Materials and Methods

Study area and sample collection

The soil samples examined here were collected from rice fields in the Wanshan Hg mining area in September 2010. The Wanshan district is located in the eastern part of Guizhou province, southwest China (Fig. 1), which is a typical hilly and karstic terrain with a subtropical monsoon humid climate, an average annual rainfall of 1200 to 1400 mm and a perennial mean temperature of 17°C. The Hg mining site is the largest Hg deposit in China, with mining activities first initiated in the Qin Dynasty (circa 221 B.C.). Large-scale mining however, officially ceased in 2001. A long-term historic discharge of waste and atmospheric precipitation from the Hg mine has resulted in serious Hg contamination of the rice fields. We selected four sites (referred to as S1, S2, S3 and S4) downstream of the Hg mine. S1, S2 and S3 are located in the Wanshan district, while S4 is located in the Tongren district without Hg contamination and is used as a control site in the current study, as it is in close proximity to the Wanshan district. We sampled soils from rice fields at different depths (0-20 cm, 20-40 cm, 40-60 cm and 60-80 cm) with three replicates across the four sites. Overall, 48 soil samples

were collected and passed through a 2.0 mm sieve. One sub-sample was stored at -20 °C for THg, MeHg and molecular analyses, while another sub-sample was air-dried for general chemical analyses. Some basic soil chemical characteristics from the four sites are listed in Table S1 [15].

Soil chemical analysis

Soil pH was determined using a soil to distilled water ratio of 1:2.5, and soil organic matter (OM) was determined using the K_2CrO_7 oxidation titration method. Ammonium in was extracted with 2 M KCl solution and determined using a Continuous Flow Analyser (SAN++, Skalar, Holand). Soil SO_4^{2-} was extracted with deionized water in a ratio 1:5 and measured via ion chromatography using an AS14 column and an ECD50 conductivity detector (Dionex, USA). For THg analysis, soil samples were digested with HNO_3+HCl (10 ml, 1:1 v/v) in a teflon tube at 100°C for 2 hrs and the Hg concentration in the solution was measured using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). MeHg in the soil samples was extracted using $CuSO_4$-methanol/solvent extraction, and the MeHg content was determined using HPLC-ICP-MS [15, 32].

Soil DNA extraction and pyrosequencing of the bacterial 16S rRNA gene

Total genomic DNA was isolated from 0.5 g soil samples using the UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol. The concentration and quality of the extracted DNA were assessed photometrically using a Nanodrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Bacterial 16S rRNA genes were amplified using the 27F primer with the 454 Life

Sciences 'A' sequencing adapter and the modified 519R primer with an 8 bp barcode sequence and the 454 Life Sciences 'B' sequencing adapter [33]. The 25 µl amplification mix contained 2 µl 5-fold diluted template DNA (1-10 ng), 0.5 ml each primer (10 mM) and 12.5 µl SYBR-Premix ExTaq (TaKaRa Biotechnology, Dalian, China). The cycling conditions included initial denaturation at 94°C for 2 min and 30 cycles of 45 s denaturation at 94°C, 45s annealing at 55 °C, and 1 min extension at 72°C, followed by an elongation of 7 min at 72°C. The resulting amplicons obtained from the triplicate independent PCR assays for each sample were pooled and purified using the Wizard SV Gel and PCR Clean-Up Kit (Promega, San Luis Obispo, CA, USA). The purified PCR products from different samples were combined in approximately equimolar proportions into a single aliquot at a final concentration of 100 ng μl^{-1}, and then pyrosequenced from the fusion adapter A using the Roche 454 GSFLX Titanium platform (Roche Diagnostics, Branford, CT, USA) at the Chinese National Human Genome Centre, Shanghai, China.

Quantification of 16S rRNA gene

Abundance bacterial 16S rRNA gene were quantified using TaqMan assays using the primers BACT1369FB (5'-CGGTGAATACGTTCYCGG-3') and PROK1492R (5'-GGWTACCTTGTTACGACTT-3') and the probe TM1389F (5'-CTTGTACACACCGCCCGTC-3') [34]. 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). Amplification conditions were as follows: 94 °C for 1 min, 40 cycles of 20 s at 94 °C, 30 s at 59 °C, and 30 s at 72 °C, followed by plate reads at 83 °C. The quantitative PCR (qPCR) analysis was conducted on an iCycler iQ5 thermocycler (Bio-Rad Laboratories, Hercules,CA,

USA). A melting curve analysis was performed at the end of the qPCR runs to determine the specificity of the amplification reactions. To prepare the standard curves, 16S rRNA 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-V is 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.

Sequencing data analysis

Sequences generated from the pyrosequencing of bacterial 16S rRNA gene amplicons were processed using the QIIME pipeline (v1.2) [35]. Operational taxonomic units (OTUs) were defined using a 97% sequence similarity cut-off. We accounted for differences in the sampling effort among samples by randomly subsampling 1000 sequences per sample 1000 times. In brief, sequences that were longer than 200 bp were denoised with the Denoiser algorithm [36] and clustered into OTUs at a 97% pairwise identity with the seed-based uclust algorithm [37]. After the chimeras were removed via Chimera Slayer [38], representative sequences from each OTU were aligned to the Green genes imputed core reference alignment [39] using PyNAST. The taxonomic identity of each representative sequence was determined using the RDP Classifier.

Statistical analyses

The observed richness and Fait Phylogenetic Diversity (Faith's PD) were used to estimate the

bacterial α-diversity by calculating the OTU richness at a 97% sequence similarity level. A nonlinear regression analysis was performed to test the changes in the α-diversity indices with the THg and MeHg levels. The relative abundance of bacterial taxa was calculated by comparing the number of sequences classified as bacterial taxa versus the number of bacterial 16S rRNA gene sequences per sample. We used linear regression analyses to test the changes of the relative abundance of the taxa along the soil Hg gradient. A Canonical Correspondence Analysis (CCA) (Canoco 4.5 for Windows) was used to explore the relationship between the various detected species and soil variables, including the soil THg, MeHg, pH, OM, SO_4^{2-}, NH_4^+ and depth. The variables that were included in the model were chosen by forward selection at the 0.05 baseline. The significance of the constrained ordination process was tested using a Monte Carlo permutation test. Bivariate correlations were conducted to link different parameters. P<0.0 5 was considered the significant level. All of these analyses were performed using the R statistical software (http://www.r-project.org).

Results

Bacterial community structure at the four sites

The observed richness and Faith's PD were correlated to the increased Hg (e.g.THg and MeHg) concentrations at the OTU (97% sequence similarity) level (Fig. 2). At the low Hg levels, the α-diversity increased with the elevated THg and MeHg concentrations and reached a plateau above 4.86 mg kg^{-1} THg or 18.62 ng g^{-1} MeHg, respectively. However, the OM and SO_4^{2-} contents also correlated with all of the examined observed richness and Faith's PD (data not shown). We included the main soil variables in the CCA model to explain the impacts of soil variables and depth on bacterial community structure, in which SO_4^{2-}, THg, MeHg and

depth were significant (P<0.01) while pH, OM and NH_4^+ were not significant (P>0.05) (Fig. 3). Among the explanatory variables, SO_4^{2-} was the most important factor for species composition. Soil THg was the second most important impact variable, followed by MeHg and soil depth. The samples were mainly grouped according to the sites, indicating that the bacterial community structures shifted among the different sites along the Hg gradients.

Bacterial abundance

The bacterial abundance in the examined paddy soils was assessed by the log-transformed copy number of the 16S rRNA gene g^{-1} dry soil (Fig. 4a). The average bacterial abundance ranged from 4.46×10^7 to 3.96×10^9 copies g^{-1} dry soil in all of the examined samples. The bacterial abundance at the 0-40 cm depth was higher than that at the 40-80 cm depth at the four sites. There was no significant correlation between the bacterial abundance and the THg level. However, significant positive correlations were observed between the copy number of the 16S rRNA gene and the OM (Fig. 4b) and MeHg contents (Fig S1).

Distribution of bacterial phylotypes

In total, more than 12 microbial phyla were detected from the paddy soils across the four sites. *Proteobacteria* was most abundant phylum in all of the examined sites, followed by *Chloroflexi*, *Actinobacteria* and *Acidobacteria*. These phyla were dominant (>5% relative abundance) in the soils, accounting for greater than 78.5% of the bacterial sequences. In addition, *Nitrospirae*, *Bacteroidetes*, *Firmicutes*, *Gemmatimonadetes*, *Planctomycetes*, and *Verrucomicrobia* were present in most soils at a low abundance, and their distribution along the soil profile at the sites is shown in Fig. 5. Interestingly, the relative abundance of *Gemmatimonadetes* tended to increase, while that of *Nitrospirae* declined with the elevated

THg and MeHg concentrations (Fig. 6). Moreover, Increased THg and MeHg concentrations both significantly reduced the relative abundance of *Deltaproteobacteria* and *Gammaproteobacteria* at the class level, which accounted for a maximum of 20% and 8% relative richness, respectively (Fig. S2). At the order level, *Anaerolineales*, *envOPS12*, *Hydrogenophilales* and *SBR1031* were detected at a greater than 1% abundance, which increased with the ascending THg and MeHg gradients (Fig. S3, Fig. S4).

Discussion

Effects of Hg contamination on the diversity, abundance and structure of bacterial community

Understanding the responses of bacterial diversity to environmental disturbance has long been of major interest to microbial ecologists. Here, we considered Hg contamination a disturbing factor to investigate its influence on bacterial diversity in the paddy soils around an Hg mining area in China. We found that the THg and MeHg had a similar impact on the bacterial α-diversity (e.g. observed richness and Faith's PD). Interestingly, the indices of diversity tended to increase at the relatively low Hg (e.g. THg and MeHg) levels and then approach a plateau despite the fact that the soil THg and MeHg concentrations continued to increase. This pattern supports, at least partially, the classic intermediate disturbance hypothesis that originated from macro-ecology, suggesting that intermediate disturbance could result in high biodiversity [40]. The relatively high diversity of bacterial communities might maintain stability of soil function because stressed systems are more stable than those which are not stressed, as the latter have acquired physiological adaptations that enable these systems to maintain function in the face of additional stresses [41, 42]. Here, the paddy soils were

stressed by Hg contamination, and the threshold for the bacterial diversity plateau was above 4.68 mg kg^{-1} of THg and 18.62 ng g^{-1} for MeHg, respectively. Namely, the bacterial community might be more stable above the observed Hg level in the examined soils. These findings could also be crucial in evaluating the chronic effects of Hg pollution on soil microbial community *in situ*.

In the present study, no significant correlation between the abundance of 16S rRNA gene and the THg contents in the soils may suggest that long-term Hg contamination did not impact total bacterial abundance. Our result agreed to, at least in part, the previous studies suggesting that elevated soil THg contents did not result in any significant changes in the abundance of bacterial ammonia oxidizers [12] or sulphate-reducing microorganism [15]. This was could be associated with soil Hg bioavailability in the examined soil, which may better explain the effect of Hg disturbance on soil bacterial abundance compared to the THg. In addition, long-term Hg contamination may reduce the relative abundance of Hg-vulnerable species, but the abundance of potential Hg-resistant species might increase to maintain ecological stability [43]. It is not surprising to observe a significant positive correlation between the OM contents and the bacterial abundance (Fig. 4b), which supports previous observations indicating the contribution of OM to bacterial growth [44, 45]. On the other hand, the different OM values among the four sites themselves might overshadow the impact of THg on microbial communities. It is highly possible since OM might not only furnish more substrate for microbes but also inhibit the toxicity of Hg especially MeHg by complexation [46]. Therefore, the significant positive correlation between MeHg and bacterial abundance (Fig S1) may not reflect any ecological significance, and this correlation just resulted from its

association with OM as explained above. However, we found the shifts in the bacterial community structure of paddy soils along Hg gradient. Many studies have suggested that Hg stress strongly affects the soil microbial function and community [7, 12]; however, to date, there are still only few studies regarding the effects of long-term Hg contamination on the bacterial community in paddy soils using next generation sequencing techniques. The results of CCA analysis demonstrate that the bacterial community structure varied across the four sites, implying the important roles of Hg pollution in influencing bacterial community structure. This finding is in line with a recent laboratory study demonstrating that THg and MeHg contamination distinctly changed the soil bacterial community structure [8, 47]. However, other variables such as SO_4^{2-} also played important role in shaping bacterial community structure. This observation is not surprising because a previous study had suggested that SO_4^{2-} strongly influenced the bacterial community of pristine paddy soils [48], although the influencing mechanism still remains unclear. In addition, depth is an important factor influencing the soil bacterial community [49], and this could be attributed to the various soil parameters along the soil depth.

Effects of Hg contamination on the distribution of bacterial phylotypes

Understanding phylogenetic relatedness may provide further insight into the mechanisms of microbial responses to environmental change, such as environmental filtering and competition, that shape local communities [50]. In this study, we found that *Proteobacteria* was the most abundant phyla in the paddy soils at the four sites, which is in accordance with most of the previous studies of paddy soils [51, 52]. We identified some groups that were sensitive to both the THg and MeHg at the levels of phylum, class and order, suggesting that these bacteria

might be potential bioindicators for monitoring Hg contamination *in situ*. For instance, a decrease in *Nitrospirae* richness was observed along the THg and MeHg gradients, implying that this phylum might be vulnerable to Hg stress. This result is in accordance with our earlier study that demonstrated that *Nitrospirae* was sensitive to Hg stress during short-term laboratory exposure [12]. Similarly, *Nitrospirae* was also found to be sensitive to arsenic contamination in soils [53]. Our findings could also be helpful in understanding the impacts of Hg on microbial nitrification (e.g., by *Nitrospirae*) in the soils. However, *Gemmatimonadetes* was stimulated by the long-term Hg stress, and a similar pattern was also observed along copper gradients under long-term field exposure (unpublished data). One possible reason for this result could be that metal stress severely affects sensitive species [54] and decreases their competition ability, resulting in an increase in the relative abundance of metal-resistant species to maintain ecological stability [46]. Similar patterns were found in the order of *Anaerolineales*, *envOPS12*, *SBR1031*, and *Hydrogenophilales*, whose abundances increased with elevated THg and MeHg concentrations. A previous study suggested that the Hg resistance gene (*merA*), responsible for tolerance to inorganic Hg^{2+}, was mainly distributed in *Proteobacteria*, *Actinobacteria*, and *Firmicutes* [16], as well as in archeal groups [55]. Nevertheless, our results might imply that the *merA* gene is more widely distributed in the other microbial groups, because less impact of Hg on these Hg-resistant-like bacteria (e.g. *Gemmatimonadetes*) was observed. Therefore, it is very necessary to investigate the diversity of *merA*-containing microbes in these paddy soils in the future. Overall, we found that MeHg had a similar impact pattern as THg did on relative richness of all the target groups in those examined samples. These findings further highlight the importance of both

THg and MeHg in influencing distribution of bacterial phylotypes in the paddy soils.

In conclusion, this study provides novel insight into the impact of historic Hg contamination on bacterial communities in paddy soils. Given Hg stress as a disturbing factor, the relationship between bacterial α-diversity and the Hg pollution levels supports the intermediate disturbance hypothesis with the peak biological diversity under intermediate disturbance. Here, we also emphasise the relationship between the THg and MeHg and the distribution of bacterial taxa. Hg-sensitive groups were identified and may be used as bioindicators for Hg monitoring *in situ*, while Hg-resistant microbes could be helpful in exploring Hg biotransformation in paddy habitats. Our findings are critical for predicting the response of the bacterial community to global Hg transportation or change. Hopefully, large-scale studies covering Hg contaminated soils from different properties of climate and geography will emerge.

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Figure legends

Figure 1 Map of the study area and sampling locations in the Wanshan Hg mining area (S1, S2, S3 and S4 represent Site 1, Site 2, Site 3, and Site 4, respectively).

Figure 2 Alpha-diversity of the bacterial communities in the paddy soils. (a) Observed richness along total Hg (THg) gradient; (b) Observed richness along methylmercury (MeHg) gradient; (c) Fait Phylogenetic Diversity (Faith's PD) along THg gradient; (d) Faith's PD along MeHg gradient.

Figure 3 Canonical Correspondence Analysis (CCA) based on bacterial community structure and environmental parameters of the THg, MeHg, SO_4^{2-} and depth in the soils. The meanings of the symbols are as follows: solid circle represents S1, empty circle represents S2, solid right triangle represents S3, and empty right triangle represents S4.

Figure 4 (a) Bacterial abundance based on the 16S rRNA gene sequences copy numbers in the soils; (b) Regressions between the bacterial abundance and soil organic matter.

Figure 5 Relative abundances of the dominant bacterial phyla in all the four sites along the soil profiles. A, B, C and D represents depth 0-20 cm, 20-40 cm, 40-60 cm and 60-80 cm, respectively. Relative abundances are based on the proportional frequencies of those DNA sequences that could be classified at the phylum level.

Figure 6 The relative abundance of bacterial phyla along the THg and MeHg gradients.

Figure 1

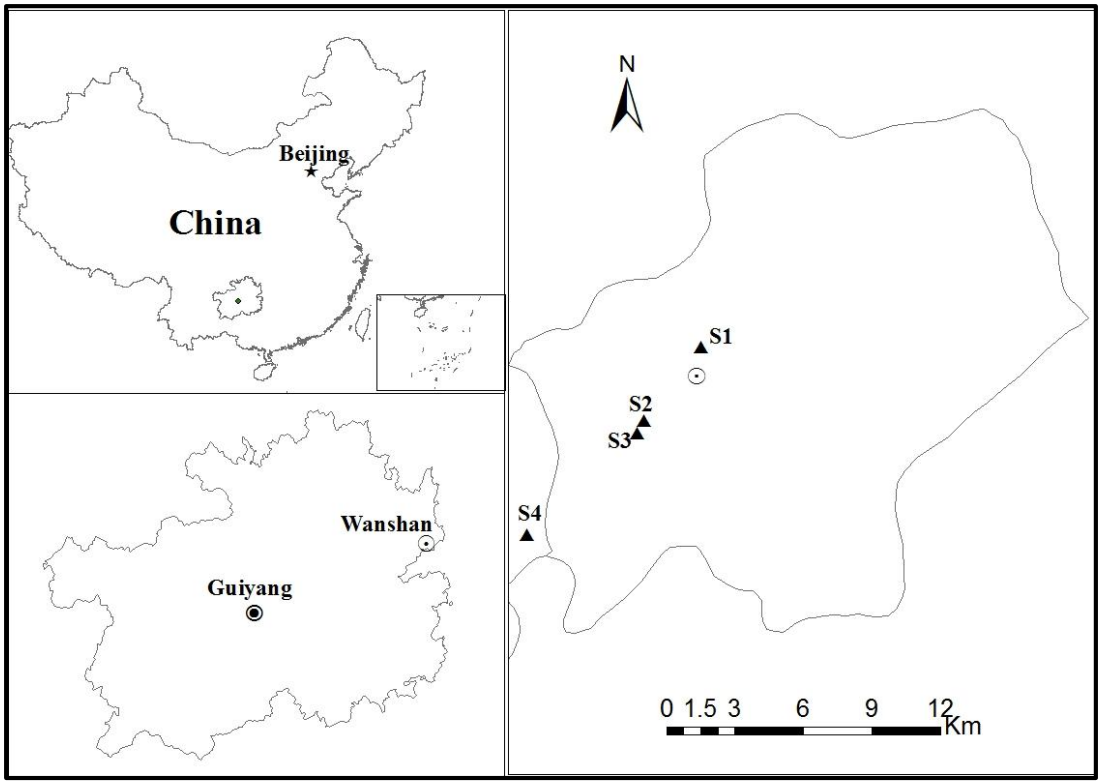


Figure 2

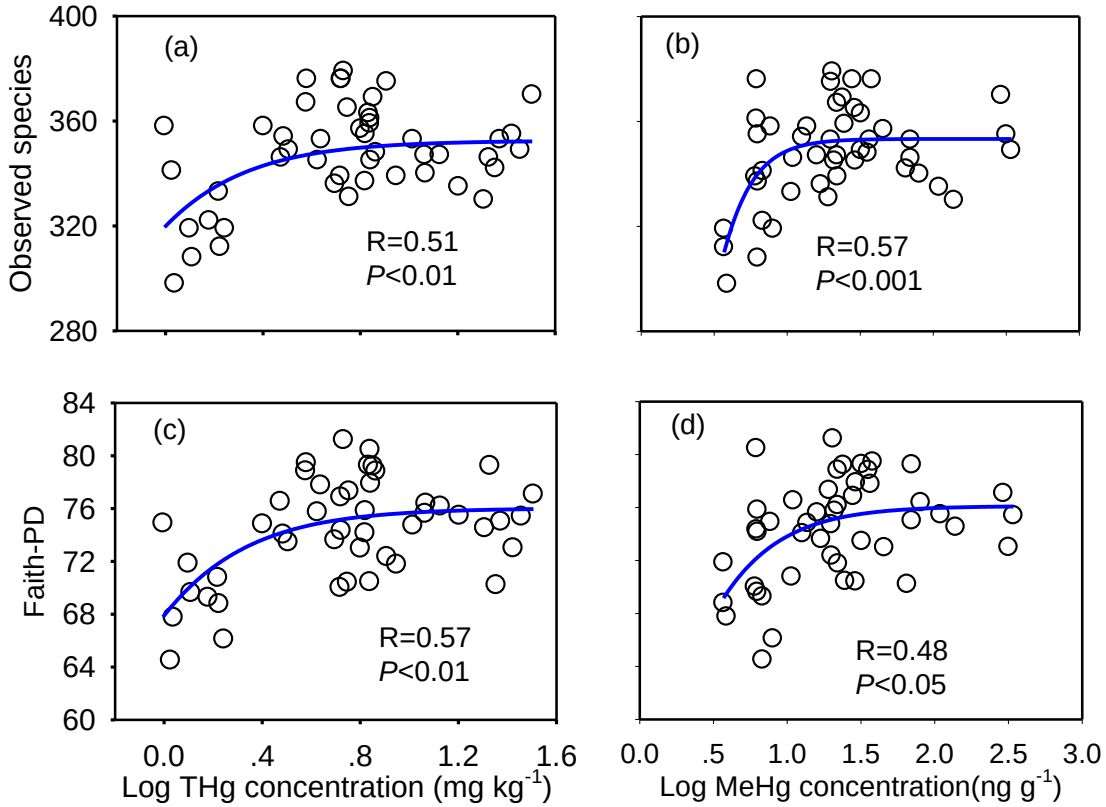

(a)
(b)
(c)
(d)
Observed species
Faith-PD
400
360
320
280
84
80
76
72
68
64
60
R=0.51
P<0.01
R=0.57
P<0.001
R=0.57
P<0.01
R=0.48
P<0.05
0.0
.4
.8
1.2
1.6
Log THg concentration (mg kg-1)
0.0
.5
1.0
1.5
2.0
2.5
3.0
Log MeHg concentration(ng g-1)

Figure 3

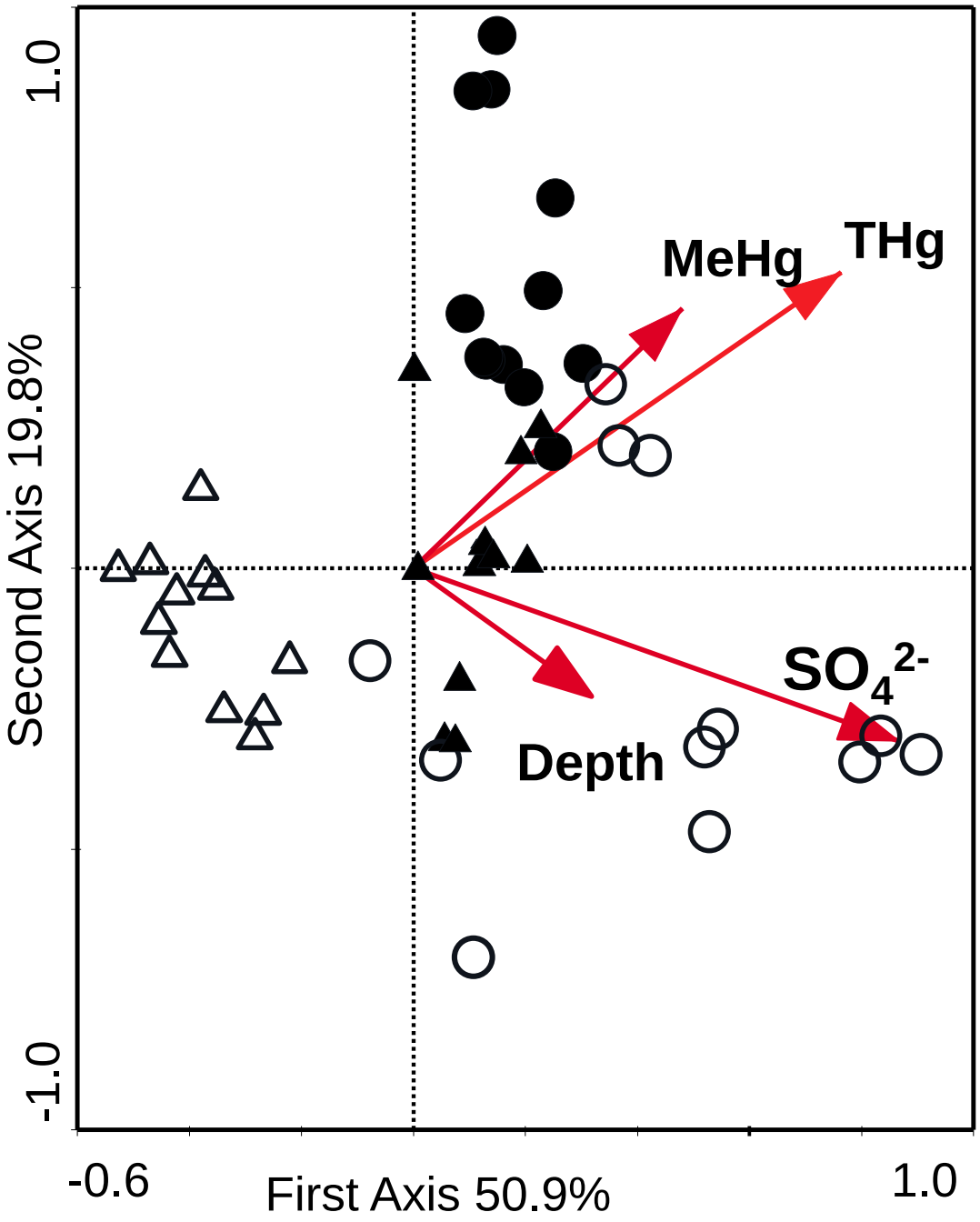


Figure 4

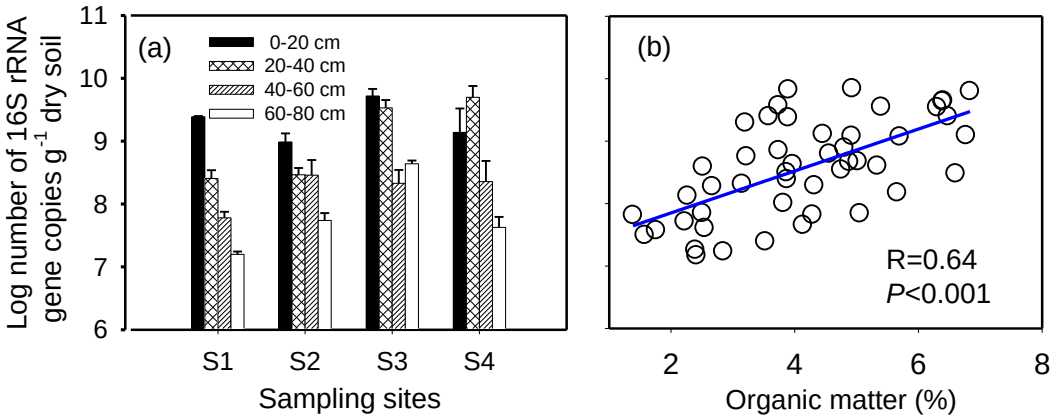

(a)
0-20 cm
20-40 cm
40-60 cm
60-80 cm
Log number of 16S rRNA gene copies g-1 dry soil
11
10
9
8
7
6
S1
S2
S3
S4
Sampling sites
(b)
R=0.64
P<0.001
2
4
6
8
Organic matter (%)

Figure 5

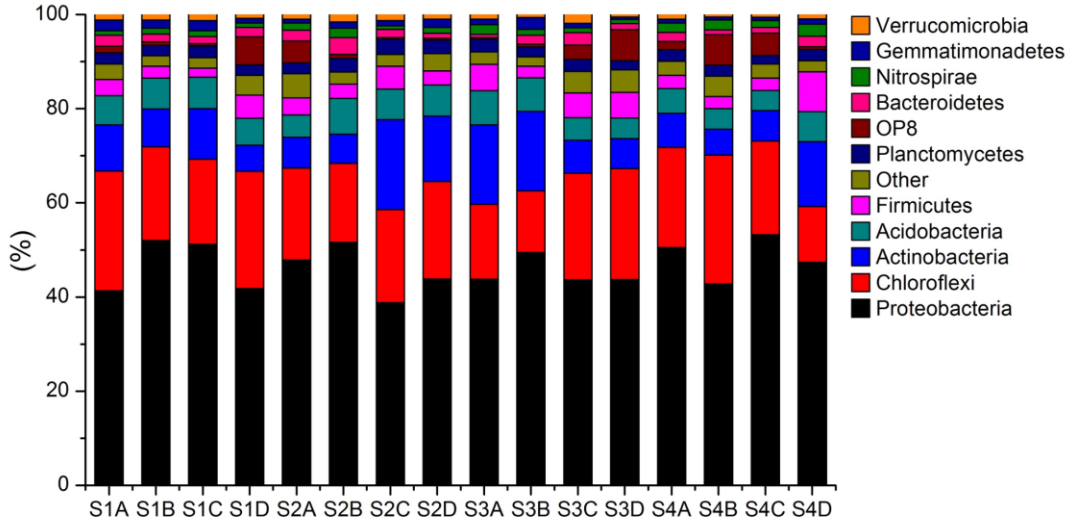
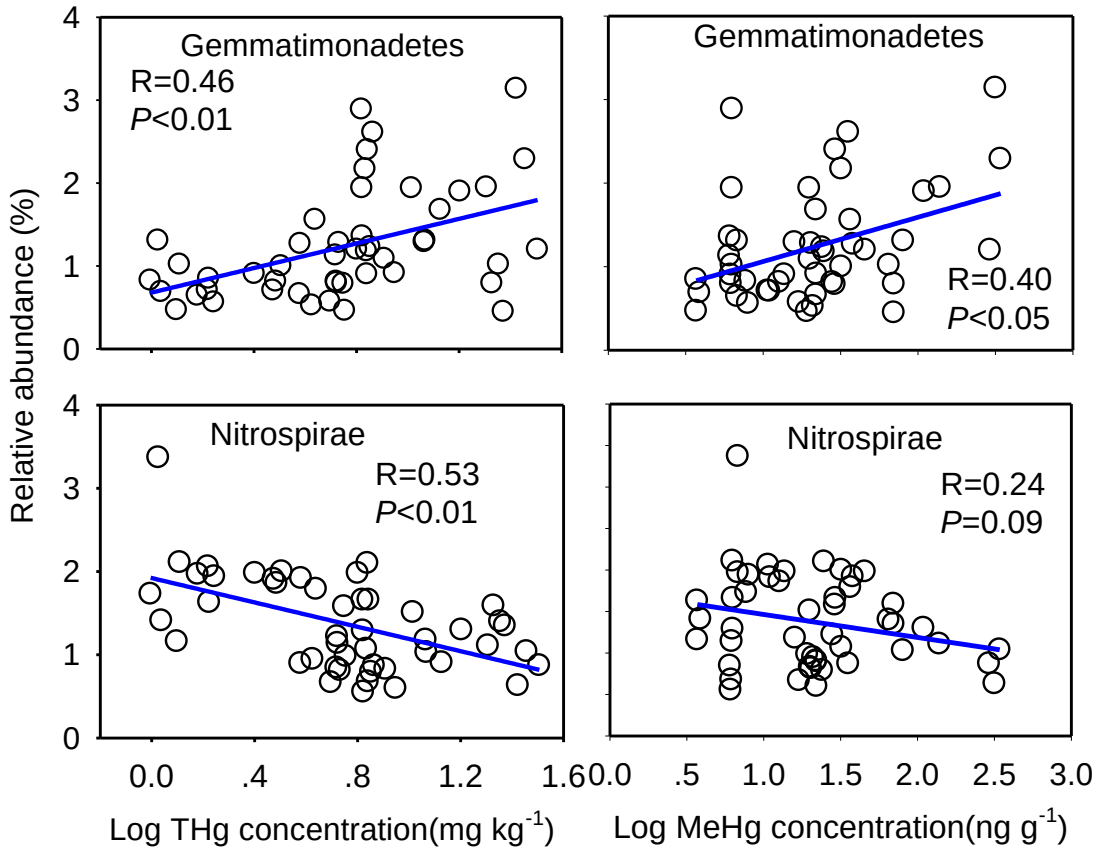

100
80
60
40
20
0
(%)
S1A S1B S1C S1D S2A S2B S2C S2D S3A S3B S3C S3D S4A S4B S4C S4D
Verrucomicrobia
Gemmatimonadetes
Nitrospirae
Bacteroidetes
OP8
Planctomycetes
Other
Firmicutes
Acidobacteria
Actinobacteria
Chloroflexi
Proteobacteria

Figure 6


Gemmatimonadetes
R=0.46
P<0.01
Gemmatimonadetes
R=0.40
P<0.05
Nitrospirae
R=0.53
P<0.01
Nitrospirae
R=0.24
P=0.09
Relative abundance (%)
Log THg concentration(mg kg-1)
Log MeHg concentration(ng g-1)