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Phylogenetic analysis of bacterial community composition in sediment contaminated with multiple heavy metals from the Xiangjiang River in China

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ABSTRACT

Understanding the ecology of sediments that are contaminated with heavy metals is critical for bioremediating these sediments, which has become a public concern over the course of the development of modern industry. To investigate the bacterial community composition of sediments that are contaminated with heavy metals in the Xiangjiang River, a total of four sediment samples contaminated with multiple heavy metals were obtained, and a culture-independent molecular analysis, polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP), was performed. The results revealed that heavy metal pollution affected the sediment microbial community diversity, and the greatest species diversity appeared in the moderately polluted sediment X sample. The dominant family in these sediments includes α-*Proteobacteria*, β-*Proteobacteria* and *Firmicutes*. Moreover, α-*Proteobacteria* was significantly increased with increases in heavy metal. A redundancy analysis (RDA) also confirmed this phenomenon.
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1. Introduction

Heavy metal contamination is a global environmental problem that has been caused by the development of metal processing, tanneries and electroplating industries (Jiang et al., 2010). The accumulation of multiple heavy metals in the environment may significantly influence public health and damage ecosystems through different pathways (Guo et al., 2009; Sun et al., 2010; Yoon et al., 2006). Microorganisms, as an essential component of ecosystems, are involved in organic material degradation, the release of nutrients, biogeochemical cycling and the maintenance of soil structure (Brandt et al., 2010; Goberna et al., 2005; Tian et al., 2008). However, the environmental microbial community composition, structure and diversity were unclear until molecular approaches were invented and applied to characterizing microbial species and assemblages. These techniques significantly increased our understanding of environmental microbial communities and the relationships between plants, animals and other factors (Green et al., 2008).

Previous studies have reported that microorganisms are far more sensitive to heavy metal stress than plants growing in the same area, and they have different sensitivities to metal toxicity (Giller et al., 1998, 2009; Sun et al., 2012). Heavy metals may change microbial biomass, activities and structure (Epelde et al., 2010; Giller et al., 1998; Jiang et al., 2010; Stephen et al., 1999; Wang et al., 2010; Yang et al., 2006). Bacterial communities can be sensitive indicators for contaminant stress, particularly metal contamination (Sun et al., 2012). However, little attention has been devoted to the diversity and structure of indigenous microbial populations within the contaminated sediments of the Xiangjiang River. The Xiangjiang watershed is the most developed and urbanized region in the province (Chai et al., 2010). In the basin, mining and the metallurgical industry are well-developed, and the Xiangjiang River is being subjected to serious pollution due to emissions of chromium, cadmium, lead, zinc and mercury in the Hunan Province (Chai et al., 2010; Guo et al., 2010). The pollution threatens human health and the balance of aquatic ecosystems, economic development and social prosperity.

This study describes the bacterial diversity in the Xiangjiang River's contaminated sediments by sequencing 16S rDNA genes in clone libraries. We focused on 16S rDNA because of its universal distribution among all prokaryotes, the presence of diverse species-specific domains, and its reliability in inferring phylogenetic relationships. (Pace, 1997). Four sediment samples that were contaminated with different degrees of multiple heavy metals were studied, and the main objectives of this study were threefold: (1) document the predominant bacterial community structure and diversity differences in the contaminated sediment from the Xiangjiang River in Zhuzhou City using PCR–RFLP analysis from the total bacterial DNA extracted from the sediment; (2) provide

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a valuable basis for further investigation on heavy metal-resistant and metal-sensitive bacteria to assess the usefulness of microbial indicators for evaluating the health and function of sediment, and (3) potentially assist in situ bioremediation of metal contaminated sediment and optimize the process of bioleaching to support river restoration decisions with the knowledge of accurate natural populations.

2. Materials and methods

2.1. Site description and sampling

The Xiangjiang River, one of the tributaries of the Yangtze River in Hunan province (where there is a typically hot, humid, tropical climate with an annual mean precipitation of 1400 mm), originates on Guangxi Haiyang Mountain. It drains an area of approximately 94,600 km^2 and has a total length of approximately 856 km. Contaminated sediments for the present study were collected from Zhuzhou, an industrial town in Hunan Province, in September, 2010. Large numbers of non-ferrous metal and chemical enterprises have located in this area over a period of approximately 50 years, contaminating the Xiangjiang River with their pollutants. Four different sediments (0–10 cm depth) were sampled for this study (Fig. 1): H and X samples were taken at the Xiawan Port (the most polluted tributary of the Xiangjiang River); the F sample was collected at the confluence of the Xiangjiang River and Xiawan Port; the S sample (a control site) was collected 6 km upriver of F site in the Xiangjiang River. The sample at each site was homogenized and divided into two parts. One part was temporarily stored at 4 °C for microbial community analysis, and the other was air-dried and then processed with a 2 mm sieve to remove root fragments and large particles.

2.2. Physicochemical and microbiological parameters

2.2.1. Physicochemical parameters

The selected physicochemical properties of the sediments were analyzed using standard methods. The pH of the sediments was measured using a 1:2.5 (sediment:distilled water) sediment slurry. The total organic carbon (% TOC) content was determined by loss-on-ignition, combusting at 450 °C for 4 h in a Muffle Furnace and at 105 °C for several hours. The total concentrations of metals in the sediment were analyzed with an Inductively Coupled Plasma Mass Spectrometer (ICP-MS, Agilent 7500 series, USA). The extractable fraction of metals was obtained using 1 M $MgCl_2$ (pH = 7.0) (Akcay et al., 2003) and then analyzed with ICP-MS.

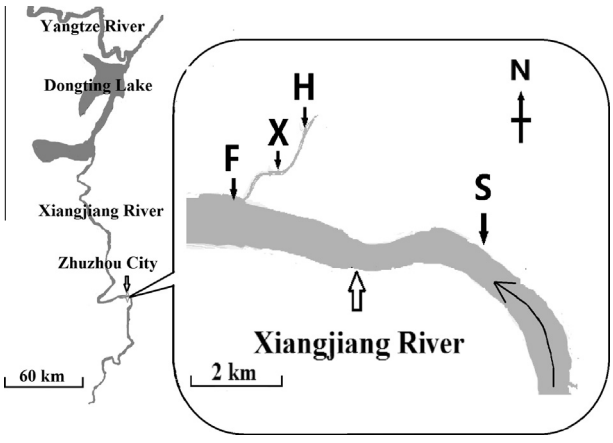

Fig. 1. Map showing the location of the sample sites in the Xiangjiang River.

2.2.2. DNA extraction and 16S rDNA gene amplification

Bacterial DNA was extracted from the sediments using a protocol described by Zhou et al. (1996). 16S rDNA was then amplified by PCR with a thermocycler using an initial denaturing step of 4 min at 95 °C followed by 32 cycles of 1 min at 94 °C, 1 min of annealing at 55 °C, a 90 s extension at 72 °C, and a final polymerization step of 72 °C for 10 min. Each reaction mixture consisted of 5 μl of 10 × reaction Buffer (Mg^{2+} plus), 1 μl of dNTP (10 mmol l^{-1}), 1 μl of each primer: forward primer (27f, 5′-AGAGTTTGATCCTGGC TCAG-3′, 5 μmol l^{-1}) and reverse primer (1492r, 5′- GGTTACCT TGTTACGACTT-3′, 5 μmol l^{-1}) (Konstantinidis et al., 2003), 0.25 μl of Taq polymerase (Fermentas, MBI) and 2 μl of DNA extraction products. Double-distilled water was added until its final volume reached 50 μl. The resulting PCR products were run on a 1.0% low-melting-point agarose gel in tris–acetate-buffer and analyzed by staining with ethidium bromide (EB) under UV light. The bands of the expected size (approximately 1500 bp) were cut off and purified with a E.Z.N.A.™ Gel Extraction Kit (OMEGA, USA).

2.2.3. Cloning and RFLP analysis

The purified PCR products were ligated into pGM-T plasmid vectors (TIANGEN) and then transformed into *Escherichia coli* TOP10 cells. Ampicillin-resistant transformants were selected on blue–white screening and grown overnight in the plates with ampicillin (80 mg ml^{-1}), IPTG (50 mg ml^{-1}) and X-gal (15 mg ml^{-1}). In total, 180 white colonies were randomly selected. Then, white colonies were randomly selected and re-amplified by PCR with vector primers M13 forward (−20) (5′-GTA AAAC-GACGGCCA G-3′) and M13 reverse (5′-CAGGAAACAGCTATGAC-3′). Next, 180 of the positive, reamplified products from each library were digested by the restriction endonuclease *Hin*61 and *Msp*I (Fermentas, MBI) at 37 °C overnight. The system for the reaction of *Msp*I and *Hin*61 digestion consists of 1 μl buffer, 0.1 μl of each enzyme, and 6 μl of purified clone PCR products. Double-distilled water was added until its final volume reached 10 μl. The digestion products were separated by gel electrophoresis (3 h, 80 V) in 3.0% low-melting-point agarose in tris–acetate-buffer and analyzed by staining with EB under UV illumination. The banding patterns were grouped into an operational taxonomic unit (OTU) based on the DNA banding pattern of individual clones. Each banding pattern found on the agarose gel constitutes one distinct OTU based on how the restriction enzyme cuts the PCR product. The representative OTUs were submitted for sequence analysis.

2.2.4. Sequencing and phylogenetic analysis

In total, 105 unique clones were sequenced by the Beijing Genomics Institute (BGI), and 96 sequences were initially estimated using the BLASTN facility at the National Center for Biotechnology Information (NCBI Taxonomy ID: 48184). The closest 16S rDNA gene sequences were aligned with CLUSTALX 1.83. A phylogenetic tree was constructed by the neighbor joining method, using MEGA3.1, and the robustness of the phylogeny was tested with a bootstrap analysis with 1000 iterations.

2.2.5. Nucleotide sequence accession numbers

All of the sequences described in this study have been submitted to GenBank under accession numbers HQ132379–HQ132474.

2.3. Statistical analysis

We used the geochemical characteristics of the samples presented in Table 1, except for pH and TOC, as environmental data for ordination by principal component analysis (PCA) to examine the correlations between sample and site variables (Canoco, version 4.5, biometric – Plant Research International, the Netherlands, for Windows XP). The geochemical variables were *z* transformed to

Table 1
Physico-chemical characteristics analysis[a] of the four sites.

Sample	Physiochemical variable									
	Cd	Cu	Zn	Pb	Cr	Hg	As	Mn	pH	TOC (%)
X	75	238	2273	778	115	56	108	52	7.66	8.62
H	90	239	2034	569	115	79	114	45	7.21	11.95
S	20	70	300	100	60	0	115	84	7.25	2.66
F	160	290	6200	500	70	15	123	78	8.80	3.50

[a] Total organic carbon (TOC) and metal concentrations are given in milligrams per kilogram.

Table 2
Indices to describe the genetic diversity of the microbial community.

Shannon–Weaver index	Simpson-index	Margalef-index	Pielou-index
$H' = -\sum_{i=1}^{S} \frac{n_i}{N} \ln(\frac{n_i}{N})$	$D = 1 - \sum \frac{n_i(n_i-1)}{N(N-1)}$	$D_{Mg} = \frac{S-1}{\ln N}$	$E = \frac{1-\sum_{i=1}^{S}(\frac{n_i}{N})^2}{1-1/S}$

Note: where n_i is the number of OTU in the same sample, N the number of individuals sampled, and S the number of species.

convert all measurements to the same scale prior to further analysis using the formula: $Z = (x_i - \bar{x})/s$, where x_i is the sample value, $\bar{x}$ is the mean of all samples, and s is the standard deviation (Van Nostrand et al., 2009). Coverage was calculated following Good (Good, 1953) with the equation: $C = (1 - n_1/N) \times 100$, where C is the homologous coverage, n_1 is the number of clone types occurring only once and N is the total number of clones examined. This program also used the Shannon–Weaver index (H'), the Simpson index (D), the Margalef index (D_{Mg}) and the Pielou index (E) to estimate phylotype diversity. The equations used are provided in Table 2.

A redundancy analysis (RDA) was conducted to examine the main factors (extractable Cd, Zn, Hg) affecting community structure. RDA correlates each species to the environmental variables by selecting the linear combination of environmental variables that returns the smallest residual sum of squares. The statistical significance of all of the canonical axes combined was calculated using a rectangular spatial grid (2×2) with a Monte Carlo permutation test (500 randomized runs). Data were standardized by applying a square root transformation and then a logarithmic transformation. We quantified the proportion of the variation in the values of the sediment's physicochemical properties could be attributed to the individual and microbial populations. All of the multivariate analyses were completed with Canoco.

3. Results and discussion

3.1. Sediment chemical properties

The partial physicochemical characteristics of the sediments are listed in Table 1. The extent of the contamination differs greatly among the sites. Sample S had the lowest heavy metal concentration and X and H had moderate metal levels. It is noteworthy that sample F had the highest concentrations of zinc, cadmium, and copper and was the most heavily contaminated site. This may be explained by the fact that it had the highest pH (8.80) because under high pH, heavy metals generally exist in compounds, limiting their spread to the surrounding environment, and more heavy metals form insoluble compounds in the sediment. This sample was located at the confluence of the Xiangjiang River and Xiawan Port. The minimal amount of river flow during dry season cannot dilute the heavy metals in the sediment. The pH values in samples H and S were approximately 7.2, and sample X was 7.6. These were

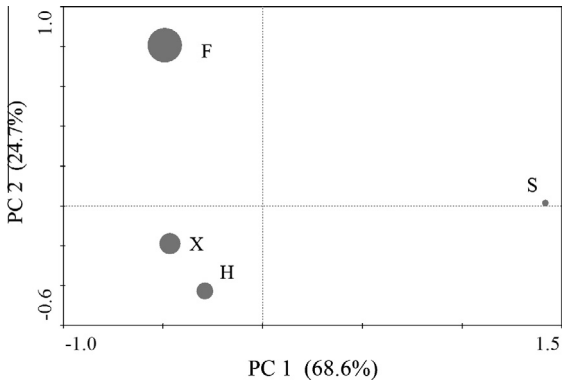

Fig. 2. Principal component analysis (PCA) of samples geochemical variables, axis 1 and axis 2 respectively accounted for 68.6% and 24.7% of the variance.

also indicated by the principal component analysis (PCA), which describes 93.3% of the variation among the environmental data explained by the first axis (PC1) and the second axis (PC2) (Fig. 2). The dimensions (axes) in Fig. 2 have no special significance and can be rotated or mirrored without influencing the relative distances between the points. Figs. 1 and 2 indicate that the closer the sites were to each other, the smaller the physico-chemical differences between the sites. The geochemistry had a regular distribution that was influenced by the impact of the water flow direction. Moreover, the TOC of the four samples was different; H had the highest TOC (11.95%), and S had the lowest (2.66%). This result does not agree with Seidemann (1991) and Valsecchi et al. (1995)), who found that metals and organic C were closely positively related. The sites were also contaminated by organic substance to varying degrees.

3.2. Clone libraries, RFLP analysis, and biodiversity

16S rDNA products of the expected size (approximately 1500 bp) were successfully amplified from community genomic DNA from the four samples. After T–A cloning, a total of 647 16S rDNA positive colonies were recovered from all of the samples, and then these clones were screened with the RFLP analysis. The RFLP analysis revealed extensive diversity in the 16S rDNA. To test whether the analyzed clones can represent the bacterial community in each sample, the coverage of all of the clone libraries (Table 3) was calculated. The results revealed that the data can represent the real status of bacterial communities in those habitats.

The 96 unique clones were subjected to 16S rDNA gene sequence analysis, sequenced, and then used to construct a phylogenetic tree (Fig. 3). Phylogenetic analysis revealed bacterial sequences belonging to nine known divisions: α-*Proteobacteria* (23.4%), β-*Proteobacteria* (22.6%), γ-*Proteobacteria* (3.6%), δ-*Proteobacteria* (5.5%), ε-*Proteobacteria* (0.4%), *Firmicutes* (29.7%), *Bacteroidetes* (3.2%), *Actinobacteria* (6.5%), *Chloroflexi* (4.2%) and unidentified bacteria (1%). Most of the bacterial clones were related to uncultivated

Table 3
The total detected number and coverage of clone sequences and diversity indices for bacterial.

Sample	X	H	S	F
No. of clone	170	165	166	146
Coverage (%)	95	92	91	98
Shannon Weaver (H')	1.81	1.45	1.62	1.24
Simpson (D)	0.80	0.67	0.76	0.67
Margalef (D_{Mg})	1.74	1.23	1.22	1.03
Pielou (E)	0.89	0.78	0.88	0.80

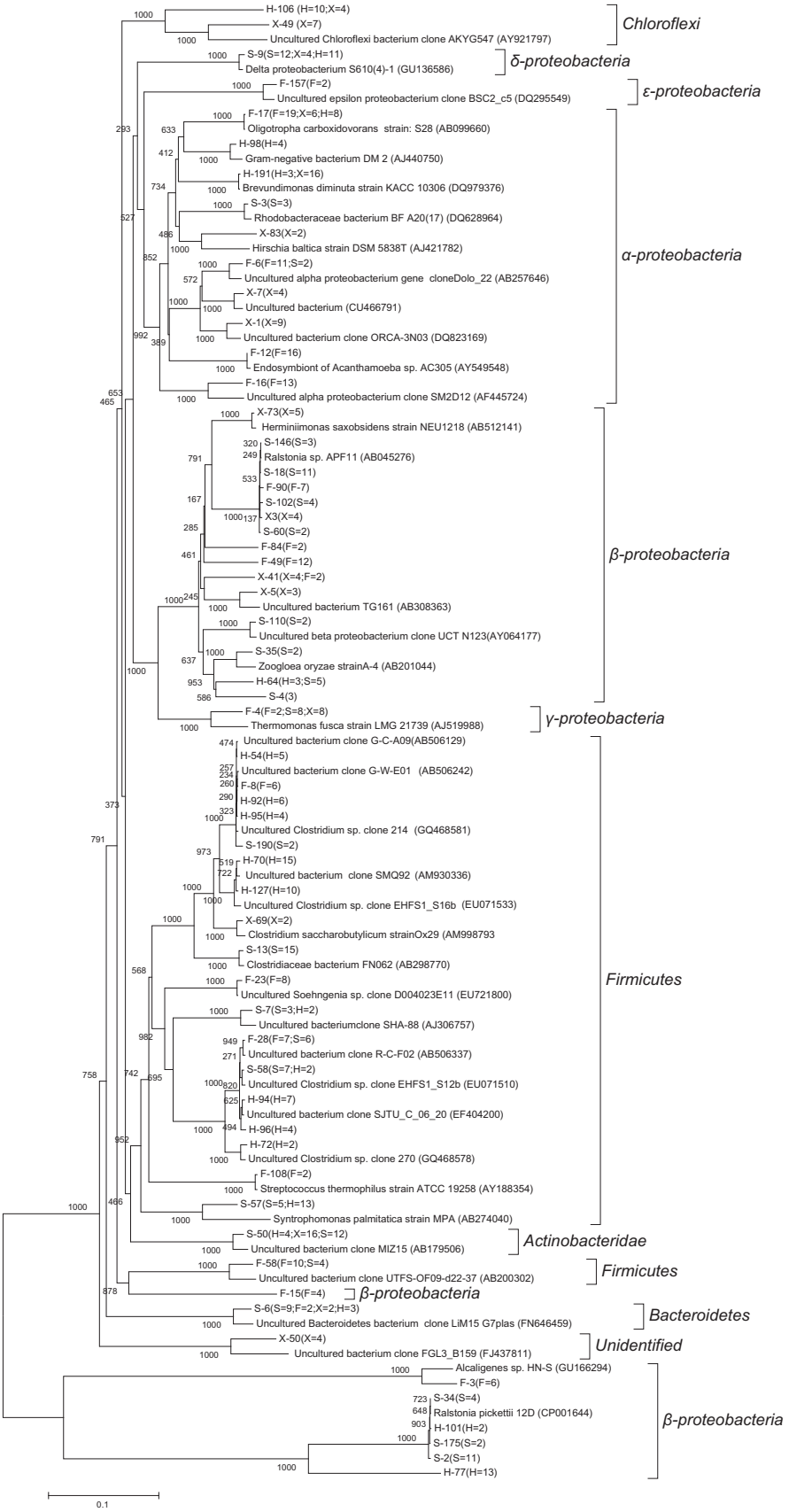

Fig. 3. Neighbor-joining bacterial phylogenetic tree construction from partial 16S rDNA sequences. Clones from four samples are coded as S, H, X F. One access number for each OTU is shown on the tree. Bootstrap values (1000 replicates) higher than 50% are shown. Scale bar represents the 10% substitution percentage.

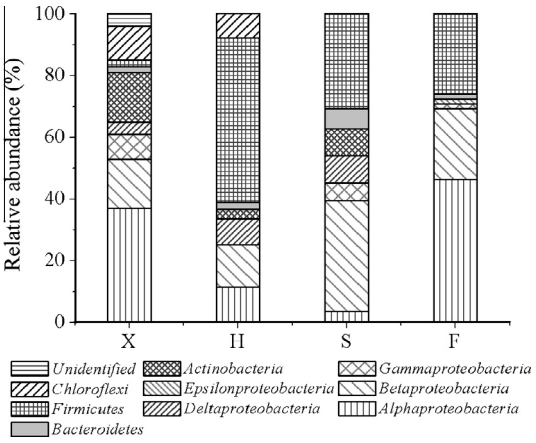

Fig. 4. Relative distribution of each phylotype in the total bacteria in the PCR–RFLP clone libraries representing the different samples.

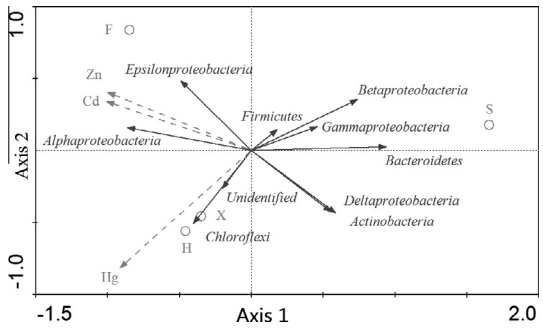

Fig. 5. Ordination diagrams from RDA of bacterial with environmental variables represented as dashed lines and species represented as solid lines. And samples are represented as circles. Both percentage variance of species data and species–environment relation are 89.8% and 9.9% for axis 1 and axis 2.

environmental microorganisms (Fig. 3), several clones found in heavy metal and organic contaminated sludge. The relative abundance of the clones related to each of the major groups was calculated based on the number of clone sequences, and results show that the microbial community structure is highly diverse and heterogeneous in four samples (Figs. 3 and 4). Both α- and β-*Proteobacteria*, and *Firmicutes* are the dominant families. The distribution of phyla differed between the four sites, depending on the different physicochemical characteristics. It was interesting to note that α-*Proteobacteria* increased as the heavy metal increased. From Fig. 4, α-*Proteobacteria* constitutes 46.5% of F library, 37.0% of X library, 11.5% of H library, and only 3.6% of S library. This phenomenon was discovered by R A. Sandaa using in situ hybridization (Sandaa et al., 2001). γ- and β-*Proteobacteria* clustered in the same branch, possibly consanguineously grouped together, and δ-*Proteobacteria* existed in three samples, except sample F contained sulfate-reducing prokaryotes. We detected a substantial number of β-*Proteobacteria* sequences that were members of the Ralstonia class and relatively few sequences of *Firmicutes* that were related to the Syntrophomonas class. Four sequences from sample X clustered with unclassified sequences, representing a novel phylogenetic lineage. Moreover, ε-*Proteobacteria* was only found in sample F (1.6%). *Chloroflexi* existed in samples H and X (7.6%, 11.0%, respectively) and may be a positive correlation with Hg, as tested by RDA (Fig. 5).

The microbial community appeared more diverse in subject X than in others based on an inspection of the richness and evenness of the clone distribution across the phylogenetic tree (Fig. 3 and Table 3). The results indicated that heavy metal contamination changed the structure of microbial community to a certain extent. The maximum index is sample X, and the minimum index is sample F. However, there was not a simple, negative relationship between heavy metal contamination and the genetic diversity of sediment microbial communities. The results are inconsistent with previous findings (Feris et al., 2003; Joynt et al., 2006; Li et al., 2006). Moreover, the richness of samples H and S is approximately equal. This similarity is consistent with microbial function redundancy among species in communities (McGrady-Steed et al., 1997; Nannipieri et al., 2003). The microbial populations have complicated interactions, for example association and competition. The effects of heavy metal do not act simply on one species, but on microbial populations in the sediment. The presence of heavy metals changed the predominant bacterium and also changed the properties of the microorganisms. Several investigations have proven that both structural and functional diversity in polluted soil can greatly improve (Yrjälä et al., 2010), and bacterial communities can exhibit structural and functional resilience to metals (Brandt et al., 2010). These properties protect other species and increase the abundance of rare species. Therefore, microbial diversity, richness and evenness are not linearly related to heavy metals.

Total metal content is not a good indicator of the availability and mobility of heavy metals in sediments because factors such as pH and others can strongly influence metal bioavailability. For this reason, $MgCl_2$-exchangeable Zn, Cd, and Hg were selected as the main factors (Akcay et al., 2003). Moreover, the total sediment metal content exceeded the soil environmental quality standards by several factors, and the metals with the highest exchangeable percentage content were Cd, Zn, and Hg. The R project's mantel test suggested that the exchangeability of these three metals was significantly correlated with the microbial community. All species were used for RDA, along with three selected environmental variables. The results are presented in graphical form in Fig. 5. Plots can be interpreted qualitatively, where the length of the line indicates how much variance was explained by that factor, and the direction of the arrows for individual environmental factors indicates an increasing concentration of that factor. The species arrows pointing in approximately the same direction as the environmental factor arrows indicate a high positive correlation (the longer the species line, the stronger the relationship). Zn and Cd were positively correlated with ε-*Proteobacteria* and α-*Proteobacteria* and negatively correlated with δ-*Proteobacteria* and *Actinobacteria*. Similarly, Hg was positively correlated with *Chloroflexi* and negatively correlated with *Firmicutes,* β-*Proteobacteria* and γ-*Proteobacteria*. The sensitivity of ε-*Proteobacteria* and δ-*Proteobacteria* to Zn and Cd suggests the potential for use of these bacteria as sediment quality indicators for Zn and Cd contamination. Moreover, *Chloroflexi* and γ-*Proteobacteria* may be the indicators for Hg contamination. This result may be due to the heredity of the species and the result of long-term adaptation to the circumstances.

4. Conclusions

The studied sediments of the Xiangjiang River were alkaline and severely polluted with Cd, As and Zn, while Hg contamination was heavy in Xiawan Port. The PCA revealed that the order of the pollution level was F > X > H > S. The phylogenetic analysis of subjects provided invaluable information about the ecological impact of heavy metals on the bacterial community and suggested that heavy metal pollution affected sediment microbial community diversity. However, there were not simply negative relationships between heavy metal contamination and the genetic diversity of sediment microbial communities, and the largest species diversity appeared in the moderately polluted sample X.

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