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RECEIVED 03 February 2023

ACCEPTED 11 April 2023

PUBLISHED 27 April 2023

CITATION

Liao J, Xu Y, Zhang Z, Zeng L, Qiao Y,
Guo Z, Chen J, Jia B, Shang C and Chen S
(2023) Effect of Cu addition on
sedimentary bacterial community structure
and heavy metal resistance gene
abundance in mangrove wetlands.
Front. Mar. Sci. 10:1157905.
doi: 10.3389/fmars.2023.1157905

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Effect of Cu addition on sedimentary bacterial community structure and heavy metal resistance gene abundance in mangrove wetlands

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Introduction: Mangroves are a unique ecosystem, but are often affected by heavy metal pollution. Bacterial communities in mangrove sediments usually respond to heavy-metal pollution stress by expressing specific heavy-metal resistance genes (HMRGs).

Methods: Multivariate statistics were performed on Metagenomics as well as environmental data to evaluated the effects of Cu addition on the sedimentary bacterial community structure.

Results and discussion: Using metagenomics technology, we found that the Shannon and Chao1 indices of natural sediments were significantly higher than in sediments with artificially added Cu. Addition of Cu significantly altered bacterial community structure and diversity at the phylum, class and order levels in sediments. The relative abundance of HMRGs in Cu added sediments was lower than that in natural sediments. Environmental factors that affect bacterial communities also affect HMRGs. However, the contribution of heavy-metal pollution to the change in HMRG abundance was much lower than its contribution to the change in the bacterial community, indicating that the relationship between HMRG abundance and heavy-metal pollution is complex and needs to be comprehensively studied.

KEYWORDS

Cu addition, heavy metal resistance genes, mangrove sediments, environmental factor, bacterial diversity

1 Introduction

Mangroves occur all over the world and represent a special ecosystem composed of woody plants distributed in tropical and subtropical tidal zones or transition zones between ocean and fresh water (Kathiresan and Bingham, 2001; Udechukwu et al., 2015). The unique acidic, organic-rich and hypoxic-reducing sedimentary environment of mangroves makes deposition of various pollutants from tides, rivers and rainstorms more likely than in the general intertidal zone (Alongi et al., 1993). Among these pollutants, heavy metals have attracted attention due to their high toxicity, non-degradability and bioaccumulation throughout the food chain (Dudani et al., 2017). Heavy metals, together with other environmental factors, influence the structure of sedimentary bacterial communities, which represent a vital component of the mangrove ecosystem (Hu et al., 2022). Changes in the sedimentary environment of mangroves can directly or indirectly affect the diversity, composition and metabolic activities of bacterial communities (Shannon et al., 2003; Lee et al., 2014; Echavarrri-Bravo et al., 2015; Balakrishnan et al., 2017). When mangroves are polluted by excessive amounts of heavy metals, the microorganisms in sediments usually respond by expressing specific heavy-metal resistance genes (HMRGs). These genes have functions such as expelling heavy metals from cells *via* transporters, transforming heavy metals into non-toxic forms, heavy-metal precipitation and chelation, and biological adsorption (Bouskill et al., 2007; Gamal et al., 2019). The expression level of HMRGs in microorganisms can reflect their tolerance to heavy metals. Indeed, microbes with a more robust heavy-metal resistance system likely represent an opportunity for use in bioremediation of heavy metals in polluted environments (Roosa et al., 2014), including in mangroves.

Many studies have reported HMRGs in bacterial resistance systems, with specificity for cadmium (*cadB*), chromium (*chrA*), lead (*pbrA*), copper (*cusA*) and nickel (*nika*) (Roosa et al., 2014; Chen et al., 2020), for example. However, there are few studies on the relationship between heavy metals and the representation of HMRGs in mangrove bacterial communities. Of all heavy metal pollution we chose Cu to add to natural mangrove sediments. This is because Cu was one of the most heavily polluted heavy metals in the mangrove sediment of South China (Chen et al., 2021; Hu et al., 2022), highlighting the importance of studying the response of the bacterial community to Cu. Moreover, previous studies have suggested that Hg and Cd, as well as Cu, were among the top three most widely recognized metals in heavy metal pollution studies of coastal wetlands (Li et al., 2022). However, adding Hg or Cd to the environment can cause serious pollution while Cu was less harmful and essential to some microorganisms (Zhang et al., 2020). Therefore, we chose Cu as the targeted heavy metal to explore how Cu addition to natural mangrove sediments changes bacterial community structure and the representation of HMRGs (at the DNA level) in these communities. The research results not only enrich our understanding of bacterial populations and bacterial HMRGs in mangrove sediments, but also have theoretical and practical significance for the bioremediation of pollution in mangroves.

2 Materials and methods

2.1 Cu-addition experiment

The Cu-addition experiment was carried out in the Golden Bay Mangrove Reserve in Beihai, Guangxi province (109.22° N, 21.42° E; 'Beihai mangrove' for short). Five sampling sites (GX1-5) in Beihai mangrove were selected in *Bruguiera gymnorhiza* mangrove forests. GX1, GX2, and GX3 are located at the same location, approximately 10 meters apart, while GX4 and GX5 are located at another location. Two locations were 100 meters apart with the same distance from the sea, approximately 50 meters. GX1, GX2 and GX3 were the control group with no artificial metal addition. GX4 was watered once a month with 1 L of 200 mg/L Cu solution. GX5 was watered once a month with with 1 L of 400 mg/L Cu solution. After three years of treatment, triplicate sedimentary samples were collected from all five sites; hence, a total of nine samples were collected from GX1, GX2 and GX3, and six samples from the Cu-added sites GX4 and GX5.

Surface sediments were collected in November 2020 in sealed polyethylene bags. The 15 surface sediment samples were stored in an incubator with ice packs and delivered to the laboratory within 2 h of sampling. Each sample was divided into three parts: the first part was sealed and stored at 4°C until further analysis; the second part was dried at 60°C for the determination of physical and chemical parameters; and the third part was sealed in dry ice and stored at -80°C for metagenome sequencing.

2.2 Analytical methods

2.2.1 Physicochemical properties of sediments

Following methods in Hu et al. (2022), a series of physicochemical parameters such as grain size composition, pH, total phosphorus (TP), and total nitrogen (TN) were measured. Grain size composition was measured with a Beckman Coulter LS 13320 particle size analyzer (Beckman Coulter Inc., Germany). Sediment pH was measured by pH meter, after mixing sediments with deionized water in a mass ratio of 1:5. Samples were oven dried under 55°C, grounded and desiccated before TP and TN analyses. TN were determined using a PE 2400 II elemental analyzer (Perkin-Elmer, Waltham, Massachusetts, USA); TP was determined using an automatic discrete analyzer (Cleverchem 380; Dechem-Tech, Hamburg, Germany). For pH and grain size composition, all samples were measured in triplicate to check reproducibility and accuracy. For other analyses, the samples were measured in triplicate resulting in a relative deviation of less than 7%.

2.2.2 Measurements of total metals and their speciations

Contents of five heavy metals (Cr, Ni, Cu, Zn, and Pb) were analyzed in the sediment samples. These metals were selected based on their toxicity and spatiotemporal concentrations reported previously by Chen et al. (2021). Approximately 0.2 g of the dried sediment sample was digested in a microwave digestion system

(Ethos One, Milestone, Italy) with a mixture of 7 mL 65% HNO₃ and 1 mL 20% H₂O₂ (Feng et al., 2017). The volume was made up to 50 mL with Milli-Q water, and this mixture was used for the determination of the total concentration of heavy metals. Next, the speciation of heavy metals in sediments was characterized using the BCR sequential extraction method (Tessier et al., 1979; Hall et al., 1996; Hall and Pelchat, 1999; Rauret et al., 1999). The heavy metals were classified as an acid-extractable fraction (denoted CrAcid, NiAcid, CuAcid, ZnAcid and PbAcid), a reducible fraction (CrReduc, NiReduc, CuReduc, ZnReduc and PbReduc), an oxidizable fraction (CrOxid, NiOxid, CuOxid, ZnOxid and PbOxid) and a residue fraction (CrResid, NiResid, CuResid, ZnResid and PbResid) (Figure S1 shows the detailed analytical procedure). All heavy-metal solutions were tested by inductively coupled plasma optical emission spectroscopy (ICP-OES; Optima 7000DV, Perkin-Elmer, Waltham, MA, USA). All the samples were measure in triplicate resulting in a relative deviation of less than 10% and a recovery ranging of 85% - 110%.

2.2.3 Methods to evaluate heavy metal pollution

The heavy metal pollution assessment methods used in this study include a comprehensive potential ecological risk index (RI), based on the total amount of heavy metals (Hakanson, 1980), and the ratio of secondary phase to primary phase (RSP), based on the speciation of heavy metals (Wang et al., 2011; Zhang et al., 2019). The RI used both measured total concentrations and background concentrations of metals, thereby taking into account the sensitivity of biological communities and heavy-metal toxicity (Zhang et al., 2019). The RI was calculated using Equation (1):

$$RI = \sum_{i=1}^n E_r^i = \sum_{i=1}^n T_r^i \cdot \frac{C^i}{C_n^i} \quad (1)$$

where C^i is the measured content of metal i in sediment, and C_n^i is the metal's concentration in background sediment. We used pre-industrial metal contents as the background values (Cr=60mg/kg, Ni=30mg/kg, Cu=30mg/kg, Zn=80mg/kg, Pb=25mg/kg) (Hakanson, 1980). T_r^i is the toxicity response coefficient of each metal as follows: Cr=2, Ni=2, Cu=5, Zn=1, Pb=5 (Hakanson, 1980). The RSP is generally used to distinguish natural and man-made sources of heavy metals (Wang et al., 2011). The formula for RSP is as follows:

$$RSP = M_{sec}/M_{prim} \quad (2)$$

where M_{sec} represents the content of heavy metals in the secondary phase of soil, which is calculated from the sum of the acid-extractable fraction, reducible fraction and oxidizable fraction; and where M_{prim} represents the heavy-metal content in the primary phase and is calculated from the residue fraction. When $RSP \leq 1$, there is no pollution; $1 < RSP \leq 2$ represents slight pollution; $2 < RSP \leq 3$ means moderate pollution; and $RSP > 3$ represents heavy pollution.

2.2.4 DNA extraction and metagenome sequencing

Sediment microbial DNA was extracted from 1 g fresh soil samples using the FastDNA spin kit for soil (MP bio, Santa Ana, CA, USA), according to the manufacturer's instructions (Ren et al., 2016). The Qubit 2.0 fluorometer (Thermo Fisher Scientific) was used to assess the quality and purity of the DNA extracts. There were three replicates for each soil sample to obtain sufficient DNA for shotgun metagenome sequencing. An Illumina small fragment (350 bp) library was constructed and its effective concentration (>3 nM) determined using the Qubit 2.0 fluorometer, while its quality was assessed by qPCR. Paired-end sequencing was performed on an Illumina NovaSeq6000 sequencing platform at Berry Genomics Technology Co., Ltd., Beijing, China. Sequence data had been uploaded in the NCBI SRA (Sequence Read Archive) database with project number PRJNA945508.

2.2.5 Processing of sequencing data and analysis of bacterial community structure

Raw sequencing reads were filtered to enhance the reliability and quality of subsequent analysis, as described by Zhang et al. (2017). The obscure bases, adapter sequences, and reads that were below 50 bp were removed. The obtained clean reads were assembled into contigs using Megahit (<https://hku-bal.github.io/megahit/>) with the optimal k-mer parameter. Subsequently, the sequencing data was compared to the Gene Catalogue to identify unigenes. DIAMOND software (version 0.9.21) was used to BLAST the unigenes against the NR database (<https://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/>), and the lowest common ancestor algorithm was used to annotate the sequences. The abundance and community structure of the samples were determined after assigning, as far as possible, kingdom, phylum, class, order, family, genus and species based on the annotation results. The alpha diversity of sediment microorganisms was assessed by calculating the Shannon (Magurran, 1988) and Chao1 indices (Chao, 1984) (using R vegan software). The Shannon index was calculated as follows:

$$H' = -\sum P_i \times \ln P_i \quad (3)$$

where H' is the Shannon index of the microorganisms, and P_i is the ratio of the i th species to the total number of species in the sample. The Chao1 index was calculated as follows:

$$S_1^* = S_{obs} + (a^2/2b) \quad (4)$$

where S_1^* is the estimated Chao1 index, S_{obs} is the actual number of species, a is the number of species with an individual number of 1, and b is the number of species with an individual number of 2.

2.2.6 Annotation and abundance calculation for HMRGs of sediment microorganisms

Using DIAMOND software, the unigenes were compared with KEGG (<https://www.kegg.jp/>), eggNOG (<http://eggno5.embl.de/#/app/home>), CAZyme (<http://www.cazy.org/>), CARD (<https://>

card.mcmaster.ca/), PHI (<http://www.phi-base.org/>), GO (<http://geneontology.org/>) to obtain annotation information and gene abundance information for each sequence. Due to the large number of databases and huge quantity of annotation information, we selected data returned from the KEGG database, which integrates genomic, chemical and system function information, for subsequent analysis. To compare the abundance of each annotated gene across the various samples, the relative abundance was calculated as follows:

$$G_k = \frac{r_k}{L_k} \times \frac{1}{\sum_{i=1}^n \frac{r_i}{L_i}} \quad (5)$$

where G is the relative gene abundance, r is the number of reads of matched genes, and L is the length of gene fragments.

2.3 Quality assurance and quality control

Plastic utensils were mainly used for sampling; metal utensils were avoided as much as possible to avoid contamination of core sediments. All sampling instruments were cleaned with 5% nitric acid solution before use. All samples were tested in triplicate to check for reproducibility and to evaluate the precision of measurements.

2.4 Statistical analysis

Excel was used to record and process raw data on environmental factors, the structural composition of bacterial communities, and the relative abundance of annotated genes. Based on the species annotation and abundance distribution of sediment microorganisms at the phylum, class and order levels, species distribution maps at these taxonomic levels were generated using R. Spearman correlation analysis was conducted using the R vegan package to determine the correlation between various environmental factors in sediments and the dominant bacterial communities at the phylum, class and order levels. Canonical correlation analysis was carried out using R to reveal the dynamic relationship between heavy-metal pollution and bacterial communities at the phylum, class and order levels. By reference to the KEGG database, HMRGs were selected and the distribution map of bacterial HMRGs was drawn using R. Correlations between various environmental factors of sediments and bacterial HMRGs were determined by Spearman correlation analysis using R vegan. Canonical correlation analysis was conducted using R to reveal the relationship between heavy-metal pollution and the distribution of bacterial HMRGs in sediments.

3 Results and discussion

3.1 Distribution characteristics of heavy metal pollution in mangrove sediments

3.1.1 Physicochemical parameters of mangrove sediments

It can be seen from the physicochemical parameters (Table S1) that, at all five sites, the sediments were mainly silt in most cases,

with the silt content ranging from 12.40% to 96.50%, while the content of clay was in the range 2.75%-17.30%, and the sand content ranged from 3.51% to 87.60%. TP ranged from 0.13 to 0.44 mg/g, while TN content ranged from 0.03% to 0.06%. The pH values of all sediment samples were moderately acidic, ranging from 6.51 to 6.99. The addition of Cu did not change the physicochemical parameters of GX4 and GX5 significantly (tested by single factor ANOVA; for all physicochemical parameters, $P > 0.05$). This is consistent with the report of Cai et al. (2013), which showed that the addition of metals did not significantly affect soil grain size.

3.1.2 Speciation of heavy metals in mangrove sediments

Following Cu addition, the total Cu content in samples from sites GX4 and GX5 was significantly higher than in other samples (i.e. GX1-3), as expected. Thus, the content of Cu ranged from 4.79 to 40.06 mg/kg in the order GX5 > GX4 > GX1 ≈ GX3 ≈ GX2 ($F = 530.186$ and $P < 0.001$, according to single-factor ANOVA). Other metals showed variable patterns. For example, the content of Cr across all sediments ranged from 7.77 to 28.66 mg/kg in the order GX5 ≈ GX3 ≈ GX1 ≈ GX2 > GX4 ($F = 9.680$; $P = 0.002$). The Ni content ranged from 2.18 to 11.59 mg/kg and showed essentially the same order, i.e. GX5 ≈ GX1 ≈ GX3 ≈ GX2 > GX4 ($F = 4.065$; $P = 0.033$). For Zn, the content ranged from 2.58 to 15.14 mg/kg, but with a different order, i.e. GX5 > GX4 ≈ GX1 ≈ GX2 > GX3 ($F = 66.055$; $P < 0.001$). Pb content, which ranged from 2.59 to 9.88 mg/kg, also showed a different order, i.e. GX5 > GX1 ≈ GX3 ≈ GX4 ≈ GX2 ($F = 4.241$; $P = 0.029$).

The speciation patterns of Cr, Ni, Zn, and Pb did not much differ between the Cu-addition samples and control samples (Figure 1; Table S2). For example, in all samples Cr was mainly found in the residue fraction, followed by the reducible fraction, while there was relatively little of this metal in the acid-extractable and oxidizable fractions. Ni was mostly in the residue fraction, followed by the acid-extractable fraction, the reducible fraction and the oxidizable fraction. Zn was mainly identified in the acid-extractable fraction, followed by the reducible fraction, the oxidizable fraction and the residue fraction. Finally, Pb was present mainly in the reducible fraction, followed by the oxidizable fraction, the residue fraction and the acid-extractable fraction. However, Cu speciations differed between the Cu-addition and natural samples. In the natural samples, i.e. GX1, GX2 and GX3, Cu was mainly in the reducible and residue fractions, followed by the oxidizable fraction and the acid-extractable fraction. In the Cu-addition samples, GX4 and GX5, Cu was mainly found in the reducible fraction, followed by the acid-extractable fraction, the residue fraction and the oxidizable fraction. The differences in speciation fractions were expected since the more active Cu^{2+} solution CuSO_4 were added in GX4 and GX5.

The total heavy-metal content in the natural samples GX1, GX2, GX3 was much lower than the background levels of heavy metals in Guangdong Province (Table S3). A possible explanation for this observation is that the mangroves in the Beihai mangrove are far from urban development centers and therefore the pollution caused

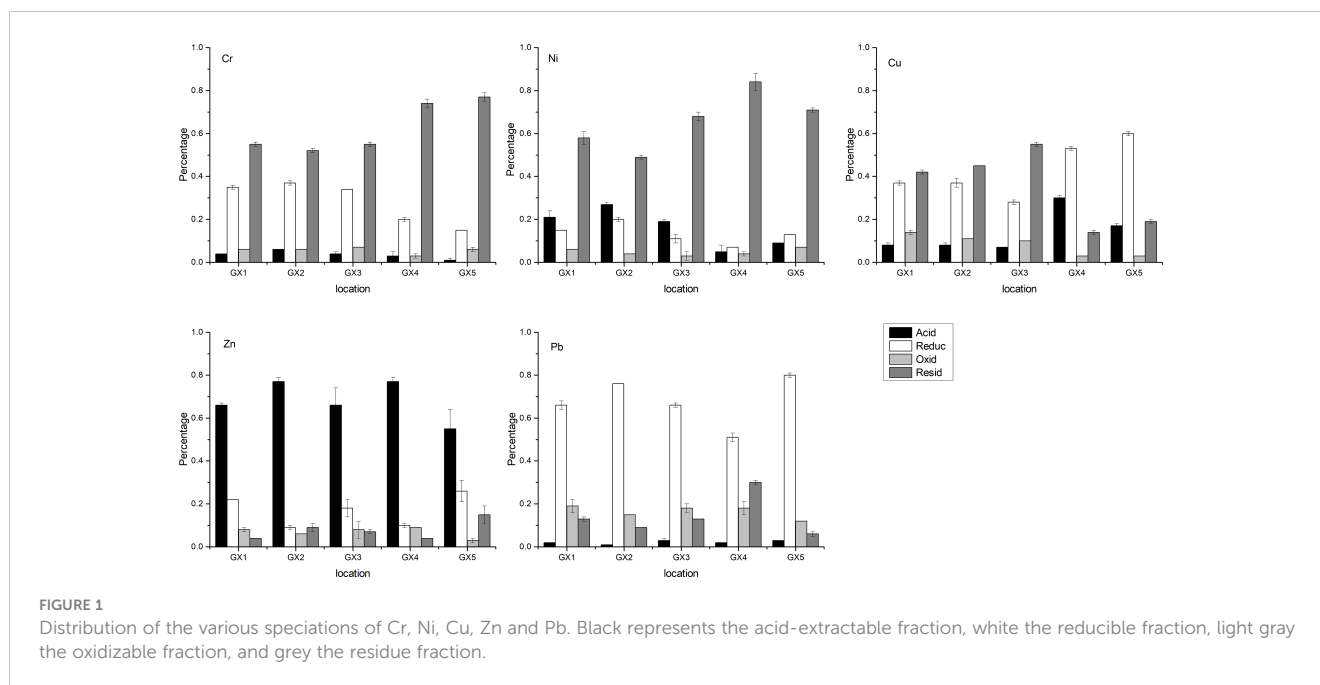


FIGURE 1

Distribution of the various speciations of Cr, Ni, Cu, Zn and Pb. Black represents the acid-extractable fraction, white the reducible fraction, light gray the oxidizable fraction, and grey the residue fraction.

by human activities is not severe. In the natural sediments of Beihai mangrove, Cr and Ni are mainly in the residue fraction, while Pb and Cu are predominantly in the reducible fraction (Figure 1), which is consistent with the findings of Cordeiro et al. (2015) in Guanabara Bay, Brazil. In addition to the reducible fraction, Cu is mainly found in the residue fraction in GX1, GX2 and GX3, indicating that the artificially added Cu in GX4 and GX5 mainly exists in the reducible fraction. It can be seen that under different conditions, the relative preponderance of a particular heavy metal species may change; in this context, it should be noted that there is the possibility of mutual transformation among the various heavy metal species (Ramos et al., 1999; Jayachandran et al., 2018).

3.1.3 Assessment of heavy metal pollution in mangrove sediments

The potential ecological risk assessment results for heavy metals in the mangrove sediments of Beihai mangrove are shown in Table 1. It can be seen from the table that in GX1 RI-Pb had the highest value at 1.56, while RI-Zn had the lowest value at 0.08. In GX2, the values for RI-Ni and RI-Cu were the highest, both being 0.94; RI-Zn had the lowest value at 0.07. In GX3, RI-Cu was the

highest, with a value of 1.19, while RI-Zn showed the lowest value at 0.05. In GX4, RI-Cu was the highest, with 5.00; RI-Cr returned the lowest value at 0.30. In GX5, RI-Cu had the highest value at 6.43, while RI-Zn was lowest at 0.18. It can be seen that all samples fall into the slight ecological risk category, in the following order: GX5>GX4>GX1>GX3>GX2.

RSP, the ratio of secondary phase to primary phase of the various fractions of metals, allows natural sources of heavy metals to be distinguished from anthropogenic sources (Table 2). Our results showed that all samples were heavily polluted by Zn and Pb. As expected, the Cu addition sites GX4 and GX5 were heavily polluted by Cu, while the three natural sites showed no Cu pollution. There was no Cr pollution at any of the sites tested.

All sites presented a slight ecological risk because of the heavy pollution with Zn and Pb, consistent with the observations that the residue fractions of both Zn and Pb were relatively low compared to the other three fractions (Table 2). A possible reason is that Beihai mangrove is far from urban areas, but the booming tourism industry and increased commercialization in the area in recent years may also cause man-made Zn and Pb pollution. Pb pollution mainly comes from automobile exhaust emissions and battery

TABLE 1 The potential ecological risk assessment for individual heavy metals in Beihai mangrove.

Sample	E_r^i					RI	Degree of ecological risk
	Cr	Ni	Cu	Zn	Pb		
GX1	0.56	1.32	1.21	0.08	1.56	4.73	Slight
GX2	0.55	0.94	0.94	0.07	0.79	3.29	Slight
GX3	0.57	1.04	1.19	0.05	1.04	3.89	Slight
GX4	0.30	0.45	5.00	0.09	0.87	6.71	Slight
GX5	0.76	1.48	6.43	0.18	1.57	10.42	Slight

processing (Prosi, 1989), while Zn pollution largely results from coal combustion, automobile tire wear and manufacturing machinery (Shi et al., 2019). In summary, the relatively high RSP values for Zn and Pb observed in this study represent a significant ecological risk that cannot be ignored in Beihai mangrove.

3.2 Relationship between bacterial diversity and heavy metal pollution in mangrove sediments

3.2.1 Bacterial community structure in mangrove sediments

Metagenomic sequencing and annotation of all samples in this study revealed a total of seven kingdoms, 183 phyla, 186 classes, 443 orders, 855 families, 2,756 genera and 15,779 species (excluding unmatched and unclassified). The top 20 bacterial phyla in the mangrove sediments of Beihai mangrove are shown in Figure 2A.

The average proportion of the top 10 bacterial phyla ranked as follows: Proteobacteria > Bacteroidetes > Chloroflexi > Planctomycetes > Actinobacteria > Gemmatimonadetes > Cyanobacteria > Acidobacteria > Verrucomicrobia > Ignavibacteriae; their average proportions were 64.14%, 7.89%, 4.55%, 4.28%, 4.09%, 3.41%, 3.07%, 2.46%, 1.47% and 0.67%, respectively. The top 20 bacterial classes are shown in Figure 2B and the average proportion of the top 10 bacterial classes ranked as follows: Alphaproteobacteria > Gammaproteobacteria > Deltaproteobacteria > Betaproteobacteria > Gemmatimonadetes > Flavobacteriia > Anaerolineae > Actinobacteria > Actinobacteria > Acidimicrobia; their average proportions were 26.87%, 14.55%, 13.44%, 5.13%, 4.06%, 3.38%, 3.07%, 2.36%, 1.92% and 1.39%, respectively. The top 20 bacterial orders are shown in Figure 2C and the average proportion of the top 10 orders ranked as follows: Rhodobacterales > Sphingomonadales > Planctomycetia > Myxococcales > Gemmatimonadetes > Flavobacteriales > Rhizobiales > Desulfobacterales > Chromatiales >

TABLE 2 The ratio of secondary and primary phases of heavy metal speciation in Beihai mangrove.

Sample	heavy metals	RSP	Ecological risk level
GX1	Cr	0.81	no pollution
	Ni	0.71	no pollution
	Cu	1.39	mild pollution
	Zn	23.09	heavy pollution
	Pb	6.54	heavy pollution
GX2	Cr	0.92	no pollution
	Ni	1.03	mild pollution
	Cu	1.24	mild pollution
	Zn	10.88	heavy pollution
	Pb	10.49	heavy pollution
GX3	Cr	0.82	no pollution
	Ni	0.48	no pollution
	Cu	0.82	no pollution
	Zn	13.89	heavy pollution
	Pb	6.47	heavy pollution
GX4	Cr	0.35	no pollution
	Ni	0.20	no pollution
	Cu	6.37	heavy pollution
	Zn	22.65	heavy pollution
	Pb	3.36	heavy pollution
GX5	Cr	0.29	no pollution
	Ni	0.40	no pollution
	Cu	4.24	heavy pollution
	Zn	5.71	heavy pollution
	Pb	15.22	heavy pollution

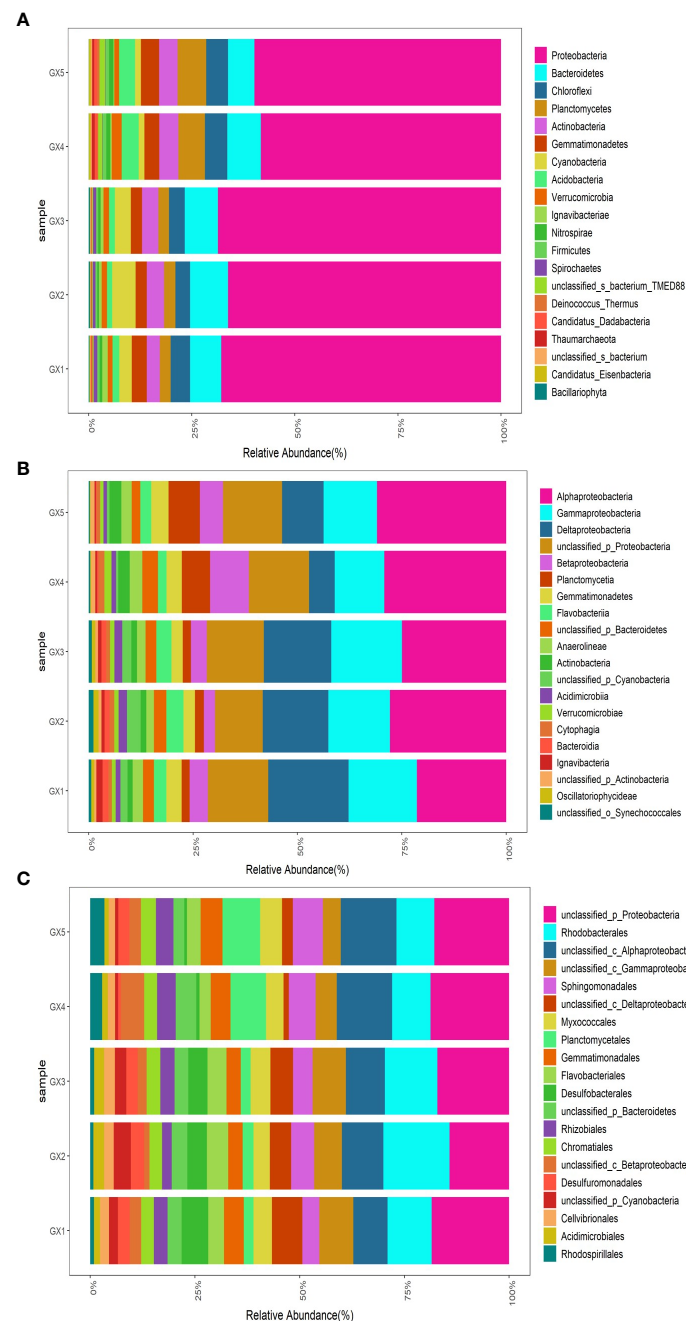


FIGURE 2

The proportion of top 20 microbial communities at the phylum (A), class (B) and order (C) level in the sediments of Beihai mangrove.

Desulfuromonadales > Desulfuromonadales; their average proportions were 11.42%, 5.57%, 4.95%, 4.50%, 4.30%, 3.88%, 3.52%, 3.40%, 3.20% and 2.40%, respectively.

In the sediments of Beihai mangrove, the dominant bacterial communities at the phylum level were Proteobacteria, Bacteroidetes and Chloroflexi, which is consistent with the report of Imchen et al. (2017), who found similarly dominant bacterial communities at the phylum level at various sites in four different mangroves in India. These workers found Proteobacteria, Bacteroidetes and Actinobacteria to be the dominant groups of bacteria, although the ranking order in terms of proportion of the community differed

slightly across the various sites. Nevertheless, Imchen et al. found that Proteobacteria was the most prevalent group of bacteria at multiple locations. In our study, Proteobacteria was also the predominant bacterial group in both natural and Cu-added sediments, accounting for ~64% of the total, a much larger proportion than other bacterial phyla.

The dominant bacterial communities at the class level in sediments are Gammaproteobacteria, Deltaproteobacteria, Alphaproteobacteria and Betaproteobacteria among the Proteobacteria; Flavobacteriia in phylum Bacteroidetes; and Gemmatimonadetes in Gemmatimonadetes, while the ranking

order of community proportion in each sample was slightly different. Wang et al. (2020) reported similar findings when studying mangrove sediments in Longhai, China. They found that the dominant bacterial communities at the class level in all sediments were also Gammaproteobacteria, Deltaproteobacteria, Alphaproteobacteria, Anaerolineae, Gemmatimonadetes, and so on, yet the proportion of each group differed from our findings.

At the order level, the dominant bacterial community was Rhodobacterales in class Alphaproteobacteria, although the proportions were similar to those of other dominant bacterial groups, albeit different in each sample. Liu et al. (2019) also found Rhodobacterales to be the dominant microflora in sediments from different areas during a study of mangroves in Hainan.

3.2.2 Alpha diversity in mangrove sediments

The Shannon diversity of sediment microorganisms in mangroves in Beihai mangrove is shown in Figure 3. The Shannon index of GX3 was the highest among all samples at 21.92, while GX4 had the lowest value at 21.15. The Shannon index values of GX1, GX2 and GX3 (natural sediments) were all significantly higher than those of GX4 and GX5 (Cu addition). The

Chao1 index calculations for sediment microorganisms are shown in Figure 3. The pattern of Chao1 index values for all samples replicated that for the Shannon index, with the Chao1 index of GX3 having the highest value and GX4 the lowest value. Similarly, the Chao1 index values for GX1, GX2 and GX3 (natural sediments) were all significantly higher than those for GX4 and GX5 (Cu addition).

The Shannon diversity index can reflect community richness and community homogeneity, while the Chao1 index is more sensitive to the presence of rare communities within the overall bacterial population. Hence, the abundance and number of rare communities in GX1, GX2 and GX3 (natural sediments) in Beihai mangrove were much higher than those in GX4 and GX5 (Cu addition). A possible reason for this observation is that the high concentration of Cu added to the GX4 and GX5 sediments over a short period of time had a significant impact on the diversity of the bacterial community. Low heavy-metal content can stimulate bacterial growth, while high heavy-metal content is likely to inhibit bacterial growth (Wu et al., 2019). Pradhan et al. (2020) also found that a short period of heavy-metal pollution significantly reduced bacterial diversity, possibly because the bacterial community was unable to adapt to the stress induced by this

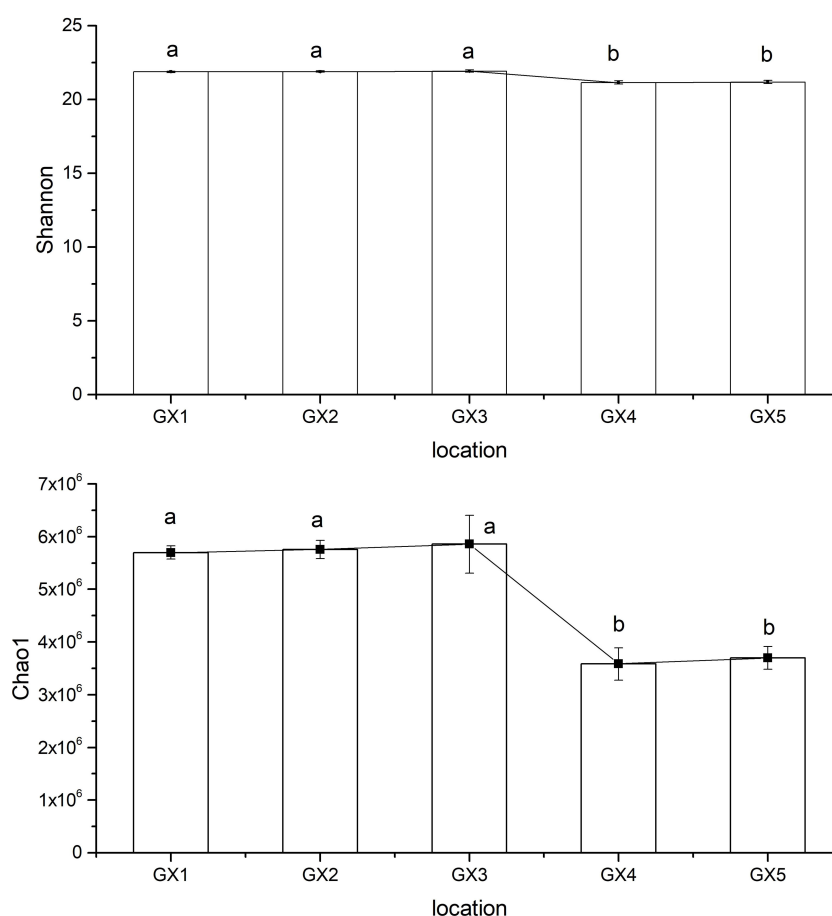


FIGURE 3

Shannon index (Top) and Chao1 index (Bottom) of sedimentary bacterial community in Beihai mangrove; (a, b) indicate significant differences between samples. Data were evaluated by single-factor ANOVA and were divided into two groups (a, b). Data in group (a) were significantly higher than data in group (b).

pollution. On the other hand, the variation in trend of both the Shannon index and the Chao1 index across the various sediments from Beihai mangrove is consistent, indicating that the richness of the overall bacterial community is mainly determined by the number of rare species.

3.2.3 Correlation analysis of heavy metal pollution and bacterial diversity

Spearman correlation analyses were performed to determine the relationships between heavy-metal pollution and bacterial diversity (Table 3). The Shannon index of sediment microorganisms was significantly positively correlated with TN, CrAcid, NiAcid, CrReduc and CrResid and significantly negatively correlated with total Cu, total Zn, CuAcid, CuReduc, CuOxid, CuResid, ZnAcid, ZnResid, RI-Cu, RI-Zn and RI. The Chao1 index was significantly positively correlated with NiAcid, CrReduc, TN and CrResid, and significantly negatively correlated with total Cu, CuAcid, CuReduc, CuOxid, CuResid, ZnAcid, ZnResid, RI-Cu, RI, Zn and RI-Zn.

It can be seen that the bacterial diversity index of sediments in Beihai mangrove is affected by various environmental factors. The Shannon index can represent the diversity and homogeneity of community distribution, while the Chao1 index mainly reflects the number of rare bacterial communities. We observed that both the Shannon and Chao1 indices were negatively correlated with the total amount of Cu and its speciations, the total amount of Zn, ZnAcid, ZnResid, RI-Cu, RI-Zn and RI. This is consistent with the

findings of Pradhan et al. (2020), which showed that heavy-metal contamination can affect the number of rare microorganisms in soil.

Spearman correlation analyses were further performed to determine the relationships between metal pollution and the dominant bacterial communities at the phylum, class and order levels (Table 4). The dominant bacterial community at the phylum level in the mangrove sediments of Beihai mangrove is affected by various environmental factors (Table 4a). Proteobacteria, the phylum that forms the largest proportion of the overall community, is associated with pH, total amount of Cu, total amount of Zn, CrAcid, NiAcid, CuAcid, PbAcid, CrReduc, CuReduc, ZnReduc, CrResid, CuResid, ZnResid, RI-Cu, RI-Zn and RI. These results indicate that the bacterial community representing the largest proportion of the overall population at the phylum level was more obviously affected by heavy-metal pollution than the dominant taxonomic groups defined at other levels (i.e. class and order). Moreover, we found that when the total amount of heavy metals was correlated with the dominant bacterial community, some species of heavy metals were also correlated with this community. On the other hand, the metal species could be correlated with the dominant bacterial community even if the total amount of an individual heavy metal showed no such correlation (Oliveira and Pampulha, 2006). Therefore, metal speciations seemed to be more important environmental factors than total heavy-metal contents in terms of their influence on the bacterial community.

We also found that Proteobacteria and Bacteroidetes are negatively correlated with most of the environmental factors in the Beihai mangrove, while Armatimonadetes and Nitrospirae are positively correlated with most environmental factors. These observations indicate that heavy-metal pollution can have different effects on different bacterial communities at the same level (Oliveira and Pampulha, 2006; Basak et al., 2016). The diversity of some bacterial communities increased under the influence of heavy metal pollution, while conversely the diversity of other bacterial communities decreased. This might be explained by the different physiological and genetic characteristics of different bacterial communities. For example, a biotin-binding protein called bacterial avidin is found mainly in Proteobacteria and Bacteroidetes, which might be an important factor in the resistance of these taxonomic groups to environmental change (Laitinen et al., 2021). Compared with other dominant bacterial communities, the correlation in all samples between Nitrospira and environmental factors was extremely significant ($P < 0.01$) (Table 4b). However, Nitrospira accounts for only a very small proportion of the top 20 bacterial communities. A possible reason for these correlations is that, compared with other heterotrophic dominant groups, Nitrospira species are more sensitive to toxic substances such as heavy metals (Ferhan et al., 2010). Thus, Pb inhibits bacterial growth by damaging the cell membrane and interrupting the transport of nutrients. Cu can interfere with proteins or enzymes in bacterial cells. Oxidative stress imposed on Nitrospira by redox reactions involving Cr can damage proteins and DNA (Belliveau et al., 1987; Yeager, 1991; Vaiopoulou and Gikas, 2012). At the order level, we observed the same pattern as at the phylum and class levels, i.e. that the metal speciations influence

TABLE 3 Correlation coefficient (r) values from Spearman correlation analysis between the environmental factors and alpha microbial diversity index in Beihai mangrove.

Environmental factors	Shannon	Chao1
TN	0.649**	0.523*
Cu	-0.760**	-0.756**
Zn	-0.732**	-0.546*
CrAcid	0.523*	0.506**
NiAcid	0.613*	0.651**
CuAcid	-0.806**	-0.846**
ZnAcid	-0.804**	-0.729**
CrReduc	0.581*	0.692**
CuReduc	-0.814**	-0.753**
CuOxid	-0.807**	-0.796**
CrResid	0.588*	0.518*
CuResid	-0.661**	-0.793**
ZnResid	-0.726**	-0.649**
RI-Cu	-0.760**	-0.756**
RI-Zn	-0.721**	-0.547*
RI	-0.771**	-0.729**

* indicates a significant correlation ($P < 0.05$); ** indicates an extremely significant correlation ($P < 0.01$).

TABLE 4A Spearman correlation between heavy metal pollution and dominant microbial communities at the phylum level in Beihai mangrove.

	Bacteroidetes	Proteobacteria	Cyanobacteria	Bacillariophyta	Nitrospirae	Firmicutes	Armatimonadetes
pH	_*	_**	ns	_*	ns	*	**
Cu	_**	_**	_**	_**	**	**	**
Zn	_**	_**	_**	_**	**	**	**
CrAcid	**	**	**	**	_**	_*	_**
NiAcid	**	**	**	**	_**	_**	_**
CuAcid	_**	_**	_**	_**	**	**	**
ZnAcid	_**	_**	_**	_**	**	**	**
CrReduc	*	*	*	**	_**	_*	_*
CuReduc	_**	_**	_**	_**	**	**	**
ZnReduc	_**	_*	_*	_*	*	*	ns
ZnOxid	ns	ns	_*	_**	ns	*	**
CrResid	_**	_**	_**	_*	*	*	*
CuResid	_**	_**	_**	_**	**	**	**
ZnResid	_**	_**	_**	_**	*	**	*
RI-Cu	_**	_**	_**	_**	**	**	**
RI-Zn	_**	_**	_**	_**	*	**	*
RI	_**	_**	_**	_**	**	**	**

the make-up of the bacterial community more than the total amount of each heavy metal (Table 4c). Thus, taking all the above correlation analyses into account, it can be concluded that the influence of heavy metal speciation on the sediment microflora is much greater than that of the total amount of heavy metals. This is consistent with the report of Wasilkowski et al. (2014) and suggests that heavy metal speciation is a key factor affecting bacterial community structure.

3.2.4 Canonical correlation analysis of heavy metal pollution and bacterial diversity in mangrove sediments

Canonical correlation analysis (CCA) was used to analyze the impact of environmental factors on the bacterial community. Environmental factors with significant correlations ($P < 0.05$) to the bacterial community variables were selected to draw the arrows in Figure 4 and the length of the arrows represents the intensity of the influence of the environmental factor on the bacterial community. CCA 1 and 2 contributed 85.57% of the bacterial community variation at the phylum level. At the phylum level, GX4 and GX5 were mainly affected by the total amount of Cu and RI-Cu, while GX1, GX2 and GX3 were mainly affected by clay, silt, sand, RI-Cr and RI-Ni (Figure 4A). At the class level, CCA 1 and 2 contributed 82.44% of the bacterial variation (Figure 4B). GX4 and GX5 were mainly affected by the total amount of Cu, RI-Cu, the total amount of Zn, and RI-Zn; GX1, GX2 and GX3 were mainly affected by sand, RI-Cr and RI-Ni. At the order level, CCA 1 and 2 contributed 82.28% of the bacterial variation (Figure 4C). GX4 and GX5 were mainly affected by the the total amount of Cu,

RI-Cu, the total amount of Zn and RI-Zn; GX1, GX2 and GX3 were mainly affected by sand, RI-Cr and RI-Ni.

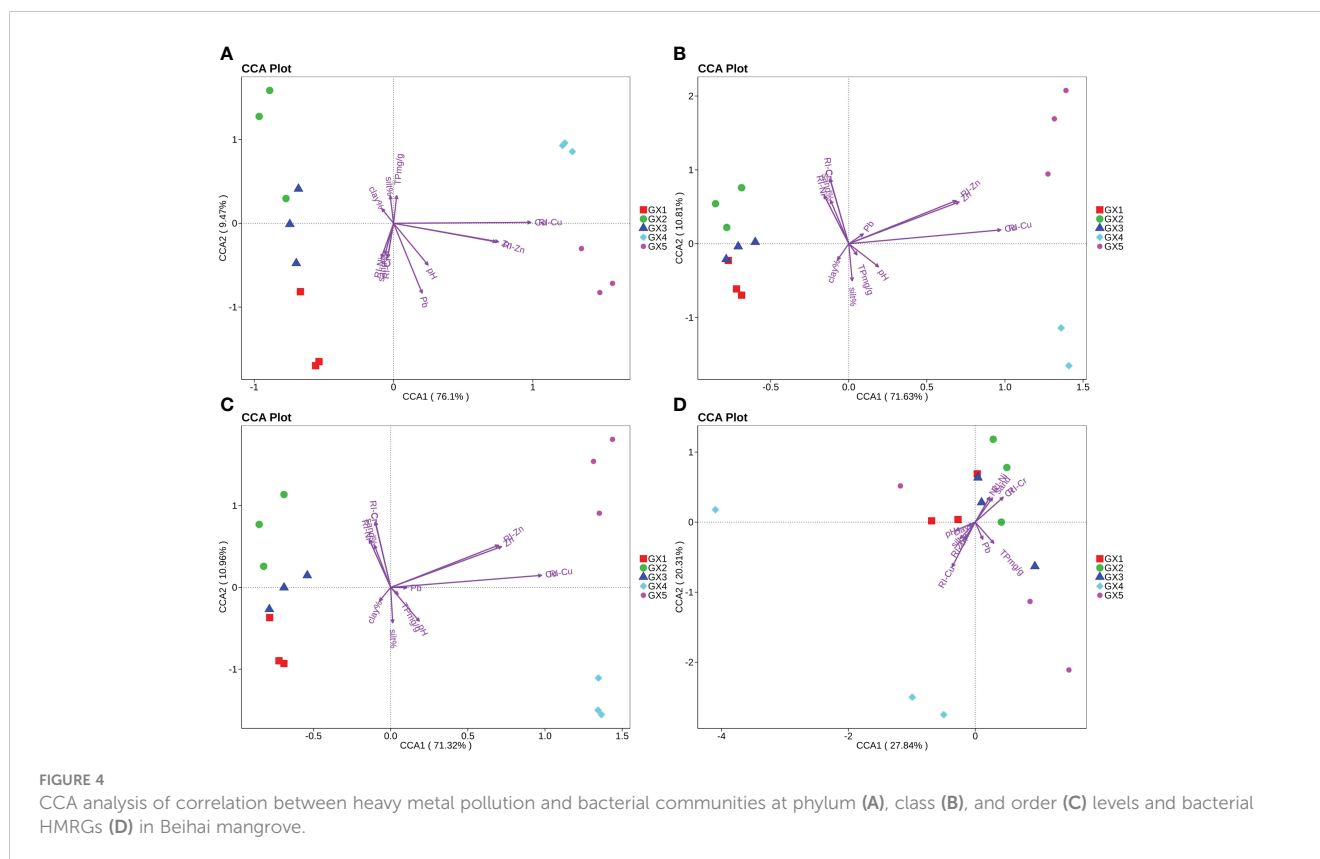
CCA analyses indicated that the effects of environmental factors on the bacterial community structure of Beihai mangrove sediments were different at the level of phylum, class and order. In natural sediments GX1, GX2 and GX3, the bacterial community structure was mainly affected by grain size (especially the content of sand), RI-Cr and RI-Ni. In GX4 and GX5 (Cu addition), the bacterial community structure at the level of phylum, class and order was mainly affected by the total amount of Cu, RI-Cu, the total amount of Zn and RI-Zn, indicating that the artificial addition of Cu over a short period can significantly change the bacterial community structure at various levels. The contributions of heavy-metal pollution to the bacterial community changes at phylum, class and order levels were all more than 80%, indicating that heavy-metal pollution was the key factor affecting the bacterial community structure of Beihai mangrove. On the other hand, we observed that the variations in bacterial community structure were more related to RI-metal values than metal speciations, which was not consistent with the results of the correlation analyses. One explanation might be that the correlation analysis determines the influence of a single environmental factor on the dominant bacterial flora, while the CCA analysis reflects the key factors that affect all bacterial communities. Nevertheless, our observations suggest that the RI values of metals play important roles in bacterial community structure variation. Li et al. (2021) reported similar findings when studying forest sediments, i.e. that RI index values accurately quantify the toxicity level of heavy metals and reflect the changes in bacterial community structure much more clearly than the total

TABLE 4B Spearman correlation between heavy metal pollution and dominant microbial communities at the class level in Beihai mangrove.

	Alphaproteobacteria	Gammaproteobacteria	Flavobacteriia	Bacteroidia	Deltaproteobacteria	Betaproteobacteria	Nitrospira
pH	-.**	-.*	-.**	ns	ns	*	*
Cu	-.*	-.**	-.**	-.**	-.*	ns	**
Zn	ns	-.**	-.*	-.**	ns	ns	**
CrAcid	**	*	**	*	ns	-.**	-.**
NiAcid	ns	*	**	*	*	-.**	-.**
CuAcid	ns	-.**	-.**	-.**	-.**	**	**
ZnAcid	-.**	-.**	-.**	-.*	ns	**	**
CrReduc	ns	*	**	**	**	-.*	-.**
CuReduc	-.*	-.**	-.**	-.**	-.*	ns	**
ZnReduc	-.*	ns	ns	-.*	ns	ns	*
CuOxid	-.*	-.**	-.**	-.**	-.*	*	**
ZnOxid	-.*	ns	-.*	ns	ns	**	*
CrResid	-.**	-.*	-.*	-.*	ns	ns	*
CuResid	ns	-.*	-.**	-.**	-.**	ns	**
ZnResid	-.*	-.**	-.**	-.*	ns	ns	*
RI-Cu	-.*	-.**	-.**	-.**	-.*	ns	**
RI-Zn	ns	-.**	-.*	-.**	ns	ns	*
RI	-.*	-.**	-.**	-.**	ns	ns	**

TABLE 4C Spearman correlation between heavy metal pollution and dominant microbial communities at the order level in Beihai mangrove.

	Rhodobacterales	Cellvibrionales	Desulfuromonadales	Chromatiales	Flavobacteriales	Desulfobacterales	Burkholderiales
pH	-.**	-.**	ns	-.**	-.**	ns	ns
Cu	-.**	-.**	-.**	-.**	-.**	-.**	**
Zn	-.**	-.**	-.*	-.**	-.*	-.*	ns
CrAcid	**	**	**	*	**	ns	-.**
NiAcid	**	*	**	**	**	*	-.**
CuAcid	-.**	-.**	-.**	-.**	-.**	-.*	**
ZnAcid	-.**	-.**	-.**	-.**	-.**	ns	**
CrReduc	**	**	**	*	**	**	-.**
CuReduc	-.**	-.**	-.**	-.**	-.**	-.**	**
ZnReduc	-.*	-.*	ns	ns	ns	ns	ns
CuOxid	-.**	-.**	-.**	-.**	-.**	-.**	**
ZnOxid	-.*	ns	-.**	-.*	-.*	ns	**
CrResid	-.**	-.**	ns	-.*	-.*	ns	ns
CuResid	-.**	-.**	-.**	-.**	-.**	-.**	**
ZnResid	-.**	-.**	-.*	-.**	-.**	ns	ns
RI-Cu	-.**	-.**	-.**	-.**	-.**	-.**	**
RI-Zn	-.**	-.**	-.*	-.**	-.*	-.*	ns
RI	-.**	-.**	-.**	-.**	-.**	-.**	**



amount of specific metals. In addition to the effect of heavy metals, we found that grain size, the content of organic matter and pH also affect the bacterial community structure in sediments (Lee et al., 2014; Zhou et al., 2017). Indeed, grain size plays an important role in our samples, possibly because sediments with different particle sizes have different structural compositions, which affect the biogeochemical processes that microorganisms participate in, and thereby the bacterial community structure (Huang et al., 2002).

3.3 Relationship between bacterial HMRGs and heavy metal pollution in mangrove sediments

3.3.1 Relative abundance of bacterial HMRGs in sediments

In this study, the same 4,143 genes annotated by reference to the KEGG database were found across all 15 sediment samples. Of these, 13 bacterial HMRGs that were highly represented and widely distributed in all samples were selected for further study. Coincidentally, these 13 HMRGs were mentioned as the most common genes in contaminated sediments by a previous review Roosa et al. (2014). These comprised two Cr-resistance genes (*chrA* and *chrR*), three Ni-resistance genes (*cnrA*, *ddpA* and *nccA*), three Cu-resistance genes (*copB*, *cusA* and *cusB*), three Zn-resistance genes (*czcB*, *czcD* and *pqqL*) and two Pb-resistance genes (*pbrB* and *pbrT*). The heavy-metal resistance genes and their corresponding gene numbers in the KEGG database are shown in Table S4.

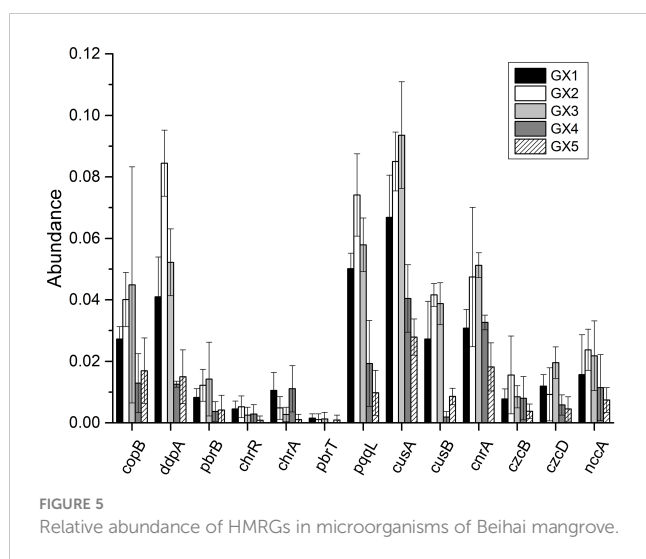
The distribution of bacterial HMRGs in Beihai mangrove sediments is shown in Figure 5. Twelve of the selected HMRGs (with the exception of *pbrT*, which was not present in GX4) were found in all samples, with the relative abundance of *cnrA*, *ddpA*, *copB*, *cusA*, *cusB* and *pqqL* being higher than other genes. The relative abundance of *chrA* and *pbrT* was highest in GX1 with values of 0.0115 and 0.0015, respectively, while *chrR*, *ddpA*, *nccA*, *cusB*, *czcB* and *pqqL* showed the highest relative abundance in GX2. The relative abundance of *cnrA*, *copB*, *cusA*, *czcD* and *pbrB* was highest in GX3. The relative abundance of *chrA*, *chrR*, *cnrA*, *nccA*, *cusA*, *czcB*, *czcD* and *pqqL* was lowest in GX5, while the relative abundance of *ddpA*, *copB*, *cusB* and *pbrB* was lowest in GX4. It can be seen that the abundance of all HMRGs in GX1, GX2, and GX3 (natural sediments) was higher than that in GX4 and GX5 (Cu addition).

The observation that HMRGs in Cu-added sediments were less abundant than that in natural sediments seems to be contradicted by the fact that an increase in heavy metal content results in a greater representation of HMRGs (Chen et al., 2019). On the other hand, this is consistent with the bacterial diversity index of GX1, GX2 and GX3 being higher than that of GX4 and GX5 (Figure 3). We speculate that the relative paucity of HMRGs in GX4 and GX5 is likely to be related to the decrease in bacterial diversity in these samples. Sun et al. (2021) reported a similar finding that the distribution of HMRGs varies with changes in bacterial diversity. The HMRG with the highest relative abundance across all sediments was *cusA*, with values ranging from 0.0279 to 0.0935. This is consistent with the report of Imchen et al. (2018), who found

that the abundance of HMRGs varied in mangrove sediments with different heavy metal contents (India, Brazil, and Saudi Arabia), yet *cusA* was always the most abundant in all sediments. This gene is involved in the dual-regulation mechanism of copper resistance in *Escherichia coli*, and many regulatory genes (such as *cusR* and *cusS*, etc.) control its expression (Munson et al., 2000). Moreover, *cusA* forms part of a complex that directly exports Cu from the cell, and is preferentially expressed compared with other Cu-resistance genes in bacterial communities (Besaury et al., 2014). At the same time, the relative abundance of *ddpA* and *pqqL* in sediments was only lower than that of *cusA*, which may be because *ddpA* encodes the binding protein of an ABC-superfamily transport pump (nickel transport system) in *E. coli* (Haddadin and Harcum, 2010), while *pqqL* encodes a periplasmic zinc metalloproteinase (Grinter et al., 2019). The relative abundance of *chrA*, *chrR*, *czcB*, *czcD* and *pbrT* was significantly lower than other HMRGs. It may be that *chrA* and *chrR* are present in *Bacillus amyloliquefaciens* and *Pseudomonas putida*, respectively, species that are sensitive to Cr^{6+} but not Cr^{3+} (Chihomvu et al., 2015). The *czcB* and *czcD* genes are found in *Cupriavidus (Ralstonia) metallidurans* and jointly encode RND heavy-metal efflux pumps; they can generate comprehensive resistance to Zn and Cd, although their sensitivity to Cd is greater than that to Zn (Legatzki et al., 2003). However, none of the samples tested in this study contained Cd and consequently there were few Cd-related HMRGs. *pbrT* was the least distributed HMRG in all sediments, which may be attributed to the fact that *pbrT* is found on an endogenous plasmid (pMOL30) in *C. metallidurans* CH34, which belongs to the Burkholderiaceae family in the order Burkholderiales. Burkholderiales was not one of the top 20 bacterial communities at the order level in any sediment sample, accounting for a very small proportion of the overall community.

3.3.2 Spearman correlation analysis of heavy metal pollution and bacterial HMRG representation

Spearman correlation analysis showed that there was no correlation between the abundance of individual bacterial



HMRGs and the physicochemical parameters of sediments, total amount of Pb and its speciation and RSP; therefore, only correlations between HMRG levels and Cr, Ni, Cu, Zn and their speciations, and RI values are presented (Figure 6). For Cr and its speciations, *chrA* was negatively correlated with the total amount of Cr, *pqqL* was negatively correlated with CrResid, and *cusA* and *ddpA* were positively correlated with CrAcid (Figure 6A). For Ni and its speciations, *cusB* and *ddpA* were significantly positively correlated with NiAcid (Figure 6B). Many correlations were observed between Cu and its speciations and HMRGs (Figure 6C). For example, *pqqL*, *ddpA* and *cusA* were significantly negatively correlated with the total amount of Cu and its speciations; *cnrA* was significantly negatively correlated with the total amount of Cu, CuReduc, and CuOxid; *cusB* was significantly negatively correlated with Cu and its speciations; *copB* was significantly negatively correlated with Cu and all its speciations; *pbrB* was negatively correlated with the total amount of Cu, CuAcid, CuReduc and CuOxid; *czcB* was negatively correlated with the total amount of Cu, *chrR* was negatively correlated with CuResid, *nccA* was negatively correlated with CuAcid, CuReduc and CuOxid, and *cnrA* was negatively correlated with CuReduc; *cusB* was negatively correlated with the total amount of Cu, and *copB* was negatively correlated with the total amount of Cu, CuAcid and CuReduc. For Zn and its speciations, *pqqL* and ZnAcid were significantly negatively correlated; *nccA* was negatively correlated with ZnAcid; *cusB* was negatively correlated with ZnAcid and ZnOxid; *cusA* was negatively correlated with the total amount of Zn, ZnAcid and ZnResid; *cnrA* was negatively correlated with the total amount of Zn, ZnAcid, ZnReduc and ZnResid; *pqqL* was negatively correlated with the total amount of Zn; *ddpA* was negatively correlated with ZnAcid and ZnResid; and *czcB* was negatively correlated with ZnResid. Moreover, correlation analyses were conducted between metal RI values and HMRGs and only negative correlations were observed. For example, *cusA* was significantly negatively correlated with RI-Zn; *pqqL*, *ddpA* and *cusA* were significantly negatively correlated with RI and RI-Cu, and *cnrA* was significantly negatively correlated with RI. The *cusB* and *copB* genes were negatively correlated with RI and RI-Cu, while *pqqL* was negatively correlated with RI-Zn, *cnrA* was negatively correlated with RI-Cu and RI-Zn, and *chrA* was negatively correlated with RI-Cr. In contrast, *czcD* and *pbrT* were not associated with any environmental factors.

The correlations between HMRGs and the specific metals were not constant (Figure 6). For example, the Cu-resistance genes *copB*, *cusA* and *cusB* were negatively correlated with the total amount of Cu and its speciations, which was consistent with the report of Besaury et al. (2014), who showed that the representation of Cu-resistance genes decreases with increasing Cu concentration. It is our conjecture that the duration of three years of copper supplementation was inadequate for the bacterial community to develop a greater number of copper-resistant genes. In contrast, there was no correlation between any of the environmental factors tested and either the Ni-resistance gene *nccA* or the Zn-resistance gene *czcB*, which may be because *nccA* can comprehensively counteract Ni, Co and Cd accumulation, but the action of *nccA*

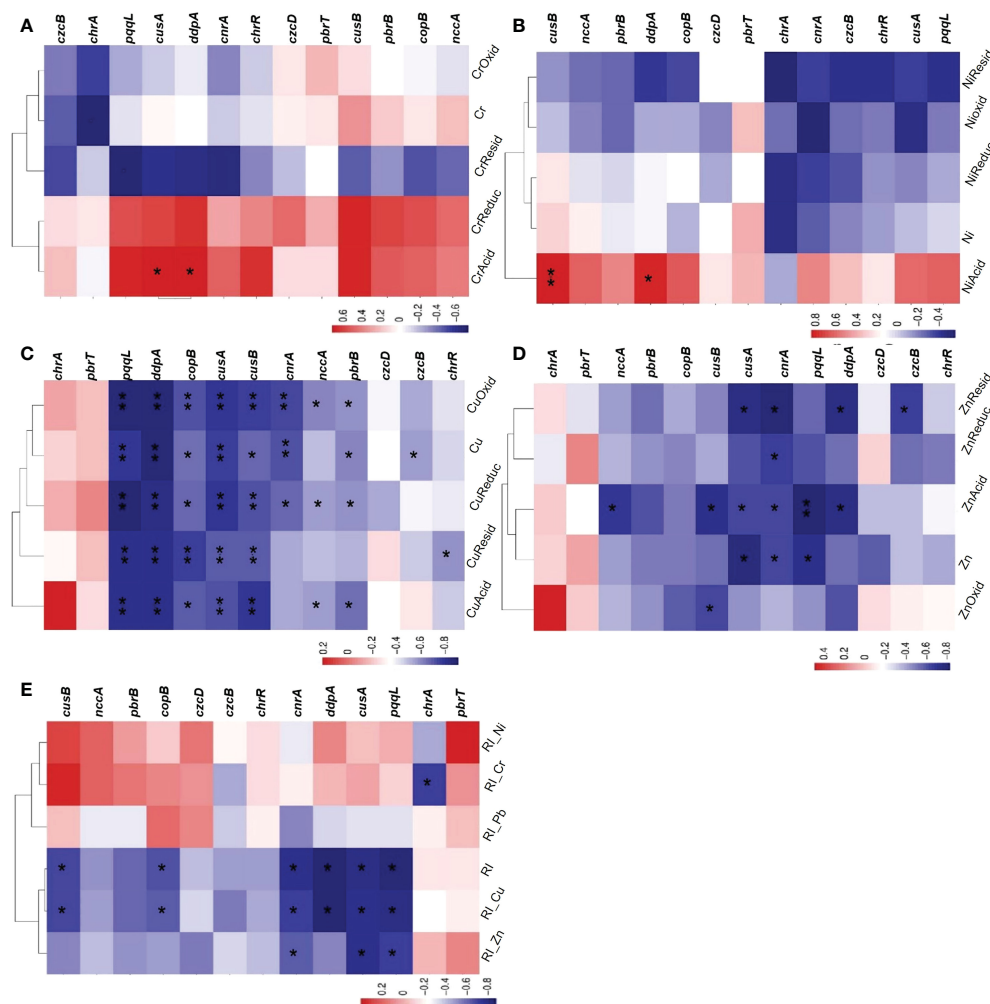


FIGURE 6

Spearman correlation analyses of HMRGs with Cr and its speciations (A), Ni and its speciations (B), Cu and its speciations (C), Zn and its speciations (D), and RI values of five metals (E). Asterisks represent significant correlations where * indicates $P < 0.05$ and ** indicates $P < 0.01$.

has no obvious effect on the total amount of Ni (Vaiopoulou and Gikas, 2012).

Interestingly, Pb speciations showed no correlation with HMRG abundance, including *pbrB* and *pbrT*. A previous study found that *pbrB* and *pbrT* were only sensitive to Pb^{2+} rather than Pb^{4+} (Borremans et al., 2001), because *pbrB* is involved in the export of Pb^{2+} from cells, and *pbrT* encodes the protein that takes up Pb^{2+} . Given the above result, we can speculate that there was little Pb^{2+} in our samples. Another paradoxical finding is that the Cr-resistance gene *chrR* was not affected by the total amount of Cr or its speciations. A possible explanation is that Cr mainly exists as Cr^{3+} , and *chrR* is only sensitive to Cr^{6+} (Chihomvu et al., 2015).

A further point of interest is that metal abundance can be correlated with the presence of HMRGs related to other, non-cognate metals. For example, the total amount of Cu was not only correlated with the Cu-resistance genes *cusB* and *copB*, but also with *cnrA*, *pbrB* and *czcB*. Sun et al. (2021) found similar correlations between the abundance of a Zn-resistance gene (*czcA*) and the total amount of Cu and Cr. This indicates that the bacterial community can develop cross-tolerance to several heavy metals simultaneously

when subjected to the stress of a single heavy metal, which may be because the same genetic element (such as a plasmid or transposon) can carry different resistance genes, resulting in gene co-selection (Seiler and Berendonk, 2012; Andrea et al., 2016; Durrant et al., 2019). In summary, there were no clear rules governing correlation between heavy metal pollution and the abundance of particular HMRGs. Thus, the abundance of HMRGs may be influenced by various environmental factors.

3.3.3 Canonical correlation analysis of heavy metal pollution and bacterial HMRG representation

CCA was performed on bacterial HMRGs and environmental factors and showed that clay, silt, sand, TP, pH, total amount of Cr, Ni, Zn, and Pb, RI-Cr, RI-Ni, RI-Cu and RI-Zn all affected HMRG abundance in sediments (Figure 4D). In the CCA analysis, the contribution rate of axis 1 and axis 2 to bacterial HMRG abundance in Beihai mangrove was 48.15%, indicating that the above environmental factors had an important impact on the representation of bacterial HMRGs. In addition, levels of

sediment microorganism HMRGs in GX1, GX2 and GX3 were mainly affected by sand, total amount of Cr and Ni, RI-Cr and RI-Ni, while the HMRGs in GX4 and GX5 were mainly affected by RI-Cu. It was also noted that GX4 and GX5 mapped further from related environmental factors than GX1, GX2 and GX3 (Figure 4D), indicating that the influence of these environmental factors on HMRG representation at GX1, GX2 and GX3 in Beihai mangrove was greater than their influence on HMRG representation at sampling sites GX4 and GX5.

In combination with the results of section 3.2.4, it is clear that both environmental factors and HMRGs affect community structure at the level of phyla, class and order. These observations further demonstrate that resistance to heavy metals is a common feature of many microorganisms subjected to heavy-metal stress, and that heavy-metal pollution effects on the bacterial community also modify the diversity and abundance of individual HMRGs (Sun et al., 2021). At the same time, the speciation of heavy metals and the RSP in all samples had no effect on changes in the bacterial community and HMRG representation, indicating that RI, which includes a heavy-metal toxicity coefficient in the formula for its calculation, can accurately quantify the toxicity level of heavy metals (Li et al., 2021), and can more fully and clearly reflect the impact of heavy-metal pollution on bacterial community structure and HMRG representation. The CCA results show that the contribution rate of heavy-metal pollution to the changes in the bacterial community was always more than 80%, while the contribution to HMRG abundance was only 48.15% (Figure 4), further suggesting that HMRG representation is influenced by other factors. For example, a large number of studies on HMRGs indicate that HMRGs and antibiotic resistance genes (ARGs) are located on the same genetic element, and heavy metals can exert selective pressure not only on HMRGs, but also on ARGs. This selection pressure may lead to cross-selection of heavy metal and antibiotic resistance, potentially resulting in co-occurrence of HMRGs and ARGs (Knapp et al., 2011; Ji et al., 2012; Seiler and Berendonk, 2012; Pal et al., 2015; Andrea et al., 2016; Durrant et al., 2019).

4 Conclusion

In this study, we designed a Cu-addition experiment to explore the effect on HMRG representation and bacterial community structure in mangrove sediments experiencing heavy-metal pollution stress. With the addition of Cu, total Cu contents increased significantly, as did its reducible fraction, in comparison to natural sediments. The Cu stress affected the Chao1 index rather than the Shannon index, indicating that Cu pollution influences bacterial diversity in the Beihai mangrove mainly by affecting the number of rare species. Spearman correlation analysis indicated that the speciations of heavy metals were more suitable for the analysis of the relationship between a single dominant bacterial community and heavy-metal pollution than the total amount of metal. CCA analysis showed that heavy-metal pollution could explain most of the changes in bacterial community structure in Beihai mangrove. Thus, the addition of Cu significantly changed the bacterial community structure at the phylum level. The metal speciations and RI could better explain the changes in bacterial community. Furthermore, we

compared the differences in individual HMRG levels between Cu stress and natural conditions in Beihai mangrove sediments. Whether Cu was added or not, *cusA* was the most abundant HMRG in all samples, while *pbrT* was the least abundant, indicating that HMRG representation was relatively consistent in mangrove sediments although the addition of Cu resulted in lower HMRG abundance generally. CCA analysis showed that environmental factors that affect bacterial community structure can also affect HMRG abundance, and resistance to heavy metals is a common feature of many microorganisms under heavy-metal stress. However, the effect of heavy-metal pollution on HMRG representation in Beihai mangrove was weaker than that of heavy-metal pollution on bacterial community structure, probably due to the co-occurrence of HMRGs and ARGs. In summary, during a three-year Cu-addition experiment, we found that the bacterial community structure and the abundance of particular HMRGs was affected by the addition of pollutants. More specific factors, such as metal speciations or RI, had a greater impact on bacterial characteristics than the total metal content.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

JL and YX contributed to the experiment and the first draft of the manuscript. ZZ, LZ, YQ, ZG, JC, BJ, and CS contributed to the manuscript revision. SC contributed to the final draft of the design of the experiment, the statistical analysis, the final draft of the manuscript and the funding acquiring. All authors contributed to the article and approved the submitted version.

Funding

CAS Key Laboratory of Science and Technology on Operational Oceanography (No.OOST2021-07). Shenzhen Science and Technology Program (JCYJ20210324093409025). Guangdong Province Undergraduate University Teaching Quality and Teaching Reform Project([2020]19).

Acknowledgments

We would like to thank the staff of Guangdong Neilingding Futian National Natural Reserve and Guangxi Beihai Mangrove Reserve for their support in sampling. We are also grateful for the support from the public service platform of instruments and equipment of College of Life Sciences and Oceanography in Shenzhen University.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2023.1157905/full#supplementary-material>

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