



Copper and cadmium co-contamination affects soil bacterial taxonomic and functional attributes in paddy soils[☆]

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ARTICLE INFO

Keywords:

Copper
Cadmium
Co-occurrence pattern
Functional genes
Metagenomics

ABSTRACT

Microorganisms inhabiting heavy metal-contaminated soils have evolved specific metabolic capabilities to survive, which has the potential for effective bioremediation. However, the ecological consequence of copper (Cu) and cadmium (Cd) on bacterial taxonomic and functional attributes of rice field remains unclear. Here, we selected paddy soils along a polluted river in southern China to evaluate the role of Cu and Cd contaminant fractions in regulating bacterial co-occurrence patterns. We also assessed the effects of these heavy metal fractions on the relative abundance of functional genes using shotgun metagenomic analysis. Soil Cu and Cd concentrations in paddy soils gradually decreased from upstream to downstream of the river, and had a greater impact on bacterial communities and metabolic potentials than soil general properties. Soil Cu and Cd contamination led to drastic changes in the cumulative relative abundance of ecological modules in bacterial co-occurrence networks. Bacteria associated with *AD3*, *HSB_OF53-F07* (both belonging to Chloroflexi), *Roku-bacteriales*, and *Nitrospira* were identified as tolerant to Cu and Cd contamination. The Cu and Cd contaminant fractions were positively correlated with the genes involved in metal resistance, carbon (C) fixation, nitrification, and denitrification, but negatively correlated with the genes related to nitrogen (N) fixation. These results indicated that soil Cu and Cd pollution not only enriched metal resistant genes, but also affected genes related to microbial C and N cycling. This is critical for facilitating microbiome bioremediation of metal-contaminated paddy soils.

1. Introduction

Heavy metal pollution from human activities poses a serious threat to biodiversity and the function of terrestrial and aquatic environments, food security, and global human health (Gall et al., 2015; Alava et al., 2017). Copper (Cu) and cadmium (Cd) contamination is of worldwide concern due to their wide distribution and potential exposure to populations (Rizwan et al., 2016). For instance, Cu has reached contaminated levels in urban soils in Italy and Mexico (Imperato et al., 2003; Morton-Bermea et al., 2009). In addition, it is reported that nearly 33.54% farmland sites and 44.65% urban sites were polluted with Cd

based on a literature survey with 355 farmland soil samples and 219 urban soil samples (Yuan et al., 2021). Increased Cu and Cd inputs to soils can have dramatic impacts on microbial communities and associated ecosystem functions (Luo et al., 2019). Importantly, soil microorganisms can drive various soil processes, such as promoting the cycling of carbon (C), nitrogen (N), and phosphorus (Fierer et al., 2012). Previous work from a long-term experiment showed that Cu pollution changed soil microbial communities (Zhang et al., 2022b). Studies based on short-term incubation experiments supported the idea that Cd amendments altered soil microbiomes and their ecological functions (Afzal et al., 2019; Zhao et al., 2020). However, the role of Cu and Cd

[☆] This paper has been recommended for acceptance by Yucheng Feng.

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co-contamination in shaping the taxonomic and functional attributes of agricultural soil microbes remains largely unexplored.

The strong co-occurrence of microbial taxa in ecological networks is generally referred to as ecological clusters or modules (Delgado-Baquerizo et al., 2018). Various microbial ecological modules tend to prefer particular environmental conditions. Furthermore, these ecological modules are likely to affect the maintenance of soil biological activity, nutrient cycling, and ecological service (Hartman et al., 2018). For example, certain ecological cluster constructed from dominant diazotrophic phylotypes is correlated with N fixation rates under long-term fertilization scenarios (Fan et al., 2019). de Menezes et al. (2015) reported strong correlations between microbial taxa within some ecological clusters and soil pH, ecosystem types, and nutrient availability. However, despite the importance of these ecological clusters for understanding the innate soil microbiota, little is known about how these ecological clusters respond to Cu and Cd contamination in the field (Afzal et al., 2019). Network analysis has been used to reveal the complex interactions of microbes under various conditions, such as sediments (Yin et al., 2015) and plants (Hartman et al., 2018). Constructing an ecological network is crucial to identify changes in microbial interactions and tolerant soil microbes under Cu and Cd contamination.

The metabolic capabilities of soil microbiome, their activities and performances responses to Cu and Cd gradients remain unclear. This information can provide direction for developing bioremediation strategies of polluted agricultural soils, but this work is challenging due to the highly diverse soil microbial communities (Delgado-Baquerizo et al., 2018). Recent advances in omics-related techniques can track the functional attributes of microbial communities and then identify the sensitive traits to soil Cu and Cd contamination gradients (Kelly et al., 2021). For example, high-throughput sequencing and metagenomics approaches revealed a great potential for arsenic biotransformation even in the low-arsenic environments in five paddy soils in southern China (Xiao et al., 2016). Liu et al. (2018) used metagenomics to investigate the genetic profiles of microbial communities in typical mercury-impacted regions in China, and found that mercury pollution affected soil element cycling and mercury transformation. These studies using omics techniques have broadened our understanding of the response of microbial functional traits to soil heavy metals.

This study aimed to clarify how multiple soil taxonomic and functional attributes responded to Cu and Cd contamination at a regional scale. We hypothesized that environmental variables changed metabolic potential through altering bacterial communities, and that heavy metal-tolerant microbes inhabited Cu and Cd contaminated sites. For this purpose, we collected paddy soils from Cu- and Cd-impacted agricultural areas polluted by long-term river sewage irrigation in southern China. Thus, this study area provided an excellent natural environment to empirically evaluate the response of indigenous microbes to soil Cu and Cd content gradients. A systematic analysis combining environmental variables analysis, 16 S rRNA gene and metagenomics sequencing was performed. The objectives of our study were (i) to reveal the diversity and community of bacteria inhabiting Cu- and Cd-contaminated soils, (ii) to identify core heavy metal-resistant or sensitive bacterial taxa, and (iii) to explore the impact of Cu and Cd pollution on metabolic potential with a focus on metal resistance and C and N cycling.

2. Materials and methods

2.1. Site description and soil collection

The survey was conducted in the Xiancha River basin in Jiangxi province, southern China (Fig. S1). This area has a subtropical monsoon climate with a mean annual temperature of 18.6 °C and mean annual precipitation of 1726 mm. With more than 80 years of tungsten mining history (Guo et al., 2022; Zhang et al., 2022a), mine discharges into the Xiancha River basin caused severe Cu and Cd pollution in paddy fields.

In November 2020, we sampled soils from 23 sites in this area (designated XCHB01-23, Table S1), which was geographically divided into three zones: 6 sites in the upstream (Zone I), 10 sites in the midstream (Zone II), and 7 sites in the downstream (Zone III). The utilization types of sampling sites are all paddy fields, and the cropping system is typical rice-rice rotation. At each site, four replicate soil samples were collected, and five cores in the 0–15 cm soil layer were pooled together to form a composite sample. A total of 92 soil samples were collected from the rice fields after harvest. All soil samples were homogenized and sieved through a 2-mm mesh to remove crop residues. Each sample was subdivided into two subsamples. One subsample was stored at –80 °C for DNA extraction and microbial analyses, while the other subsample was stored at 4 °C for determination of environmental variables.

2.2. Measurement of environmental variables

For the determination of total Cu, Cd, Pb, As, Zn, Ni, and Cr (M_{tot} , M stands for metals, hereinafter), soil samples were fully digested with trace mixed acids ($\text{HF-HNO}_3\text{-HClO}_4$) in a microwave digestion system. The bioavailable fraction of these metals (M_{bio}) was extracted using diethylene-triamine pentacetic acid (DTPA). Then, the total and bioavailable heavy metals in the final solution were analyzed by an inductively coupled plasma-mass spectrometer (Thermo Fisher, USA). Blanks and certified reference materials of GBW07429 were used for quality control, and recoveries of different heavy metals ranged from 96% to 103%. The analyses of soil general properties such as pH, total carbon (TC), and total nitrogen (TN) are provided in the supplementary material.

2.3. Illumina sequencing of 16 S rRNA gene

Total soil DNA was isolated from 0.5 g of soil using the PowerSoil® DNA Isolation Kit (Mo Bio Laboratories, CA, USA) following the manufacturer's protocol. DNA content and quality were confirmed by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The V3–V4 hypervariable region of 16 S rRNA gene was amplified using primer set 338F/806 R (Du et al., 2018). Amplicon sequencing was performed using an Illumina NovaSeq platform. Raw reads were subsequently processed using Quantitative Insights Into Microbial Ecology (QIIME2) (Bolyen et al., 2019), and DADA2 method was employed for filtering, denoising, splicing, primers and chimera removal (Callahan et al., 2016). At the end of the workflow, an amplicon sequence variant (ASV) table was obtained. Each ASV was taxonomically assigned against the SILVA database using the RDP classifier (Quast et al., 2013). Each sample was rarified to the same sequence depth for downstream analysis.

2.4. Metagenome sequencing and analysis

Thirty-six soils (12 soils from each of zones I, II, and III) were selected for metagenomics sequencing from our 92 samples (Table S1). After analysis for Cu and Cd, these samples were chosen to yield a wide range of Cu and Cd concentrations (15.17–267.26 mg kg⁻¹ for Cu and 0.10–2.93 mg kg⁻¹ for Cd). Metagenome libraries were constructed using the NEB Next Ultra DNA Library Prep Kit for Illumina (New England Biolabs, MA, USA) following the manufacturer's protocol. Library quality was assessed by Qubit 3.0 Fluorometer (Life Technologies, Grand Island, NY) and Agilent 4200 (Agilent, Santa Clara, CA) systems. Sequencing was performed using the Illumina HiSeq X-ten platform (paired-end 150 bp reads). A total of 494.35 Gb raw sequence data was generated (3,295,638,542 raw reads) from the 36 metagenomes (Table S2). Raw reads were quality-controlled using Trimmomatic v0.36 (Bolger et al., 2014) and then assembled into longer contigs using MEGAHIT v1.0.6 (Li et al., 2015). Open reading frames (ORFs) were predicted for contigs ≥500 bp with MetaGeneMark v3.38 (Zhu et al., 2010). Reads from each sample were mapped to contigs using BBMap

(<http://jgi.doe.gov/data-and-tools/bbttools/>) to calculate relative abundance. Contigs were searched against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database for functional analysis (Kanehisa and Goto, 2000). See Table S2 for details of the bioinformatics analysis. Raw sequencing data of 16 S rRNA and metagenomes were available in the NCBI Sequence Read Archive under accession number PRJNA908650.

2.5. Statistical analysis

Statistical comparisons of test parameters between sampling zones were performed using one-way analysis of variance (ANOVA) combined with Duncan's test, and $P < 0.05$ was considered significant. Alpha diversity was assessed using the Observed species, Chao1, Simpson, and Shannon in QIIME2. Principal coordinate analysis (PCoA) was analyzed based on the weighted UniFrac distance matrices using the 'vegan' package in R (Lozupone et al., 2011). An analysis of similarity (ANOSIM) was performed to test the significance of beta diversity. Linear or nonlinear regression was used to assess the associations between soil Cu and Cd contaminant fractions and Simpson index, cumulative relative abundance of modules, and relative abundance of selected bacterial taxa. We conducted random forest (RF) analyses using the 'randomForest' package in R to predict the associations between environmental variables and microbial attributes.

Three co-occurrence networks were established in our study. The interactions between environmental variables and individual microbial taxa (ASVs) and functional genes were performed with the 'igraph' package in R. The results were visualized using the Gephi. To identify ecological modules of bacterial taxa, bacteria-bacteria interaction networks were established. The cutoff values for microbial interactions were Spearman correlation >0.7 and $P < 0.001$. Ecological clusters in

the network were detected using the greedy modularity optimization method. The Fruchterman-Reingold layout with 10^4 permutations in the 'igraph' package was used to visualize the network. We used counts per million (CPM) computed using the "trimmed means of M" (TMM) method to express the cumulative relative abundance of modules. The ASVs confirmed by indicator species analysis and likelihood ratio test were defined as zone-sensitive ASVs whose abundance varied between zones.

3. Results

3.1. Soil heavy metals and general properties

Soil Cu and Cd concentrations in zone I and most of soil Cd concentrations in zone II exceeded the screening standard values for farmland soil contamination in China (GB 15618–2018; Table S3). Therefore, Cu and Cd were selected for further analysis. Total Cu and total Cd (Cu_{tot} and Cd_{tot}) concentrations were relatively higher in zone I compared to zones II and III (Fig. 1). Significantly higher Cd_{tot} concentrations were observed in zone II compared to zone III. Bioavailable Cu and bioavailable Cd (Cu_{bio} and Cd_{bio}) accounted for 19.1% and 60.6% of Cu_{tot} and Cd_{tot} , respectively (Fig. 1). Also, the concentrations of Cu_{bio} and Cd_{bio} were higher in zone I than in other zones (Fig. 1).

The paddy soils were acidic with pH values ranging from 4.4 to 6.2, especially the strongly acidic zone III (4.63 ± 0.03 , Table S4). The highest TC and TN contents were found at zone II, while no differences were found between zone I and zone III. In contrast, zone I exhibited significantly higher TP and DOC concentrations compared to other zones. The highest nitrate concentration was observed in zone III, whereas ammonium and DON decreased in zone III soils. The differences in soil AP concentrations between the three zones were not significant.

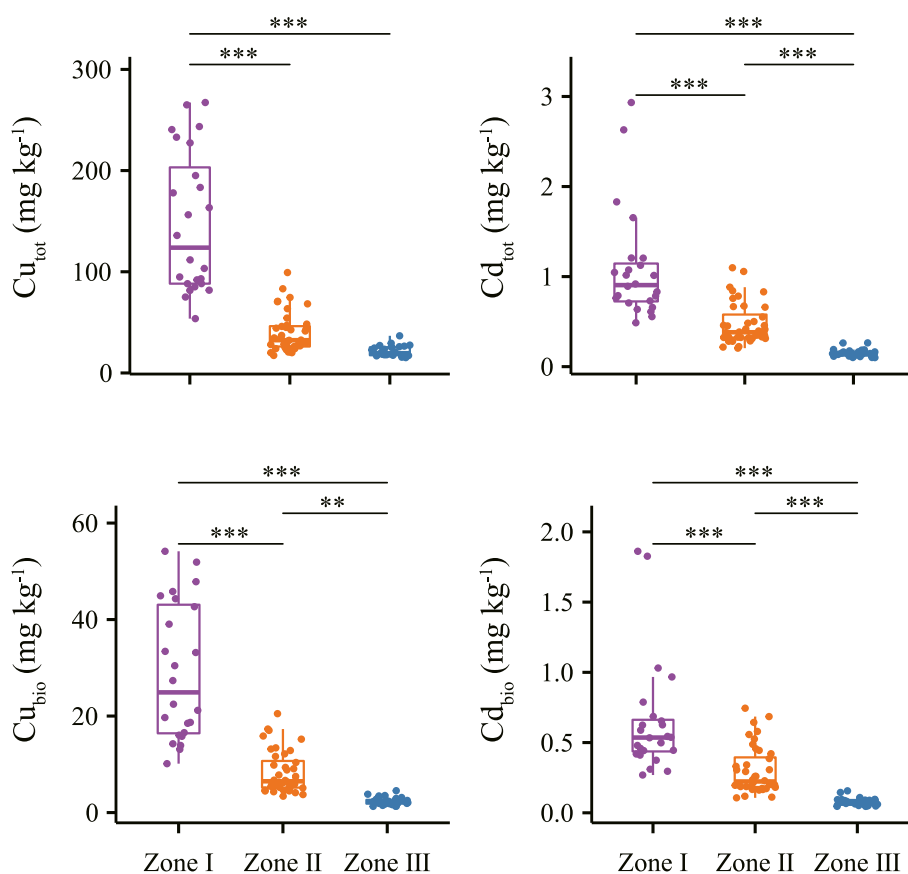


Fig. 1. Boxplots show the concentrations of total and bioavailable Cu and Cd in the three sampling zones. ** and *** indicate significant differences at $P < 0.01$ and <0.001 as determined by Duncan's test, respectively.

3.2. Bacterial community analysis

Among the bacterial alpha diversity indices, the Shannon and Simpson indices were relatively lower in zone I than in zones II and III (Fig. 2a). However, there were no significant differences in the Observed species and Chao1 indices among the three sampling zones (Fig. 2a). PCoA analysis showed that the bacterial members clustered according to the sampling zones (Fig. 2b). ANOSIM tests were also performed to support the PCoA results.

The top ten most abundant phyla in all soils were Proteobacteria (27.9%), Chloroflexi (23.1%), Acidobacteria (21.5%), Actinobacteria (8.1%), Nitrospirae (5.0%), Bacteroidetes (3.5%), Gemmatimonadetes (2.2%), Rokubacteria (1.6%), Patescibacteria (1.3%), and Verrucomicrobia (1.0%) (Fig. S2). A clear zone-specific distribution of bacterial phyla was observed. For example, Chloroflexi, Actinobacteria, Gemmatimonadetes, and Rokubacteria were relatively enriched in zone I. Proteobacteria and Nitrospirae were significantly enriched in zone II, whereas Patescibacteria were strongly enriched in zone III (Fig. S2). At the genus level, the relative abundance of 15 genera was elevated (>1%) in paddy soils (Fig. S3). *AD3* (4.8%), *Subgroup_2* (2.1%), *HSB_OF53-F07* (1.7%), and *Rokubacteriales* (1.0%) were significantly enriched in zone I, while *KD4-96* (4.1%), *BSV26* (2.1%), *Bryobacter* (1.6%), *Subgroup_7* (1.4%), and *SC-I-84* (1.1%) showed significantly higher relative abundance in zones II and III (Fig. S3).

3.3. Correlations between bacterial communities and environmental variables

The RF model of the effect of environmental variables on bacterial alpha diversity (represented by the Simpson index) indicated that 65% of the variation could be explained using the model (Fig. 3a). Among the selected environmental variables, the effects of Cu_{tot} , Cu_{bio} , TP, Cd_{tot} , ammonium, and Cd_{bio} on the Simpson index were the highest. Regression analysis exhibited that soil Cu and Cd contaminant fractions were linearly and negatively correlated with Simpson index, implying that heavy metal pollution could decrease bacterial diversity (Fig. 3b).

Further analyses were conducted to investigate the interactions between environmental variables and individual bacterial taxa (ASVs). The co-occurrence networks indicated that Cu_{tot} , Cd_{tot} , Cu_{bio} , and Cd_{bio} were probably the most important variables shaping bacterial communities according to the largest nodes (Fig. 4). In contrast, the nodes

associated with nitrate, DON, pH, TP, ammonium, DOC, and TC were relatively small, suggesting that these variables could have less impact on the bacterial assemblage. The network consisted of seven modules, namely modules I-VII (Fig. 4). Nodes in the same module often shared many similar traits. Importantly, the variables related to Cu and Cd were only grouped into modules I and II, respectively (Fig. 4).

3.4. Bacterial co-occurrence network

Soil bacterial taxa were classified into three major ecological clusters, consisting of bacterial taxa that were strongly co-occurring with each other (modules #1, #2, and #3; Fig. 5a). Interestingly, the three zones were dominated by distinct ecological clusters (Fig. 5b). Zone I exhibited a relatively higher cumulative relative abundance of module #1 compared to other zones. However, the cumulative relative abundance of modules #2 and #3 was the highest in soils from zone III. The zone-sensitive ASVs from zone I accounted for 94.6% in module #1 ASVs, whereas that proportion from zone III in modules #2 and #3 was 68.7% and 82.8%, respectively (Fig. 5a). The cumulative relative abundance of module #1 was significantly and positively correlated with Cu_{tot} and Cd_{tot} concentrations (Fig. S4). In contrast, exponential negative associations were found between the cumulative relative abundance of module #2 and Cd_{tot} concentrations and between the cumulative relative abundance of module #3 and Cu_{tot} and Cd_{tot} concentrations (Fig. S4). According to RF analysis, soil heavy metal variables had significant effects on the cumulative relative abundance of modules (Fig. S5). Soil general properties were also important predictors of bacterial communities, although their relative importance varied greatly by taxa and module. See Table S5 for the Pearson correlations between bacterial attributes and environmental variables.

All ecological clusters were formed by multiple genera (Fig. 5c). For example, *HSB_OF53-F07* and *AD3* dominated in module #1, *Saccharimonadales* and *BSV26* in module #2, and *RBG-13-54-9*, *Bryobacter*, *Candidatus_Koribacter*, and *Subgroup_2* in module #3, respectively. The relative abundances of several genera were linearly or exponentially correlated with soil Cu and Cd gradients. The relative abundances of *AD3*, *HSB_OF53-F07*, *Rokubacteriales*, and *Nitrospira* were linearly and positively correlated with Cu_{tot} and Cd_{tot} concentrations (Fig. 6). In contrast, the relative abundances of *BSV26*, *Bryobacter*, *Pajaroellobacter*, and *WPS-2* were exponentially and negatively correlated with Cu_{tot} and Cd_{tot} concentrations (Fig. S6).

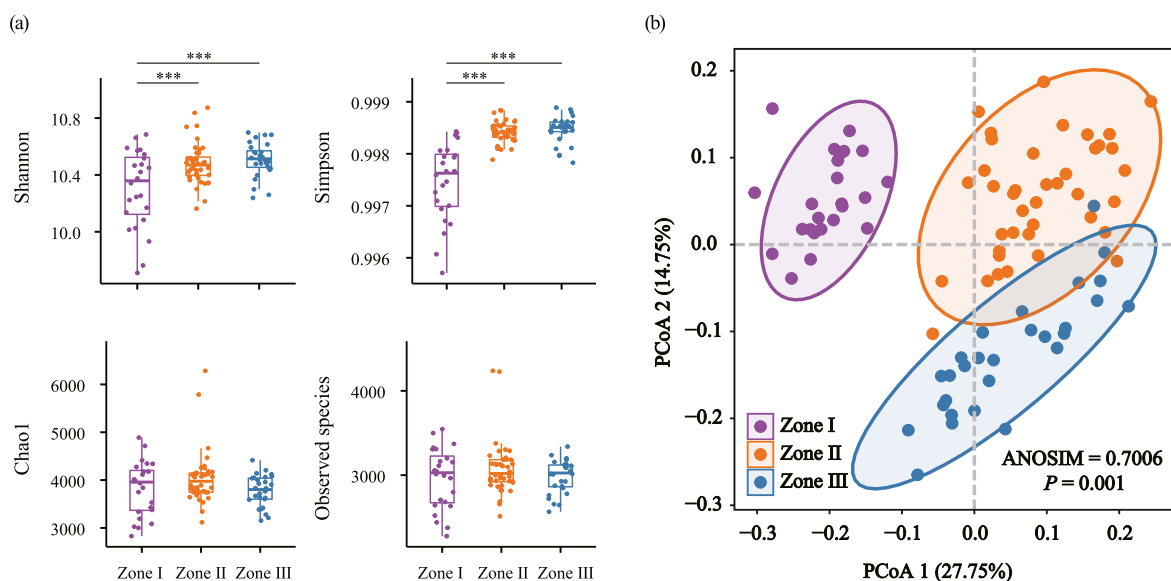


Fig. 2. (a) Bacterial alpha diversity metrics for the three sampling zones. **** indicates a significant difference at $P < 0.001$ as determined by Duncan's test. (b) Principal coordinate analysis (PCoA) of the weighted UniFrac distance matrices of bacterial communities from the three zones.

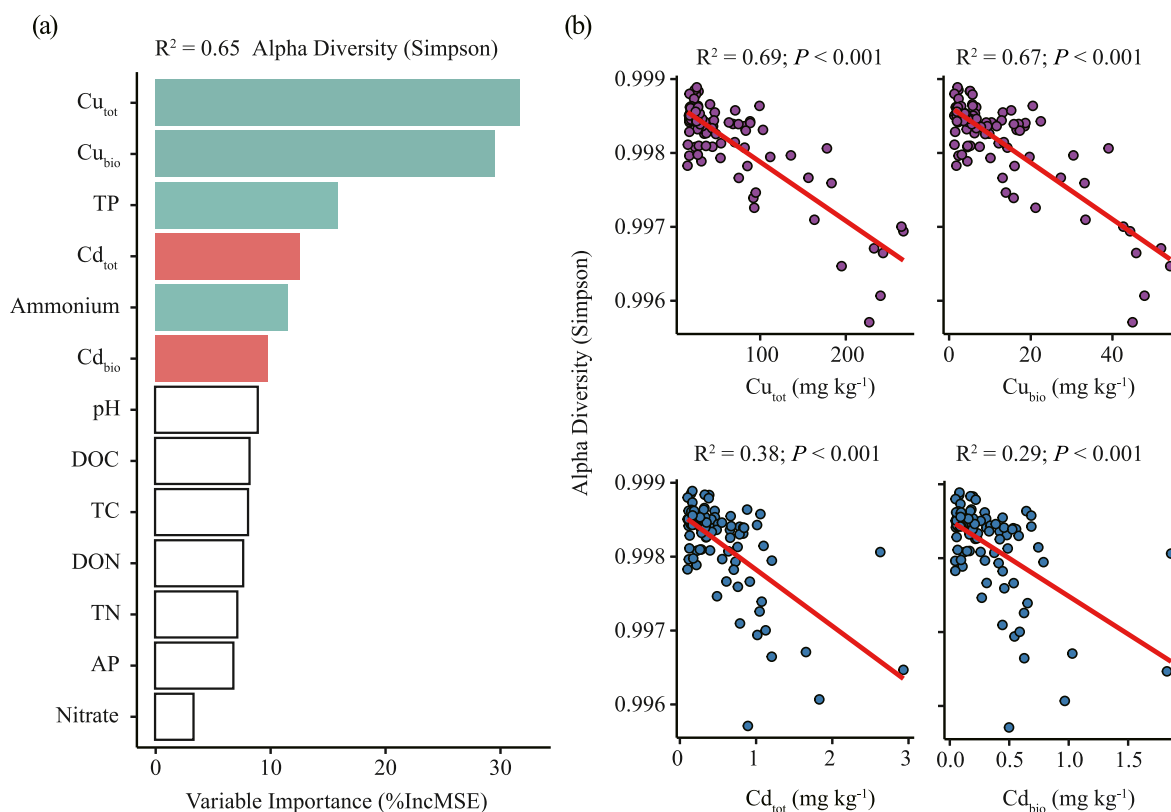


Fig. 3. (a) Random Forest model output relating environmental variables to the Simpson index. Green, red, and white bars denote $P < 0.05$, $P < 0.10$, and $P > 0.10$, respectively. (b) Regression between the concentrations of total and bioavailable Cu and Cd and the Simpson index. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.5. Metabolic potential revealed by metagenomic analysis

The microbial metabolic potential of paddy soils was inferred from the genes and pathways identified in the metagenomes. The genes associated with Cu and Cd resistance were selected for further analysis (Fig. 7). Cu and Cd resistance genes were detected in all soils. However, the relative abundance of these genes varied across the three zones. Most gene clusters related to Cu and Cd resistance, including *copAB*, *pcoBCD*, *ccmF*, *cusABFR*, *cueR*, *csr*, *zntA*, *czcABCD*, *cadC*, and *zipB*, showed significantly higher relative abundances in zone I than other zones (Fig. 7a). Most of Cu- and Cd-related genes in different sampling zones were linked to *Candidatus Sulfofopaludibacter*, *Bradyrhizobium*, and *Candidatus Sulfofotelmatobacter* (Fig. 7b).

Microbial metabolic related genes involved in C (C fixation) and N cycling were analyzed (Figs. S7 and S8). Six C fixation pathways have been identified in the 36 analyzed metagenomes. The reductive citric acid cycle (rTCA) was the most abundant, whereas the reductive acetyl-CoA pathway (Wood-Ljungdahl pathway) was the least abundant (Fig. S7a). Most C fixation pathways were associated with *Bradyrhizobium*, *Candidatus Sulfofopaludibacter*, and *Pseudolabrys* (Fig. S7b). The genes related to nitrification (e.g. *amoABC* and *hao*) and the genes related to denitrification (e.g. *narGH*, *napAB*, *nirK*, and *nosZ*) showed significantly higher relative abundances in zone I compared to other zones (Fig. S8a). However, the lowest relative abundance of nitrogenase gene *nifDHK* was observed in zone I. Most of the N cycling genes were associated with *Bradyrhizobium*, *Candidatus Sulfofopaludibacter*, and *Anaeromyxobacter* (Fig. S8b).

The interactions between environmental variables and functional genes related to Cu and Cd resistance and C and N cycling were investigated. Thus, Cu_{tot}, Cd_{tot}, Cu_{bio}, and Cd_{bio} had substantial effects on the selected functional genes, followed by TP, nitrate, pH, ammonium, AP, DOC, TN, TC, and DON (Fig. 8). The network contained four major

modules, namely modules I-IV (Fig. 8). Interestingly, the Cu and Cd fractions had similar effects on the functional genes since they were all located within module I. The genes involved in Cu and Cd resistance (e.g. *copAB*, *pcoBCD*, *ccmF*, *cusABFR*, *cueR*, *csr*, *zntA*, *czcABCD*, *cadC*, and *zipB*), C fixation (e.g. the hydroxypropionate-hydroxybutyrate cycle (3-HP/4-HB) and the 3-Hydroxypropionate bi-cycle (3-HP)), nitrification (e.g. *amoABC* and *hao*), and denitrification (e.g. *narGH*, *napAB*, *nirKS*, and *nosZ*) were significantly and positively correlated with one or more Cu and Cd fractions (Fig. 8). However, all genes involved in N fixation (e.g. *nifDHK*) were significantly and negatively correlated with Cu and Cd concentrations.

4. Discussion

4.1. Bacterial communities influenced by environmental variables

Long-term sewage irrigation and contamination by Cu and Cd in the Xiancha River basin have had a profound impact on the indigenous microbial community. Microbial diversity is a crucial indicator for assessing ecosystem function (Bowles et al., 2014). We found that Cu and Cd pollution had a negative effect on bacterial diversity (according to the Simpson index) through RF model and regression analysis (Fig. 3). Biodiversity loss will reduce the ecological services provided by indigenous microbes (Yan et al., 2017). This result is in contrast to previous studies where an increase in soil bacterial diversity or no response to heavy metals was reported (Chodak et al., 2013; Wu et al., 2017). For instance, some metals such as Fe, Ni, and Cd can accelerate microbial metabolism and their toxicity varies with dose (Oladipo et al., 2018). In this study, Cu and Cd stress may inhibit or lead to the death of sensitive microbes and the reduction of total bacterial species, thereby decreasing the genetic diversity of microorganisms (Marques et al., 2009).

The selection pressure of Cu and Cd contamination resulted in

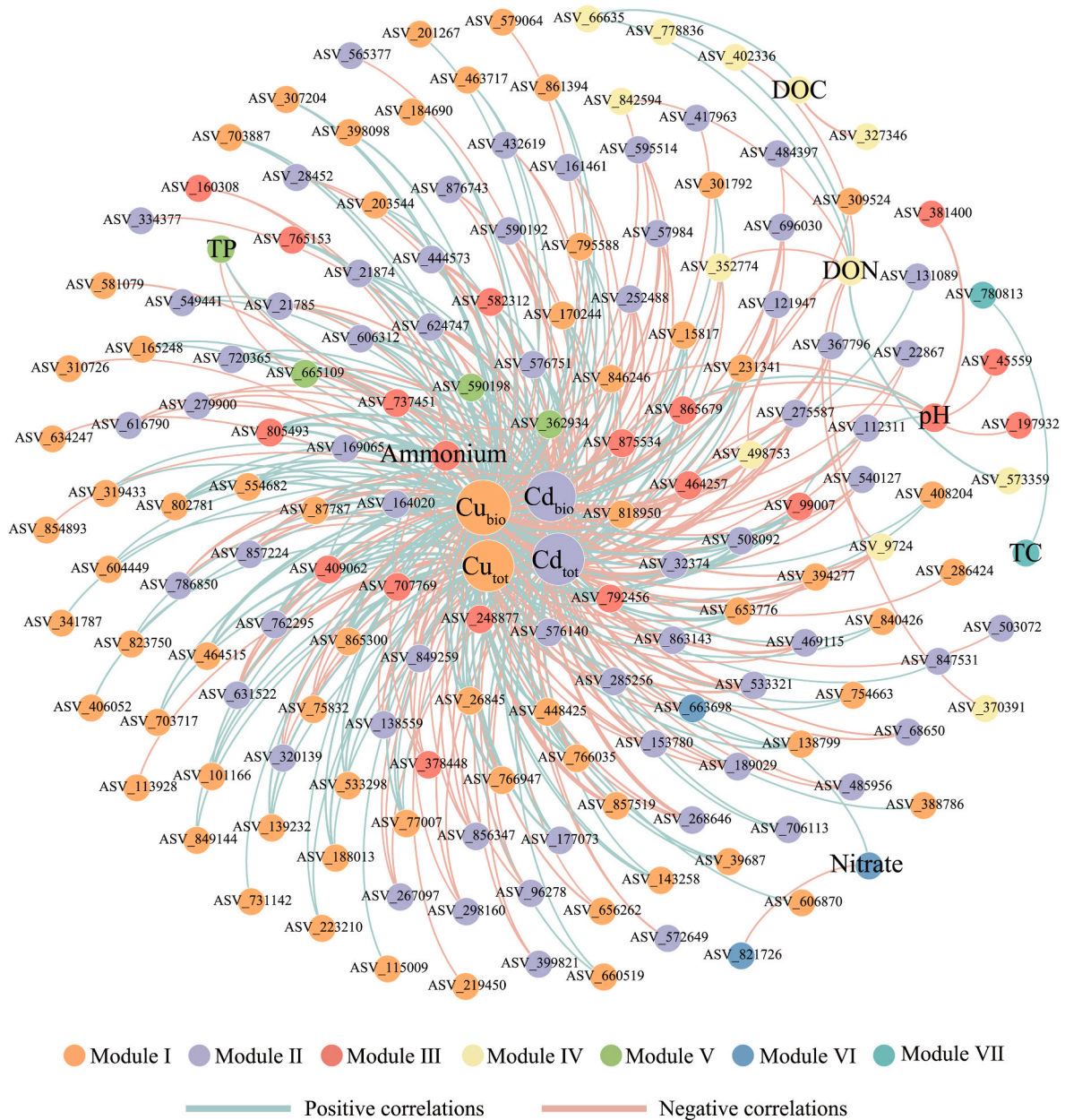


Fig. 4. Co-occurrence network analysis showing the correlations between environmental variables and bacterial taxa (ASVs). Green and red lines indicate significant positive and negative connections ($|\text{Spearman correlation}| > 0.6, P < 0.05$), respectively. The size of each node is proportional to the number of connections (i.e., degree). The thickness of the edges is proportional to the Spearman's correlation coefficient. The network is colored by module, and nodes clustered in the same module share the same color. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

distinct phylogenetic clustering of bacterial populations (Fig. 2b). This suggested that heavy metals play a critical role in microbial community assembling, which was consistent with results from other contaminated soils (Wu et al., 2017; Li et al., 2021). In detail, bacterial community dynamics were also observed for different gradients of heavy metal contamination (Figs. S2 and S3). This could be due to the inhibition of metal-sensitive microbes, which could promote the growth of resistant bacteria (Xu et al., 2018). The adaptation mechanisms of these bacterial groups were related to different microbial life activities. In this study, Proteobacteria, Chloroflexi, and Acidobacteria were the predominant bacterial phyla in the paddy soils (Fig. S2). The community composition was similar to other soil types, but the proportion of populations varied across soil types (Chodak et al., 2013). Chloroflexi, Actinobacteria, Gemmatimonadetes, and Rokubacteria were the most active bacterial populations in the heavily contaminated zones, suggesting that they

were adapted to Cu and Cd co-contaminated environments, whereas Patescibacteria was sensitive. For example, Chloroflexi is ubiquitous in extreme environments and is essential for sugar respiration, fermentation, and carbon dioxide fixation (Hug et al., 2013). However, Patescibacteria is susceptible to heavy metal contamination, which may be related to reduced metabolic potential and stress response due to reduced genome (Tian et al., 2020). Metal-resistant microbes can compensate for the loss of sensitive species to maintain stable micro-ecological conditions (Awasthi et al., 2014).

The interactions between bacterial communities and environmental variables were visualized using co-occurrence networks (Fig. 4). It was observed that Cu and Cd related species had larger sizes than soil general properties, suggesting that contamination may have a greater impact on the innate soil microbiome. Bioavailable Cu had a greater impact on individual bacterial groups than total Cu, although Cu_{bio} only

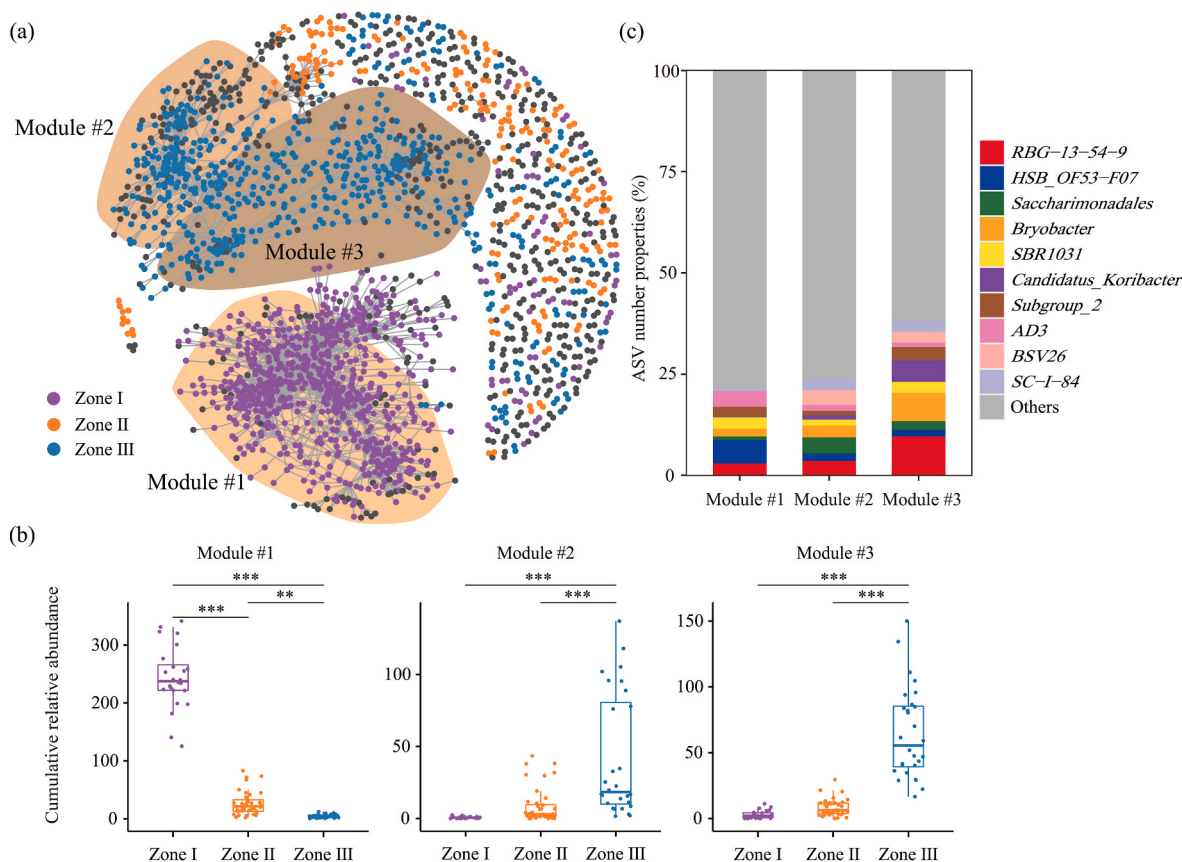


Fig. 5. (a) Co-occurrence network visualizing significant correlations (Spearman correlation >0.7 , $P < 0.001$; indicated with gray lines) of biological interactions. ASVs are colored according to their association with different sampling zones, and black ASVs are insensitive to sampling zones. Shaded areas represent the network modules #1–3. (b) Cumulative relative abundance (as counts per million, CPM; y-axis in $\times 1000$) of modules in the three sampling zones. ** and *** indicate significant differences at $P < 0.01$ and <0.001 as determined by Duncan's test, respectively. (c) Amplicon sequence variant (ASV) number properties of bacterial genera in the three modules.

constituted a minor proportion of the total concentration. The bioavailable fraction is readily released from the soil matrix and absorbed by microbial cells (Sullivan et al., 2013). This harsh environment may have forced the indigenous microbiota to evolve survival strategies. Indeed, Cu and Cd fractions showed various strong positive correlations with the ASVs related to the genera *AD3* (e.g. ASV_248,877, ASV_795,588, and ASV_865,300), *HSB_OF53-F07* (e.g. ASV_231,341, ASV_554,682, ASV_737,451, ASV_802,781, and ASV_818,950), *Rokubacteriales* (e.g. ASV_170,244 and ASV_582,312), and *Nitrospira* (e.g. ASV_653,776, ASV_720,365, ASV_805,493, and ASV_87,787), suggesting that these microbes were tolerant to Cu and Cd pollution.

4.2. Changes in the distribution of bacterial ecological clusters and core populations

Microbial interactions become particularly important in regulating microbial adaptation to metal pollution (Frossard et al., 2018). Our RF analysis revealed that Cu and Cd contaminant fractions had a significant effect on the ecologically preferential modules (Fig. S5). Notably, increased soil Cu and Cd pollution significantly altered the pattern of cumulative relative abundance in some modules (Fig. S4). Ecological modules associated with heavy metals are likely to cluster metal-resistant or sensitive microbes (Liu et al., 2018). For example, the cumulative relative abundance of module #1 increased along Cu and Cd gradients in the study area. This module was mainly composed of *AD3*, *HSB_OF53-F07*, *Rokubacteriales*, and *Nitrospira*, whose relative abundances were positively correlated with Cu and Cd (Fig. 5c and 6). These genera were relatively abundant in zone I (Fig. S3), and they may have a

special interest for tolerance in contaminated environments and provide services to other members of the bacterial community. In contrast, elevated Cu and Cd concentrations decreased the cumulative relative abundance of modules #2 and #3. The genera *BSV26*, *Bryobacter*, *Pajaroellobacter*, and *WPS-2* occurred in these modules and enriched in zones II and III. The negative relationships between the relative abundance of these bacterial genera and Cu and Cd concentrations suggested that they were sensitive to contaminated habitats (Fig. S6). These findings revealed that the gradient of Cu and Cd concentrations shifted the distribution in bacterial modules. Consistent with previous investigations (Delgado-Baquerizo et al., 2018; Liu et al., 2018), other environmental predictors also strongly correlated with the cumulative relative abundance of modules.

The genera *AD3* and *HSB_OF53-F07* (both belonging to Chloroflexi) may have high resistance to heavy metal toxicity, which makes them superior to heavy metal-polluted habitats (Liu et al., 2021; Lin et al., 2022). However, the physiological roles of the two taxa in Cu and Cd co-contaminated soils remain unclear. The existence of these taxa indicates a strong adaptation of the novel bacteria to extreme environments. Uncultured *Rokubacteriales* can tolerate multiple metal (loid)s in contaminated arable regions through membrane adsorption, antioxidant system, and intracellular complexation (Shahid et al., 2017; Liu et al., 2022). *Rokubacteriales* has also been reported to have the potential for aerobic respiration, fermentative metabolisms, and nitrogen respiration (Becraft et al., 2017). In this study, heavy metal contamination altered the relative abundance of *Rokubacteriales*, which may affect C and N cycling in soils. Also, *Nitrospira* exhibits a high tolerance to Cd due to its overproduction of electron-transporting cytochrome c-like protein (Lin

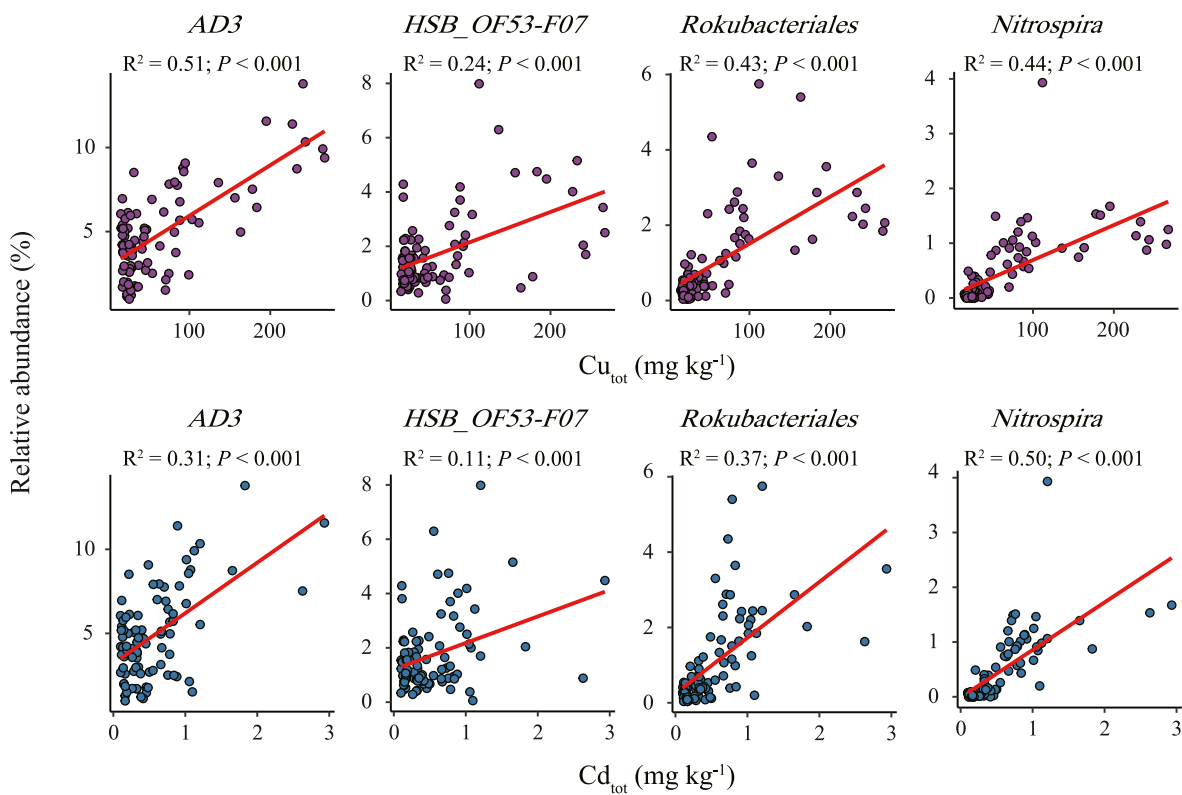


Fig. 6. Regression between soil total Cu and Cd concentrations and the relative abundance of selected bacterial genera.

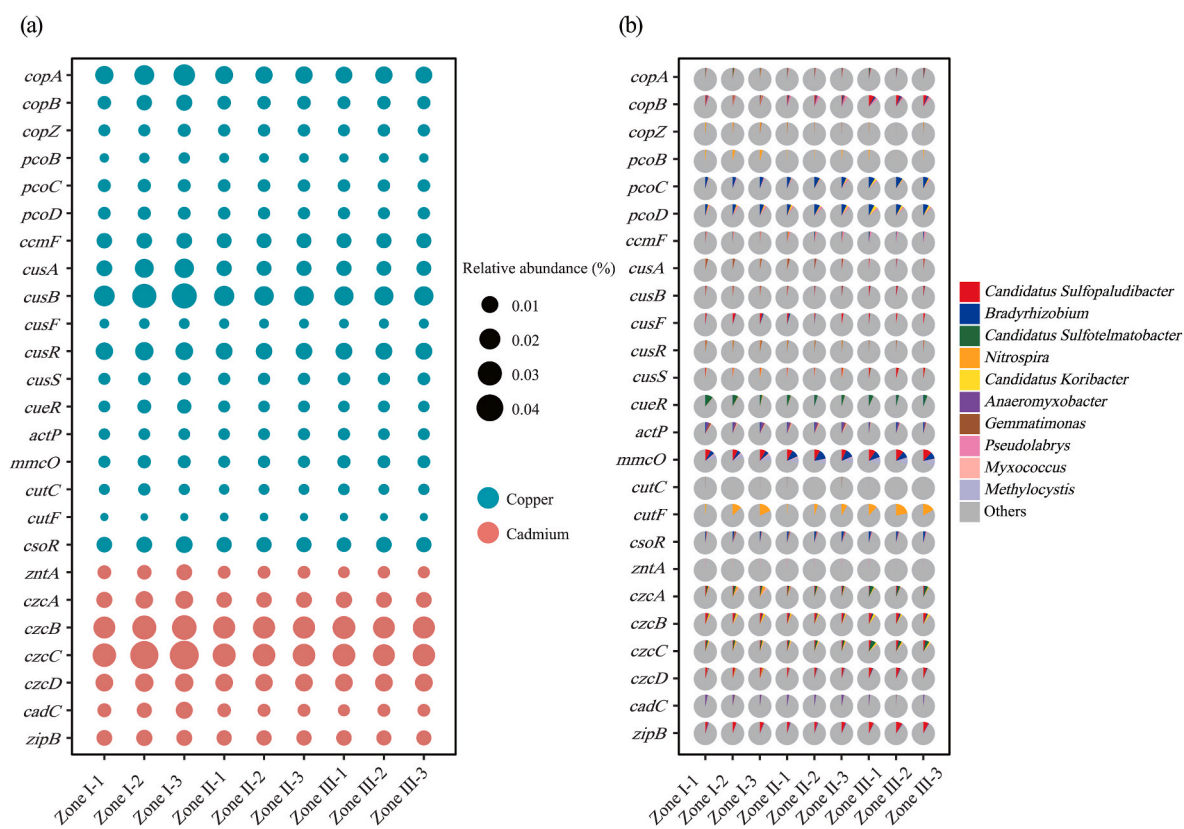


Fig. 7. (a) Percentage of reads attributed to Cu and Cd resistance genes in 36 shotgun metagenomic sequencing libraries. (b) Taxonomy of reads attributed to Cu and Cd resistance genes in 36 shotgun metagenomic sequencing libraries at the genus level.

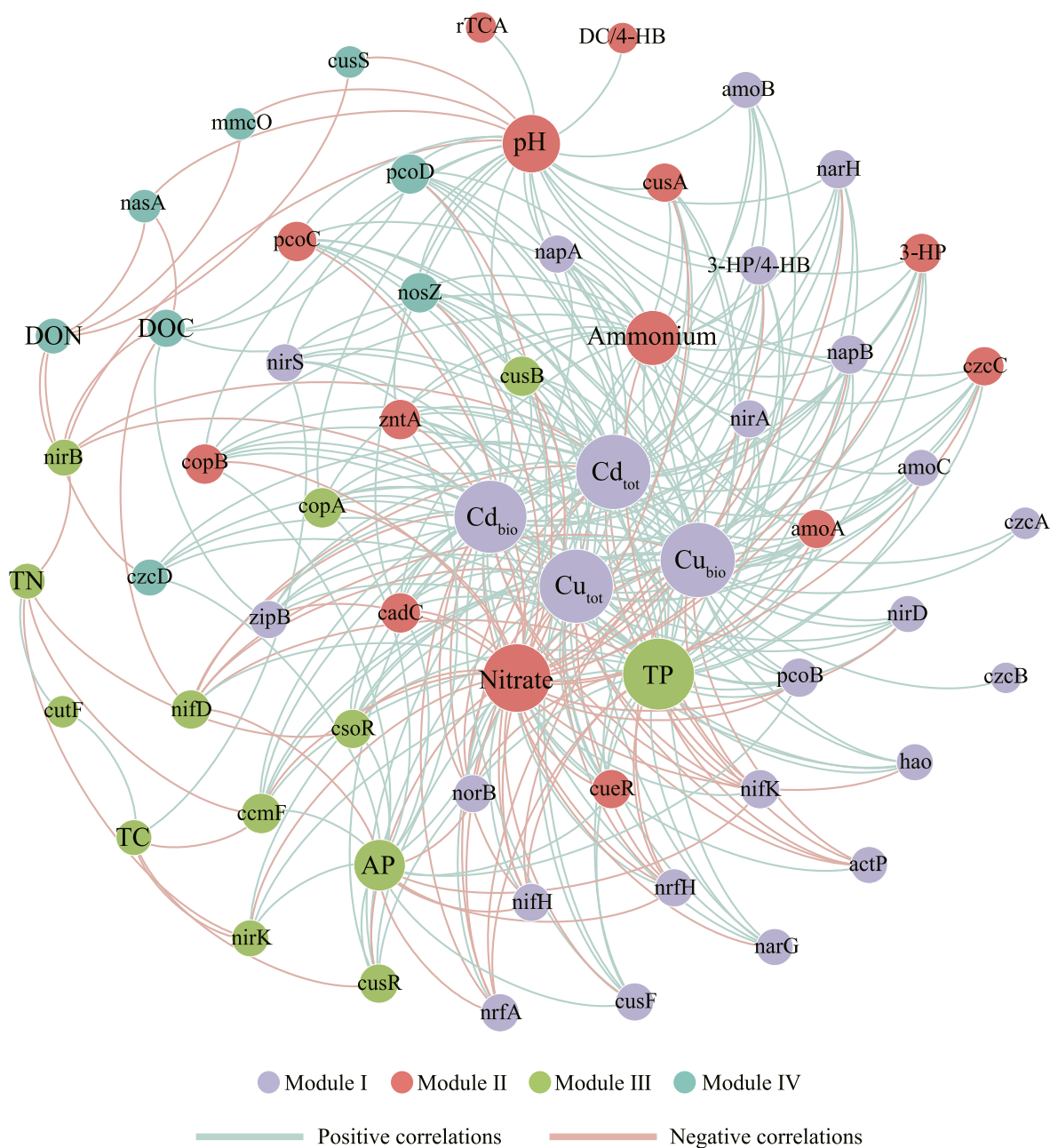


Fig. 8. Co-occurrence network analysis showing correlations between environmental variables and selected functional genes relating to Cu and Cd resistance and C and N cycling. Green and red lines indicate significant positive and negative connections ($|\text{Spearman correlation}| > 0.4, P < 0.05$), respectively. The size of each node is proportional to the number of connections (i.e., degree). The thickness of the edges is proportional to the Spearman's correlation coefficient. The network is colored by module, and nodes clustered in the same module share the same color. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

et al., 2021). This finding was consistent with our results that *Nitrospira* was significantly abundant in zone I. The high adaptability of these bacterial taxa to Cu and Cd contamination made them promising candidates for bioremediation. In contrast, some genera, such as *BSV26*, *Bryobacter*, *Pajaroellobacter*, and *WPS-2*, reduced exponentially with soil Cu and Cd gradients. These genera are out-competed by the predominant bacterial taxa, probably because they are sensitive to heavy metals. Changes in heavy metal-resistant and sensitive species altered the distribution of bacterial modules, and shifts of these modules may be an adaptation of bacteria to Cu and Cd contamination.

4.3. Effects of environmental variables on metabolic potential of microorganisms

Indigenous soil microbiomes exposed to toxic heavy metals have evolved different immune mechanisms and metabolic processes to adapt and withstand harsh environments (Ojuederie and Babalola, 2017). The relative abundance of metal resistant genes can reflect the survival strategies of these microorganisms. In this study, the gene clusters for Cu and Cd were selected to investigate microbial response to Cu and Cd contaminant fractions. The *copAB*, *pcoBCD*, *ccmF*, *cusABFR*, *cueR*, and *csor* genes were abundant in zone I (Fig. 7), and they were positively correlated with Cu fractions (Fig. 8). Among these selected genes, *copA* and *copB* encoding putative ATPases are associated with copper

transport (Navarro et al., 2013). A gene cluster consisting in *cusA* and *cusB*, related to a system of the resistance nodulation cell division family, can extract copper from cells using proton motive force (Chong et al., 2016). Also, cobalt-zinc-cadmium resistance genes of the *czc* operon (*czcA*, *czcB*, *czcC*, and *czcD*) were detected. These genes are responsible for the expression of chemiosmotic efflux pumps that expel the ions Co^{2+} , Zn^{2+} , and Cd^{2+} from the cytoplasm (Xavier et al., 2019). The increased relative abundance of resistance genes may be the main reason why the microbiome is able to survive high Cu and Cd concentrations. The taxonomic assignments of Cu and Cd resistance genes indicated that *Candidatus Sulfoaludibacter*, *Bradyrhizobium*, and *Candidatus Sulfoelmatobacter* had great potential to resist Cu and Cd contamination.

Microbe-mediated C and N cycling is essential for microbial growth and reproduction. Metal contamination affects the biogeochemical cycling of these critical elements through microorganisms (Zhang et al., 2021). C fixation was a strategy used by indigenous microbes to survive in contaminated environments (e.g. zone I). Among the detected C fixation pathways, rTCA was the prevalent C fixation pathway, whereas the Wood-Ljungdahl pathway was in the lowest relative abundance (Fig. S7a). The rTCA requires only two ATP equivalents to form pyruvate and requires less energy than other C fixation pathways with at least 5 ATPs, with the exception of the Wood-Ljungdahl pathway (Berg, 2011; Bar-Even et al., 2012). Therefore, the high energy efficiency of rTCA could explain its dominance in contaminated soils. The 3-HP/4-HB and 3-HP exhibited positive correlations with all of Cu and Cd fractions (Fig. 8), indicating that Cu and Cd pollution might enrich these related genes. This result was similar with a previous study showing that the 3-HP/4-HB and 3-HP were positively correlated with one or more of the arsenic and antimony contaminant fractions (Li et al., 2021). 3-HP has been reported to be dominant under extreme environmental conditions (Wei et al., 2023), which may account for its enrichment under metal contamination. In addition, some green non-sulfur bacteria of the Chloroflexi phylum are the main players in 3-HP (Shih et al., 2017). The increased relative abundance of Chloroflexi under Cu and Cd co-contamination may contribute to this pathway.

N fixation is particularly interesting because the N fixers involved in this process can provide bioavailable N to microbes and plants (Dos Santos et al., 2012). Previous studies have reported strong sensitivity of N fixers to metals in diverse environments (Kouchou et al., 2017; Sun et al., 2018). Such findings were consistent with our observations that, for example, the *nifDHK* genes encoding nitrogenase were negatively related to Cu and Cd fractions (Fig. 8). This suggested that Cu and Cd contamination could decrease N fixation efficiency through inhibiting the expression of related genes. Most of the N fixers in paddy soils were associated with *Geobacter*, *Bradyrhizobium*, and *Anaeromyxobacter*, these genera with some species have been reported as N-fixing organisms (Bahulikar et al., 2014). Soil Cu and Cd contamination may affect other N metabolisms. Several studies found that soil nitrification and denitrification genes were highly depressed by metal stress (Wang et al., 2018; Afzal et al., 2019). However, our results showed that the relative abundances of *amoABC*, *hao* (nitrification), *narGH*, *napAB*, *nirKS*, and *nosZ* (denitrification) were positively correlated with Cu and Cd (Fig. 8). Thus, the nitrifying and denitrifying populations have undergone adaptation and reproduction in response to Cu and Cd pollution. This may be related to multiple adaptation processes and resistance mechanisms of microorganisms. For example, some surviving populations of ammonia oxidizers can thrive through upregulating metal resistance genes and encoding multiple metal ion efflux proteins (Spang et al., 2012). Our findings revealed that increased Cu and Cd might enhance nitrification and denitrification activities and further accelerate N loss from paddy soils. It demonstrated the importance of decreasing heavy metal contamination in soils for future sustainable agriculture objectives. However, correlation analyses only implied the potential relationships between metals and microbes. In future studies, short- and long-term incubation experiments are needed to elucidate how and why soil microbial community and function change in response to Cu

and Cd stress. It is worth noting that genomic data only depicted the microbial metabolic potential. Transcriptomic or proteomic analyses are needed to clarify the in situ expression of these processes. In addition, there is a lack of information reported on agronomic parameters such as crop and soil management, which may influence the results on environmental drivers of microbial communities.

5. Conclusions

This study explored the response of microbes to Cu and Cd pollution in paddy soils. Soil Cu and Cd pollution significantly affected the taxonomic and functional attributes of microbes through changing bacterial diversity, the relative abundance of ecological clusters and functional genes. The Cu and Cd contaminant fractions were negatively correlated with bacterial alpha diversity, and these fractions had a greater impact on microbial communities and metabolic potentials than soil general properties. The relative abundances of *AD3*, *HSB_OF53-F07*, *Rokubacteriales*, and *Nitrospira* were positively correlated with Cu and Cd concentrations, while the relative abundances of *BSV26*, *Bryobacter*, *Pajarollobacter*, and *WPS-2* decreased along the Cu and Cd gradients. The changes in bacterial taxa indicated adaptation to heavy metals. The shotgun metagenomics analysis demonstrated that metal resistance genes may facilitate the survival of microbiomes in highly contaminated soils. Cu and Cd contamination affected microbial C and N fixation capacity, nitrification and denitrification processes. Overall, our results suggested that Cu and Cd contamination not only led to the enrichment of metal resistance genes but also influenced soil C and N cycling. This work improves our understanding of the microbial potential for implementing bioremediation strategies in paddy soils.

Credit author statement

Yifan Guo: Conceptualization, Formal analysis, Investigation, Writing - original draft, Visualization. **Shulan Cheng:** Methodology, Data curation. **Huajun Fang:** Conceptualization, Supervision, Funding acquisition, Writing - review & editing, Visualization. **Yan Yang:** Investigation, Visualization. **Yuna Li:** Investigation. **Fangying Shi:** Investigation. **Yi Zhou:** Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This research was funded by the Strategic Priority Research Program of the Chinese Academy of Sciences (Nos. XDA28130100, XDA200204020, XDA23060401), the Second Tibetan Plateau Scientific Expedition and Research Program (STEP) (No. 2019QZKK1003), National Natural Science Foundation of China (Nos. 41977041, 31770558), the “Thousand Talents Plan” Project of High-End Innovative Talents of Qinghai Province (No. TTP-PHEITQP-2019), and the “Unveiling the List of Hanging” and Technology Project of Jinggangshan Agricultural High-tech Industrial Demonstration Zone (No. 20222–051244).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2023.121724>.

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