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Heavy metal pollution decreases the stability of microbial co-occurrence networks in the rhizosphere of native plants

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Soil microorganisms play an important role in maintaining soil quality and function, although the response of soil microbial biodiversity to heavy metals has been extensively investigated, the microbe-microbe associations under the influence of both native plant species and extremely high heavy metal contamination are not well documented. We examined the diversity and composition of microbial communities and the physicochemical properties in the rhizosphere of three native plant species, *Carex breviculmis*, *Buddleja davidii*, and *Artemisia annua* growing on and around a Pb-Zn waste heap with a nearly 100-year history of natural recovery. Both plant species and heavy metals influence soil microbial diversity and composition. *C. breviculmis* and *A. annua* showed a prominent advantage in increasing rhizosphere microbial diversity and richness as well as network complexity compared with plant *Buddleja davidii* at severely contaminated soil, which was mainly related to the accumulation of soil nutrients such as soil organic carbon (SOC), total nitrogen ammonium nitrogen and nitrate nitrogen rather than a reduction in heavy metal concentrations. Moreover, the heavy metal concentration and soil nutrient levels significantly affected the microbial groups affiliated with Proteobacteria, Chloroflexi, Ascomycota, and Basidiomycota, in which those affiliated with Chloroflexi and Ascomycota were positively associated with heavy metals. Soil microbial network on the Pb-Zn waste heap exhibited higher average degree and a higher proportion of positive links than those around the waste heap, and thus soil microbial structure became more complexity and unstable with increasing heavy metal pollution.

KEYWORDS

trace element, Pb-Zn waste heap, biodiversity, co-occurrence patterns, plant-soil interaction

Introduction

Over the past several decades, prosperous mining activities have promoted the development of the Chinese economy. However, they have generated vast amounts of solid waste, which results in the degradation of the surrounding ecosystem (Becerra-Castro et al., 2012; Li et al., 2021). Li (2006) and Liu et al. (2020) reported that mining activities alone have generated approximately 3.2 Mha of wasteland and that this value is increasing by 46,700 ha/year in China. Waste materials contain large amounts of heavy metals and metalloids that often reach toxic levels and are susceptible to dispersing into the adjacent ecosystem *via* wind dispersion and water erosion (Luo et al., 2019), imposing a threat to life, including humans, plants, and microorganisms (Epelde et al., 2014). Some heavy metals and metalloids, such as Pb, Cd, Cr, and As, are nonessential for living organisms. However, because of their reactivity with thiols or other groups, these heavy metals can interfere with physiological processes even at low concentrations by disturbing enzymatic reactions, triggering cell membrane damage, and modifying deoxyribose nucleic acid (DNA) structure (Dubey et al., 2018; DalCorso et al., 2019). In China, soil heavy metal contamination resulting from mining activities has become a severe environmental problem, especially in areas rich in nonferrous metal resources (Li Q. et al., 2020).

Heavy metals impact ecosystems through a variety of effects on plants, soils, and microbial communities (Azarbad et al., 2013; Barra Caracciolo and Terenzi, 2021). Metalliferous soils, such as waste heaps, are often sandy and dry, with low organic matter and nutrient availability, as well as elevated heavy metal contents (Borymski et al., 2018). This leads to strong selective pressure driving the evolution and adaptation of plants and microorganisms (Epelde et al., 2014). In this context, some metal-tolerant plants have been the focus of previous studies (Becerra-Castro et al., 2012; Elbehiry et al., 2020) because of their biological mechanisms that resist and tolerate heavy metals. Pseudo-metallophyte populations of *Carex breviculmis* (Lei and Duan, 2008; Xu et al., 2020), *Buddleja davidii* (Margui et al., 2006), and *Artemisia annua* (Petriccione et al., 2013; Inelova et al., 2021) are metal-tolerant plant species that have exhibited good abilities in colonizing Pb, Zn, and Cd contaminated soils. Soil microorganisms play an important role in maintaining soil quality and function by decomposing organic matter, participating in nutrient cycling, and reducing heavy metal toxicity (Banning et al., 2011). Heavy metal/metalloids alter various metabolic processes of microorganisms, including blocking essential functional groups, displacing other metal ions, and inactivating biological molecules (Chen et al., 2020). The effect of heavy metal contamination on soil microbial communities is usually adverse, resulting in the reduction of microbial biomass and activity, the disturbance of specific taxa, and shifts in the composition of the microbiome (Azarbad et al., 2013; Epelde

et al., 2014). Although previous studies have revealed that soil microbial structure, composition, and diversity, can change under heavy metal contamination, it is unclear how microbe-microbe associations change with increasing heavy metal pollution, especially when considering the complex interactions between heavy metal/metalloid-contaminated soils and plants (Borymski et al., 2018; Chun et al., 2021). Therefore, identifying inter-taxa correlations is crucial for comprehensively understanding microbial community structures and predicting the response of soil ecosystems to future degenerative and pollution processes (Cheng et al., 2020).

In addition to the phytoextraction and phytostabilization of soil heavy metals, metal-tolerant plants strongly affect soil microbial biomass and activity through the rhizosphere effect, which is thought to arise from the deposition of various organic compounds, including amino acids, carbohydrates, and flavonoids, from plants into soils (Haichar et al., 2008; Montiel-Rozas et al., 2016). The nutrient-rich microenvironment provided by rhizosphere deposition allows certain microbial communities to thrive, as different plant species produce different exudates with specific compositions (Haichar et al., 2008; Borymski et al., 2018). Ali et al. (2013) reported that the metabolic activities of rhizosphere microorganisms are 10–100 times higher than those in bulk soil because of the exudates secreted by plants. Metal-tolerant plants select specialized metal-tolerant microorganisms to inhabit their rhizosphere, with a preference for plant growth-promoting bacteria that enhance the phytoremediation of heavy metal-contaminated soils (Hamidpour et al., 2019; Li Z. et al., 2020). Therefore, the interactions between metal-tolerant plants and accompanying microorganisms, as well as the surrounding environment, play key roles in maintaining nutrient cycling and element turnover (Epelde et al., 2010). On this basis, it is important to study the biodiversity of rhizosphere microbial communities linked with plant-soil-microbe interactions to better understand soil functions impacted by both heavy metal pollution and plant species to improve phytoremediation of mine sites in the future.

Northwestern Guizhou, China, is an area rich in nonferrous metal resources, especially lead zinc ore. This region has an approximately 300-year-old history of zinc mining and smelting using indigenous methods, and large volumes of Pb-Zn waste (more than 20 million tons by 2000) have been left and piled onto the surrounding farmland with almost no safety treatment (Yang et al., 2016). In this study, we examined a Pb-Zn waste heap with a nearly 100-year history, which has been naturally colonized by some native wild plant species (*C. breviculmis*, *B. davidii*, and *A. annua*) with limited human disturbance. We hypothesized that plant species will significantly affect rhizosphere microbial richness, diversity and interaction, and that soil microbe-microbe association will be different on and around the Pb-Zn waste heap. To test this hypothesis, we collected the rhizosphere of the aforementioned plant species both on and

around the Pb-Zn waste heap. Heavy metals/metalloids and soil properties were quantified, and microbial diversity was measured using high-throughput sequencing to: 1) assess the response of soil properties to plant species and sampling sites, including soil nutrients and heavy metal contents, as well as the diversity and composition of microbial communities; 2) explore the major environmental factors contributing to soil microbial community variations; and 3) profile the microbial association in the rhizosphere soil of three metal-tolerant plants on and around the Pb-Zn waste heap. The results of this study will provide crucial insights into the restoration of heavy metal-contaminated soils.

Methods and materials

Study site

An abandoned Pb-Zn waste heap located in the northwest of the Guizhou Province, China (26°54'N, 104°54'W) was used as the study site. The local climate is subtropical humid, with a mean annual precipitation of 800–1000 mm and an average temperature of 10 °C. Rainfall is irregularly distributed throughout the year, with 55% occurring from June to August. This area is famous for its long history of Zn smelting using indigenous methods, which generated large amounts of waste slag (more than 20 million tons by 2000) and formed numerous Pb-Zn waste heaps (Yang et al., 2016). Waste slag from Zn smelting is a mixture of ore smelting slag and small amounts of unburned cinders and contains high levels of toxic heavy metals, including Cu, Pb, Zn, and Cd (Luo et al., 2019).

Sample collection

At the time of this study, the selected Pb-Zn waste heap is nearly 100 years old, during which period it has been restored naturally, almost without human interference. *C. breviculmis*, *B. davidii*, and *A. annua* are common native wild plant species on and around the Pb-Zn waste heap. *C. breviculmis* is the dominant perennial herb. *A. annua* is a late-successional plant in the herbaceous stage, and *B. davidii* is a pioneer plant in the shrub stage.

Four replicate rhizosphere samples of *C. breviculmis*, *B. davidii*, and *A. annua* were collected on and around the abandoned Pb-Zn waste heap. Relatively isolated plants were selected to reduce the impact of nearby plants and consistent environmental conditions were ensured to reduce the impact of environmental heterogeneity, when collecting the rhizosphere of each plant. The rhizosphere soil samples were collected by shaking off the loosely attached bulk soil

adhered to the root systems. In addition, unvegetated soil was collected from the Pb-Zn waste heap as a control (CK). Each sample was divided into two subsamples after all roots, stones, and litter were removed. One subsample was immediately stored at -80 °C for microbiological analysis, and a second subsample was air-dried for physicochemical analysis. Sampling sites on and around the Pb-Zn waste heap were defined as S1 and S2, respectively, and the rhizosphere samples of *C. breviculmis*, *B. davidii*, and *A. annua* were defined as CB, BD, and AA, respectively.

Physicochemical analyses

Soil organic carbon (SOC), total nitrogen (TN), total phosphorus (TP), available phosphorus (AP) ammonium nitrogen (AN), nitrate nitrogen (NN), soil pH, and heavy metals were quantified using standard methods and detailed descriptions are provided in the Supplementary Materials.

DNA extraction, sequencing, and data processing

DNA was extracted from 0.5 g of each soil sample using a FastDNA Spin Kit (MP Biomedicals, Cleveland, United States) following the manufacturer's specifications. DNA extracts were diluted ten-fold and assessed for quality and quantity using a spectrophotometer (NanoDrop ND-1000, NanoDrop Technologies, Wilmington, United States). The integrity of the DNA extracts was confirmed by 1% agarose gel electrophoresis. The bacterial 16S rRNA and fungal 18S rRNA genes were amplified using the primer pairs 338F/806R (Deng et al., 2018) and ITS1F/ITS2R (Bellemain et al., 2010), respectively. The primers were tagged with unique barcodes for each sample. A PCR was performed in a 30 µL solution containing 2 µL of sterile ultrapure water, 15 µL of Phusion Master Mix (2X), 3 µL of 6 µM primer, and 10 µL of template DNA (5–10 ng). A PCR was conducted at 98 °C for 60 s, followed by 30 cycles of 98 °C for 10 s, 50 °C for 30 s, 72 °C for 30 s, and 72 °C for 5 min. The amplicons were purified and high-quality libraries were generated using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, United States) following the manufacturer's recommendations. All samples were pooled in equimolar amounts and sequenced on an Illumina HiSeq PE300 platform.

Bacterial and fungal sequences were analyzed using the UPARSE pipeline (Edgar 2013). Briefly, paired-end reads were merged into single sequences, after which the chimeras were removed using the UCHIME algorithm (Caporaso et al., 2010). Sequences with 97% similarity were clustered into operational taxonomic units (OTUs). Low-abundance OTUs

were eliminated from the OTU table if they did not present at least two counts across all samples in the experiment. Bacteria were identified using the Silva reference database (<http://www.arb-silva.de>) with the RDP classifier (Wang et al., 2007) and fungi were identified using the Unite database (<https://unite.ut.ee/>) with the BLAST tool in QIIME (Abarenkov et al., 2010).

Statistical analysis

Soil properties, microbial diversity, and abundance at each sampling site were evaluated among the three plant species, using a one-way analysis of variance, followed by an LSD post hoc test at $p < 0.05$. Soil properties, microbial diversity, and abundances between the two sampling sites were compared using a t -test ($p < 0.05$). The Shannon and Richness indices were used to represent plant diversity, and Chao1 and Shannon indices were used to indicate the richness and diversity of the soil microbial communities. A two-way ANOVA was conducted to determine the individual and interacting effects of plant species and sampling sites on each soil property, bacterial and fungal composition and α -diversity. Principal Co-ordinates Analysis (PCoA) and an ANOSIM test based on the Bray–Curtis distances of the sequencing data was used to assess differences in the structures of the microbial communities. Mantel tests based on Bray–Curtis distance similarities calculated at the OTU (operational taxonomic unit) level were used to identify the plant and soil factors correlated with microbial community composition. A hierarchical partitioning was used to estimate the importance of individual and group of environmental variables (edaphic variables and heavy metals) in predicting soil microbial communities. Statistical tests and graphic plotting were performed using the packages *vegan*, *reshape2*, *sem*, *packfor*, *picante*, and *ggplot2* in R v.4.0.3 (R Development Core Team, 2019).

Networks were constructed for the microbial communities for bare soils and rhizosphere soils of three metal-tolerant plants at S1 and S2. All pairwise Sparse Correlations for Compositional (SparCC) data among microbial nodes using the FastSpar algorithm with 1000 bootstraps and 1000 permutations were calculated to control the false discovery rate. A robust correlation coefficient of $|r| > 0.9$ and $p < 0.01$ were retained to construct networks. The networks were visualized using Gephi (version 0.9.2; <https://gephi.org/>). The nodes in the networks represent OTUs, in which the node size is proportional to the number of connections (degrees), and the node color represents microbial taxonomy. The edges between each pair of nodes (i.e., links) correspond to positive or negative correlations between the nodes.

Results

Soil characteristics and heavy metal contents

In general, plant species significantly affected the soil nutrient contents, especially SOC, TN, and pH, while heavy metals significantly differed between the two sampling sites (Table 1). The rhizosphere of AA exhibited the highest contents of SOC (11.669 and 15.656 g kg⁻¹) and TN (4.367 and 4.534 g kg⁻¹) compared with the other two plant species at both S1 and S2 and that in the non-vegetated soil (CK) at S1. The pH of the CB rhizosphere was the highest at S1 and S2, and was significantly higher than that of CK at S1. Overall, the heavy metal content and pH values in the rhizosphere of all three plant species had higher values at S1 than at S2.

Abundance and diversity of soil bacteria and fungi

A total of 1,558,675 high-quality sequences of bacteria and 1,840,010 high-quality sequences of fungi were obtained. The richness (Richness index) and diversity (Shannon index) of the soil bacterial communities in the rhizosphere of plant BD were significantly lower than in plants CB and AA but were not significantly different from that of the CK at S1; similarly, the richness (Richness index) and diversity (Shannon index) of the soil fungal communities in the rhizosphere of plant BD were significantly lower than in AA but were not significantly different from that of the plant CB and CK at S1. The diversity of the bacterial community was significantly higher in the rhizosphere of plants CB and BD than in AA, and the richness of the fungal community was significantly higher in the rhizosphere of plant CB than in plant BD at S2. Moreover, the richness and diversity of the bacterial and fungal communities in the rhizosphere of plant BD were significantly lower at S1 than at S2 (Table 2).

Overall patterns of soil bacterial and fungal communities

A total of eight soil bacterial phyla (relative abundances >1%) were obtained, decreasing in relative abundance as follows: Actinobacteria (25.7%), Proteobacteria (21.2%), Chloroflexi (19.4%), Acidobacteria (15.6%), Gemmatimonadota (3.6%), Myxococcota (2.5%), Planctomycetota (2.1%), and Firmicutes (1.9%) (Figure 1 and Supplementary Table S1). Plant species had a significant effect on Proteobacteria and Chloroflexi, of which Proteobacteria showed a significantly higher relative abundance in the rhizosphere of AA than in CB at both S1 and S2, while Chloroflexi exhibited the opposite trend. Between sampling sites, the relative abundance of Proteobacteria in the rhizosphere of

TABLE 1 Physico-chemical parameters and heavy metal content in sampled soils.

Sampling sites	Plant species	SOC (g kg ⁻¹)	TN (g kg ⁻¹)	TP (g kg ⁻¹)	AP (mg kg ⁻¹)	An (mg kg ⁻¹)	NN (mg kg ⁻¹)	pH	Moisture	Cu (mg kg ⁻¹)	Zn (mg kg ⁻¹)	As (mg kg ⁻¹)	Cd (mg kg ⁻¹)	Pb (mg kg ⁻¹)
S1	CK	8.2 ± 0.4bc	1.0 ± 0.1b	0.1 ± 0.0a	7.1 ± 0.9a	3.6 ± 0.6a	11.2 ± 1.4b	5.8 ± 0.1c	0.3 ± 0.0a	411.7 ± 46.8a	47,879.5 ± 8747.3a	1407.8 ± 148.4a	40.6 ± 9.0ab	6315.9 ± 1246.5a
	CB	6.3 ± 0.511c	1.0 ± 0.2b	0.4 ± 0.1a	8.0 ± 0.8a	3.6 ± 0.3a*	12.1 ± 1.1b	6.5 ± 0.1a*	0.3 ± 0.0a	343.4 ± 34.3a*	44,561.9 ± 5149.7a*	1155.9 ± 206.5a*	56.8 ± 11.4a*	4212.8 ± 472.6a*
	BD	9.3 ± 0.6bc*	1.9 ± 0.5b	0.2 ± 0.1a	8.2 ± 1.3a	3.8 ± 0.3a	10.5 ± 0.8b	6.2 ± 0.1ab	0.3 ± 0.0a	366.2 ± 19.0a*	48,827.2 ± 3614.7a*	1729.4 ± 75.8a*	30.1 ± 4.8ab*	4398.3 ± 697.7a*
	AA	11.7 ± 1.0a	4.4 ± 0.6a	0.3 ± 0.1a	9.3 ± 1.6a	4.5 ± 0.5a	22.4 ± 2.4a	6.0 ± 0.1bc*	0.4 ± 0.1a	408.9 ± 54.7a*	35,966.8 ± 5848.9b*	1408.0 ± 250.5a*	21.4 ± 3.3b*	6714.7 ± 1070.6a*
S2	CB	8.8 ± 1.0B	2.4 ± 0.2B	1.6 ± 1.2A	5.4 ± 0.47A	6.3 ± 0.3A	18.1 ± 2.5A	5.8 ± 0.3A	0.4 ± 0.0A	203.1 ± 15.1A	22,295.0 ± 4118.2A	624.9 ± 81.5A	28.8 ± 3.6A	2897.6 ± 605.8A
	BD	15.9 ± 1.9A	2.8 ± 0.2B	0.6 ± 0.1A	6.1 ± 1.1A	6.3 ± 0.9A	17.0 ± 2.7A	5.7 ± 0.2 A B	0.3 ± 0.1A	222.4 ± 23.6A	1609.5 ± 334.8B	51.0 ± 16.5B	3.3 ± 0.7B	128.6 ± 68.0B
	AA	15.7 ± 0.8A	4.5 ± 0.6A	0.7 ± 0.1A	8.4 ± 1.7A	7.0 ± 0.7A	14.5 ± 1.5A	5.1 ± 0.1B	0.5 ± 0.0A	167.4 ± 8.9A	3344.7 ± 303.143B	58.2 ± 16.3B	6.0 ± 0.9B	340.3 ± 110.3B
Two-way ANOVA														
	Plant species	<0.001	<0.001	0.192	0.222	0.276	0.070	<0.001	0.025	0.772	0.008	0.450	<0.001	0.080
	Sampling site	<0.001	0.035	0.009	0.081	<0.001	0.344	<0.001	0.047	<0.001	<0.001	<0.001	<0.001	<0.001
	Sampling site×Plant species	0.175	0.410	0.046	0.767	0.992	0.002	0.366	0.567	0.188	0.017	0.002	0.457	0.002

CB, BD, and AA respectively represented *C. breviculmis*, *B. davidii*, and *A. annua*. S1, sample site on the zinc smelter waste heap; S2, sample site around the zinc smelter waste heap. Mean values (mean ± se, n = 4) followed by different lowercase letters presented significant differences of sampled soils collected on the zinc smelter waste heap (S1), by different capital letters represent significant differences of sampled soils collected near the zinc smelter waste heap (S2). SOC, soil organic carbon; TN, total nitrogen; TP, total phosphorus; AP, available phosphorus; AN, ammonium nitrogen; NN, nitrate nitrogen * indicates significant differences between S1 and S2. $p < 0.05$ is considered as a statistical difference (LSD, test). The significant p values ($p < 0.05$) from two-way ANOVA, are in bold.

TABLE 2 Soil bacterial and fungal diversities for bare soils and rhizosphere soils of three metal-tolerant plants at S1 and S2.

Sampling Sites	Plant Species	Bacterial Community			Fungal Community		
		Richness	Shannon	Chao1	Richness	Shannon	Chao1
S1	CK	1141.8 ± 133.0ab	8.3 ± 0.3ab	1351.6 ± 118.0a	225.3 ± 39.0ab	4.5 ± 0.6a	246.2 ± 41.3ab
	CB	1311.3 ± 77.9a	8.7 ± 0.1a	1477.7 ± 99.4a	258.5 ± 35.7ab	4.4 ± 0.6a	282.5 ± 42.5ab
	BD	970.3 ± 115.7b	7.8 ± 0.4b	1201.2 ± 106.1a	188.8 ± 37.8b	3.6 ± 0.6a	205.2 ± 45.6b
	AA	1299.3 ± 41.3a	8.6 ± 0.1a	1483.7 ± 37.7a	308.3 ± 23.2a	4.5 ± 0.1a	339.9 ± 35.2a
S2	CB	1430.3 ± 14.3A	8.8 ± 0.1A	1645.3 ± 8.2A	425.5 ± 11.1A*	5.5 ± 0.4A	449.5 ± 13.4A*
	BD	1389.3 ± 58.5A*	8.9 ± 0.1A*	1550.6 ± 54.1A*	355.3 ± 18.3B*	5.6 ± 0.2A*	370.0 ± 23.3B*
	AA	1147.3 ± 35.0A*	8.1 ± 0.3B	1334.3 ± 27.1B*	375.5 ± 27.7 A B	4.9 ± 0.4A	431.3 ± 21.3 A B
Two-way ANOVA							
Plant species		0.074	0.042	0.253	0.357	0.594	0.140
Sampling site		0.027	0.117	0.035	<0.001	0.003	<0.001
Sampling site×Plant species		<0.001	0.003	0.001	0.013	0.201	0.041

CB, BD, and AA respectively represented *C. breviculmis*, *B. davidii*, and *A. annua*. S1, sample site on the zinc smelter waste heap; S2, sample site around the zinc smelter waste heap. Mean values (mean ± se, n = 4) followed by different lowercase letters presented significant differences of sampled soils collected on the zinc smelter waste heap (S1), by different capital letters represent significant differences of sampled soils collected near the zinc smelter waste heap (S2). * followed after letters indicates significant differences between S1 and S2. *p* < 0.05 is considered as a statistical difference (LSD, test). The significant *p* values (*p* < 0.05) from two-way ANOVA, are in bold.

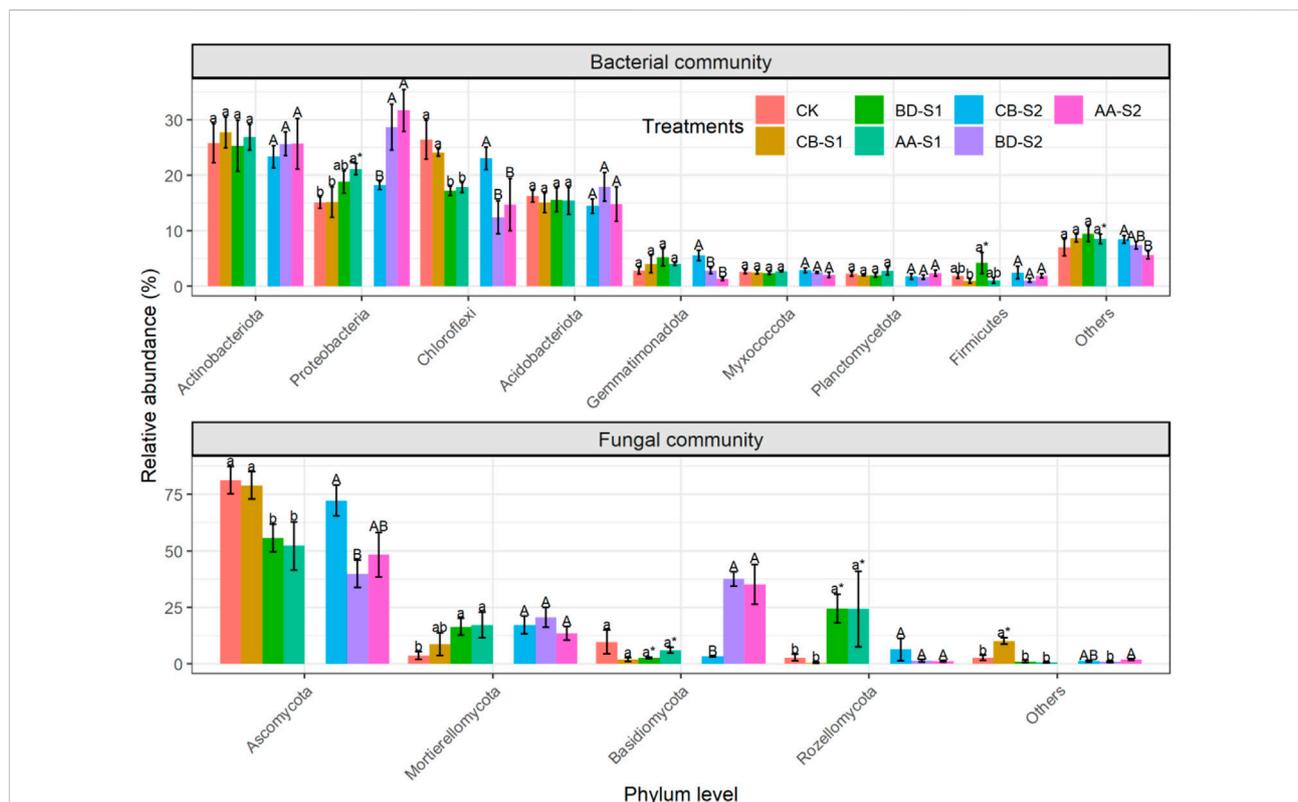
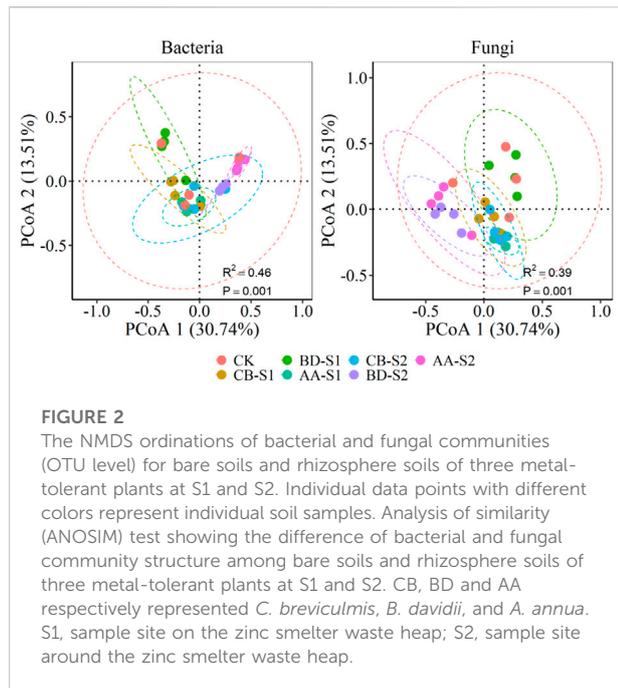


FIGURE 1 Soil microbial composition at the phylum level for bare soils and rhizosphere soils of three metal-tolerant plants at S1 and S2. CB, BD and AA respectively represented *C. breviculmis*, *B. davidii*, and *A. annua*. S1, sample site on the zinc smelter waste heap; S2, sample site around the zinc smelter waste heap. Mean values (mean ± se, n = 4) followed by different lowercase letters presented significant differences of sampled soils collected on the zinc smelter waste heap (S1), by different capital letters represent significant differences of sampled soils collected near the zinc works (S2). * followed after letters indicates significant differences between S1 and S2. *p* < 0.05 is considered as a statistical difference (LSD test).



AA was significantly lower at S1 than at S2. Regarding the fungal community, four fungal phyla with a relative abundance >1% were obtained, including Ascomycota (61.2%), Mortierellomycota (13.8%), Basidiomycota (13.7%), and Rozellomycota (8.6%). The relative abundance of Ascomycota was significantly higher and that of Rozellomycota was significantly lower in the rhizosphere of CB than in the rhizospheres of BD and AA at both S1 and S2. For the rhizospheres of BD and AA, the relative abundances of Basidiomycota and Rozellomycota were significantly lower and higher, respectively, at S1 than at S2.

PCoA accompanied with ANOSIM test showed results that rhizosphere bacterial and fungal community composition was significantly different among plant species as well as between sampling sites (Figure 2). Furthermore, pairwise ANOSIM test showed that both bacterial and fungal community from S1 were different from those of S2. Rhizosphere bacterial and fungal community of plants CB and BD were not significantly different but they were significantly distinct from those of plant AA at S1. Moreover, rhizosphere bacterial community of plant BD and rhizosphere fungal community of plants CB and BD were significantly different from those of CK. At sampling site of S2, rhizosphere bacterial and fungal community of plant CB were significantly different from those of plants BD and AA, which showed no significant difference (Supplementary Table S2). Additionally, some soil microbial functional profiles also differed significantly between S1 and S2 (Supplementary Figure S1). For example, the proportion of bacterial functional profiles

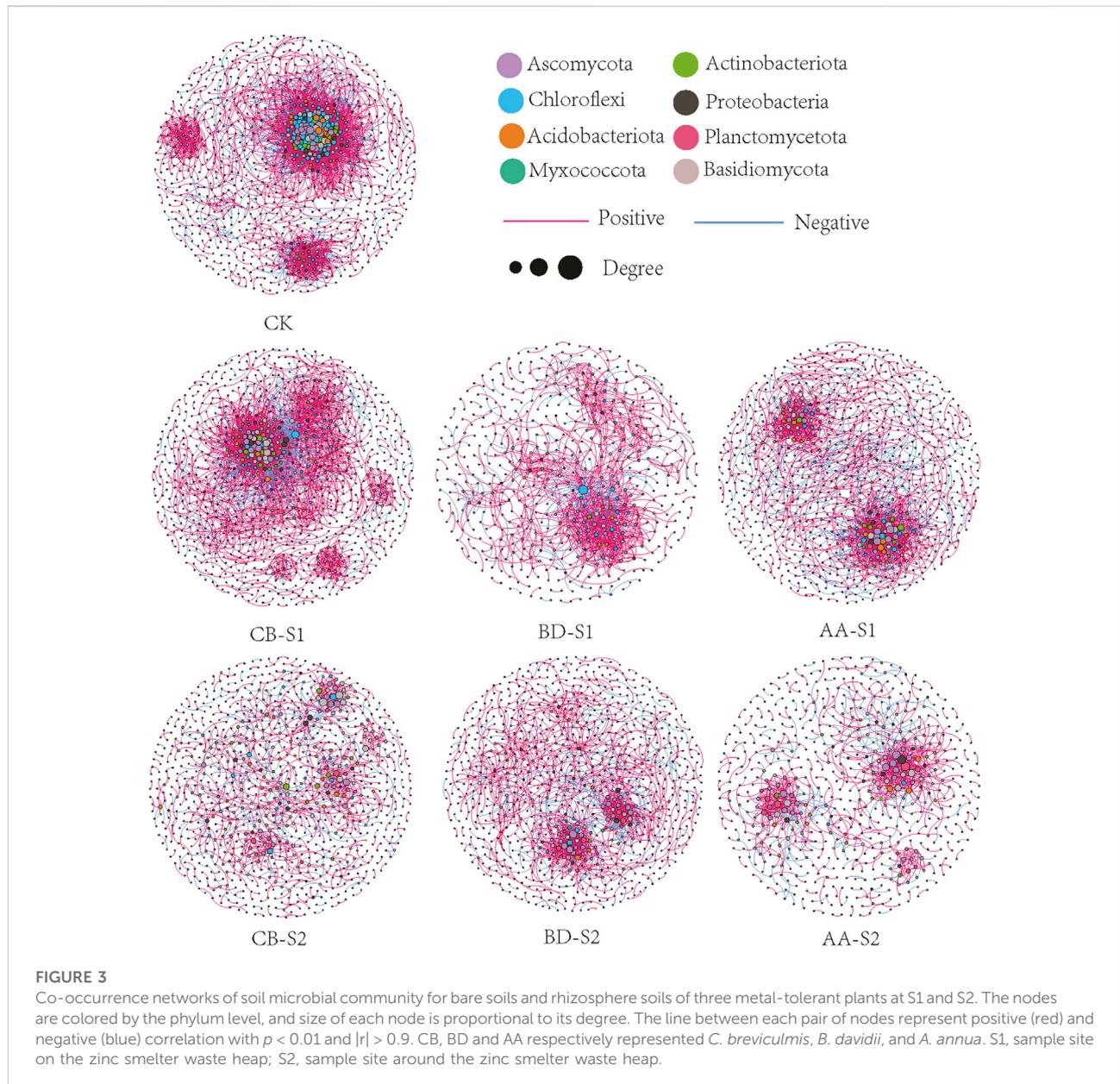
related to glycine, serine, and threonine metabolism and the fungal functional profiles related to endophyte-plant pathogens were remarkably higher at S1 than at S2. Conversely, samples from S2 had significantly higher fungal functional profiles related to endophyte-soil saprotrophs, animal pathogen-clavicipitaceous endophyte-fungal parasites, wood saprotrophs and plant saprotroph-wood saprotrophs, as compared with those from S1.

Co-occurrence networks for rhizosphere microbial community of three metal-tolerant plants

Co-occurrence networks of soil microbial community for bare soils and rhizosphere soils of three metal-tolerant plants at S1 and S2 were constructed (Figure 3), and topological properties were calculated to describe the complex pattern of network and to compare the real network with an identically sized Erdős-Rényi random network (Table 3). Overall, the structure properties of the real networks were greater than the Erdős-Rényi random networks, indicating that the former are more clustered than the latter. Soil microbial network of CK was the largest (more edges) and most complex (higher average degree) compared with rhizosphere microbial networks of three plants at S1. Furthermore, rhizosphere microbial networks of plant CB and AA were larger and more complex than that of plant BD at S1, but rhizosphere microbial networks showed no obvious different among plant species at S2. Compared with site S2, rhizosphere microbial networks at S1 were larger and more complex, and the ratio of positive to negative edges of the microbial network was higher. Moreover, there were fewer nodes affiliated with Ascomycota but more nodes affiliated with Chlooflexi, Planctomycetota and Myxococcota at S1 compared with S2 (Supplementary Table S3). Ascomycota, Actinobacteriota, Chloroflexi, Actinobacteriota and Rozellomycota were the keystone taxa in networks for bare soils and rhizosphere soils of three metal-tolerant plants at S1 and S2 (Supplementary Table S4).

Relating soil microbial communities to environmental factors

The Mantel test results for the correlation between community composition and environmental variables revealed that both the bacterial and fungal communities were significantly correlated with heavy metals (Cu, Zn, As, Cd and Pb). In addition, the bacterial community had significant correlations with SOC, TN, AN, pH, and moisture, while the fungal community only had significant correlations with SOC and AN. Additionally, there was a significant positive correlation within indices of soil nutrients, which were negatively correlated and heavy metals



(Supplementary Figure S2). In general, the edaphic variables showed a higher relative importance in predicting both bacterial and fungal communities compared with heavy metals, and edaphic variables of SOC, AN, NN and pH together with heavy metals of Zn, As and Pb were important predictors of bacterial and fungal communities compared with the other environmental variables (Figure 4). The correlation heatmaps showed that all heavy metals and pH were negatively correlated with Proteobacteria and Basidiomycota, and positively correlated with Ascomycota and Chloroflexi. Conversely, SOC, TN, and AN were significantly positively correlated with Proteobacteria and Basidiomycota and negatively correlated with Ascomycota (Figure 5).

Discussion

Native plant species influence on soil nutrients

In this study, the heavy metal contamination levels at S1 and S2 exceeded the allowable limits set by Chinese standards for agricultural soils (Ministry of Ecological Environment of the People's Republic of China, 2018), in which the concentrations of multiple heavy metals at S1 were at least two times higher than those at S2. Thus, S1 was defined as “severely contaminated soil,” while S2 was defined as “moderately contaminated soil.” Our results show that AA had a better ability to increase the SOC and TN contents in

the rhizosphere, but most heavy metal contents were not significantly affected by plant species at S1. The differences in rhizosphere SOC and TN contents among the three studied plant species could be attributed to differences in the quantity and quality of root exudates and the organic litter inputs of each plant species (Bardgett et al., 2014). However, although there was no obvious difference in the heavy metal contents among the three plant species, this does not indicate that the plants had no effect on the total metal concentrations. This is mainly because the background heavy metal content value in the Pb-Zn waste heap is extremely high, and any changes in heavy metal content caused by plant colonization would be comparatively insignificant, rendering them statistically undetectable. Moreover, the phytoremediation of heavy metals generally involves one or more mechanisms such as phytoaccumulation, phytostabilization, phytodegradation, phytovolatilization, and hydraulic control, depending on the plant species (Muthusarayanan et al., 2018). Thus, phytoremediation is more relevant to the bioavailability of heavy metals (Fu et al., 2019) than their absolute abundance. However, in this study, only the heavy metal contents of the soil samples were measured. Therefore, the response of the heavy metal speciation distributions to plant species must be further studied to confirm these findings. Nevertheless, we found that the contents of Zn and Cd in the rhizosphere of AA was significantly lower than CK at S1, which is consistent with the findings of Inelova et al. (2021) and Petriccione et al. (2013), who found that *A. annua* has a large accumulation capacity for heavy metals, especially Cd and Zn. Note that the difference among the heavy metal content among the three plant species at S2 was mainly attributable to differences in sampling

location rather than the effect of plant species because *C. breviculmis* was growing closer to S1 than the other plants.

Native plant species altered rhizosphere microbial composition and diversity

Certain plant species have the ability to take up or immobilize heavy metal contaminants in soils while enriching rhizosphere nutrients by transporting various organic compounds (such as amino acids, organic acids, and phenolic compounds) from plants into the soil (Borymski et al., 2018; Muthusarayanan et al., 2018). Plant characteristics and rhizosphere effects greatly contribute to changes in the rhizosphere microbial community (Borymski et al., 2018; Barra Caracciolo and Terenzi, 2021). Our results showed that, at S1, the richness and diversity of the bacterial communities of BD were lower than those of CB and AA but were not significantly different from those of the CK, and the fungal community showed a similar trend. The difference in soil microbial community among plant species was mainly the result of an accumulation of soil nutrients rather than a reduction in heavy metal concentrations, which was supported by our findings that the edaphic variables showed a higher relative importance in predicting both bacterial and fungal communities compared with the heavy metal variables, especially the SOC, AN, NN and pH (Figure 4). The different effects of plant species on the rhizosphere microbial community were also verified by the NMDS results, which showed that the rhizosphere bacterial and fungal community were significantly different

TABLE 3 Topological properties of empirical networks and Erdős–Rényi random networks for bare soils and rhizosphere soils of three metal-tolerant plants at S1 and S2.

	Network Properties	S1				S2		
		CK	CB	BD	AA	CB	BD	AA
Real networks	Total edges	7443	3196	1188	2026	1201	1523	1029
	Positive edges	7120	2722	978	1670	917	1242	804
	Negative edges	323	474	210	356	284	281	225
	positive/negative edges	22.04	5.74	4.66	4.69	3.23	4.42	3.57
	Total nodes	963	981	546	895	954	905	634
	Average degree	15.46	6.52	4.35	4.53	2.52	3.37	3.25
	Average path length	4.33	4.57	5.16	11.72	7.96	12.21	4.91
	Degree centrality	0.13	0.07	0.14	0.06	0.02	0.04	0.05
	Betweenness centrality	0.04	0.06	0.24	0.25	0.04	0.23	0.03
	Clustering coefficient	0.35	0.24	0.26	0.2	0.13	0.22	0.19
Erdős–Rényi random networks	Modularity	0.29	0.52	0.60	0.67	0.87	0.78	0.76
	Average path length	2.79	3.88	4.42	4.64	7.15	7.82	5.49
	Clustering coefficient	0.02	0.01	0.01	0.01	0.00	0.00	0.01
	modularity	0.18	0.30	0.40	0.38	0.59	0.63	0.49

CB, BD, and AA respectively represented *C. breviculmis*, *B. davidii*, and *A. annua*. S1, sample site on the zinc smelter waste heap; S2, sample site around the zinc smelter waste heap.

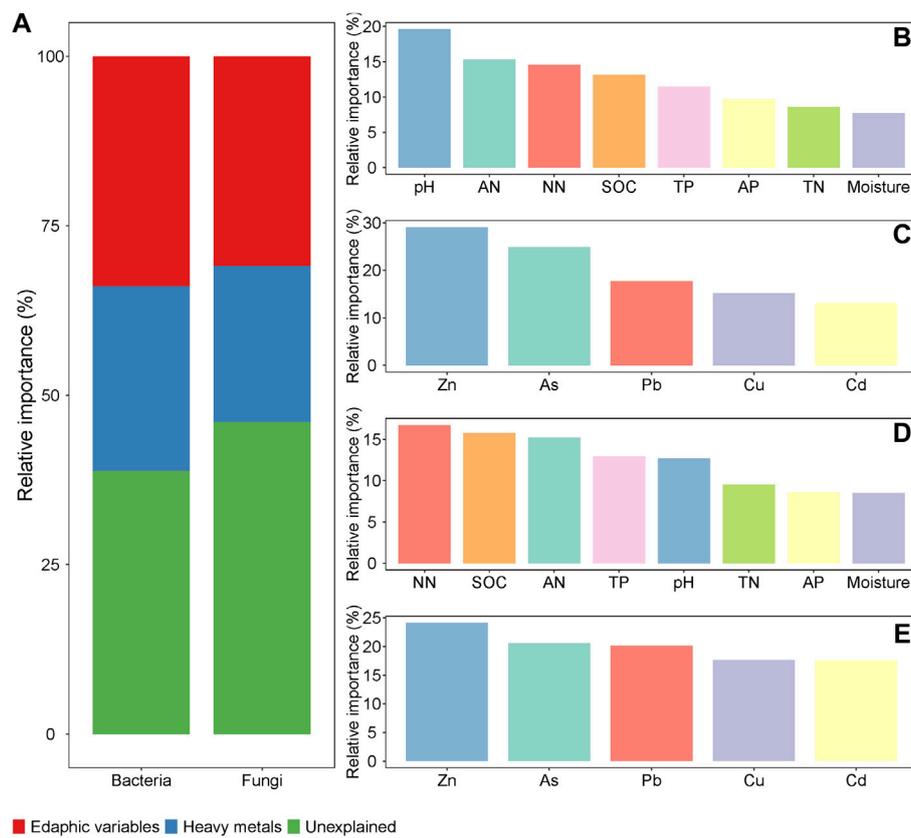


FIGURE 4

The relative importance of individual and group of environmental variables in predicting soil microbial communities. (A) group of edaphic variables and heavy metals variables in predicting soil microbial communities; (B) individual edaphic variables in predicting bacterial communities; (C) individual heavy metal variables in predicting bacterial communities; (D) individual edaphic variables in predicting fungal communities; (E) individual heavy metal variables in predicting fungal communities. SOC, soil organic carbon; TN, total nitrogen; TP, total phosphorus; AP, available phosphorus; AN, ammonium nitrogen; NN, nitrate nitrogen.

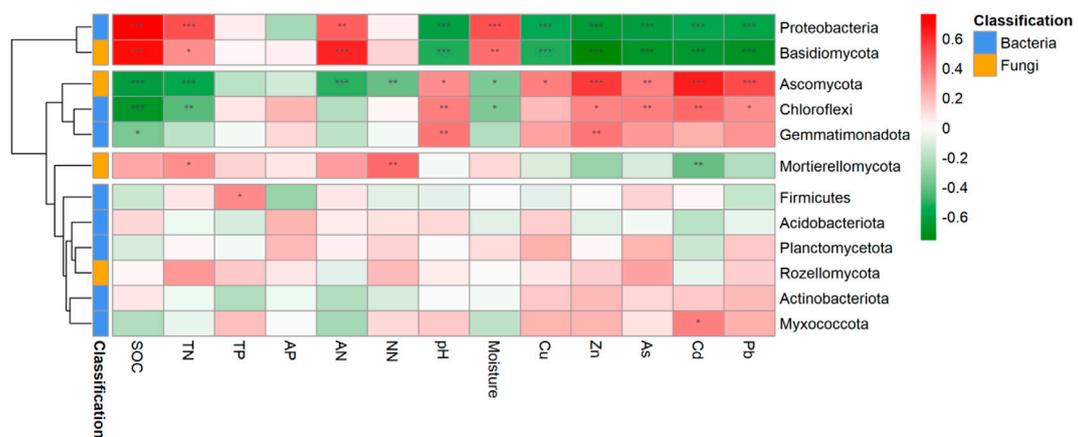


FIGURE 5

Correlation heat maps for the correlation between community composition and environmental variables at the phylum level. The left dendrogram linkages represent clustering results of these microbial groups. SOC, soil organic carbon; TN, total nitrogen; TP, total phosphorus; AP, available phosphorus; AN, ammonium nitrogen; NN, nitrate nitrogen. *, <0.05; **, <0.01; ***, <0.001.

among species both at S1. [Borymski et al. \(2018\)](#) suggested that a nutrient-deficit microenvironment was not effective in attracting and stimulating microbial groups around their roots. Several other previous studies also demonstrated that the rhizosphere effect usually increases microbial diversity ([Azarbad et al., 2013](#); [Zhao et al., 2020](#)). Notably, we found the soil bacterial and fungal α -diversity in CK was not significantly different from that in the rhizosphere of the three plant species at S1. This discrepancy may be because the S1 sampling site is on an artificial Pb-Zn waste heap that has undergone natural restoration for nearly a hundred years, and *C. breviculmis* and *A. annua* are dominant pioneer species with a large degree of coverage, which may cause its rhizosphere effect to influence the soils uncovered by plants. Moreover, as heavy metal contamination has persisted for nearly a hundred years, the soil microbes have adapted to heavy metal contamination and increased their diversity and richness ([Bourceret et al., 2016](#)).

The rhizosphere bacterial composition also responded to plant species, in which Proteobacteria showed a significantly higher relative abundance in the rhizosphere of AA, as compared with CB at both sites, while Chloroflexi showed the opposite trend. As Proteobacteria are usually considered copiotrophic r-strategists and prefer to utilize fresh photosynthates ([Bastida et al., 2016](#)), the observed increase in its relative abundance resulted from the higher amount of photosynthates in the rhizosphere of AA than that in CB. Chloroflexi are slow-growing oligotrophic bacteria that are particularly resistant to heavy metals ([Rastogi et al., 2011](#)). Herein, the significantly higher relative abundance of Chloroflexi in the rhizosphere of CB was related to its high content of heavy metals, especially Zn and Cd. Regarding fungal composition, the dominant phylum of Ascomycota was significantly higher and Rozellomycota had a significantly lower relative abundance in the rhizosphere of CB than in that of BD and AA at both S1 and S2. The dominance of Ascomycota in the rhizosphere of all three plant species reveals its strong tolerance and profound impact on certain ecological and biogeochemical conditions under severe heavy metal contamination ([Lin et al., 2020](#)). Both Ascomycota and Rozellomycota are heavy metal-tolerant fungi ([Iram et al., 2009](#); [Lin et al., 2019](#)), and improvements in soil quality usually weaken the dominance of Ascomycota ([Yuan et al., 2020](#)). Meanwhile, Rozellomycota has been found to be positively affected by soil organic matter ([Lin et al., 2019](#)). In this study, nutrients, such as SOC and TN, were relatively higher for BD and AA ([Table 1](#)), which contributed to the differences in the Ascomycota and Rozellomycota abundances in the rhizosphere of each plant species.

Soil microbial structure becomes more unstable with increasing heavy metal pollution

Microbes do not thrive in isolation but instead interact and adapt to survive in harsh environments ([Faust and Raes, 2012](#); [Chun et al., 2021](#)). Soil microbial networks unravel the associations among

network members and reflect the response of the microbial community to environmental changes ([Banerjee et al., 2019](#)). Co-occurrence networks of soil microbial community for bare soils and rhizosphere soils of three metal-tolerant plants at S1 and S2 showed that rhizosphere microbial networks of plant CB and AA were larger and more complex than that of plant BD at S1, but rhizosphere microbial networks showed no obvious difference among plant species at S2. This indicates that interactions or niche-sharing in rhizosphere microbial community of plant CB and AA are maximal relative to plant BD at the severely contaminated soil, however, influence of plant species on microbial interactions are reduced at the moderately contaminated soil. Soil microbial communities with a large proportion of positively connected members are unstable because members may respond in tandem with environmental changes ([Deng et al., 2012](#)). In this sense, soil microbial networks at S1 had a higher number of edges and higher ratio of positive to negative edges relative to S2 indicate that soil microbial communities at S1 are less stable with multi-metal contamination increasing. A possible explanation for these findings is that the association in microbial communities were strengthened under harsh environmental conditions, such as heat, drought, and heavy metal pollution ([Zaefarian et al., 2021](#)). In this study, the extremely high content of multiple heavy metals at S1 affected the microbial community, forcing some metal-tolerant bacteria to survive *via* stronger positive cooperation and trophic interaction, in which such cooperation may cause the formation of a microbial biofilm ([Li Q. et al., 2020](#); [Chun et al., 2021](#)). However, this assumption must be corroborated by further studies. In addition, soil temperature at S1 are higher than S2 because it is located on a lead-zinc waste heap, and the warm environment has been demonstrated to activate dormant microbial members and strengthen the interactions among microbial communities ([Chen et al., 2012](#)). One potential limitation of co-occurrence network analysis is that species associations based on correlation analysis can yield spurious results and cannot be automatically interpreted as interactions, consequently, it may not be possible to comprehensively depict the microbial interactions under real-world conditions ([Jiao et al., 2021](#)). Even so, the information about negative/positive correlations between taxa is still essential for estimating potential species interrelationships within complex environments, and they are frequently used to investigate microbial interconnection patterns ([Banerjee et al., 2018](#); [Jin et al., 2022](#)).

Both heavy metal pollution and soil nutrients lead to changes in microbial groups

Microbes interact with plants to adapt to and alter soil environments ([Chun et al., 2021](#)). In this study, the rhizosphere microbial community was influenced by heavy metals (Pb, Zn and As), soil nutrients (SOC, TN, NN and AN), moisture, and pH ([Supplementary Figure S2](#) and [Figure 4](#)). Moreover, the

relationship between heavy metals, pH, and the microbial community was opposite to the relationship between soil physical properties and the microbial community (Figure 5). Similar results have been reported in previous studies (Haichar et al., 2008; Li C. et al., 2020; Lopez-Marcos et al., 2020), and the opposing effects of heavy metals and soil nutrients on soil microbial communities may be one of the driving forces for maintaining soil microbial community stability. Metal-contaminated environments exert strong selective pressure on microbial compositions by affecting microbial metabolism, including the production of oxidative stress or the destruction of microbial proteins and DNA (Mejias Carpio et al., 2017). This results in the evolution of several metal-tolerant microbial groups with better metal resistance mechanisms, such as extracellular and intracellular sequestration, exclusion by permeability barriers, and enzymatic detoxification (Epelde et al., 2014). Bacterial groups affiliated with Chloroflexi and fungal groups affiliated with Ascomycota were positively associated with almost all the heavy metals involved in this study, indicating that they are tolerant to heavy metal pollution and play leading roles in the bioremediation of heavy metals. Additionally, soil nutrients (especially SOC, TN, NN and AN), moisture, and pH play important roles in shaping soil microbial communities (Figure 3). Although microorganisms participate in the decomposition of soil organic matter and the generation of available nitrogen and phosphorus, microbial metabolisms are affected by soil moisture and pH, which alter extracellular enzyme activity and the absorption rate of soluble substrates (Schimel and Schaeffer, 2012; Calcioglu et al., 2018).

Conclusion

Both plant species and heavy metals influence soil microbial diversity and composition. The influence of native plant species on the microbial structure and diversity of soils contaminated with different degrees of heavy metal pollution was mainly the result of an accumulation of soil nutrients rather than a reduction in heavy metal concentrations. Herein, AA and CB showed relatively higher rhizosphere microbial diversity, richness and network complexity than BD at the severely contaminated soil (S1), which was mainly because of a significant accumulation of soil nutrients, including SOC, TN, AN and NN. Moreover, the heavy metal concentration and soil nutrient levels significantly affected the microbial groups affiliated with Proteobacteria, Basidiomycota, Ascomycota, and Chloroflexi. In particular, the microbial groups affiliated with Chloroflexi and Ascomycota were tolerant to heavy metals and may play an important role in the bioremediation of heavy metals, as they were positively associated with almost all the heavy metals involved in this study. Effect of plant species on rhizosphere microbial networks was more obvious under higher heavy metal pollution. Furthermore, microbial networks at S1 exhibited higher average degree and a higher proportion of positive links than those

at S2, indicating that soil microbial structure became more complexity and unstable with increasing heavy metal pollution.

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

CS, GW and XK initiated and designed the research, collected the materials, and performed the experiments. CS and PW obtained funding for this study. CS analyzed the data and wrote the manuscript. All authors read and approved the manuscript.

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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/fenvs.2022.979922/full#supplementary-material>

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