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Responses of soil fungal taxonomic attributes and enzyme activities to copper and cadmium co-contamination in paddy soils

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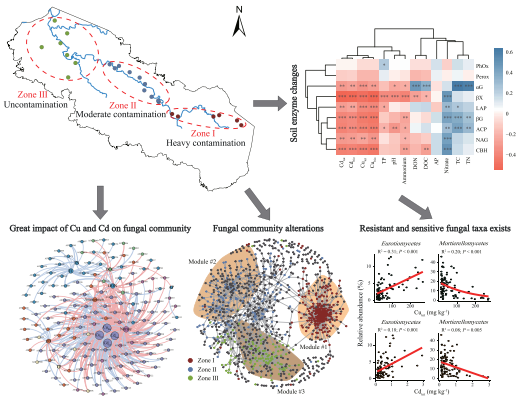
HIGHLIGHTS

- Cu and Cd markedly affect fungal diversity, composition, and co-occurrence pattern.
- Heavy metals have a greater impact on fungal community than soil general properties.
- Cu and Cd are significantly correlated with resistant and sensitive fungal taxa.
- Hydrolase activities can be used as indicators for Cu and Cd co-contamination.

GRAPHICAL ABSTRACT



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ABSTRACT

Excess heavy metals, especially copper (Cu) and cadmium (Cd), are common in paddy soils in the red soil hilly areas of southern China. Microorganisms are regulators of soil organic matter accumulation and pollutant transformation. Clarifying the effects of Cu and Cd accumulation on microbial community composition and function is a prerequisite for bioremediation of paddy soil contamination. However, it remains unclear how Cu and Cd contamination affects soil fungal taxonomic attributes and microbial-mediated biogeochemical processes in paddy soils. Here, soil heavy metals, fungal community composition, and soil enzyme activities were determined in paddy fields downstream of a typical mining area in southern China, and the effects of Cu and Cd co-contamination on fungal community diversity and co-occurrence networks, as well as the associations between them were assessed. The concentrations of Cu and Cd in paddy soils decreased from upstream to downstream of the river, and were positively correlated with the Shannon index of fungal communities. Soil Cu and Cd concentrations exhibited a greater impact on the structure and assembly of fungal communities than soil general properties. Increases in soil Cu and Cd concentrations were correlated with drastic changes in the cumulative relative abundance of ecological clusters in fungal co-occurrence networks. Soil Cu and Cd concentrations were positively correlated with the relative abundances of *Eurotiomycetes*, *Pezizomycetes*, *Ustilaginomycetes*, and *Kickxellomycetes*, respectively, whereas negatively correlated with hydrolase activities related to carbon, nitrogen, and phosphorus cycles. These results confirmed in the field that long-term Cu and Cd enrichment significantly altered the structure and diversity of fungal communities in the subtropical paddy soils, thereby affecting soil nutrient transformation and organic matter accumulation. This can also provide a basis for the bioremediation of heavy metal pollution in paddy soils.

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1. Introduction

Heavy metals in soils are mainly derived from human activities such as mining, smelting, agriculture, and power generation, and accumulate globally (Sherman et al., 2012; Shi et al., 2019). Unlike organic pollutants, heavy metals are not degraded but bio-accumulate in soils and sediments, thereby threatening environmental safety and public health (Rehman et al., 2018). Among the heavy metals, copper (Cu) and cadmium (Cd) have received much attention due to their high toxicity, wide distribution, and persistence in agricultural soils (Sheldon and Menzies, 2005). For instance, excessive Cu (109–1313 mg kg^{-1}) has been found in paddy soils around mining areas in South China (Zhuang et al., 2009); furthermore, 29.4 % of agricultural soils in China are contaminated with Cd based on a meta-analysis (Huang et al., 2019). In addition, elevated Cu and Cd inputs into the environment not only impair soil biodiversity and its associated ecosystem functions, but also affect human health through the food chain (Haider et al., 2021).

Soil microorganisms are involved in various ecosystem processes such as nutrient cycling (Fierer, 2017). They are vulnerable to changes in the external environment, which can have a huge impact on ecosystem function (Liu et al., 2020a). However, most studies on the relationship between soil heavy metals and microorganisms have only considered bacterial communities (Li et al., 2021; Wu et al., 2022). For example, Liu et al. (2015) reported that Cu was identified as one of the main factors explaining changes in bacterial assemblages. Cadmium reduced the richness and/or diversity of bacterial species in the underlying red soil (Song et al., 2021). In contrast, the combined effects of Cu and Cd contamination on soil fungal communities have not been well evaluated. Fungi play crucial roles in organic matter decomposition, some of which interact with plants to enhance plant nutrient uptake and plant tolerance to metal stress (Zeilinger et al., 2016). Therefore, exploring fungal community composition is helpful for developing bioremediation strategies in Cu and Cd co-contaminated agricultural soils.

Members of the soil microbiome co-occur strongly in ecological networks, referred to as ecological clusters or modules (de Menezes et al., 2015). These ecological modules, following particular environmental preferences, are expected to have multiple effects on regulating soil biogeochemical processes and ecological functions (Delgado-Baquerizo et al., 2018). For example, the extent of interactions within certain ecological modules among archaea, bacteria, and fungi correlates with nutrient cycling and storage (Creamer et al., 2016). However, the response of these ecological modules in soil fungi to Cu and Cd gradients remains largely unexplored, although they are crucial for understanding soil microbiome (Shi et al., 2016). The construction and analysis of ecological networks are beneficial to determine shifts in microbial interactions and to identify resistant- or sensitive-microbes under heavy metal contamination.

Soil enzyme activity is sensitive to heavy metal stress and is directly related to soil functions associated with carbon, nitrogen, and phosphorus cycling. Therefore, soil enzyme activity is used as a bioindicator of soil quality and ecological health (Liu et al., 2020b; Wahsha et al., 2017). For example, specific enzyme activity linearly decreases with increasing heavy metal concentrations, especially for arylsulfatase with Cd, Pb, and Zn (Aponte et al., 2020). Also, several studies have documented no significant correlation between soil enzyme activity and heavy metal contamination (Tripathy et al., 2014; Zhang et al., 2010). The response of enzyme activity to heavy metal stress depends on soil properties, metal type and concentration. Kandziora-Ciupa et al. (2016) observed that soil acid phosphatase and β-glucosidase activities were highly affected by soil pH in heavy metal contaminated soils. Although the effect of environmental factors on enzyme activity is recognized, the cause-effect relationship between soil key enzymes and heavy metals has not been elucidated to date. This has hindered the application of enzymes as bioindicators. Furthermore, various enzyme activities respond differently to heavy metals (Yang et al., 2016), so it is important to find indicator enzymes for soil Cu and Cd co-contamination.

In this study, we hypothesized that soil fungal community was niche-driven, and that there were heavy metal-resistant microorganisms and soil enzymes sensitive to Cu and Cd co-contamination. We collected paddy soils from southern China where paddy fields have been chronically polluted by mine drainage. Environmental variables analysis, ITS amplicon sequencing, and microbial fluorometric assay were combined to reveal microbial responses to heavy metal contamination. The objectives of this study were (i) to investigate the effects of Cu and Cd co-contamination on fungal diversity and co-occurrence network in paddy soils, (ii) to identify the potential fungal taxa that were resistant or sensitive to heavy metals, and (iii) to elucidate the response of major soil hydrolase and oxidoreductase activities to Cu and Cd co-contamination.

2. Materials and methods

2.1. Study area and soil sampling

This study was conducted in the Xiancha River basin, located in Taihe County, Jiangxi Province, China (Fig. S1). This area characterizes a subtropical monsoon climate with mean annual temperature of 18.6 °C and mean annual precipitation of 1726 mm. Most of agricultural land is paddy fields. About 80 years of river sewage irrigation induced by tungsten mining have led to severe Cu and Cd contamination of paddy soils. Soil samples were collected from 23 sites along the Xiancha River, with six, ten, and seven sites distributed in the upstream (Zone I), midstream (Zone II), and downstream (Zone III) of the basin. In November 2020, four fields were selected as replicates at each site, and five topsoil cores (0–15 cm) were collected from each field. The five soil cores were then pooled together and homogenized into a composite sample per field. Therefore, a total of 92 paddy soil samples were collected after the rice harvest. All soil samples were passed through a 2 mm sieve to remove plant residues and gravel. The sieved soils were subsequently separated into two portions: one was stored at −80 °C for microbial and enzyme activity assays, and the other was stored at 4 °C for environmental variable determination.

2.2. Determination of heavy metals and soil general properties

Total heavy metals including Cd, As, Cu, Cr, Pb, Ni, and Zn (hereafter referred to as M_{tot}) were extracted using HF-HNO_3-$HClO_4$, whereas bioavailable fraction of these metals (hereafter referred to as M_{bio}) were extracted using extraction solution (0.005 M DTPA-0.1 M TEA-0.01 M $CaCl_2$). The concentrations of total and bioavailable heavy metals were determined using inductively coupled plasma-mass spectrometer (Thermo Fisher, USA). The certified reference material (GBW07429, China Standard Research Center) and blank were used for quality control, and the recovery rate of different heavy metals was between 96 % and 103 %.

Soil total carbon (TC) and total nitrogen (TN) concentrations were determined by the combustion method on a CHNOS analyzer (Vario Max; Elementar, Langenselbold, Germany). Total phosphorus (TP) and available phosphorus (AP) concentrations were determined using Olsen's method (Olsen et al., 1954). Soil pH was measured in a 1: 2.5 soil: water suspension using a pH meter (Mettler Toledo, Switzerland). Dissolved organic carbon (DOC) was extracted by deionized water in a soil/water ratio of 1: 10 and determined on a TOC analyzer (Liqui TOCII, Elementar, Germany). Soil samples were extracted in 2 M KCl solution (soil: water 1: 10) and the concentrations of ammonium, nitrate, and total dissolved nitrogen were determined using a continuous flow Analyzer (AA3, SEAL Company, Germany). Dissolved organic nitrogen (DON) was equal to the difference between total dissolved nitrogen and total inorganic nitrogen (ammonium and nitrate).

2.3. DNA extraction, PCR amplification, and Illumina sequencing

Genomic DNA was extracted from 0.5 g soil samples using the PowerSoil® DNA Isolation Kit (Mo Bio Laboratories, CA, USA) according to the manufacturer's instructions. The concentration and quality of the extracted DNA were determined by spectrophotometer analysis (NanoDrop 2000, Thermo Fisher Scientific, USA). The fungal ITS1 region was amplified with the primers ITS1-F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2 (5′-GCTGCGTTCTTC-ATCGATGC-3′) (Nottingham et al., 2018). The

purified PCR amplicons were sequenced on an Illumina NovaSeq platform (paired-end 250-bp mode).

Raw sequencing data were filtered and analyzed using QIIME2 (Bolyen et al., 2019), and DADA2 was used for primers removal, denoise, filtering, splicing, and chimera removal (Callahan et al., 2016). Subsequently, representative sequences of amplicon sequence variant (ASV) and a feature table were generated, and singleton ASVs were removed. The taxonomic information was assigned to each ASV based on the UNITE database (version 8.0) for ITS classifier training (Koljalg et al., 2013). All samples were resampled to the same sequencing level to avoid sequencing depth effects on fungal community. Raw sequencing data obtained in this study were archived in the NCBI Sequence Read Archive under accession number PRJNA832480.

2.4. Soil enzyme assays

Heavy metal pollution alters fungal community composition, thereby affecting soil ecosystem functions, especially soil organic matter accumulation and nutrient cycling. Therefore, the activities of hydrolases and oxidoreductases related to soil carbon, nitrogen, and phosphorus cycles were determined using a modified microplate method (Table S1, German et al., 2011; Saiya-Cork et al., 2002). The hydrolase activity was determined using a microplate fluorometric protocol. Briefly, 2 g fresh soil was homogenized in 125 ml of acetate buffer (50 mM) using a vortex oscillator (Vortex-6, QILINBEIER), and then a magnetic stirrer was used to keep the suspension uniform. The buffer, suspension, references (10 μM), and appropriate substrates (200 μM) were added to the wells of a black 96-well microplate in eight replicates per sample. The microplates were incubated at 25 °C for 4 h in the dark, and fluorescence was quantified on a microplate reader (SynergyH4, BioTek) with 365 nm excitation and 450 nm emission filters. Soil phenol oxidase and peroxidase were measured colorimetrically in clear 96-well microplates. The microplates were incubated at 25 °C for 18 h in the dark, and the absorbance at 460 nm was measured using the same microplate reader. Soil enzyme activity in this study was expressed as nmol reacted substrate g^{-1} soil h^{-1}.

2.5. Co-occurrence network analysis

Two co-occurrence networks were constructed in this study. The network of environment-microbe interactions was calculated in the 'igraph' package with a cutoff of Spearman correlation coefficient $|r| > 0.4$ and $P < 0.001$. These results were visualized using Gephi (Bastian et al., 2009). The other co-occurrence network was constructed using the collected 92 soil samples to identify the main ecological clusters of fungal taxa. We calculated all pairwise Spearman correlations between ASVs and visualized the positive and significant correlations ($r > 0.5$ and $P < 0.001$). Modules were identified using the *greedy optimization of modularity* algorithm. The network was visualized using the Fruchterman-Reingold layout with 10^4 permutations in 'igraph' package. The cumulative relative abundance of each ecological cluster was expressed as counts per million (CPM) calculated by the trimmed means of M-values method (TMM). We used complementary methods, including indicator species analysis and likelihood ratio test, to identify the ASVs that differ in abundance between zones, i.e. zone-sensitive ASVs.

2.6. Statistical analysis

Before statistical analysis, Shapiro-Wilks test and Levene's test were used to assess the normality and homogeneity of variance, respectively. One-way analysis of variance (ANOVA) combined with Duncan's test was used to assess difference in test parameters between sampling zones, and a $P < 0.05$ was statistically significant. Alpha diversity indices including the Shannon, Simpson, Chao1, and Observed species, were calculated using QIIME2. Principal coordinate analysis (PCoA) and analysis of similarity (ANOSIM) were performed to analyze differences in fungal community across zones based on the Bray-Curtis distance matrix using the 'vegan' R package. Pearson correlations between environmental variables and alpha diversity indices as well as between environmental variables and soil enzyme activities were depicted in heatmaps using the 'pheatmap' R package. In addition, the relationships between total Cu and Cd concentrations and the cumulative relative abundance of ecological clusters, relative abundance of selected fungal taxa, as well as soil enzyme activities were fitted with linear or exponential models. Random forest (RF) analysis was performed to assess the relationships between the environmental variables and the cumulative relative abundance of ecological clusters using the 'randomForest' package.

3. Results

3.1. Soil environmental variables

The concentrations of Cu and Cd were above local background values and were therefore selected for further analysis (Table S2). Total Cu and Cd (Cu_{tot} and Cd_{tot}) concentrations were significantly higher in zone I than in zones II and III of the basin. Bioavailable Cu (Cu_{bio}) only accounted for a proportion of Cu_{tot}, with an average of 19.1 % (Fig. 1). However, the proportion of bioavailable Cd (Cd_{bio}) to Cd_{tot} averaged 60.6 % (Fig. 1). The distribution of Cu_{bio} and Cd_{bio} was similar to the observed trends for Cu_{tot} and Cd_{tot}, with significantly higher concentrations in zone I relative to other zones.

All soil samples were acidic with a pH range of 4.4 and 6.2, and soil pH values were significantly lower in zone III than in other zones. Soil TC and TN concentrations were significantly higher in zone II than in zones I and III, whereas soil TP and DOC concentrations in zone I were the highest. In addition, soils in zone III had the highest nitrate concentration and the lowest ammonium and DON concentrations. There were no significant differences in soil AP concentration among the three sampling zones.

3.2. Fungal diversity and community composition

Among the alpha diversity indices, the Shannon index was significantly higher in zones I and II than in zone III, whereas the Simpson index was not significantly different among the three zones (Fig. 2). The Chao1 and observed species indices of fungi were significantly higher in zone II than in other zones. PCoA analysis revealed that the fungal communities of the three zones were clearly separated (Fig. 3). The ANOSIM test based on the Bray-Curtis matrix supported the PCoA results (globe $R = 0.5104$, $P = 0.001$), suggesting that heavy metal and edaphic factors reshaped soil microbiomes.

The dominant phyla in all soil samples were *Ascomycota* (36.4 %), *Basidiomycota* (18.5 %), *Mortierellomycota* (14.5 %), and *Rozellomycota* (3.0 %), accounting for >72 % of the total fungal communities (Fig. S2). The relative abundance of *Ascomycota* was significantly higher in zone I than in zone III. The relative abundance of *Basidiomycota* in zone II and *Mortierellomycota* in zone I was the lowest, and there was no significant difference in the relative abundance of *Rozellomycota* among the three zones. At the class level, the relative abundances of *Sordariomycetes*, *Agaricomycetes*, *Mortierellomycetes*, *Dothideomycetes*, *Leotiomycetes*, *Eurotiomycetes*, and *Tremellomycetes* were elevated (>1 %) (Fig. S3). Distinct microbial taxa were enriched in different zones. *Eurotiomycetes*, *Pezizomycetes*, *Ustilaginomycetes*, *Saccharomycetes*, *Olpidiomycetes*, and *Kickxellomycetes* were significantly enriched in zone I, *Mortierellomycetes* in zones II and III, and *Tremellomycetes* in zone III.

3.3. Relationships between environmental variables and fungal communities

Substantial variation in environmental variables across the basin was observed, which facilitated to investigate the relationships between environmental variables and native microbiota. Soil pH was positively correlated with soil fungal diversities (Fig. 4). The four metal-related variables (Cu_{tot}, Cd_{tot}, Cu_{bio}, and Cd_{bio}) were positively correlated with the Shannon index, but not with other diversity indices (Fig. 4). Also, soil TN and DON

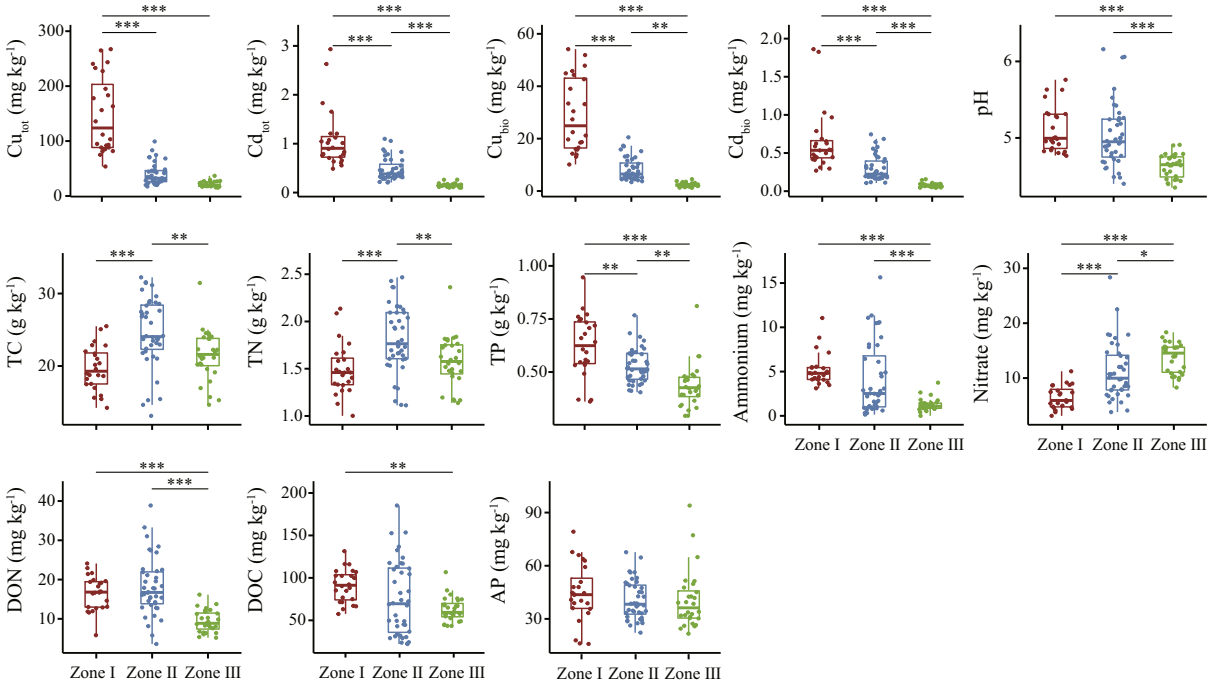

Fig. 1. Box plots showing the distribution of environmental variables across the three sampling zones. *, **, and *** indicate significant differences in means based on one-way ANOVA and Duncan's post hoc test at $P < 0.05$, <0.01, and <0.001, respectively.

concentrations were positively correlated with the Chao1 and Observed species indices (Fig. 4).

The co-occurrence networks revealed the interactions between individual phylotypes (ASVs) and environmental variables. Soil heavy metals, i.e. Cu_{tot}, Cd_{tot}, Cu_{bio}, and Cd_{bio}, had the greatest effects on fungal communities, followed by nitrate, DON, TC, ammonium, pH, TN, DOC, TP, and AP (Fig. 5). The co-occurrence networks consisted of six modules according to the connection profile (Fig. 5). Distinct modules indicated the existence of different microbial functions, and nodes clustered within a module had some similarities. Interestingly, four metal-related variables

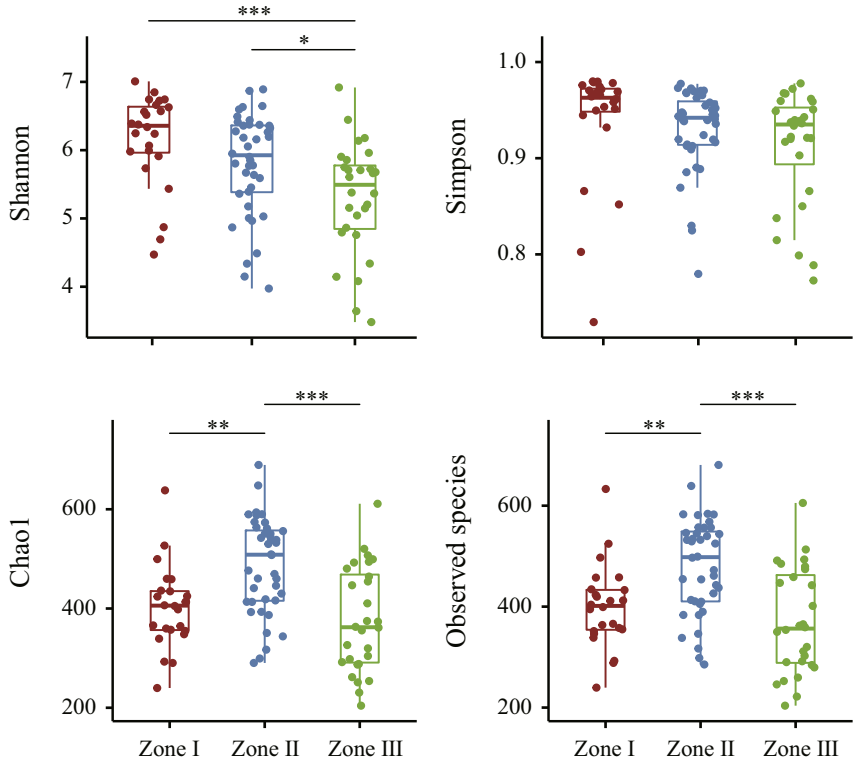

Fig. 2. Box plots showing fungal alpha diversity indices across the three sampling zones. *, **, and *** indicate significant differences in means based on one-way ANOVA and Duncan's post hoc test at $P < 0.05$, <0.01, and <0.001, respectively.

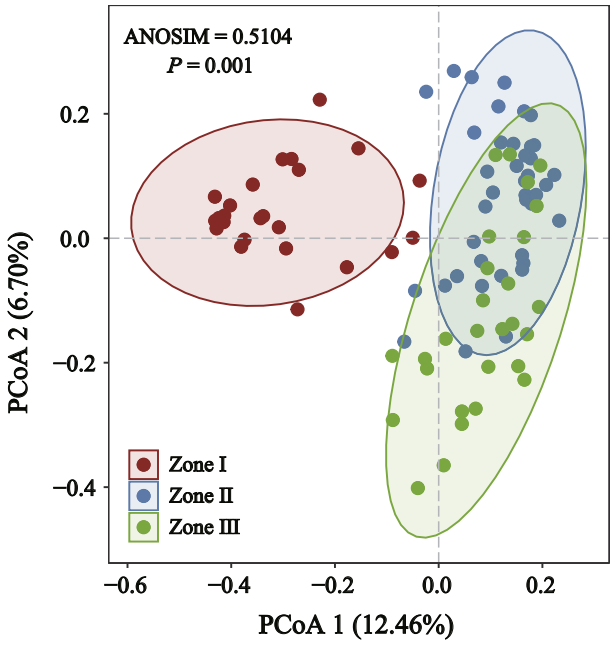

Fig. 3. Principal Coordinates Analysis (PCoA) of Bray-Curtis distance matrices of fungal ITS amplicons across the three sampling zones.

(Cu_{tot}, Cd_{tot}, Cu_{bio}, and Cd_{bio}) were located in the same module (i.e. module I, Fig. 5), indicating that the metal-related environmental variables had similar and marked effects on soil fungal communities.

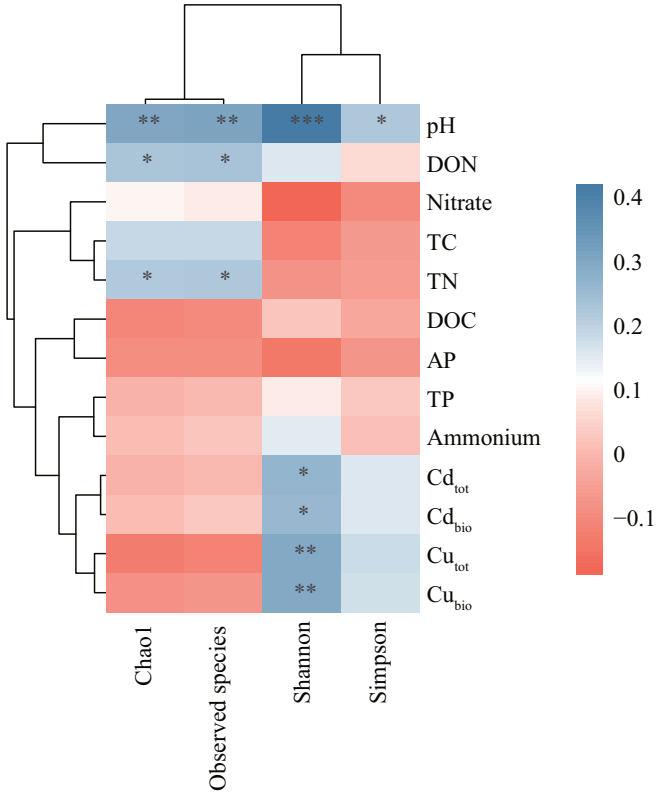

Fig. 4. Heatmap of Pearson correlation between environmental variables and fungal alpha diversity indices. Negative and positive Pearson's correlation coefficients are represented in red and blue color, respectively. *, **, and *** indicate $P < 0.05$, <0.01, and <0.001, respectively.

3.4. Fungal co-occurrence patterns

Soil fungal taxa could be grouped into three major ecological clusters (modules #1, #2, and #3), which strongly coexisted with each other (Fig. 6a). Different ecological clusters dominated the three sampling zones downstream of the mining site (Fig. 6b). Specifically, the cumulative relative abundance of module #1 was significantly higher in zone I than in zones II and III, whereas the cumulative relative abundance of modules #2 and #3 was the highest in zones II and III (Fig. 6b). The proportion of zone-sensitive ASVs of zones I, II, and III in modules #1, #2, and #3 to the total module ASVs was 92.7 %, 35.8 %, and 52.3 %, respectively (Fig. 6a). The responses of the cumulative relative abundance of ecological clusters to heavy metals were different (Fig. S4). For example, significant linear positive relationships were found between the cumulative relative abundance of module #1 and the concentrations of Cu_{tot} ($R^2 = 0.38$, $P < 0.001$) and Cd_{tot} ($R^2 = 0.51$, $P < 0.001$). However, the cumulative relative abundance of module #3 was exponentially negative correlated with the concentrations of Cu_{tot} ($R^2 = 0.16$, $P < 0.001$) and Cd_{tot} ($R^2 = 0.24$, $P < 0.001$). Random forest analysis revealed that total and bioavailable Cu and Cd contents were the main predictors of the cumulative relative abundance of ecological clusters (Fig. S5). Also, soil general properties were important for fungal community, but their relative importance was largely depended on taxa and modules. The Pearson correlations between environmental predictors and the cumulative relative abundance of three ecological clusters were shown in Table S3.

Each ecological cluster contained multiple fungal taxa attributing to different classes (Fig. 6c). *Sordariomycetes*, *Eurotiomycetes*, and *Dothideomycetes* were the most dominant classes in module #1, whereas *Leotiomycetes* predominated in module #2 and *Agaricomycetes* and *Mortierellomycetes* in module #3. However, some fungal classes were only found in specific ecological clusters, such as *Orbiliomycetes*, *Ustilaginomycetes*, *Rozellomycotina_cls_Incertae_sedis*, *Pezizomycetes*, *Malasseziomycetes*, and *Kickxellomycetes* in module #1, *Rhizophydiomycetes*, *Cystobasidiomycetes*, and *Exobasidiomycetes* in module #2, and *Archaeorhizomycetes* and *Pucciniomycetes* in module #3. The relative abundance of some classes was linearly or exponentially correlated with the concentrations of Cu_{tot} and Cd_{tot} (Fig. 7). For example, the relative abundances of *Eurotiomycetes*, *Pezizomycetes*, *Ustilaginomycetes*, and *Kickxellomycetes* were positively correlated with the concentrations of Cu_{tot} and Cd_{tot}, whereas the relative abundance of *Mortierellomycetes* was negatively correlated with the concentrations of Cu_{tot} and Cd_{tot}, respectively.

3.5. Soil enzyme activities

The activities of β-glucosidase (βG), N-Acetyl-glucosaminidase (NAG), acid phosphatase (ACP), β-xylosidase (βX), cellobiohydrolase (CBH), α-glucosidase (αG), L-leucine aminopeptidase (LAP), phenol oxidase (PhOx), and peroxidase (Perox) were determined. The activities of βG, ACP, βX, CBH, αG, and LAP were significantly lower in zone I than in zones II and III, whereas the activities of ACP, βX, and CBH were significantly lower in zone II than in zone III (Fig. S6). The NAG activity showed significantly increased in soils of zone III (Fig. S6). In contrast, the activities of two oxidoreductases (PhOx and Perox) were relatively higher in zone II than in other two zones (Fig. S6).

Pearson correlations demonstrated that hydrolases were more sensitive to heavy metals relative to oxidoreductases, and were negatively correlated with the concentrations of Cu_{tot}, Cd_{tot}, Cu_{bio}, and Cd_{bio} (Fig. 8). Regression analysis revealed that the activities of all hydrolytic enzymes were linearly or exponentially related to the concentrations of Cu_{tot} and Cd_{tot}, whereas no significant relationship between oxidoreductase activities and heavy metals was observed (Fig. S7). In addition, soil pH had negative effects on soil enzyme activities, especially for ACP ($P < 0.01$), βX ($P < 0.001$), and αG ($P < 0.05$) (Fig. 8). Soil ammonium was negatively correlated with most of hydrolases except LAP, while soil nitrate was positively correlated with most of hydrolases except αG. Significant positive correlations were observed between the concentrations of TC and TN and the activities of

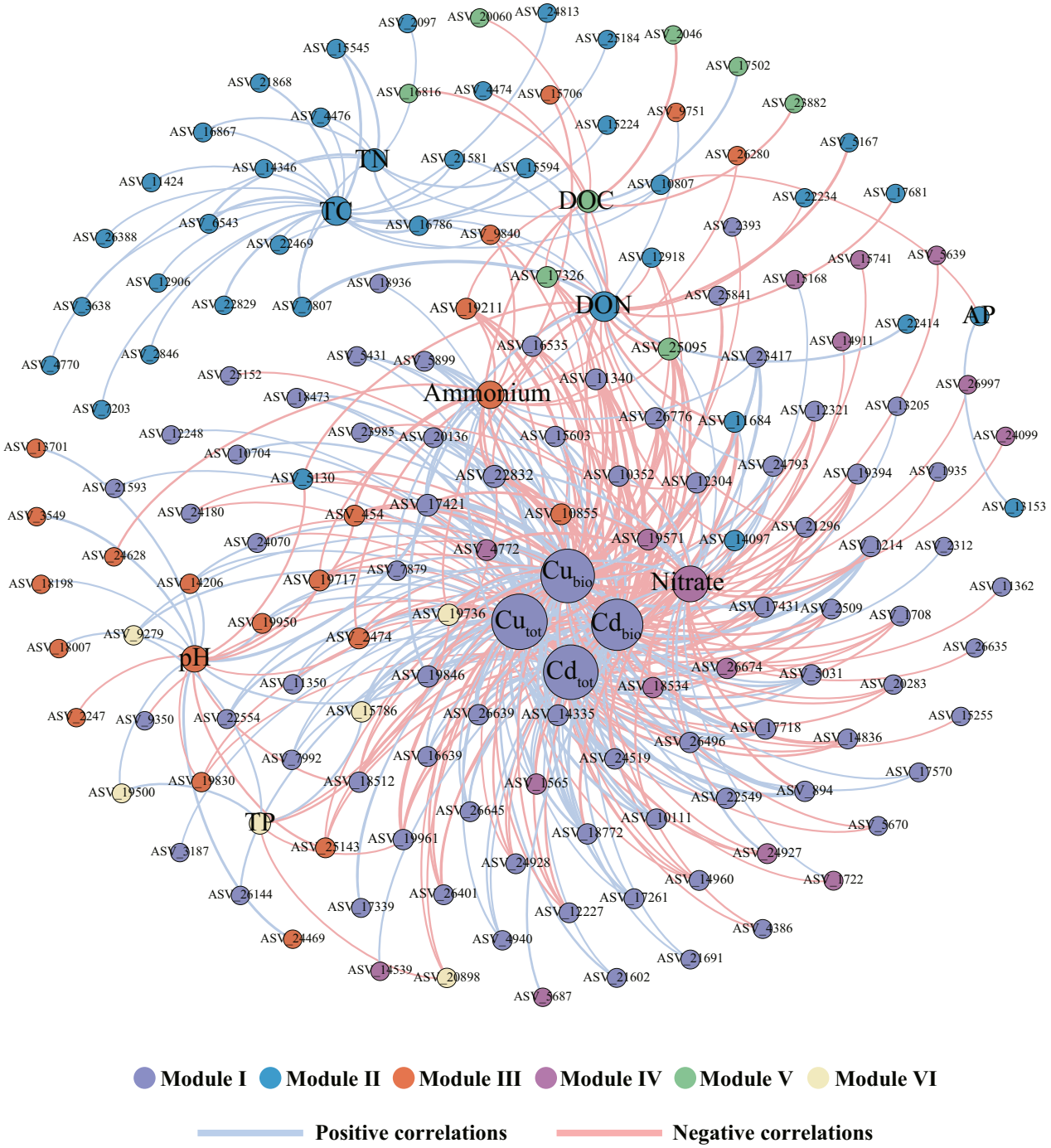

Fig. 5. Co-occurrence network of the environment-microbe correlations. Blue and red lines present significant positive and negative connections ($|r| > 0.4$, $P < 0.001$), respectively. The thickness of the edges is proportional to the strength of correlation. The size of a node is proportional to the number of connections. The color of nodes represents the cluster of interactions.

ACP, βG, and αG, as well as between TC concentration and LAP activity, respectively.

4. Discussion

4.1. Effects of environmental variables on fungal communities

The Xiancha River basin has been heavily contaminated with Cu and Cd from long-term sewage irrigation. Elucidating the impacts of Cu and Cd on native microbial community is essential to understand their ecological roles in the restoration of Cu and Cd co-contamination farmlands. The diversity indices of soil fungi in the three sampling zones were significantly different (Fig. 2), and the pollutant fractions had positive effects on the Shannon index (Fig. 4). However, our observations are inconsistent with previous studies, in which heavy metals have been reported to decrease soil microbial diversity (Frossard et al., 2017; Sun et al., 2020). For example, a significant reduction in fungal diversity was observed in soils contaminated with heavy Cu compared to normal soils (Zhang et al., 2022). The paddy soils examined in this study were chronically contaminated with Cu and Cd, and consequently, the fungal communities could have had sufficient time to develop resistance to the heavy metal stresses (Lin et al., 2020). Alternatively, increasing Cu and Cd concentrations might increase fungal diversity

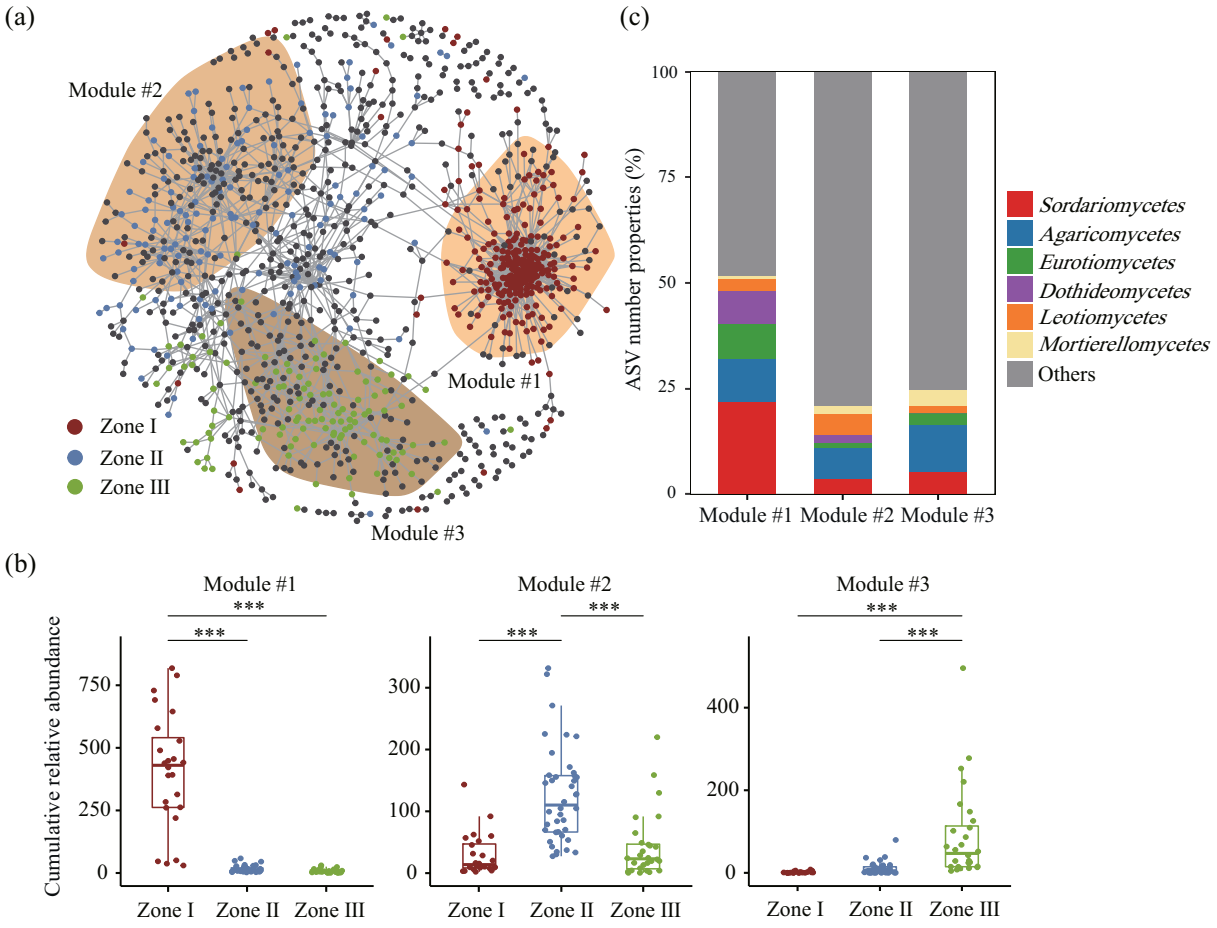

Fig. 6. (a) Network diagram with nodes colored by their association to the different sampling zones. The shaded areas represent the three main ecological clusters (modules #1, 2, and 3). (b) Cumulative relative abundance (as counts per million, CPM; y-axis in $\times 1000$) of modules in the three sampling zones. *** indicates a significant difference based on one-way ANOVA and Duncan's post hoc test at $P < 0.001$. (c) Amplicon sequence variant (ASV) number properties of fungi taxa in the three main ecological clusters.

through competitive release of subordinate microbial taxa. These results indicated that Cu and Cd contamination shaped the diversity of soil fungi, thereby affecting ecosystem function. In addition, soil pH has been proved to be an important determinant of microbial diversity (Wei et al., 2019). Our results also revealed that pH was significantly correlated with all fungal diversity indices. Soil nutrients play crucial roles in microbial growth, such as energy metabolism, cell division, and protein synthesis (Shahid et al., 2017). This was confirmed by the positive correlations between fungal diversity and soil TN and DON concentrations. Soil pH and dissolved N are the key drivers influencing fungal community composition (Hu et al., 2017). In general, soil fungi have wider pH range 4.0–8.3 compared with soil bacteria, and fungi thrive well under high nitrogen conditions (Philippot et al., 2013). Soil pH regulates soil nitrogen transformation rates and nitrogen availability, and NO_3^- and NH_4^+ directly influence soil fungal communities due to the alteration of N availability, ion toxicity, and osmotic potential (Omar and Ismail, 1999).

Our results showed that soil fungal community segregated significantly under varying levels of heavy metal contamination (Fig. 3). Several previous studies have also reported profound impacts of heavy metals on microbial community assembly (Mohammadian et al., 2017; Yu et al., 2021). Simultaneously, soil fungi adapt to heavy metal contamination by altering the community composition (Zhao et al., 2019). Our results showed that soil Cu and Cd co-contamination significantly affected fungal community composition (Figs. S2 and S3). The contrasting responses of these taxonomic groups could be related to the physiological characteristics of fungi. Long-term heavy metal stress reduces the abundance of sensitive fungi and stimulates the growth of tolerant fungi, thereby rebuilding fungal communities (Singh et al., 2014). For example, *Ascomycota*, the most abundant and widespread fungus in soil (Al-Sadi et al., 2017), increased drastically in the heavily contaminated zones (Fig. S2). This result indicated that *Ascomycota* was highly tolerant to heavy metals. In contrast, *Mortierellomycota* was less abundant in zone I. This was consistent with a previous study showing a higher relative abundance of *Mortierellomycota* in healthy soils (Yuan et al., 2020). Changes in the relative abundances of *Ascomycota* and *Mortierellomycota* would largely influence ecological processes mediated by the two groups.

To further investigate the response of individual fungal taxa to environmental variables, especially in relation to Cu and Cd contaminations, the environment-microbe interactions were visualized through co-occurrence networks (Fig. 5). The Cu and Cd fractions (e.g. Cu_{tot}, Cd_{tot}, Cu_{bio}, and Cd_{bio}) were larger in sizes than other nodes, indicating that heavy metal contamination had a greater impact on native fungal community than soil general properties. Although bioavailable Cu was only 19.1 % of total Cu, both Cu_{tot} and Cu_{bio} had similar impacts on individual fungal populations. Bioavailable fraction is readily released from soil substrates and then transported into microbial cells (Wang et al., 2020). Consistent with a previous study (Mohammadian et al., 2017), there were numerous fungal taxa showing multiple connections with heavy metal contamination. In particular, the ASVs associated with the classes *Eurotiomycetes* (e.g. ASV_18534, ASV_23417, and ASV_24519), *Ustilaginomycetes* (e.g. ASV_15786), *Pezizomycetes* (e.g. ASV_4772), and *Olpidiomycetes* (e.g. ASV_7992) were positively correlated with the Cu and Cd fractions. These classes were significantly enriched in zone I (Fig. S3), indicating that their enrichments could be related to Cu and Cd contamination.

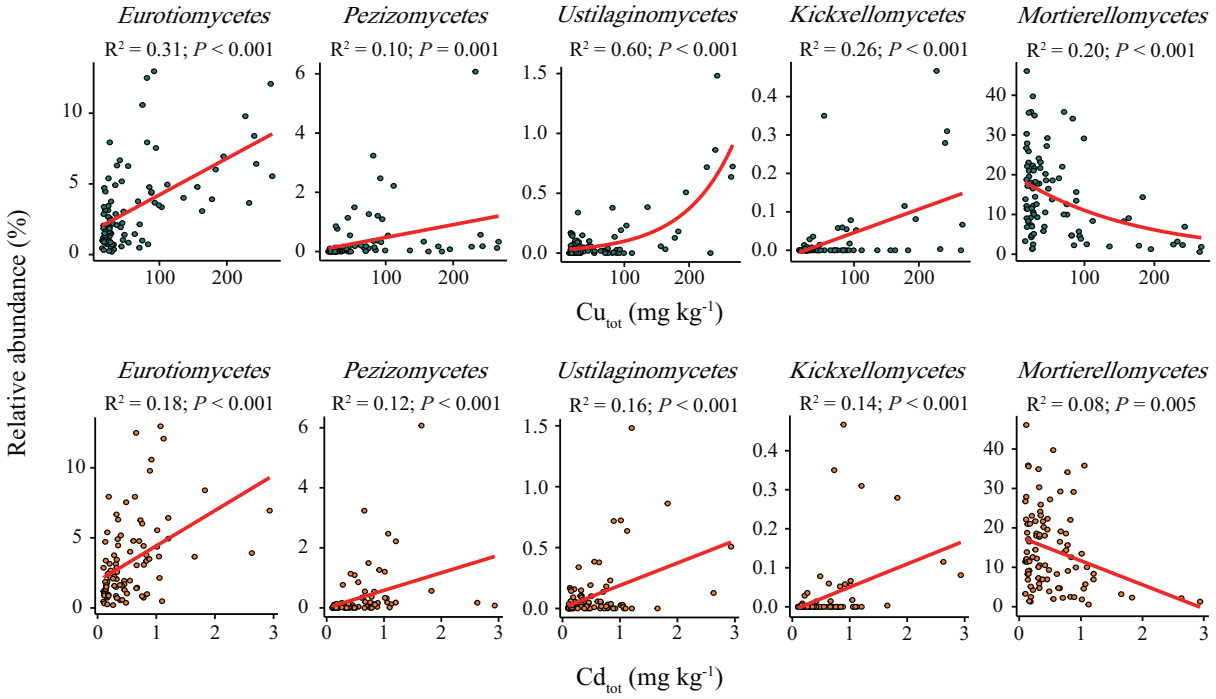

Fig. 7. Regression between soil total Cu and Cd concentrations and relative abundance of selected fungal taxa.

4.2. Alterations of fungal co-occurrence patterns and crucial populations

Fungal interactions are particularly crucial for revealing the mechanisms of fungi respond to environmental stresses (Wang et al., 2019). For example, changes in microbial interactions enhance microbial adaptation to heavy metal contamination and drought (de Vries et al., 2018; Zhang et al., 2022). After considering other environmental predictors, random forest analysis showed that Cu and Cd fractions were generally significant predictors of the ecological clusters within fungal co-occurrence networks (Fig. S5). Therefore, the increase in soil Cu and Cd concentrations also caused great changes in the co-occurrence networks of fungal communities in the study area. Interestingly, the cumulative relative abundance of some ecological clusters changed significantly with increasing Cu and Cd contaminations (Fig. S4). For example, elevated Cu and Cd concentrations tended to increase the cumulative relative abundance of module #1, but decrease the cumulative relative abundance of module #3. These results indicated that the distribution of fungal ecological clusters could be altered by changes in the two pollutants in the paddy soils. Other environmental predictors were also important for the cumulative relative abundance of ecological clusters (Fig. S5), which had been documented in previous studies (de Menezes et al., 2015; Delgado-Baquerizo et al., 2018).

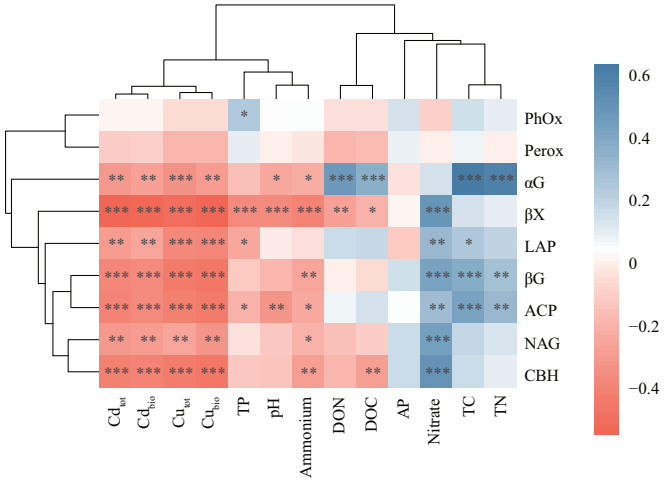

Fig. 8. Heatmap of Pearson correlation between the environmental variables and soil enzyme activities. Negative and positive Pearson's correlation coefficients are represented in red and blue color, respectively. *, **, and *** indicate $P < 0.05$, <0.01, and <0.001, respectively.

Distinct fungal taxa were present in each ecological cluster (Fig. 6c). Ecological preference modules susceptible to heavy metals tended to cluster microorganisms that were resistant or sensitive to heavy metal contamination (Liu et al., 2018). Analysis of crucial fungal populations in the co-occurrence networks at the species level brought in unclassified data, so we performed further analyses at the class level. Importantly, Cu and Cd contamination was significantly correlated with the relative abundance of several fungal classes in different ecological clusters (Fig. 7). For example, *Eurotiomycetes*, *Pezizomycetes*, *Ustilaginomycetes*, and *Kickxellomycetes* occurred in module #1, and their relative abundance increased with Cu and Cd concentrations. These classes were significantly enriched in heavily contaminated regions (e.g. zone I, Fig. S3), indicating that these microorganisms might be tolerant of heavy metals and adapt to survive and reproduce in such environments. In contrast, *Mortierellomycetes* dominated in module #3 and enriched in low-pollutant regions (e.g. zones II and III), and its relative abundance was negatively related to Cu and Cd concentrations. These results suggested its sensitivity to Cu and Cd contamination.

The class *Eurotiomycetes* exhibits obvious metal resistance as it is abundant in mine-contaminated soils (Ye et al., 2020). A number of fungal strains belonging to the class *Eurotiomycetes* are isolated, and they are likely correlated with the presence of abundant metal ions in the culture medium (Muggia et al., 2017). Especially, members of this class, such as *Aspergillus niger* and *Penicillium* sp., appear to evolve specific catabolic activities to use pollutants as nutrients and energy sources (Mohammadian et al., 2017). The class *Pezizomycetes* has been reported as a leading group in trace metal contaminated areas with high concentrations of Cd, Cu, Zn,

and Pb (Yung et al., 2021). This finding confirmed our results that *Pezizomycetes* was resistant to Cu and Cd co-contamination. Similarly, *Kickxellomycetes* was significantly enriched at zone I, probably due to its tolerance to heavy metals (Sun et al., 2022). However, less information was available on the mechanisms underlying the response of *Ustilaginomycetes* to Cu and Cd. Therefore, these fungal taxa were highly adaptable to heavy metal contamination and might have great potential for bioremediation. In addition, our results suggested that elevated soil Cu and Cd decreased the relative abundance of *Mortierellomycetes*. This class is highly sensitive to soil bioavailable Cd (Shi et al., 2020). Changes in resistant and sensitive fungal taxa represented an alternation in major ecological clusters, and a shift in co-occurrence patterns could be a way for fungi to adapt to Cu and Cd co-contamination.

4.3. Effects of environmental variables on soil enzyme activities

Soil enzymes catalyze a variety of critical reactions in microbial life processes, including nutrient cycling and detoxification of xenobiotics (Burns et al., 2013). Therefore, investigating soil enzyme activities under Cu and Cd co-contamination is important to identify changes in soil ecological functions and pollution status (Hagmann et al., 2015). Here, Cu and Cd contamination strongly and negatively affected the activities of all hydrolytic enzymes (Figs. 8 and S7). This indicated that heavy metal contamination could reduce the rate of carbon, nitrogen, and phosphorus cycling in soils. The inhibition of hydrolytic enzyme activities by Cu and Cd attributed to multiple processes, including causing protein denaturation, forming complexes with the substrate, and affecting enzyme synthesis, etc. (Hinojosa et al., 2004). However, the activities of oxidoreductases (PhOx and Perox) were not sensitive to the Cu and Cd fractions. These results revealed that hydrolases rather than oxidoreductases could be used as a sensitive indicator of Cu and Cd co-contamination, which was inconsistent with other reports (Yang et al., 2016). Also, soil general properties significantly affected the enzyme activity (Fig. 8). For example, soil pH is a key environmental variable for enzyme activity, as it can influence the dissociation condition of enzymatic active sites and enzyme stability. In line with previous investigations (Fang et al., 2017; Xian et al., 2015), soil total carbon and nitrogen promoted several hydrolase activities. Overall, these results provide a comprehensive enzymatic view of their role in regulating nutrient cycling in Cu and Cd co-contaminated soils. Unfortunately, our study only depicted the effects of Cu and Cd contamination on soil fungal communities and enzyme activities. Future research should isolate and cultivate the heavy metal resistant taxa to clarify their bioremediation functions.

5. Conclusions

This study assessed the responses of soil microbiomes and enzymes to long-term Cu and Cd co-contamination in paddy soils in southern China. Cu and Cd contamination significantly affected multiple taxonomic microbial attributes, including fungal diversity, community composition, and the cumulative relative abundance of ecological clusters. The concentrations of total and bioavailable Cu and Cd were positively correlated with fungal alpha diversity. Heavy metal contamination altered fungal community composition, and the impact of metal contaminant fractions on fungal communities was greater than soil general properties. The changes in ecological clusters of fungal co-occurrence networks potentially affect ecosystem function. The relative abundance of *Eurotiomycetes*, *Pezizomycetes*, *Ustilaginomycetes*, and *Kickxellomycetes* increased with Cu and Cd concentrations, whereas a negative correlation between *Mortierellomycetes* and Cu and Cd pollutants was found. The changes in fungal taxa reflected adaptation to heavy metal-contaminated soils. Also, heavy metal contamination inhibited the activities of hydrolases, thereby influencing soil carbon, nitrogen, and phosphorus cycles. Overall, this study promotes the understanding of the ecological response to Cu and Cd co-contamination in paddy soils, which could provide evidence for improving soil productivity and performing bioremediation of heavy metal contamination.

CRediT authorship contribution statement

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.157119.

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