Initial responses of influential microbial taxa to metal elements lead to alterations in the forest soil microbial community structure and function

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July 15, 2022

Abstract

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Running title: Effects of metals on soil microbial community

Keywords: C:N ratio; community structure; forest ecosystem; metal element; microbial function

Article Type: Letter

Abstract length: 150 words

Main text length: 4977 words

Number of References: 77

Number of Figures/Tables: 6

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Author contributions

XW and YL designed the study; HX, XW, MZ, QY, and SD collected the data; XW, JY, JC, XL, and DD analyzed the data and performed the statistical analyses; XW and YL wrote the manuscript with input from all authors.

Data availability

The raw data are available in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (accession numbers: PRJNA-bacteria and PRJNA-fungi). Once accepted, all data will be available on Dryad.

Abstract

As primary drivers of underlying microbial changes, soil metals have been extensively studied in agroecosystems. However, while their contributions to forest soil microbial processes are crucial for maintaining tree biodiversity, they remain poorly understood. Based on the analysis of 1287 soil samples collected from a 20 ha forest plot, we show that seven metal elements (Al, Ca, Cu, Fe, Mg, Mn, and Zn) shape microbial community structure and function by initially altering influential microbial taxa. Microbial α-diversity and community structure responded differently to these elements at low *vs.* high C:N ratios, pH, and water content. Moreover, these elements also affected microbial functional guilds (e.g., phosphorus and sulfur metabolism, ectomycorrhizae, plant pathogens, and wood saprotrophs) via sensitive microbial taxa. This study advances our capacity to predict belowground microbial processes by revealing the fundamental importance of metals in forest soils, with important implications for better conserving forest biodiversity under global climate change.

Introduction

Forests, which host much of the planet's terrestrial biodiversity, play a paramount role in global ecosystems and buffer against climate change (Bonan 2008; Zhang *et al.* 2018c). In forest ecosystems, tree growth, development, and reproduction are regulated by metal element availability, including essential macronutrients (e.g., Ca and Mg) and micronutrients (e.g., Cu, Fe, Mn, and Zn), as well as numerous non-essential but beneficial elements (e.g., Al; Bojorquez-Quintal *et al.* 2017). These elements must be mobilized from the soil matrix and absorbed by the roots in the form of metal ions (DalCorso *et al.* 2014). Moreover, as one of the main drivers underlying soil microbial community change, metal elements may also exert persistent stress on soil microbial communities, thereby impacting belowground biodiversity that comprises up to a quarter of the Earth's species (Beattie *et al.* 2018; Wagg *et al.* 2019; Rogiers *et al.* 2021). As any soil disturbance may disrupt microbial activity (Bissett *et al.* 2013), understanding the responses of microbial communities to soil metal elements is a fundamental ecological issue for maintaining belowground biodiversity and ecosystem stability. Although numerous studies have been conducted on the roles and influence of metal elements on soil microbes in terrestrial ecosystems, these have focused particularly on the contamination and bioremediation of heavy metals in farmlands (Toth *et al.* 2016; Rodríguez Eugenio 2018; Hou *et al.* 2020a), while their importance and contribution to soil microbial processes in unmanaged forest ecosystems with high biodiversity remain largely unexplored. Furthermore, a robust prediction of future forest belowground biodiversity requires a mechanistic understanding of how metal elements influence microbial community structure and function in forest soils.

As fundamental determinants of tree biodiversity (John *et al.* 2007) and microbial

organic nutrient acquisition (Sinsabaugh *et al.* 2009), metal elements exert different regulating effects on soil microbes depending on their types and amounts (Memoli *et al.* 2018), thereby driving different physiological processes of microbial life (Liu *et al.* 2018). Specifically, some microelements with essential biological functions (Marschner & Kalbitz 2003; Bowker 2006) are required in smaller amounts by plants (<100 $mg \cdot kg^{-1}$ dry weight; e.g., Cu, Fe, Mn, and Zn; DalCorso *et al.* 2014). These elements are involved in plant metabolic processes (Hansch & Mendel 2009; Hou *et al.* 2020b), regulate plant growth and physiological activity (DalCorso *et al.* 2014; Hou *et al.* 2020b), ensure the organic matter cycle, and maintain soil functionality (Memoli *et al.* 2018). Moreover, they are also components of enzyme prosthetic groups that catalyze redox processes, form enzyme-substrate complexes, and enhance enzyme reactions (Radujkovic *et al.* 2021). Other microelement metals (>1000 $mg \cdot kg^{-1}$ dry weight) are essential for plant growth (DalCorso *et al.* 2014), among which Ca regulates global soil fungal diversity (Leho Tedersoo *et al.* 2014) and affects fungal community composition in forest soils, whereas Mg^{2+} influences the richness of soil bacterial taxa (e.g., Actinobacteria, Bacteroidetes, Chloroflexi, and Proteobacteria; Xia *et al.* 2016; Liu *et al.* 2018). Furthermore, plants must acquire a moderate amount of metal elements for healthy growth; if any of these elements are in short supply, symptoms of nutrient deficiencies and increased susceptibility to disease can appear (DalCorso *et al.* 2014). For instance, Mn- and Cu-deficient plants are more susceptible to root-infecting pathogens and fungal diseases, respectively (DalCorso *et al.* 2014), whereas Zn deficiency is widespread in plants growing in acidic conditions (Hansch & Mendel 2009; DalCorso *et al.* 2014). Notably, excessive accumulation of some metal elements can be toxic to plants. For example, increasing soil Al^{3+} concentrations with acid deposition can cause Al phytotoxicity, and excess Al can also

lead to oxidative stress because of the manifestation of reactive oxygen species (ROS; Yamamoto *et al.* 2003). Although previous studies have enriched our collective knowledge regarding the effects of metal elements on plants, most have primarily focused on their indispensability and toxicity.

As the second genome of plants and facilitators of soil ecosystem change (Coban *et al.* 2022; Zhang *et al.* 2022), soil microbes dominate terrestrial soil habitats (Bardgett & van der Putten 2014; Bahram *et al.* 2018). Furthermore, soil microbes afford primary functions for the formation and maintenance of soil structure and fertility (Bronick & Lal 2005; Rogiers *et al.* 2021); thus, variations in the microbial community may lead to significant changes in soil ecosystems (Rogiers *et al.* 2021). However, among the numerous soil factors that predict microbial community change, the effects of metal elements on microbial community structure and function may be confounded by other soil properties. In addition to the direct filtering effects of predominant soil properties on microbes (e.g., C:N ratio, pH, and water content [WC]), there are complex interrelationships between microbial communities and metal elements in highly heterogeneous forest soils. For instance, Al^{3+} in acidic soils can interfere with the metabolic activities of soil microbes, and thus have a toxic effect on plants (Zhang *et al.* 2022). As a defense mechanism, some microbial taxa, especially Al-tolerant microbes (e.g., *Klebsiella* and *Serratia*), can reduce Al toxicity via absorbing, adsorbing, and secreting organic acids that extracellularly chelate Al^{3+} (Mora *et al.* 2017; Zhang *et al.* 2022). Additionally, soil microbial activity can regulate the availability of metal elements and mediate important biogeochemical cycles of various elements (He *et al.* 2010; Memoli *et al.* 2018). For example, microbes associated with Fe biogeochemical cycles (e.g., Fe (III)-reducing bacteria with extensive metabolic capacity) can be coupled with the oxidation of organic

matter and the reduction of Fe (III) oxyhydroxide to dissolve elements associated with Fe (III) minerals (e.g., As, Cr, Cd, Pb; Dubinsky *et al.* 2010; Memoli *et al.* 2018).

In general, to better explore the potential ability of metallic elements in a contiguous natural ecosystem, it is essential to investigate the linkages between forest soil metals and belowground microbes. Accordingly, in the present study, 1287 soil samples collected from a 20 ha subtropical forest dynamic plot were analyzed to assess how metal elements influence microbial community structure and function. Considering that solubility, biological availability, and activity of soil metals are dependent upon soil properties (McBride 1989; Kabata-Pendias 2004), the differences in microbial α-diversity, community structure, and function were also explored in response to metal elements under relatively low *vs.* high C:N ratios, pH values, and WC. We hypothesized that: (i) soil metals can shape microbial community structure and function by initially altering the influential microbial taxa (i.e., microbial biomarkers and keystone taxa) and (ii) soil metals can influence microbial functional guilds by regulating various sensitive microbial taxa (i.e., microbial indicator taxa) at relatively low *vs.* high C:N ratios, pH values, and WC (Figure 1).

Materials and Methods

Study site

The study site was located at Tiantong National Field Observation Station for Forest Ecosystems, Zhejiang Province, South China (29°48′ N, 121°47' E). This 20 ha (500 × 400 m) forest plot was established in 2008 as a component of the Forest Global Earth Observatory (ForestGEO) network (https://forestgeo.si.edu/; Qiao *et al.* 2020). It is a typical subtropical evergreen broadleaved forest, with a subtropical monsoon climate consisting of humid–hot summers and dry–cold winters (Zhou *et al.* 2020). The

161 average annual temperature is 16.2°C, and precipitation is 1374 mm (Hu *et al.* 2020).
162 The forest soil is clay loam, with 6.8% sand, 55.5% silt, and 37.7% clay (Zhou *et al.*
163 2020), and the most common tree species include *Castanopsis fargesii* Franch.,
164 *Schima superba* Gardn, and *Castanopsis carlesii* (Hemsl.) Hayata (Hu *et al.* 2020).

165 *Soil sampling and analyses*

166 Soil samples were collected in September 2018 for molecular analysis of soil
167 microbes and physicochemical analyses. The plot was divided into 500 quadrats (20
168 m × 20 m). Following the Center for Tropical Forest Sciences criterion for soil sample
169 collection (John *et al.* 2007), the intersection of the southwest corner of each quadrat
170 was selected as the sampling base point, and two sampling points (2, 5, and 8 m away
171 from the base point in each selected direction) were selected randomly (Figure S1).
172 Following the removal of surface debris, four soil cores from 0–10 cm in depth (5 cm
173 diameter) were randomly selected within 1 m around each selected sampling point,
174 and mixed to comprise a single sample. In total, 1287 soil samples were obtained.
175 Each sample was sealed in a sterile sampling bag, and shipped to the laboratory in
176 iceboxes immediately following collection. Upon arrival, all samples were sieved to 2
177 mm, and subdivided into three subsamples: one stored at -80°C before extracting
178 DNA for molecular analyses of soil microbes, a second partially air- or oven-dried for
179 analyses of soil physicochemical properties, and a third stored at 4°C for analyses of
180 ammonium (NH_4^+-N) and nitrate (NO_3^--N) content. Measurements of pH, WC, and 11
181 soil elements, including C, N, P, K, Al, Ca, Cu, Fe, Mg, Mn, and Zn, were conducted.
182 Further detailed descriptions of soil physicochemical analyses are available in the
183 supplementary information.

184 *Soil microbial DNA extraction and PCR amplification*

185 Total DNA was extracted from 0.5 g of soil for each sample using a MagPure Soil

DNA KF Kit according to the manufacturer's instructions (Magigene Biotechnology Co., Ltd. Guangzhou, China). DNA quality was assessed using 1% agarose gels, and its concentration and purity were determined using a NanoDrop One (Thermo Fisher Scientific, Waltham, MA, USA). The bacterial V4 region of the 16S rRNA gene was amplified with the universal primers 515F and 806R. The fungal first internal transcribed spacer (ITS1) region was targeted using the universal primers ITS5-1737F and ITS2-2043R. The primers were synthesized by Invitrogen (Carlsbad, CA, USA). PCR amplification was performed in triplicate using a BioRad S1000 (Bio-Rad Laboratory, Hercules, CA, USA) in a 50 μL reaction system that included 25 μL of 2× Premix Taq (Takara Biotechnology Co. Ltd., Dalian, China), 1 μL of each primer (10 μM), and 3 μL of DNA template (20 $ng \cdot \mu L^{-1}$). DNA samples were amplified under the following PCR conditions: 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and elongation at 72°C for 30 s, and fluorescence intensity at 72°C. The PCR product length and concentration were determined following 1% agarose gel electrophoresis. PCR products were mixed in equidensity ratios according to GeneTools Analysis (*v*.4.03.05.0, SynGene), and PCR products were combined and purified using an EZNA® Gel Extraction Kit (Omega Bio Tek, Norcross, GA, USA). Sequencing libraries were generated using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA, USA) following the manufacturer's protocol, and appropriate index codes were added. The purified amplicons were pooled in equimolar concentrations, and paired-end sequenced on an IlluminaHiseq2500 platform (Guangdong Magigene Biotechnology Co., Ltd., Guangzhou, China) according to standard protocols.

Sequence data processing

The Quantitative Insights into Microbial Ecology (QIIME, *v*.1.8.0) pipeline was used

211 to process the sequencing data. The raw 16S rRNA and ITS gene sequencing reads
212 were demultiplexed, quality-filtered in Trimmomatic (Bolger *et al.* 2014), and merged
213 using FLASH (Magoc & Salzberg 2011) under the following criteria: (i) only
214 overlapping sequences > 10 bp were assembled accordingly; (ii) the maximum
215 mismatch ratio of the overlap region was 0.1, and reads that could not be assembled
216 were discarded; and (iii) samples were distinguished according to the barcode and
217 primers using Mothur software (v.1.35.1) (Schloss *et al.* 2009), and sequence
218 direction was adjusted. Two mismatches were allowed by the barcode, with a
219 maximum number of three mismatches. Barcodes and primers were then removed and
220 effective clean tags were obtained. Operational taxonomic units (OTUs) were
221 clustered using USEARCH at a 97% similarity level (Edgar 2010), while singleton
222 OTUs and chimeric sequences were identified and removed. Representative
223 sequences for each bacterial and fungal OTU were taxonomically assigned according
224 to the Ribosomal Database Project (RDP) using the Silva (https://www.arb-silva.de/)
225 and Unite databases (http://unite.ut.ee/index.php), respectively. Ultimately, 8373
226 bacterial OTUs and 11,961 fungal OTUs were assigned after deleting OTUs with
227 sequence numbers < 20 across all samples. Furthermore, the sequences of all samples
228 were rarefied according to the minimum sequence number to correct for differences in
229 sequencing depth among samples.

230 *Statistical analyses*

231 Sixteen soil factors were grouped into three categories, namely metallic elements (Al,
232 Ca, Cu, Fe, Mg, Mn, and Zn), soil properties (soil organic carbon [OC], pH, and WC),
233 and macronutrients (available potassium [AK], available phosphorous [AP],
234 ammonium [NH_4^+-N], nitrate [NO_3^--N], total nitrogen [TN], and total phosphorus
235 [TP]; Figure S2). The direct and indirect effects of these soil factors on microbial *α*-

diversity, community structure, and function were then estimated using structural equation models (SEMs). Path model fit was verified via path analysis using the "*lavaan*" package in R (v.4.2.0) (Rosseel 2012). To improve normality, all variables were standardized through Z transformation (mean = 0, SD = 1) using the "scale" function in R. Microbial community structure and function were represented by the first principal coordinate (PCo1) to effectively avoid the "double zero" effect, and the first principal component (PC1) after Hellinger conversion was used to represent standardized soil factors. Specifically, the models should ideally meet the following criteria: nonsignificant Chi-square test ($p > 0.05$), goodness-of-fit index (GFI) > 0.90, and root mean square error of approximation (RMSEA) < 0.08 (Schermelleh-Engel 2003).

The random forest model (RF) was used to identify major soil predictors of microbial α-diversity and community structure (Breiman 2001). To estimate the importance of soil factors, increases in node purity (IncNodePurity) of variables were used, where higher IncNodePurity values imply more important predictors (Breiman 2001; Jiao *et al.* 2018). The RF analyses were performed using the "*randomForest*" package, and the significance of each soil factor for microbial α-diversity and community structure was assessed using the "*rfPermute*" package in R (Jiao *et al.* 2018). Model significance and cross-validated R^2 values were assessed with 500 permutations of the response variable using the "*A3*" package in R (Jiao *et al.* 2018). Biomarkers of metal elements and microbial functions were identified using 10-fold cross-validation implemented with the "rfcv" function in the R package "*randomForest*," with five repeats (Zhang *et al.* 2018a). Keystone taxa were determined using the microbial ecological network analysis method (see supplementary file for details).

Bacterial function profiles were generated using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2; Douglas *et al.* 2020), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used for functional gene annotation (Kanehisa & Goto 2000). Bacterial functions were divided into metabolic functions, genetic information processing, organismal systems, cellular processes, and environmental information processing at KEGG functional gene-annotated level 1. Furthermore, metabolic functions were classified into broad and specialized metabolic functions, as described by Xun *et al.* (2021). Co-occurrence network patterns of bacterial functions (KEGG functional gene annotated level 3) were built following strong (Spearman's correlation coefficient $r > 0.8$) and significant ($p < 0.01$) correlations. To reduce the possibility of false positives, the Benjamini-Hochberg's false discovery rate (FDR) correction was performed across the dataset (Benjamini & Hochberg 1995). A correlation matrix was constructed using the '*psych*' package in R, and network visualization and topology analyses were carried out on the Gephi interactive platform (*v.*0.9.2). Additionally, bacterial metabolic functions were specifically divided into C, N, P, and S metabolism. According to the FUNGuild database, fungal OTUs were categorized into guilds (i.e., animal pathogens, arbuscular mycorrhizae, ectomycorrhizae, plant pathogens, and wood saprotrophs) according to confidence levels of "highly probable" and "probable" (http://www.stbates.org/guilds/app.php; Nguyen *et al.* 2016).

We hypothesized that the diversity and structure of microbial communities would differ in response to metal elements across different levels of soil properties. To test this, all samples were sorted by C:N ratio, pH, and WC. Subsequently, the bottom and top 50% of samples were classified as relatively "low" and "high" levels, respectively. Correlations between microbial α-diversity and community structure for metal

elements at relatively low and high levels of soil properties were analyzed using the "*ggcor*" and "*dplyr*" packages in R. Spearman's correlation analyses between metal element composites and microbial functions were then performed separately at each soil property level.

Furthermore, the potential distinct effects of influencing indicator microbes on microbial functions at different levels of soil properties were explored. The significance of metal element variables was determined by ANOVA and distance-based redundancy analyses (db-RDA; Legendre & Anderson 1999) in the '*ggvegan*' R package, whereby the leading metal elements with significant impacts on microbial functions were identified. Microbial functions and metal elements were Hellinger converted and log-transformed, respectively. Metal elements with collinearity were removed according to variance inflation factor (VIF) analysis, while the lowest AIC values were detected with step models, and significance tests were performed using ANOVA with 999 permutations. Moreover, to determine the sensitive microbial taxa in different environmental preferences (i.e., low *vs.* high C:N ratios, pH values, or WC), linear discriminant analysis (LDA) coupled with effect size measurements (LEfSe) analysis were performed using Wekemo Bioincloud (https://www.bioincloud.tech). Fungal OTUs were further trimmed by retaining the OTUs with average relative abundances ≥ 0.0001% across all samples to reduce the complexity, whereas all bacterial OTUs were selected.

Results

Predictors of microbial α-diversity and community structure

After model comparison and averaging, SEMs (Figure S3) were used to gain a system-level understanding of the primary drivers underlying microbial *α*-diversity

and community structure (Figure 2a; Figure S4; Tables S1–S4). The results revealed that 65.2% and 52.6% of the variation in bacterial and fungal community structures, respectively, could be explained by soil factors (Figure 2a). Further, RF indicated that metal elements were the most important variables for predicting bacterial α-diversity and fungal community structure (Figure 2b). Moreover, metal elements, biomarkers, and keystone taxa jointly explained 90.1% and 94.6% of the variation in bacterial and fungal community structure, respectively (Figure 2d). Nevertheless, standardized path coefficients further showed that keystone taxa exhibited higher predictive power than biomarkers in bacterial community structure and fungal α-diversity (Figure 2d).

Effects of metal elements on microbial functions

Network analyses detected functional gene co-occurrence patterns, which were gathered into one major gene aggregate consisting primarily of genes exerting broad and specialized metabolic functions, defined here as “metabolic processes” (Figure 3a). The SEM showed that the initial responses of influential microbial taxa to metal elements altered the “metabolic processes” (Figure 3b). Significant indirect effects of metal elements on specialized and broad metabolic functions were observed through the linkages of influential microbial taxa (Figure 3b). Metal elements and influential microbial taxa together explained 52.6% and 54.5% of the variation in specialized and broad metabolic functions, respectively (Figure 3b). In fungi, correlation network analysis revealed positive correlations between metal elements and fungal pathogens as high as 70.41% (Figure 3c). Based on pathway analysis results, metal elements exerted significantly direct or indirect effects on plant pathogens and animal pathogens based on the linkages of influential microbial taxa, including keystone taxa, as well as the biomarkers of metal elements and pathogenic fungi (Figure 3d). Furthermore, metal elements and influential microbial taxa together explained 70.4%

and 55.6% of the variation in plant pathogens and animal pathogens, respectively (Figure 3d).

Linkages between metal elements and microbial communities by soil property

Overall, microbial α-diversity and community structure were closely related to all seven metal elements at both levels of soil properties (Figure 4). After removing outliers, all correlations between metal element composites and microbial functions were significant, except for ectomycorrhizae at relatively low C:N ratios and high pH, or wood saprotrophs at relatively high C:N ratios and low pH (Figure 5; Figures S5, S6). Specifically, both P and S metabolism exhibited opposite linear correlations with the metal element composite at relatively low *vs.* high C:N ratios (Figure 5). Additionally, plant pathogens at different C:N ratios with metal element composites also showed opposite correlations, as did ectomycorrhizae at different levels of WC (Figure 5; Figure S6). Furthermore, sensitive microbial taxa were linked with the leading metal elements and microbial functions (Figure 6; Figure S7–S9). Generally, the leading metal elements could influence microbial functional guilds via regulating sensitive microbial taxa, and their influences were dependent upon the levels of soil properties.

Discussion

With ongoing global climate change, the concomitant acidification of soil (acid deposition) and enrichment of soil nitrogen (nitrogen deposition) are expected to change soil microbial communities in forest ecosystems (Tian *et al.* 2017; Zhang *et al.* 2018b; Yu *et al.* 2022). Accordingly, understanding the effects of metal elements on soil microbial processes is critical for enhanced conservation of tree biodiversity in forest ecosystems, but significant gaps in scientific understanding remain. Here, 1287

soil samples from a subtropical forest plot were used to decipher the importance of the initial responses of influential microbial taxa to metal elements in altering microbial community structure and function, in-line with our first hypothesis. Supporting our second hypothesis, disparate responses of microbial communities to various metal elements were observed at relatively low *vs.* high levels of C:N ratios, pH, and WC. Previous studies may have overlooked certain contributions of metal elements, given that they are rarely measured across large spatial scales, whereas the present findings help fill this gap in knowledge, and increase the collective understanding surrounding the ecological importance of metal elements on microbial processes in a contiguous natural ecosystem. Accordingly, the linkages between belowground microbial diversity and soil abiotic factors were further explored at the level of metal elements and influential microbial taxa, thus improving the maintenance of aboveground biodiversity and plant productivity, particularly in the face of global climate change.

Geochemical elements may be the major factors influencing microbial assemblages (Liu *et al.* 2018), as it has been shown that minor changes in the availability of metal elements can have strong effects on individual soil organisms, communities, and even entire ecosystems (Luo *et al.* 2016). Here, the SEMs revealed that keystone taxa showed a stronger regulatory effect than that of biomarkers on bacterial community structure and fungal α-diversity (Figure 2d). This finding is consistent with previous studies showing that keystone taxa are highly connected in the microbiome and play a crucial role in maintaining microbial community structure and function (Paine 1995; Lynch & Neufeld 2015; Banerjee *et al.* 2018). As the structure and functioning of the entire microbial community may change drastically if such taxa are eliminated, they are widely referred to as "ecosystem engineers" (Mills 1993; Banerjee *et al.* 2018; Yue *et al.* 2019); however, the effects of metal elements on biomarkers were much

386 higher than on keystone taxa here (Figure 2d). This suggested that biomarkers for
387 specific soil factors are more responsive to metal elements than keystone taxa for
388 general soil factors. Recent analyses by the current authors also showed that keystone
389 taxa initially respond to changes in soil abiotic factors, after which they respond to
390 other species through hierarchical interactions, thus influencing the structure of the
391 whole microbial community (unpublished). Accordingly, biomarkers that play a
392 predictive role for metal element functioning as specific soil factors may be more
393 susceptible to initial impacts than keystone taxa, and thus transmit these effects to
394 keystone and other taxa.

395 Soil microbes play important roles in soil carbon and nutrient cycling (Dove *et al.*
396 2021a; Dove *et al.* 2021b), and the biogeochemical cycling of soil elements is
397 essential for maintaining ecosystem functions (Ochoa-Hueso *et al.* 2021). In bacteria,
398 specialized metabolic functions may involve multiple steps by a series of functionally
399 or taxonomically specific microbial taxa, whereas broad metabolic functions related to
400 intracellular metabolism can be accomplished within a single cell (Xun *et al.* 2021).
401 Considering that identified biomarkers of metabolic processes were strongly collinear
402 with specialized and broad metabolic functions (Figure S10), the top three important
403 OTUs shared by specialized and broad metabolic functions were selected to illustrate
404 the enormous bridging effect using a minimum number of bacterial taxa. Although
405 these top three OTUs positively affected metabolic processes, they were negatively
406 correlated with metal elements (Figure 3b). Consequently, metal elements reduced the
407 occurrence of specialized and broad metabolic functional genes, which appear to have
408 negative impacts on ecosystem multifunctionality and microbiome stability (Xun *et*
409 *al.* 2021). Fortunately, keystone taxa were not significantly affected by metal
410 elements, and showed the opposite regulatory effects on metabolic processes

411 compared with the top three important OTUs (Figure 3b). This suggested that
412 keystone taxa can partially alleviate the negative impacts of metal elements on the
413 forest ecosystems. The SEM further revealed the positive effects of metal elements on
414 pathogenic fungi through the bridging role of influential microbial taxa (Figure 3d),
415 highlighting the pathogenicity and potential detrimental effects of metal elements on
416 forest ecosystems.
417 Indeed, an intricate balance of mineral nutrients is required for plant growth and
418 reproduction (DalCorso *et al.* 2014). Meanwhile, soil microbial communities are
419 jointly driven by various factors (Liu *et al.* 2018). Owing to their specific functional
420 traits, different soil microbe functional taxa may have different interdependencies
421 with specific soil factors. The bioavailability of metal elements is controlled by
422 complex interactions between soil abiotic and biotic factors, which are in turn
423 influenced by soil physicochemical properties and microbiomes (Rogiers *et al.* 2021).
424 Soil organic matter is the key determinant of soil fertility and nutrient availability
425 (Bunemann *et al.* 2018; Radujkovic *et al.* 2021). Metal elements, such as Cu, Mn, and
426 Zn, notably crucial for cell growth, redox homeostasis, synthesis of biomolecules, and
427 animal immunocompetence in acidic soils, were all positively correlated with soil
428 organic matter content (Muthusamy *et al.* 2012). Also, nitrogen is important for
429 uptake and translocation of certain micronutrients, particularly Zn (Cakmak *et al.*
430 2010; Erenoglu *et al.* 2011; Gupta *et al.* 2016; Radujkovic *et al.* 2021). Soil microbes
431 require a specific C:N ratio to maintain their activities (Karhu *et al.* 2022).
432 Furthermore, the soil C:N ratio is the major predictor for fungal biomass, as well as
433 the relative abundance and composition of gene functions (Bahram *et al.* 2018). In the
434 present study, microbial α-diversity and community structure did show different
435 correlations with metal elements at relatively low *vs.* high levels of C:N ratios (Figure

436 4). A potential explanation for this is that different levels could alter the living
437 environments and activities of soil microbes, thus leading to changes in their
438 associations with metal elements. Additionally, bacteria predominate in low C:N ratio
439 soils, while fungi-dominated food webs are characterized by higher ratios (van der
440 Heijden *et al.* 2008; Ochoa-Hueso *et al.* 2021; Karhu *et al.* 2022). This can be used to
441 interpret and explain the results showing that metal elements exhibited higher
442 correlations with bacterial functions and lower correlations with fungal functions in
443 relatively low compared to high C:N ratio soils (Figure 5).
444 Other than the C:N ratio, soil microbial communities are known to be responsive to
445 differences in pH (Smenderovac *et al.* 2022), which is widely recognized as an
446 important soil property in altering microbial community diversity and functions
447 (Lammel *et al.* 2018; Tripathi *et al.* 2018). Increasing acidic precipitation
448 accompanying global climate change could reduce soil pH, thus increasing the
449 availability of metal elements, and reshaping microbial community structure and
450 association patterns (Zhang *et al.* 2022). Variations in pH are related to "spillover
451 effects" on metal elements (e.g., availability of Al, Cu, Fe, Mn, and Zn), while the
452 effects of pH on the community structure indirectly affect elemental availability
453 (Lammel *et al.* 2018). In neutral or weakly alkaline soils, the availabilities of metal
454 elements are relatively low (Tian *et al.* 2020), whereas most metal elements are
455 soluble in acidic conditions, thereby increasing their toxic potential. For instance,
456 soluble Zn was shown to increase at low pH, especially in soils with lower soluble
457 organic matter content (Broadley *et al.* 2007); Mn is more soluble as Mn^{2+} in acidic
458 soils, thereby increasing the risk of Mn poisoning (Lammel *et al.* 2018). Moreover,
459 Al_2O_3 is easily dissolved at a pH < 4.0, increasing free Al^{3+} in soils and potentially
460 harming plants owing to Al phytotoxicity (Muthusamy *et al.* 2012). Similar to the

variable responses of microbes to different C:N ratios, soils with low pH could reduce bacterial fitness but promote fungal growth (Rousk *et al.* 2010). Moreover, soil WC is of paramount significance for controlling microbial activities and regulating the rate of mineralization (Paul *et al.* 2003). Although the difference in microbial functions under different WC were not as obvious as that under different C:N ratios and pH, the WC could also affect the relationships between metal elements and microbial community α-diversity as well as structure (Figure 4). Collectively, soil C:N ratios as a nutrient index of microbes directly affect microbial activity, whereas pH can indirectly affect microbial activity by changing the availability of metal elements. These findings suggest that other soil factors must be considered to fully understand the roles of metal elements in predicting microbial community changes across highly heterogeneous forest soils.

Overall, the results of this study indicated that soil microbes are clearly diverse in their response to metal elements across different levels of soil properties. Possible indirect mechanisms may also be important factors. The SEMs illustrated that the intricate relationships between leading metal elements, sensitive microbial taxa, and microbial functions (Figure 6; Figure S9) support the latter hypothesis that various indicators are regulated at relatively low *vs.* high levels of C:N ratios, pH values, and WC. Notably, Mn is the best explanatory variable of microbial function for most variable conditions (Table S6), and potentially plays a key role in monitoring the functioning of forest ecosystems. Mn ion not only is implicated in litter decomposition (Keiluweit *et al.* 2015), but also is involved in various cellular functions, such as energy metabolism, gene expression regulation, hormone synthesis, and perception; thus, it is essential for plant growth and development (DalCorso *et al.* 2014).

Taken together, the present study emphasizes the importance of the initial responses of influential microbial taxa to metal elements, including micro- and macronutrients, as well as non-essential, but beneficial elements, in altering the community structure and function of forest soil microbes. Although observational studies such as this cannot fully disentangle causal relationships, these results highlight the potential undervalued role of metal elements, and influential microbial taxa in forest soils. Subsequent manipulation experiments should focus on the linkages of metal elements (especially Mn) and influential microbial taxa, in combination with different levels of C:N ratios and pH, to further reveal the fundamental importance of metal elements in determining forest tree productivity.

Acknowledgements

The work was supported by the National Natural Science Foundation of China [31670531 and 32071645], and the Fundamental Research Funds for the Central Universities.

Declaration of competing interest

The authors declare no competing financial interests.

Supplementary information

Tables **S1–S8** and Figures **S1–S10**.

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693

Figures

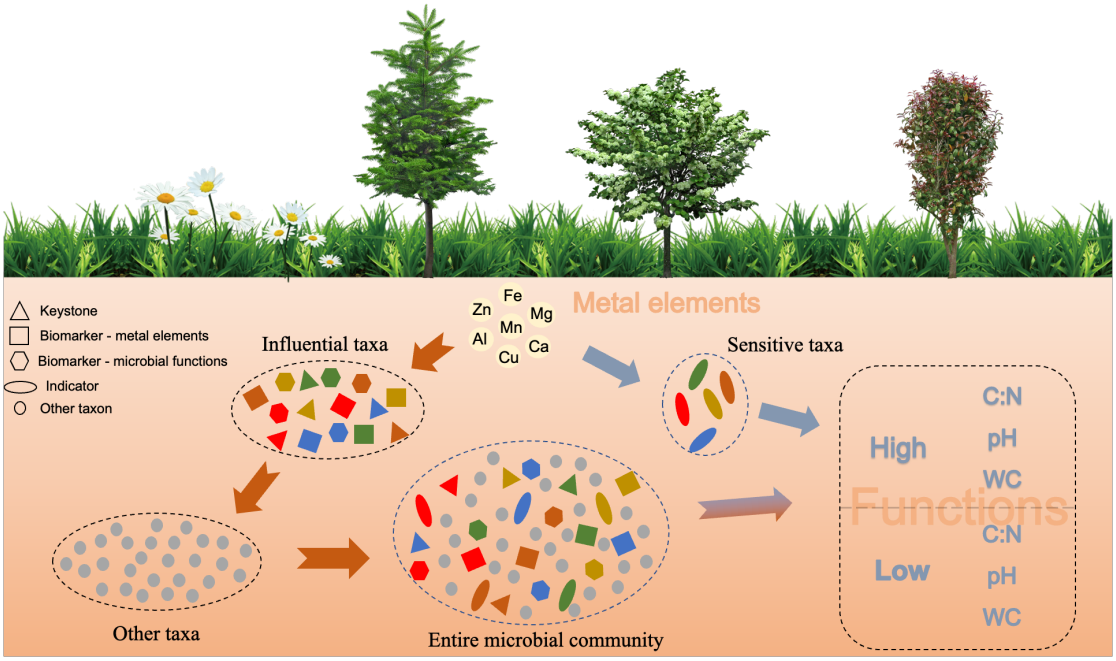


Figure 1. Study hypotheses. The initial responses of influential taxa to metal elements are proposed to alter forest soil microbial community structure and function differently under high *vs.* low C:N ratios, pH, and water content (WC).

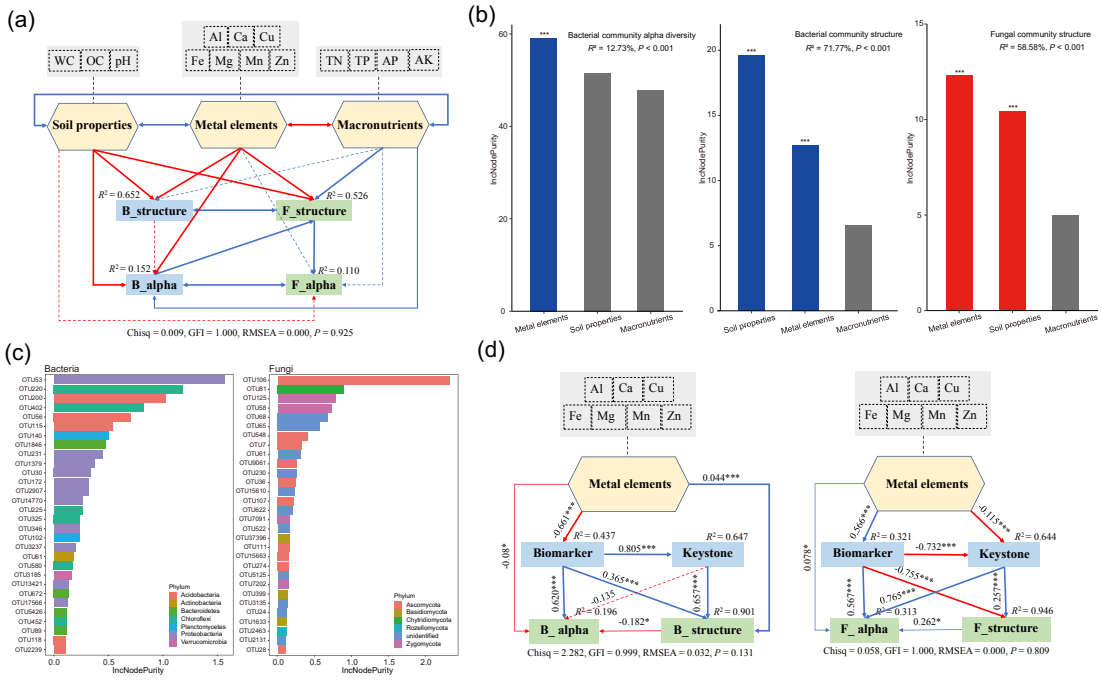
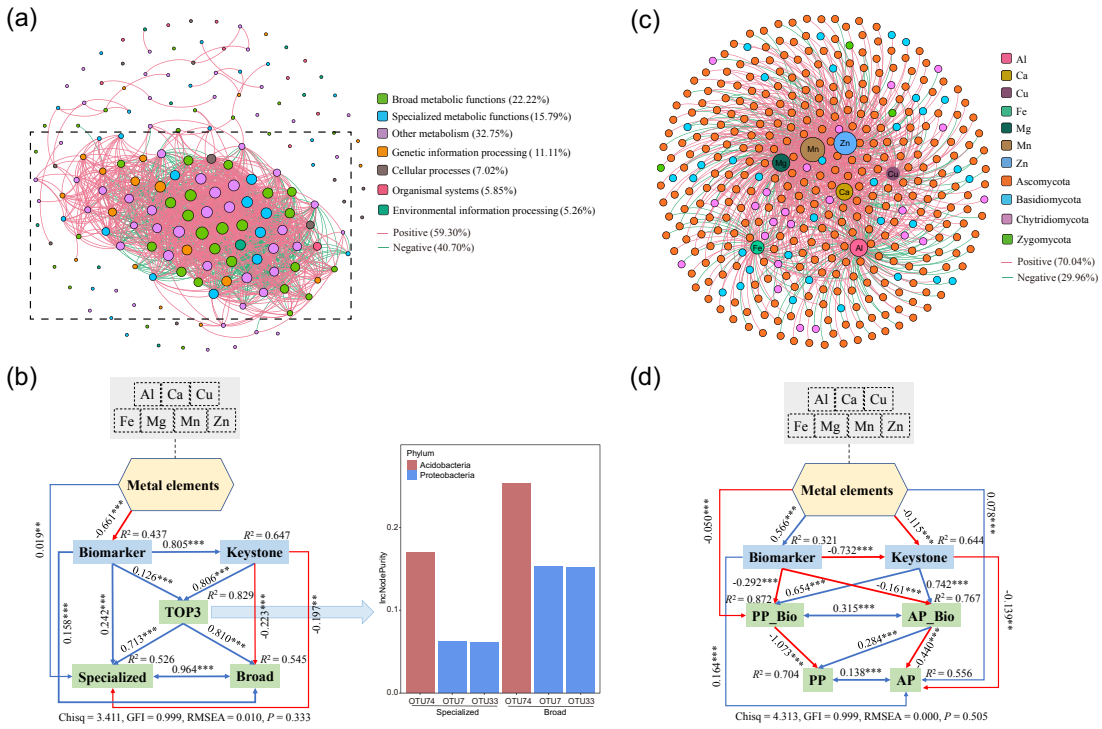


Figure 2. Effects of metal elements on microbial *α*-diversity and community structure. (a) Structural equation model reflecting the direct and indirect effects of metal elements, soil properties, and macronutrients on microbial *α*-diversity and community structure. Hexagons represent compound variables, and boxes represent observed variables. Solid and dashed arrows indicate significant and nonsignificant relationships, respectively. Blue and red arrows indicate positive and negative relationships, respectively. Arrow width is proportional to the strength of standardized path coefficients. R^2 values denote the proportion of variance explained for each variable. The standardized path coefficients are shown in Supplementary Table 1. WC, water content; OC, organic carbon; TN, total nitrogen; TP, total phosphorus; AP, available phosphorus; AK, available potassium; B_structure, bacterial community structure; F_structure, fungal community structure; B_alpha, bacterial *α*-diversity; F_alpha, fungal *α*-diversity. **(b)** Major soil predictors of microbial community. The accuracy importance measure was computed for each tree and averaged over the forest (500 trees). Increases in node purity (IncNodePurity) of variables were used to estimate the importance of these predictors, and higher IncNodePurity values imply

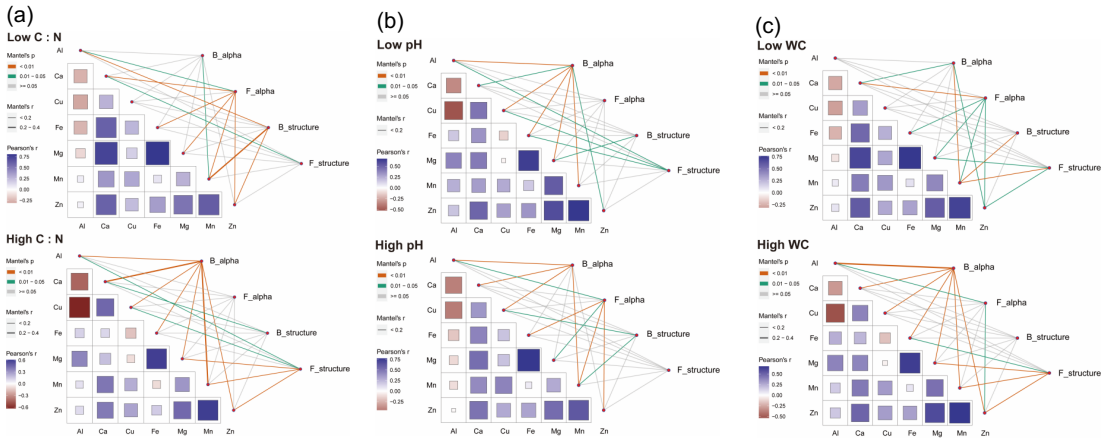
more important predictors. *** indicates $P < 0.001$. **(c)** Top 30 important OTUs of metal elements were identified by applying Random Forests regression of their relative abundances in metal element composites, and they are ranked in descending order of importance to the accuracy of the model. **(d)** Structural equation models reflecting the direct and indirect effects of metal elements and influential taxa (including biomarkers and keystone taxa) on microbial α-diversity and community structure. *** indicates $P < 0.001$ and * indicates $P < 0.05$.



723

724 **Figure 3. Effects of metal elements on microbial functions. (a)** Co-occurrence
725 network patterns of bacterial functions with strong (Spearman's correlation coefficient
726 r > 0.8) and significant (*P* value < 0.01) correlations. Nodes are colored according to
727 KEGG functional gene annotated level 3. The size of each node is proportional to the
728 number of degrees. The edges in the networks depict interactions (red color = positive
729 interaction; green color = negative interaction). Dotted line frames encompass the
730 gene aggregates. **(b)** Structural equation model reflecting the direct and indirect
731 effects of metal elements and influential taxa (including biomarkers of metal
732 elements, keystone taxa, and the top three important OTUs of specialized and broad
733 metabolic functions) on specialized and broad metabolic functions. Solid and dashed
734 arrows indicate significant and nonsignificant relationships, respectively. Blue and red
735 arrows indicate positive and negative relationships, respectively. Arrow width is
736 proportional to the strength of standardized path coefficients. R^2 values denote the
737 proportion of variance explained for each variable. TOP3, the top three important
738 OTUs of specialized and broad metabolic functions. *** indicates $P < 0.001$ and **

739 indicates $P < 0.01$. **(c)** Network relationship between metal elements and fungal
740 pathogens. The pathogenic OTUs are colored according to phylum level. The size of
741 each node is proportional to the number of degrees. The edges in the networks depict
742 interactions (red color = positive interaction; green color = negative interaction). **(d)**
743 Structural equation model reflecting the direct and indirect effects of metal elements
744 and influential taxa (including keystone taxa and the biomarkers of metal elements
745 and pathogenic fungi) on plant pathogens and animal pathogens. PP, plant pathogens;
746 AP, animal pathogens. PP_Bio, the biomarkers of plant pathogens; AP_Bio, the
747 biomarkers of animal pathogens. *** indicates $P < 0.001$ and ** indicates $P < 0.01$.



748

749 **Figure 4**. **Correlations of microbial *α*-diversity and community structure with**

750 **metal elements between relatively low and high C:N ratios (a), pH (b), and water**

751 **content (WC) (c).** Edge width corresponds to the Mantel's r value, and the edge color

752 denotes the statistical significance. Pairwise correlations of these variables are shown

753 with a color gradient denoting Pearson's correlation coefficient. WC, water content;

754 B_structure, bacterial community structure; F_structure, fungal community structure;

755 B_alpha, bacterial *α*-diversity; F_alpha, fungal *α*-diversity.

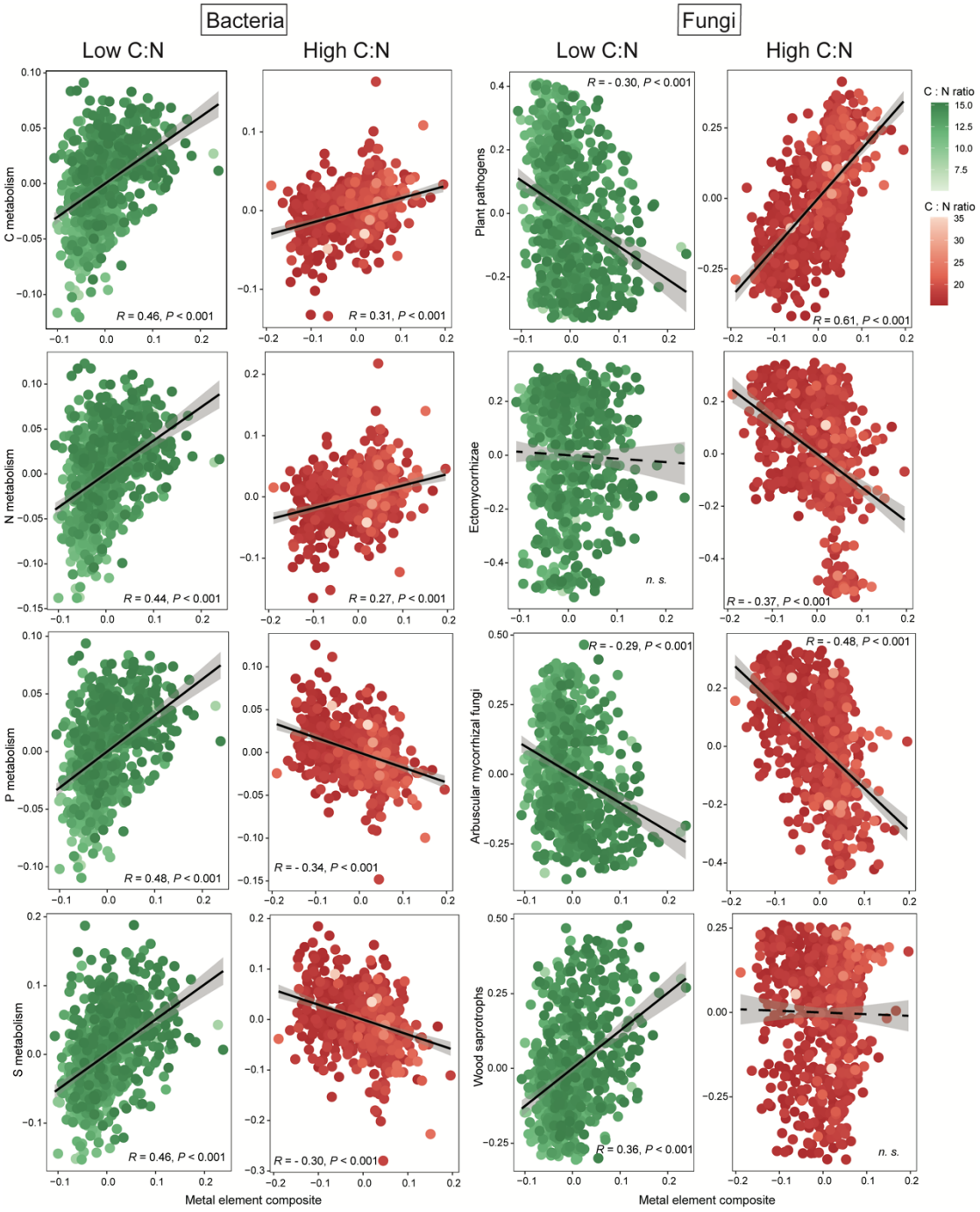


Figure 5. **Correlations between the metal element composite (Al, Ca, Cu, Fe, Mg, Mn, and Zn) and microbial functions at relatively low *vs*. high C:N ratios.** Bacterial functions include C, N, P, and S metabolism. Fungal functions include plant pathogens, ectomycorrhizae, arbuscular mycorrhizal fungi, and wood saprotrophs. n.s., no significant difference.

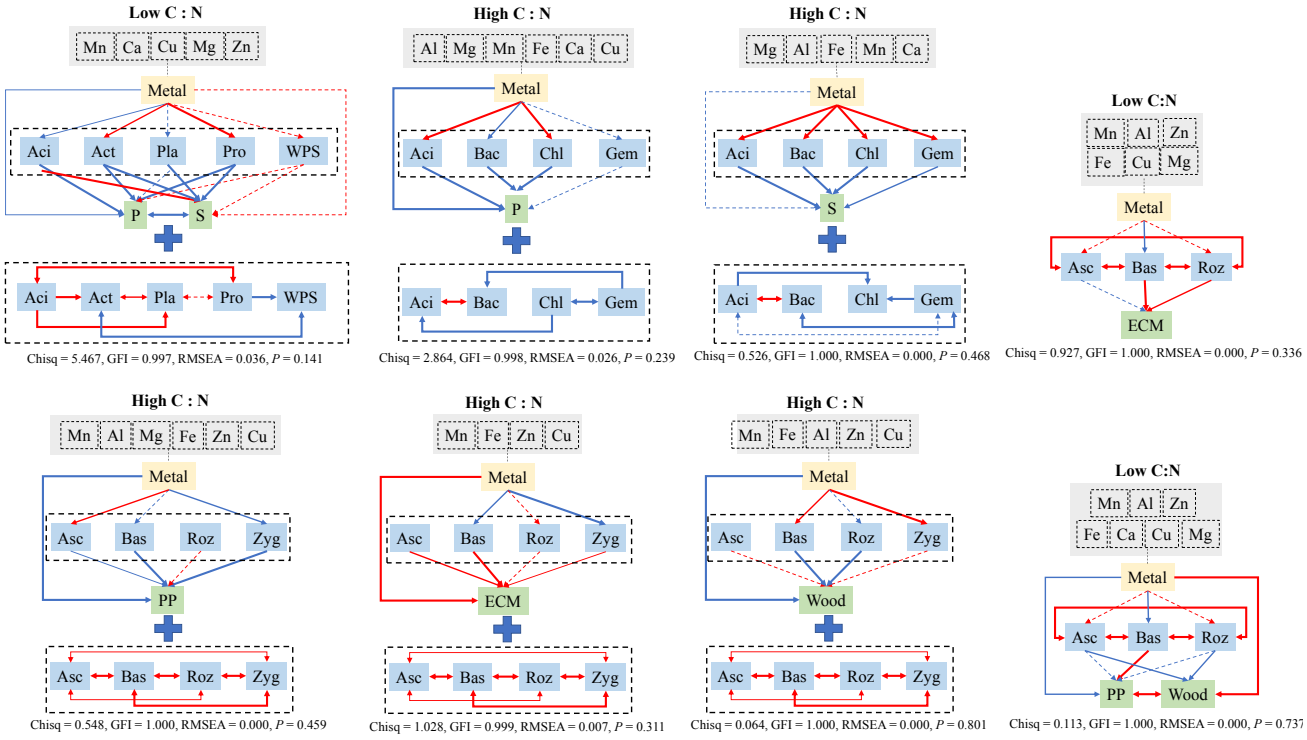


Figure 6. **Structural equation models reflecting the direct and indirect effects of the leading metal elements and sensitive taxa on P and S metabolism for bacteria and plant pathogens, wood saprotrophs, and ectomycorrhizal fungi at relatively low *vs*. high C:N ratios.** Solid and dashed arrows indicate significant and nonsignificant relationships, respectively. Blue and red arrows indicate positive and negative relationships, respectively. Arrow width is proportional to the strength of standardized path coefficients. The standardized path coefficients are shown in Supplementary Table 7. Aci, Acidobacteria; Act, Actinobacteria; Pla, Planctomycetes; Pro, Proteobacteria; WPS, WPS-2; Bac, Bacteroidetes; Chl, Chloroflexi; Gem, Gemmatimonadetes. Asc, Ascomycota; Bas, Basidiomycota; Roz, Rozellomycota; Zyg, Zygomycota; P, phosphorus metabolism; S, sulfur metabolism. PP, plant pathogens; Wood, wood saprotrophs; ECM, ectomycorrhizae.