

## Response of rare and abundant rhizosphere microbial communities to inoculated rhizobium in cadmium-contaminated soil phytoremediation

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### ABSTRACT

Soil microorganisms play an important role in maintaining ecosystem balance and exerting ecological functions. The taxonomic and functional changes in rare and abundant communities during in bioremediation of cadmium-contaminated soils and their contributions to the remediation of ecosystem functions remain elusive despite the significance of rare and abundant microbial taxa in maintaining soil ecological function. *Mesorhizobium loti* HZ76-inoculated seedlings of the *Robinia pseudoacacia* were used to create a symbiotic system. The effects of rhizobium inoculation on rare and abundant rhizosphere microbial populations were investigated in pot experiments. Despite having minimal effects on the  $\alpha$ -diversity of rare and abundant microbial communities, inoculation altered microbial community composition and functional gene abundance. For example, abundant phyla such as Proteobacteria were enriched following inoculation, whereas rare phyla such as Ascomycota were depleted. After inoculation, the abundance of *nifH* gene increased, while the abundance of *amoA*, *amoB*, *nirS*, *nirK* and *nosZ* gene decreased. Rare bacterial populations were more susceptible to the inoculated rhizobium's environmental alterations. In rare communities compared to abundance communities, the  $\alpha$ -diversity was higher. Stochastic processes predominated in the communities of uncommon and abundant microorganisms. Furthermore, keystone taxa, particularly rare bacterial taxa (Gemmatimonadaceae and Saprospiraceae) and rare fungal taxa (Ascomycota), more mediates nitrogen cycle processes and is directly associated to the abundance of nitrogen functional genes. Together, our findings demonstrate the different ways that rare and abundant microbial species react to rhizobia inoculation and their connections to the nitrogen cycle.

### 1. Introduction

High concentrations of heavy metals, such as cadmium (Cd), are released into the soil environment in the course of industrial and mining development. The heavy metals have complex physical and chemical effects in the soil, leading to soil fertility decline, poor crop growth and development, and impaired ecosystem stability (Ayangbenro and Babalola, 2017). Furthermore, such heavy metals can persist in the soil, bioaccumulate in plant tissues, and enter the food chain, with risks to human health (Wang et al., 2021). Numerous researchers have explored heavy metal remediation technologies for green development and environmental conservation, and applied them in ecological restoration

of mining areas. The major restoration methods include soil exchange (physical), chemical solidification and soil leaching (chemical), and plant extraction and microbial remediation (biological) (Ayangbenro and Babalola, 2017; Song et al., 2017). Compared with the physical and chemical restoration methods, the biological methods (especially microbe-enhanced phytoremediation) are advantageous in terms of their economic costs, environmental friendliness, and capacity to be applied in the restoration of relatively large areas (Shah and Daverey, 2020).

Generally, mining soils contaminated with heavy metals have poor fertility and nitrogen (N) deficiency (Hu et al., 2015; Liu et al., 2018; Munoz-rojas et al., 2016), which severely limit vegetation restoration

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efforts at the local scale. In recent years, the legume–rhizobium symbiotic system has emerged as a powerful tool for the remediation of soils contaminated with heavy metals. The plant–microorganism system has attracted considerable attention from environmental researchers and managers because of its capacity to promote soil N cycling and nutrient accumulation under heavy metal pollution (Abdelkrim et al., 2019; Fagorzi et al., 2018; Ren et al., 2019; Shoaib et al., 2021; Zhang et al., 2021). Many rhizobia, which are considered plant growth-promoting bacteria, are symbionts of legume crops and benefit their host plants by fixing atmospheric N in the soil. In addition, some rhizobia that exhibit heavy metal resistance are used to improve phytoremediation efficiency in heavy metal-contaminated soils, because these bacteria can drive soil redox reactions through N fixation or release of chelators and ferrocarrriers (Ashraf et al., 2017). Such an approach can both increase soil fertility and enhance heavy metal phytoextraction or phytostabilization (Fagorzi et al., 2018). Therefore, the symbiotic system formed by rhizobia and legumes has great application potential in the remediation of heavy metal-contaminated mining soils.

The rhizosphere is an active zone for the exchange of energy and matter (especially N, a key factor limiting plant growth) between plants and the soil. Therefore, it is critical to explore the underlying mechanisms of rhizosphere N cycling to facilitate vegetation restoration in contaminated mining soils (Liu et al., 2018; Munoz-Rojas et al., 2016). Soil N cycling is a complex biological process driven by specific enzymes encoded by functional genes, such as *nif* (N fixation), *amo* (nitrification), and *nir*, *nos*, *nap*, and *nar* (denitrification). N cycling could alter plant root behavior and rhizosphere environmental conditions (Hu et al., 2017; Zheng et al., 2016). In addition, variations in soil pH, organic matter, root exudates, and pollutants could arise in the rhizosphere due to the dynamic interactions between plant roots and soil microorganisms (Coskun et al., 2017; Mushinski et al., 2019). The mechanisms of N cycling in rhizosphere soil have been explored extensively in diverse fields of research spanning soil science, ecology, plant nutrition, and environmental science (Finzi et al., 2015; Henneron et al., 2020; Wei et al., 2019). However, previous research on rhizosphere N cycling has mainly been restricted to farmland and aquatic environments, with the bacterial phylum Proteobacteria (Choi et al., 2021; Wang et al., 2020) and fungal phylum Ascomycota (Gorfer et al., 2011; Inselsbacher et al., 2010) being reported to play pivotal roles in N fixation, nitrification, or denitrification processes. Currently, the role of rhizosphere microorganisms (especially those associated with N cycling) in rhizobium-enhanced phytoremediation of heavy metal-contaminated mining soils remains unclear.

Generally, there are more rare microbial taxa with low abundance and fewer abundant microbial taxa with high abundance in nature (Du et al., 2020; Zhang et al., 2018). The rare and abundant microorganisms exhibit distinct responses to environmental disturbance and inhabit various ecological niches (Jiao and Lu, 2020; Gao et al., 2020; Liang et al., 2020). Consequently, rare and abundant taxa exhibit differences in their distribution patterns, ecological function, and functional traits (Jia et al., 2018; Jiao and Lu, 2020). Compared with rare microbial taxa, abundant microbial communities occupy more important ecological niches and perform vital ecological functions (Rivett and Bell, 2018). Nevertheless, rare microbial communities are major contributors to community diversity and functional gene diversity (Du et al., 2020), in addition to participating in soil nutrient cycling, ecosystem stabilization, external disturbance resistance, and ecological restoration (Jousset et al., 2017; Lynch and Neufeld, 2015). Specifically, soil ecosystem functions in a subtropical agroecosystem were found to be mainly influenced by rare microbial groups (Xue et al., 2020). However, in a Cd-contaminated paddy soil inoculated with *Pseudomonas chenduensis* (a heavy metal-resistant bacterium), rare microbial communities exhibited greater variations but played fewer roles compared with abundant microbial communities in the bioremediation process (L J Li et al., 2019). In addition, following *in-situ* chemical stabilization of Cd-contaminated farmland soil, rare and abundant microbial sub-communities exhibited

prominent differences in terms of community composition, niche width, and environmental responses (Xu et al., 2021). Similarly, rare and abundant microbial sub-communities could exhibit different dynamics and functional roles in rhizobium-enhanced phytoremediation of Cd-contaminated mining soil, and such dynamics need to be investigated.

In the present study, we hypothesized that during rhizobium-enhanced phytoremediation of heavy metal-contaminated mining soils, soil–plant–microorganism interactions could alter edaphic factors in the rhizosphere; then, the altered rhizosphere environment would reshape the rare and abundant microbial sub-communities and regulate the abundance of N-cycling genes. To test the hypotheses, we constructed a legume–rhizobium symbiotic system with black locust (*Robinia pseudoacacia*) and *Mesorhizobium loti* HZ76. *R. pseudoacacia* is a model legume plant that exhibits improved adaptability to adverse environments following rhizobial inoculation, with great potential applications in the remediation of heavy metal-contaminated soils (Fan et al., 2018b; Zhang et al., 2021). *M. loti* HZ76 is a rhizobial strain with plant growth-promoting effects and heavy metal resistance, which has previously been isolated from the root nodules of *R. pseudoacacia* growing in a mining area (Fan et al., 2018a). The *R. pseudoacacia*–*M. loti* symbiotic system was used to set up greenhouse pot experiments with Cd-contaminated mining soil. The aims of the present study were to (i) investigate the changes in the rhizosphere environment and analyze the responses of rare and abundant microbial sub-communities induced by rhizobium-enhanced phytoremediation, and (ii) explore the gene functions and identify the rare and abundant keystone taxa associated with N cycling.

## 2. Material and methods

### 2.1. Sampling area and experimental design

The sampling area was located near an abandoned lead-zinc mine in Hanzhong, Shaanxi Province, China. In July 2020, soil samples were collected from a depth of 0–20-cm after litter removal. Cd pollution levels were classified (none: 0.08 mg kg<sup>-1</sup>, low: 0.39 mg kg<sup>-1</sup>, and high: 1.72 mg kg<sup>-1</sup>; Table S1) according to the Chinese National Standard — Soil Environmental Quality Risk Control Standard for Soil Pollution of Agricultural Land (GB 15618-2018). The soil samples were sieved and air-dried, with 1.2 kg filled in each plastic pot (14-cm height × 23-cm diameter).

Three factors (pollution level [n = 3], legume planting [n = 2], and rhizobial inoculation [n = 2]) were considered in the experiments, and a total of 12 treatments were assigned randomly. Details on the test treatment design are provided in Supplementary Information Method S1. *R. pseudoacacia* seeds (Yangling Nongyuan Seed Management Department, Northeast China Seeds) were surface-sterilized and pre-germinated as described previously (Hao et al., 2012). In the planting treatments, six seedlings with root lengths of 1 cm were transferred to each pot (14-cm height × 23-cm diameter). Rhizobial inoculation was carried out when the first true leaves emerged, by adding 2 mL of *M. loti* HZ76 cell suspension (10<sup>8</sup> CFU mL<sup>-1</sup>) to each seedling (Ramsay et al., 2006). An equal volume of sterilized water was added to the planting treatment without inoculation. All pots (three replicates per treatment with 12 pots per replicate) were maintained in a greenhouse at day/night temperatures of 30/21 °C. Soil moisture content was maintained at 70% field capacity by frequently adding deionized water.

### 2.2. Sample collection and physicochemical analysis

After 60 days of growth, the whole plants were taken out of pots, and loose chunks of soil were gently patted and shaken off the roots. Ultrasonic cleaning and centrifugal collection were carried out to obtain the soil attached to the roots within 1–2 mm as rhizosphere soil (Zgadaj et al., 2016). In addition, the 0–5-cm surface soil in unplanted pots was

collected as non-rhizosphere soil. Part of the collected soil was stored at  $-80^{\circ}\text{C}$  for DNA extraction and amplicon sequencing analysis. The other part was used for the determination of soil pH, total N (TN), soil organic matter (SOM), nitrate-N (NO<sub>3</sub>-N), ammonium-N (NH<sub>4</sub><sup>+</sup>-N), total phosphorus (TP), and Cd content after air-drying and sieving (Bao, 2000). All plants were divided into aboveground and underground parts and placed in sterile ziplocked bags. The gathered plant tissue is cleaned using ultrasonic technology until the distilled water is crystal clear. Any surface water is then dried using absorbent paper before the plant tissue is weighed again (fresh weight). And then oven-dried at  $105^{\circ}\text{C}$  for 0.5 h, followed by  $70^{\circ}\text{C}$  until constant weight. Subsequently, the dry weight was determined. The dry plant samples were ground for use in the determination of TN and Cd using standard testing methods (Bao, 2000).

### 2.3. DNA extraction, high-throughput sequencing, and real-time quantitative PCR (qPCR)

Total genomic DNA was extracted from 0.5-g soil samples using the soil Fast DNA SPIN kit (MP Biomedicals, Santa Ana, CA, USA). The primer set 515F (GTGCCAGCMGCCGCGGTAA)/806R (GGACTACHVGGGTWTCTAAT) with barcode was used to amplify the V4–V5 region of bacterial 16S ribosomal RNA genes (Walters et al., 2011). The primer set ITS5-1737F (GGAAGTAAAGTCGTAACAAGG)/ITS2-2043R (GCTGCGTTCATCGATGC) was used to amplify the first internal transcribed spacer region of fungal ribosomal RNA gene in each sample (Lu et al., 2013). The procedures of PCR amplification and sequencing of bacterial and fungal genes are detailed in Supplementary Methods S2. DNA libraries were constructed and a 250-bp paired-end protocol was used for sequencing on an Illumina NovaSeq platform (Illumina, USA). Sequences were analyzed using Uparse v7.0.1001 (<http://drive5.com/uparse/>) and clustered into operational taxonomic units (OTUs) at 97% similarity. The Silva 132 (<https://www.arb-silva.de/documentalio/release-132/>) and UNITE (<https://unite.ut.ee/>) databases were used for annotation of bacterial and fungal OTUs, respectively. OTUs were classified as rare (<0.01% of the community) or abundant (>0.1% of the community) according to their relative abundances (Jiao, 2020; Zheng et al., 2021).

The qPCR primers were designed for eight functional genes related to N fixation (*nifH*), nitrification (*amoA* and *amoB*), and denitrification (*nirK*, *nirS*, *napA*, *narG*, and *nosZ*). The primers were packaged into thin-layer metal alloy nanopore arrays to obtain high-throughput qPCR arrays. The 16S rRNA gene (F525/R907) was used as the reference gene with HT-qPCR on a SmartChip Real-time PCR system (WaferGen Biosystems in Fremont, USA) in accordance with Zheng's approach (Zheng et al., 2018). Three copies of each sample were amplified, and the reproducibility of QMEC was examined. The SmartChip qPCR program eliminated results with numerous melting peaks or amplification efficiencies between 80% and 120%. For further investigation, the data with a threshold cycle (CT) of 31 were employed. The ratio of a functioning gene's abundance to the abundance of the 16S rRNA gene was used to define relative gene abundance.

### 2.4. Statistical analysis

All data were analyzed using R v4.0.3 (<https://www.R-project.org/>). After testing the data for normality (Shapiro-Wilk test) and homogeneity of variance (Bartlett test), comparisons between sample pairs were performed using the *t*-test or Wilcoxon rank-sum test, and comparisons among multiple groups were performed using one-way Analysis of Variance (ANOVA) or Kruskal-Wallis test. The  $\alpha$ -diversity indices of microbial communities were calculated using the R package 'vegan' (Dixon, 2003). Principal coordinate analysis (PCoA) based on Bray-Curtis distances was used to analyze community differences between samples, and the major factors driving the differences were identified using Permutational Multivariate Analysis of Variance (PerMANOVA) (Anderson and Walsh, 2013). Spearman's correlation coefficients

between edaphic factors or microbial community structure and functional gene abundances were calculated. A Mantel test was used to identify the major edaphic factors that influenced the structures of rare and abundant communities for bacteria and fungi.

To characterize the phylogenetic diversity between samples, the beta-mean nearest taxa distance ( $\beta\text{MNTD}$ ) and beta-nearest taxa index ( $\beta\text{NTI}$ ) were calculated using the 'comdistnt' function of a R toolkit, 'Picante' (Kembel et al., 2010).  $\beta\text{NTI}$  and Bray-Curtis-based Raup-Crick ( $\text{RC}_{\text{bray}}$ ) were used to distinguish between deterministic processes (homogeneous selection:  $\beta\text{NTI} < -2$ ; variable selection:  $\beta\text{NTI} > 2$ ) and stochastic processes (dispersal limitation:  $|\beta\text{NTI}| < 2$ ,  $\text{RC}_{\text{bray}} > 0.95$ ; homogeneous dispersal:  $|\beta\text{NTI}| < 2$ ,  $\text{RC}_{\text{bray}} < -0.95$ ) in microbial community assembly (Stegen et al., 2012, 2013). Linear regression analysis was used to determine the correlations among  $\beta\text{NTI}$ , edaphic factors, and community diversity. The co-occurrence networks of bacterial and fungal communities were constructed based on Spearman's rank correlations among OTUs ( $|r| > 0.6$ ,  $P < 0.01$ ) using the R 'Hmisc' package (Banerjee et al., 2018). The network structure was explored and visualized using the Gephi v0.9.3 interactive platform (Fan et al., 2019). According to Banerjee's technique (Banerjee et al., 2018), high mean degree, high proximity centrality, and low betweenness centrality were identified as a threshold for determining keystone taxa in microbial communities based on network topological features.

## 3. Results

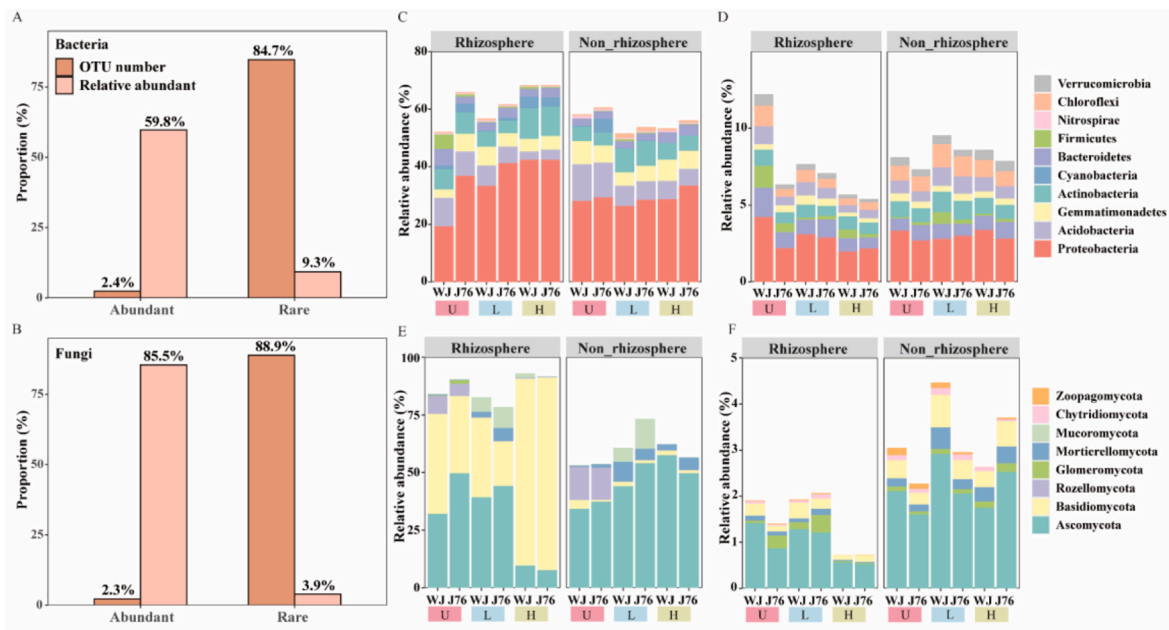
### 3.1. The effects of legume-rhizobium symbiosis on soil chemical properties

Without considering rhizobial inoculation, 60 days of treatment with legume planting decreased NO<sub>3</sub>-N significantly in the rhizosphere under all three Cd pollution levels. In contrast, both TN and SOM increased under the non-pollution level, and NH<sub>4</sub><sup>+</sup>-N increased under the pollution levels, whereas pH increased under both non-pollution and low pollution levels ( $P < 0.05$ ). Compared with the uninoculated treatment, the effects of rhizobial inoculation on TN and SOM varied with Cd pollution level. Following inoculation, TN and SOM decreased under non-pollution and low pollution levels, with relatively high values observed under the high pollution level (Table S2). In addition, inoculation enhanced root nodule formation and Cd accumulation in plant (Table S3).

### 3.2. Distribution and composition of rare and abundant taxa

Among the bacteria, rare taxa accounted for 84.7% of total OTUs, although their mean relative abundance per sample was only 9.3%. In contrast, abundant bacteria accounted for a small proportion of OTUs (2.4%), despite having a distinctively high mean relative abundance per sample (59.8%) compared with that of rare bacteria (Fig. 1A). Similar to the situation in the bacteria, rare fungi OTUs accounted for the majority of total OTUs (88.9%), with a mean relative abundance of only 3.9%. Conversely, abundant fungi accounted for 2.3% of total OTUs, whereas their mean relative abundance was 85.5% (Fig. 1B).

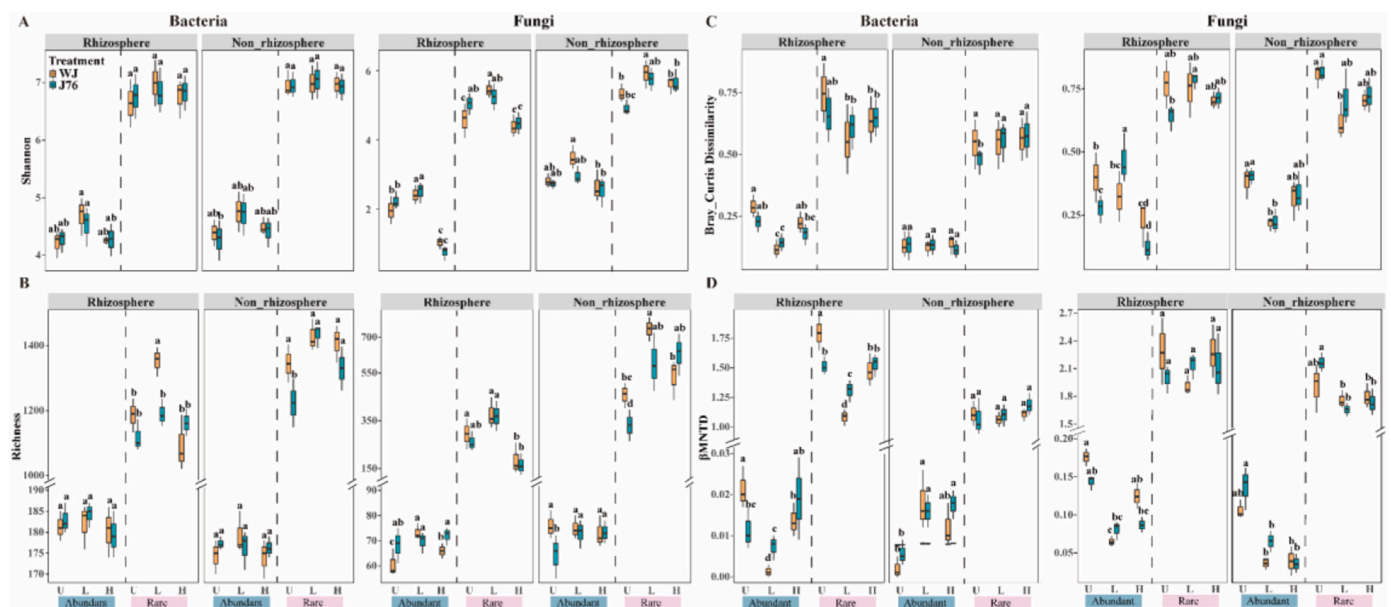
In the case of bacteria, the most dominant phyla in the abundant community were Proteobacteria (32.6%) and Acidobacteria (7.3%; Fig. 1C), which were also observed in the rare community (2.9% and 1.0%, respectively; Fig. 1D). Without considering rhizobial inoculation, legume planting decreased the relative abundances of abundant Proteobacteria and Gemmatimonadetes under the non-pollution level, but increased them under the pollution level. Planting also increased the relative abundances of most rare phyla under the non-pollution level and only decreased the relative abundance of rare Chloroflexi under the pollution level. Furthermore, rhizobial inoculation increased the relative abundance of abundant Proteobacteria and decreased that of rare Proteobacteria under the non-pollution and low pollution levels. However, inoculation had little effect on the relative abundances of rare and abundant phyla under the high pollution level (Fig. 1C and D).



**Fig. 1.** Distributions and compositions of abundant and rare microbial sub-communities in Cd-contaminated mining soil samples of different treatments. The numbers and proportions of abundant and rare taxa of (A) bacteria and (B) fungi in all samples are shown at the operational taxonomic unit (OTU) level. The relative abundances of abundant and rare taxa of (C, D) bacteria and (E, F) fungi in different treatments are shown at the phylum level. U, L, and H indicate non-, low, and high levels of Cd pollution levels, respectively. Rhizosphere and Non\_rhizosphere indicate planting *Robinia pseudoacacia* and no planting, respectively. WJ and J76 indicate without inoculation and inoculation with *Mesorhizobium loti* HZ76, respectively.

With respect to fungi, the most dominant phyla in the abundant community were Ascomycota (38.3%) and Basidiomycota (25.6%; Fig. 1E), whereas Ascomycetes was dominant in the rare community (1.6%; Fig. 1F). The relative abundances of major phyla were significantly different between the rhizosphere and non-rhizosphere (Table S4). Without considering rhizobial inoculation, legume planting increased the relative abundance of abundant Basidiomycota, but

decreased those of abundant and rare Ascomycota and rare Basidiomycota under the three pollution levels. In the rhizosphere, rhizobial inoculation increased the relative abundance of abundant Ascomycota and decreased that of rare Ascomycota under non-pollution and low pollution levels. Meanwhile, the relative abundances of abundant and rare Basidiomycota decreased, while those of abundant and rare Mortierellomycota increased under the low pollution level. However,



**Fig. 2.** Variations in  $\alpha$ - and  $\beta$ -diversities of abundant and rare microbial sub-communities between different treatments.  $\alpha$ -diversity was calculated using (A) Shannon index and (B) Richness index;  $\beta$ -diversity was calculated based on (C) Bray-Curtis dissimilarity; and (D) beta-mean nearest taxa distance ( $\beta$ MNTD) was used to characterize the phylogenetic diversity between samples. Bars indicate standard deviation of the means, with different letters above the bars indicating significant differences between treatments (Kruskal-Wallis test). U, L, and H indicate non-, low, and high levels of Cd pollution, respectively. Rhizosphere and Non\_rhizosphere indicate planting *Robinia pseudoacacia* and no planting, respectively. WJ and J76 indicate without inoculation and inoculation with *Mesorhizobium loti* HZ76, respectively.



rhizobial inoculation had minimal effect on the relative abundances of rare and abundant phyla under the high pollution level (Fig. 1E and F).

### 3.3. Diversity and structure of rare and abundant sub-communities

The  $\alpha$ -diversity indices (Richness and Shannon) of both bacteria and fungi were higher in rare sub-communities than in the abundant sub-communities (Fig. 2A and B). Rhizobial inoculation had minimal effect on the  $\alpha$ -diversity of all sub-communities in both the rhizosphere and non-rhizosphere soils. In addition, the  $\beta$ -diversity and  $\beta$ MNTD of the rare sub-communities were higher than those of the abundant sub-communities (Fig. 2C and D). According to ANOVA results, Cd pollution level or legume planting affected the  $\alpha$ -diversity of all four sub-communities significantly, with the exception of the Shannon index in the case of rare bacteria (Table S5).

PCoA plots can illustrate the separation of abundant bacterial and fungal sub-communities on the second and first axes, respectively, based on legume planting or not; the rare bacterial and fungal sub-communities could be roughly separated on the first and second axes, respectively, based on the level of Cd pollution (Fig. S1). PerMANOVA results corroborated that pollution level and legume planting were the major factors driving community structure variation in each of the four sub-communities (Table S6).

To identify the major edaphic factors influencing microbial community structure, the Mantel test was performed for each sub-community. The structures of all four sub-communities were significantly correlated with soil pH, TN, and SOM ( $P < 0.005$ ). In addition, the structures of rare bacterial, and abundant bacterial, and rare fungal sub-communities were all significantly correlated with soil NH<sub>4</sub>-N, whereas the structure of the abundant fungal sub-community was correlated with soil NO<sub>3</sub>-N ( $P < 0.05$ ; Table 1).

### 3.4. Assembly of rare and abundant sub-communities

RC<sub>bray</sub> and  $\beta$ NTI were used to distinguish between deterministic and stochastic assembly of microbial communities. Irrespective of legume planting or rhizobial inoculation, stochastic processes governed the assembly of all sub-communities (Fig. 3A and B; Fig. S2). The rare sub-communities were dominated by homogeneous dispersal, whereas 50% and 92% of the abundant bacterial and fungal sub-communities, respectively, were mediated by homogeneous dispersal, and the remaining proportions were dominated by unknown processes (Fig. 3C and D).

Linear regression analysis was used to determine the influence of edaphic factors and community diversity on the assembly of different microbial sub-communities. Bray-Curtis dissimilarity ( $R^2 = 0.297$ ), Richness index ( $R^2 = 0.479$ ), soil pH ( $R^2 = 0.242$ ), and Shannon index ( $R^2 = 0.750$ ) are the factors that influenced the  $\beta$ NTI of abundant bacteria, rare bacteria, abundant fungi, and rare fungi, respectively ( $P \leq 0.001$ ; Fig. S3) the most.

**Table 1**

Mantel tests of the correlations between edaphic factors and the structures of rare and abundant microbial sub-communities (OTU level) in Cd-contaminated mining soil samples after 60 days of treatment. pH, soil pH; TN, total nitrogen; SOM, soil organic matter; TP, total phosphorus; NO<sub>3</sub>-N, nitrate-nitrogen; NH<sub>4</sub>-N, ammonium-nitrogen.

Factors	Bacteria				Fungi			
	Abundant		Rare		Abundant		Rare	
	R <sup>2</sup>	P	R <sup>2</sup>	P	R <sup>2</sup>	P	R <sup>2</sup>	P
pH	0.491	<b>0.0001</b>	0.519	<b>0.0001</b>	0.150	<b>0.0100</b>	0.377	<b>0.0001</b>
TN	0.239	<b>0.0002</b>	0.139	<b>0.0056</b>	0.309	<b>0.0001</b>	0.315	<b>0.0001</b>
SOM	0.165	<b>0.0028</b>	0.154	<b>0.0050</b>	0.184	<b>0.0019</b>	0.217	<b>0.0001</b>
TP	0.040	0.2621	-0.119	0.5278	0.112	<b>0.0396</b>	0.123	<b>0.0252</b>
NO <sub>3</sub> -N	0.025	0.3408	0.058	0.2168	0.123	<b>0.0319</b>	0.060	0.1752
NH <sub>4</sub> -N	0.156	<b>0.0094</b>	0.142	<b>0.0203</b>	0.018	0.3302	0.100	<b>0.0390</b>

### 3.5. Responses of functional genes associated with nitrogen cycling

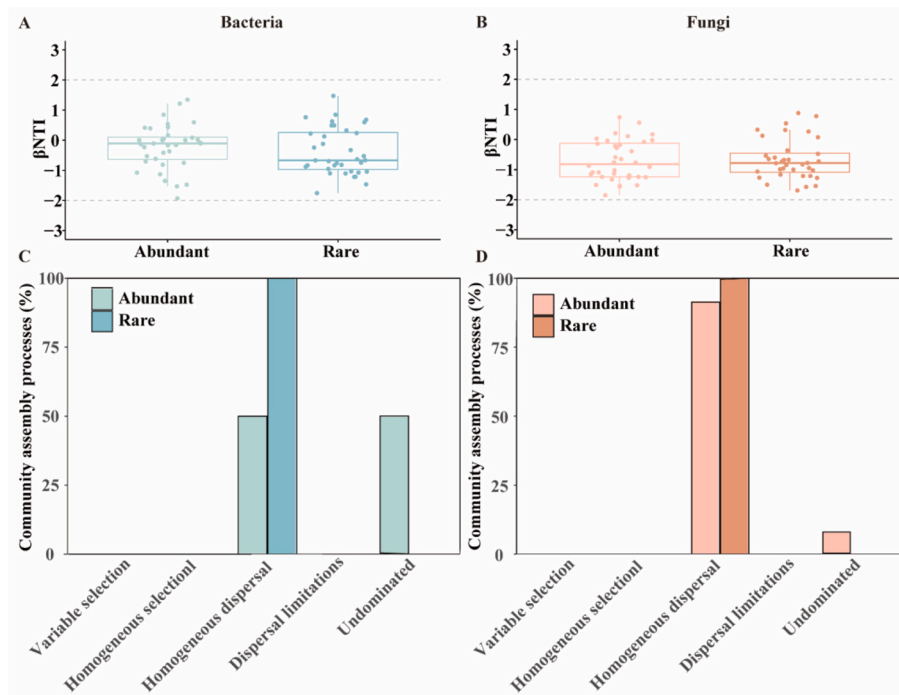
qPCR data showed distinct responses of N-cycling genes in the rhizosphere to rhizobial inoculation when compared with those of the uninoculated treatment. Upon inoculation, the abundance of N fixation gene (*nifH*) increased and the abundances of nitrification genes (*amoA* and *amoB*) and denitrification genes (*nirS*, *nirK*, *nosZ*, *napA*, and *narG*; Fig. 4) decreased. In addition, the abundances of N-cycling genes in the rhizosphere were affected by pollution level. Most notably, the abundance of the *nifH* gene was significantly higher in the rhizosphere under high pollution than under non-pollution and low pollution levels (Fig. 4). In non-rhizosphere soil, different pollution levels significantly affected the abundances of *amoA*, *amoB*, *nirS*, and *napA*; however, inoculation had almost no effect on functional genes.

Spearman's correlation analysis was used to identify the relationship between edaphic factors and functional gene abundance, irrespective of pollution level. In the rhizosphere, *nifH* and *napA* gene abundances were significantly negatively correlated with soil TN and SOM, whereas *amoA* and *narG* gene abundances were significantly and positively correlated with soil pH ( $P < 0.05$ ; Fig. 5). Under different pollution levels, the correlations between functional gene abundances and edaphic factors varied with legume planting and rhizobial inoculation (Fig. S4).

### 3.6. Co-occurrence patterns of bacterial and fungal sub-communities

The effects of legume planting and rhizobial inoculation treatments on microbial co-occurrence relationships were studied using co-occurrence networks (Fig. 6). In bacterial communities, there were significantly more positive correlations among taxa following legume planting (mean for rhizosphere/non-rhizosphere: 66.1%/58.5%), and the network complexity in rhizosphere soil was markedly lower following inoculation (mean clustering coefficient: 0.48–0.38; Table 2, Fig. 6A). Similar results were observed in fungal communities; that is, irrespective of legume planting, positive correlations among taxa dominated the whole network, with network complexity in rhizosphere soil decreasing considerably following inoculation (mean clustering coefficient: 0.76–0.70; Table 2, Fig. 6B) when compared with that under no inoculation.

Distinct keystone taxa were identified in the legume planting and rhizobial inoculation treatments (Table S7). Without considering rhizobial inoculation, abundant and rare taxa accounted for 80% and 20% of keystone bacterial OTUs in non-rhizosphere soil (mainly Proteobacteria), respectively, whereas the keystone OTUs in rhizosphere soil comprised equal proportions of abundant and rare taxa (50% each, mainly Proteobacteria and Gemmatimonadetes). All the keystone bacterial OTUs in the rhizosphere without inoculation were rare taxa, with a few belonging to  $\alpha$ -Proteobacteria. However, most of the keystone bacterial OTUs in the rhizosphere with inoculation were abundant taxa, mainly belonging to  $\gamma$ -Proteobacteria (Fig. 6A, Table S7). Furthermore, the keystone fungal OTUs in non-rhizosphere and rhizosphere soils were all rare taxa (mainly Ascomycota), irrespective of inoculation. A small



**Fig. 3.** Assembly processes of rare and abundant microbial sub-communities based on the values of  $\beta$ -nearest taxon index ( $\beta$ NTI) and Bray-Curtis-based Raup-Crick (RCbray). Boxplots show the  $\beta$ NTI patterns of all pairwise (A) bacterial and (B) fungal sub-communities between different treatments. Histograms show the relative contributions of deterministic and stochastic processes to the assembly of (C) bacterial and (D) fungal sub-communities.

number of keystone fungal OTUs in the rhizosphere without inoculation were identified as Basidiomycota; however, following inoculation, members of Mortierellomycota were found to be keystone OTUs (Fig. 6B, Table S7).

### 3.7. Relationships between keystone taxa, edaphic factors, and nitrogen-cycling genes

To identify the influence of edaphic factors on keystone taxa, Spearman's correlation analysis was performed on the data of different treatments (Fig. 7). Generally, in rhizosphere bacteria, TN, SOM, TP, and NO<sub>3</sub>-N mainly influenced the relative abundances of keystone OTUs without inoculation, whereas soil pH and NH<sub>4</sub>-N primarily influenced the relative abundances of keystone OTUs with rhizobial inoculation. In addition, all edaphic factors prominently influenced the relative abundances of keystone fungal OTUs in the rhizosphere ( $P < 0.01$ ).

Furthermore, the potential roles of keystone taxa in rhizosphere N cycling were investigated based on Spearman's correlations (Fig. 7). The correlations between the relative abundances of keystone OTUs and the abundances of N-cycling genes differed in the legume planting and rhizobial inoculation treatments. In addition, the relative abundances of keystone bacterial OTUs in the rhizosphere were significantly negatively correlated with *nifH* and *nosZ* gene abundances in the uninoculated treatment, with most of them being significantly negatively correlated with *narG* gene abundance in the inoculated condition ( $P < 0.01$ ). In both the inoculated and uninoculated treatments, the relative abundances of keystone fungal OTUs in the rhizosphere were significantly negatively correlated with *nifH* gene abundance and positively correlated with *narG* gene abundance ( $P < 0.05$ ).

## 4. Discussion

Numerous studies have demonstrated that rhizobial inoculation can improve phytoremediation efficiency in agroecosystems (Duan et al., 2022; Jian et al., 2019; Teng et al., 2015). In the present study, we observed that inoculation with *M. loti* HZ76 increased root nodule

formation, and Cd accumulation in *R. pseudoacacia* seedlings in Cd-contaminated mining soil (Table S3), which broadens the potential applications of the rhizobium-enhanced phytoremediation technology. In addition, rare and abundant microbial taxa inhabit different ecological niches and play distinct roles in the maintenance of ecosystem stability and functioning (Du et al., 2020; Jia et al., 2018). Our previous study demonstrated structural and functional variations in rhizosphere bacterial communities associated with *R. pseudoacacia* in response to inoculation with *M. loti* HZ76 (Fan et al., 2018b). In the present study, we verified our hypothesis that rhizobial inoculation could alter microbial community structure and regulate the N cycling functions of rare and abundant bacteria and fungi in the rhizosphere of *R. pseudoacacia* during phytoremediation of Cd-contaminated mining soil. The results enhance our understanding of microbial responses to rhizobium-enhanced phytoremediation of mining soils, and could facilitate the restoration of N-deficient soil ecosystems polluted by heavy metals due to mining activity.

### 4.1. Effects of rhizobial inoculation on structure and composition of rare and abundant microbial sub-communities in the rhizosphere

In the present study, we observed higher  $\alpha$ -diversity in the rare bacterial and fungal sub-communities than in the respective abundant sub-communities during phytoremediation of Cd-contaminated mining soil (Fig. 2). The observation is consistent with the  $\alpha$ -diversity patterns of rare and abundant microbial sub-communities under *in-situ* remediation of Cd-contaminated farmland soil with chemical stabilizers (Xu et al., 2021). Notably, rhizosphere inoculation with *M. loti* HZ76 had minimal effect on  $\alpha$ -diversity in the rare and abundant sub-communities of rhizosphere bacteria and fungi. However, compared with the abundant taxa, the rare bacteria were more affected by inoculation in terms of Richness index (Fig. 2). The results show that rare taxa are more sensitive to environmental conditions and have a narrower environmental response threshold compared with that of abundant taxa (Du et al., 2020; M Li et al., 2019).

The community dissimilarity analysis results demonstrated that

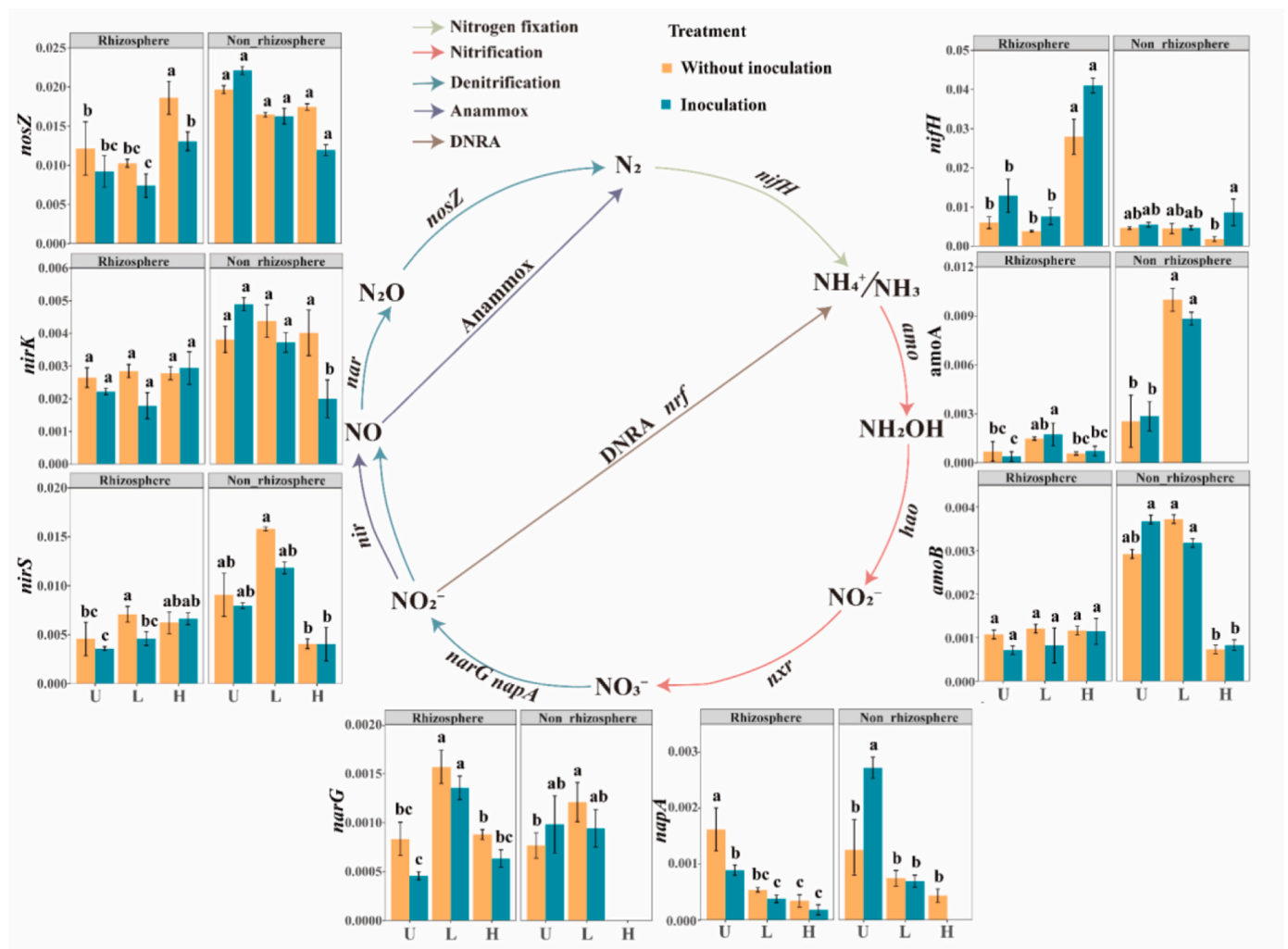


Fig. 4. Abundances of nitrogen-cycling genes in different treatments. Data of functional gene abundances are presented as mean ± standard deviations, and different letters above the bars indicate significant differences between treatments (Kruskal-Wallis test). U, L, and H indicate non-, low, and high levels of Cd pollution, respectively. Rhizosphere and Non\_rhizosphere indicate planting *Robinia pseudoacacia* and no planting, respectively. WJ and J76 indicate without inoculation and inoculation with *Mesorhizobium loti* HZ76, respectively.

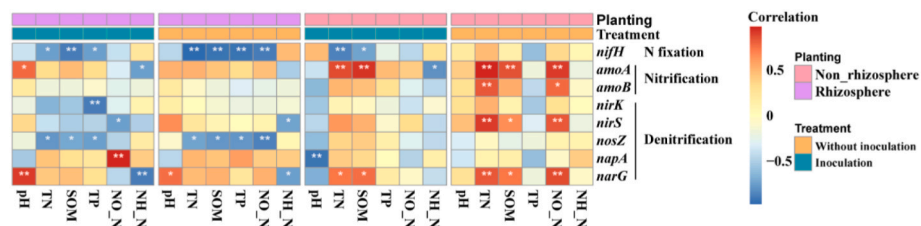
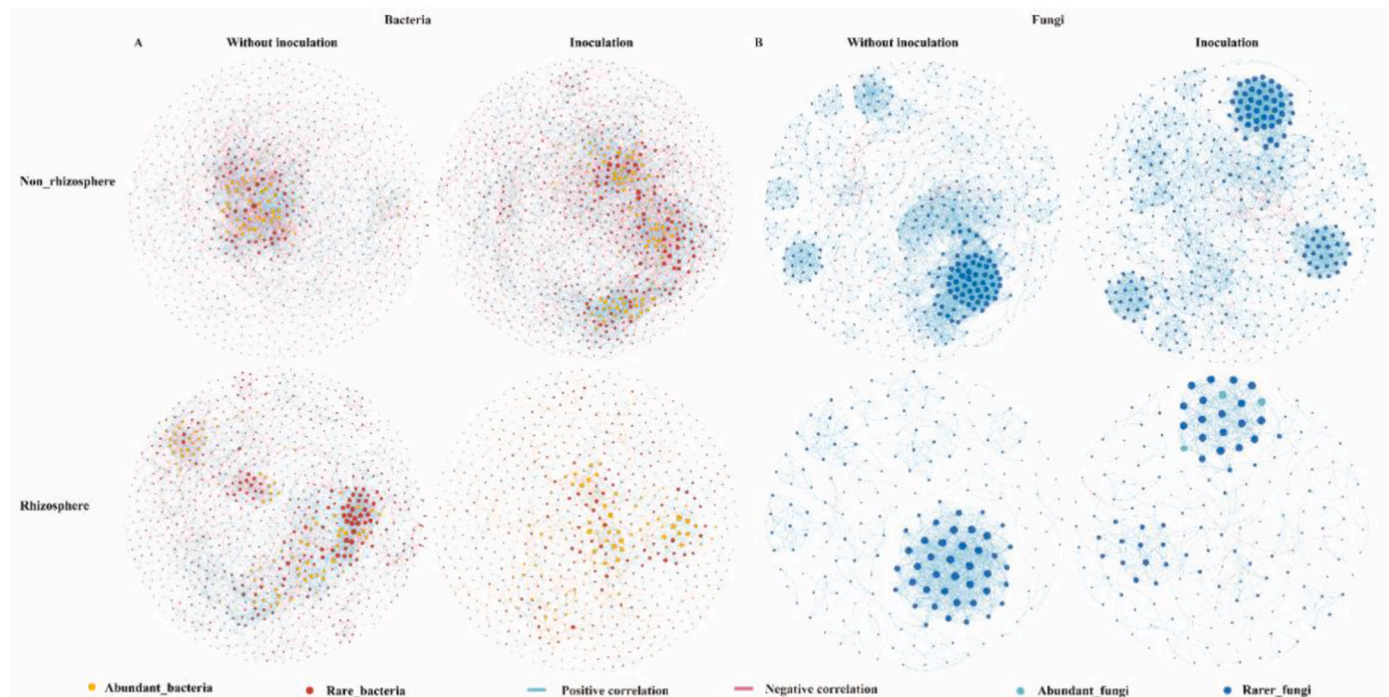


Fig. 5. Relationships between the abundances of nitrogen-cycling genes and edaphic factors in different treatments. TN, total nitrogen; TP, total phosphorus; SOM, soil organic matter; NH<sub>4</sub>-N, ammonium-nitrogen; and NO<sub>3</sub>-N, nitrate-nitrogen (\*, P < 0.05; \*\*, P < 0.01).

pollution level was the primary factor affecting the structure of rare and abundant bacterial and fungal sub-communities, followed by legume planting. Inoculation with *M. loti* HZ76 had minimal effect on microbial community structure; nevertheless, it altered the relative abundances of major phyla, such as Proteobacteria and Ascomycota (Fig. S1, Table S6). According to pertinent studies, Proteobacteria can adapt to their environment and have a high metabolic capability in heavy metal-polluted environments. Through biological transformation, it can alter the status of heavy metal ions, lessen the toxicity of heavy metals to the environment, and assist plants in enhancing heavy metal resistance and improving heavy metal absorption (Zhang et al., 2019). Inoculation with

rhizobia can stimulate the release of organic acids by plant roots (Atemkeng et al., 2011). Similar to the result in a previous study (Xu et al., 2021), soil pH was the predominant edaphic factor influencing microbial community structure in the present study (Table 1). In addition, inoculation with the rhizobial strain altered soil TN and SOM contents in the rhizosphere (Table S2), both of which significantly influenced microbial community structure, based on Mantel tests (Table 1). A potential reason is that symbiotic root nodules supply N to legume plants, while plant roots release high amounts of N and P into the soil, improving soil quality (Peoples et al., 2009). According to these findings, *R. pseudoacacia*'s phytoremediation of Cd-contaminated soil



**Fig. 6.** Keystone taxa and their relationships with environmental variables and nitrogen-cycling genes. Co-occurrence networks of (A) bacterial and (B) fungal sub-communities were constructed at the operational taxonomic unit (OTU) level. The connection represents a significant correlation between two OTUs ( $|r| > 0.6$ ,  $P < 0.01$ ). The size of each node is proportional to the number of connections (i.e., degree), and the thickness (i.e., edge) of each connection between two nodes is proportional to the value of Spearman's correlation coefficient. OTUs with high connectivity and compactness centrality are defined as keystone taxa.

**Table 2**

Key topological features of microbial co-occurrence networks in different treatments. Rhizosphere, planting *Robinia pseudoacacia*; Non\_rhizosphere, no planting; WJ, without inoculation; J76, inoculation with *Mesorhizobium loti* HZ76; OTUs, operational taxonomic units.

	Bacteria				Fungi			
	Rhizosphere		Non_rhizosphere		Rhizosphere		Non_rhizosphere	
Rhizobial inoculation	WJ	J76	WJ	J76	WJ	J76	WJ	J76
Nodes	899	681	873	1063	192	173	590	545
Edges	4344	1250	3840	6039	883	477	3934	3265
Average degree	9.664	3.671	8.797	11.362	9.198	5.514	13.336	11.982
Diameter	21	28	22	21	9	11	20	15
Density	0.011	0.005	0.010	0.011	0.048	0.032	0.023	0.022
Modularity	0.743	0.839	0.581	0.671	0.549	0.708	0.687	0.783
Mean clustering coefficient	0.481	0.384	0.411	0.434	0.761	0.696	0.649	0.638
Average path length	6.792	8.806	6.684	6.333	3.501	2.862	7.867	5.491
Positive (%)	66.6%	65.5%	56.5%	60.6%	99.9%	98.5%	98.0%	98.2%
Negative (%)	33.4%	34.5%	43.5%	39.4%	0.1%	1.5%	2.0%	1.8%
Abundant OTUs (%)	16.6%	20.1%	18.0%	15.3%	12.5%	5.2%	11.7%	11.7%
Rare OTUs (%)	83.4%	79.9%	82.0%	84.7%	87.5%	94.8%	88.3%	88.3%

had little impact on both rare and abundant bacterial and fungal sub-communities. It might stimulate plant development by helping leguminous plants fix nitrogen or by boosting the number of helpful microbes for metal resistance, which would increase plant Cd enrichment and absorption.

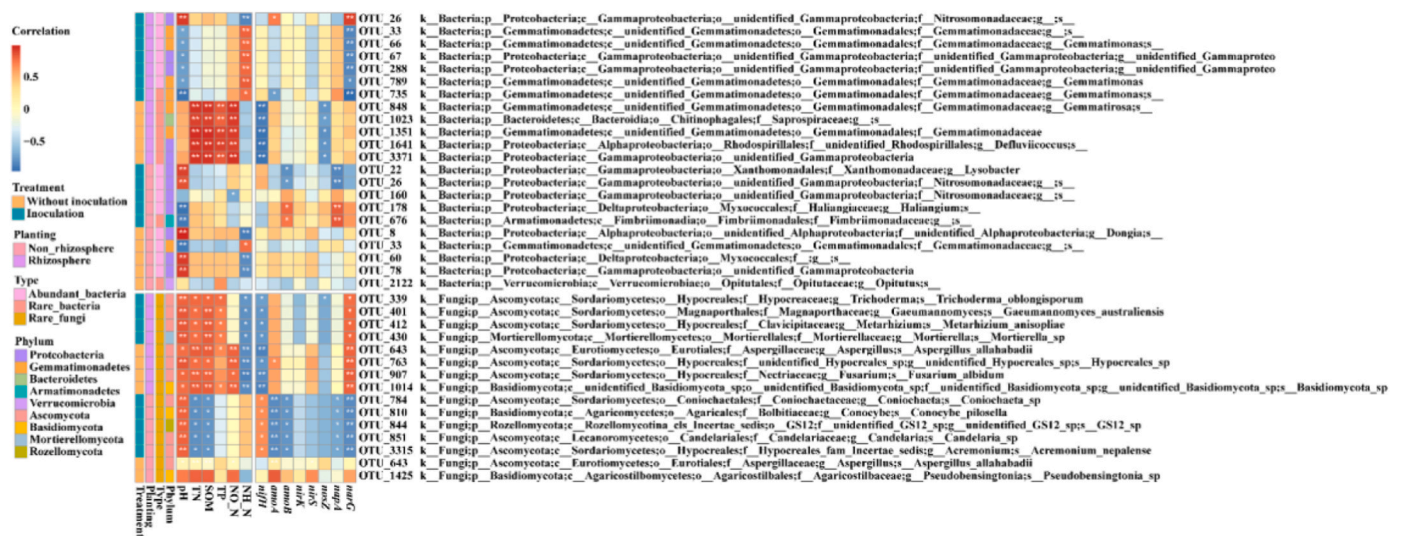
#### 4.2. Assembly of rare and abundant microbial sub-communities is mediated by different processes

Microorganisms play key roles in ecosystem processes and biogeochemical cycles through mutual interaction networks, facilitating various ecosystem services. It is essential to investigate the assembly processes of microbial communities under rhizobium-enhanced phytoremediation with *R. pseudoacacia* to facilitate vegetation restoration in mining areas. Our results indicated that stochastic processes (mainly homogeneous dispersal) governed the assembly of rare and abundant

bacterial and fungal sub-communities (Fig. 3), with legume planting and rhizobial inoculation having limited effects (Fig. S2). The findings suggest that the legume-rhizobium symbiotic system established by *R. pseudoacacia* and *M. loti* HZ76 is an environmentally friendly restoration method with minimal disturbance to the soil ecosystem based on a microbial community assembly perspective.

Identifying the factors influencing microbial community assembly is an integral part of microbial ecology research. The  $\beta$ NTI regression analysis results demonstrated that species richness and diversity were the major factors influencing the assembly of microbial sub-communities in the experimental conditions (Fig. S3). Previously, the same factors have been reported to influence the assembly of rare bacterial and fungal sub-communities in apple orchard soil under different mulching practices (Zheng et al., 2021). However, in the previous study, deterministic processes (e.g., homogeneous selection) dominated the assembly of rare microbial communities, perhaps because long-term





**Fig. 7.** Relationships between keystone operational taxonomic units (OTUs), edaphic factors, and nitrogen-cycling genes in different treatments based on Spearman's correlation analysis. TN, total nitrogen; TP, total phosphorus; SOM, soil organic matter; NH<sub>4</sub>-N, ammonium-nitrogen; and NO<sub>3</sub>-N, nitrate-nitrogen (\*, P < 0.05; \*\*, P < 0.01).

fertilization and nutrient inputs (C, N, and P) considerably influenced soil fertility (especially C and N) and organic matter mineralization. The experimental soil in the present study was collected from an abandoned mining area with minimal human disturbance. The soil microorganisms could have formed stable communities through filtering and selection in the local environment. Therefore, short-term legume planting and rhizobial inoculation had minor effects on edaphic factors and microbial α-diversity (Table S2, Fig. 2).

**4.3. Effects of rhizobial inoculation on abundance of functional genes associated with rhizosphere nitrogen cycling**

Under the experimental conditions in the present study, inoculation with *M. loti* HZ76 tended to enhance N fixation capacity while attenuating nitrification and denitrification capacity in the rhizosphere, based on the changes in functional gene abundances (Fig. 4). The results are consistent with those of a recent study on rhizosphere N cycling under various agronomic practices (Singh et al., 2021). In the case of serious N deficiency in mining soils, *R. pseudoacacia*, a leguminous tree species, can actively synthesize specific signal substances (e.g., progesterone) and release them into the rhizosphere through the roots. Consequently, a large number of rhizobia are recruited to the rhizosphere, where they receive signal substances that trigger gene expression reactions to form mature N-fixing nodules (Redmond et al., 1986). So we speculated that in addition to soil physicochemical differences, the better nitrogen fixation and repair effects of legume-rhizobia at high pollution levels were also the main factors that caused *nifH* gene abundance at high pollution level to be significantly higher than that at low pollution level and no pollution. The Cd enrichment and nodule number of *R. pseudoacacia* plants under high pollution level were significantly higher than those under low pollution level and no pollution, which supported our conjecture.

Similar to the findings of previous studies (Abdelkrimet et al., 2019; Fan et al., 2018b; Jian et al., 2019), we observed increased numbers of legume root nodules following rhizobial inoculation (Table S3). Such root nodules are N fixation sites for rhizobia. The rhizobia inhabiting root nodules convert atmospheric N directly into ammonia that plants can absorb. As can be shown, rhizobium inoculation can enhance the ability of the rhizosphere to fix nitrogen and raise soil nitrogen accumulation, which will encourage plant development and increase Cd enrichment and soil absorption. There were significant correlations between *nifH* gene abundance and soil TN and SOM contents in the

rhizosphere (Fig. 5), and considerable changes in both nutrient contents following the inoculation (Table S2). In addition, the rhizosphere *nifH* gene abundance significantly increased after implantation (Fig. 4), and the enriched Cd content of robinia acacia increased (Table S3). These results indicate that the legume-rhizobia biois system changes the abundance of functional genes related to rhizosphere nitrogen cycle by changing soil nutrient status, so as to promote plant growth and plant absorption of Cd to improve the phytoremediation effect.

The activity, diversity, and abundance of N cycling-associated microorganisms could reflect the intensity of soil N cycling (Teng et al., 2015; Nelson et al., 2016). According to our results, inoculation with *M. loti* HZ76 reduced the abundance of *amoA* and *amoB* in the rhizosphere (Fig. 4), which corroborates the effects of rhizobial inoculation on ammonia-oxidizing microorganisms in the rhizosphere of crop plants under intercropping (Zhang et al., 2015). Soil nitrification potential has been reported to be positively correlated with *amoA* and *amoB* gene abundance (Sims et al., 2012). In addition, in the present study, we observed that rhizobial inoculation altered the pH of rhizosphere soil (Table S2), whereas Zheng et al. (2016) observed that the activity and diversity of ammonia-oxidizing microorganisms driving ammonia oxidation were mainly affected by soil pH. Accordingly, rhizobial inoculation can reduce nitrification capacity in the rhizosphere of legume plants by lowering functional gene abundance and soil pH levels.

We also observed that rhizobial inoculation increased the *nirK* + *nirS*/*nosZ* ratio (Table S8), which signifies a high capacity of nitrous oxide (N<sub>2</sub>O) production from denitrification (Kong et al., 2017). Following rhizobial inoculation, the abundance of the *nosZ* gene in the rhizosphere decreased when compared with that in the uninoculated treatment (Table S8; Fig. 4). The *nosZ* gene encodes N<sub>2</sub>O reductase, which modulates the conversion of N<sub>2</sub>O into N<sub>2</sub>, and *nosZ* gene deletion is considered to be a major determinant of greenhouse gas emissions from agricultural soils (Bakken et al., 2012). Consequently, inoculation with *M. loti* HZ76 could increase N<sub>2</sub>O emissions from Cd-contaminated soil during phytoremediation by *R. pseudoacacia*, which is consistent with the findings observed following soybean inoculation with *Bradyrhizobium* strains (Akiyama et al., 2016; Melissa et al., 2022). Conversely, rhizobial inoculation could reduce denitrification capacity and negatively affects N<sub>2</sub>O production, so that it remains unclear whether inoculation could increase net N<sub>2</sub>O emissions.

#### 4.4. Important roles of rare and abundant taxa in rhizosphere nitrogen cycling

In the present study, inoculation with *M. loti* HZ76 improved the modularity degree of microbial co-occurrence networks but reduced network complexity, in both rhizosphere and non-rhizosphere soils (Table 2, Fig. 6). As rhizobial inoculation decreased soil TN and SOM contents (Table S3), soil microorganisms were likely to adjust their interspecies interactions in response to environmental change (Gurmesa et al., 2022). This study also found a positive correlation between dominant bacterial and fungal networks, and the positive correlation was enhanced after inoculation (Fig. 6, Table 2). Network positive and negative correlations affect the stability and function of microbial communities. In general, a positive correlation means that species have the same environmental needs, are mutually beneficial to each other or cooperate with each other to cope with the environment (Yuan et al., 2021). This suggests that microorganisms may respond to external cadmium stress through mutual cooperation and metabolic reactions.

According to our results, rare and abundant taxa played distinct and non-trivial roles in the regulation of rhizosphere N cycling in Cd-contaminated soil (Fig. 7), which is consistent with previous findings in terrestrial and aquatic environments (Jousset et al., 2017; Lynch and Neufeld, 2015). Positive correlations were found between these keystone taxa, suggesting mutual cooperation. Keystone taxa are located at key positions in microbial co-occurrence networks and play pivotal roles in community stability maintenance (Toju et al., 2018). Keystone taxa have been demonstrated to be closely associated with functional genes involved in ecological processes (Banerjee et al., 2018; Chen et al., 2019). In addition, significant correlations between the abundances of keystone taxa and functional genes associated with N fixation, nitrification, and denitrification were observed in the rhizosphere in the present study, based on network and correlation analyses (Fig. 7). These results suggest that inoculating rhizobium may also affect rhizosphere nitrogen cycling by changing microbial interaction patterns, so as to promote plant growth and improve plant remediation of heavy metal contaminated soil.

In the present study, the rare taxa in bacterial communities, mainly from the families Gemmatimonadaceae and Saprospiraceae, regulated N fixation and denitrification. Conversely, the abundant taxa, mainly derived from the families Gemmatimonadaceae and Nitrosomonadaceae, mediated nitrification (Table S7, Fig. 7). It has been reported that Bacteroidetes, Proteobacteria, and Gemmatimonadetes play key roles in nitrification, denitrification (Choi et al., 2021; Sun et al., 2018; Zhang et al., 2021), and N fixation (Inoue et al., 2015; Wang et al., 2020) in sewage environments and farmland ecosystems. In the case of fungal communities, rhizosphere N cycling was regulated mainly by rare taxa rather than abundant taxa (Fig. 7, Table S7). Fungal regulation of NO<sub>3</sub>-N assimilation has been studied extensively, especially in the phyla Ascomycota and Basidiomycota (Gorfer et al., 2011; Inselsbacher et al., 2010). In the present study, we observed that the major fungal taxa involved in rhizosphere N fixation and denitrification were the genera *Aspergillus*, *Fusarium*, *Trichoderma*, *Gaeumannomyces*, and *Metarhizium*, all belonging to the phylum Ascomycota. The results further demonstrate that keystone taxa in the total bacterial community and abundant fungal community play a central role in mediating rhizosphere N cycling by regulating functional gene abundance (Banerjee et al., 2018; Toju et al., 2018).

## 5. Conclusions

In the present study, we report the microbial mechanisms underlying a symbiotic system established using *R. pseudoacacia* and *M. loti* HZ76 for the phytoremediation of Cd-contaminated mining soil, with a focus on rhizosphere N cycling. After 60 day of legume planting, root nodule number, and tissue Cd accumulation were increased by rhizobial inoculation depending on the Cd pollution level. Cd Pollution level and

legume planting were the major factors driving variation in rhizosphere microbial community  $\alpha$ -diversity and structure, whereas rhizobial inoculation mainly influenced microbial community composition and network interactions. Stochastic processes (primarily homogeneous dispersal) governed the assembly of rare and abundant microbial sub-communities, irrespective of legume planting or rhizobial inoculation. At the gene level, rhizobial inoculation enhanced N fixation and decreased nitrification and denitrification capacity in the rhizosphere, and keystone taxa such as Proteobacteria (bacteria) and Ascomycetes (fungi) mediated rhizosphere N cycling. The results of the present study enhance our understanding of microbe-driven N cycling in the rhizosphere during enhanced phytoremediation, and could facilitate the ecological restoration of N-deficient mining soils with heavy metal pollution.

## Declaration of competing interest

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

## Data availability

The authors are unable or have chosen not to specify which data has been used.

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## Appendix B. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.rhisph.2023.100734>.

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