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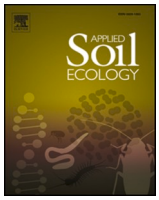

Short-term N addition in a *Pinus tabuliformis* plantation: Microbial community composition and interactions show different linkages with ecological stoichiometry

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ABSTRACT

Increasing nitrogen (N) deposition severely impacts terrestrial biogeochemical cycles by altering the stoichiometry of ecological components. Although microbes are known to play an important role in biogeochemical cycles, the mechanisms how soil microbes drive nutrient cycling remain elusive under N deposition. Therefore, we investigated changes in microbial community diversity, composition, and interactions, and elucidated the relationship among microbial community responses, soil available nutrients, and ecological stoichiometry resulting from two years of N addition to a *Pinus tabuliformis* plantation on the Loess Plateau at four rates of N addition (0 (N0), 3.0 (N3), 6.0 (N6), and 9.0 (N9) g N m^{-2} y^{-1}). N addition significantly influenced microbial composition, decreasing the relative abundance of *Acidobacteria* and *Basidiomycota* along N addition gradients and increasing the relative abundance of *Ascomycota* from N3 to N9. Along N addition gradients except N3, bacterial interactions increased from 62.70% to 73.38%, whereas interactions between bacterial and fungal communities decreased from 34.44% to 24.43%. Among all microbial interactions, the positive ones accounted for a larger proportion (over 55%), indicating a predominance of mutualism under all N addition treatments. Changes in the microbial composition were correlated with soil resource stoichiometry factors, including soil organic carbon: soil total N (SOC:TN) and SOC: soil total phosphorus (SOC:TP), whereas the topological network features were correlated with ammonium N (NH_4^+-N), nitrate N (NO_3^--N), β-1,4-*N*-acetylglucosaminidase (NAG), alkaline phosphatase (AP), and eco-enzymatic stoichiometry. Therefore, the soil variables that caused changes to microbial composition and interactions were different. In this sense, microbial community compositions were more easily affected by soil resource stoichiometry, whereas microbial interactions were more easily affected by soil available nutrients. In addition, changes to microbial interactions could mediate microbial metabolism via eco-enzyme expression.

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1. Introduction

Nitrogen (N) inputs into terrestrial ecosystem from anthropogenic sources are 30% higher than those from natural sources, 10 times higher than anthropogenic inputs 100 years ago, and continuously increasing (Galloway et al., 2004; Canfield et al., 2010). Ecological components interact with each other under continuous N inputs, forming a dynamic balance process and driving element cycles. Microbes play a vital role in this dynamic process under N deposition by mediating soil biogeochemical cycling (Bardgett et al., 2008). Many previous studies have focused on how N addition impacts microbes by changing soil conditions. However, because of variations in climate, soil conditions, and soil microbial properties among ecosystems, experimental N addition may have different effects on microbial communities (Treseder, 2008; Ramirez et al., 2010; Li et al., 2016; Liu et al., 2016). For example, the ratio of fungi to bacteria decreases with increasing available soil N content under N addition because fungi have lower N demands than bacteria (Zhao et al., 2015). Increased plant biomass caused by N enrichment can lead to increased C inputs into the soil from plant roots which increases microbial respiration (Zang et al., 2017). Therefore, the mechanisms how soil microbes govern these effects and drive element cycles remain uncertain. Recently, ecological stoichiometry, using elemental ratios as common currency, can determine trophic dynamics and elemental cycling across all trophic levels within an ecosystem (Schindler, 2003), thereby illustrating the key changes to C, N, and P cycling in the ecosystem. Wan et al. (2015) found that the soil C:N ratio is a major determinant of the soil microbial community structure. In addition, when microbes decompose organic material, TN and NO_3^--N become the main drivers influencing bacterial community composition; thus, the soil C:N ratio drives changes in bacterial community composition (Guo et al., 2018). Therefore, using ecological stoichiometry to identify soil factors that impact microbial communities may help to reveal how microbes drive the element cycles.

The traditional approach for determining the factors influencing bacterial and fungal communities mostly involves measuring abiotic factors, such as soil nutrients and pH, whereas relatively little is known about the effects of biotic interactions in shaping microbial communities (Xiao et al., 2018). However, fungi and bacteria share the same habitats and frequently interact in soils (de Boer et al., 2005; de Menezes et al., 2017). Therefore, when analyzing changes to microbial community composition and diversity caused by N addition, the response of biotic interactions cannot be ignored. In this study, network analysis was used to elucidate the soil bacterial-fungal community interactions along N addition gradients. Some previous studies have concluded that biotic interactions are resource driven, such as by soil organic carbon (SOC) and iron; therefore, changes in resources may affect the nature of interactions of microorganisms (Ma et al., 2016; Banerjee et al., 2016). In addition, Hoek et al. (2016) found that nutrient enrichment shifted the interactions among microbial communities from mutualism to competition. However, whether soil stoichiometry changed by N addition and how soil variables would impact microbial interactions under N addition, thus shifting microbial community, remains poorly understood.

As reported by previous studies, severe N deposition not only directly increases soil N enrichment and decreases C:N (Zhou et al., 2016), but also indirectly influences plant diversity and microbe communities, thus changing soil C and P contents and their stoichiometry in nutrient cycling (Liu and Greaver, 2010). Once soil substrates are changed by N deposition, microbes vary their allocations of resources to C-, N- or P-acquiring enzymes, adjusting their metabolism, to maintain a dynamic balance between the demand of the microbial community and supply of soil resources (Allison et al., 2011). For example, fungi prefer a substrate with higher C:N (Kuhar et al., 2008; Zhao et al., 2015) and adjust their own strategies of nutrient acquisition with higher C metabolism capacities under N addition. However, some studies have revealed that the community composition and diversity of soil microbes determines the microbial metabolism and function of the soil ecosystem and nutrient cycling (Carney et al., 2007). Other studies have concluded that changes to the microbial community are not significantly correlated with changes in eco-enzymatic activities due to the functional redundancy of the microbial community (Azarbad et al., 2015; Banerjee et al., 2016). Zhang et al. (2019) illustrated how microbes regulate their metabolism to adapt to soil conditions changed by N addition from the perspective of ecological stoichiometry. Therefore, how changes in microbial community composition, diversity, and interactions caused by soil properties contribute to changes in eco-enzymatic activities and eco-enzymatic stoichiometry under short-term N addition remains unknown.

Pinus tabuliformis is one of the largest artificial forest species in the Loess Plateau and the preferred species for maintaining ecosystem function in this region and meeting the demands of local economic materials. The *P. tabuliformis* forest was originally deficient in active N, but is now experiencing severe N deposition (Liang et al., 2016). To investigate how the microbial community mediates microbial metabolisms via eco-enzymatic expression in response to changes in soil properties, we design an experiment based on N addition along a gradient. We hypothesize that: 1) microbial community composition and diversity will be affected by altered soil stoichiometry under N addition; 2) microbial interactions will also be impacted by altered soil stoichiometry under N addition; and 3) changes to microbial community compositions and interactions can mediate microbial metabolism, inducing changes in eco-enzymatic activities and eco-enzymatic stoichiometry.

2. Materials and methods

2.1. Site description

The study area of Tielongwan plantation is located in Yichuan County (36°04′N, 110°15′E) in northern Shaanxi Province, China. This region has a semiarid climate with mean annual precipitation of 584.4 mm and mean annual temperature of 9.7 °C. Sixty percent of the rainfall occurs between July and September. The soil in our experimental site is gray forest soil (Gray Luvisol, FAO soil classification). The elevation of this plantation ranges from 860 m to 1200 m, with a landscape of rolling hills and slopes from 20° to 25°.

The artificial *P. tabuliformis* forest was established in 1966, with an area of 600 ha. The zonal vegetation is temperate deciduous broad-leaved forest. The main trees are *P. tabuliformis*, with *Populus davidiana*. Shrubs including *Lespedeza davurica*, *Elaeagnus umbellata*, *Rcsa xanthine*, *Spiraea salicifolia*, and *Caragana korshinskii* are also present, along with *Carex lanceolate* herbs distributed sporadically throughout the forest.

2.2. Experimental design and sample collection

The experiment was conducted with one-factor randomized design comprising four treatments. N was applied at rates of 0 g N m^{-2} y^{-1} (N0), 3 g N m^{-2} y^{-1} (N3, low N addition), 6 g N m^{-2} y^{-1} (N6, middle N addition), and 9 g N m^{-2} y^{-1} (N9, high N addition) with four replicates. Each plot had an area of 10 × 10 m^2, with a 5 m-wide buffer strip separating each plot. The rates of N addition were determined based on global N-deposition levels (Bobbink et al., 2010) and the amounts of N soil received in the Loess Plateau (Wei et al., 2010). N was added in the form of urea ($CO(NH_2)_2$) four times a year in April, June, August, and October from 2014. The plots with N treatment, i.e., N3, N6, and N9, received the corresponding dose of urea solution one day prior to rainfall to reduce ammonia volatilization, while N0 received the same volume of water applied at N3, N6, and N9 without urea.

After two years of N addition, soil samples were collected in September 2015 from depths of 0–20 cm. In each plot, four soil cores from randomly selected locations were mixed to form a composite sample. Small stones, roots, and litter were removed from the composite samples, and the soil was divided into three subsamples. One subsample was air-dried and sieved through 0.25-mm mesh for physicochemical

analysis, and another subsample was transferred immediately in bags with ice to a super-cold refrigerator (−80 °C) for DNA extraction and enzyme activity analysis.

2.3. Soil physicochemical analysis

The H_2SO_4-$K_2Cr_2O_7$ and Kjeldahl methods were conducted to measure SOC and TN, respectively (Bremner and Mulvaney, 1982). The soil TP was determined colorimetrically after digestion with H_2SO_4 and $HClO_4$ (Schade et al., 2003). The soil NH_4^+-N and NO_3^--N in filtered 2.0 M KCl extracts of fresh soil sample were measured with a flow injection autoanalyzer (Alpkem, OI Analytical, USA). The soil pH was determined in 1:2.5 (soil:water) solutions. The soil available P (aP) was measured by molybdenum-antimony colorimetry with $Na(HCO_3)_2$ extracts (Olsen and Sommers, 1982). The soil physicochemical properties are shown in Table S1.

2.4. Soil eco-enzymatic activity analysis

The activity of the C-acquiring enzyme (β-1,4-glucosidase [BG]), N-acquiring enzyme (β-1,4-*N*-acetylglucosaminidase [NAG]), and P-acquiring enzyme (alkaline phosphatase [AP]) was determined following the method described by Saiya-Cork et al. (2002), with modifications based on German et al. (2011). The detailed method and data can be found in the study by Zhang et al. (2019). In brief, 1 g soil stored at −80 °C was added to 125 ml of 50 mM sodium acetate buffer (pH 8.5) to obtain sample suspensions for all enzymes. The suspensions added with fluorescent substrates were then incubated for 2 h. After incubation, fluorescence was measured using a fluorometer (SpectraMax M2, Molecular Devices, USA) set at 365 nm excitation and 450 nm emission.

2.5. DNA extraction, Illumina MiSeq high-throughput sequencing, and sequence processing

Soil samples from four replicates of each treatment (N0, N3, N6, and N9) were used for DNA extraction, PCR, and high-throughput sequencing. Microbial DNA was extracted from 0.25 g soil using a TIANamp Soil DNA Kit (Tiangen Biotech Co., Ltd., Beijing, China). After being diluted tenfold, the quality and quantity of DNA extraction were confirmed by 1% agarose gel electrophoresis and a spectrophotometer (NanoDrop ND-1000, NanoDrop Technologies, Wilmington, USA). The genes of the bacterial V3–V4 region, 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′), and fungal ITS1 region, 1737F (5′-GGAAGTAAAAGTCGTAACAAGG-3′)/2043R (5′-GCTGCGTTCTTCATCGATGC-3′), were amplified by polymerase chain reaction (PCR) using primers. The PCR reactions contained 2 μl sterile ultrapure water, 15 μl Phusion Master Mix (2×), 3 μl of 6 μM primers, and 10 μl template DNA (5–10 ng). The PCR amplification was performed as follows: 98 °C for 60 s; 30 cycles of 98 °C for 10 s, 50 °C for 30 s, 72 °C for 30 s, and 72 °C for 5 min. Following this, the PCR products were verified by 2% agarose gel electrophoresis. The PCR products were mixed in equal density ratios then purified using a Qiagen Gel Extraction Kit (Qiagen, Germany). The purified amplicons were then sequenced on an Illumina HiSeq2500 platform.

The quality of sequences was filtered and chimera-checked using Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al., 2010). After chimeric sequences were identified and removed to obtain effective tags, the remaining sequences were clustered by UPARSE (Edgar, 2013) and assigned to operational taxonomic units (OTUs) with 97% similarity. Finally, taxonomic information for each representative sequence was obtained from the GreenGenes Database13_5 using the RDP classifier (Wang et al., 2007) algorithm. The average reads obtained for bacteria and fungi were 59,348 and 64,991, respectively. Rarefaction curves for bacteria and fungi were generated in Fig. S1. Shannon and Simpson indices were calculated to assess the microbial diversity, and Chao1 and ACE richness indices were calculated to estimate the species richness at the OTU level.

2.6. Network analysis

To minimize the complexity of the network and promote understanding of the core soil microbial community, OTUs with relative abundance ≥0.1% were selected for network analysis. The co-occurrence of OTUs from the soil bacterial and fungal communities under N addition was analyzed using Pearson correlation with R v.3.6.0. The cutoff correlation coefficient (similarity threshold) was set at 0.8, and the adjusted *P*-value for correlation was 0.001. The nodes in this network represent OTUs, and the edges that connect these nodes represent interactions between OTUs. Network topological properties were calculated with Gephi 0.9.2 software and network images were generated with Cytoscape 3.7.1. Degree represents the number of nodes connected to a given node; betweenness centrality represents the number of shortest paths through a node; closeness centrality represents the number of steps required from a given node to approach all other nodes; and clustering represents the probability of the adjacent nodes of a node being connected.

2.7. Calculations and statistical analyses

Homogeneity of variance tests of soil physicochemical parameters were conducted, and these indices met the normal distribution. Then the differences in soil physicochemical parameters in response to different N addition gradients were analyzed by one-way analysis of variation (ANOVA), followed by multifractal comparison using Duncan's tests. ANOVA and multifractal comparison significant were established at $P < 0.05$, and both analyses were performed using SPSS 20.0. The community similarities were calculated by Jaccard's index. The differential bacterial and fungal OTUs responsible for community differences between N treatments and no N addition were identified by the DESeq2 package in R v.3.6.0 (Love et al., 2014) as microbial indicator OTUs of N-added soil. Differential expression using DESeq2 was conducted to provide OTU counts from N0 soil as a control, and the Wald test was used to determine significance (adjusted *P* value < 0.1). The indicator OTUs used to show community differences were also visualized by Cytoscape (Shannon et al., 2003). Principal coordinate analyses (PCoA) were used to evaluate the overall differences in the structures of the bacterial and fungal communities based on Bray-Curtis distances using R v.3.6.0. The Mantel test for the correlation between microbial community composition and soil variables, soil microbial biomass, and eco-enzymatic properties, and Spearman's rank correlation test for the correlation between topological features of co-occurrence and soil factors were both conducted with R v. 3.6.0. The figures were drawn using OriginPro 9.0.

3. Results

3.1. Diversity and composition of bacterial and fungal communities

The number of bacterial OTUs and fungal OTUs, ranging from 2763 to 2899 and 661 to 818, respectively, did not show significant differences among each treatment (Table 1). N addition had no significant effect on bacterial diversity indices (Shannon and Simpson) (Table 1a). The bacterial richness indices (Chao1 and ACE) for N6 displayed numerically maximum values, and were significantly higher than those for N0. In contrast, the fungal community indices exhibited the opposite results, i.e., both fungal Shannon and Simpson indices exhibited maximum values for N6, significantly higher than minimum values for N3, and N addition had no significant effect on richness indices (Chao1 and ACE).

Proteobacteria and *Basidiomycota* were found to be the dominant bacterial and fungal phylum, respectively, in all treatments (Fig. 1).

Table 1
α-Diversities of (a) bacterial and (b) fungal communities under different levels of N addition.

(a)

Treatments	OTUs	Shannon	Simpson	Chao1	ACE	Coverage (%)
N0	2763 ± 64.41a	9.37 ± 0.08a	0.995 ± 0.001a	3116.19 ± 96.09b	3196.53 ± 97.37b	0.985
N3	2792 ± 123.47a	9.35 ± 0.19a	0.995 ± 0.001a	3157.89 ± 126.60ab	3254.39 ± 118.87ab	0.985
N6	2899 ± 121.37a	9.39 ± 0.13a	0.995 ± 0.00a	3349.08 ± 212.00a	3454.65 ± 181.19a	0.983
N9	2898 ± 59.16a	9.45 ± 0.07a	0.996 ± 0.001a	3304.35 ± 63.20ab	3424.87 ± 101.07a	0.983

(b)

Treatments	OTUs	Shannon	Simpson	Chao1	ACE	Coverage (%)
N0	791 ± 155.87a	5.15 ± 0.86a	0.891 ± 0.072ab	771.46 ± 140.28a	790.58 ± 146.59a	0.997
N3	661 ± 55.95a	3.70 ± 0.46b	0.771 ± 0.049b	689.38 ± 94.36a	713.31 ± 102.40a	0.997
N6	818 ± 177.51a	5.40 ± 0.70a	0.925 ± 0.037a	820.71 ± 210.69a	840.03 ± 210.98a	0.997
N9	802 ± 159.99a	4.95 ± 0.82a	0.866 ± 0.137ab	793.82 ± 149.35a	833.26 ± 149.35a	0.996

Note: Different letters indicate significant differences of the same parameter among different treatments at the $P < 0.05$.

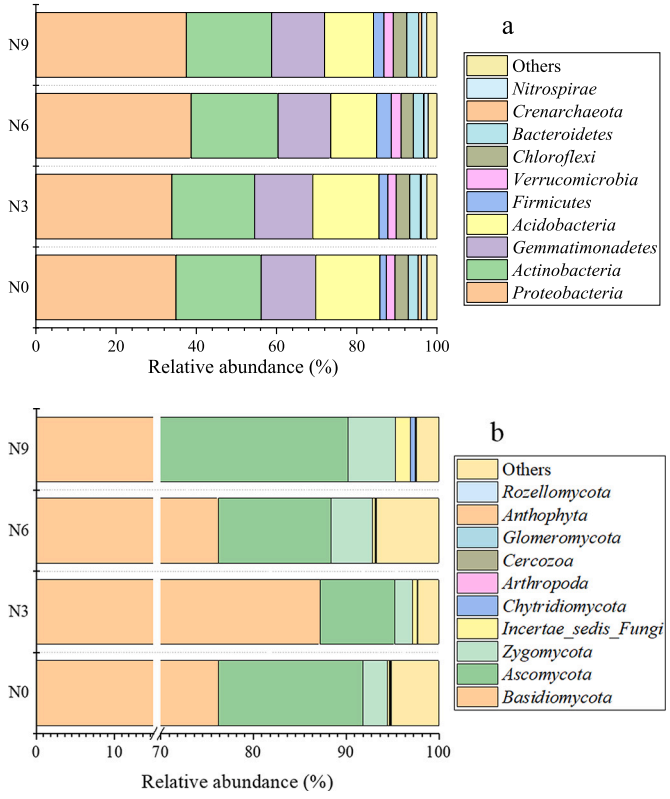
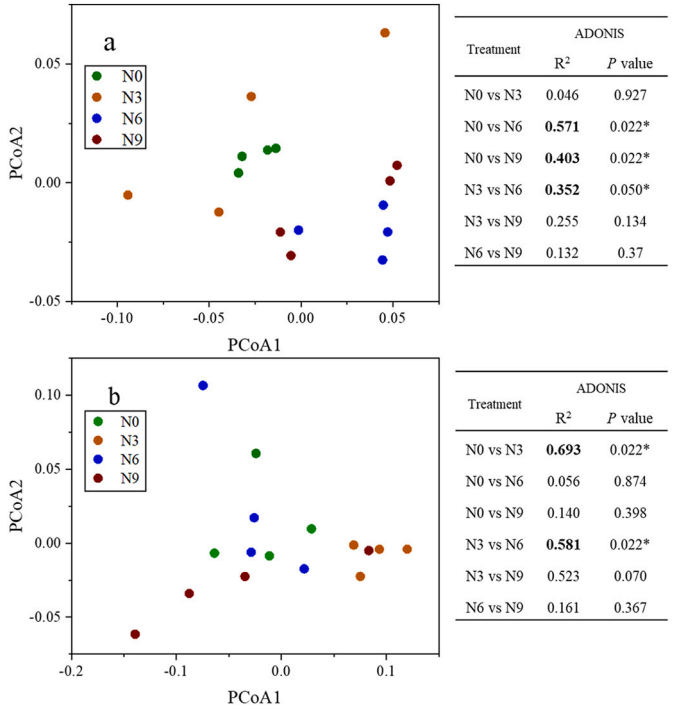


Fig. 1. Relative abundance of (a) bacterial and (b) fungal communities at the phylum level.



Treatment	ADONIS	
	R^2	*P* value
N0 vs N3	0.046	0.927
N0 vs N6	**0.571**	0.022*
N0 vs N9	**0.403**	0.022*
N3 vs N6	**0.352**	0.050*
N3 vs N9	0.255	0.134
N6 vs N9	0.132	0.37

Treatment	ADONIS	
	R^2	*P* value
N0 vs N3	**0.693**	0.022*
N0 vs N6	0.056	0.874
N0 vs N9	0.140	0.398
N3 vs N6	**0.581**	0.022*
N3 vs N9	0.523	0.070
N6 vs N9	0.161	0.367

Fig. 2. Principal coordinates analysis (left panel) and ADONIS (right panel) of community composition and relative abundance of the top 10 taxonomic groups at the phylum level based on (a) bacterial and (b) fungal phyla under different N addition levels. N0-1, N0-2, N0-3, and N0-4 represent N0 treatments; N3-1, N3-2, N3-3, and N3-4 represent N3 treatments; N6-1, N6-2, N6-3, and N6-4 represent N6 treatments, and N9-1, N9-2, N9-3, and N9-4 represent N9 treatments.

Furthermore, *Basidiomycota* displayed numerically maximum values for N3 and was positively correlated with SOC (Fig. S2). At bacterial and fungal phylum level, PCoAs and Adonis tests were conducted to show the impacts of N addition on bacterial and fungal communities (Fig. 2). For bacterial communities, N0 and N3 (low N addition) and N6 and N9 (high N addition) had similar bacterial community structures, while N0 had a clearly different bacterial structure compared to N6 and N9 (Fig. 2). For fungal communities, N0 tended to have a fungal structure similar to N6, whereas N3 tended to have a similar structure to N9.

3.2. Soil microbial community indicators and similarities under N addition

A total of 23, 26, and 64 microbial OTUs (compared with N0), which were defined as indicator OTUs, were present in N3-, N6-, and N9-treated soil, respectively (Fig. 3). Most identifiable indicators were fungal OTUs. Among the OTUs in N3 vs N0, there was only one bacterial OTU, belonging to *Actinobacteria*. Bacterial indicator OTUs identified in N9 vs N0 belonged to more phyla: *Acidobacteria*, *Actinobacteria*, *Gemmatimonadetes*, *Firmicutes*, and *Nitrospirae*.

The ln-transformed microbial community similarity between N9 vs N6 was the numerically maximum value, and had no significant difference with the similarities of N6 vs N0, N3 vs N0, and N9 vs N0 (Fig. S3). However, ln-transformed microbial community similarity between N6 vs N0 was significantly higher than that of N9 vs N3. The linear regression between ln-transformed microbial community similarity and N addition contents exhibited no significant correlation.

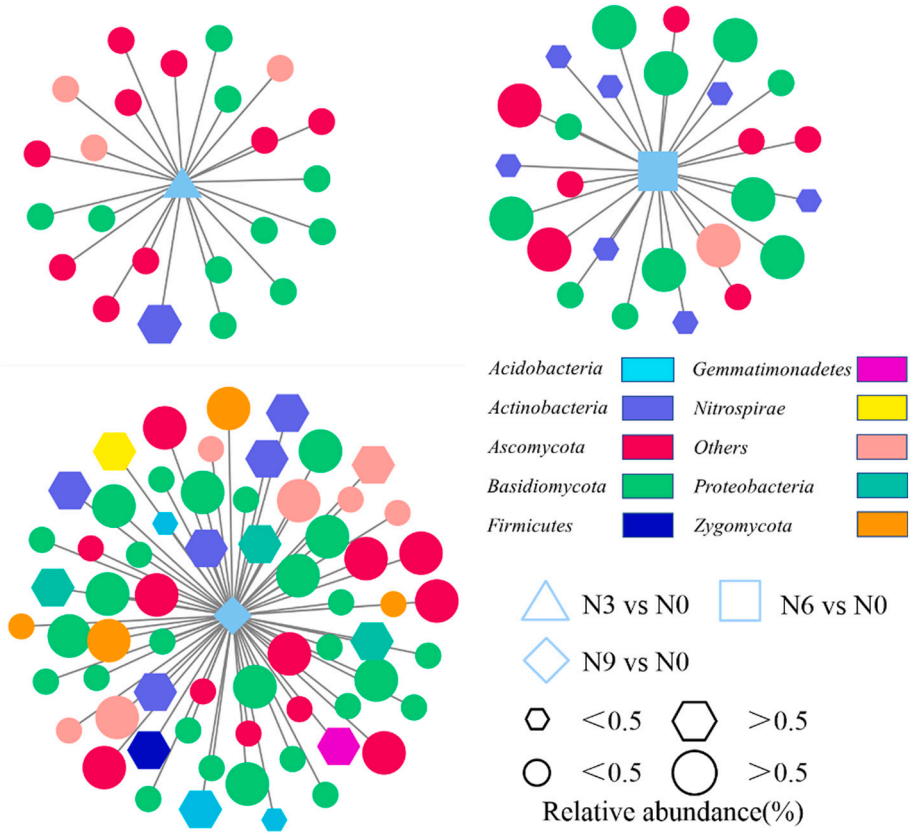


Fig. 3. Indicator OTUs of N-treated soils compared to N0 soil. Hexagons Circles represent bacterial OTUs and represent fungal OTUs. The sizes of each circle and hexagon represent the relative abundance.

3.3. Network differences among N addition treatments

The distinct patterns of microbial co-occurrence networks under different N treatments are visualized in Fig. 4a–d. The properties of microbial co-occurrence networks, like numbers of OTUs selected to detect the bacteria-fungi interactions and numbers of associations among selected OTUs, are summarized in Table S2. Overall, numbers of bacterial nodes, bacterial OTUs selected for co-occurrence networks, were higher than numbers of fungal nodes. Furthermore, among all the associations, the positive ones accounted for a higher proportion than negative one under N treatments. In addition, the number of associations between nodes for each phylum pair was counted and shown in the left panel of Fig. 4. As the dominant bacterial phylum, *Proteobacteria* had the most relationships with other phyla. The relationship between the dominant bacterial and fungal phylum, *Proteobacteria* and *Basidiomycota*, changed in N3 from negative to positive.

The betweenness centrality first increased, then decreased, with a maximum value at N6 (Table S2). The closeness centrality showed the opposite tendency, first decreasing then increasing, with the lowest value at N6. Another index showing network association, the clustering coefficient, initially increased then decreased, reaching a maximum at N6. However, clustering coefficients at N3 and N6 did not show a significant difference.

3.4. Correlation among soil variables, microbial community properties, and eco-enzymatic properties

SOC, TN, and TP and soil available nutrients were insignificantly correlated with overall microbial community composition ($P > 0.05$) (Table 2). Soil stoichiometry, soil SOC: TN (SC:N), and soil SOC:TP (SC:P) exhibited a significant relationship with the overall microbial community composition. In previous study, soil stoichiometry showed insignificant correlation with eco-enzymatic properties (Zhang et al., 2019). In addition, microbial community composition had no significant correlation with eco-enzymatic properties. The correlation of the top ten bacterial and fungal phyla with soil variables, soil microbial biomass, and enzymatic activities and their stoichiometries was determined (Fig. S2). SOC were positively and negatively correlated with *Basidiomycota* and *Ascomycota*, respectively. Soil-available nutrients, NH_4^+-N and NO_3^--N, had a significantly positive relationship with *Acidobacteria* and *Firmicutes*. In addition, soil C:N and soil C:P were positively correlated with *Basidiomycota* and negatively correlated with *Ascomycota*.

We then examined the correlations between the network topological features and contents and stoichiometry of soil under N addition (Fig. 5). Soil available nutrients, NH_4^+-N, became an important factor, showing a significant correlation with some topological features, average closeness centrality, average harmonic closeness centrality, clustering, and triangle ($P < 0.001$). Another soil available nutrient, NO_3^--N, was positively correlated with eigencentrality and negatively correlated with harmonic closeness centrality. However, the degree centrality, closeness centrality, and betweenness centrality did not show significant correlations with the majority of soil variables and stoichiometry. In addition, harmonic closeness centrality was positively correlated with NAG:AP, but negatively correlated with BG:NAG. However, eigencentrality exhibited the opposite relationships, i.e., a negatively correlation with SMN:P and NAG:AP and a positive correlation with BG:NAG.

4. Discussion

4.1. Effect of N addition on microbial communities

In this study, the microbial community similarity responds nonlinearly to concentration differences brought about by N addition. This is supported by Liu et al. (2020), who found that bacterial diversity

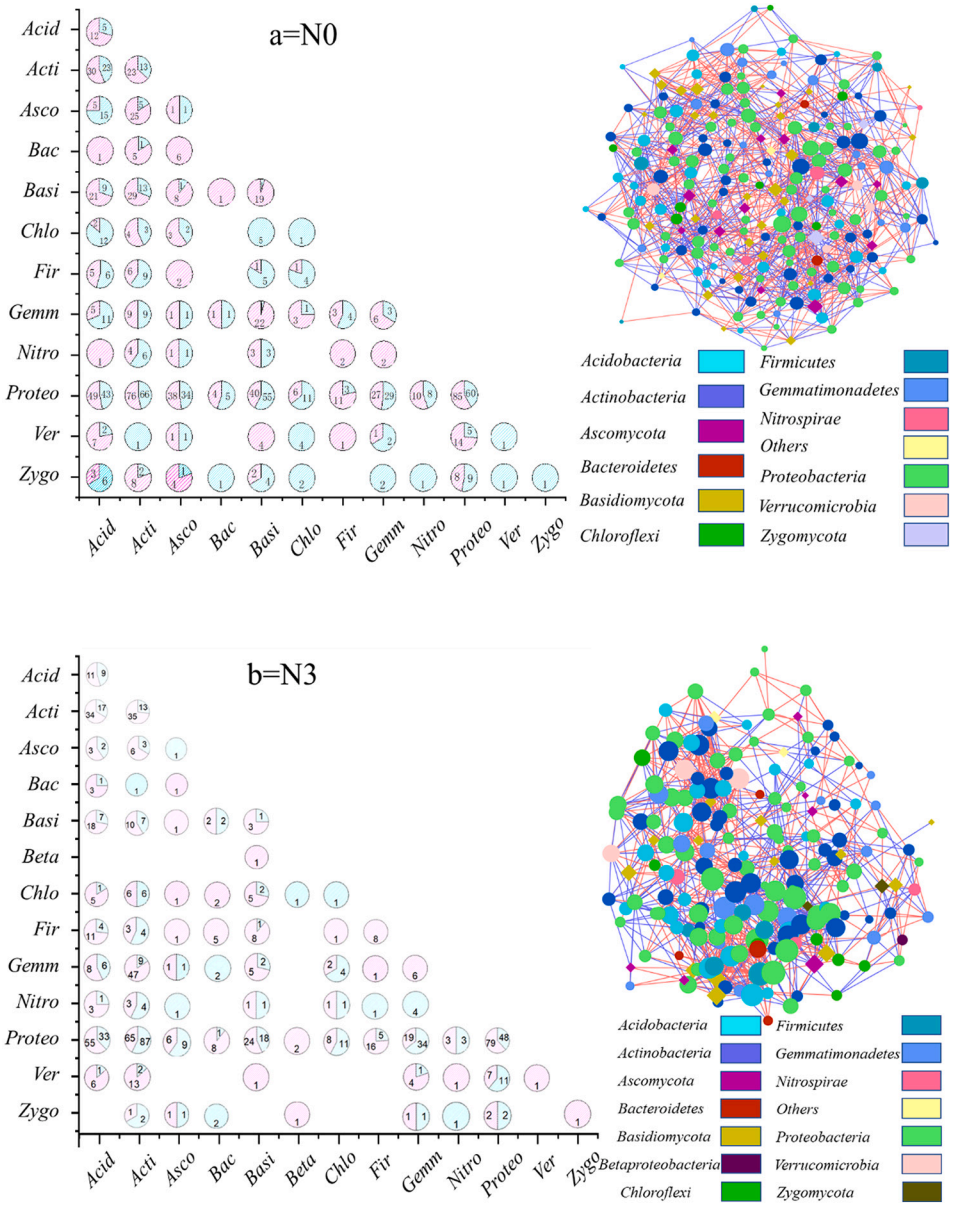


Fig. 4. Overview of the co-occurrence networks (right panel) for soil microbial communities under N addition and the number of associations (left panel) among different phyla. a, b, c, and d represent different N addition levels of N0, N3, N6, and N9, respectively. In right panel, *Acid*, *Acti*, *Asco*, *Bac*, *Basi*, *Beta*, *Chlo*, *Cren*, *Fir*, *Gemm*, *Incer*, *Nitro*, *Proteo*, *Ver*, and *Zygo* represent *Acidobacteria*, *Actinobacteria*, *Ascomycota*, *Bacteroidetes*, *Basidiomycota*, *Betaproteobacteria*, *Chloroflexi*, *Crenarchaeota*, *Firmicutes*, *Gemmatimonadetes*, *Incertae_sedis_Fungi*, *Nitrospirae*, *Proteobacteria*, *Verrucomicrobia*, and *Zygomycota*, respectively. Each node represents an operational unit (OTU). The size of the node represents the degree of node relevance. Pie charts in the left panel represent the relative abundance of positive and negative associations among different bacterial and fungal phyla in the networks. Red circles represent positive relationships among the taxa; blue circles represent negative relationships among the taxa; and spaces without circles represent no significant relationships among the taxa. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

responds nonlinearly to N inputs. However, this phenomenon is unlike the influence of distance or elevation on microbial community similarity, the farther or higher the distance or elevation (Zhang et al., 2015; Ma et al., 2016), the lower the community similarity. This is because N addition would have multiple impacts on microbial growth, composition, and function (Zhang et al., 2018), and excessive N addition would inhibit organism growth, showing a unimodal relationship with organism properties. In addition, Treseder (2008) revealed that the microbial properties influenced by N addition were mainly determined by the duration and content of N received by soil rather than the direct N deposition level. These suggest the microbial community similarity cannot respond linearly to differences of N addition dose.

Although the numbers of bacterial and fungal OTUs did not exhibit a significant difference, the diversity indices (Shannon and Simpson) of the fungal community at N3 were significantly lower than those under other treatments. This may be caused by the increased relative abundance of *Basidiomycota* at N3 (Fig. 1) and the lack of a significant difference between fungal PLFA contents at N3 and N0 (Fig. S4), which reduced the relative abundance of other fungal taxa or even led to the disappearance of some fungal groups. With increasing N addition, the richness of the bacterial community (Chao1 and ACE) tended to increase. In addition, bacterial PLFA contents increased along N addition gradients (Fig. S4). This increase in bacterial communities may be related to the improvement of poor soil conditions (Wang et al., 2004),

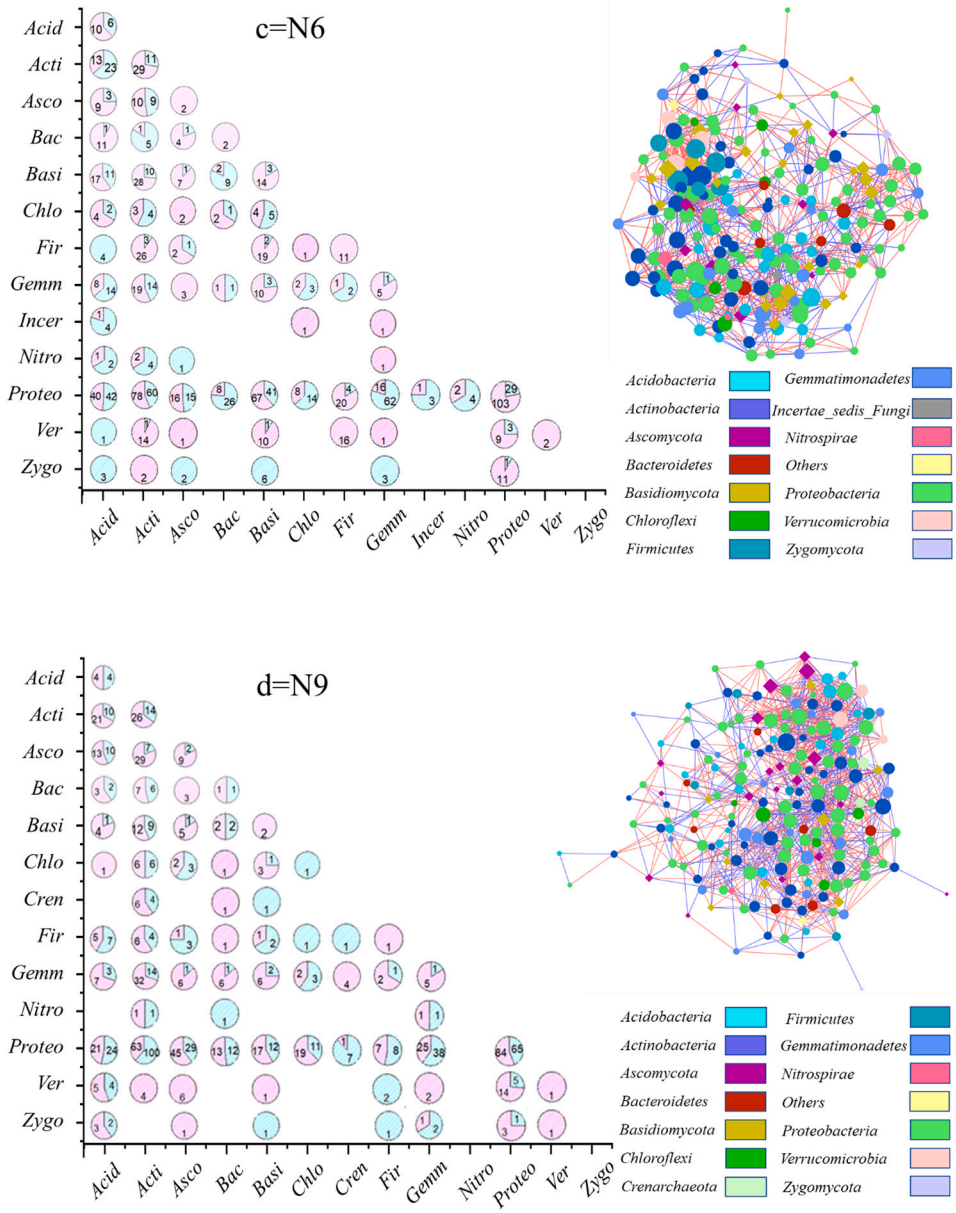


Fig. 4. (*continued*).

Table 2
Mantel test results for the correlation between microbial community composition and soil variables, soil microbial biomass, and eco-enzymatic properties based on bacterial and fungal phyla under N addition.

Parameters	Microbial community		Parameters	Microbial community		Parameters	Microbial community	
	r	*P*		*r*	*P*		*r*	*P*
SOC	0.195	0.068	SMBC	**0.385**	**0.001**	BG	0.180	0.154
TN	−0.042	0.553	SMBN	0.070	0.177	NAG	−0.121	0.681
TP	−0.096	0.678	SMBP	**0.377**	**0.003**	AP	−0.045	0.496
SC:N	**0.393**	**0.040**	SMC:N	−0.248	0.988	BG:NAG	0.336	0.073
SC:P	**0.365**	**0.003**	SMC:P	**0.338**	**0.002**	BG:AP	0.348	0.059
TN:P	−0.105	0.688	SMN:P	−0.085	0.763	NAG:AP	−0.143	0.761
aP	−0.084	0.760	NO_3^--N	−0.015	0.441	CUE:NUE	−0.009	0.465
NH_4^+-N	0.005	0.425	pH	−0.114	0.740			

Note: SC:N represents SOC:TN ratio; SC:P represents SOC:TP ratio; TN:P represents TN:TP ratio; SMBC, N, and P represent soil microbial biomass C, N, and P; SMC:N represents SMBC:SMBN ratio; SMC:P represents SMBC:SMBP ratio; SMN:P represents SMBN:SMBP ratio; aP represents available phosphorus; BG represents β-1,4-glucosidase; NAG represents β-1,4-*N*-acetylglucosaminidase; AP represents alkaline phosphatase; BG:NAG represents ratio of activities of β-1,4-glucosidase to β-1,4-*N*-acetylglucosaminidase; BG:AP represents ratio of activities of β-1,4-glucosidase to alkaline phosphatase; NAG:AP represents ratio of β-1,4-*N*-acetylglucosaminidase to alkaline phosphatase; CUE:NUE represents ratio of microbial C-use efficiency (CUE) to microbial N-use efficiency (NUE). Bold numbers indicate significant correlation ($P < 0.05$).

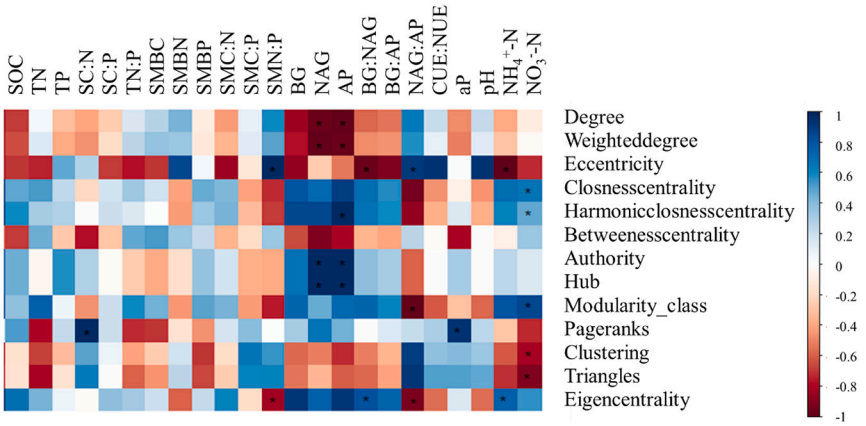


Fig. 5. Correlation between network topological features and soil factors, microbial biomass, eco-enzymatic activities, and eco-enzymatic stoichiometries. SC:N represents SOC:TN ratio; SC:P represents SOC:TP ratio; TN:P represents TN:TP ratio; SMBC:N represents SMBC:SMBN ratio; SMBC:P represents SMBC:SMBP ratio; SMBN:P represents SMBN:SMBP ratio; BG:NAG represents ratio of activities of β-1,4-glucosidase to β-1,4-*N*-acetylglucosaminidase; BG:AP represents ratio of activities of β-1,4-glucosidase to alkaline phosphatase; NAG:AP represents ratio of β-1,4-*N*-acetylglucosaminidase to alkaline phosphatase; CUE:NUE represents ratio of microbial C-use efficiency (CUE) to microbial N-use efficiency (NUE). Symbol "*" indicate significant correlation ($P < 0.05$).

which was found by a previous study of microbes which were in an N-limited status (Zhang et al., 2019). N addition increased soil nutrients and alleviated N limitation to some degree for microbes, thus increasing the bacterial richness indices.

Studies have illustrated that N addition not only influences the microbial diversity (Zeng et al., 2016; Wang et al., 2018), but also induces changes in the microbial community composition (Leff et al., 2015). Despite being the dominant bacterial phylum in our study, *Proteobacteria* did not show significant differences among the N addition treatments. The changes of *Proteobacteria*, regulating several steps in the denitrification process (Shapleigh, 2011), did not show the similar tendencies from various studies with N addition. For example, Li et al. (2016) found N addition reduced relative abundance of *Proteobacteria*, while Liu et al. (2016) noticed N addition promoted the relative abundance of *Proteobacteria*. In addition, microbial taxa with different strategies for nutrient acquisition, such as *Acidobacteria* belonging to the oligotrophic taxa and *Firmicutes* belonging to the copiotrophic taxa, are also impacted by N addition. N addition significantly decreased the relative abundance of *Acidobacteria* in our study, which was consistent with the results of Liu et al. (2016) and Ramirez et al. (2012). The relative abundance of *Firmicutes* increased across the N addition gradients which was consistent with the results of Ramirez et al. (2010) and Bergmann et al. (2011). These results are supported by the oligotrophic hypothesis (Leff et al., 2015) that nutrient-rich conditions induced by N addition are beneficial for copiotrophs (*Firmicutes*), but not good for oligotrophic bacterial growth (*Acidobacteria*). Among the fungal communities, *Basidiomycota* was the dominant fungal phylum. In this study, the relative abundance of *Basidiomycota* was highest at N3, which was consistent with the results of Wang et al. (2019). This is because *Basidiomycota* is typically saprotrophic and very sensitive to decomposing organic matter (Curlevski et al., 2010), especially plant litter with a high lignin content such as forest (Blackwood et al., 2007). In this study, the relative abundance of *Basidiomycota* was positively correlated with SOC content and SC:N (Table S1, and Fig. S2). Therefore, relative abundance of *Basidiomycota* highest at N3 was due to the highest SOC content and SC:N ratios at N3.

Generally, the indicator OTUs represented the fungal community (*Basidiomycota*, *Ascomycota*, and *Zygomycota*) more than the bacterial community (*Proteobacteria*, *Actinobacteria*, *Gemmatimonadetes*, *Acidobacteria*, and *Firmicutes*) (Fig. 3), revealing that the fungal community composition was more influenced by N addition than the bacterial community composition, based on a fine taxonomic level. This may be related to differences in the ranges of microbial C:N between bacteria and fungi. That is, because fungal C:N ratios are higher than those of bacteria (Zhao et al., 2015), fungi are better able to take advantage of organic matter with higher C:N ratios, thus preferentially adapting to live in an environment with high soil C:N ratios. As revealed by Goldfarb et al. (2011), microbial abundances increase in preferred substance. Therefore, the reduction of soil C:N caused by N addition could influence fungal taxa on finer taxonomic levels.

4.2. Interactions among bacterial and fungal communities under N addition

Co-occurrence networks with bacterial and fungal OTUs were used to reveal the interactions among OTUs and the shifts in their interactions along N addition gradients. The interactions between different bacterial OTUs account for a high proportion of all interactions, which may be because bacterial OTUs were more abundant than fungal OTUs (Guo et al., 2018). In addition, the interactions among bacterial and fungal communities were generally dominated by positive correlations, indicating that, under N addition, the relationships maintained by these communities was mutualism. Hoek et al. (2016) found that microbial interactions tended to exhibit mutualism when soil nutrients were poor. This was supported by our previous results which stated that the microbes were in a N-limited status (Zhang et al., 2019), thus maintaining mutualism in this study. The percentage of between bacterial interactions was highest at N3, with interactions under the other three treatments exhibiting an increasing tendency along the N addition gradients. The interactions between bacterial and fungal OTUs were lowest at N3; however, interactions under the other three treatments showed a decreasing tendency along the N addition gradients. These changes in the interactions at N3 highly correlated with the increasing abundance of *Basidiomycota* which decreased the fungal diversity. In addition, this increasing pattern of interactions among bacterial communities and decreasing pattern of interactions between bacterial and fungal communities indicated a strengthening of bacterial-bacterial interactions and a weakening of bacterial-fungal interactions. This may be related to the increase in bacterial richness indices prior to reaching an N threshold level. Bacteria have a much shorter turnover; therefore, bacteria respond rapidly to changes in soil factors (Guo et al., 2018). In addition, fungi prefer substrates with higher C:N, and N addition decreases C:N, which inhibits fungal growth, therefore weakening bacterial-fungal interactions.

4.3. Microbial community composition and interactions mediating ecological stoichiometry under N addition

Microbial community structures can be altered to some degree by short-term N addition (Liu et al., 2016). According to a previous study, short-term N addition partially alleviated the N-limited status for microbes; however, the microbial community remained in homeostasis (Zhang et al., 2019). Different factors triggered changes in the microbial community composition, for example, pH (Rousk et al., 2010), NO_3^--N (Guo et al., 2018), and SOC (Li et al., 2017). In this study, the microbial composition was not typically influenced by SOC, whereas soil stoichiometry tended to impact microbial composition. This was supported by

Wan et al. (2015), who found that the soil C:N ratio is the major determinant of soil microbial community. In addition, the overall changes in microbial composition were not correlated with eco-enzymatic activities and eco-enzymatic stoichiometry. This is not supported by studies that found microbial community composition has a key impact on soil function (Stark et al., 2014; Chen et al., 2019). This may because of functional redundancy of the microbial community, with some studies finding that changes to microbial composition were not significantly correlated with eco-enzymatic activities (Azarbad et al., 2015; Banerjee et al., 2016). The ability to mediate microbial function in response to soil conditions is dependent on specific functional groups and not on the overall changes in the microbial community (Banerjee et al., 2016). This was partly confirmed by the fact that *Proteobacteria* was significantly correlated with β-1,4-glucosidase (BG) and β-1,4-*N*-acetylglucosaminidase (NAG) (Fig. S2), and the group identified by the predicted function for controlling chitin deposition was correlated with NAG (Fig. S5; Table S3). In addition, some microbial taxa were influenced by a greater number of soil factors and soil stoichiometry. For example, *Acidobacteria* was influenced not only by soil stoichiometry, SC:N, and SN:P but also by the soil-available nutrients aP, NH_4^+-N, and NO_3^--N. Therefore, overall changes in microbial community compositions could be impacted by soil stoichiometry. For specific microbial groups, these changes tend to mediate element cycles.

A correlation analysis was conducted between topological features of bacterial-fungal community co-occurrence with soil properties directly impacted by N addition to reveal the main factors influencing bacterial-fungal community interactions. In addition, another correlation analysis was conducted between topological features of bacterial-fungal community and eco-enzymatic stoichiometry. This was done to illustrate the mechanism of bacterial and fungal interactions that mediate the microbial metabolism in response to changes in soil conditions caused by N addition. The interactions tended to be influenced by soil available nutrients (NH_4^+-N and NO_3^--N), while the microbial community composition was driven by soil stoichiometry. This indicates that the interactions among bacterial-fungal communities are more sensitive than microbial community compositions to changes in soil nutrients. This is because the interactions among bacterial and fungal microbial communities are mainly resource driven (Banerjee et al., 2016). The interactions were correlated with eco-enzymatic activities and eco-enzymatic stoichiometry, indicating that they play a more important role in mediating specific microbial metabolism via eco-enzymatic properties than the entire microbial community. This finding is supported by Ma et al. (2016), who found that microbial interactions have a greater contribution to soil functions than species diversity.

5. Conclusion

The results of this study indicate that short-term N addition has a larger effect on the fungal community than on the bacterial community at a fine taxonomic level. Moreover, microbes maintain mutualism under all N treatments in order to gain the nutrients to meet their substrate requirements. Microbial community composition and interactions were resource driven by different factors. Microbial community composition was mainly impacted by soil stoichiometry, whereas microbial interactions were more sensitive than the overall microbial community composition to soil available nutrients. In addition, due to functional redundancy, changes in microbial community composition did not show significant relationships with eco-enzymatic activities and stoichiometry, even though some microbial taxa and interactions tend to mediate eco-enzymatic activities and stoichiometry. Our study contributes to a better understanding of the mechanisms by which the microbial community mediates nutrient cycling under short-term N addition.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2022.104422.

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