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Impact of 36 years of nitrogen fertilization on microbial community composition and soil carbon cycling-related enzyme activities in rhizospheres and bulk soils in northeast China

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ABSTRACT

Nitrogen (N) deposition can change ecosystem functions but little is known of long-term N-deposition and rhizosphere effects on the microbial community composition and enzymes activities related to the carbon (C) cycle in the black soil common to northeastern China. Here, we studied two enzyme activities involved in C cycles and microbial community composition in both the rhizosphere and bulk soil from a long-term (36-year) fertilization field experiment. N-addition significantly decreased bacterial abundance and phenol oxidase activity, but enhanced fungal abundance and peroxidase activity, in both the rhizosphere and bulk soil. The fungal diversity exhibited more obvious shifts than the bacterial diversity after long-term N-addition, resulting in significantly decreased bacterial and fungal diversity levels, except for bacterial diversity in the rhizosphere, which was not significantly changed. Moreover, the enzyme activities and the bacterial and fungal abundance levels were higher in the rhizosphere than in the bulk soil, suggesting a rhizosphere effect on microbial activities involved in the C cycle. There was a significant difference in the microbial community compositions among different N-addition levels. A lesser β-diversity response to N-addition was observed in the rhizosphere than in the bulk soil, and the responses of fungal communities were greater than those of bacterial communities. Our findings suggested that rhizosphere effects and fertilization regimes both have significant influences on microbial communities and soil enzyme activities, and that fungi were more sensitive than bacteria in responding to N-deposition.

1. Introduction

Human activities have increased nitrogen (N) availability in ecosystems (Vitousek et al., 1997; Galloway et al., 2008). Over the past 100 years, extensive agricultural intensification has inputted a large amount of N fertilizer into agricultural farmlands, and this trend is projected to reach 135 Tg N in 2050 (Galloway et al., 2008; Fowler and Sutton, 2013). This has resulted in the degradation of ecosystem structures and functions, including soil acidification, changes in nutrient availability and loss of biodiversity (Guo et al., 2010; Zhou et al., 2015). The elevated N bioavailability could also decrease lignin in leaf litter, alter soil carbon (C) turnover and increase the susceptibility of plants to secondary stresses (Phoenix et al., 2012). However, the effects of long-term and elevated N fertilization were assessed in experiments lacking plants (Fierer et al., 2009; 2012; Zhou et al., 2015; Zeng et al., 2016) and only bacterial or fungal diversity was monitored (Shrestha et al., 2010; Paungfoo-Lonhienne et al., 2015). Plant exudates can select and enrich special bacterial taxa, modify bacterial activities (Ai et al., 2013; 2015) and fungal communities in the rhizosphere (Paungfoo-Lonhienne et al., 2015), and this influence is known as the rhizosphere effect. Nevertheless, our understanding of the mechanisms underlying the rhizosphere's microbial feedback in response to global climate change and the mechanisms protecting plants from abiotic and biotic stresses are poorly understood.

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A high N fertilizer input influences the N ratio and the C substrates that directly stimulate heterotrophic microorganisms (Phoenix et al., 2012). Many soil microbes are directly related to soil biogeochemical processes and play prominent roles in soil C cycling (Bardgett et al., 2008). The N-deposition decreases the microbial activities in soil. For example, N fertilization decreases microbial respiration by 36–46% in the rhizospheric soils of three hardwood trees (Phillips and Fahey, 2007). N fertilizer can also change the metabolic activities of microbial communities in decomposing soil C pools (Mack et al., 2004; Ramirez et al., 2012). There are two hypotheses regarding the impacts of N fertilizer on the C cycle: i) microbes decompose soil organic matter at maximal rates when the ratio of C and N matches their demands, and increasing N availability decreases soil C storage (Melillo et al., 1982; Sterner and Elser, 2002; Mack et al., 2004); and ii) the "microbial nitrogen mining" hypothesis states that increases in N availability are likely to lead to a net decrease in soil organic materials' decomposition rates because soil microbes use labile C to decompose recalcitrant organic matter to facilitate N acquisition (Craine et al., 2007). In addition to changes in microbial activity, N fertilizer also alters the rhizosphere's microbial community composition. For instance, Paungfoo-Lonhienne et al. (2015) found that N fertilizer modifies the composition of fungal communities in the rhizosphere, with a potential negative impact on C cycling and an increase in the abundance levels of pathogens. However, Giagnoni et al. (2015) found that microbial composition and activities were both influenced by N forms and plant species, suggesting that the microbial responses to N fertilization are frequently mixed and lack consistency (Zeng et al., 2016).

The N enrichment significantly decreases soil pH (Guo et al., 2010), with effects on microbial and enzyme activity levels (Sinsabaugh, 2010). In soil, extracellular enzyme activities mediate the degradation, transformation and mineralization of soil organic matter and are considered to be good indicators of soil C decomposition (Sinsabaugh, 1994; Sinsabaugh et al., 2008). Phenol oxidase and peroxidase are produced by microbes for a variety of purposes, including ontogeny, defense and the acquisition of C and N (Sinsabaugh, 2010). Those two enzyme activities are decreased under fertilization (Carreiro et al., 2000; Waldrop et al., 2004), but phenol oxidase activities are both stimulated, or remained constant, in other studies (Allison et al., 2008; Sinsabaugh, 2010; Li et al., 2013a). However, the interaction between root exudates and long-term N fertilization influences on enzyme activities, and the relationship between enzyme activities and changed microorganism communities is still unknown.

At present, although the impacts of N fertilizer on the soil microbial community have been widely reported in recent years (Fierer et al., 2009; 2012; Zhou et al., 2015), limited information is available for addressing the rhizosphere microbiomes and their roles in decomposing soil organic matter and protecting plants from soil acidification and secondary stresses caused by N fertilizers. Long-term field experiments allow the study of fertilization effects. As a result, since 1980, we conducted a long-term fertilizer experiment in a black soil area with different doses of N fertilizer in Harbin City in northeastern China. The aims of this study were (i) to investigate responses of soil bacterial and fungal community composition and diversity under different levels of N fertilization, and (ii) to assess the relationships between microbial diversity, microbial activity, dominant groups and properties of bulk and rhizospheric soils. To achieve our goals, we collected and compared bulk and rhizosphere soil samples to identify alterations in microbial activities and diversity levels between the two soil compartments. High-throughput sequencing and quantitative PCR were used to characterize the bacterial and fungal communities quantitatively and qualitatively. It was hypothesized that the microbial activity levels and composition would vary along an N-addition gradient in the two compartments and that the bacterial and fungal communities' respond differently to N-addition.

2. Material and methods

2.1. Experimental design, soil sampling and soil property determinations

The experimental site and soils have been described previously (Wei et al., 2008). Briefly, the experimental field, started in 1980, is in Harbin City, Heilongjiang Province, P. R. China (45°40′N, 126°35′E). Soils were collected from three treatments with three replicates: (i) no fertilization (CK), (ii) 150 kg urea ha^{-1} y^{-1} (N_1) and (iii) 300 kg urea ha^{-1} y^{-1} (N_2). The bulk and rhizosphere soil samples were collected in July 2015, when the rhizosphere effects were considered to be most pronounced (Cheng et al., 2003). For rhizospheric soil, 10 maize plants with their roots were extracted from the middle of each plot (to avoid border effects), and the loosely adhering soil was shaken off. The remaining adhering soil was carefully collected and mixed thoroughly as a single rhizosphere sample. For the bulk soil, 10 soil cores (5 cm in diameter) from the plough layer (0–20 cm) adjacent to the plants were collected from each plot, and mixed thoroughly as a single bulk soil sample. Thus, six samples of each treatment were collected, and a total of 18 soil samples (3 × 6) were taken. The fresh samples were transported to the laboratory on ice. The samples were sieved through a 2.0-mm sieve and stored at room temperature and −80 °C for chemical and molecular analyses, respectively. Soil pH was determined with a glass combination electrode using a 1:1 soil to water ratio (Li et al., 2013b). Soil total C (TC) and total N (TN) were determined using a CN analyzer (Vario Max CN, Elementar, Hanau, Germany). Soil organic material (SOM) was determined according to Strickland and Sollins (1987). Nitrate N (NO_3^--N) and ammonium (NH_4^+-N) were determined by extracting the soil with 2 M KCl and then their concentrations were determined using a flow injection auto-analyzer (FLA star 5000 Analyzer, Foss, Denmark). Available potassium (AK) and available phosphorus (AP) were measured according to Olsen (1954) and Helmke et al. (1996), respectively. Phenol oxidase and peroxidase activities were determined according to Saiya-Cork et al. (2002).

2.2. DNA extraction and quantitative PCR

Soil genomic DNA was extracted from 0.25 g of bulk and rhizosphere soil samples using a Power Soil DNA Isolation Kit (MOBIO, Carlsbad, CA, USA) according to the manufacturer's instructions. Six successive extractions of microbial DNA from replicate soil samples were combined to minimize the DNA extraction bias (Ding et al., 2016). The quantitative PCR (qPCR) method was used to measure the abundance of the soil bacteria and fungi with the primer sets 515F-806R (Peiffer et al., 2013) and ITS4-ITS5 (Schoch et al., 2012), respectively. The sequences of primers are detailed in Table S1. The 16S rRNA and internal transcribed spacer (ITS) gene quantities were measured by preparing plasmid DNA from the clone with the correct insert using a Miniprep kit (Qiagen, Germantown, MD, USA). The R^2 of the standard curve was > 0.99. The reactions were carried out in triplicate with DNA extracted from each soil sample using an ABI Real-Time 7500 system (Applied Biosystems, Waltham, MA, USA) and SYBR Green detection (FastFire qPCR PreMix, TIANGEN BIOTECH, Beijing, China). The bacterial-to-fungal (B/F) ratio was calculated using the 16S rRNA and ITS gene copy numbers (Wurzbacher et al., 2014).

2.3. PCR amplification and high-throughput sequencing

The DNA was purified using a DNeasy Tissue kit (Qiagen, Valencia, CA, USA) and checked on a 1.0% agarose gel. The V3–V4 hypervariable region of 16S rRNA gene was amplified using the primers 515F and 806R as recommended by Peiffer et al. (2013). Partial ITS amplicons were produced using the primer set ITS1F -ITS2 (Ghannoum et al., 2010; Sun et al., 2016), which is the universal DNA barcode marker for the molecular identification of fungi (Schoch et al., 2012; Blaalid et al.,

2013). For 16S rRNA, gene amplifications conditions were as follows: 2 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 50 °C and 1 min at 72 °C, followed by a final elongation step of 6 min at 72 °C. Amplification of the ITS gene was identical, except there were 35 cycles and 30 s of elongation rather than 1 min at the end of each cycle (Sun et al., 2016). Amplicons were gel purified using the PureLink® Quick Gel Extraction Kit (Invitrogen, Carlsbad, CA, USA). The products were pooled in equimolar concentrations using the quantitative DNA-binding method for bacteria and fungi, separated and then sequenced on the Illumina HiSeq PE250 platform. Sequence data were deposited in the NCBI Sequence Read Archive under the accession number: SRX2992452.

2.4. Sequence classification and operational taxonomic unit (OTU) analysis

The bacterial 16S rRNA and fungal ITS sequences were analyzed using QIIME (Caporaso et al., 2010). The reads were assigned to samples according to their unique barcodes (Table S2), and were quality-filtered using the fastq_filter command (split_libraries_fastq.py) according to the following criteria: (i) the reads were trimmed with a threshold average quality score ≤19 over a 3-bp window size: (ii) only reads with a ≥10-bp overlap and less than 0.1 mismatches were combined, and sequences that could not be assembled were removed (Magoč and Salzberg, 2011). The combined sequences were checked by UCHIME (Edgar et al., 2011) to remove potential chimeric sequences using Silva and Unite databases for 16S rRNA and ITS gene sequences, respectively. OTU clustering was carried out using the cluster_otus command in the UPARSE pipeline at a 97% similarity level (Edgar, 2013). The RDP classifier (Caporaso et al., 2011) was used to select representative sequences for each OTU and assign taxonomic data from the Silva database for 16S rRNA genes (Quast et al., 2013) and UNITE + INSDC fungal ITS database (Abarenkov et al., 2010) to each representative sequence at the 80% and 60% thresholds, respectively. Singletons, non-bacterial and non-fungal OTUs were removed, and the OTU abundance levels were normalized based on the sample with the least number of sequences. All subsequent analyses were performed according to the normalized data.

2.5. Statistical analyses

The α-diversity levels of the Shannon, ACE, Chao1 and Observed-species indices were calculated using QIIME software (Version 1.7.0). For the soil properties, the α-diversity levels and the relative abundances of bacterial or fungal communities were determined using a one-way ANOVA. A two-way ANOVA was used to compare the soil fractions (rhizosphere and bulk soil) and fertilizer treatments. The comparison of treatment means was achieved by Tukey's procedure using SPSS statistical software (Version 19.1, Chicago, IL, USA). Pearson's correlation analyses were performed to assess the relationships among soil properties, enzyme activities, and the soil microbial communities. To visualize the shift in the bacterial and fungal β-diversity, a principal coordinate analysis was used based on the UniFrac distance matrix. In all of the tests, a P-value < 0.05 was considered to be statistically significant.

3. Results

3.1. Effects of N-addition on soil properties and soil enzyme activities

N-addition increased soil TN, NO_3^--N, SOM and TC concentrations, while it decreased the soil pH in both the rhizospheres and the bulk soils (Table 1). Although the soil NH_4^+-N concentration was significantly increased by N-addition in the bulk soil, it was not significantly changed by N-addition in the rhizosphere. In addition, soil properties (AP, AK, TN, NO_3^--N and NH_4^+-N) of the rhizospheric soils were similar or, in some cases, significantly lower than those in the bulk soils. In contrast, the soil TC concentration was higher in the rhizosphere than in the bulk soil samples. Soil pH values in the rhizosphere were lower in the CK and N_1 treatments, but higher in the N_2 treatment, than those of bulk soils.

The rhizosphere and N-addition affected soil enzyme activities (Fig. 1; Table 2). Compared with CK, long-term N fertilization (N_1 and N_2) significantly decreased the phenol oxidase activity in both the rhizosphere and bulk soils (Fig. 1a). In the rhizosphere, N_2 decreased the phenol oxidase activity by 21%, while N_1 decreased it by 16% compared with CK. In contrast, peroxidase activities in the rhizosphere were significantly increased by the N_2 treatment, but in the bulk soil they were not significantly changed by N-addition (Fig. 1b). In addition, the two enzyme activities of the rhizospheric soil were slightly greater than those of the bulk soils, indicating a positive rhizosphere effect.

3.2. Effects of N-addition on soil microbial abundance

We used qPCR to determine the bacterial and fungal abundances. The number of 16S rRNA gene copies ranged from 2.1×10^9 to 4.0×10^9 (per g soil) (Fig. 2a). In the bulk soil, the bacterial abundance was reduced by N-addition, indicating its negative effect on the number of 16S rRNA gene copies. In contrast, N-addition did not have a significant ($P = 0.758$) effect on bacterial abundance in the rhizospheric soil. Additionally, a significantly positive correlation was observed between bacterial abundance and phenol oxidase activity ($R^2 = 0.751$, $P < 0.01$) (Table S3).

The number of ITS gene copies ranged from 0.88×10^5 to 5.09×10^5 (per g soil) and was higher in the rhizospheres than in the bulk soils (Fig. 2b). The rhizosphere, fertilization and their interaction had significant effects on fungal abundance (Table 2). Unlike bacteria, the fungal abundance was increased across N gradients in the bulk soil, with the greatest abundance being observed under N_2-treatment conditions; however, in the rhizospheric soil, the N_1 treatment had the greatest abundance of ITS gene copies. Additionally, the fungal abundance was positively correlated with peroxidase activity ($R^2 = 0.636$, $P < 0.01$) and C/N ($R^2 = 0.980$, $P < 0.01$).

The B/F ratios were greater in bulk than in rhizospheric soils (Fig. 2c), and decreased significantly as the N gradient increased (Fig. 2c). Additionally, significantly positive correlations were observed with soil pH ($R^2 = 0.487$, $P < 0.05$) and TN ($R^2 = 0.619$, $P < 0.01$) levels. However, the opposite trend was observed for TC ($R^2 = -0.660$, $P < 0.01$) and C/N ($R^2 = -0.721$, $P < 0.01$) values (Table S3).

3.3. Effects of N-addition on microbial α-diversity

In total, 901,056 bacterial and 1,123,834 fungal effective sequences were obtained. In one sample, there was between 35,570 and 62,862 bacterial sequences and between 37,233 and 63,671 fungal sequences. The Good's coverage values were in the range of 96.8%–97.9% and 99.2%–99.6% at the 97% similarity cut-off for bacterial and fungal communities, respectively, indicating that the current numbers of sequence reads were sufficient to capture the microbial diversity in all of the soil samples.

Long-term N fertilization distinctly impacted microbial α-diversity in rhizosphere and bulk soils (Table 3). Bacterial diversity (Shannon index) in bulk soil was decreased by N fertilization, but in the rhizosphere it was not significantly changed by N-addition. However, the rhizospheric bacterial diversity indices, including Observed species, ACE, Chao1 and Shannon, were all slightly higher in the N_1 treatment than in the other treatments, although the difference was not significant ($P > 0.05$) (Table 3). The fungal diversity index (Shannon), as well as richness indices (Chao1 and ACE), which were significantly lower in rhizospheres than in bulk soils, were decreased by N-addition both in the rhizosphere and in the bulk soil.

Table 1
Properties of maize rhizospheres and bulk soils after different long-term N-addition treatments.

Treatments	Soil fractions	pH (1:1water)	AP (mg kg^{-1})	AK (mg kg^{-1})	TC (%)	NH_4^+-N (mg kg^{-1})	NO_3^-N (mg kg^{-1})	TN (g kg^{-1})	SOM (g kg^{-1})
CK	Bulk soil	6.30 ± 0.13a	10.63 ± 2.03b	165.7 ± 13.0a	1.76 ± 0.04c	18.60 ± 0.12ab	2.26 ± 0.88bc	2.27 ± 0.01b	2.56 ± 0.04b
	Rhizosphere	6.28 ± 0.02a	10.04 ± 2.03b	143.1 ± 1.6ab	1.91 ± 0.09b	18.25 ± 0.05b	0.43 ± 0.05e	1.82 ± 0.02d	2.60 ± 0.004b
N_1	Bulk soil	5.51 ± 0.08b	10.04 ± 0.52b	165.2 ± 9.6a	1.95 ± 0.04b	18.63 ± 0.06ab	3.84 ± 0.73b	2.38 ± 0.02a	2.77 ± 0.03a
	Rhizosphere	5.41 ± 0.08b	9.54 ± 0.77b	128.5 ± 6.5ab	2.10 ± 0.05a	18.20 ± 0.02b	1.14 ± 0.71de	1.87 ± 0.05d	2.73 ± 0.03a
N_2	Bulk soil	4.67 ± 0.20c	19.58 ± 4.95a	156.6 ± 35.5ab	1.84 ± 0.02ab	19.06 ± 0.50a	13.15 ± 5.78a	2.42 ± 0.04a	2.75 ± 0.05a
	Rhizosphere	4.92 ± 0.06c	14.56 ± 3.27ab	119.5 ± 8.4b	1.96 ± 0.04b	18.15 ± 0.03b	1.53 ± 0.60 cd	2.01 ± 0.12c	2.76 ± 0.07a

Soil properties were calculated for each replicate of fertilizer treatment and bulk soil/rhizosphere samples (n = 3). Data are the means ± standard deviations. Different letters indicate significant differences among fertilizer treatments. Soil factors indicated included Available phosphorus (AP), Available potassium (AK), Total carbon (TC), Total nitrogen (TN), Organic matter (OM) and TC:TN (C:N). Fertilizer regimes: no fertilization (CK), 150 kg urea ha^{-1} y^{-1} (N_1) and (300 kg urea ha^{-1} y^{-1} (N_2).

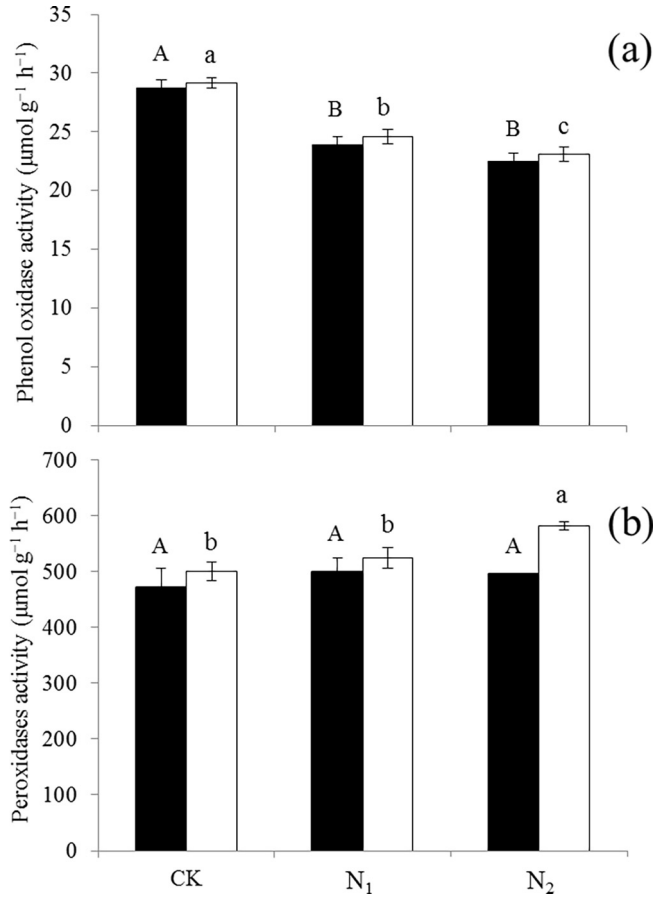

Fig. 1. Effects of different N-addition treatments on (a) phenol oxidase activity and (b) peroxidase activities in the bulk soils (black bars) and rhizospheres (white bars). Vertical bars represent the standard deviations (n = 3). Different capital letters indicate significant differences among fertilizer treatments in the bulk soils, while different lower-case letters indicate significant differences among fertilizer treatments in the rhizospheres soils at $P < 0.05$ (Tukey's test).

3.4. Effects of N-addition on microbial community composition

The relative abundances of different phyla and classes (bacterial and fungal) in rhizospheres and bulk soils are shown in Fig. 3, and the different abundances of detected bacterial and fungal families are shown in Table 4 and Tables S4 and S5. The bacterial phyla of Proteobacteria, Acidobacteria and Actinobacteria occupied 78.3% and 79.7% of the total sequences in the bulk soil and rhizospheric soil, respectively, followed by Gemmatimonadetes (6.45% bulk soil, 4.74% rhizosphere), Verrucomicrobia (3.43% bulk soil, 3.74% rhizosphere), Bacteroidetes (2.58% bulk soil, 3.62% rhizosphere), Firmicutes (1.67% bulk soil, 1.07% rhizosphere), Nitrospirae (1.55% bulk soil, 1.21% rhizosphere) and Planctomycetes (1.17% bulk soil, 1.30% rhizosphere) (Fig. 3a). For the fungi, all of the samples were dominated by phylum Ascomycota, occupying 67.3% and 87.5% of the bulk soil and rhizospheric soil, respectively. Basidiomycota (14.7% bulk soil, 5.8% rhizosphere), Zygomycota (10.9% bulk soil, 4.7% rhizosphere), Chytridiomycota (2.6% bulk soil, 0.3% rhizosphere), Glomeromycota (0.8% bulk soil, 0.2% rhizosphere) and Cercozoa (0.2% bulk soil, 0.1% rhizosphere) were also present in both soils (Fig. 3b).

Here, we only focused on bacterial and fungal families that differed significantly among different N-additions in the rhizosphere and bulk soils. At the family level (relative abundance > 0.5% in at least one sample), both the rhizosphere and bulk soil samples contained 11 bacterial families that were differentially abundant between the different levels of N-addition (Table 4). For example, Oxalobacteraceae and Comamonadaceae, belonging to phylum Proteobacteria, were significantly more abundant in the control bulk soil and rhizosphere. In contrast, Koribacteraceae and Gaiellaceae, which belong to the phyla Acidobacreria and Actinobacteria, respectively, were significantly more abundant in N-addition treatments than in the control bulk soil and rhizosphere (Table S4). For the fungi (relative abundance > 0.1% in at least one sample), there were 5 and 17 fungal families that were differentially abundant between different levels of N-addition in the bulk soil and rhizosphere, respectively (Table 4). For example, Trichocomaceae was significantly more abundant in the N-addition samples than in the control bulk soil and rhizosphere, while Chaetomiaceae was only more abundant in the rhizospheres of N-addition samples. In contrast, Lasiosphaeriaceae was more abundant in the control than in the bulk soils and rhizospheres of N-addition samples (Table S5).

3.5. OTU-level microbial β-diversity analysis

The variations in microbial communities caused by soil fractions and fertilization were analyzed using a principal coordinate analysis based on UniFrac metrics. The results are presented in Fig. 4, indicating a clear shift of bacterial and fungal community compositions among different fertilization and soil fractions. The microbial community compositions in rhizosphere and bulk soils were generally distributed along PC1, while the communities under different N-addition conditions were distributed along PC2 (Fig. 4a and b). Furthermore, bacterial UniFrac distances (Fig. 4c and d) between different N-additions were generally lower than those of fungi (Fig. 4e and f) both in rhizospheres and bulk soils. Fungal and bacterial community UniFrac distances in the rhizosphere were shorter than in the bulk soil (Fig. 4).

4. Discussion

4.1. The abundances of 16S rRNA and the ITS gene

N fertilization strongly decreased the bacterial 16S rRNA gene abundance but increased the fungal ITS gene abundance in the bulk soil, which corroborated other results (Treseder, 2008; Zhou et al.,

Table 2
Two-way ANOVA of soil biological properties in two soil fractions (rhizosphere and bulk soil) and three different N-addition treatments with three replicates each (n = 18).

	Soil fraction (rhizosphere or bulk soil)		Fertilizer treatments		Soil fraction × fertilizer treatments	
	F	*P*	*F*	*P*	*F*	*P*
Bacterial population	2.878	0.116	**30.543**	**< 0.001**	**12.807**	**0.001**
Fungi population	**1504.642**	**< 0.001**	**80.789**	**< 0.001**	**52.337**	**< 0.001**
Bacteria-to-fungi radio	**668.603**	**< 0.001**	**135.173**	**< 0.001**	**78.518**	**< 0.001**
Peroxidase activity	**23.599**	**< 0.001**	**10.169**	**0.003**	**4.304**	**0.039**
Phenol oxidase activity	3.976	0.069	**154.876**	**< 0.001**	0.051	0.951

Bold values are significant at $P < 0.05$ (Tukey's test).

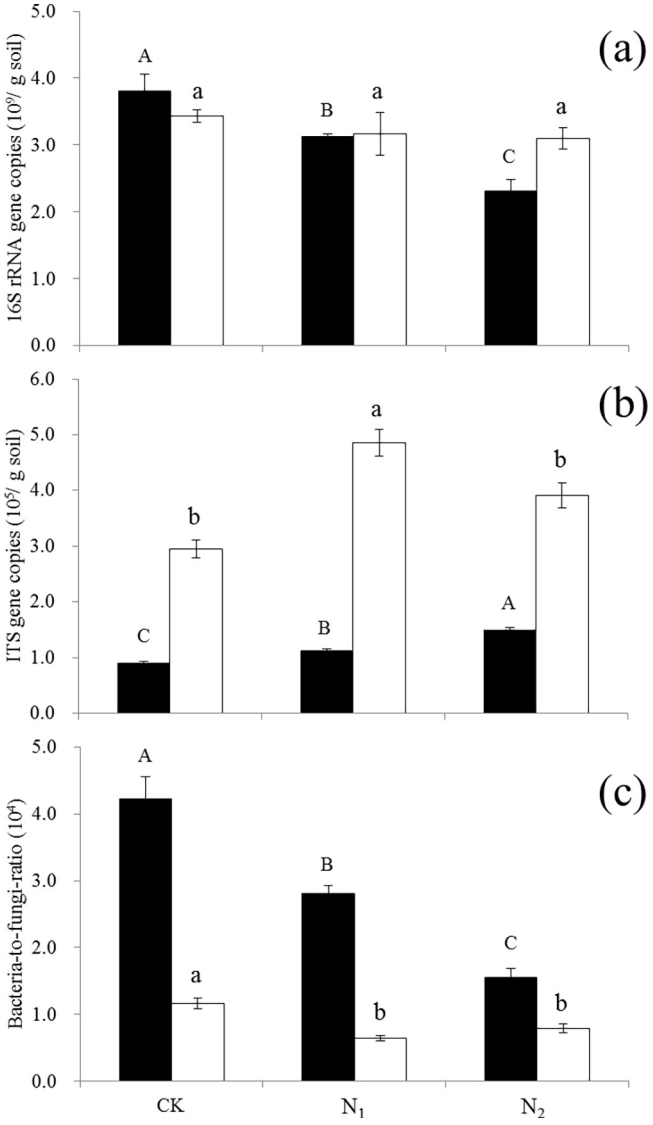

Fig. 2. Effects of different N-addition treatments on bulk soils (black bars) and rhizospheres (white bars) based on (a) 16S rRNA gene copy numbers, (b) ITS gene copy numbers and (c) bacteria-to-fungi ratios. Vertical bars represent the standard deviations (n = 3). Different capital letters indicate significant differences among fertilizer treatments in the bulk soils, while different lower-case letters indicate significant differences among fertilizer treatments in the rhizospheres soils at $P < 0.05$ (Tukey's test).

2015; Zhou et al., 2016). However, in the rhizosphere, N fertilization had no significant effect on bacterial 16S rRNA gene abundance (Fig. 2a), which was in agreement with Turner and Newman (1984). This may reflect changes in the quantity or quality of the resources in the rhizosphere, or the root and soil surfaces available for adhesion (niches). The relocation of C to roots and its release through both rhizodeposition and respiration increase with N-addition. For example, after a large N addition, relatively more ^{14}C is released by roots, as indicated by greater percentages of translocated ^{14}C found in root-soil respiration and soil residues (Liljeroth et al., 1990). However, Zhu et al. (2016) found that the total abundance levels of sugars, sugar alcohols and phenolics were positively correlated with the N rate. Those changes in the rhizosphere may stably maintain the bacterial abundance in the rhizosphere after long-term N addition. The fungal ITS' abundance, which was greater in the rhizosphere than in the bulk soil, was significant increased by N-addition to the bulk soil. However, in the rhizosphere, the fungal abundance was increased by the N_1 treatment but was not significantly affected by the N_2 treatment compared with the control (Fig. 2b), which was in good agreement with Denef et al. (2009). This can be explained by the fungi preferring the high C/N substrate to the low one (Zhou et al., 2016). Moreover, our results also confirmed that the fungal ITS' abundance was positively correlated to the C/N ratio ($R^2 = 0.908$, $P < 0.01$) in the soil (Table S3).

In our study, N-addition had negative effects on the B/F ratios in both the rhizosphere and bulk soils (Fig. 2c). This was consistent with a previous study of Zhou et al. (2016). In the rhizosphere, the decreasing B/F ratio was mainly caused by an increase in the fungal abundance because the bacterial abundance remained approximately constant as the N fertilization dose increased (Fig. 2). As previously documented, the B/F ratio tends to be higher at a lower C/N level (Frey et al., 1999; Fierer et al., 2009) or a higher pH (Bååth and Anderson, 2003). Similar results were found in our study, namely the B/F ratio was also significantly positively correlated with the soil pH and significantly negatively correlated with the C/N ratio (Table S3).

It is frequently hypothesized that a lower B/F ratio is indicative of a more sustainable agricultural system (De Vries et al., 2006). In most cases, fungi dominate under no-tillage, whereas bacteria dominate under conventional tillage (Beare et al., 1992). In our study, long-term N fertilization decreased the B/F ratio, indicating that the soil microbial composition was directly or indirectly affected by high N-addition inputs (Dean et al., 2014; Zhou et al., 2015; Zeng et al., 2016). We found that the B/F ratio was positively correlated with soil TN ($R^2 = 0.619$, $P < 0.01$), and fungal abundance was negatively correlated with soil mineral N (NH_4^+) ($R^2 = -0.684$, $P < 0.01$), which suggests that fungi-dominated niches led to a faster mineralization of N, as well as a slightly lower microbial retention of mineral N (NH_4^+) (Rousk and Frey, 2015; Zhou et al., 2016).

4.2. Bacterial and fungal α-diversities

The α-diversities of bacteria and fungi are significantly reduced following long-term consecutive years of N fertilization in bulk soils (Paungfoo-Lonhienne et al., 2015; Zhou et al., 2015; Zhou et al., 2016). Consistently, the Shannon diversity estimates of bacteria and fungi in the bulk soil were both significantly decreased by 36 years of N-addition (Table 3). However, in the rhizosphere, of the Shannon, Observed

Table 3
Estimators of bacterial and fungal diversity and richness in two soil fractions under different N-addition treatments.

Fertilizer treatments	Soil fraction	Bacteria				Fungi			
		Observed species	Diversity[1]	Richness[2]		Observed species	Diversity	Richness	
			Shannon	Chao1	ACE		Shannon	Chao1	ACE
CK	Bulk soil	2811 ± 230a[3]	9.28 ± 0.12a	3753 ± 693a	3771 ± 638a	1136 ± 43a	7.30 ± 0.16a	1351 ± 136a	1348 ± 112a
	Rhizosphere	2497 ± 73a	9.03 ± 0.03ab	2907 ± 127a	3010 ± 159a	761 ± 91bc	4.61 ± 0.25 cd	934 ± 168b	976 ± 162b
N_1	Bulk soil	2732 ± 39a	8.99 ± 0.12ab	3699 ± 43a	3752 ± 72a	942 ± 85ab	6.11 ± 0.10ab	1076 ± 183ab	1124 ± 163ab
	Rhizosphere	2826 ± 157a	9.23 ± 0.07ab	3686 ± 504a	3805 ± 491a	635 ± 34 cd	4.50 ± 0.28 cd	804 ± 69bc	828 ± 63bc
N_2	Bulk soil	2740 ± 278a	8.89 ± 0.20b	3732 ± 553a	3788 ± 613a	767 ± 119bc	4.92 ± 0.92bc	1006 ± 177ab	1023 ± 179ab
	Rhizosphere	2777 ± 1 53a	8.96 ± 0.17ab	3716 ± 541a	3865 ± 516a	452 ± 74d	3.50 ± 0.36d	564 ± 101c	572 ± 95c
Two-way ANOVA[4]	Fertilizer	F = 0.866	**F = 5.204**	F = 1.277	F = 1.533	**F = 26.885**	**F = 24.544**	**F = 9.149**	**F = 10.822**
		P = 0.445	***P* = 0.024**	P = 0.314	P = 0.251	***P* < 0.001**	***P* < 0.001**	***P* = 0.004**	**P = 0.002**
	Soil fraction	F = 0.540	F = 0.092	F = 1.696	F = 0.905	**F = 77.361**	**F = 85.868**	**F = 30.295**	**F = 34.087**
		P = 0.477	P = 0.767	P = 0.217	P = 0.360	***P* < 0.001**	***P* < 0.001**	***P* < 0.001**	**P < 0.001**
	Interaction	F = 2.356	**F = 5.157**	F = 1.534	F = 1.552	F = 0.328	F = 3.716	F = 0.598	F = 0.491
		P = 0.137	***P* = 0.024**	P = 0.255	P = 0.251	P = 0.727	P = 0.055	P = 0.566	P = 0.624

Results from a two-way ANOVA analysis are presented as *F*- and *P*-values [Bold values are significant at $P < 0.05$ (Tukey's test)].
[1] Base on Shannon diversity index.
[2] Base on Chao1 and abundance-based coverage estimator (ACE) richness indices.
[3] Data are the means ± standard deviations and different letters indicate significant differences among different soils.

species, ACE and Chao1 estimates showed that the bacterial α-diversity was not significantly changed, while the fungal α-diversity was significantly reduced ($P < 0.05$) by the 36 years of N-addition. Thus, the N fertilizer affected the taxonomic diversity of bacterial and fungal communities differently in the wheat rhizosphere. For the rhizospheric bacterial community, all of the α-diversity indices (including Observed species, ACE, Chao1 and Shannon) of the N_1 treatments were slightly higher for other treatments. This could result from a decrease in the dominant bacteria (Fig. 3a). Many rare species that are below the detection limit of a molecular assessment can become detectable when dominant species are reduced in their relative abundance. Alternatively, rare microbial species that are insensitive to the disturbance can find new available niches after disturbances and increase in relative abundance, providing an-additional explanation for the slight increase in α-diversity after the N-fertilization disturbance. However, in the rhizosphere, the fungal diversity levels under N-addition treatments were lower than that of the control, and this was consistent with Paungfoo-Lonhienne et al. (2015). The decrease in fungal α-diversity in the rhizosphere after an N fertilization disturbance might be a consequence of an increase in the abundance of dominant fungal groups

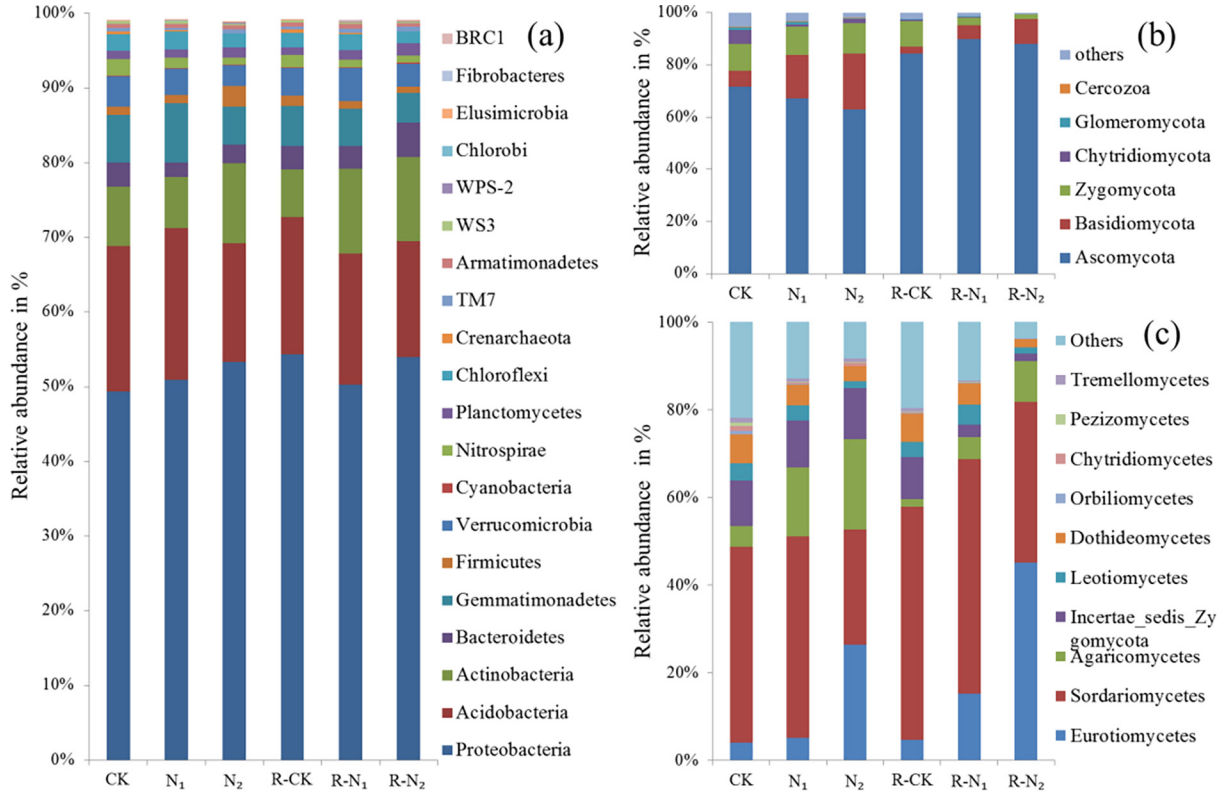

Fig. 3. Community composition of major (a) bacterial; (b) fungal phyla; and (c) classes of fungi across soils from different fertilizer regimes and soil fractions. CK, N_1, and N_2 indicate different N treatments in the bulk soil; R-CK, R-N_1, and R-N_2 indicate different N treatments in the rhizosphere. Values are the means of three replicates from each sample. Operational taxonomic units were clustered at the 97% similarity level.

Table 4
Effects of N-addition on bacterial (relative abundance > 0.5%) and fungal (relative abundance > 0.1%) communities in bulk soils and rhizospheres.

Bacterial families	Bulk soil		Rhizosphere		Fungal families	Bulk soil		Rhizosphere	
	F	*P*	*F*	*P*		*F*	*P*	*F*	*P*
Oxalobacteraceae	**7.05**	**0.027**	1.78	0.247	Trichocomaceae	**6.43**	**0.032**	**10.72**	**0.010**
Sphingomonadaceae	4.41	0.066	**5.87**	**0.039**	Mortierellaceae	0.10	0.908	**10.54**	**0.011**
Comamonadaceae	**21.28**	**0.002**	**91.62**	**< 0.001**	Chaetomiaceae	3.44	0.101	**5.88**	**0.039**
Moraxellaceae	0.08	0.927	**8.94**	**0.016**	Xylariaceae	1.86	0.235	**6.50**	**0.031**
Koribacteraceae	**68.72**	**< 0.001**	**9.44**	**0.014**	Lasiosphaeriaceae	**10.95**	**0.010**	**61.86**	**< 0.001**
Gaiellaceae	**12.58**	**0.007**	**11.09**	**0.010**	Microascaceae	3.84	0.084	**42.08**	**< 0.001**
Streptomycetaceae	2.51	0.161	**8.62**	**0.017**	Incertae_sedis_Hypocreales	**6.45**	**0.032**	**7.77**	**0.022**
Chitinophagaceae	**18.88**	**0.003**	0.05	0.952	Pleosporaceae	2.06	0.208	**8.87**	**0.016**
Bacillaceae	4.40	0.067	**21.33**	**0.002**	Ceratostomataceae	**10.90**	**0.010**	5.15	0.050
Solibacteraceae	**18.61**	**0.003**	4.26	0.071	Incertae_sedis_Leotiomycetes	2.20	0.193	**8.92**	**0.016**
Acidobacteriaceae	**7.40**	**0.024**	3.35	0.088	Magnaporthaceae	4.30	0.069	**13.83**	**0.006**
Ellin5301	**6.93**	**0.028**	4.94	0.054	Herpotrichiellaceae	0.18	0.179	**183.78**	**< 0.001**
Nitrospiraceae	**9.90**	**0.013**	**22.07**	**0.002**	Incertae_sedis_Xylariales	4.38	0.067	**67.75**	**< 0.001**
Bradyrhizobiaceae	2.35	0.176	**10.01**	**0.012**	Bionectriaceae	1.10	0.391	**6.52**	**0.031**
Ellin6075	**11.95**	**0.008**	2.56	0.157	Cystofilobasidiaceae	0.33	0.729	**5.96**	**0.037**
Syntrophobacteraceae	**6.16**	**0.035**	1.77	0.248	Chaetosphaeriaceae	2.79	0.139	**10.89**	**0.010**
Pseudomonadaceae	0.45	0.655	**10.58**	**0.011**	Glomeraceae	**9.33**	**0.014**	**6.99**	**0.027**
Nocardioidaceae	0.84	0.478	**27.06**	**0.001**	Incertae_sedis_Ascomycota	0.53	0.614	**16.88**	**0.003**

Bold values are significant at $P < 0.05$.

(Fig. 3b and c) and of some fungal species being more sensitive to N fertilization (Table 3).

4.3. Microbial community composition

Compared with bacteria, fungi showed a greater response to N fertilization. For example, the UniFrac distances in fungal communities between different doses of N fertilizer were greater than in the bacterial communities (Fig. 4). Given the limited resource availability for microbial growth in soils, the greater response observed in soil fungi was in agreement with evidence that fungi serve as the degraders of lignin and cellulose in plant residues and the soil OM in soils (Schneider et al., 2012) and have a relatively higher sensitivity to changes in soil OM (Cline and Zak, 2015). Although few studies have compared microbial community changes in both soil fungi and bacteria in respond to N fertilization, fungi appear to respond to changes associated with soil nutrient status (Lauber et al., 2008). In our study, the most abundant fungal phyla, Ascomycota, which are the key decomposers in agricultural soils, clearly increased in the rhizosphere, and they flourished in response to straw additions to soil (Ma et al., 2013). The results indicated that members of the Ascomycota are particularly vulnerable to high nutrient levels. For example, the abundance levels of Eurotiomycetes and Sordariomycetes were greater in rhizospheres than in bulk soils, and were favored in the high urea treatments in this study (Fig. 3). The shifts in their abundance levels may in turn affect the soil C decomposition (Xiong et al., 2014). However, bacteria appear to be more sensitive to soil pH (Table S3) (Lauber et al., 2008; Zhou et al., 2015). For example, the relative abundances of Acidobacteria and Actinobacteria were significantly ($R^2 = 0.561$ and -0.688, respectively; both $P < 0.05$) correlated with soil pH in this study, as found previously (Zhou et al., 2015). Moreover, in the rhizosphere, fungal and bacterial community responses to N fertilization were less than in the bulk soil (Fig. 4). This is not surprising because plant roots could stabilize the rhizosphere by buffering soil acidity, adjusting soil nutrient levels and changing the microbial abundance and composition (Tables 1 and 2, Fig. 3) (Bais et al., 2006; Peiffer et al., 2013). In our study, we found that the ranges for soil pH and for some soil nutrients were smaller than in the bulk soil than in the rhizosphere. For example, the soil pH in bulk soil ranged from 6.30 to 4.67, while in the rhizosphere, the soil pH ranged from 6.28 to 4.92 (Table 1).

4.4. Enzyme activities

In our study, the activity of the phenol oxidase was significantly reduced by N fertilization (Fig. 1a), which was in agreement with other studies (Allison et al., 2008; Jian et al., 2016). Phenol oxidase is a nonspecific enzyme that breaks down recalcitrant polymers, such as lignin and humic acids (Allison et al., 2008). The activity of this microbial source enzyme (Phillips et al., 2011) was positively correlated with soil bacterial abundance (Table S3). This may be because some of soil microbial groups could produce this enzyme and exert its function to break down SOM, resulting in the acquisition of C and N (Sinsabaugh, 2010). In addition, if mineral N is abundant, then microbes should reduce their allocation to enzymes that acquire complex N (Sinsabaugh and Moorhead, 1994). In the rhizosphere, the mineral N (NH_4^+-N and NO_3^--N) concentration was lower and the phenol oxidase activity was higher than those in the bulk soil (Table 1; Fig. 1). However, the peroxidase activity, which was higher in the rhizosphere than in the bulk soil, was significantly increased by N-addition (Fig. 1b). This result was inconsistent with previous conclusions that N-addition decreased peroxidase activity (Meier et al., 2015). Here, the increase in the peroxidase activity was mostly likely due to N-addition promoting microbial genera with known pathogenic traits (Zhou et al., 2016), and some microbial species secreted this enzyme to defend against a pathogen (Sinsabaugh, 2010). Thus, the peroxidase could not only be involved in biogeochemical cycling but could also act as an antimicrobial defense (Rabinovich et al., 2004; Sinsabaugh, 2010; Baldrian, 2014). Furthermore, the peroxidase activity was positively correlated with fungal abundance ($R^2 = 0.636$, $P < 0.01$) (Table S3), which was in good agreement with Allison et al. (2008).

4.5. Correlations between abundance groups and enzyme activities

Changes in microbial community composition could influence the enzymatic potential, although this phenomenon appeared to attenuate from community to functional levels (Patra et al., 2005). Fungi, the main group of soil microorganisms actively secreting oxidative enzymes (Keiblinger et al., 2012; Schneider et al., 2012; Baldrian, 2014), can decompose plant litter and release C to the soil (Purahong et al., 2016). This is consistent with the fungal abundance being significantly correlated with soil TC (Table S3). Phenol oxidase and peroxidase are involved in lignin degradation, carbon mineralization and defense (Sinsabaugh, 2010). Here, the phenol oxidase activity correlated with a

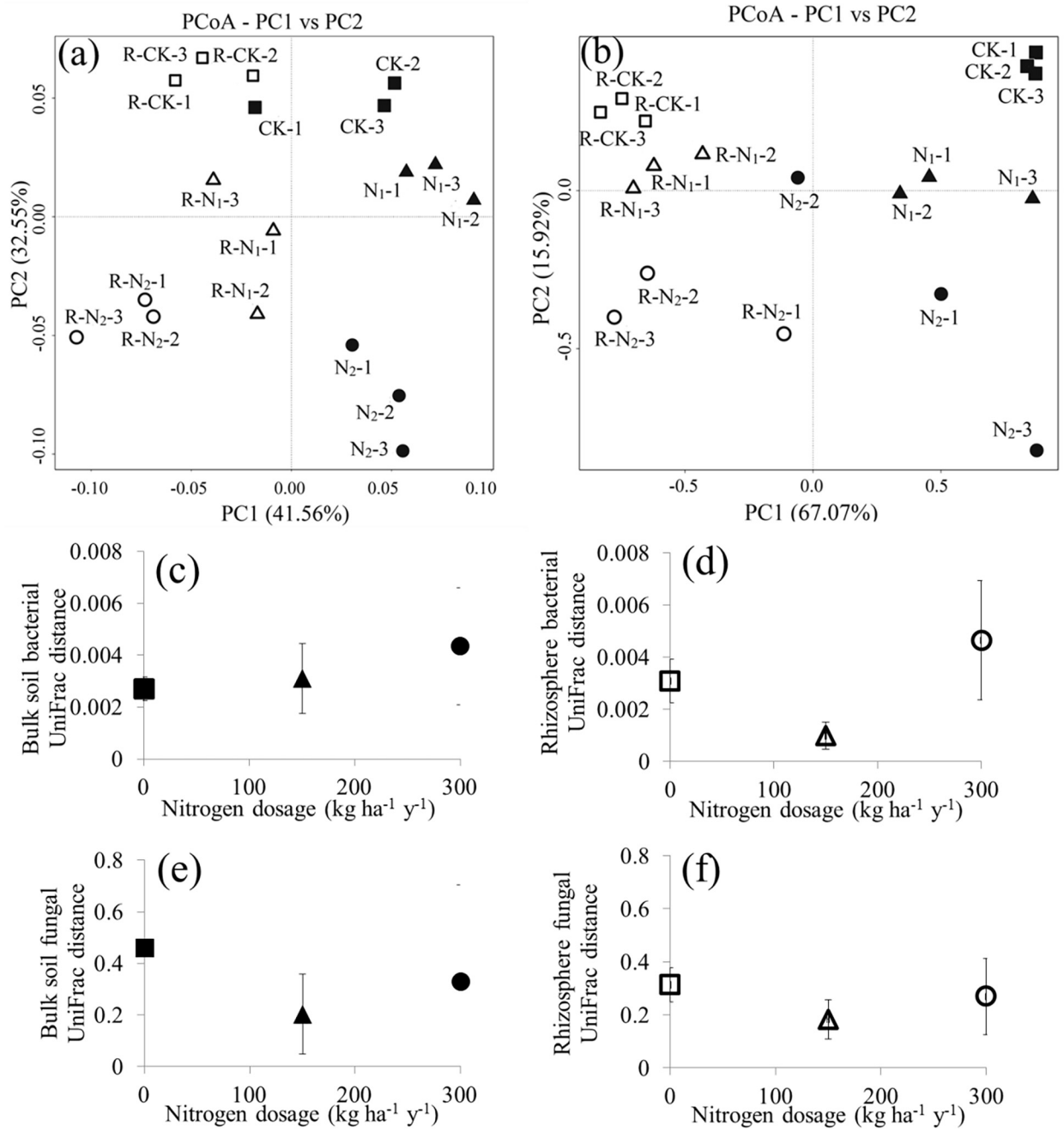

Fig. 4. Principal coordinates analysis of the bacterial (a) and fungal (b) phylogenetic composition of bulk soils and rhizospheres under different N-addition treatments, and the effects of N-addition on bacterial UniFrac distances in bulk soils (c) and rhizospheres (d), as well as fungal UniFrac distances in bulk soils (e) and rhizospheres (f). Phylogenetic distances were calculated based on the weighted UniFrac distance metric.

significant increase in the relative abundance of Lasiosphaeriaceae, while peroxidase activity positively correlated with an abundance of Trichocomaceae and Chaetomiaceae (Table 5), suggesting that these three fungal families may contribute to litter decomposition. Previous findings also confirmed that Trichocomaceae could produce diverse enzymes that decompose plant litter (Geiser et al., 2006; Houbraken and Samson, 2011). Additionally, *Penicillium herquei* species, which belong to the Trichocomaceae family, have a protofarming symbiosis with *Euops chinensis* that contributes to disease suppression (Wang et al., 2015). Recent experiments by Miura et al. (2015), in which multiple *Lasiosphaeriaceae* species were identified during the decomposition of sugarcane leaf litter, suggested that *Lasiosphaeriaceae* species/strains contribute to the decomposition of plant litter, possibly through the production of phenol oxidase and laccase (Luis et al., 2004). However, these correlations between the relative abundances of specific microbial species and the alterations of ecosystem functions, may lead to misinterpretations. For example, although some species of Chaetomiaceae can secrete enzymes that biodegrade plant litter (Saito et al., 2003), the family also includes numerous soil borne, saprotrophic, endophytic and pathogenic fungi that adapt to various growth conditions and living niches (Zámocký et al., 2016) and do not provide plants protection against disease or increase the nutrients available to plants. Thus, in microbiome studies, the microbes' ecological functions should be validated using specific microbe at specific times or in specific environment, or preferably use synthetic community approaches (Lebeis et al., 2015). The latter holds much promise because many

Table 5
Pearson's correlations between soil enzyme activities and abundant families (relative abundance > 1%).

	Family	Peroxidase	Phenol oxidase
Fungi	Trichocomaceae	0.643**	−0.624**
	Nectriaceae	0.064	0.246
	Mortierellaceae	−0.535*	0.269
	Chaetomiaceae	0.632**	−0.179
	Lasiosphaeriaceae	−0.536*	0.856**
Bacterial	Oxalobacteraceae	0.249	0.447
	Sphingomonadaceae	−0.455	−0.345
	Comamonadaceae	−0.266	0.865**
	Koribacteraceae	0.332	−0.717**
	Gaiellaceae	0.300	−0.839**
	Xanthomonadaceae	0.141	−0.398
	Chitinophagaceae	−0.167	0.369
	Solibacteraceae	0.186	−0.733**
	Rhodospirillaceae	−0.218	−0.341

** Correlation is significant at the 0.01 level.
* Correlation is significant at the 0.05 level.

microorganisms exert their functions through interactions among specific species in a community context (Garbeva et al., 2011; Tyc et al., 2014). Hence, more holistic approaches (including network analyses) are needed in soil and plant microbiome research, taking into account the concerted, dynamic and spatially separated actions of multiple microbiome members, not only microorganisms.

5. Conclusion

This study demonstrates that long-term N-addition distinctly changes soil properties and the microbial community's abundance and composition, and these changes may result in a significantly shift in microbial activities associated with soil C cycling. The change in the microbial diversity after long-term N-addition was mainly caused by species evenness. The compositions of the bacterial and fungal communities in the rhizosphere were significantly different from those in the bulk soil. The responses of bacterial and fungal communities to N-addition were not the same: fungal community's response to N-addition was greater than that of the bacterial community, and the rhizosphere effects decreased the microbial changes caused by N-addition. Thus, 36 years of N-addition changed the microbial community, and the rhizosphere's microbes had more significant positive effects on C cycling and N levels in the soil, as well as protecting crops from pathogens. More studies need to be conducted to elucidate the mechanisms that bacterial and fungal communities use to cope with the stress caused by global environmental changes, such as N-deposition.

Competing interests

The authors declare no conflict of interest.

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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.2018.12.019.

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