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Effects of Nitrogen Deposition on Nitrogen-Mineralizing Enzyme Activity and Soil Microbial Community Structure in a Korean Pine Plantation

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Abstract

To predict the effects of nitrogen deposition on nitrogen-mineralizing enzyme activity and soil microbial community structure in artificial temperate forests in northern China, we studied the soil properties, nitrogen-mineralizing enzyme activity, and microbial community structure in the soil of a Korean pine plantation in which different concentrations (0, 20, 40, 80 kg N ha^{-1} $year^{-1}$) of ammonium nitrate were applied for 5 consecutive years. The results showed that nitrogen addition at different concentrations did not significantly affect the soil pH. High nitrogen addition (80 kg N ha^{-1} $year^{-1}$) significantly increased the soil organic matter, ammonium nitrogen, and nitrate nitrogen content in the Korean pine plantation, and ammonium nitrogen was the key factor that influenced the soil fungal community structure. The urease activity under the moderate nitrogen addition treatment (40 kg N ha^{-1} $year^{-1}$) was significantly lower than that under the control (0 kg N ha^{-1} $year^{-1}$), and the protease activity in the three treatments was also significantly lower than that in the control. There was no significant correlation between microbial community structure and the four mineralizing enzymes. After nitrogen addition at different concentrations, the Simpson and Shannon indexes of soil bacteria decreased significantly under low nitrogen addition (20 kg N ha^{-1} $year^{-1}$), but the α-diversity index of soil fungi did not show significant differences under nitrogen addition. The microbial community composition was significantly changed by the different treatments. PLS-DA analysis showed that *Tardiphaga* was an important genus that made the greatest contribution to the differences in bacterial community composition among treatments, as was *Taeniolella* for fungal community composition. The low level of nitrogen addition inhibited nitrogen mineralization in the Korean pine plantation by reducing the relative abundances of Nitrosomonadaceae and Betaproteobacteriales and by reducing the abundances of symbiotrophic fungi. Berkelbacteria and Polyporales were bacteria and fungi, respectively, that changed significantly under the high nitrogen addition treatment (80 kg N ha^{-1} $year^{-1}$). This study provides more data to support predictions of the changes in nitrogen-mineralizing enzyme activity and microbial community structure in artificial temperate forest soils in response to increased nitrogen deposition.

Keywords Nitrogen deposition · Korean pine plantation · Microbial community structure · Nitrogen-mineralizing enzymes · Soil properties

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Introduction

With the rapid development of industrialization, fossil-fuel consumption, artificial fertilizer application, nitrogen-fixing legume cultivation, and animal husbandry, the content of active nitrogen in the global terrestrial ecosystem has increased by more than two times since the Industrial Revolution and is predicted to increase further in many areas around the world [1, 2]. Atmospheric N deposition around the world has increased from 24.42 to 106.3 Tg N $year^{-1}$ from the 1960s to 2000s, an increase of 3.4 times, and may increase continuously; it is expected to reach 200 Tg N $year^{-1}$ in 2050 [3–5]. China has become the third largest area of increased nitrogen deposition in the world [4], and N deposition has increased significantly, from 13.2 kg N ha^{-1} $year^{-1}$ in the 1980s to 23 kg N ha^{-1} $year^{-1}$ in the 2015 [6]. The average annual increase was 0.04 g N m^{-2} [7]. In a nitrogen-deficient forest ecosystem, increased nitrogen deposition can relieve nitrogen limitation in the system and promote the N absorption efficiency, photosynthetic capacity, and carbon-fixing capacity of plants in the system [8], further improving plant productivity [9]. However, when the nitrogen deposition content exceeds the ecosystem threshold, it also causes some negative effects on the ecosystem; for example, high nitrogen deposition will lead to soil acidification [10], imbalances in soil nutrient storage, changes in the soil microbial community structure [11], decreased biomass [12], etc.

Soil microorganisms are an important driver of nutrient cycling in forest ecosystems [13]. In general, changes in the abundance and composition of microbial communities are accompanied by changes in their functional activities [14]. Nitrogen deposition can affect soil microorganisms by changing soil nutrients, thus influencing the activity of the soil microbial population and the composition and diversity of the microbial community [15]. Studies have shown that nitrogen deposition can affect microbial community composition and diversity by changing the soil physicochemical properties [16].

Extracellular enzymes secreted by soil microorganisms can decompose organic matter into small molecules that can be absorbed by plants [17], and these enzymes participate in various biochemical processes in the soil. Their activity roughly reflects the relative strength of biochemical processes under certain soil ecological conditions. The measurement of soil enzyme activities can be used to indirectly understand the transformation of certain substances in the soil. Research has shown that urease, protease, L-asparaginase, and L-glutaminase are enzymes that are related to soil nitrogen mineralization [18]. Research by Khorsandi and Nourbakhsh [19] showed that the activities of urease, L-asparaginase, and amidohydrolase can affect nitrogen mineralization processes in soil. In addition, nitrogen deposition can increase the available nitrogen content in the soil and reduce the activity of enzymes related to nitrogen mineralization [20]. However, the impacts of nitrogen deposition on the activity of nitrogen-mineralizing enzymes in northern forest soils are not clear.

Korean pine (*Pinus koraiensis*) is a coniferous species and is a national second-class key protected wild plant in China that is a remnant from the Tertiary. The Korean pine forest is a typical temperate forest type in northeastern China, and large areas of natural forest have been transformed into plantations due to forest harvesting. This forest type plays an important role in carbon and oxygen absorption, climate regulation, water conservation, windbreak and sand fixation, environmental protection, and biodiversity stability. There is a great deal of variety fields in published reports on effects of nitrogen deposition on *Pinus koraiensis* in this site [21–23], i.e., soil and leaf C:N:P stoichiometry, soil nitrogen dynamics and greenhouse gas emissions, and soil N transformations and N loss. However, the responses of soil microbial community structure to nitrogen deposition in Korean pine plantations are less well understood.

To determine whether increased nitrogen deposition changes the soil nitrogen content and causes soil acidification, thus influencing the structure of the soil microbial community in Korean pine plantations, this study investigates the response of soil microbial community structures and mineralization enzyme activities to nitrogen deposition through a simulated nitrogen deposition incremental experiment and then identifies the key soil factors that drive these changes. We hope to provide data to further clarify the response mechanisms of forest soil microorganisms in the context of global climate change.

Materials and Methods

Site Description

The sample site is located in the Liangshui National Nature Reserve, Dailing district, Yichun city, Heilongjiang Province (47° 10′ 50″ N, 128° 53′ 20″ E) in Northeast China. This region is located on the east slope of the Dali Range in the southern Xiaoxing'an Mountains, where the landform is characterized by low mountains and hills at elevations from 280 to 707 m. The region experiences a temperate continental monsoon climate, with an annual average temperature, precipitation, and evaporation of − 0.3 °C, 676 mm, and 805 mm, respectively. The frost-free period and snowfall period are 100–120 days and 130–150 days, respectively. In the Chinese classification system, the soil is a dark brown forest soil, equivalent to Humaquepts or Cryoboralfs [24]. The growing season is from May to October, and the total nitrogen deposition during the growing season and annual bulk nitrogen deposition is 11.4 kg N ha^{-1} and 12.9 kg N ha^{-1},

respectively [23]. The research object was a Korean pine plantation that was cut down in 1953 and planted the following year through afforestation. The plantation has a basal area of 41.6 m^2 ha^{-1} and a mean diameter at breast height of 16.0 cm.

Experimental Design

The simulated N deposition experiment was initiated in May 2014 in a Korean pine plantation, and 12 plots of four treatments with three replicates were laid out in a completely randomized block design. Each plot was 5 m × 30 m and was bounded by a 10 m wide buffer strip. N was added as ammonium nitrate (NH_4NO_3) solution at four N levels: control (WN: without N addition), low-N (LN: 20 kg N ha^{-1} $year^{-1}$), moderate-N (MN: 40 kg N ha^{-1} $year^{-1}$), and high-N (HN: 80 kg N ha^{-1} $year^{-1}$). N was applied to the plots at mid-month from June to September starting in 2014 [23]. The amount of NH_4NO_3 required for each treatment was dissolved in 20 L of water, and the solutions were sprayed evenly on the forest floor using a backpack sprayer. The control soil was treated with an equal volume (20 L) of water.

Collection of Soil Samples

Soil samples were collected in early October 2018. After carefully removing litter, soil samples were taken using a soil corer (0–10 cm deep, 5 cm inner diameter) from 5 random points across each plot and mixed to yield one composite sample per plot. Thus, 3 samples of each treatment were collected, and a total of 12 soil samples (3 × 4) were taken. The soil samples were stored in airtight poly-propylene bags and placed in an icebox at 4 °C during transportation to the laboratory. The soil samples stored in the icebox were sieved through a 2 mm sterile sieve and divided into three parts: one part was preserved at − 80 °C for the extraction of soil microbial DNA, one part was dried for the determination of soil chemical properties, and one part was preserved at 4 °C for the determination of soil enzyme activity.

Experimental Method

Soil Property Determinations

The pH value of the soil was obtained by measuring a mixture with a soil:water ratio of 2.5:1 with a pH meter (FiveEasyFE20, Shanghai, China). The soil organic matter (SOM) content was determined by oxidation volumetry of potassium dichromate [25]. The soil ammonium nitrogen (NH_4^+-N) and nitrate nitrogen (NO_3^--N) were determined by MgO-diesel alloy distillation. The molybdenum antimony colorimetric method was used to determine the soil available phosphorus (AP) and total phosphorus (TP); flame spectrophotometry was used to determine the soil available potassium (AK) and total potassium (TK).

Soil Enzyme Activity Determination

The urease activity in the soil was measured by the sodium phenoxide-sodium hypochlorite colorimetric method, and the results were expressed as the number of milligrams of NH_3-N released in 1 g of soil after incubation at 37 °C for 24 h (mg g^{-1}). The ninhydrin colorimetric method was used to determine the protease activity in soil, and the results were expressed in milligrams of glycine released from 1 g of soil after incubation at 37 °C for 24 h (mg g^{-1}). Five grams of sieved soil was placed in a 50 mL Erlenmeyer flask, 0.2 mL toluene and 9 mL THAM buffer were added, and then 1 mL 0.5 M asparagine and glutamine solution was added. The mixture was sealed and incubated at 37 °C for 2 h. After incubation, 50 mL of KCl-Ag_2SO_4 was added, and the solution was filtered to determine the NH_4^+-N content in the filtrate. The activity of L-asparaginase and L-glutaminase (mg g^{-1}) was expressed by the number of mg of NH_4^+-N released from 1 g soil after incubation at 37 °C for 2 h [26, 27].

Soil DNA Extraction and High-Throughput Sequencing

Soil genomic DNA was extracted from 1 g root perimeter soil samples using an E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's instructions. Qualitative detection of soil genomic DNA was performed with 1% agarose gel electrophoresis and DNA purity and concentration detection was performed using a Nanodrop 2000 spectrophotometer. (5′-ACTCCTACGGGAGG CAGCA-3′) and (5′-GGACTACHVGGGTWTCTAAT-3′) were used as bacterial v3-v4 region primer sequences, and (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and (5′-GCTG CGTTCTTCATCGATGC -3′) were used as fungal ITS1 region primer sequences for PCR amplification. The amplified products were recovered and purified, and the sequencing library was prepared after fluorescence quantification. Finally, the sequencing was performed based on the Illumina MiSeq platform at Frasergen Biotechnology Co., Ltd (Wuhan, China).

Bioinformatics Analysis

After sequencing, QIIME software (quantitative insights into microbial ecology, v1.8.0, http://qiime.org/) [28] was used to obtain high-quality clean tags. A sequence length ≥ 160 bp was required, and fuzzy bases were not allowed. Sequences with > 1 5′ primer base mismatches and sequences with > 8 continuous identical bases were also eliminated. Then, we applied Usearch (v5.2.236, http://www.drive5.com/usearch/) through QIIME software (v1.8.0, http://qiime.org/) to check

for and remove chimeric sequences and finally obtain the required sequences. OTU clustering was carried out at the 97% similarity level for the effective sequences, and the classification status of OTUs after clustering was identified in the Silva [29] and Unite [30] databases for bacteria and fungi, respectively.

Statistical Analysis

After collating the data in Microsoft Excel 2016, Statistical Product and Service Solutions 22.0 (SPSS Inc., Chicago, IL, USA) was used to statistically analyze the soil indexes of broad-leaved Korean pine forest under different nitrogen deposition treatments. One-way ANOVA was used to test the significance of differences in soil properties and soil enzyme activities under different nitrogen deposition treatments (Fisher's LSD test, $P < 0.05$). The autocorrelation between soil physical and chemical properties and soil enzyme activities was calculated by Pearson's test (two-tailed) at two significance levels, $P < 0.05$ and $P < 0.01$. Pie charts and relative abundance maps of bacterial and fungal phyla with average relative abundances greater than 1% were plotted in Origin 8.0 software. Using MOTHUR software, the metastats statistical algorithm (http://metastats.cbcb.umd.edu/) [31] was used to test the sequence quantity (i.e., the absolute abundance) difference between the samples (groups) of each taxon at the phylum and genus levels. The results are shown in the form of a violin chart. The "fat and thin" areas of the violin reflect the density of the sample data distribution (the wider the width, the more samples correspond to the sequence number). Functional prediction for the fungi was performed with FUNGuild. Using the Galaxy online analysis platform (http://huttenhower.sph.harvard.edu/galaxy/), the relative abundance matrix at the genus level was submitted for LEfSe analysis. Based on the species abundance matrix and the sample grouping data, a PLS-DA discriminant model was constructed using the mixomics package in R (3.6.0). The vegan and ggplot2 packages in R were used to take the soil properties and soil enzymes as explanatory variables and the bacteria and fungi with significant differences between treatments as species variables to perform a redundancy analysis (RDA) and draw RDA diagrams.

Results

Soil Properties

The effects of nitrogen deposition on soil properties as determined through one-way ANOVA are shown in Table 1. The different nitrogen deposition levels had no significant effects on soil pH, available phosphorus, available potassium, or total phosphorus in the Korean pine plantation. Compared with properties under WN, the contents of SOM and NO_3^--N in the soil decreased, followed an increase with increasing N application. The SOM content in the HN treatment was significantly higher than that in the LN treatment ($P < 0.05$), and the NO_3^--N content in the HN treatment was significantly higher than that in the MN treatment ($P < 0.05$). The TK content in the soil showed a decreasing trend with increasing N application. The TK content in the HN treatment was significantly lower compared with WN ($P < 0.05$).

The Pearson correlation analysis showed that the SOM content was significantly positively correlated with the soil NO_3^--N ($r = 0.728$, $P < 0.01$) and NH_4^+-N ($r = 0.721$, $P < 0.01$) contents, and the NO_3^--N content was significantly positively correlated with the AP content ($r = -0.773$, $P < 0.01$), while the other soil factors were not significantly correlated with each other (Table S1).

Nitrogen-Mineralizing Enzyme Activities

The changes in nitrogen-mineralizing enzyme activities in the Korean pine plantation under the different nitrogen deposition treatments were not consistent (Fig. 1). Compared with those under WN, the urease and protease activities showed decreasing trends with increasing nitrogen deposition, and the urease activity decreased significantly in the MN treatment ($P < 0.05$), by 39.58%. In addition, protease activity decreased significantly ($P < 0.05$) compared with that under WN across the LN, MN, and HN treatments ($P < 0.05$), by 76%, 84%, and 56%, respectively. However, nitrogen deposition had no significant effects on L-asparaginase or L-glutaminase activity.

The Pearson correlation analysis found that protease activity was significantly positively correlated with soil NO_3^--N ($r = 0.807$, $P < 0.01$) and AP ($r = 0.769$, $P < 0.01$), while L-glutaminase activity was significantly negatively correlated with TK ($r = -0.720$, $P < 0.01$) (Table S1).

Soil Microbial Diversity and Community Structure

High-Throughput Sequencing Results

The quality control process for the original data resulted in 498,600 effective sequences for bacteria and 385,030 effective sequences for fungi. By drawing a dilution curve (Fig. S1), the sequencing amount of each treatment was determined to have reached saturation, and the sequencing depth fully met the test requirements. OTU clustering of the quality-controlled effective sequences was performed according to a sequence similarity threshold of 97%, and the results were shown in Fig. 2. Among the four treatments, bacteria shared 2,788 OTUs (Fig. 2a) and fungi shared 76 OTUs (Fig. 2b).

Table 1 Effects of nitrogen deposition on the soil properties of a Korean pine plantation

	WN without N addition	LN 20 kg N ha^{-1} $year^{-1}$	MN 40 kg N ha^{-1} $year^{-1}$	HN 80 kg N ha^{-1} $year^{-1}$
pH	6.49 ± 0.19a	6.50 ± 0.16a	6.46 ± 0.14a	6.35 ± 0.28a
SOM (%)	15.60 ± 2.64ab	13.79 ± 5.29b	14.51 ± 4.58ab	19.84 ± 4.61a
NH_4^+-N (mg kg^{-1})	17.01 ± 2.88b	17.85 ± 4.11b	20.24 ± 6.84b	38.52 ± 15.76a
NO_3^--N (mg kg^{-1})	21.01 ± 4.83a	15.34 ± 5.29ab	14.17 ± 2.48b	20.69 ± 4.80a
AP (mg kg^{-1})	1.26 ± 0.37a	0.83 ± 0.33a	1.10 ± 0.38a	1.03 ± 0.26a
AK (mg kg^{-1})	322.15 ± 29.32a	313.67 ± 48.36a	282.56 ± 59.65a	302.35 ± 56.56a
TP (g kg^{-1})	1.89 ± 0.44a	1.39 ± 0.44a	1.21 ± 0.64a	1.65 ± 0.57a
TK (g kg^{-1})	62.76 ± 7.36a	57.81 ± 4.50ab	55.40 ± 2.85ab	53.71 ± 7.34b

The value represents the mean ± SD. Different letters in the same row of data in the table indicate significant differences among different treatments in the level of $P < 0.05$

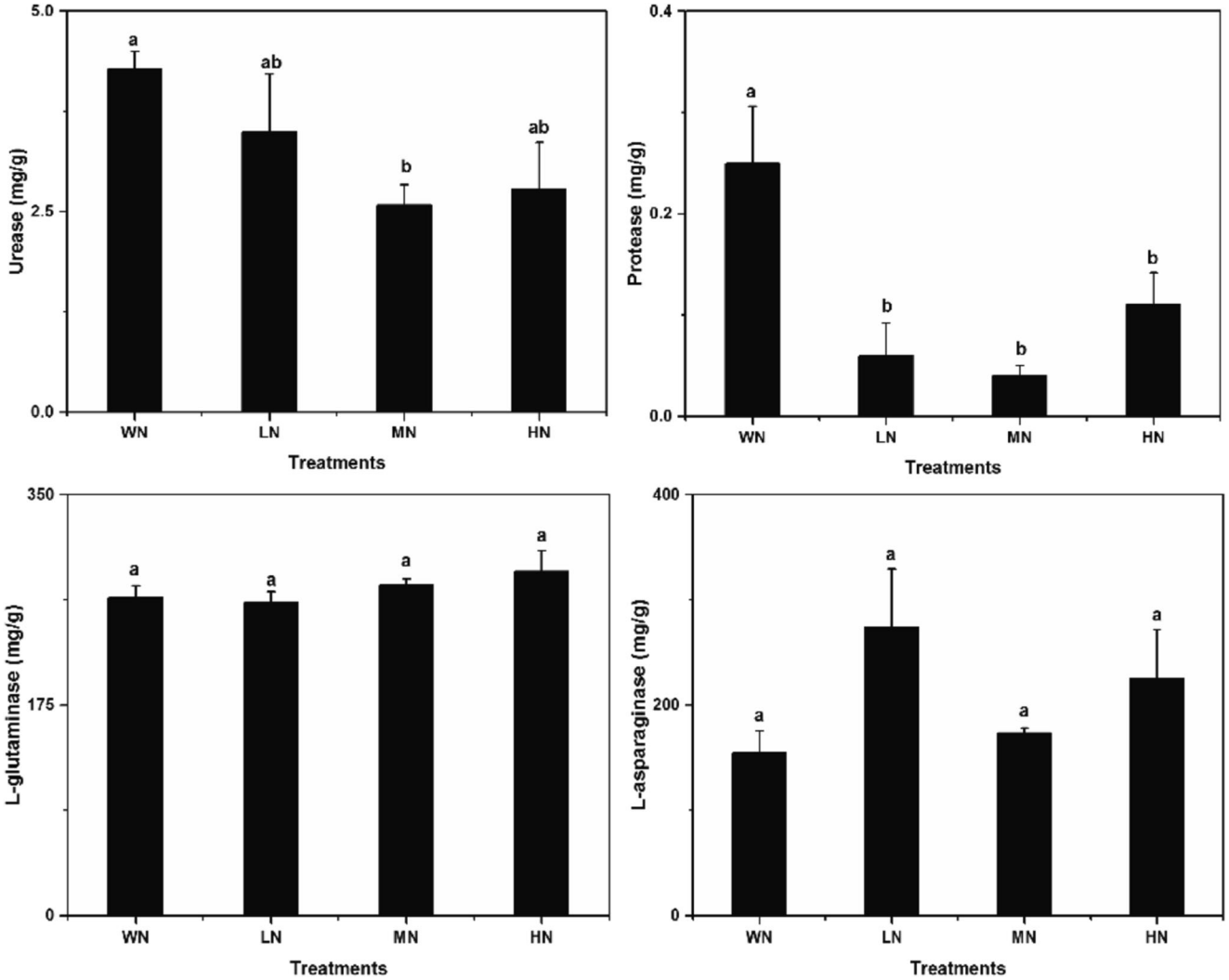

Fig. 1 Effects of nitrogen deposition on nitrogen-mineralizing enzyme activities in a Korean pine plantation. Note: The error line in the figure is standard error, and the different letters above the histogram indicate significant differences between differences at the level of $P < 0.05$. WN represents without N addition; LN represents 20 kg N ha^{-1} $year^{-1}$; MN represents 40 kg N ha^{-1} $year^{-1}$; HN represents 80 kg N ha^{-1} $year^{-1}$. The same abbreviations appear below

α-Diversity of the Soil Microbial Community

There were no significant differences in the ACE and Chao1 indexes for soil bacteria among the treatments (Table 2). The LN- and HN-treated soil samples had the minimum and maximum values for richness indexes respectively, among which the minimum values of LN treatment were 3,278 and 3,263, and the maximum values of HN treatment were 3,801 and 3,764. The Simpson index and Shannon index of the LN treatment were significantly lower than those of the other three treatments ($P < 0.05$), and the Simpson index was positively correlated with the NO_3^--N content ($r = 0.698$, $P < 0.05$) (Table S2).

There was no significant effect of different nitrogen deposition on the α-diversity indexes of soil fungi, among which the ACE index and Chao1 index had the maximum and minimum values in the WN and LN treatments, respectively. The maximum values of WN treatment for richness indexes were 271 and 274, the minimum value of LN treatment was 166, and both of the abovementioned treatments were positively correlated with AP ($r = 0.624$, $P < 0.05$; $r = 0.637$, $P < 0.05$). Simpson's index and Shannon's index had the maximum values in the LN treatment, which were 0.973 and 6.12, respectively, and both indexes were positively correlated with TK ($r = 0.670$, $P < 0.05$; $r = 0.576$, $P < 0.05$) (Table S2).

With the increase in nitrogen deposition, the soil microbial community changed adaptively such that the richness of bacteria was higher than that of the control and the richness and diversity of fungi were lower than those of the control.

Community Structure of Soil Microorganisms

According to the results of the OTU classification and the taxonomic status identification, the relative abundances in all treatments at the phylum level were Proteobacteria (30.0–46.0%), Acidobacteria (22.8–31.9%), Chloroflexi (4.3–14.3%), Verrucomicrobia (2.3–19.6%), Actinobacteria (4.8–8.7%), Gemmatimonadetes (2.8–5.3%), Rokubacteria (1.4–4.2%), Bacteroidetes (0.9–2.2%), Planctomycetes (0.9–2.2%), and Nitrospirae (0.5–1.5%). The above ten phyla accounted for 97.1% of the total bacterial abundances (Fig. S2A).

The relative abundances of the above ten phyla were shown in Fig. 3a. Except for Verrucomicrobia and Gemmatimonadetes, the relative abundances of the other phyla did not have significant differences among treatments. Compared with those under WN, the average relative abundances of Verrucomicrobia and Gemmatimonadetes in the LN treatment increased by 235.64% and 28.74%, respectively. The relative abundances of Proteobacteria and Chloroflexi were the lowest in the LN treatment. In addition, the relative abundance of Acidobacteria decreased with increasing nitrogen deposition concentration, while that of Actinobacteria increased with increasing nitrogen deposition concentration.

At the phylum level, the relative abundances of fungi in all treatments were Basidiomycota (12.5–80%), Ascomycota (7.9–62.4%), Mortierella (2.0–26.9%), Cryptomycota (0.5–5.3%), and unidentified (0.3–6.9%), and the above five phyla accounted for 90.6% of the total abundance of fungi (Fig. S2B).

The relative abundances of the above five phyla were shown in Fig. 3b. The relative abundance of Basidiomycota first decreased and then increased with increasing nitrogen deposition, and there was a significant difference in Basidiomycota abundance between the MN and LN treatments. Compared with that under WN, the average relative abundance of Basidiomycota increased by 85.98% in the LN treatment, while that of Ascomycota, Mortierella, and Rozellomycota decreased by 35.78%, 29.98%, and 19.37%, respectively.

Metastats Analysis in Different Treatments

The Metastats results showed that there were significant differences in the bacterial community in 9 phyla and 106 genera. The abundances of the top 20 bacterial genera with the most significant differences among treatments were shown in Fig.

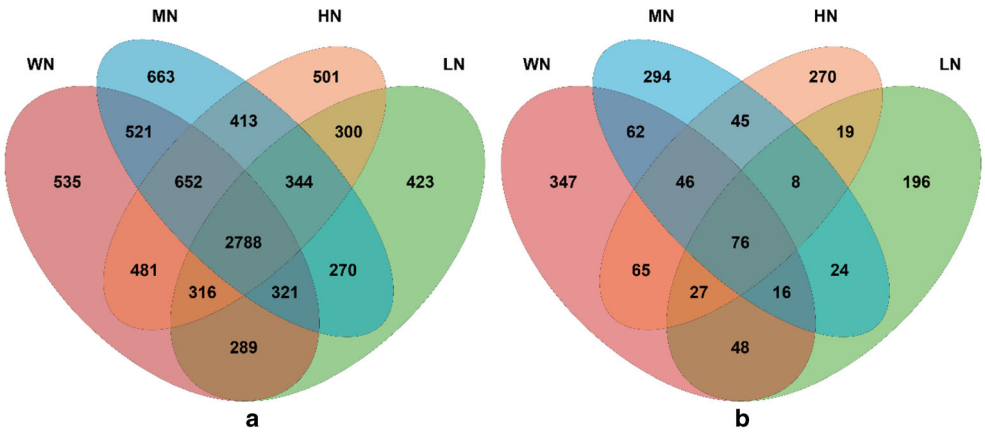

Fig. 2 OTU numbers for the four nitrogen deposition treatment

Table 2 α-Diversity indexes of soil bacterial and fungal communities among four nitrogen deposition treatments in a Korean pine plantation

Treatments	Bacteria				Fungi			
	Richness[1]		Diversity[2]		Richness		Diversity	
	ACE	Chao1	Simpson	Shannon	ACE	Chao1	Simpson	Shannon
WN	3696 ± 713a[3]	3654 ± 733a	0.998 ± 0.0001a	10.50 ± 0.19a	271 ± 116a	274 ± 120a	0.919 ± 0.080a	5.63 ± 0.88a
LN	3278 ± 1097a	3263 ± 1084a	0.996 ± 0.0007b	10.10 ± 0.09b	166 ± 33a	166 ± 33a	0.973 ± 0.009a	6.12 ± 0.40a
MN	3471 ± 144a	3430 ± 153a	0.998 ± 0.0003a	10.52 ± 0.20a	223 ± 63a	223 ± 63a	0.848 ± 0.192a	4.94 ± 2.09a
HN	3801 ± 561a	3764 ± 567a	0.998 ± 0.0004a	10.43 ± 0.08a	218 ± 119a	218 ± 119a	0.831 ± 0.227a	4.99 ± 1.82a

[1] The richness is mainly based on ACE and Chao1 index

[2] Diversity is mainly based on Simpson and Shannon index

[3] The data in the table is mean ± standard deviation, and different letters in the same column indicate significant differences between different treatments. WN represents without N addition; LN represents 20 kg N ha^{-1} $year^{-1}$; MN represents 40 kg N ha^{-1} $year^{-1}$; HN represents 80 kg N ha^{-1} $year^{-1}$

4. *HSB_OF53-F07* belongs to Chloroflexi, *Nakamurella* belongs to Actinobacteria, and the rest belong to Proteobacteria.

In the HN treatment, the abundances of four genera were significantly lower than those in the WN treatment, namely, *Dokdonella*, *Aquaspirillum_arcticum_group*, *Pajaroellobacter*, and *Massilia*; the abundances of four genera were significantly higher than those in the LN treatment, namely, *Bradyrhizobium*, *Nitrosospira*, *P3OB-51*, and *Pseudolabrys*; and the abundances of eight bacterial genera were significantly lower than those in the MN treatment, namely, *Bosea*, *Herminiimonas*, *Labrys*, *Massilia*, *Methylocella*, *Nakamurella*, *Ramlibacter*, and *Tardiphaga*.

Nitrosospira and *P3OB-51*, two bacterial genera that can participate in ammonia oxidation reactions, belong to Nitrosomonadaceae and Betaproteobacteriales. The average relative abundance of them decreased significantly in the LN treatment ($P < 0.05$) compared with that in the WN treatment, while in the MN and HN treatments, although a certain amount of recovery was observed, it was still lower than that in the WN treatment. LN treatment may inhibit the mineralization process by reducing the abundance of microorganisms that are involved in the ammonia oxidation process. As the nitrogen content in the soil continues to increase, these microorganisms develop tolerance, so their content is restored to a certain extent (Fig. 6a).

There were significant differences in abundances among the 3 phyla and 34 genera in the fungal community, and the abundances of the top 20 genera with the most significant differences among treatments were shown in Fig. 5. *Amanita*, *Anthracoidea*, *Cortinarius*, *Cutaneotrichosporon*, *Malassezia*, *Russula*, *Sebacina*, *Sporobolomyces*, and *Tomentella* are in Basidiomycota; *Archaeorhizomyces*, *Cladophialophora*, *Lecanicillium*, *Sphaerulina*, *Taeniolella*, *Trichocladium*, *Trichoderma*, and *Tumularia* are in Ascomycota; *Mortierella* is in Mortierellomycota; and *Exophiala* and *Diversispora* are in Glomeromycota.

In the HN treatment, three fungal genera had significant differences in abundances from the WN treatment; the abundance of *Sporobolomyces* was significantly lower than that in

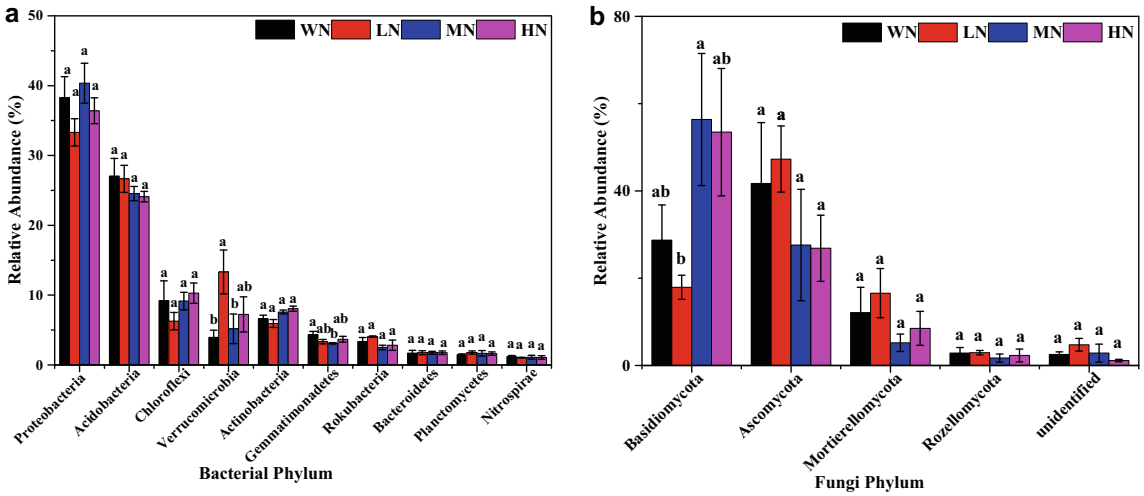

Fig. 3 Relative abundances of soil microorganisms in a Korean pine plantation at the phylum level

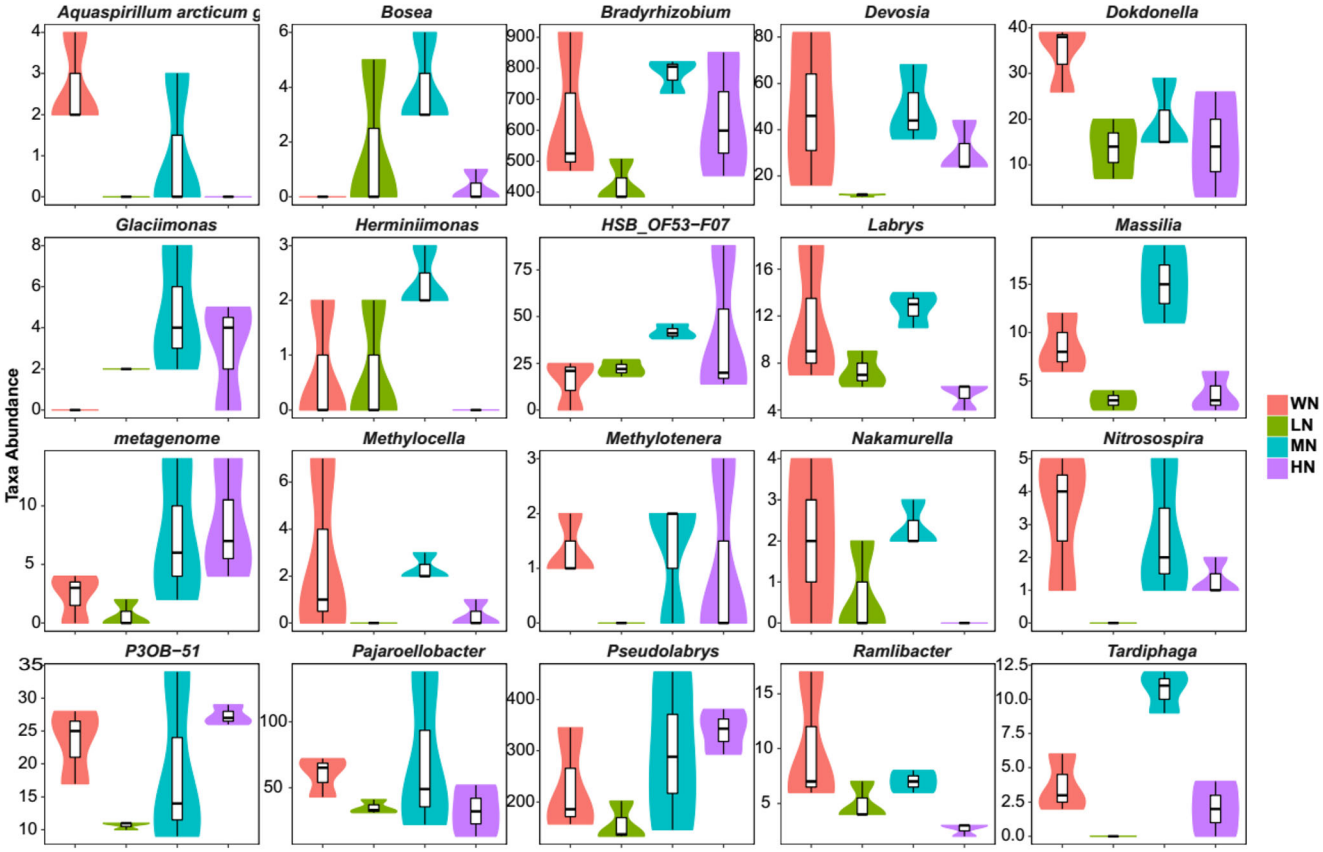

Fig. 4 Distribution of the abundances of the 20 bacterial genera with the most significant differences among the four nitrogen deposition treatments in the Korean pine plantation

the WN treatment, and the abundances of *Diversispora* and *Spitherulina* were significantly higher than those in the WN treatment. There were 6 genera of fungi that showed significant differences in abundances between HN and LN; the abundances of 2 genera were significantly lower than those under LN, i.e., *Trichoderma* and *Taeniolla*, and the abundances of 4 genera were significantly higher than those under LN, i.e., *Diversispora*, *Cutaneotrichosporon*, *Exophiala*, and *Sphaerulina*. Three fungal genera had significant differences in abundances between the HN and MN treatments; the abundance of *Diversispora* was significantly higher than that in the MN treatment, and the *Tumularia* and *Trichocladium* abundances were significantly lower than those in the MN treatment.

FUNGuild was used to predict the trophic modes of the soil fungal community under different treatments (Fig. 6b). The above 20 bacterial genera mainly include symbiotrophs, saprotrophs, pathotrophs, and others (pathotroph-saprotrophs, saprotroph-symbiotrophs). Among these four trophic modes, symbiotrophs was the most common. The LN treatment decreased the relative abundance of symbiotroph microorganisms compared with that in the other three treatments, while the other three trophic modes showed the opposite trends.

Screening of Key Microbial Taxa in Different Treatments

LEfSe analysis coupled with LDA was conducted to examine the effects of nitrogen deposition on taxa (from phylum to genus). The results showed that 7 taxa in bacterial community distinguished the four treatments, and LDA scores of them were greater than 2 (Fig. 7a). At the genus level, a total of 6 clades were detected, including *Ramlibacter* from WN and *Serratia*, *Tardiphaga*, *Massilia*, *Bdellovibrio*, and *Metanome* from MN. Only the HN treatment was characterized by the class Berkelbacteria. Berkelbacteria could be used as an indicator taxon in response of soil bacteria to high nitrogen deposition in Korean pine plantations.

Only 1 fungal taxon distinguished the four treatments with LDA scores greater than 4 in the fungi community (Fig. 7b). Only the HN treatment was characterized by the order Polyporales, which could be used as an indicator taxon in response of soil fungi to nitrogen deposition in Korean pine plantations. According to FUNGuild function prediction, Polyporales is a typical wood rot fungus, and it has no significant correlation with the four mineralizing enzymes in this study. Therefore, the changes in the abundances of saprophytic fungi were caused by the increasing nitrogen deposition and did not affect soil nitrogen mineralization.

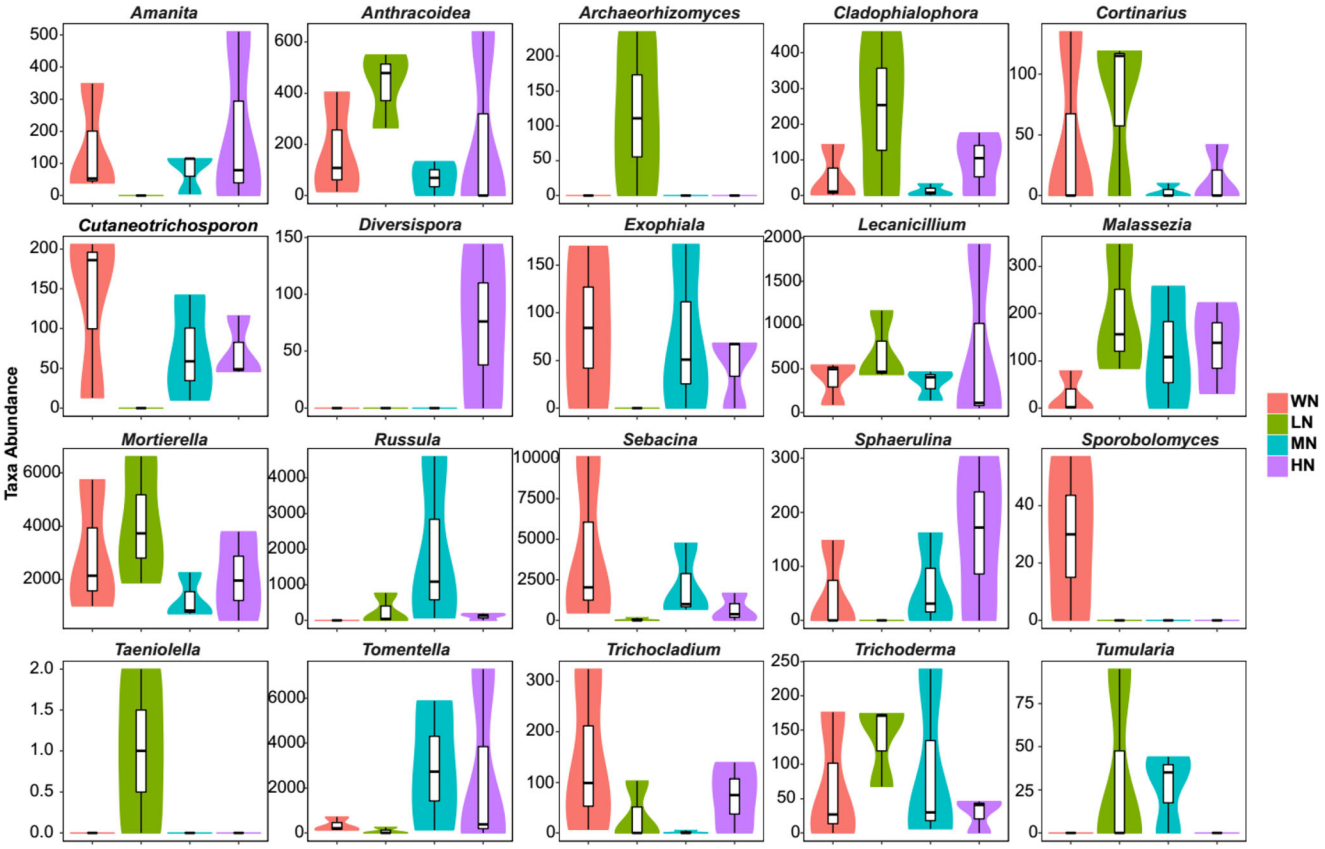

Fig. 5 Distribution of the abundances of the 20 fungal genera with the most significant differences among the four nitrogen deposition treatments in a Korean pine plantation

PLS-DA Analysis of the Soil Microbial Community

A PLS-DA discriminant model was built based on a taxon abundance matrix and sample grouping data (Fig. 8). For the bacterial community (Fig. 8a), the X-axis did not separate different treatments from the control but separated the LN and MN treatments, while the Y-axis separated different treatments from the control. Based on the separation distance, LN and MN had the greater impacts on the bacterial community than HN treatment. By calculating the VIP values of each taxon, *Tardiphaga* (VIP1 = 2.38, VIP2 = 1.94) in Proteobacteria was shown to be the bacterial genus that made the greatest contribution to the differences between treatments.

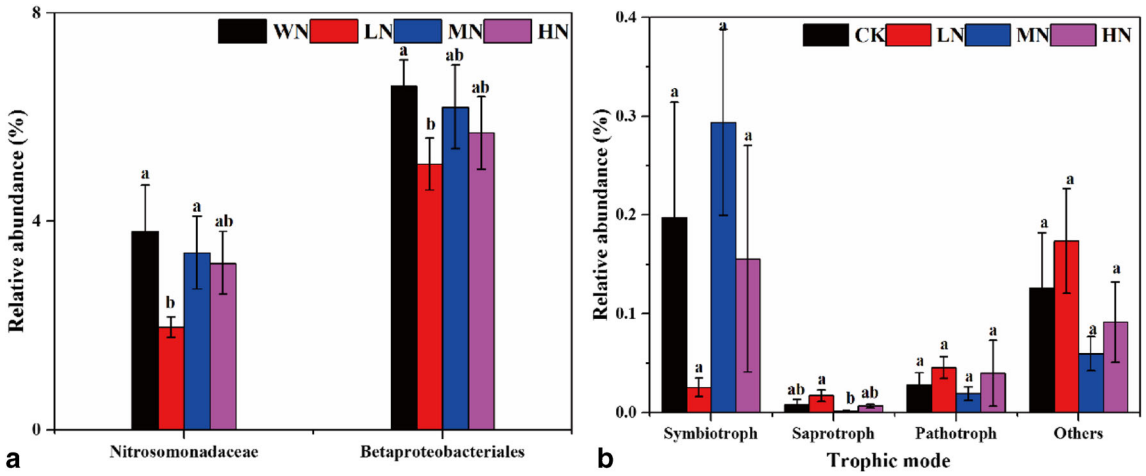

Fig. 6 Relative abundances of functional microorganisms under different treatments. (**a**) The relative abundances of bacteria related to ammonia oxidation. (**b**) The relative abundances of fungi with different trophic modes

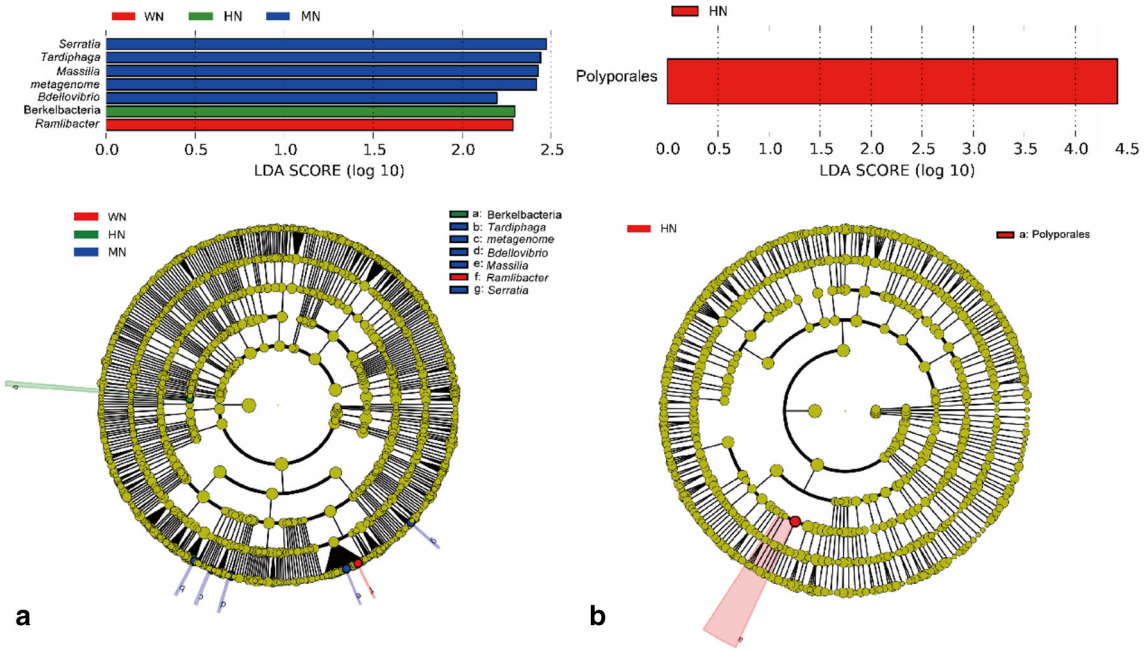

Fig. 7 The taxonomic differences between treatments based on a phylogenetic tree in a Korean pine plantation. (**a**) The LEfSe map of bacteria. (**b**) The LEfSe map of fungi. Lefse map is a linear discriminant analysis based on the composition of sample taxonomy according to different grouping conditions. In the four treatments, $P < 0.05$, LDA > 2 as the standard, communities with significant differences at each classification level are found to be represented by bar chart. The vertical coordinate of the bar chart is the classification unit with significant difference between groups, and the horizontal coordinate is the bar chart to display the logarithm score value of LDA difference analysis of the corresponding classification unit intuitively, and sort according to the score value. The longer the length is, the more significant the difference of the classification unit is, so as to describe the difference between them in different groups of samples. If the LDA > 0, it means the flora and phase. We should deal with the positive correlation and the negative correlation. The different colors of the bar chart indicate the sample groups with higher abundance corresponding to the taxon. In the bacterial community, red indicates the higher flora in WN treatment, blue indicates the higher flora after MN treatment, and green indicates the higher flora after HN treatment; in the fungal community, red indicates the higher flora after HN treatment. Pie chart can intuitively show the difference information of samples at each classification level, and the classification units with significant differences correspond to the bar chart one by one

For the fungal community (Fig. 8b), the X-axis separated different treatments from the control, and the result of Y-axis was similar with that of X-axis; the community structure of MN was more similar with that of HN. Calculating the VIP values of each taxon showed that *Taeniolella* (VIP1 = 2.09, VIP2 = 1.63) in Ascomycetes was the fungal genus that made the greatest contribution to the differences between treatments.

Redundancy Analysis

The 20 bacterial genera and 20 fungal genera that showed significant differences between the treatments were subjected to a redundancy analysis with soil factors, and the results are shown in Fig. 9. The double-sequence RDA diagram in Fig. 9a showed that 54.57% of the total bacterial community variation could be explained by the soil factors; RDA1 explained 29.03% of the total variation, and RDA2 explained 25.54% of the total variation. The double-sequence RDA diagram in Fig. 9b showed that 58.55% of the total variation of the fungal community could be explained by the soil factors; RDA1 explained 32.58% of the total variation, and RDA2 explained 25.97% of the total variation.

The Mantel test analysis of soil chemical factors and soil bacterial and fungal community structures under the different treatments revealed that the changes in bacterial community structure were not significantly related to any soil factors, and the changes in fungal community structure were only significantly related to the NH_4^+-N content ($r = 0.302$, $P = 0.024$) (Table 3).

Discussion

Effects of Simulated Nitrogen Deposition on the Soil Properties of Korean Pine Plantations

In this study, soil pH did not vary significantly between the different treatments, which is consistent with the results of other studies [32] on the effects of nitrogen application on farmland soils in Northeast China over 36 years. However, this result goes against the research results of Shi [33] from an Inner Mongolia temperate

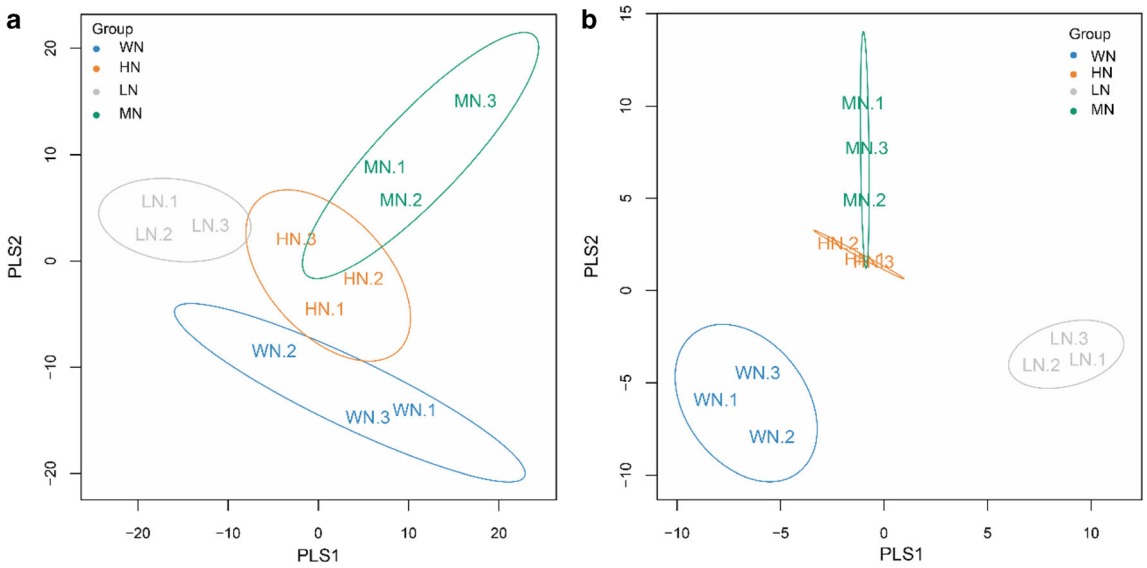

Fig. 8 PLS-DA analysis of the microbial community among different treatments

grassland. This may be because the nitrogen deposition in this area is within the buffer range of the soil pH [34].

Under the LN and MN treatments, there was no significant change in the SOM content, but the HN treatment significantly increased the soil organic matter content compared with LN treatment. This is consistent with the results of a study [35] on the effects of nitrogen application on the soil organic carbon content in tropical monsoon evergreen broad-leaved forests in Dinghushan over 13 consecutive years. The increase in organic matter accumulation may be due to the decrease in the mineralization rate under nitrogen application [36]. In addition, the increase in nitrogen input increased the aboveground productivity of forest plants and slowed the decomposition rate of organic carbon [37, 38]. At the same time, it reduced the distribution of underground carbon, increased the amount of plant litter and root exudates, reduced the rate of litter decomposition, increased the stability of lignin compounds, and thus promoted the accumulation of soil organic carbon [39–42]. In addition, nitrogen application changes the community structure and function of soil fungi, thereby increasing soil organic carbon accumulation [43]. Studies have shown that the nitrogen deposition threshold for temperate coniferous forests and subtropical forests (coniferous forests and broad-leaved forests) is lower than 100 kg N ha^{-1} $year^{-1}$ [44]. But there are few reports on the threshold of nitrogen deposition in the temperate zone of Northeast China. We also hypothesized that the significant increase of SOM in HN treatment may be due to the nitrogen deposition value breaking the threshold value in this area.

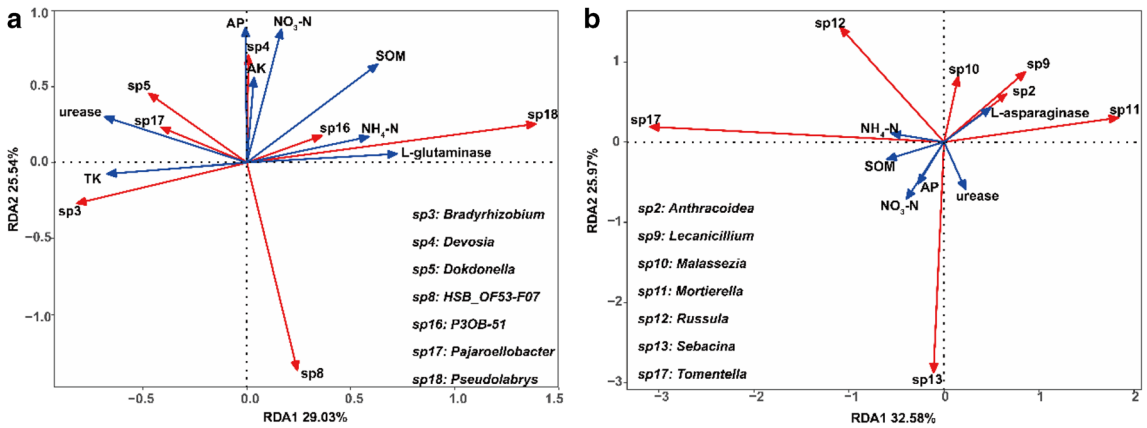

Fig. 9 Redundancy analysis of soil microorganisms and environmental factors in Korean pine plantations. (**a**) The RDA diagram of bacteria. (**b**) The RDA diagram of fungi. In the bacterial and fungal communities, 20 genera with significant differences between treatments were selected for redundant analysis, but only 7 genera had strong correlations with environmental factors, respectively

Table 3 Mantel test analysis of soil properties, nitrogen-mineralizing enzymes, and soil microbial community structure in a Korean pine plantation

		pH	SOM	NH^+-N	NO_3^--N	AK	AP	TK	TP	Urease	Protease	L-glutaminase	L-asparaginase
Bacteria	*r*	− 0.068	0.116	0.018	0.052	− 0.082	0.110	− 0.097	− 0.056	0.005	0.230	− 0.020	0.231
	p	0.679	0.158	0.412	0.298	0.726	0.247	0.762	0.656	0.428	0.059	0.527	0.06
Fungi	*r*	− 0.062	0.110	*0.302**	0.096	− 0.015	0.145	− 0.188	− 0.049	− 0.049	− 0.027	− 0.056	− 0.027
	p	0.644	0.184	*0.024*	0.235	0.494	0.236	0.902	0.580	0.582	0.523	0.577	0.494

Asterisk indicates significant correlation ($P < 0.05$); SOM, AK, AP, TK, and TP indicate soil organic matter, available potassium, available phosphorus, total potassium, and total phosphorus respectively

Effect of Simulated Nitrogen Deposition on Nitrogen-Mineralizing Enzyme Activities in the Korean Pine Plantation

In this study, the effects of the different nitrogen deposition levels on the nitrogen-mineralizing enzyme activities in the soil were inconsistent. This inconsistency may have been due to the initial nitrogen levels of the soil and plants [45], the amount of nitrogen applied [46], the nitrogen addition cycle [47], the type of exogenous nitrogen applied, the soil microorganisms [48], the direct or indirect effects of soil nutrients, or other factors.

Compared with urease under WN, the urease activity under MN treatment was significantly lower, which is consistent with the results of Song [49] in the Daxinganling permafrost region. Microorganisms can inhibit the production of urease by absorbing excess nitrogen to form by-products [49]. In contrast, studies have shown [50] that nitrogen application did not inhibit soil urease activity, probably because the increase in soil available nitrogen content did not fully meet the requirements of plant and microbial growth.

The protease activity in the LN, MN, and HN treatments decreased significantly with increasing nitrogen application, which is consistent with the study of Bowles [51] in agroecosystems. This likely occurred because Korean pine plantations in this area experience nitrogen limitation [22]. After the application of nitrogen, more nitrogen is available to soil microorganisms and plants, and there is no need for microorganisms to decompose organic matter and release nitrogen through mineralization [52].

Some studies have shown that amino acids (Glu, Gln, Asn, and Arg) can be considered cellular sensors of N status [53] and that their biosynthesis in plant leaves is very sensitive to atmospheric N deposition [54]. In our research, L-asparaginase and L-glutaminase were shown to produce Gln and Asn. However, nitrogen deposition did not have a significant effect on L-asparaginase or L-glutaminase, and there was no significant correlation between the soil microbial communities and the soil enzymes. These two amino acids in soil may not be sensitive to nitrogen deposition, which would explain the lack of a significant effect on either enzyme. Further research is needed on the coupled relationship between enzymes and microorganisms.

Effects of Simulated Nitrogen Deposition on Soil Microbial Diversity and Community Structure in Korean Pine Plantations

Soil microbial diversity is a sensitive indicator of changes in soil quality and reflects the overall dynamics of microbial communities. The HN treatment in this study did not significantly affect soil bacterial or fungal diversity. Studies have shown that nitrogen deposition can inhibit soil bacterial diversity [55], but some researchers believe that a certain concentration of nitrogen can promote the growth of bacterial communities and increase soil bacterial community diversity [56]. In this study, the different levels of nitrogen deposition did not significantly affect the richness of soil bacterial communities (ACE and Chao1) in the Korean pine plantation, but the soil bacterial diversity indexes (Simpson and Shannon) decreased significantly in the LN treatment compared with those in the control. However, there were no significant differences in bacterial communities between the MN and HN treatments and the WN control, which is inconsistent with previous research results. High nitrogen deposition may lead to an increase in the available nitrogen content in the soil, which leads to the rapid growth of nitrogen-loving microorganisms [57]. Therefore, the diversity of soil bacterial communities in the MN and HN treatments increased compared to that in the LN treatment. The soil fungal community abundance in northern spruce fir and Korean pine forests in the Xiaoxing'an Mountains reportedly increased due to the addition of nitrogen fertilizer. An increase in the amount of fertilizer applied did not produce a significant change. As shown in Table S2, the richness indexes for soil fungi (ACE and Chao1) were significantly positively correlated with AP, and the diversity indexes (Simpson and Shannon) were significantly positively correlated with TK. AP and TK did not change significantly in this study, which may be one of the reasons for the lack of significant changes in soil fungal community diversity.

Proteobacteria and Acidobacteria are the main phyla of forest soil bacteria. They were also the dominant bacteria in the soil bacterial community of the studied Korean pine plantation. This may be due to the adaptability of Proteobacteria

to a wide range of soil pH values, from acidic soils to alkaline environments [58]. Although Acidobacteria can survive in humid and acidic environments [59, 60], nitrogen deposition did not cause obvious soil acidification problems in this study, and the short-term nitrogen application may not have affected the Acidobacteria population in the soil. *Tardiphaga* is an endophytic rhizobia bacterium that can coexist with *Vavilovia formosa* (Stev.) Fed [61]. and can effectively promote plant growth. *Tardiphaga* is the genus that had the greatest impact on the bacterial community structure of this Korean pine plantation. Nitrogen deposition may change the abundances of nitrogen-fixing bacteria in Korean pine plantations. Berkelbacteria is a rhizospheric microorganism that can effectively degrade organic matter, release nutrients, and promote plant growth. It was positively correlated with the HN treatment and showed significant changes among treatments.

Numerous studies have shown that nitrogen application can reduce fungal biomass [62] and change the structure of soil fungi [11, 63]. In this study, the relative abundance of Basidiomycota decreased and then increased with increasing nitrogen deposition, which is consistent with the change in soil organic matter content. This is because ammonium nitrogen has an extremely significant correlation with soil organic matter, and high nitrogen deposition may indirectly affect the abundances of dominant bacterial groups by affecting the NH_4^+-N content and thus the soil organic matter content. The relative abundance of Ascomycota first increased and then decreased with increasing nitrogen deposition, which may be because high nitrogen deposition is not conducive to the growth of Ascomycota [64]. *Taeniolella* is a saprophyte in Ascomycetes that showed a significant difference in abundance in the LN treatment; it can effectively promote litter decomposition and had the greatest impact on the structure of the fungal community in this study. Polyporales, which showed a significant difference in abundance in the HN treatment, is an important class of wood rot fungi in the phylum Basidiomycetes [65] and is able to decompose cellulose and hemicellulose. Polyporales can also effectively promote material cycling in forest ecosystems [66]. This may be one of the reasons for the increase in soil organic matter content in this study. In nitrogen-limited forest ecosystems, ectomycorrhizal fungi can help plants absorb nitrogen in the soil. In forests of Korean pine, which is a typical ectomycorrhizal tree species, low nitrogen deposition levels (20 kg N ha^{-1} $year^{-1}$) will lift the nitrogen limitation on forest ecosystems, and the microbial abundance of symbiotrophs will decrease. Some studies have shown that carbohydrates are distributed to fungi in the form of glutamate under at high nitrogen levels [67], but some fungi require glutamate and a small amount of protein and serine to grow in soil that is high in inorganic nitrogen [68]. This may be the reason for the increase in symbiotroph fungi in the MN and HN treatments. The effects of high nitrogen deposition on symbiotic fungi require further study.

Conclusions

In the context of increasing global nitrogen deposition, high levels of nitrogen deposition (80 kg N ha^{-1} $year^{-1}$) did not cause soil acidification in a Korean pine plantation in Northeast China but significantly affected the content of organic matter, ammonium nitrogen, and nitrate nitrogen in the soil. The ammonium nitrogen content was the key factor causing the changes in the soil fungal community structure of the Korean pine plantation. The increase in nitrogen deposition inhibited urease and protease activities but had no significant effects on L-glutamine or L-asparaginase activity.

The low nitrogen deposition treatment (20 kg N ha^{-1} $year^{-1}$) significantly decreased the Simpson index and Shannon index of soil bacteria, and the Simpson index and Shannon index in soil fungi increased. However, the ACE and Chao1 indexes for soil fungi decreased under the LN treatment. With the increase in nitrogen deposition in the future, the soil bacterial richness of Korean pine plantations may increase, and the richness and diversity of fungi may decrease. Low nitrogen deposition (20 kg N ha^{-1} $year^{-1}$) can decrease the relative abundance of ammonia-oxidizing bacteria (Nitrosomonadaceae and Betaproteobacteriales) and reduce the abundances of symbiotrophic fungi.

The increase in nitrogen deposition produced significant effects on the bacterial and fungal community structure. *Tardiphaga* was an important genus that made the greatest contribution to the differences in bacterial community structure among treatments, as was *Taeniolella* for fungal community structure. Berkelbacteria and Polyporales can be used as indicator taxa for soil bacterial and fungal communities in Korean pine plantations under increasing nitrogen deposition.

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Compliance with Ethical Standards

Competing interests The authors declare that they have no competing interests.

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