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The impact of nitrogen deposition on nitrogen metabolism in ryegrass lawn with different soil nutrient levels

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Nitrogen deposition is a crucial factor in global change, which is widespread across various regions globally. It has drawn extensive attention due to its direct modification of soil nitrogen retention and nitrogen species distribution, thereby influencing nitrogen metabolism across entire ecosystems. Previous studies on its influence on nitrogen metabolism have not reached a consensus. In an urban ryegrass lawn mesocosm experiment, we set two levels of nitrogen deposition and soil nutrients respectively, aiming to study the impacts of these factors on the N-cycling process through metagenomic analysis. The results demonstrated nitrogen deposition increased nitrification, nitrogen fixation, denitrification, and dissimilatory nitrate reduction, but decreased assimilatory nitrate reduction in the nitrogen metabolism process by changing soil nitrogen availability and the abundance of N-cycling functional genes in the soil microbial community. The soil nutrient levels exhibited effects opposite to those of nitrogen deposition, negatively impacting nitrification, denitrification, and nitrogen fixation in the nitrogen metabolism process. This work further elucidates the impacts of nitrogen deposition on the ecological functions of the ryegrass lawn with different soil nutrient levels, and predicts the potential impacts of intensified nitrogen deposition on these ecological functions. It provides valuable theoretical support for understanding and evaluating complex ecological interactions.

Keywords Nitrogen deposition, Nitrogen metabolism, Soil nutrient level, N-cycling functional genes

Since the Industrial Revolution, atmospheric nitrogen deposition and agricultural nitrogen fertilizer application have sharply increased in terrestrial ecosystems. On the basis of nitrogen deposition data, the atmospheric nitrogen deposition rate in China from 2011 to 2015 was estimated to be 20.4 ± 2.6 kg N ha^{-1} yr^{-1}, which is 30% higher than that in North America and Europe[1], and this rate is expected to gradually increase in the next few decades[2]. Research has shown that nitrogen retention caused by nitrogen deposition or other processes has multiple effects on plants, and soil environments, such as decreased plant diversity; soil acidification; the accumulation of excess nutrients[3,4]; and changes in the soil microbial community composition[5,6]. Although nitrogen retention's impact on ecosystems has been widely studied, key aspects such as nitrogen retention's impact on the ecological functions of soil microorganisms, especially on the soil nitrogen cycling process, remain unclear.

The rate of nitrogen retention in soil depends on the amount of external nitrogen introduced (including nitrogen deposition, fertilization, soil nitrogen storage) and the potential for internal nitrogen transformation and release (mainly microbial processes). Both of these processes significantly affect nitrogen retention in soil, and they interact and synergize with each other[7]. Many studies suggest that nitrogen deposition and/or fertilization directly alter the availability and content of soil nutrients[8,9] and indirectly alter the abundance and structure of soil microbial communities[10–13] and plant biomass, community structure, and diversity[14]. Nitrogen deposition is believed to increase soil nitrogen availability in areas where soil nitrogen is relatively scarce and is also believed to potentially lead to soil acidification in areas where soil nitrogen is abundant[9,15,16]. These different impacts of nitrogen deposition often result in varying outcomes in different ecosystems. For example, in N-deficient areas,

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nitrogen deposition can lead to an increase in plant productivity, whereas in N-rich areas, nitrogen deposition can reduce plant productivity or have a less significant impact. Yuan et al.[17] reported that nitrogen deposition can significantly affect the abundance and activity of soil microorganisms; however, these effects depend on the degree of nitrogen deposition. A low level of nitrogen deposition can increase the abundance and activity of soil microorganisms, whereas a high level of nitrogen deposition dose not[17]. Ye et al. [18] noted that different degrees of nitrogen deposition have different impacts on the abundance of soil microorganisms. In their study, at low levels of nitrogen deposition, the number of soil bacteria increased, whereas at high levels of nitrogen deposition, the increase was not significant. Frey et al. [19] reported that nitrogen deposition can decrease the abundance of bacteria in temperate hardwood and pine forests, where the soil nutrient level is high relative to that in other ecosystems. Local soil nitrogen retention and the degree of nitrogen deposition are important factors determining the effects of nitrogen deposition.

Many related studies have shown that the level of soil nutrients also affects plant biomass and diversity, as well as soil microbial community structure and functions[20–22]. Humbert et al.[23] reported that the increase in soil nutrients caused by nitrogen fertilization is beneficial for increasing plant biomass but has a negative effect on plant diversity in mountain grasslands. Yang et al.[24] reported that the increase in soil nutrients caused by nitrogen fertilization can stimulate the activity of soil bacterial species that specialize in performing the different steps of the denitrification process and can differentially modify the diversity and composition of nitrite reductase (*nirK* and *nirS*) and nitrous oxide reductase (*nosZ*) gene-carrying denitrifying bacterial communities. These findings further confirmed that soil nitrogen retention can affect plant and soil properties and the soil microbial community. Therefore, when nitrogen deposition occurs in soils with different nutrient levels, its effects on plants, soil properties, and soil microbial communities inevitably vary.

The potential for nitrogen metabolism in soil microbial communities is an important indicator of N-cycling and related trends in ecosystems. Quantifying and characterizing N-cycling functional genes can provide information on N-cycling microbial communities and the soil microbial N-cycling potential[25]. Thus, N-cycling functional gene information can be used to determine the N-cycling potential and nitrogen metabolism trends in ecosystems. Macrogenomic sequencing is currently considered an effective method for analyzing the gene functions of microbial communities[26]. Zhao et al.[27] reported that differences in nitrogen fixation capacity and nitrogen utilization efficiency among different types of soil environments, which were mainly caused by differences in microbial community structure, especially in the ammonia-oxidizing archaea family Nitrososphaeraceae and Nitrospira-like nitrite-oxidizing bacteria. The subsequent metagenomic analysis results confirmed this finding: differences in nitrogen fixation capacity and nitrogen utilization efficiency were caused by significant differences in microbial community structure and N-cycling functional gene abundance[28]. However, our understanding of the impact of nitrogen deposition on nitrogen metabolism in soils with different nutrient levels is still unclear.

In this study, to gain a better understanding of how nitrogen deposition affects N metabolism in soils with different soil nutrient levels, we established two gradients based on the current global levels of nitrogen deposition and soil nutrients. Ryegrass lawn ecosystems, which are commonly found in urban areas, were selected as the research objects in this study. We employed metagenomic sequencing to analyze the impact of different nitrogen deposition conditions on the abundance, structure, and functions of soil microbial communities in soils with different nutrient levels, particularly on the abundance of N-cycling functional genes. Our aims are to investigate (1) how nitrogen deposition influences the abundance, structure, and function of the soil microbial community, especially the N-cycling microbial community, in soils with different nutrient levels; (2) the differences in N-cycling trends in soils with different nutrient levels and under different degrees of nitrogen deposition; and (3) the N-cycling functional potentials that are most sensitive to nitrogen deposition and soil nutrient levels. This work helps us to further understand the effects of changes in nitrogen deposition and local soil nutrient levels on soil microbial community nitrogen metabolism and provides a theoretical basis for enhancing the intensity of nitrogen deposition response strategies in soil environments with different nutrient levels.

Materials and methods

Plant cultivation and study site

An urban ryegrass lawn ecosystem was selected as the object in this study. Ryegrass (*Lolium perenne* L.) seeds used in this study were harvested and stored by our own laboratory, with a germination rate of 98%. First, seeds were sown in a germination tray. After germination, when the plants reached the three-leaf stage, strong and highly consistent ryegrass plants were selected and transplanted them into pots (diameter: 15.5 cm; height: 13.5 cm), with four plants per pot. The soil used for planting was collected from a local, non-forested area in Yantai, Shandong, China. The soil nutrient level was relatively low, with an alkali-hydrolyzable nitrogen content of 3.450 mg kg^{-1} and an available phosphorus content of 0.234 mg kg^{-1}.

This study was conducted in a greenhouse with natural lighting, a temperature range of 24–28 °C, and a humidity range of 65–85%. Watering was carried out every three days. Except for nitrogen deposition and soil nutrient addition, all other management measures for seedling planting were the same.

Nitrogen deposition method

Ammonium nitrate was selected as the additive for simulating nitrogen deposition. On the basis of global nitrogen deposition levels (ranging from 1 to 93.6 kg ha^{-1} yr^{-1}, but commonly ranging from 1 to 30 kg ha^{-1} yr^{-1})[29], the nitrogen addition of 14 kg ha^{-1} yr^{-1} and 28 kg ha^{-1} yr^{-1}were chosen as the low and high nitrogen deposition level, respectively. Moreover, the levels of nitrogen deposition set in this study are also commonly adopted by other researchers, e.g. the nitrogen deposition degrees ranged from 8 to 72 kg ha^{-1} yr^{-1} in the work of Yuan et al.[30]. The pots used for plant cultivation (outer diameter: 15.5 cm, inner diameter: 14.7 cm, bottom diameter: 11.5 cm, height: 13.5 cm) can hold approximately 1.5 kg of soil, which is equivalent to covering an area of 0.03 m^2 of land with a depth of 10 cm. Therefore, in accordance with the preset nitrogen deposition addition levels,

the amounts of ammonium nitrate was added as follows: 0 mg NH_4NO_3 kg^{-1} soil for no nitrogen deposition, 40 mg NH_4NO_3 kg^{-1} soil for low nitrogen deposition, and 80 mg NH_4NO_3 kg^{-1} soil for high nitrogen deposition. Fifteen days after transplanting at the three-leaf stage, the addition amount of the corresponding treatment were evenly divided into four parts and sprayed four times above the flowerpots of the cultivated plants by spraying to simulate atmospheric nitrogen deposition[30–33]. The interval between each spraying was 7 days. Eight replicates were set for each treatment.

Soil nutrient level regulation

Nitrogen and phosphorus are the main nutrients in the soil. Because the addition of nitrogen fertilizer alone might lead to nitrogen absorption barriers due to phosphorus deficiency, we selected urea and phosphorus pentoxide, two commonly-used chemical fertilizers, as a kind of soil nutrient regulator to regulate the soil nutrient levels. On the basis of the local soil nutrient levels, a gradient consisting of three soil nutrient levels (no soil nutrient addition, 0 mg CH_4N_2O kg^{-1} soil + 0 mg P_2O_5 kg^{-1} soil, low soil nutrient addition, 60 mg CH_4N_2O kg^{-1} soil + 12 mg P_2O_5 kg^{-1} soil, and high soil nutrient addition, 180 mg CH_4N_2O kg^{-1} soil + 36 mg P_2O_5 kg^{-1} soil) was set for studying the effects of the soil nutrient level. The corresponding additives were uniformed mixed with the soil and placed in pots for plant cultivation. During the once-every-three-days watering process, as the plants grow continuously, the nutrients mixed in the soil can be gradually absorbed by the plant roots. There were eight replicates for each treatment.

Sampling of plant materials and soil

After 2 months of cultivation, the plants were removed from the soils in the pots, and the roots were shaken to remove the soil adhering to the roots. The plants were then carefully rinsed in water, and the water was removed with filter paper. Each plant was divided into aboveground and underground parts from the surface of the ground and weighed separately to determine the fresh weight. Each part of the plants was first dried in a drying oven at 105 °C for 20 min, and then 80 °C until a constant weight was reached. After drying, the plant parts were weighed again to determine the dry weight.

The soil was shaken, and the plant roots were immediately placed in sterile tubes for DNA extraction and soil microbial community analysis, while another portion of the soil was naturally dried in the dark for 2 days and then sieved through a 2 mm sieve for soil property determination.

Determination of soil properties

The pH value and electrical conductivity of soils were determined following a conventional approach, by measuring the soil suspension prepared with a soil-to-water ratio of 5:1 (mass-to-volume ratio). The pH value was measured using a METTLER TOLEDO SevenCompact™ S210 pH meter (Shanghai, China). The electrical conductivity was measured with a INESADDS-307A conductivity meter (Shanghai, China). The soil alkali-hydrolysable nitrogen content was determined via the conventional alkaline hydrolysis diffusion method. A soil sample (5 g) was placed in a diffusion dish, and 20 ml of 1 mol l^{-1} NaOH solution was added to the sample in a 40 °C constant bath for the alkaline hydrolysis reaction. Moreover, 4 ml of 2% H_3BO_3 solution was added to the inner chamber of the diffusion dish for the absorption of the released NH_3. After 24 h, the amount of nitrogen absorbed in the H_3BO_3 solution was titrated with 0.005 mol l^{-1} H_2SO_4 solution.

The soil available phosphorus content was determined via the conventional $NaHCO_3$ extraction molybdenum antimony colorimetric method. The soil pH and soil conductivity were determined with a Mettler Toledo S210 pH meter (Mettler Toledo, Shanghai, China) and a Yidian DDS-307A conductivity meter (Yidian, Shanghai, China), respectively.

DNA amplification and metagenomics analysis

DNA isolation and analysis were performed from 0.5 g soil for each sample by employing the MP FastDNA™ SPIN Kit for Soil DNA Extraction (MP Biomedicals, USA) according to the manufacturer's instructions. The integrity and concentration of the isolated DNA were analyzed via electrophoresis and a Nanodrop 2000 instrument (Thermo Fisher Scientific, USA), respectively. The DNA samples were fragmented into pieces with an average size of approximately 400 bp by a Covaris ME220 (Covaris, USA). These fragments were subsequently used to construct paired-end libraries. Paired-end sequencing was performed via an Illumina NovaSeq/HiSeq 2 × 150 bp platform (Illumina, Inc., Shanghai, China). Microbial data were analyzed via a free online platform (http://cloud.geneskybiotech.com/index.html#/tools/all) provided by Genesky Biotechnologies Inc., Shanghai, 201315, China. The original sequencing data were submitted to the Genome Sequence Archive (GSA, Genomics, Proteomics Bioinformatics 2021) of the National Genomics Data Center (Nucleic Acids Res 2022), China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences, and the assigned accession number is CRA010448.

After obtaining the sequencing data, the Fast QC software was employed to conduct quality assessment on the raw sequencing data. Sequences containing N base were removed, those with the proportion of high-quality bases less than 60% were excluded, the low-quality bases at both ends of the sequences were eliminated, and sequences with a length less than 100 bp were also discarded. MetaSPAdes was utilized to assemble the Clean Reads of each sample into Contigs. Subsequently, bwa-mem (with default parameters) was adopted to align the Clean Reads to the Contigs to calculate the sequence assembly efficiency. Then, the assembly results of each sample were combined, and Contigs longer than 500 bp were retained. Thereafter, MMseqs2 was utilized to eliminate redundancies from the assembly results. Next, MetaGeneMark (with default parameters) was employed to predict the gene structure, and Genes longer than 100 bp were preserved. The Clean Reads were aligned to the Contigs using bwa-mem (with default parameters), and then the R software was employed to calculate the expression levels of the transcripts per million (TPM) values of each Contig. Based on the predicted relationship

between Contigs and Genes, the TPM values of the expression levels of each gene were obtained. MMseqs2 was used to align the nucleotide sequences of the non-redundant Contigs/Genes to the species nucleotide sequence database, and then DIAMOND was used to align the nucleotide sequences of the non-redundant genes to the species protein sequence database. According to the predicted relationship between Contigs and Genes, the optimal nucleotide alignment results were preferentially selected, and the protein alignment results were chosen secondly. Currently, the database comprises Bacteria (GTDB), Archaea (GTDB), Fungi (NCBI RefSeq), and Viruses (NCBI RefSeq). DIAMOND was used to align the nucleotide sequences of the non-redundant genes to the functional protein sequence database, and the protein alignment results with sequence similarity ≥ 80% and coverage ≥ 90% were selected. After the gene composition of the soil microbial community was obtained through metagenomic analysis, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used for functional annotation and pathway analysis in this study. The following simple pathway impact map of nitrogen metabolism was drawn based on the latest nitrogen metabolism pathway map in KEGG (https://www.kegg.jp/pathway/map00910), tracing the nitrogen metabolism-related genes.

Statistical analysis

All the data were reported in the form of the mean values of the replicated experiments for each treatment, with the number of replications ranging from three to eight times. Origin 2022 (OriginLab, USA) was used to construct the graphs. SPSS was used to analyze the variance in the data, and $P < 0.05$ was considered to indicate statistical significance. Permutational multivariate analysis of variance of the soil bacterial community via Bray distance was performed with R software. Linear discriminant effect size (LEfSe) analysis was performed according to the linear discriminant analysis (LDA) score of the soil bacterial taxa with significant differences ($P < 0.05$) among the different treatments via the software Lefse version 1.0.0. Circos maps of nitrogen metabolism-related genes showing significant differences under different treatments were generated via the software Circos version 0.69–6. The correlation network analysis diagram shows the pairwise correlations among various features or indicators, which was completed using the software R version 1.14. Each point represents a feature or indicator, and in this study, we refer to the abundance of the corresponding bacterial phylum. The size of the point is positively correlated with the relative abundance, and different colors represent different annotation information. The red connection lines represent strong positive correlations, the green connection lines represent strong negative correlations, and the gray ones represent weak correlations.

Results

Responses of plant biomass to nitrogen deposition and soil nutrient levels differ

The effect of nitrogen deposition on plant biomass varies in soils with different nutrient levels (Table 1). Moreover, the effect of nitrogen deposition on plant biomass is more significant than that of soil nutrient level. In soils with low and moderate nutrient levels, the level of nitrogen deposition is crucial, and a low level of nitrogen deposition can promote the accumulation of plant biomass, whereas a high level of nitrogen deposition dose not. In soils with high nutrient levels, both low and high nitrogen deposition inhibit the accumulation of plant biomass. Without nitrogen deposition, the promoting effect of the soil nutrient level on plant biomass was more obvious. However, when nitrogen deposition occurs, the promoting effect of the soil nutrient level on plant biomass becomes obvious.

Impact of nitrogen deposition on soil nitrogen availability

In soils with different nutrient levels, the soil alkali-hydrolysable nitrogen content increases with increasing nitrogen deposition (Table 2). This trend was most pronounced in low-nutrient soils but not in moderate- or high-nutrient soils. Regardless of nitrogen deposition, there are significant differences in the alkali-hydrolysable nitrogen contents in soils with different nutrient levels, with higher nutrient levels indicating higher alkali-hydrolysable nitrogen contents. However, as the degree of nitrogen deposition increases, the difference in alkali-hydrolysable nitrogen content caused by different soil nutrient levels becomes less significant. The one-way ANOVA results indicate that nitrogen deposition and soil nutrient levels can significantly affect soil nitrogen availability ($P < 0.001$, Table S1). Two-way ANOVA revealed that nitrogen deposition and the soil nutrient level had interactive effects on the soil alkali-hydrolysable nitrogen content ($P < 0.001$, Table S1).

Both nitrogen deposition and soil nutrient levels significantly affect the soil bacterial community.

The abundance, composition, α diversity, and β diversity of the soil bacterial communities were analyzed under different nitrogen deposition and nutrient treatments (Figs. 1, 2, 3, S1 and S2, Tables 3 and 4). The results showed that nitrogen deposition can significantly affect the abundance and composition of bacterial communities in soils at all nutrient levels (Fig. 1). Soil nutrient levels can also significantly affect the soil microbial community under different degrees of nitrogen deposition (Fig. 1). According to the analysis of similarities (ANOSIM), the no-, low-, and high-nitrogen deposition treatment clusters presented significant differences on the basis of the Bray distance algorithm ($P = 0.006$, number of permutations = 9999). The differences among the treatments with no, low, and high soil nutrient levels were significant according to the Bray distance algorithm ($P = 0.0002$, number of permutations = 9999). The effects of nitrogen deposition and the soil nutrient level on the soil bacterial community were also reflected in the interrelationships between the soil bacterial phyla (Fig. 3, Tables 3 and 4). As nitrogen deposition increased, the interrelationships between soil bacterial phyla increased (Fig. 3, Table 3), but the positive correlation decreased (Table 4). As the soil nutrient level increased, the interrelationships between the soil bacterial phyla decreased (Fig. 3, Table 3), but the positive correlation increased (Table 4).

Both nitrogen deposition and soil nutrient levels significantly affect soil nitrogen metabolism.

Since nitrogen deposition and soil nutrient levels significantly affect the soil alkali-hydrolysable nitrogen contents and soil bacterial communities, we paid special attention to the responses of the abundance of the phylum Nitrospirota related to nitrogen metabolism, gene expression in the nitrogen metabolism pathway, and

Treatment	Total plant biomass (g)				Plant aboveground biomass (g)				Plant belowground biomass (g)			
	Regardless of soil nutrient levels	Low soil nutrient level	Moderate soil nutrient level	High soil nutrient level	Regardless of soil nutrient levels	Low soil nutrient level	Moderate soil nutrient level	High soil nutrient level	Regardless of soil nutrient levels	Low soil nutrient level	Moderate soil nutrient level	High soil nutrient level
Regardless of nitrogen deposition degrees		2.62 ± 0.78	2.90 ± 0.27	3.21 ± 0.32		1.56 ± 0.37	1.97 ± 0.23	2.17 ± 0.34		1.06 ± 0.42	0.93 ± 0.15	1.04 ± 0.11
No nitrogen deposition	2.82 ± 0.47	2.29 ± 0.10	2.63 ± 0.00	3.52 ± 0.43	1.92 ± 0.44	1.40 ± 0.11	1.79 ± 0.08	2.57 ± 0.32	0.90 ± 0.09	0.89 ± 0.08	0.84 ± 0.08	0.95 ± 0.11
Low nitrogen deposition	3.32 ± 0.36	3.79 ± 0.11	3.24 ± 0.17	2.92 ± 0.10	2.09 ± 0.17	2.10 ± 0.02	2.32 ± 0.18	1.83 ± 0.03	1.23 ± 0.31	1.69 ± 0.11	0.91 ± 0.07	1.08 ± 0.13
High nitrogen deposition	2.59 ± 0.57	1.76 ± 0.12	2.84 ± 0.22	3.17 ± 0.13	1.69 ± 0.35	1.17 ± 0.04	1.82 ± 0.06	2.09 ± 0.21	0.90 ± 0.26	0.59 ± 0.09	1.02 ± 0.25	1.08 ± 0.08

Table 1. Total plant biomass under different treatments.

Treatment	Alkali-hydrolysable nitrogen content (mg kg^{-1})			
	Regardless of soil nutrient levels	Low soil nutrient level	Moderate soil nutrient level	High soil nutrient level
Regardless of nitrogen deposition degrees	36.94 ± 0.71	30.34 ± 0.76	30.47 ± 0.56	44.45 ± 0.48
No nitrogen deposition	27.06 ± 0.68	21.91 ± 0.20	22.02 ± 0.45	37.26 ± 0.38
Low nitrogen deposition	36.12 ± 0.73	27.35 ± 0.35	33.89 ± 0.28	47.12 ± 0.57
High nitrogen deposition	42.07 ± 0.48	41.76 ± 0.84	35.49 ± 0.44	48.96 ± 0.55

Table 2. Soil alkali-hydrolysable nitrogen contents under different treatments.

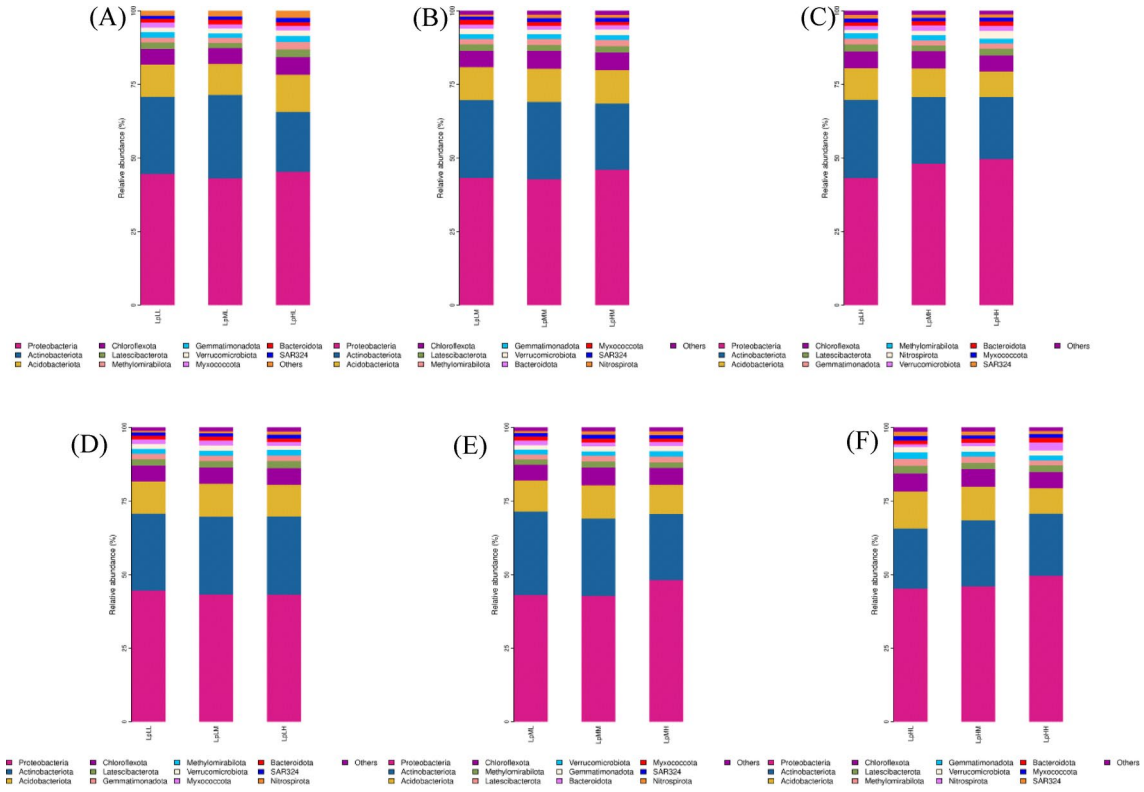

Fig. 1. The relative abundance of bacteria in soils with low (**A**), moderate (**B**), and high (**C**) nutrient levels with different nitrogen deposition levels, and with different nutrient levels under no (**D**), low (**E**), and high (**F**) nitrogen deposition levels. LpLL, LpML, and LpHL in (**A**) indicate the treatments with low soil nutrient levels under no, low, and high nitrogen deposition levels, respectively. LpLM, LpMM, and LpHM in (**B**) indicate the treatments with moderate soil nitrogen levels under no, low, and high nitrogen deposition levels, respectively. LpLH, LpMH, and LpHH in (**C**) indicate the treatments with high soil nitrogen levels under no, low, and high nitrogen deposition levels, respectively. LpLL, LpLM, and LpLH in (**D**) indicate the treatments with no nitrogen deposition and with low, moderate, and high nutrient levels, respectively. LpML, LpMM, and LpMH in (**E**) indicate the treatments with low nitrogen deposition and low, moderate, and high nutrient levels, respectively. LpHL, LpHM, and LpHH in (**F**) indicate the treatments with high nitrogen deposition and low, moderate, and high nutrient levels, respectively.

nitrogen metabolism functions related to nitrogen deposition and soil nutrient levels. In soils at all nutrient levels, the abundance of the phylum Nitrospirota was significantly altered by nitrogen deposition (Fig. 4). Similarly, soil nutrient levels also caused significant changes in the abundance of the phylum Nitrospirota, except under low nitrogen deposition (Table 5).

When analyzed in detail according to different soil nutrient levels, it was found that nitrogen deposition had a significant impact on the nitrogen metabolism pathway only in soils with low nutrient levels; in the soils with medium and high nutrient levels, nitrogen deposition had no significant impact on the nitrogen metabolism pathway (Table 6). However, in soils with different nitrogen deposition rates, soil nutrient levels had different effects on nitrogen metabolism processes. When no and low nitrogen deposition occurred, the soil nutrient level did not significantly affect the nitrogen metabolism pathway, whereas under high nitrogen deposition, the soil nutrient level impacted the nitrogen metabolism pathway (Table 6).Linear discriminant effect size (LEfSe) analysis also confirmed that the abundance of nitrogen metabolism-related bacteria under high soil nutrient levels and high nitrogen deposition rates was significantly greater than that under the other treatments (Fig. 5).

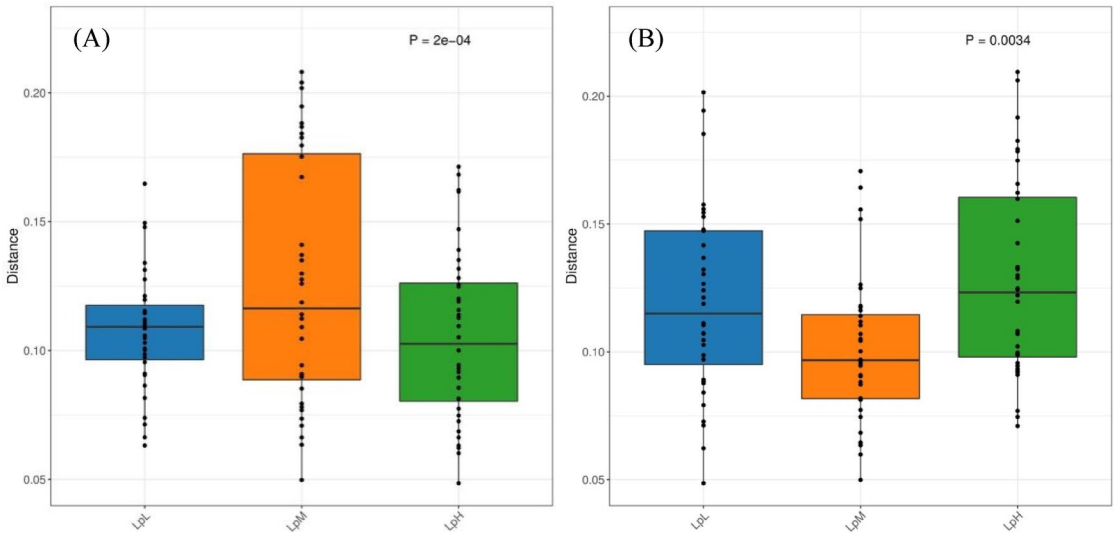

Fig. 2. Bray distances based on permutational multivariate analysis of variance among treatments with different degrees of nitrogen deposition (**A**) and different soil nutrient levels (**B**). LpL, LpM, and LpH indicate the treatments with no, low, and high nitrogen deposition, respectively. LpL, LpM, and LpH indicate the low, moderate, and high soil nutrient level treatments, respectively.

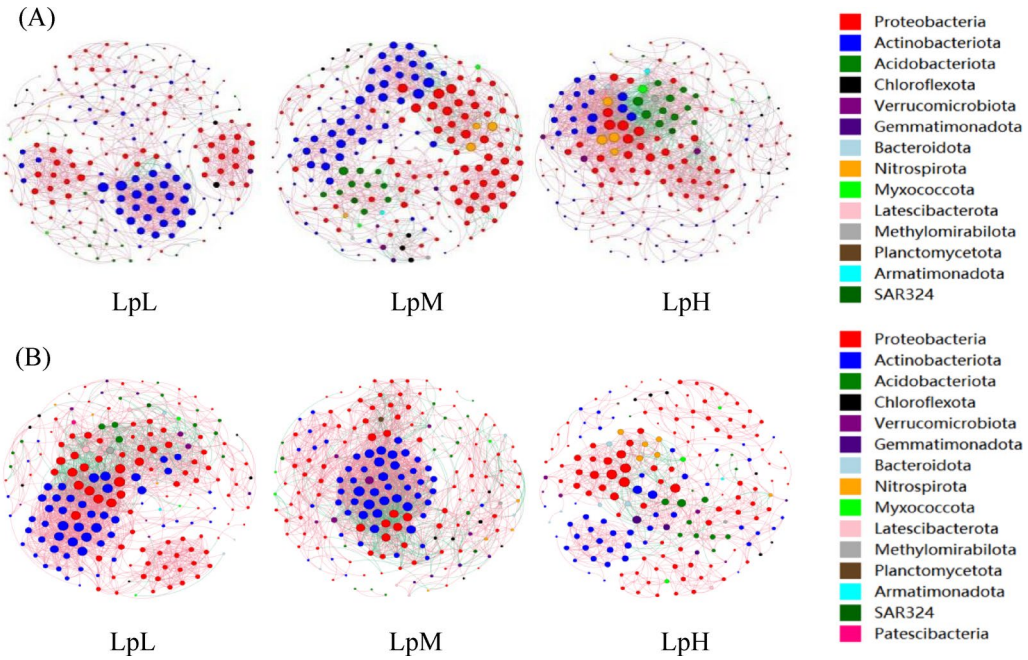

Fig. 3. Correlation analysis of soil bacterial phyla under different nitrogen deposition levels (**A**) and soil nutrient levels (**B**). LpL, LpM, and LpH indicate the no, low, and high nitrogen deposition/low, moderate, and high soil nutrient level treatments, respectively. The red connection lines represent strong positive correlations, the gree connection lines represent strong negative correlations, and the gray ones represent weak correlations. Only the correlations between phyla with relative abundances ≥ 0.005 are displayed.

Although nitrogen deposition did not have a significant effect on the nitrogen metabolism pathway, some genes related to the nitrogen metabolism pathway differed under the different nitrogen deposition treatments. The ANOVA analysis results revealed that 12 genes were significantly different among 48 nitrogen metabolism-related genes (Fig. S3, Table 7). The results from the nitrogen metabolism pathway impact map suggested that nitrogen deposition did indeed have some effect on the nitrogen metabolism pathway, mainly via the stimulation of nitrification, nitrogen fixation, denitrification, and dissimilatory nitrate reduction processes and the inhibition of the assimilatory nitrate reduction process at low nitrogen deposition rates, whereas the inhibition of the

Bacterial phyla	Nitrogen deposition			Bacterial phyla	Soil nutrient level		
	LpL (%)	LpM (%)	LpH (%)		LpL (%)	LpM (%)	LpH (%)
Proteobacteria	46.35	48.22	50.53	Proteobacteria	47.40	47.42	50
Actinobacteriota	30.73	27.92	24.21	Actinobacteriota	28.12	28.35	25
Acidobacteriota	7.81	7.61	7.89	Acidobacteriota	7.29	7.73	6.77
Chloroflexota	3.65	3.55	3.16	Chloroflexota	3.12	3.09	3.12
Verrucomicrobiota	3.12	2.54	3.16	Verrucomicrobiota	3.12	3.09	2.60
Bacteroidota	2.08	1.52	2.11	Gemmatimonadota	2.08	2.06	2.08
Nitrospirota	1.56	2.03	2.63	Bacteroidota	2.08	2.06	2.60
Gemmatimonadota	1.56	2.03	2.11	Nitrospirota	1.56	2.06	3.12
Myxococcota	1.04	1.52	1.05	Myxococcota	1.56	1.55	1.56
Methylomirabilota	0.52	0.51	0.53	Latescibacterota	1.04	1.03	1.04
Latescibacterota	0.52	1.02	1.05	Methylomirabilota	0.52	0.52	0.52
Planctomycetota	0.52	0.51	0.53	Planctomycetota	0.52	0.52	0.52
SAR324	0.52	0.51	0.53	Armatimonadota	0.52	–	0.52
Armatimonadota		0.51	0.53	SAR324	0.52	0.52	0.52
				Patescibacteria	0.52	–	–
Positive correlation ratio	95.73	87.97	82.10	Positive correlation ratio	83.87	74.47	89.66
Negative correlation ratio	4.27	12.03	17.90	Negative correlation ratio	16.13	25.53	10.34

Table 3. Proportions of soil bacterial phyla in the correlation analysis and the proportions of bacteria at different positions with negative correlations under nitrogen deposition and soil nutrient treatments. LpL, LpM, and LpH indicate the no, low, and high nitrogen deposition/low-, moderate-, and high-level nitrogen fertilizer treatments, respectively. – indicates that this bacterial phylum has not been detected.

Samples	Node	Edges	Average degree	Clustering coefficient	Average diameter	Modularity	Statistical inference	Density
Nitrogen deposition								
LpL	192	913	9.51	0.659	14	0.709	2750.95	0.05
LpM	197	1164	11.817	0.609	14	0.729	3543.353	0.06
LpH	190	1352	14.232	0.63	11	0.571	4260.302	0.075
Soil nutrient level								
LpL	192	1748	18.21	0.57	13	0.57	4900.99	0.1
LpM	194	1837	18.94	0.6	9	0.55	4861.33	0.1
LpH	192	841	8.76	0.64	15	0.81	2849.93	0.05

Table 4. The detailed parameters of the correlation network. LpL, LpM, and LpH indicate the no, low, and high nitrogen deposition/low-, moderate-, and high-soil nutrient treatments, respectively.

nitrogen fixation process occurred under high nitrogen deposition rates. Therefore, nitrogen deposition could cause the accumulation of nitrate in soils, which might be one of the main reasons leading to an imbalance in soil ecology (Fig. 6). The soil nitrogen level significantly affected the nitrogen metabolism pathway, which was represented by 15 genes (Fig. S4, Table 8). Among these genes, the expression of 13 genes increased as the soil nitrogen level increased, whereas that of the *nifD* and *nifK* genes decreased (Fig. 7). The results from the nitrogen metabolism pathway impact map suggested that nitrogen fertilizer application could stimulate nitrification and denitrification processes and inhibit nitrogen fixation, thus resulting in the accumulation of nitrogen in the studied ecosystem.

Correlations among plant biomass, soil alkali-hydrolysable nitrogen content and Nitrospirota abundance under different treatments.

On the basis of the above results, we determined that nitrogen deposition and soil nutrient levels significantly affect plant growth, the soil alkali-hydrolysable nitrogen content, the soil microbial community structure, the abundance of nitrogen metabolism-related bacterial phyla, and the abundance of nitrogen metabolism function-related genes. We analyzed the correlations among plant biomass, soil alkali-hydrolysable nitrogen content and the abundance of Nitrospirota in the soil bacterial community under different treatments (Fig. 8). The results indicated that nitrogen deposition affected the nitrogen metabolism process by altering the correlations among the three factors. Under the different nitrogen deposition treatments, the soil alkali-hydrolysable nitrogen content was positively correlated with the abundance of Nitrospirota in the soil bacterial community, whereas the correlations between the plant biomass and the soil alkali-hydrolysable nitrogen content and the abundance of Nitrospirota in the soil bacterial community varied with the degree of nitrogen deposition. In the absence of nitrogen deposition, plant biomass, soil alkali-hydrolysable nitrogen content and the abundance of Nitrospirota

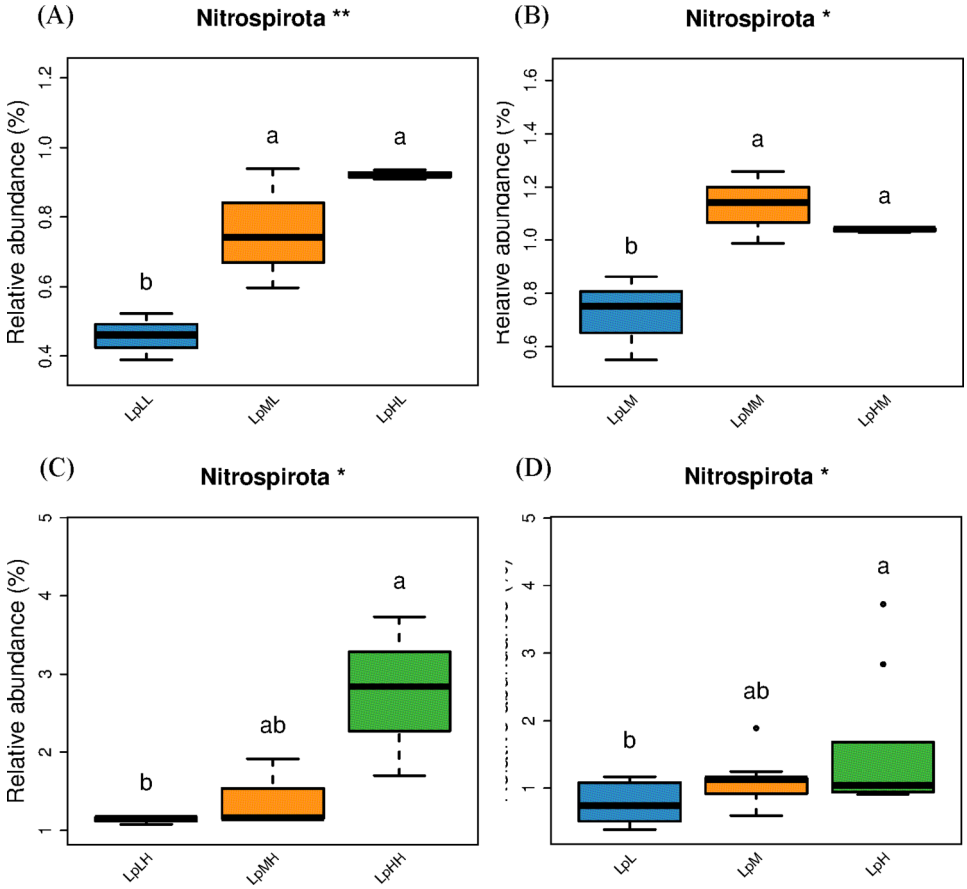

Fig. 4. The relative abundance of the phylum Nitrospirota in soils with no, low, and high nitrogen deposition levels at low (**A**), moderate (**B**), and high (**C**) nutrient levels at all soil nutrient levels (**D**).

Taxon	Relative abundance (%)			F value	*P* value	Significance
Nitrospirota	LpLL	LpLM	LpLH			
	0.456	**0.722**	**1.135**	**33.558**	**0.001**	***
	LpML	LpMM	LpMH			
	0.760	1.120	1.392	3.795	0.086	
	LpHL	LpHM	LpHH			
	0.922	**1.037**	**2.747**	**8.951**	**0.016**	*
	LpL	LpM	LpH			
	0.713	**0.958**	**1.768**	**8.322**	**0.002**	**

Table 5. The relative abundance of the phylum Nitrospirota at low (LpxL), moderate (LpxM), and high (LpxH) nutrient levels under no (LpLx), low (LpMx), and high (LpHx) nitrogen deposition and all nitrogen deposition (Lpx) treatments. Significant values are in bold. *** indicates a significance less than or equal to 0.001. ** indicates a significance less than or equal to 0.005. * indicates a significance less than or equal to 0.05.

in the soil bacterial community were positively correlated with each other. After nitrogen deposition occurred, plant growth was inhibited. As nitrogen deposition intensified, the interrelationships among the three influencing factors became blurred (Fig. 8), indicating that the ecosystem cycles were imbalanced. With low nutrient levels, the soils could not support normal plant growth (Fig. 9). In sols with moderate nutrient levels, the three factors had a mutually beneficial positive correlation. However, in high-nutrient soils, nutrient levels are sufficient for normal plant growth, and excess available nitrogen is not conducive to the continuous accumulation of plant biomass. Moreover, there was no significant correlation among the three factors, and the ecosystem cycles were imbalanced.

Treatment	F value	*P* value	Significance
Effect of nitrogen deposition			
All soil nutrient levels	0.546	0.586	
Low soil nutrient level	5.201	0.049	*
Moderate soil nutrient level	0.215	0.812	
High soil nutrient level	0.904	0.454	
Effect of soil nutrient level			
All nitrogen deposition levels	4.567	0.021	*
No nitrogen deposition	0.226	0.804	
Low nitrogen deposition	0.860	0.469	
High nitrogen deposition	28.828	0.001	***

Table 6. Effects of nitrogen deposition and soil nutrient level on nitrogen metabolism in the soil bacterial community under different treatments according to the ANOVA results.

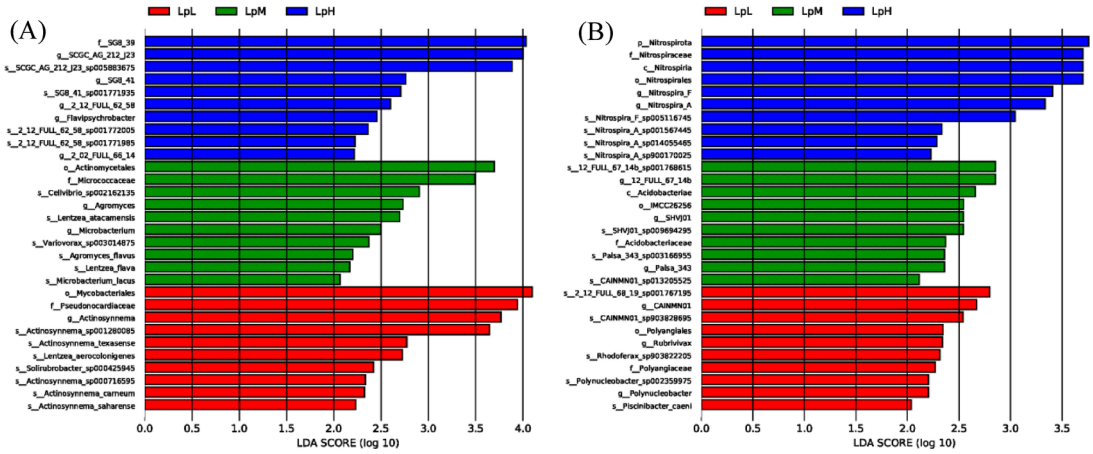

Fig. 5. Linear discriminant analysis (LDA) scores of soil bacterial taxa with significant differences ($P < 0.05$) among the different nitrogen deposition (**A**) and nutrient level (**B**) treatments |LDA| > 2 was the significant difference filtering threshold. LpL, LpM, and LpH indicate the no, low, and high nitrogen deposition/low, moderate, and high nutrient level treatments, respectively.

No	ID	Description	ANOVA			The change rate (%) of gene abundance	
			f. value	p. value	p. adjust (BH)	Low nitrogen deposition (%)	High nitrigen deposition (%)
1	K10945	pmoB-amoB; methane/ammonia monooxygenase subunit B	6.92	0.00	0.12	74.12	182.37
2	K02588	nifH; nitrogenase iron protein NifH	4.10	0.03	0.25	20.51	−34.57
3	K02591	nifK; nitrogenase molybdenum-iron protein beta chain [EC:1.18.6.1]	5.70	0.01	0.16	26.24	−51.81
4	K00376	nosZ; nitrous-oxide reductase [EC:1.7.2.4]	2.79	0.08	0.39	13.92	−12.17
5	K00362	nirB; nitrite reductase (NADH) large subunit [EC:1.7.1.15]	3.73	0.04	0.28	7.63	−10.90
6	K00363	nirD; nitrite reductase (NADH) small subunit [EC:1.7.1.15]	1.78	0.19	0.59	84.99	36.53
7	K02586	nifD; nitrogenase molybdenum-iron protein alpha chain [EC:1.18.6.1]	4.71	0.02	0.20	21.77	−51.00
8	K02305	norC; nitric oxide reductase subunit C	1.09	0.35	0.74	322.78	265.63
9	K10946	pmoC-amoC; methane/ammonia monooxygenase subunit C	3.94	0.03	0.26	27.51	76.66
10	K00372	nasA; assimilatory nitrate reductase catalytic subunit [EC:1.7.99.–]	7.59	0.00	0.11	−2.83	−9.82
11	K15876	nrfH; cytochrome c nitrite reductase small subunit	1.89	0.17	0.57	43.48	76.58
12	K00360	nasB; assimilatory nitrate reductase electron transfer subunit [EC:1.7.99.–]	3.78	0.04	0.27	−2.67	−27.51

Table 7. ID numbers in the KEGG database showing significant differences among different nitrogen deposition levels according to ANOVA analysis ($P < 0.05$) and their detailed descriptions.

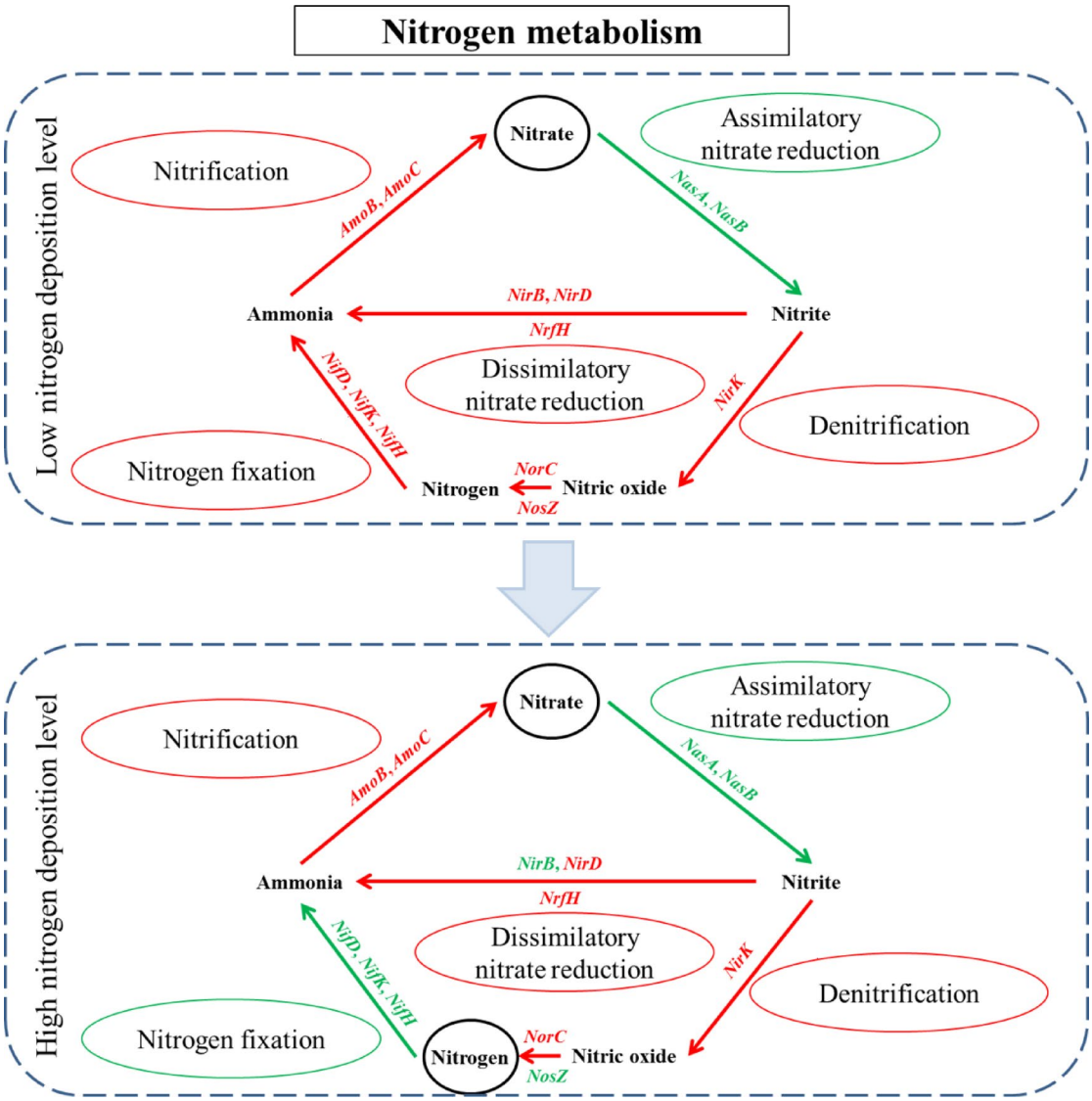

Fig. 6. Nitrogen metabolism pathway impact map under different nitrogen deposition levels on the basis of nitrogen metabolism-related genes whose expression significantly differed. Red indicates promotion, and green indicates inhibition. The black circle indicates the nitrogen species that readily accumulate in the nitrogen metabolism pathway.

Discussion

Previous studies have demonstrated that the effects of nitrogen deposition are jointly influenced by many environmental factors, such as differences in ecosystem types, regions, and degree of nitrogen deposition. Nitrogen deposition can alter soil nutrient availability, nutrient species composition, and soil microbial communities[9,10,34]. As nitrogen deposition intensifies, previously insignificant effects become increasingly significant[35]. Moreover, different regions exhibit diverse responses even to the same degree of nitrogen deposition[36].

. In our study, soil nitrogen availability varied with the degree of nitrogen deposition, which was consistent with the results of most previous studies[8,35,37]. Soil nitrogen availability is positively related to the amount of nitrogen deposited[38], but the amount of nitrogen stored in the soil significantly affects the response of soil nitrogen availability to nitrogen deposition[22]. This finding was also reflected in our study: under low soil nutrient levels, nitrogen deposition significantly affected soil nutrient availability, whereas high original soil nutrient levels induced a relatively large flexible space of available nutrient contents in soils, and the available nitrogen level was not easily affected by the additional nitrogen introduced by nitrogen deposition.

Nitrogen deposition can indirectly change the abundance of N-cycling functional genes in soil microbial communities and the nitrogen metabolism process through its impact on the availability of soil nutrients[22,35,39]. Our experimental results also yielded similar findings, as the abundance of nitrogen metabolism-related soil bacterial communities increased as the soil nutrient level increased. However, this pattern was affected by external nitrogen deposition in the present study: the abundance, composition, diversity and functions of the soil bacterial community were significantly affected by nitrogen deposition. In particular, the abundance of

No	KEGG ID	Description	ANOVA			The change rate (%) of gene abundance	
			f. value	p. value	p. adjust (BH)	Moderate soil nutrient level (%)	High soil nutrient level (%)
1	K00262	E1.4.1.4, gdhA; glutamate dehydrogenase (NADP +) [EC:1.4.1.4]	4.22	0.03	0.13	2.48	26.74
2	K00368	nirK; nitrite reductase (NO-forming) [EC:1.7.2.1]	7.78	0	0.04	4.67	39.73
3	K00370	narG, narZ, nxrA; nitrate reductase/nitrite oxidoreductase, alpha subunit [EC:1.7.5.1 1.7.99.–]	9.99	0	0.02	6.13	29.65
4	K00371	narH, narY, nxrB; nitrate reductase/nitrite oxidoreductase, beta subunit [EC:1.7.5.1 1.7.99.–]	12.52	0	0.01	8.36	35.63
5	K00374	narI, narV; nitrate reductase gamma subunit [EC:1.7.5.1 1.7.99.–]	9.69	0	0.02	1.91	48.02
6	K00459	ncd2, npd; nitronate monooxygenase [EC:1.13.12.16]	13.92	0	0.01	−2.02	30.54
7	K01743	E4.2.1.1; carbonic anhydrase [EC:4.2.1.1]	5.08	0.01	0.09	−80.10	185.20
8	K02305	norC; nitric oxide reductase subunit C	3.54	0.05	0.18	35.12	455.20
9	K02568	napB; nitrate reductase (cytochrome), electron transfer subunit	4.8	0.02	0.1	111.79	185.51
10	K02586	nifD; nitrogenase molybdenum-iron protein alpha chain [EC:1.18.6.1]	3.83	0.04	0.16	−11.01	−56.14
11	K02591	nifK; nitrogenase molybdenum-iron protein beta chain [EC:1.18.6.1]	4.42	0.02	0.12	−17.16	−57.94
12	K10535	hao; hydroxylamine dehydrogenase [EC:1.7.2.6]	12.81	0	0.01	32.48	130.23
13	K10944	pmoA-amoA; methane/ammonia monooxygenase subunit A [EC:1.14.18.3 1.14.99.39]	10.21	0	0.02	33.69	103.05
14	K10945	pmoB-amoB; methane/ammonia monooxygenase subunit B	7.64	0	0.04	81.90	195.44
15	K10946	pmoC-amoC; methane/ammonia monooxygenase subunit C	5.96	0.01	0.06	23.34	88.43

Table 8. ID numbers in the KEGG database showing significant differences among different nitrogen fertilizer application levels according to ANOVA analysis ($P < 0.05$) and their detailed descriptions.

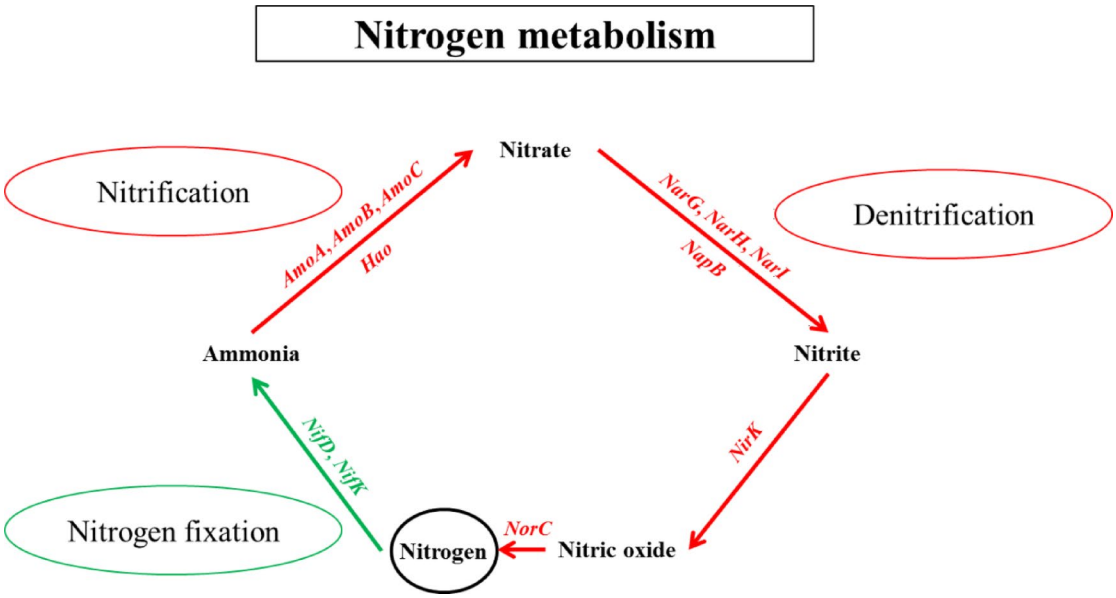

Fig. 7. Nitrogen metabolism pathway impact map under different soil nutrient levels drawn from nitrogen metabolism-related genes whose expression significantly differed. Red indicates promotion, and green indicates inhibition. The black circle indicates the nitrogen species that readily accumulate in the nitrogen metabolism pathway.

the phylum Nitrospirota, which is related to nitrogen metabolism, was significantly correlated with nitrogen deposition and soil nitrogen availability. In nutrient-poor soil, nitrogen deposition stimulated nitrogen metabolism, whereas in fertile soils, this stimulating effect was less significant. This might be because the amount of nitrogen originally present in soils with low nutrient levels was insufficient to meet the needs for plant growth. When additional nitrogen was introduced, the nitrogen metabolism process was stimulated, which resulted in an increase in the abundance of genes involved in the nitrogen metabolism pathway. In soils in which the nutrient levels were sufficient to meet the basic needs for plant growth and in which nitrogen metabolism was already active, additional nitrogen input did not significantly affect the nitrogen metabolism process.

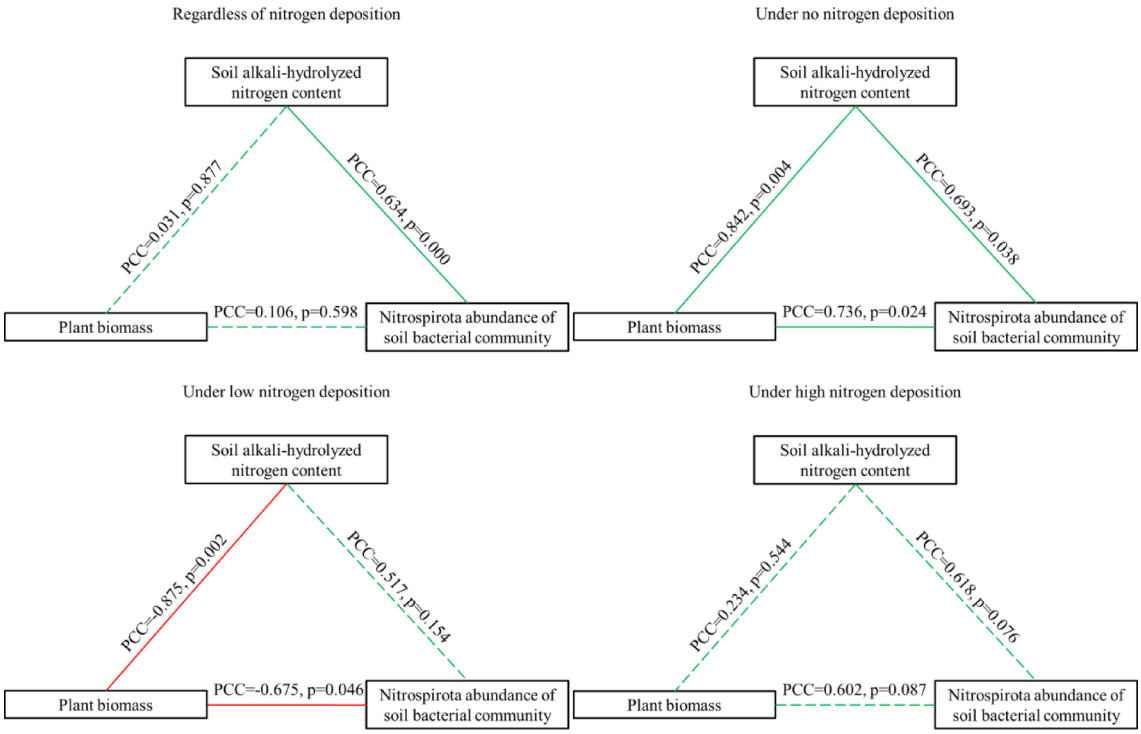

Fig. 8. Correlations among plant biomass, soil alkali-hydrolysable nitrogen content and Nitrospirota abundance in the soil bacterial community under different nitrogen deposition treatments. PCC indicates Pearson's correlation coefficient, and p indicates the p value in the statistical analysis. Red indicates a negative correlation, and green indicates a positive correlation. The solid line represents a significant relationship between the two influencing factors at the ends, whereas the dashed line represents a nonsignificant relationship between them.

Soil nutrient levels could also affect the response of the abundance of Nitropirota to nitrogen deposition, which was one of the important findings of our present work. Under no or low nitrogen deposition, differences in soil nutrients did not cause significant changes in the abundance of soil bacterial communities related to nitrogen metabolism. However, under high nitrogen deposition, differences in soil nutrients had a significant effect on the abundance of nitrogen metabolism-related bacterial communities. This occurred because under both no and low nitrogen deposition conditions, the nitrogen content in soils was within the normal range; thus, the nitrogen metabolism pathway was not significantly promoted or inhibited. However, high nitrogen deposition caused the soil nitrogen content to exceed the normal range, stimulating nitrogen metabolism. By comparing their effects, we concluded that when the soil nutrient level was relatively low, nitrogen deposition affected mainly soil nitrogen metabolism; whereas, when there was no or low nitrogen deposition, the soil nutrient level affected mainly soil nitrogen metabolism. When the soil nutrient level and nitrogen deposition were both high, the impact of the soil nutrient level on nitrogen metabolism was greater than that of nitrogen deposition.

Moreover, nitrogen deposition can affect plant growth through both the direct impact of nitrogen deposition on soil nutrient availability and its indirect impact on soil microbial community abundance, composition, and functions[40–42]. Due to the differences in plants' nutrient acquisition and utilization strategies as well as the varying degrees of nitrogen deposition, nitrogen deposition have positive, negative and neutral effects on plant growth[43–45]. In the present study, the nitrogen input from nitrogen deposition directly provided the nutrients needed for plant growth in nutrient-poor soils, increased the abundance of nitrogen metabolism-related genes in the soil microbial community, promoted nitrogen metabolism, and thus enhanced the plant growth. However, as the degree of nitrogen deposition increased, even in nutrient-poor soils, the soil nutrient distribution was disturbed, the reciprocal relationships among plants were disrupted, and the interactions among soil properties, soil microbial communities, and the ecosystem became imbalanced. Thus, high nitrogen deposition had no promoting effect on plant growth even in nutrient-poor soils. As evidenced by previous studies, the imbalance of soil nutrients caused by high nitrogen deposition is manifested in the increase in the soil N concentration and N:P ratio, which would further lead to the imbalance in N and P metabolism as well as plant growth[46].

Although this study has taken into account the coupling effects of plants, soil properties, and soil microbial communities, in the natural environment, considering that the impact of nitrogen deposition is related to multiple environmental factors, this study still has certain research limitations. In the complex natural environment, there are intricate energy exchanges among different ecosystems, and the multiple environmental factors interact and couple with each other, jointly influencing nitrogen metabolism genes[47]. Integrating a large number of published data has revealed that organic carbon and soil pH value are among the important environmental factors that

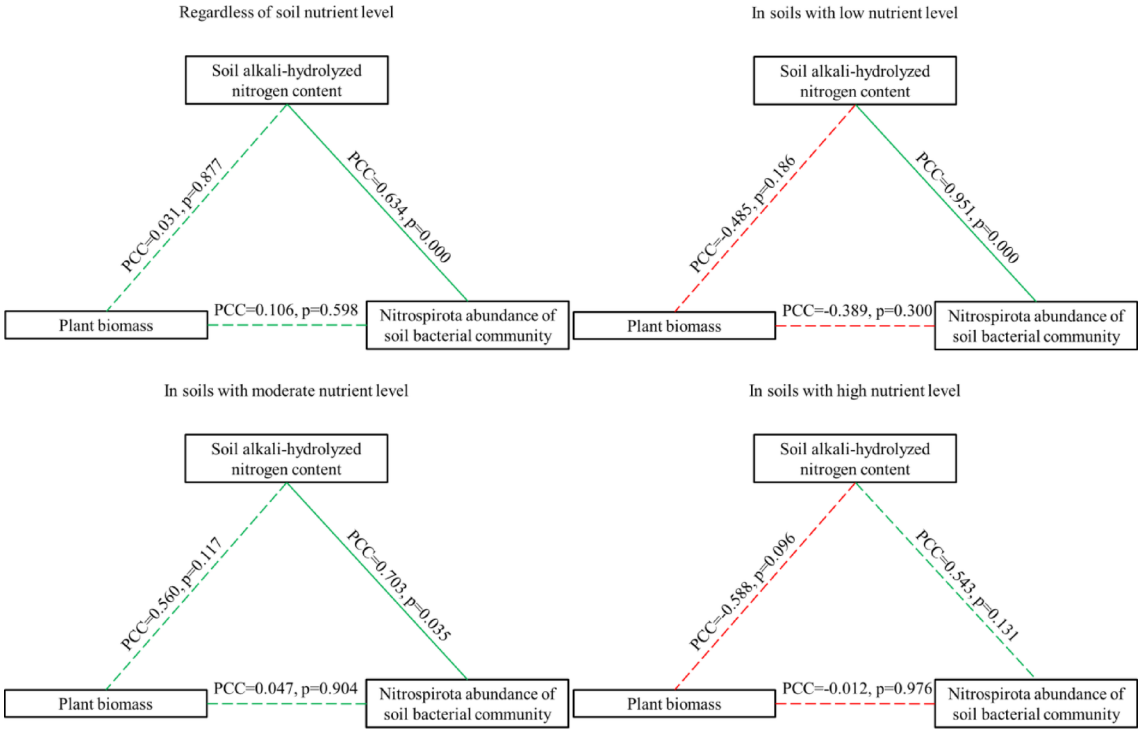

Fig. 9. Correlations among plant biomass, soil alkali-hydrolyzabke nitrogen content and Nitrospirota abundance in the soil bacterial community in soils with different nutrient levels. PCC indicates Pearson's correlation coefficient, and p indicates the *p* value in the statistical analysis. Red indicates a negative correlation, and green indicates a positive correlation. The solid line represents a significant relationship between the two influencing factors at the ends, whereas the dashed line represents a nonsignificant relationship between them.

influence the abundances of soil N-cycling genes[48]. To deeply explore the impacts of nitrogen deposition and soil nutrient levels on the nitrogen metabolism process, the measurement of time-series flux and absolute quantification of genes in the soil microbial community will undoubtedly be more illuminating. This will be the direction of our following work. In addition, although the degree and application method of nitrogen deposition selected in this study were designed to simulate natural atmospheric nitrogen deposition as much as possible, the soil type was single, and the irrigation method was also rather monotonous. The differences between this study and actual production will lead to certain limitations in the practical application of this research. This is also the reason why nitrogen deposition has diverse effects on plants, soil properties, and soil microbial communities under different external conditions. To ensure ecological security, it is necessary to accurately monitor the amount of nitrogen entering ecosystems from nitrogen deposition processes and consider local soil nutrient levels to comprehensively predict the potential impact of nitrogen deposition on various ecological processes.

Data availability

The raw sequence data reported in this paper have been deposited in genome Sequence Archive[49] in National Genomics Data Center, China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA010448) that are publicly accessible at http://ngdc.cncb.ac.cn/gsa.

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