Ecological Processes

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Nitrogen addition changes the nitrogen conversion process in forest steppe ecotone by increasing enzyme activity

Baihui Ren[1*†], Haoyan Li[1†], Daiyan Li[1†], Meng Meng[1], Jiahuan Li[1], Long Bai[1] and Yulong Feng[1*]

Abstract

Background The forest-steppe ecotone, a critical transition zone sensitive to global change, faces increasing nitrogen deposition. However, the interplay between nitrogen conversion processes and soil enzyme activity remains unclear. We investigated the effects of nitrogen addition on plant nutrient dynamics, microbial functional genes, and enzyme activity in northwest Liaoning, China.

Results Nitrogen addition significantly increased leaf nitrogen content in *Potentilla tanacetifolia* (peak under N40) and *Artemisia frigida* (peak under N40), while *Lespedeza daurica* showed a non-linear response (peak under N20). Phosphorus content remained unaffected across species. Soil enzyme activities (urease, nitrate reductase, dehydrogenase) increased with nitrogen input, with protease activity rising proportionally to nitrogen addition rate. Functional genes (*nirK*, *nifH*, AOB-*amoA*) exhibited dynamic responses: *nirK* abundance peaked under N40, *nifH* under N10, and AOB-*amoA* increased with nitrogen input. Structural equation modeling revealed that *nirK* gene abundance positively influenced enzyme activity ($\lambda = 0.512$), while *nifH* negatively correlated with leaf N/P ratios ($\lambda = -0.606$). Soil protease activity directly drove leaf N/P ratios ($\lambda = 0.734$).

Conclusions Nitrogen addition enhances plant nitrogen uptake and enzyme-driven mineralization, but species-specific responses highlight ecological trade-offs. Soil pH and protease activity are pivotal in mediating nitrogen conversion and plant nutrient stoichiometry. These findings underscore the need to integrate microbial and enzymatic dynamics into nutrient management strategies for ecotones under nitrogen enrichment.

Keywords Nitrogen addition, Plant nitrogen and phosphorus ratio, Enzyme activity, Nitrogen transformation, Functional gene of nitrogen transformation

Introduction

Anthropogenic nitrogen emissions have dramatically increased global nitrogen deposition, with China now ranking among the top three contributors worldwide (Liu et al. 2021). Elevated nitrogen deposition profoundly alters terrestrial ecosystems by enhancing soil nitrogen availability, accelerating acidification, and disrupting plant–microbe interactions (Stevens 2022). Forest-steppe ecotones, as transition zones between biomes, exhibit heightened sensitivity to such perturbations due to their unique biodiversity and climatic vulnerability (Smith et al. 2023). However, the mechanisms by which nitrogen enrichment regulates nitrogen conversion processes—particularly through enzyme-microbe-plant interactions—remain poorly understood in these ecologically critical regions.

Recent studies highlight that nitrogen addition reshapes soil microbial communities and enzyme

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activities, directly influencing nutrient cycling (Zhou et al. 2012). For instance, urease and protease activities are strongly linked to nitrogen mineralization rates, yet their responses to varying nitrogen gradients in ecotones remain inconsistent (Chen et al. 2023). While moderate nitrogen addition can enhance nitrogen-fixing bacteria, excessive nitrogen can inhibit their activity (Jorquera et al. 2014; Orr et al. 2012). Furthermore, while ammonia-oxidizing bacteria (AOB) and archaea (AOA) dominate nitrification, their functional gene abundances (e.g., *amoA*) show divergent responses to nitrogen inputs, with AOB often being more sensitive (Wang et al. 2021a, b). Such microbial shifts may cascade to affect plant nutrient stoichiometry, particularly leaf nitrogen-to-phosphorus (N/P) ratios—a key indicator of ecosystem nutrient limitation (Peng et al. 2023). However, species-specific responses complicate predictions: leguminous plants exhibit stronger nitrogen assimilation due to symbiotic nitrogen fixation, whereas non-legumes may face phosphorus limitations under high nitrogen conditions (Gao et al. 2017). This deposition not only directly affects plants but also indirectly impacts nutrient absorption by altering soil nutrient content, potentially exacerbating phosphorus limitation in grassland ecosystems (Su et al. 2019; Wang et al. 2016). These divergent responses underscore the need to disentangle the roles of enzyme-microbe-plant interactions in driving nitrogen conversion efficiency. However, few studies have integrated these components (enzyme activity, microbial genes, and plant stoichiometry) into a cohesive framework, especially in forest-steppe ecotones where nitrogen dynamics are inherently complex.

In the forest steppe ecotone in northwest Liaoning Province, a region experiencing rapid nitrogen deposition, critical knowledge gaps persist. First, it is unclear how nitrogen addition alters plant N/P ratios across dominant species with distinct nutrient strategies (e.g., legumes vs. non-legumes). Second, the interplay between soil enzyme activity and microbial functional genes under nitrogen enrichment remains underexplored. Third, whether enzyme-driven shifts in nitrogen conversion directly modulate plant nutrient profiles requires empirical validation. Addressing these questions is essential for predicting ecosystem responses to global change and informing sustainable management.

Here, we conducted a multi-level nitrogen addition experiment (0–40 kg N hm^{-2} a^{-1}) to investigate: (1) How nitrogen enrichment affects plant N/P ratios in dominant species (*Potentilla tanacetifolia*, *Lespedeza daurica*, *Artemisia frigida*). (2) The coupling between soil enzyme activity (e.g., urease, protease) and microbial functional genes (*nifH*, *amoA*, *nirK*). (3) Whether enzyme-mediated nitrogen conversion processes drive plant nutrient stoichiometry. By integrating microbial genomics, enzymatic assays, and structural equation modeling, we aim to unravel the cascading effects of nitrogen enrichment on ecosystem nutrient dynamics.

Materials and methods

Experimental design and sample collection

The study area is located in the Zhangwu village of northern Liaoning Province, China (42° 08′–42° 50′ N, 121° 53′–122° 58′ E). This region has a semiarid climate with an average annual precipitation of 450–500 mm and average annual temperature of 6.2–7.2 °C. Sixty percent of rainfall occurs between June and August. The soil at the site is classified as aeolian sandy soil (Chinese Soil Taxonomy: Sandy Ustic Cambisol), typical of the Horqin Sandy Land ecoregion. Key characteristics include low organic matter content (SOC: 5.22 ± 0.86 g kg^{-1} in control plots), weak acidity (pH: 6.21–6.69 across treatments), and limited nutrient retention capacity (TN: 0.12–0.40 g kg^{-1}; TP: 0.54–1.19 g kg^{-1}).

The experimental site was located in the transition zone from the western grassland to the eastern forest, and the vegetation is a community of trees, shrubs, and herbs. The predominant tree species are *Crataegus pinnatifida, Ulmus pumila, Populus simonii,* and *Salix matsudana.* Shrubs including *Ulmus davidiana, Armeniaca sibirica, Rhamnus parvifolia*, and *Lespedeza daurica,* are also present. Herbs include *Cleistogenes squarrosa, Potentilla tanacetifolia, Agropyron cristatum* and *Artemisia frigida.*

The experiment was conducted using a one-factor randomized design comprising four treatments. N was applied at rates of 0 (N0), 10 (N10), 20 (N20), and 40 kg N ha^{-1} yr^{-1} (N40), with five replicates per treatment. Each plot had an area of 2 × 2 m^2, with a 2 m-wide buffer strip separating each plot (see Supplementary Figure S1). The rates of N addition were determined based on global N-deposition levels. N was added in the form of urea ($CO(NH_2)_2$) three times in May, June, and August since 2016. The amount of fertilizer applied each time was 1/3 of the total amount applied throughout the year. The fertilizer was dissolved in 6 L of tap water and sprayed evenly in the quadrat using a watering can. For the N0 treatment, the same amount of water was used. The three dominant plants in this area were selected as the research objects, namely, *Potentilla tanacetifolia*, *Lespedeza daurica* and *Artemisia frigida.*

Sample collection began in August 2020. Ten intact healthy plants of each species were selected in each plot, and the selected plant samples were cut with scissors. The plant samples were dried at 108 °C for 2 h, and dried at 65 °C for 48 h. After that, the same number of complete leaves were selected, crushed with a grinder, and passed through a 60-mesh screen for the

Table 1 Soil physicochemical properties under different nitrogen addition treatments

Soil properties	Treatments			
	N0 (CK)	N10	N20	N40
TN (g kg^{-1})	0.12 ± 0.02^{c}	0.40 ± 0.08^{a}	0.12 ± 0.01^{c}	0.25 ± 0.08^{b}
TP (g kg^{-1})	0.54 ± 0.11	1.19 ± 0.54	0.97 ± 0.42	0.55 ± 0.25
N/P	0.21 ± 0.01^{d}	0.33 ± 0.05^{c}	0.36 ± 0.01^{b}	0.45 ± 0.04^{a}
SOC (g kg^{-1})	5.22 ± 0.86	5.79 ± 0.70	5.40 ± 1.61	5.16 ± 0.68
pH	6.69 ± 0.06^{a}	6.68 ± 0.04^{a}	6.46 ± 0.15^{ab}	6.21 ± 0.25^{b}
EC (S m^{-1})	16.86 ± 4.50	18.74 ± 2.75	14.51 ± 0.66	13.87 ± 2.56
AN (mg kg^{-1})	26.25 ± 3.50	33.25 ± 6.31	32.67 ± 4.40	30.33 ± 1.01
AP (mg kg^{-1})	5.00 ± 2.36	5.35 ± 4.57	5.18 ± 0.93	4.23 ± 0.83
NO_3^+–N (mg kg^{-1})	0.46 ± 0.02	0.53 ± 0.40	0.59 ± 0.16	0.58 ± 0.11
NH_4^+–N (mg kg^{-1})	5.11 ± 4.50^{b}	12.27 ± 0.73^{a}	5.81 ± 2.32^{b}	2.65 ± 0.49^{b}

TN total nitrogen, *TP* total phosphorus, *N/P* nitrogen phosphorus ratio, *SOC* soil organic carbon, *EC* soil electrical conductivity, *AN* alkali-hydrolyzable nitrogen, *AP* available phosphorus, NO_3^+*–N* nitrate nitrogen, NH_4^+*–N* represents ammonium nitrogen. Different lowercase letters indicate significant differences between different nitrogen addition treatments ($P < 0.05$)

determination of plant leaf elements. The "five-point sampling method" was used to collect soil samples, using a soil drill with a diameter of 5 cm and a depth of about 10 cm. The plant residues, weeds, stones and other debris in the soil sample are picked out, mixed evenly after natural air drying, and used for the determination of soil physical and chemical properties.

Physicochemical properties of plant and soil

The total nitrogen of plants was determined by Kjeldahl method. The plant total phosphorus was boiled by H_2SO_4-H_2O_2 and molybdenum-antimony resistance colorimetric method. All soil physicochemical property analyses (total soil nitrogen (TN), total phosphorus (TP), soil organic carbon (SOC), pH, electrical conductivity (EC), alkali-hydrolyzable nitrogen (AN), available phosphorus (AP), nitrate nitrogen (NO_3^+–N) and ammonium nitrogen (NH_4^+–N)) were performed according to Bao (2000) and the basic information is shown in Table 1.

Fluorescence quantitative PCR technique

Quantitative fluorescence PCR was used to determine the abundance of soil microorganisms. The reaction system was as follows: soil N-fixation, AOB, AOA, and denitrifying bacteria were used as the quantitative amplification primers (Table 2). The total volume of the amplification reaction system was 20 μL, and included 10 μL 2×GoTaq® qPCR Master Mix, 10 μmol/L upstream and downstream primers (0.5 μL each), 2 μL DNA template (1–10 ng), and 7 μL sterilized ultra-pure water. The enhanced 96-PCR plate was amplified on a quantitative fluorescence PCR instrument, with three replicates per sample. The amplification reaction conditions were as follows: predenaturation at 95 °C for 30 s, denaturation at 95 °C for 5 s, annealing at 60 °C for 40 s, extension at 72 °C for 30 s, and 40 cycles.

Soil enzyme activity

Urease was determined by hydrolysis method. The detection of nitrate reductase was carried out using the phenol disulfonate reaction method. The protease was quantified using the indophenol blue colorimetric

Table 2 Primers for PCR amplification

Microbial types	Primer name	Primer sequence	Fragment length
nifH	Upstream	AAAGGYGGWATCGGYAARTCCACCAC	432
	Downstream	TTGTTSGCSGCRTACATSGCCATCAT	
AOB-*amoA*	Upstream	GGGGTTTCTACTGGTGGT	491
	Downstream	CCCCTCKGSAAAGCCTTCTTC	
AOA-*amoA*	Upstream	STAATGGTCTGGCTTAGACG	635
	Downstream	CACCGTTTACTGCCAGGACT	
nirK	Upstream	GGMATGGTKCCSTGGCA	514
	Downstream	GCCTCGATCAGRTTRTGG	

method. Chlorotriphenylmethylene imidazolochloride (TTC) method was used for dehydrogenase.

Statistical analyses

The data from this experiment were subjected to statistical analysis, calculations, and charting using Microsoft Office Excel 2013. One-way analysis of variance (ANOVA) was performed using the software SPSS 22.0 (IBM Co., Armonk, NY, USA) to determine the differences of environmental factors among different N treatments. The significance between different treatments was confirmed using Tukey's HSD test at $P < 0.05$.

The OmicShare tool was used to create network heatmaps of environmental factors and soil nitrogen transformation functional gene abundance, plant nitrogen and phosphorus fractions. The effects of environmental factors on nitrogen transformation functional gene abundance were analyzed by constrained ordinal redundancy analysis (RDA) in CANOCO 5.0 software (Microcomputer Power, Ithaca, NY, USA). The effect of each variable was assessed using an RDA-based Monte Carlo test (999 permutations).

We utilized AMOS 7.0 software to conduct a structural equation model (SEM) in order to investigate the primary pathways that might elucidate the correlation of soil enzyme activity, abundance of functional genes related to nitrogen transformation and leaf N/P ratio. Initially, we constructed a prior model based on general ecological knowledge to establish causal relationships from our observed data. Subsequently, we calibrated the model using gene abundance data, soil enzyme activity, leaf N/P ratio and soil physicochemical properties. The initial assumption found support through its alignment with existing data. In cases where the initial model was rejected due to inadequate data fitting, alternative models were considered until satisfactory support was achieved. Ideally, subsequent research should confirm the inference made by the revised model. Finally, we employed v2 test, Bentler Bonnet normalization fit index, comparative fit index (CFI), and goodness of fit index (GFI) as evaluation metrics for scanning electron microscopy's goodness of fit assessment. The insignificant difference observed in v2 test ($P > 0.05$) provided evidence for optimal fitness of the selected SEM.

Results

Effects of nitrogen addition on N and P contents and N/P value of dominant plants

The nitrogen addition treatment had a significant impact on the nitrogen content of plant leaves ($P < 0.05$). As shown in Fig. 1a, leaf nitrogen content of *Potentilla tanacetifolia* exhibited a gradual increase with rising nitrogen application rates. The leaf nitrogen content of *Lespedeza daurica* initially increased and then decreased, while that of *Artemisia frigida* showed an initial decrease followed by an increase. Both *Potentilla tanacetifolia* and *Artemisia frigida* demonstrated peak leaf nitrogen content under N40 treatment, while *Lespedeza daurica* had the highest nitrogen content in leaves under N20 treatment. Amongst the three levels of nitrogen addition treatments, all three plants displayed their lowest leaf nitrogen content under the N10 treatment.

The plant species exerted a significant influence on the nitrogen content of plant leaves ($P < 0.05$). Substantial variations in leaf nitrogen content were observed among different plant species subjected to N10 and N20 treatments. Notably, *Lespedeza daurica* and *Potentilla tanacetifolia* exhibited the higher leaf nitrogen content under the N20 treatment, compared to *Artemisia frigida*. Similarly, under the N40 treatment, *Lespedeza daurica* had higher leaf nitrogen content than *Potentilla tanacetifolia* and *Artemisia frigida*.

Nitrogen addition had no significant effect on phosphorus content in plant leaves, while the effect of plant species on phosphorus content in leaves was highly significant ($P < 0.01$). Irrespective of nitrogen addition treatment, *Artemisia frigida* exhibited the highest phosphorus content in its leaves, whereas *Potentilla tanacetifolia* displayed the lowest phosphorus content (Fig. 1b).

Nitrogen addition had significant effect on the ratio of nitrogen to phosphorus in leaves ($P < 0.05$), and only *Artemisia frigida* was affected by nitrogen addition (Fig. 1c). Under N10 treatment, the ratio of nitrogen to phosphorus in *Artemisia frigida* leaves was significantly different from that under N0, N20 and N40 treatments, respectively, with an increase in the ratio corresponding to higher nitrogen addition rates. The nitrogen-to-phosphorus ratios of *Artemisia frigida* leaves under the N10, N20, and N40 treatments were significantly different from those of *Potentilla tanacetifolia* and *Lespedeza daurica*, with the lowest ratio observed.

Effects of nitrogen addition on the activity of enzymes related to nitrogen transformation process

The activities of urease, nitrate reductase, and soil dehydrogenase were significantly enhanced under nitrogen treatment compared with the control (CK). However, there was no significant difference among different nitrogen addition rates. The activity of soil protease increased proportionally with nitrogen addition rate; it was significantly higher in the N20 and N40 treatments than in the N10 treatment and CK. Although the activity of reductase also showed a slight increase with rising nitrogen addition rate, this difference was not statistically significant (Fig. 2). Therefore, soil protease

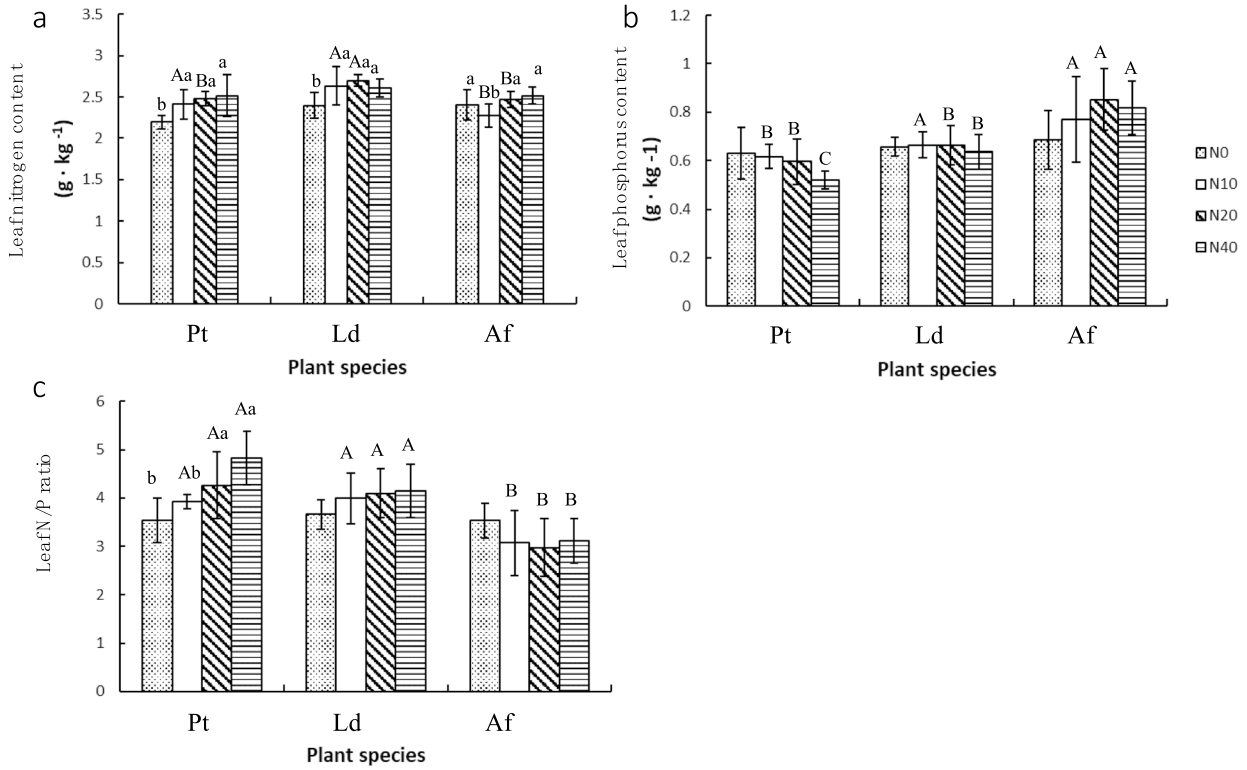


Fig. 1 Effect of nitrogen addition on leaf nitrogen (**a**), leaf phosphorus (**b**) and leaf N/P (**c**) of three plants (mean ± SD). Pt represents *Potentilla tanacetifolia*, Ld represents *Lespedeza daurica,* Af represents *Artemisia frigida*. Different lowercase letters denote the significant differences ($P < 0.05$) among nitrogen treatments within the same plant species, while different uppercase letters indicate the significant differences ($P < 0.05$) among different plant species under the same nitrogen treatment. Error bars represent the standard deviation (SD)

activity can serve as an indicator for determining nitrogen deposition levels.

Effects of nitrogen addition on nitrogen transformation functional genes

The abundance of denitrifying bacteria (*nirK*) genes ranged from 3.28×10^8 to 5.23×10^8 copies per gram of soil. The abundance increased in the N10 treatment, decreased in the N20 treatment, and then increased again in the N40 treatment, with the highest abundance observed in the N40 treatment. Overall, the abundance of denitrifying bacteria was higher in all treatments compared to that in the N0 treatment; however, these differences were not statistically significant. The abundance of nitrogen-fixing bacteria (*nifH*) genes ranged from 2.27×10^6 to 3.15×10^6 copies per gram of soil. As nitrogen application rate increased, there was an initial increase followed by a decrease in the N20 treatment and another increase in the N40 treatment, with the highest abundance found in the N10 treatment. The abundances of *nifH* genes were higher than those in control (N0) for both N10 and N40 treatments but lower for the N20 treatment; nevertheless, no significant differences were observed among treatments. The abundance of ammonia-oxidizing archaea (AOA-*amoA*) genes ranged from 5.12×10^8 to 9.03×10^8 copies per gram of soil. It was higher in the N10 treatment compared to the control, while it was lower in other nitrogen treatments than in the control. The results indicated a decrease in the abundance of AOA-*amoA* genes with increasing nitrogen addition rate. In contrast, the abundance of ammonia-oxidizing bacteria (AOB-*amoA*) genes ranged from 6.37×10^5 to 1.44×10^6 copies per gram of soil and was higher in the N10 and N40 treatments compared to the control, although this difference was not statistically significant. Notably, the number of copies of AOB-*amoA* genes in the soil was substantially lower than that of AOA-*amoA* genes (Fig. 3).

The correlations between soil nitrogen transformation functional gene abundance, plant nitrogen and phosphorus fractions, and environmental factors

NH_4^+-N was positively correlated with TN, TP and EC ($r < 0.64$, $P < 0.05$), NO_3^-–N was negatively correlated with pH ($r = -0.61$, $P < 0.05$), and SOC was positively correlated with TP ($r = 0.73$, $P < 0.01$). Soil protease

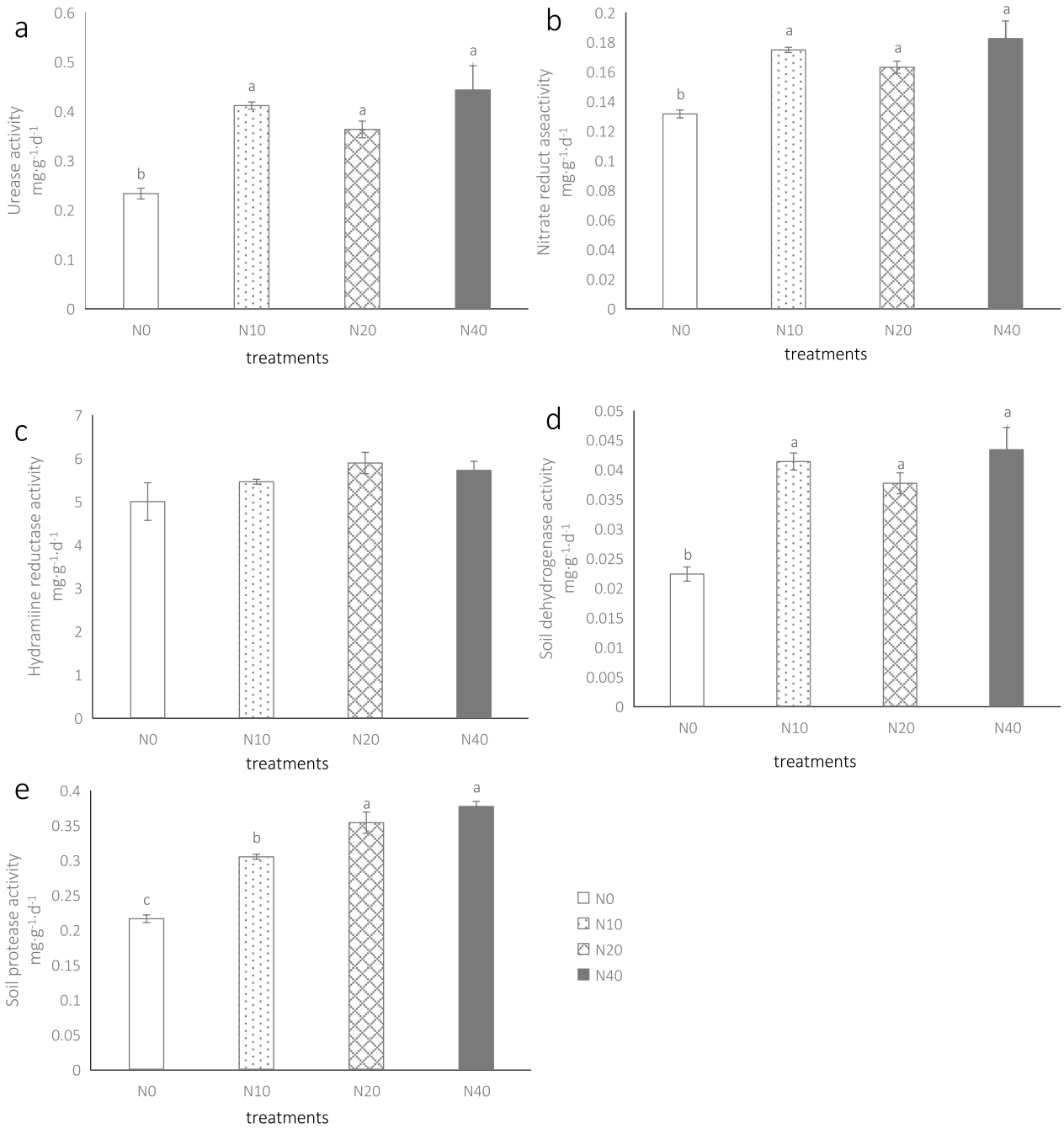


Fig. 2 Activities of five soil enzymes under different nitrogen addition rates (mean ± SD). **a** is urease activity, **b** is nitrate reductase activity, **c** is hydroxylamine reductase activity, **d** is dehydrogenase activity, and **e** is soil protease activity. Different lowercase letters denote the significant differences ($P < 0.05$) among nitrogen treatments. Error bars represent the standard deviation (SD)

activity was negatively correlated with pH ($r = -0.75$, $P < 0.05$) and positively correlated with urease activity, nitrate reductase activity and soil dehydrogenase activities ($r < 0.81$, $P < 0.01$). Mantel analysis in Fig. 4a showed that *nifH* gene abundance was significantly positively correlated with NO_3^-–N and SOC ($P < 0.05$), AOA-*amoA* gene abundance was significantly positively correlated with SOC ($P < 0.01$), and AOB-*amoA* gene abundance was significantly positively correlated with hydroxylamine reductase activity ($P < 0.05$). Figure 4b mantel analysis showed that the ratio of nitrogen and phosphorus in *Potentilla tanacetifolia* leaves was

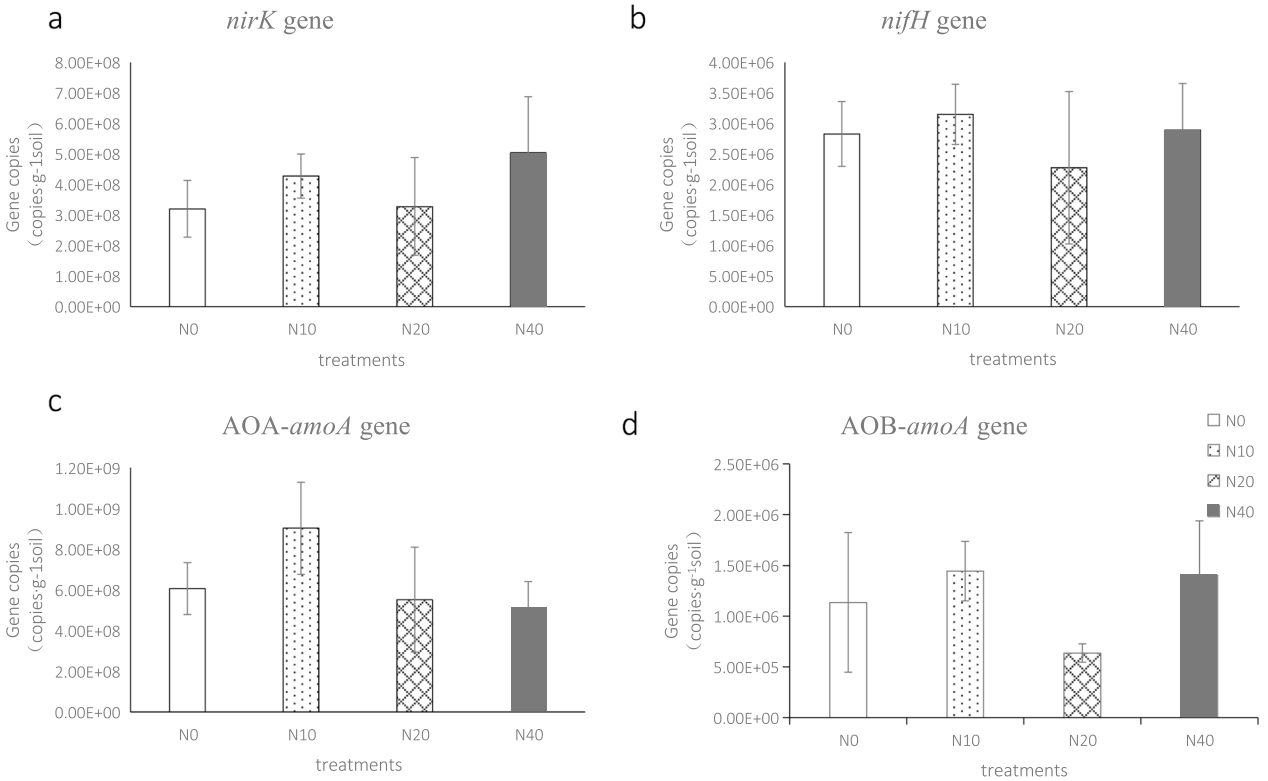


Fig. 3 Effects of nitrogen addition on the relative abundance of four functional genes (mean ± SD). **a** is denitrifying bacteria (*nirK*) gene of nitrite reduction, **b** is N-fixing bacteria (*nifH*) gene of nitrogen fixation, **c**, **d** are ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) with ammonia monooxygenase genes (*amoA*) gene. Error bars represent the standard deviation (SD)

significantly positively correlated with NO_3^-–N, pH and soil protease activities ($P<0.05$), and the ratio of nitrogen and phosphorus in *Lespedeza daurica* leaves was significantly positively correlated with AN ($P<0.05$). This indicated that NH_4^+–N and NO_3^-–N were the key factors affecting soil properties and played a leading role in the process of nitrogen conversion.

Redundancy analysis (RDA) of nitrogen transformation functional gene abundance and environmental factors

In RDA analysis, the first and second axis accounted for 83.16% and 16.84%, respectively. AOA-*amoA* gene abundance was positively correlated with phosphorus content in *Potentilla tanacetifolia*, nitrogen-to-phosphorus ratio in *Lespedeza daurica*, urease activity, and dehydrogenase activity, and negatively correlated with other factors. The correlations of abundance of *nirK* gene, AOB-*amoA* gene and *nifH* gene were the same, all positively correlated with urease activity, hydroxylamine reductase activity, soil dehydrogenase activity, phosphorus content in *Potentilla tanacetifolia* leaf, nitrogen content in *Lespedeza daurica* leaf, and nitrogen-to-phosphorus ratio in *Artemisia frigida* leaf, negatively correlated with nitrogen content in *Potentilla tanacetifolia* leaf, nitrogen-to-phosphorus ratio in *Potentilla tanacetifolia* leaf, and phosphorus content in *Lespedeza daurica* leaf. The correlation between abundance of *nirK* and AOA-*amoA* genes was the longest, indicating that its functional influence on the community was greater, occupying an important position in nitrogen transformation, and changing its abundance would affect plant stoichiometric characteristics by changing soil enzyme activity (Fig. 5).

Structural equation model analysis

The structural equation model illustrated in Fig. 6 revealed that the abundance of the *nifH* gene exerted a significant negative influence on the N/P ratio of *Potentilla tanacetifolia* ($\lambda=-0.606$, $P<0.01$), while *nirK* gene abundance demonstrated a significant positive effect on both urease and nitrate reductase activities ($\lambda=0.512$, $P<0.05$). Furthermore, the leaf N/P ratio of *Potentilla tanacetifolia* positively correlated with soil protease activity ($\lambda=0.734$, $P<0.001$). In contrast, the leaf N/P ratio of *Artemisia frigida* exhibited a significant negative relationship with hydroxylamine reductase activity ($\lambda=-0.558$, $P<0.01$), and total N showed a notable positive impact on ammonium nitrogen

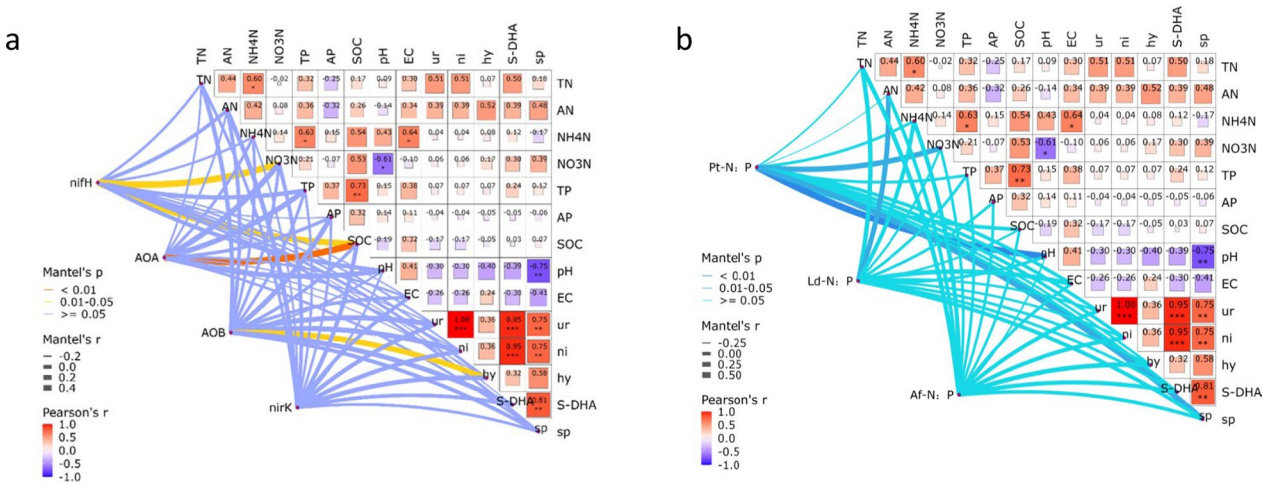


Fig. 4 Correlations between environmental factors, soil enzyme activity and functional genes of soil nitrogen conversion (**a**), leaf N/P ratio (**b**). Note: The right triangle diagram illustrates the Pearson correlation coefficients between soil physical and chemical properties and soil enzyme activity. The corresponding results of the Mantel test are presented in the left line graph. For the Mantel analysis, we utilized proportional values of soil physicochemical properties alongside enzyme activity. The width of each line reflects specific Mantel's *r* statistics, while the color denotes statistical significance based on 999 permutations. Environmental factors were compared pairwise, with the Pearson correlation coefficient represented by a color gradient. An asterisk indicates statistical significance (***$P < 0.001$; **$P < 0.01$; and *$P < 0.05$). (TN: total nitrogen, AN: alkali-hydrolyzable nitrogen, NH_4^+-N: ammonium nitrogen, NO_3^--N: nitrate nitrogen, TP: total phosphorus, AP: available phosphorus, SOC: soil organic carbon, EC: soil electrical conductivity, ur: urease activity, ni: nitrate reductase activity, hy: hydroxylamine reductase activity, S-DHA: soil dehydrogenase activity, sp: soil protease activity, nifH: N-fixing bacteria (*nifH*) gene, AOA: ammonia-oxidizing archaea with ammonia monooxygenase genes (*amoA*) gene, AOB: ammonia-oxidizing bacteria with ammonia monooxygenase genes (*amoA*) gene, nirK: denitrifying bacteria (*nirK*) gene, Pt: *Potentilla tanacetifolia,* Ld: *Lespedeza daurica,* Af: *Artemisia frigida,* N:P: nitrogen to phosphorus ratio of the leaves of the three superior plants)

concentration. Additionally, nitrate nitrogen significantly enhanced the N/P ratio of *Potentilla tanacetifolia*, and gene abundances associated with nitrogen conversion influenced leaf N/P ratios directly. Notably, there existed an interdependent relationship between leaf N/P ratios and soil enzyme activities.

Discussion

How does the addition of nitrogen affect the nitrogen and phosphorus components of plants?

Nitrogen and phosphorus are essential elements that enable plants to perform vital functions, serving as critical limiting factors for growth and development in terrestrial ecosystems. The stoichiometric ratios of these elements provide indirect insights into nutrient utilization patterns during plant growth (Xiong et al. 2020). In this study, the leaf nitrogen content of the three plants without nitrogen addition treatment was in the range of 2.19–2.40 mg g^{-1}, which was significantly lower than that of typical desert grassland plants in northern China (6.8–52.1 mg g^{-1}) (Li et al. 2010). This discrepancy can be attributed to the study area's location at the southern edge of the Horqin Sandy Land in Inner Mongolia, where aeolian sandy soils dominate. These soils have low organic matter and nitrogen content, insufficient to support optimal plant growth. Numerous studies have demonstrated that nitrogen application significantly increases leaf nitrogen content (Liu et al. 2018). In this experiment, the addition of varying rates of nitrogen revealed that the leaf nitrogen content in *Artemisia frigida* treated with N10 was significantly lower than that observed in the control group. Conversely, the leaf nitrogen content in the other three plant species exhibited a marked increase, corroborating previous findings. The observed decrease in nitrogen content in the leaves of *Artemisia frigida* may be due to the selected leaves being older, despite uniformity in size and specifications. Older leaves likely sequester more nitrogen within chloroplasts for photosynthesis, providing essential energy for plant growth, leading to reduced nitrogen levels in the leaves. Simultaneously, the data analysis from this experiment revealed that the leaf nitrogen content of *Lespedeza daurica* consistently exceeds that of *Potentilla tanacetifolia* and *Artemisia frigida*. This phenomenon can be attributed to *Lespedeza daurica* being a leguminous species capable of forming symbiotic relationships with rhizobia, enhancing its nitrogen fixation capabilities under similar environmental conditions, resulting in comparatively higher leaf nitrogen content (Bin et al. 2014).

The interplay between nitrogen and phosphorus is crucial for plant growth and development, influencing

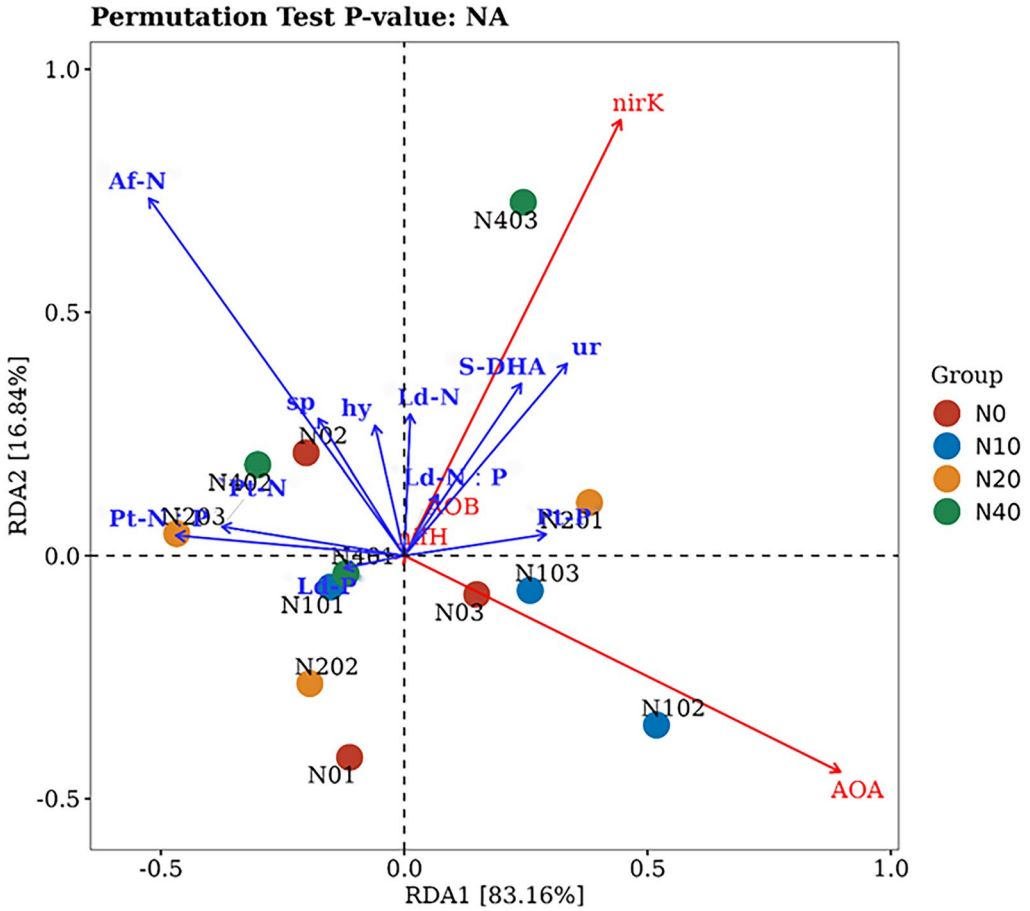


Fig. 5 RDA analysis of enzyme activity, abundance of functional genes related to nitrogen conversion and plant nitrogen and phosphorus fractions. (ur: urease activity, ni: nitrate reductase activity, hy: hydroxylamine reductase activity, S-DHA: soil dehydrogenase activity, sp: soil protease activity, nifH: N-fixing bacteria (*nifH*) gene, AOA: ammonia-oxidizing archaea with ammonia monooxygenase genes (*amoA*) gene, AOB: ammonia-oxidizing bacteria with ammonia monooxygenase genes (*amoA*) gene, nirK: denitrifying bacteria (*nirK*) gene, Pt: *Potentilla tanacetifolia*, Ld: *Lespedeza daurica*, Af: *Artemisia frigida*, N:P: nitrogen phosphorus ratio)

each other's absorption and utilization. Phosphorus plays a pivotal role in nearly all physiological processes within plants. This study revealed that the phosphorus content in the leaves of *Potentilla tanacetifolia* did not exhibit significant changes with increasing nitrogen addition rates; however, a decreasing trend was observed. This could be due to nitrogen addition treatments enhancing available nitrogen levels while soil-supplied phosphorus was insufficient to balance the elevated nitrogen within the plants. Consequently, plants must absorb and utilize more phosphorus to achieve a stable nutrient equilibrium (Li et al. 2018). The phosphorus content in the leaves of *Artemisia frigida* exhibited no significant changes following the application of varying nitrogen rates; however, a general upward trend was observed. This increase may be attributed to enhanced microbial activity induced by nitrogen addition treatments, which facilitated organic matter decomposition and increased inorganic phosphorus levels in the soil (Fujita et al. 2010). In contrast to the previous two species, the phosphorus content in the leaves of *Lespedeza daurica* exhibited a relatively stable and nearly unchanged pattern. This stability may result from the biomass growth rate exceeding the phosphorus absorption rate, leading to a "biomass dilution effect" that hinders nutrient uptake (Sterner et al. 2017).

To assess whether plant growth is constrained by nitrogen or phosphorus, the commonly used chemical index is the nitrogen-to-phosphorus (N/P) ratio in plant leaves. Studies synthesizing findings from nitrogen and phosphorus addition experiments across diverse ecosystems suggest that when the N/P ratio falls below 14, it indicates potential nitrogen limitation, while ratios above 16 suggest phosphorus limitation (Yan et al. 2008). Ecosystem diversity introduces variability in N/P ratios, influenced by factors such as study location, vegetation type, and

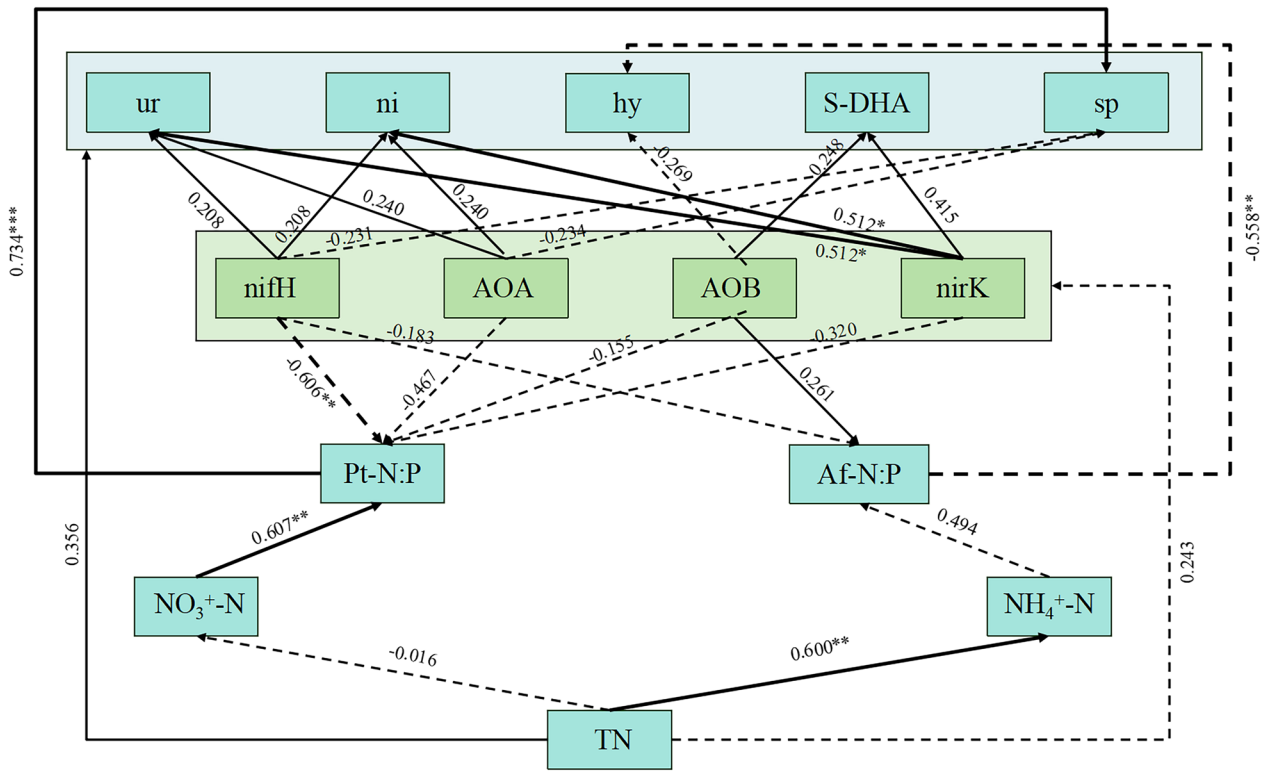


Fig. 6 Structural equation model of enzyme activity, abundance of functional genes related to nitrogen transformation and leaf N/P ratio. Note: The size of the effect was standardized, and the number next to the arrow represents the standardized regression coefficient. The arrow represented by the black solid line and dashed line represent the positive path coefficient and the insignificant path coefficient, respectively. Since the implicit covariance matrix of the model was not significantly different from the original covariance matrix (v2 test, $P > 0.05$), the model cannot be rejected (***$P < 0.001$; **$P < 0.01$; and *$P < 0.05$). (ur: urease activity, ni: nitrate reductase activity, hy: hydroxylamine reductase activity, S-DHA: soil dehydrogenase activity, sp: soil protease activity, nifH: N-fixing bacteria (*nifH*) gene, AOA: ammonia-oxidizing archaea with ammonia monooxygenase genes (*amoA*) gene, AOB: ammonia-oxidizing bacteria with ammonia monooxygenase genes (*amoA*) gene, nirK: denitrifying bacteria (*nirK*) gene, Pt: *Potentilla tanacetifolia*,, Af: *Artemisia frigida*, N:P: nitrogen phosphorus ratio)

successional stages. Therefore, assessing N/P limitations based on a single index is insufficient. Generally, a high N/P ratio suggests phosphorus limitation, while a lower ratio indicates nitrogen limitation (Liu et al. 2010). In this study, the N/P ratios of the three plant species ranged from 2.3 to 5.2, much lower than the 4.6 to 52.4 range observed in typical desert grassland plants. The relatively low N/P ratio and diminished nitrogen content in the leaves of these species may be a primary factor contributing to this phenomenon. For *Potentilla tanacetifolia*, the significant increase in leaf N/P under N40 surpassed the threshold for phosphorus limitation (> 16 in global grasslands; Güsewell 2004), yet remained below this value due to the inherently low baseline N availability in aeolian sandy soils. This paradox suggests that while nitrogen addition alleviates N limitation, it exacerbates phosphorus scarcity by stimulating biomass production without proportional P uptake—a phenomenon termed "nutrient imbalance amplification" (Fay et al. 2015). *Lespedeza daurica* (legume) maintained stable root P content despite N enrichment, likely due to rhizobial symbiosis enhancing P solubilization (Houlton et al. 2008). In contrast, *Artemisia frigida*'s stagnant growth under N40 aligns with its conservative nutrient retention strategy, prioritizing root storage over shoot growth (Bloom et al. 1985). Elevated N/P ratios in *Potentilla tanacetifolia* may grant it a competitive advantage over P-sensitive species, potentially reducing biodiversity—a pattern observed in European grasslands under chronic N deposition (Stevens et al. 2010). High N/P litter slows decomposition rates by favoring fungi over bacteria (C:N > 25; Strickland et al. 2009), creating a negative feedback loop that further restricts P cycling.

Whether there is a correlation between soil enzyme activity and soil physical and chemical properties?

Soil nitrogen mineralization is a critical and intricate process that significantly influences soil ecosystem health and plant productivity. This complex process involves multiple interacting factors, with biological components

playing a particularly important role. Soil enzymes are among the key catalysts in nitrogen conversion processes. Various types and quantities of soil enzymes participate at different stages of nitrogen transformation reactions, including ammonification, nitrification, and denitrification. For example, urease catalyzes the hydrolysis of urea into ammonia, which is then converted into ammonium ions, while nitrate reductase facilitates the reduction of nitrate to nitrite or ammonium. Abiotic factors also play a crucial role in influencing nitrogen mineralization in soils. Nutrient availability and pH levels are especially significant. The presence of nutrients directly impacts microbial activity and metabolic rates, thereby modulating enzyme activity throughout the mineralization process. Additionally, pH levels have substantial effects on specific enzyme activities. For instance, under mildly acidic conditions, certain bacterial populations may be more conducive to ammonium formation. Therefore, mobilizing substantial amounts of inorganic forms like ammonium salts for plant uptake requires multiple steps influenced by various internal and external environmental factors. According to Xue et al. (2003), urease activity can serve as a reliable indicator for assessing the mineralization rate of organic nitrogen under specific conditions. Correlation and redundancy analyses have revealed that soil pH significantly influences the activities of several key soil enzymes involved in carbon and nitrogen cycles, including urease, nitrate reductase, and protease. These findings align with previous research on enzyme activities in forest soils (Chen et al. 2023). However, the extent and direction of pH influence vary across different regions and vegetation types. On one hand, soil pH affects microbial activity due to the varying optimal ranges among different microorganisms; bacteria generally exhibit greater sensitivity to pH changes compared to fungi. On the other hand, soil pH can also influence enzyme activity by altering the conformation between enzymes and substrates, as well as their adsorption characteristics within the soil matrix.

Soil moisture conditions significantly influence microbial activity, which in turn affects enzyme secretion and functionality. Optimal soil moisture levels enhance the enzymatic activity in the soil (Henry et al. 2005). In various ecosystems, a strong correlation exists between NO_3^--N content and the abundance of functional genes involved in nitrogen cycling (e.g., *amoA*, *nirK*). Consequently, we assessed nitrogen convertase activity by measuring NO_3^-–N concentrations in this study. Our findings indicated that nitrogen addition had a marked impact on soil enzymes, with high nitrogen addition rates leading to a substantial increase in enzyme activity. This observation aligns with previous studies conducted in the alpine grasslands of northern Tibet and global meta-analyses on nutrient additions, both of which suggest that increased nitrogen supplementation correlates with enhanced soil enzyme activity (Li et al. 2022). The primary factor driving the increase in the soil N/P ratio is the rise in nitrogen content and its availability due to added nitrogen.

Soil acidification induced by nitrogen addition emerged as a critical mechanism regulating enzyme activity. Urea hydrolysis releases NH_4^+, which undergoes nitrification ($NH_4^+ \rightarrow NO_3^-$), generating H^+ ions and lowering soil pH (Zhang et al. 2012). In our study, pH decreased significantly under N40 treatment (Table 1), aligning with observed increases in urease and protease activities (Fig. 2). Urease, which catalyzes urea hydrolysis, exhibits optimal activity at pH 6.5–7.0 (Hong et al. 2022). The pH decline in N40 plots (6.21 ± 0.25) likely shifted urease conformation, enhancing its substrate affinity. Conversely, protease activity surged proportionally with nitrogen input, possibly due to increased NH_4^+ availability (a byproduct of urea decomposition) serving as both a substrate and microbial energy source (Chen et al. 2023). Notably, hydroxylamine reductase activity showed no significant response, suggesting its insensitivity to short-term pH fluctuations but potential reliance on NO_2^- availability, which remained unchanged across treatments.

Can the change of enzyme activity caused by nitrogen addition altered the microorganisms involved in the nitrogen conversion process and further affect the nitrogen and phosphorus components of plants?

Nitrogen addition altered microbial functional gene abundance, indirectly modulating enzyme production. For instance, AOB-*amoA* gene abundance increased under N10 and N40 (Fig. 3d), correlating with elevated urease activity ($\lambda = 0.512$, $P < 0.05$; Fig. 6). AOB thrive in NH_4^+-rich environments (Shen et al. 2011), and their proliferation likely enhanced ammonification, releasing NH_4^+ to fuel urease synthesis. Conversely, AOA-*amoA* declined with nitrogen input, as archaeal ammonia oxidizers are often suppressed by high NH_4^+ (Ning et al. 2015). This niche partitioning between AOB and AOA underscores pH-mediated shifts in microbial dominance, which cascade to enzyme dynamics.

The rise in protease activity under nitrogen addition (Fig. 2e) may stem from substrate priming. Proteases require organic N substrates (e.g., proteins), which increase as nitrogen addition stimulates plant litter production (Liu et al. 2018). Enhanced protease activity subsequently liberated amino acids, elevating NH_4^+ pools (Table 1) and creating a feedback loop that further promoted AOB activity. These intertwined microbial and

enzymatic responses directly impacted plant N/P ratios. For example, the negative correlation between *nifH* abundance and *Potentilla tanacetifolia* N/P ($\lambda = -0.606$, $P < 0.01$; Fig. 6) implies that nitrogen fixation compensated for soil N limitations, reducing plant reliance on N uptake and balancing N/P stoichiometry.

This study elucidates the mechanistic links between nitrogen addition and soil nitrogen dynamics; however, certain limitations warrant consideration. First, the four-year experimental period (2016–2020) may obscure long-term microbial community succession and enzymatic adaptation, as transient gene abundance shifts (e.g., AOA-*amoA*) might stabilize or reverse over decadal scales under sustained nitrogen loading. Second, while nitrogen inputs were controlled, unregulated environmental variables—notably annual precipitation (450–500 mm) and temperature fluctuations (6.2–7.2 °C)—could confound enzyme activity patterns through episodic modulation of microbial metabolism and substrate diffusion. Third, geographic specificity (single forest-steppe ecotone in semi-arid northwest Liaoning) limits extrapolation to humid or high-latitude ecotones, where divergent hydrological and thermal regimes may alter nitrogen conversion pathways. Finally, focusing solely on functional gene quantification (e.g., *nifH*, *amoA*) precludes insights into taxonomic diversity, non-culturable microbial guilds, and metabolic plasticity—critical drivers of enzymatic resilience. Future work should prioritize multi-decadal monitoring to resolve legacy effects, incorporate multifactorial designs (e.g., N and precipitation gradients) to disentangle environmental interactions, and employ metagenomic/metatranscriptomic approaches to map taxonomic-functional linkages. Cross-regional replications in contrasting biomes will further validate the universality of observed mechanisms.

Conclusion

Nitrogen addition in forest-steppe ecotones drives divergent plant stoichiometric responses and microbial-enzymatic cascades, as demonstrated by four-year experimental data. Elevated nitrogen inputs significantly increased leaf nitrogen content but not phosphorus, amplifying N/P ratios and revealing species-specific nutrient constraints. Mechanistically, urea hydrolysis and subsequent nitrification reduced soil pH, optimizing urease activity through conformational shifts, while NH_4^+ enrichment stimulated AOB-*amoA* gene proliferation, reinforcing ammonification. These enzymatic-microbial interactions directly regulated plant N/P stoichiometry, evidenced by structural equation models linking *nifH* suppression to *P. tanacetifolia* N/P reduction. To mitigate phosphorus limitation risks in non-leguminous species and acidification-driven enzymatic feedbacks, we advocate integrating soil buffering capacity into regional nitrogen management frameworks. Future studies should prioritize decadal monitoring to resolve legacy effects, employ multi-omics to map taxon-specific enzymatic drivers, and validate mechanisms across biomes to universalize ecological predictions.

Abbreviations

Pt	*Potentilla tanacetifolia*
Ld	*Lespedeza daurica*
Af	*Artemisia frigida*
TN	Total nitrogen
TP	Total phosphorus
N:P	Nitrogen phosphorus ratio
SOC	Soil organic carbon
EC	Electrical conductivity
AN	Alkali-hydrolyzable nitrogen
AP	Available phosphorus
NO_3^+–N	Nitrate nitrogen
NH_4^+–N	Ammonium nitrogen

Supplementary Information

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Additional file 1.

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Author contributions
B.R., L.B. and J.L. designed this study. D.L., H.L. and M.M. performed the laboratory analysis. H.L. and B.R. wrote the paper. All authors reviewed the manuscript and approved for publication.

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Data availability
Data will be made available on request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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