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Role of plant species and nitrogen compounds

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Abstract

Determining the abundance of N isotope (δ15N) in natural environments is a simple but powerful method for providing integrated information on the N cycling dynamics and status in an ecosystem under exogenous N inputs. However, whether the input of different N compounds could differently impact plant growth and their 15N signatures remains unclear. Here, the response of 15N signatures and growth of three dominant plants (Leymus chinensis, Carex duriuscula, and Thermopsis lanceolata) to the addition of three N compounds (NH4HCO3, urea, and NH4NO3) at multiple N addition rates were assessed in a meadow steppe in Inner Mongolia. The three plants showed different initial foliar δ15N values because of differences in their N acquisition strategies. Particularly, T. lanceolata (N2-fixing species) showed significantly lower 15N signatures than L. chinensis (associated with arbuscular mycorrhizal fungi, AMF) and C. duriuscula (associated with AMF). Moreover, the foliar δ15N of all three species increased with increasing N addition rates, with a sharp increase above an N addition rate of ~10 g N m⁻² yr⁻¹. Foliar δ15N values were significantly higher when NH4HCO3 and urea were added than when NH4NO3 was added, suggesting that adding weakly acidifying N compounds could result in a more open N cycle. Overall, our results imply that assessing the N transformation processes in the context of increasing global N deposition necessitates the consideration of N deposition rates, forms of the deposited N compounds, and N utilization strategies of the co-existing plant species in the ecosystem.

Key words: δ15N, arbuscular mycorrhiza, foliar N concentration, inorganic N, N fixation, N deposition, N compounds
1. Introduction

Human activities, such as fossil fuel combustion and increased fertilizer application, have increased the deposition of large amounts of reactive nitrogen (N) in terrestrial ecosystems, and this is expected to increase in the future (Galloway et al., 2008; Peñuelas et al., 2013). Previous studies have shown that increased soil N availability can alleviate N limitation in terrestrial ecosystems, thereby increasing plant productivity (Liang et al., 2020; Xia & Wan, 2008); however, this can also simultaneously decrease biological diversity (Stevens et al., 2004; Tian et al., 2022). To date, numerous N addition experiments have simulated the effects of increasing N deposition on plant traits and plant growth by adding an N compound, mostly inorganic N ammonium nitrate (NH$_4$NO$_3$). However, atmospherically deposited N includes various forms of N compounds (Cornell, 2011; Jia et al., 2016; Violaki et al., 2010), including the reduced and oxidized forms of inorganic N, such as ammonium bicarbonate (NH$_4$HCO$_3$), NH$_4$NO$_3$, and ammonium sulfate ((NH$_4$)$_2$SO$_4$) (Stevens & Gowing, 2014; Stevens et al., 2011), along with organic N compounds, such as urea (CO(NH$_2$)$_2$), free amino acids, and other methylated amines (Neff et al., 2002). Different N compounds can result in different soil N transformation processes, and consequently, different soil N dynamics and statuses (Cheng et al., 2019; Cowan et al., 2019; Du et al., 2021). They can also induce different extents of soil acidification (Hao et al., 2020; Liu et al., 2021; Schroder et al., 2011). Thus, the “openness” of the N cycle is highly likely to be specific to N-compounds, as N losses via NH$_3$ volatilization and denitrification are higher when the soil is less acidified with addition of weakly acidifying N compound, other conditions being equal (Pan et al., 2016; Sun et al., 2012). Further, because soil is the major nutrient source for terrestrial plants, soil properties (pH and N availability) influenced by the input of various N compounds can affect plant traits and growth differently; however, the links between these factors have rarely been assessed previously.
The natural abundance of stable N isotope (δ^{15}N) ratios is a reliable indicator for assessing N dynamics in an ecosystem (Chen et al., 2022; Kahmen et al., 2008; Sheng et al., 2019). Exogenous N inputs can increase soil ^{15}N signatures because N enrichment can stimulate N transformations (e.g., nitrification and denitrification) and N losses (e.g., NH₃ volatilization and N₂O emissions), which all discriminate against the heavier N isotope (^{15}N) (Cheng et al., 2019; Gurmesa et al., 2022a; Högberg, 1997; Robinson, 2001). Plant δ^{15}N is closely related to the ^{15}N signal of their N source (Takebayashi et al., 2010), and it increases with increased soil δ^{15}N following exogenous N inputs (Pardo et al., 2007; Yan et al., 2020). In addition to the ^{15}N signature of the soil N pool, other factors, such as N acquisition strategies (via mycorrhiza or biological N fixation), preferential uptake of certain N forms (NH₄⁺-N, NO₃⁻-N, or dissolved organic N, hereafter DON), and rooting depth of plants (Craine et al., 2015; Hobbie & Hogberg, 2012), can also modify plant δ^{15}N. For instance, the δ^{15}N value of N₂-fixing plants is usually close to 0‰ and lower than that of non-N₂-fixing plants in N-limited ecosystems, because the latter mainly relies on soil N, which is more enriched with ^{15}N than atmospheric N₂ (Unkovich et al., 1994). Similarly, mycorrhizal fungi transfer ^{15}N-depleted N to plants at low N availability, resulting in lower δ^{15}N values in mycorrhizal plants than the co-existing non-mycorrhizal plants (Hobbie & Hogberg, 2012). However, N enrichment could prevent the discrimination against ^{15}N among co-existing species, which exhibit different N acquisition strategies, by weakening the importance of the associated microbes (N₂-fixing bacteria and mycorrhizal fungi) for plant N uptake (Jach-Smith & Jackson, 2020; Skogen et al., 2011), consequently, leading to a convergence of ^{15}N signatures in plants with different N acquisition strategies. Further, depending on the N accessibility, plants acquire different N forms, each of which has a unique signature and can be impacted by exogenous N inputs (Craine et al., 2009; Kahmen et al., 2008). For instance, higher foliar δ^{15}N may reflect preferential NH₄⁺-N uptake (Falkengren-Garerup et al., 2004; Kahmen et al., 2008). Variations among different plant ^{15}N signatures within a site could also be attributed to the soil depth where N is acquired, as soil δ^{15}N
increases with increasing depths (Craine et al., 2015). Collectively, the natural abundance of $^{15}$N in plants can provide integrated information on soil N dynamics and plant N acquisition strategies.

Temperate grassland ecosystems in China are N-limited, floristically diverse, and sensitive to N enrichment (Zhang et al., 2018). Previous studies have shown that N enrichment increases leaf N concentration and stimulates aboveground biomass of plant communities, and simultaneously decreases plant species richness, consequently, altering the community structure (Bai et al., 2010; Yang et al., 2019; Zhang et al., 2016). However, whether this role of N compounds can be detected using foliar $^{15}$N signatures, is yet to be assessed, particularly at the same site.

In this study, we aimed to investigate the variations in foliar $^{15}$N signatures after adding three different N compounds during an N-enrichment field experiment conducted for six years in a meadow steppe in Inner Mongolia. Before formulating our working hypotheses, we drew a logical workflow diagram of the main variables to be measured and a brief explanation of the purpose of these measurements (Fig. S1). We then sketched a conceptual framework illustrating the major factors that could affect foliar $^{15}$N signatures under various N input rates (Fig. 1). Nitrogen cycling includes various N transformation processes, such as volatilization, nitrification, and denitrification (Fig. 1①), all of which affect $^{15}$N signatures both in soil and plant tissues. As an integrated indicator, foliar $\delta^{15}$N values reflect the openness of the N cycle in a given ecosystem. Among the existing myriad factors that influence foliar $\delta^{15}$N values, in this study, we focused only on the N acquisition strategies of plants (Fig.1②), ability/preference of plants to access different N forms (Fig.1③) and N uptake of plants at different rooting depths (Fig.1④). We hypothesized that: 1) Foliar $\delta^{15}$N would increase with N addition rates across different plant species because N inputs can stimulate most soil N cycling processes. 2) The mycorrhiza-associated, non-N$_2$-fixing plants would have higher initial foliar $\delta^{15}$N values than the N$_2$-fixing plants, but these differences would be eliminated with N addition, as the plants would source N from the same soil N pool. 3) Addition
of weakly acidifying N compounds would result in more open N cycles, which could be reflected by relatively higher $^{15}$N signatures in plant leaves.

2. Materials and methods

2.1 Study area and experimental design

The study was conducted in a meadow steppe at the Erguna Forest-Steppe Ecotone Research Station ($50^\circ 12'\ N, 119^\circ 23'\ E$) of the Institute of Applied Ecology, Chinese Academy of Sciences, Inner Mongolia, China. The mean annual precipitation and temperature in this area are approximately 360 mm and $-2.5^\circ C$ (1957–2016), respectively (Yang et al., 2019, 2022). The soil is classified as Chernozem with 49.67% sand, 31.57% silt and 18.76% clay and a pH (0–10 cm) of $6.81 \pm 0.09$ (Peng et al., 2022), according to the classification by Food and Agricultural Organization (IUSS Working Group WRB 2014). The soil organic carbon and total nitrogen content, total and available phosphorus concentration in the topsoil (0–10 cm) are 38.27 ± 0.88 g kg$^{-1}$, 3.17 ± 0.07 g kg$^{-1}$, 401.85 ± 15.50 mg kg$^{-1}$ and 5.40 ± 0.25 mg kg$^{-1}$, respectively (Peng et al., 2022). The growing season in the region extends from June to September, which receives more than 70% of the total annual precipitation. The vegetation is dominated by *Leymus chinensis*, *Carex duriuscula*, *Thermopsis lanceolata*, *Stipa baicalensis*, *Cleistogenes squarrosa*, and *Cymbaria dahurica*. Here, we selected the following three most dominant species belonging to different functional groups: a grass (*L. chinensis*) and a sedge (*C. duriuscula*), which host arbuscular mycorrhizal symbionts, and a legume (*T. lanceolata*), which hosts rhizobial symbionts. Together, these three species accounted for approximately 70% of the aboveground biomass of the plant community when the N addition treatment was initiated.

This study was a part of an ongoing long-term N addition experiment, which began in 2014. The experiment included 60 treatments with eight blocks as replicates, each consisting of six N addition
rates, that is, 0 (control), 2, 5, 10, 20, and 50 g N m$^{-2}$ yr$^{-1}$, added as five different N compounds, namely, NH$_4$HCO$_3$, urea, slow-release urea, NH$_4$NO$_3$, and (NH$_4$)$_2$SO$_4$, and two grassland use types (mown vs. non-mown). The N addition rates cover a gradient from the background N deposition rate in this area to documented amounts of N fertilizer application, along with long-term, extreme N addition experiments in the steppe ecosystem (Li et al., 2015a; Wang et al., 2019). In total, there were 480 experimental plots (10 m × 10 m), with plots separated by 1 m walkways and blocks separated by 2 m walkways. Every year in late May, N fertilizers mixed with sand (0.5 kg per plot) were applied to the plots. We used only NH$_4$HCO$_3$, urea, and NH$_4$NO$_3$ treatments in the unmown plots with six blocks in which all the three species were encompassed in the sampling quadrats. However, N additions decreased the occurrence of C. duriuscula and T. lanceolata in the study site. For this reason, we had the sample number that was less than 6 in occasional cases. Only these three N compounds were selected because they represented the reduced (NH$_4$HCO$_3$), oxidized (NH$_4$NO$_3$), and organic (urea) forms of N fertilizer. Further, we defined 0–5 g N m$^{-2}$ yr$^{-1}$ and 10–50 g N m$^{-2}$ yr$^{-1}$ as low and high N addition levels, respectively.

2.2 Sampling and measurements

We sampled healthy leaves of similar sizes at the beginning of August 2019 from 20 individuals of each species. After drying at 65 °C for 48 h, leaf samples were ball-milled to a fine powder to determine the N concentration with a Vario El cube CHNS elemental analyzer (Elementar Analysensysteme, Germany) and the natural $^{15}$N abundance in leaves was determined using an isotopic-ratio mass spectrometer (IRMS; Thermo Finnigan MAT DELTA$^+$ XP; Thermo Scientific, Germany). The aboveground biomass of the entire plant community was determined annually in every plot in mid-August from a 1 m × 1 m quadrat by cutting all vascular plants from the soil surface; subsequently, all cut plants were sorted according to species, oven-dried at 65 °C for 48 h, and weighed.
Soil samples from the surface (0–10 cm) and subsurface (10–20 cm) layers were collected in August 2019 from the plots treated with NH₄HCO₃, urea, and NH₄NO₃ using a 3-cm diameter auger. Three soil cores from each soil layer of each plot were randomly collected, homogenized into one composite sample per plot, sieved using a 2-mm mesh size sieve, and then divided into three subsamples. One subsample was stored at 4 °C for analyzing the inorganic N (NH₄⁺ and NO₃⁻) concentrations, the second subsample was stored at −80 °C for measuring the nitrogenase gene (**nifH**), and the third subsample was air-dried to determine the total soil N content. To measure inorganic N concentrations (nitrate, NO₃⁻-N, and ammonium, NH₄⁺-N; expressed as mg kg⁻¹ dry soil), approximately 10 g of fresh soil from each sample was extracted with 2 M KCl; subsequently, the mixture was shaken, filtered, and analyzed using an AA3 continuous flow analyzer (Bran+Luebbe, Germany). Previous studies implied that N₂ fixation mainly occurred in the topsoil and the soil **nifH** gene abundance sharply decreased with the soil depth (Mergel et al., 2001; Song et al., 2019). Hence, **nifH** abundance was quantified in the surface layer using a LineGene 9600 Plus real-time quantitative PCR (qPCR) detection system (Bioer Technology Co., Ltd, Hangzhou, China) using the primers PolF (5’-TGCGAYCCSAARGCBGACTC-3’) and PolR (5’-ATSGCCATCATYTCRCCGGA-3’) (Poly et al., 2001). Quantification was performed in 20 μL of reaction mixture containing 10 μL ChamQ SYBR Color qPCR Master Mix (2X), 0.4 μL of each primer (5 μM), 2 μL of template DNA, and 7.2 μL of double-distilled water (ddH₂O). The qPCR program was as follows: denaturation at 95 °C for 5 min and 40 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 40 s. The efficiency of **nifH** amplification was 92.53% with an R² value of 0.9983.

After the soil samples were air-dried and ball-milled, total soil N concentration was measured using the same elemental analyzer as that used for leaf N concentration mentioned above. Water suspensions (soil: water = 1:5, w/v) of the air-dried soil samples were used to measure the pH using a pH meter (S210 SevenCompact™, Metter, Germany).
As grasses and sedges are usually colonized by arbuscular mycorrhizal fungi (AMF) (Li et al., 2015b; Zhu et al., 2020), root samples to measure AMF colonization were collected at the beginning of August 2020 from the plots treated with the selected three N compounds. Sufficient root samples of *L. chinensis* and *C. duriuscula* were collected only up to a depth of 20 cm because their roots are mainly distributed within the 0–20 cm soil depth (Wang et al., 2017). The AMF colonization of *T. lanceolata* could not be analyzed because it is a creeping-rooted species having a deep root system (> 50 cm) with few fine roots (Wang et al., 2017). At least six individuals of each dominant species were collected per plot and cleaned with distilled water. Their roots were cut into 1-cm segments, which were subsequently soaked in 10% (w/v) KOH at 90 °C (water bath heating) for approximately 2 h. After cooling, the samples were washed with distilled water, acidified with 2% HCl at room temperature for 5 min, stained with 0.05% trypan blue at 90 °C for 15 min, and then decolorized in lactoglycerol solution (lactic acid, glycerin, and water, 1:1:1 v/v) at 20 °C for 72 h (Blanke et al., 2005; Zheng et al., 2018). For each replication, at least 50 root segments were randomly selected and permanently mounted on microscope slides in polyvinyl-lactoglycerol. The samples were then observed under a light microscope to estimate the occurrence frequency of AMF structures (hyphae, vesicles, and arbuscules) in the root samples. AMF colonization was expressed as a percentage of the total root segments scored.

**2.3 Statistical analysis**

The relative biomass of each dominant plant species was calculated in relation to the total aboveground biomass of the entire plant community in each plot. The values of foliar N concentration, relative biomass, AMF colonization rate, and soil inorganic N (NH$_4^+$-N, NO$_3^-$-N) concentrations were log-transformed before analysis to meet the assumptions of normality and homogeneity of variance. Mixed linear models (“lmer” function in the “lme4” package) were used to analyze variances in the total soil N content, pH, soil inorganic N concentration, foliar δ$^{15}$N, foliar N concentration, relative biomass, AMF colonization rate, and *nifH* gene abundance.
according to N addition rate, N compounds, soil layer or species, with the interactions among these parameters expressed as fixed factors, and the blocks as random factors. Regression models were used to determine the effects of N addition on the above mentioned responses and the relationship between the \( \text{NH}_4^+:\text{NO}_3^- \) ratio and foliar \( \delta^{15}\text{N} \). Differences in foliar \( \delta^{15}\text{N} \), foliar N concentration, and relative biomass among N compounds at low and high N addition rates were determined by two-way analysis of variance (ANOVA), followed by Duncan’s multiple range tests \( (p < 0.05) \). One-way ANOVA followed by Duncan’s test were used to compare the effects of different N compounds on soil pH and foliar \( \delta^{15}\text{N} \). “Rmisc”, “dply”, “basicTrendline”, and “ggplot2” were used to develop the models and graphs.

Further, a structural equation model (SEM) was used to quantify the direct and indirect effects of the N addition rates, types and plant species on the foliar \( \delta^{15}\text{N} \) values. We developed an a priori causal model that includes all possible pathways (Fig. S2). In the a priori model, we predicted that N addition rates, types and plant species would directly induce changes in foliar \( \delta^{15}\text{N} \) values, and indirectly impact the \( \delta^{15}\text{N} \) values via their effects on soil and plant variables. Doing this, we employed piecewise SEM using the “psem” function from the package “piecewiseSEM” (Lefcheck, 2016). We included plant species and N types in the model as categorical variables. As the “piecewiseSEM” currently did not produce standardized estimates for categorical variables, a wrapper function was used with the package “emmeans” (Lenth et al., 2020) to compute marginal means at each level of the categorical variables (Dossa et al., 2021; Marker et al., 2022). The “emmeans” function also provided pairwise comparison of the marginal means. All variables were z-scaled prior to analysis for the SEM. We created the model with linear mixed models using “lmer” function (see above). Directional-separation tests were applied to check for any missing significant paths and whether or not unnecessary insignificant paths need to be excluded. We refined the a priori model by dropping non-significant links, starting with the least significant link, and continuing stepwise until changes in the Akaike information criteria (AIC) were <2. Overall fitness
of the piecewise SEM was assessed using AIC, Fisher's C statistic and \( p \)-values (where \( p > 0.05 \) was considered adequate). All statistical analyses were performed using R 4.2.1 (R Core Team, 2022).

3 Results

3.1 Response of foliar \( \delta^{15}N \), N concentration, and relative biomass to N addition

The foliar \( \delta^{15}N \) of \( L. \ chinensis \), \( C. \ duriuscula \), and \( T. \ lanceolata \) generally increased with N addition rates, with a steep increase observed above N addition rates of 10 g N m\(^{-2}\) yr\(^{-1}\) (Fig. 2a-c, Table 1). Significantly higher \( \delta^{15}N \) values were found in plots treated with \( NH_4HCO_3 \) and urea than in plots treated with \( NH_4NO_3 \) (Fig. 2d). This trend was observed at both low (\( \leq 5 \) g N m\(^{-2}\) yr\(^{-1}\)) and high (\( \geq 10 \) g N m\(^{-2}\) yr\(^{-1}\)) N addition rates (\( p < 0.05 \); Fig. 2d). We also found that foliar \( \delta^{15}N \) varied between species, with \( L. \ chinensis \) exhibiting the highest values both before and after N addition, followed by \( C. \ duriuscula \) and \( T. \ lanceolata \). The foliar \( \delta^{15}N \) value of the species converged only after \( NH_4NO_3 \) addition.

Foliar N concentration of \( L. \ chinensis \) and \( C. \ duriuscula \) significantly increased with increasing N addition (\( p < 0.01 \)), regardless of the N compound, while that of \( T. \ lanceolata \) increased significantly only after \( NH_4NO_3 \) addition (\( p < 0.06 \); Fig. 2e–g, Table 1). The foliar N concentration was significantly higher in all species at high N addition rates (\( \geq 10 \) g N m\(^{-2}\) yr\(^{-1}\)) than that at low N addition rates (\( \leq 5 \) g N m\(^{-2}\) yr\(^{-1}\)) for urea and \( NH_4NO_3 \) additions (\( p < 0.05 \)), but not for \( NH_4HCO_3 \) addition (Fig. 2h). However, the foliar N concentrations of the three species did not significantly differ under treatments with the three N compounds at either low or high addition rates (Fig. 2h).

The relative biomass of the three investigated plants showed significantly different response patterns to increasing N addition rates but the biomass was not significantly different among the N compounds (Fig. 3, Table 1). The relative biomass of \( L. \ chinensis \) increased with increasing N
addition rates, while that of *C. duriuscula* decreased. Contrastingly, the relative biomass of *T. lanceolata* remained unaffected by N addition.

### 3.2 Response of soil physicochemical properties to N addition

Overall, the total soil N content did not significantly differ among the N compounds (Fig. S3). Nitrogen addition only increased the total N content in the subsurface (10–20 cm) and surface (0–10 cm) soil layers of the NH₄HCO₃ (R² = 0.68, *p* = 0.03) and NH₄NO₃ (R² = 0.72, *p* = 0.02) treatments, respectively, with the surface soil layer exhibiting a higher total N content than the subsurface soil layer under all three N compound treatments (NH₄HCO₃, urea, and NH₄NO₃) (Fig. S3a–c). The soil pH declined with increasing N addition rates for all three N compounds (Fig. S4). Overall, the lowest pH was found in the plots treated with NH₄NO₃, while the plots treated with NH₄HCO₃ and urea exhibited relatively higher soil pH both in the surface and subsurface soils.

Soil NH₄⁺-N and NO₃⁻-N concentrations increased with increasing N addition rates in both surface and subsurface soil layers under all three N compounds, except for NH₄⁺-N concentration in the subsurface soil layer treated with NH₄HCO₃ (Fig. 4a–f). Particularly, the NH₄⁺-N concentration in the surface soil layer was higher than that in the subsurface soil layer for both urea and NH₄NO₃ treatments. However, the NH₄⁺-N concentrations in the surface and subsurface soil layers did not differ significantly with NH₄HCO₃ addition (Fig. 4a–c, inserts, Table 2). Moreover, NO₃⁻-N concentration did not significantly differ between both soil layers with urea and NH₄NO₃ additions, but it was significantly higher in the surface soil layer with NH₄HCO₃ addition only at high N addition rates (Fig. 4d–f, inserts, Table 2). Additionally, the NH₄⁺-N concentration was significantly higher under the NH₄NO₃ treatment than in the NH₄HCO₃ and urea treatments (*p* < 0.05).

The NH₄⁺:NO₃⁻ ratio of the surface soil layer was not significantly different across all N addition rates under NH₄HCO₃ and urea treatments, but it increased significantly with N addition in the...
NH$_4$NO$_3$ treatment (Fig. 4g–i). Contrastingly, the NH$_4^+:$NO$_3^-$ ratio decreased with increasing N addition rates in the subsurface soil layer for all three N compounds. Moreover, the NH$_4^+:$NO$_3^-$ ratio was higher in the subsurface soil layer under NH$_4$HCO$_3$ addition (1.06 ± 0.09 vs. 2.09 ± 0.26 for surface and subsurface soil layers, respectively) and urea addition (1.38 ± 0.15 vs. 1.86 ± 0.25 for surface and subsurface soil layers, respectively), while it significantly increased in the surface and decreased in the subsurface soil layers with N addition in the NH$_4$NO$_3$ treatment (6.25 ± 0.81 vs. 2.82 ± 0.47 for surface and subsurface soil layers, respectively).

3.3 Response of AMF colonization rates and soil nifH gene copies to N addition

Nitrogen addition reduced AMF colonization of *L. chinensis* under urea and NH$_4$NO$_3$ addition and that of *C. duriuscula* under NH$_4$HCO$_3$ and urea additions (Fig. 5a–b, Table 1). NH$_4$NO$_3$ addition resulted in the lowest AMF colonization in these two dominant species. Particularly, AMF colonization values for NH$_4$HCO$_3$, urea, and NH$_4$NO$_3$ additions were 10.6% ± 1.7%, 31.9% ± 3.6%, and 7.5% ± 1.2%, respectively, for *L. chinensis* and 5.6% ± 1.1%, 8.6% ± 1.5%, and 4.8% ± 1.1%, respectively, for *C. duriuscula*. Soil nifH gene copies also decreased with increasing N addition rates in the NH$_4$HCO$_3$ and NH$_4$NO$_3$ treatments (Fig. 5c, Table 1), and NH$_4$NO$_3$ addition had the strongest negative effect on nifH gene copies in soil.

3.4 Structural equation modeling

The SEM for the response of foliar $\delta^{15}$N indicated a good fit with the selected data (AIC = 178.23, Fisher's C = 62.23, $p = 0.21$). The results of SEM showed that both N addition rates and types had direct and indirect (through soil pH, NH$_4^+$-N and NO$_3^-$-N concentration) effects on the foliar $\delta^{15}$N (Fig. 6). Plant species only showed direct impacts on the foliar $\delta^{15}$N, and no significant indirect effects were detected on the foliar $\delta^{15}$N via relative biomass and N acquisition strategies (AMF or nifH). Taken together, these pathways explained 74% of the total variance of foliar $\delta^{15}$N.
4. Discussion

4.1 Increase in foliar $\delta^{15}N$ with increasing N addition rates, with a steep increase after an N addition threshold of 10 g N m$^{-2}$ yr$^{-1}$

$\delta^{15}N$ increased with increasing N addition rates in all species, which was consistent with our first hypothesis. Foliar $\delta^{15}N$ is considered a surrogate of the whole-plant stable N isotopic signatures and is directly linked to soil $\delta^{15}N$ (Craine et al., 2015; Houlton et al., 2007; Kahmen et al., 2008). Therefore, the increasing trend of foliar $\delta^{15}N$ after N addition indicated that the soil N pool was enriched with $^{15}N$ after N addition was increased (Craine et al., 2015). The SEM results also showed that N addition rate had a strong effect ($R^2 = 0.82$) on the foliar $^{15}N$ signature. This could be attributed mainly to the stimulated N loss under N addition via N cycling processes, including mineralization, nitrification, denitrification, and NH$_3$ volatilization, all of which discriminate strongly against $^{15}N$ (Gurmesa et al., 2022a; Ren et al., 2017). A recent study from the same platform demonstrated that N addition significantly increased soil net N mineralization and nitrification rates by measuring N-related functional genes (Hu et al., 2021). The decrease in the NH$_4^+$:NO$_3^-$ ratios with increasing N addition rates in the subsurface soil indicated enhanced nitrification rates and a significant positive relationship between leaf $\delta^{15}N$ of plants and nitrification rates, which was consistent with previous studies on forest ecosystems (Bai et al., 2009; Gurmesa et al., 2022b; Pardo et al., 2006). Other studies also demonstrated that N addition significantly enhanced N$_2$O emissions and NH$_3$ volatilization, thereby significantly enriching the available soil N pools with $^{15}N$ under exogenous N input (Cowan et al., 2019; Kahmen et al., 2008; Xu et al., 2010). However, some studies have reported a decrease in foliar $\delta^{15}N$ with N addition (Sheng et al., 2019; Wang et al., 2021). This discrepancy could be due to different soil types and characteristics, preferential uptake of N (NH$_4^+$ vs. NO$_3^-$) by the target plants subjected to N addition, or differences in the experimental duration (Liu et al., 2017).
Notably, $\delta^{15}$N of the three plant species did not significantly change at low N addition rates (0–5 g N m$^{-2}$ yr$^{-1}$), but later increased sharply at an N input threshold of approximately 10 g N m$^{-2}$ yr$^{-1}$. This indicated possible N limitation in the plants (Bai et al., 2010) and dependency of plants on N$_2$ fixation or mycorrhizal, to some extent, for N uptake (see below), thereby potentially offsetting the increasing trend of foliar $\delta^{15}$N values induced by exogenous N inputs at low rates. When the N inputs increase progressively (exceeding 10 g N m$^{-2}$ yr$^{-1}$), N limitation is alleviated (Bai et al., 2010), and the symbiotic relationships between plants and mycorrhizal fungi or rhizobia are disrupted (Fu et al., 2022; Wei et al., 2013). Here, the N pool was the main N source for plant growth; thus, detectable differences in $^{15}$N signatures were found because the soil N became remarkably enriched with $^{15}$N, which was induced by the enhanced N turnover and discrimination against the heavier N isotope.

**4.2 Similarity in the response patterns of foliar $\delta^{15}$N of different species**

As expected, we detected different initial foliar $^{15}$N signatures in the three dominant species; moreover, foliar $^{15}$N increased almost proportionally with increasing N addition rates, which partially supported our second hypothesis. The N acquisition strategy could be considered as the most important factor that caused differences in the initial foliar $\delta^{15}$N (Craine et al., 2015), which might be the primary reason that why the N acquisition strategy did not show a significant effect on the foliar $\delta^{15}$N after N addition in the SEM. *T. lanceolata* is associated with N$_2$-fixing bacteria, whereas *L. chinensis* and *C. duriuscula* are associated with AMF. Many studies have reported that plants associated with N$_2$-fixing bacteria have lower $\delta^{15}$N values than plants that are not associated with N$_2$-fixing bacteria (Bai et al., 2009; He et al., 2009). Nitrogen addition could weaken the symbiosis between plants and mycorrhizal fungi or N$_2$-fixing bacteria, such as *Rhizobium*, because the C cost to the plant exceeds the N demand covered by microbial associates, which would cause plants to utilize the soil N pool more directly, consequently increasing the foliar $\delta^{15}$N. Indeed, our greenhouse experiment illustrated that N addition decreased the investment in nodulation and
inhibited N\textsubscript{2} fixation in \textit{T. lanceolata} (Fig. S5), although direct evidence to prove that \textit{T. lanceolata} decreased its N\textsubscript{2} fixation after N addition was lacking. Moreover, the decreased soil \textit{nifH} abundance (which included both autotrophic and symbiotic diazotrophs) after N addition indicated the negative effects of N enrichment on N\textsubscript{2} fixation (Watanabe et al., 2013; Zhou et al., 2021). Further, the decreasing AMF colonization rates in \textit{L. chinensis} and \textit{C. duriuscula} also confirmed the weak mycorrhizal dependency of N uptake in the plants after N addition.

The reasons explaining why the δ\textsuperscript{15}N differences among the plant species did not converge for N compounds at high N addition rates, at which available soil N became the main N pool remain unclear. The following mechanisms may possibly explain this unexpected trend. First, the ability to access different N forms (e.g., NH\textsubscript{4}\textsuperscript{+}-N, NO\textsubscript{3}\textsuperscript{-}-N, and DON) could have caused differences in the plant 15N signatures after N addition. Moreover, \textit{L. chinensis}, the most dominant species in the study site after N addition, could have possibly taken up a more substantial proportion of NH\textsubscript{4}\textsuperscript{+}-N that was enriched with 15N than NO\textsubscript{3}\textsuperscript{-}-N, while \textit{C. duriuscula} and \textit{T. lanceolata} could have taken up more NO\textsubscript{3}\textsuperscript{-}-N because of the relatively weak competition for NH\textsubscript{4}\textsuperscript{+}-N or preferential uptake of NO\textsubscript{3}\textsuperscript{-}-N. Li et al. (2018) confirmed that NH\textsubscript{4}\textsuperscript{+}-N was preferred over NO\textsubscript{3}\textsuperscript{-}-N to promote \textit{L. chinensis} growth. The increased δ\textsuperscript{15}N difference between \textit{L. chinensis} and \textit{C. duriuscula} (or \textit{T. lanceolata}) after NH\textsubscript{4}HCO\textsubscript{3} and urea additions also confirmed that \textit{L. chinensis} has stronger preference to NH\textsubscript{4}\textsuperscript{+}-N, because NH\textsubscript{4}HCO\textsubscript{3} and urea would lead to an extremely high NH\textsubscript{3} volatilization rate, consequently, causing NH\textsubscript{4}\textsuperscript{+}-N to be more enriched with 15N. Second, the depth at which fine roots access N may also influence plant 15N signatures. Plants with deeper roots can assimilate N from deeper soil depths, where soil is more enriched with 15N than the surface soil (Hobbie & Ouimette, 2009; Wang et al., 2017). \textit{T. lanceolata} has a relatively deep root system, with a large proportion of taproots (approximately 1m deep). Conversely, both \textit{L. chinensis} and \textit{C. duriuscula} develop dense fibrous roots with a rhizome-root system; however, the main root biomass of \textit{L. chinensis} is distributed at 0–30 cm, which is deeper than that of \textit{C. duriuscula} (at 0–10 cm) (Chen & Zhang,
Therefore, the differences in the root depth and distribution among the three species may explain the differences in the $^{15}$N signatures after N addition.

4.3 Variations in the soil pH and inorganic N concentrations because of the addition of different N compounds

After N addition, soil pH significantly decreased, while soil inorganic N concentration significantly increased, and these effects were stronger with NH$_4$NO$_3$ addition than with NH$_4$HCO$_3$ and urea additions, especially under the high N addition rates. Comparatively, the relatively weaker acidifying abilities of NH$_4$HCO$_3$ and urea may be related to the hydrolysis of these fertilizers that require H$^+$ from the soil system to produce OH$^-$, which volatilizes NH$_4^+$ as NH$_3$, and consumes the H$^+$ released from stimulated nitrification by fertilization (Lasisi & Akinremi, 2021; Soares et al., 2012; Tong & Xu, 2012; Wang et al., 2020). Generally, NH$_4$NO$_3$ is assumed to have a greater potential to decrease soil pH than NH$_4$HCO$_3$ and urea (Tian & Niu, 2015; Tkaczyk et al., 2020). Since soil pH could affect the soil microbial community structure ((Leff et al., 2015), functional gene abundance and enzymatic functions (Wang et al., 2018b; Liu & Zhang, 2019; Chen & Sinsabaugh, 2021), it may indirectly regulate soil N turnover, with different N compounds affecting N transformations differently. Nevertheless, there are other studies that suggest changes in the soil microbial activity and community structure do not align with changes in N transformation processes under N addition (Zhang et al., 2022).

In our study, N addition increased the availability of inorganic N but had marginal or no impact on the total soil N, possibly because of the large pool size of total soil N (Vourlitis et al., 2009; Zeng et al., 2010). The large differences in the NH$_4^+$:NO$_3^-$ ratio among NH$_4$HCO$_3$, urea, and NH$_4$NO$_3$ may be attributed to the differences in nitrification rates, as different types of N compounds can cause different magnitudes of changes in the soil pH (Li et al., 2020). This could further affect the soil microbial activity. Previous studies have shown that soil nitrification is more significantly enhanced by urea addition than ammonium addition (Wang et al., 2020; Zhu et al., 2013), and the
inhibition of nitrification is stronger under more acidic conditions (Li et al., 2020; Tong & Xu, 2012). Additionally, leaching may change the NH₄⁺:NO₃⁻ ratio and inorganic N availability. Geng (2021) showed that soil NO₃⁻-N increased with soil depth (0–60 cm) after NH₄NO₃ addition at the same study site. The significant increase in the NH₄⁺:NO₃⁻ ratio in the surface soil with NH₄NO₃ addition was likely caused by the suppression of nitrification at low soil pH (Li et al., 2020). Our results suggested that the significantly different soil inorganic N concentrations and pH values caused by different N addition rates and different N compounds can induce different N cycling dynamics and status, which may eventually be reflected by the changes in plant traits (especially foliar ¹⁵N signatures), because soil N is the main N source, especially for plants that are not associated with N₂-fixing bacteria.

4.4 Different foliar δ¹⁵N induced by different N compounds

Our results suggested that foliar δ¹⁵N was higher with NH₄HCO₃ and urea additions than with NH₄NO₃ addition, which was consistent with our third hypothesis. We speculate that this may be related to the differences in the “openness” of N cycling after adding different N compounds, because the N output/loss fluxes, including NH₃ volatilization, N₂O emission, and NO₃⁻ leaching, usually result in heavier ¹⁵N in soils (Robinson, 2001). Previous studies have shown that up to 64% of the applied N can be lost as NH₃, and this loss is positively related with N addition rates and soil pH (Pan et al., 2016; Ti et al., 2021). Moreover, urea-based fertilizers exhibit the highest NH₃ volatilization, while NH₄NO₃ application could reduce approximately 80% of NH₃ volatilization relative to urea application (Pan et al., 2016; Vaio et al., 2008). In addition to volatilization, nitrification could also cause different responses of foliar δ¹⁵N when different N compounds are added because NH₄HCO₃ and urea do not induce soil acidification as strongly as NH₄NO₃, and nitrification is generally higher at higher soil pH (Li et al., 2020). Moreover, higher nitrification rates could provide more volumes of “reactants” (i.e., NO₃⁻-N) for denitrification and leaching, thereby increasing the openness of the N cycle. Additionally, the δ¹⁵N difference among species
was reduced after \( \text{NH}_4\text{NO}_3 \) addition than after \( \text{NH}_4\text{HCO}_3 \) and urea additions, implying less N loss after \( \text{NH}_4\text{NO}_3 \) addition. In addition to measurements of foliar \( ^{15}\text{N} \), future research should simultaneously determine the \( ^{15}\text{N} \) signatures of soil \( \text{NH}_4^+ \) and \( \text{NO}_3^- - \text{N} \), *in situ* N transformation processes modulated by different soil microbiomes (such as biological N fixation, mineralization, nitrification and denitrification) to provide more solid evidence and a panoramic picture showing how N compounds could affect N transformation process in grasslands. \( \text{NH}_4\text{HCO}_3 \) \((1.56 \pm 0.26\%\)) and urea \((-0.76 \pm 0.11\%\)) additions resulted in lower \( \delta^{15}\text{N} \) values than \( \text{NH}_4\text{NO}_3 \) addition \((2.86 \pm 0.22\%\)) (Fig. S6), while foliar \( \delta^{15}\text{N} \) was higher under \( \text{NH}_4\text{HCO}_3 \) and urea treatments than under \( \text{NH}_4\text{NO}_3 \) treatments (Fig. 2a–d). Similar results were observed in a temperate grassland (Ren et al., 2017), where foliar \( \delta^{15}\text{N} \) increased more than in the controls, although the added urea was depleted in \( ^{15}\text{N} \) \((-3.4\%\)). Collectively, these results illustrate that differences in N cycling dynamics induced by the addition of different N compounds are more important than the \( ^{15}\text{N} \) signature of fertilizers in shaping the isotopic characteristics of soil N pool after long-term N addition.

Further, the addition of different N compounds induced significantly different foliar \( \delta^{15}\text{N} \) values. However, the foliar N concentration and relative biomass in the three species did not differ significantly with the addition of different N compounds. This might be because our experimental site was subjected to N addition for six consecutive years, and this long-term N accumulation in the soil might have predominated the effects of N compounds on some plant traits. Moreover, the three plant species showed distinctively different response patterns in their biomass based on N additions. Legumes usually exhibit higher foliar N concentrations than other species because of their \( \text{N}_2 \) fixation capacity, and most legumes are less sensitive to N supply (Adams et al., 2016; Wang et al., 2018a). This was consistent with our results wherein higher N concentrations were observed in *T. lanceolata* than in *L. chinensis* and *C. duriuscula*, and the response pattern of relative biomass and foliar N content of *T. lanceolata* to N addition was insignificant or mild. The foliar N concentrations of both *L. chinensis* and *C. duriuscula* showed a saturation tendency at N addition.
rates of $\geq 10$ g N m$^{-2}$ yr$^{-1}$, implying a transition from N to other limiting resources (e.g., light, phosphorous). As a N-nitrophilous species, *L. chinensis* became the dominant species having a greater competitive advantage under N enrichment than the other two species because of the positive relationship between its maximum photosynthetic rates and N availability (Chen et al., 2005), clonal expansion via rhizomes, and relatively high stature (Wang et al., 2004; Yang et al., 2019). The decreasing biomass of *C. duriuscula* based on the N addition rates might be related to the low light availability owing to its shorter height and its susceptibility to suppression by other taller species, such as *L. chinensis* (Wang et al., 2011; Yang et al., 2019). Nonetheless, plant N concentration and relative biomass were not as sensitive as foliar $\delta^{15}$N in reflecting different N dynamics caused by the addition of different N compounds.

**Conclusions**

Our results showed that N addition increased the plant foliar $\delta^{15}$N values of the selected plant species, independent of the N acquisition strategies, but subtle differences in the foliar $\delta^{15}$N values were likely linked to the ability or preference to access different N forms in the meadow steppe habitat. The increase in the foliar $^{15}$N signature was stronger when N addition rates exceeded the threshold of 10 g N m$^{-2}$ yr$^{-1}$. Foliar $\delta^{15}$N was greatly affected by the fertilizer type, with weakly acidifying N compounds, such as NH$_4$HCO$_3$ and urea, playing a more important role in increasing the foliar $\delta^{15}$N than strongly acidifying N compounds, such as NH$_4$NO$_3$. In grassland ecosystems limited by N, increased exogenous N inputs lead to a more open N cycle with potentially large losses of gaseous N. Our results suggested that the natural abundance of the stable N isotope in plant foliage could be a better indicator than plant biomass or N concentration in delineating the effects of N addition on N transformation processes in grassland ecosystems. Nitrogen addition rates, plant species, and N compounds should receive more research emphasis in the future to predict changes in ecosystem functions in the face of increasing atmospheric N deposition.
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Author contributions

XH, JH and XL conceived and designed the experiment. YW, GN, MH, CW, JX and GY helped to conduct the field work. YW, AL and JH analyzed the data. YW wrote the first draft, KR, RW, YJ, XH and JH revised the manuscript. All authors contributed substantially to manuscript revisions.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Data availability statement

The data that support the findings of this study are available from Dryad (https://doi.org/10.5061/dryad.fllbg79czp).
References


Gurmesa, G. A., Wang, A., Li, S., Peng, S., de Vries, W., Gundersen, P., Ciais, P., Phillips, O. L.,
Hobbie, E. A., Zhu, W., Nadelhoffer, K., Xi, Y., Bai, E., Sun, T., Chen, D., Zhou, W.,
Zhang, Y., Guo, Y., Zhu, J., Duan, L., Li, D., Koba, K., Du, E., Zhou, G., Han, X., Han,
S., & Fang, Y. (2022b). Retention of deposited ammonium and nitrate and its impact on
the global forest carbon sink. Nature Communications, 13, e3998. https://doi.org/10.1038/s41467-022-28345-1

of nitrogen fertilizer type and application rate on soil acidification rate under a wheat-maize

Hobbie, E. A., & Hobbie, J. E. (2008). Natural abundance of ${^{15}}$N in nitrogen-limited forests and
tundra can estimate nitrogen cycling through mycorrhizal fungi: a review. Ecosystems, 11, 815. https://doi.org/10.1007/s10021-008-9159-7

Hobbie, E. A., & Hogberg, P. (2012). Nitrogen isotopes link mycorrhizal fungi and plants to
nitrogen dynamics. New Phytologist, 196(2), 367-382. https://doi.org/10.1111/j.1469-
8137.2012.04300.x


https://doi.org/10.1046/j.1469-8137.1997.00808.x

in plant nitrogen acquisition within tropical forest communities. Proceedings of the
National Academy of Sciences, 104, 8902. https://doi.org/10.1073/pnas.0609935104

decreases in soil pH drive the positive effects of nitrogen addition on soil net nitrogen
https://doi.org/10.1016/j.pedobi.2021.150756

https://doi.org/10.1002/eap.2047


Table 1. Results of the linear mixed-effect model for N addition rate (Rate), N compound (Type), species (Species) and their interactions on foliar δ¹⁵N values, foliar N concentration, relative biomass of the three investigated species, AMF colonization rate and soil *nifH* gene abundance. Significant values (*p* < 0.05) are highlighted in bold. Symbol “-” in the table means data were unavailable.

<table>
<thead>
<tr>
<th>Term</th>
<th>df</th>
<th>Foliar δ¹⁵N (%)</th>
<th>Foliar N concentration (%)</th>
<th>Relative biomass (%)</th>
<th>AMF colonization rate (%)</th>
<th>nifH gene abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>F</em></td>
<td><em>p</em></td>
<td><em>F</em></td>
<td><em>p</em></td>
<td><em>F</em></td>
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<tr>
<td>Rate</td>
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<td>117.50</td>
<td><strong>&lt;0.001</strong></td>
<td>0.92</td>
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<tr>
<td>Type</td>
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<td>4.76</td>
<td><strong>0.009</strong></td>
<td>1.21</td>
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<tr>
<td>Species</td>
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<td>121.57</td>
<td><strong>&lt;0.001</strong></td>
<td>1339.36</td>
<td><strong>&lt;0.001</strong></td>
<td>868.08</td>
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<tr>
<td>Rate × Type</td>
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<td>54.91</td>
<td><strong>&lt;0.001</strong></td>
<td>8.60</td>
<td><strong>0.002</strong></td>
<td>1.23</td>
</tr>
<tr>
<td>Rate × Species</td>
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<td>12.04</td>
<td><strong>&lt;0.001</strong></td>
<td>23.05</td>
<td><strong>&lt;0.001</strong></td>
<td>45.60</td>
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<td>Type × Species</td>
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<td><strong>&lt;0.001</strong></td>
<td>2.03</td>
<td>0.09</td>
<td>6.77</td>
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</table>
Table 2. Results of the linear mixed-effect model for N addition rate (Rate), N compound (Type), soil layer (Layer) and their interactions on NH$_4$$^+$ and NO$_3$- concentrations, and NH$_4$$^+$$:$NO$_3$-.

Significant values ($p < 0.05$) are highlighted in bold.

<table>
<thead>
<tr>
<th>Term</th>
<th>df</th>
<th>NH$_4$$^+$ (mg kg$^{-1}$)</th>
<th>NO$_3$- (mg kg$^{-1}$)</th>
<th>NH$_4$$^+$$:$NO$_3$-</th>
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<td>$F$</td>
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<td>$F$</td>
</tr>
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<td>Layer</td>
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<td>6.63</td>
</tr>
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</tr>
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<td>0.91</td>
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<td>Rate × Type × Layer</td>
<td>10</td>
<td>6.46</td>
<td>$0.001$</td>
<td>1.11</td>
</tr>
</tbody>
</table>
Figure legends:

**Fig. 1** A conceptual framework showing the impacts of N addition on plant natural abundance $^{15}$N. Four major pathways/factors are listed here, including ① N input via N deposition/fertilization (light green arrows) can stimulate N cycling processes (e.g. ammonification, nitrification, denitrification) and can increase the openness of the N cycle (e.g. gaseous losses), leading to increased $^{15}$N signatures in soils; ② plant species with different N acquisition strategies (i.e. symbiotic associations) may shift their N uptake after N additions (orange arrows); ③ N additions increase inorganic N availability in soil and the ability/preference to access NH$_4^+$-N or NO$_3^-$-N (dark green arrows) will differ among species; ④ the plant root distribution also impacts plant $^{15}$N as the soil $^{15}$N signatures increases with depth (purple arrows). The dashed arrows in ② represent the negative feedback (inhibition) of the symbionts to the plant partner after N input. The “↑” and “↓” symbols in brackets indicate processes leading to either enriched or depleted $^{15}$N in soil or plants, respectively.

**Fig. 2** Effects of N addition rates of three N compounds on foliar $\delta^{15}$N values (a-c) and N concentration (e-g) of the three investigated plant species. Shown are means ± se (n = 6). $R^2$ indicates the adjusted R-square. The solid lines indicate significant relationships at $p < 0.05$, while the dashed lines indicate significant levels of $0.05 < p < 0.1$. (d) and (h) show the effects at low (add the numbers here) and high (add the numbers here) N addition rates on foliar $\delta^{15}$N and N concentration, calculated across all species. Different lowercase letters indicate significant differences between low and high N addition rates, while different uppercase letters indicate significant differences among N fertilizer types. R represents the N addition rates and T represents the N fertilizer types. Ns $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

**Fig. 3** Relationships between the relative biomass of the three investigated species and N addition rates of three N compounds (a-c). Shown are means ± se (n ≤ 6). $R^2$ indicates the adjusted R-square. The solid lines indicate significant relationships at $p < 0.05$, while the dashed lines indicate
significant levels of $0.05 < p < 0.1$. (d) shows the effects of low and high N addition rates on the relative biomass of the three investigated plant species, calculated across all three N compounds. Different lowercase letters indicate significant differences between low and high N addition rates, while different uppercase letters indicate significant differences between species. R represents the N addition rates and S represents the species. Ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Please note no data of *C. duriuscula* at 50 g N m$^{-2}$yr$^{-1}$ addition rate with urea could be obtained because no *C. duriuscula* was found at this level.

**Fig. 4** Effects of N addition rates on soil NH$_4^+$ (a-c), NO$_3^-$ (d-f) concentrations and NH$_4^+$:NO$_3^-$ ratios (g-i) of surface (0-10cm) and subsurface (10-20cm) soil layers with additions of NH$_4$HCO$_3$, urea and NH$_4$NO$_3$. Shown are means ± se ($n = 6$). R$^2$ indicates the adjusted R-square, and the solid lines indicate a significant level of $p < 0.05$, while the dashed lines indicate a significant level of $0.05 < p < 0.1$. The inserted graphs present the effects of low ($\leq 5$ g N m$^2$ yr$^{-1}$) and high ($\geq 10$ g N m$^2$yr$^{-1}$) N addition rates on the average NH$_4^+$, NO$_3^-$ concentrations and NH$_4^+$:NO$_3^-$ ratios at surface and subsurface soil layers. R represents the N addition rates and L represents the soil layers. Ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Please note the differences in the y-axes.

**Fig. 5** Relationships between N addition rate and AMF colonization rate of (a) *L. chinensis* and (b) *C. duriuscula*, (c) soil nifH quantity after addition of three N compounds. Shown are means ± se ($n \leq 6$). R$^2$ indicates the adjusted R-square, and the solid lines indicate a significant level of $p < 0.05$, while the dashed lines indicate a significant level of $0.05 < p < 0.1$.

**Fig. 6** Structural equation model (SEM) disentangling the major pathways of foliar $\delta^{15}$N responses to N addition rates of the three N compounds for three plant species (AIC = 178.23, Fisher's C = 62.23, $p = 0.21$). The green lines indicate positive correlations and the black lines represent negative correlations. Solid arrows indicate significant relationships ($p < 0.05$), and dashed grey arrows indicate non-significant pathways. R$^2$ represent the proportion of variance explained for each dependent variable in the model. Standardized coefficients for continuous variables are in boxes.
over each significant arrow. *, $p < 0.05$; **, $0.05 < p < 0.01$; ***, $p < 0.001$. Plant species and N types report estimated marginal means, which are accompanied by letters that indicate the marginal means pairwise comparison (different letters indicate significant effects between the levels, $p < 0.05$). For species, L.c, C.d and T.l represent *Leymus chinensis*, *Carex duriuscula* and *Thermopsis lanceolata*, respectively. For N types, C, U and N represent NH$_4$HCO$_3$, urea and NH$_4$NO$_3$, respectively. Please note that soil properties are based on the results from 0-10 cm as the *nifH* was measured only in that layer.
Figure 1

N deposition / fertilization

NH₃

N₂O, NO, N₂

Mycorrhizal fungi (\(^{15}N\)j)

N fixation (\(^{15}NO\))

Volatilization (\(^{15}NH\)↑)

Mineralization

\(\text{NH}_4^+\)

Nitrification (\(^{15}NH\)↑; \(^{15}NO\)↑)

\(\text{NO}_3^-\)

Processes and openness of N cycling

The ability/preference to access different N forms

\(\uparrow \delta^{15}N\) increase

\(\downarrow \delta^{15}N\) decrease

N acquisition strategies

N uptake by different root depth

\(\text{Process weakened by N inputs}\)
Figure 2

- Foliar δ15N (%)
  - L. chinensis
  - C. duriuscula
  - T. lanceolata

- NH4HCO3
  - Low rates (0-5)
  - High rates (10-50)

- urea

- NH4NO3

- Average across species
  - A
  - B

- Log10(N addition rate + 1) (g N m⁻² yr⁻¹)
  - NH4HCO3
  - urea
  - NH4NO3

- R² values:
  - R² = 0.86, p = 0.03
  - R² = 0.88, p = 0.02
  - R² = 0.96, p < 0.01

- R² values:
  - R² = 0.98, p < 0.01
  - R² = 0.73, p = 0.06
  - R² = 0.97, p < 0.01

- R² values:
  - R² = 0.80, p = 0.04
  - R² = 0.63, p = 0.10
  - R² = 0.99, p < 0.01

- R² values:
  - R² = 0.92, p < 0.01
  - R² = 0.90, p < 0.01
  - R² = 0.97, p < 0.01

- R² values:
  - R² = 0.53, p = 0.06
$$R^2 = 0.75, \ p = 0.02$$

$$R^2 = 0.82, \ p < 0.01$$

$$R^2 = 0.52, \ p = 0.07$$

$$R^2 = 0.85, \ p < 0.01$$

$$R^2 = 0.88, \ p = 0.01$$

$$R^2 = 0.49, \ p = 0.07$$

$$R^2 = 0.83, \ p < 0.01$$
Figure 4

(a) Soil $\text{NH}_4^+$ concentration (mg kg$^{-1}$) with $\text{NH}_4\text{HCO}_3$

(b) Soil $\text{NO}_3^-$ concentration (mg kg$^{-1}$) with urea

(c) Soil $\text{NH}_4^+$ concentration (mg kg$^{-1}$) with $\text{NH}_4\text{NO}_3$

- **: $R^2 = 0.57$, $p = 0.05$
- ***: $R^2 = 0.99$, $p < 0.01$
- **: $R^2 = 0.92$, $p = 0.01$
- **: $R^2 = 0.85$, $p = 0.03$
- ***: $R^2 = 0.99$, $p < 0.001$
- **: $R^2 = 0.82$, $p = 0.01$
- **: $R^2 = 0.98$, $p < 0.01$
- **: $R^2 = 0.78$, $p = 0.01$
- **: $R^2 = 0.46$, $p = 0.09$
- **: $R^2 = 0.97$, $p < 0.01$
- **: $R^2 = 0.88$, $p < 0.01$

Log$_{10}$(N addition rate + 1) (g N m$^{-2}$ yr$^{-1}$)
Figure 5

(a) AMF colonization of *L. chinensis*

- NH₄HCO₃
- Urea
- NH₄NO₃

(b) AMF colonization of *C. duriuscula*

- NH₄HCO₃
- Urea
- NH₄NO₃

(c) Soil nifH (0-10 cm)

- NH₄
- HCO³⁻
- Urea

R² values:
- a: 0.83, p < 0.01
- b: 0.43, p = 0.09
- c: 0.37, p = 0.10
- d: 0.38, p = 0.10
- e: 0.93, p < 0.01
- f: 0.73, p = 0.02
### Foliar δ¹⁵N

<table>
<thead>
<tr>
<th>Species</th>
<th>Foliar N%</th>
<th>( R^2 ) = 0.74</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMF/( nifH )</td>
<td>0.18*</td>
<td>0.21*</td>
</tr>
<tr>
<td>pH</td>
<td>( 0.56*** )</td>
<td>( 0.25* )</td>
</tr>
</tbody>
</table>

### Relative biomass

<table>
<thead>
<tr>
<th>Species</th>
<th>AMF/( nifH )</th>
<th>( R^2 ) = 0.88</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄⁺⁻NO₃</td>
<td>0.38*</td>
<td>0.38*</td>
</tr>
<tr>
<td>NO₃⁻⁻NH₄⁺</td>
<td>( 0.82*** )</td>
<td>( 0.29*** )</td>
</tr>
</tbody>
</table>

### N types

<table>
<thead>
<tr>
<th>Species</th>
<th>AMF/( nifH )</th>
<th>( R^2 ) = 0.79</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMF</td>
<td>( 0.13* )</td>
<td>( 0.27 )</td>
</tr>
<tr>
<td>pH</td>
<td>( 0.10* )</td>
<td>( -0.16*** )</td>
</tr>
</tbody>
</table>

### N rates

<table>
<thead>
<tr>
<th>Species</th>
<th>AMF/( nifH )</th>
<th>( R^2 ) = 0.93</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄⁺⁻NO₃</td>
<td>( 0.10* )</td>
<td>( -0.51*** )</td>
</tr>
<tr>
<td>NO₃⁻⁻NH₄⁺</td>
<td>( 0.82*** )</td>
<td>( -0.82*** )</td>
</tr>
</tbody>
</table>

#### Figure 6

![Diagram showing relationships between foliar δ¹⁵N, relative biomass, AMF/\( nifH \), pH, and N types and rates.]

*Significance levels: * p < 0.05, ** p < 0.01, *** p < 0.001.