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Effects of intensified freeze-thaw frequency on dynamics of winter nitrogen resources in temperate grasslands

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Abstract

In seasonal snow-covered temperate regions, winter serves as a crucial phase for nitrogen (N) accumulation through persistent mineralization processes. Climate warming has accelerated snowmelt and intensified freeze-thaw cycle frequency (FTC), potentially altering the availability of winter N sources for plants. We simulated intensified FTC regimes (increased 0, 6, and 12 cycles) in situ across two contrasting temperate grasslands, employing dual-labeled isotopes ($^{15}NH_4{}^{15}NO_3$) to quantify winter N dynamics. Our results showed that intensified FTC significantly enhanced soil net ammonification rates and inorganic N levels in early spring, while net nitrification rates remained stable. This suggests that frequent FTC may provide a substantial N source for soil microorganisms and plant growth. Notably, soil microbial biomass N increased despite microbial C limitation, indicating efficient microbial N competition that restricted plant access to winter N sources. Intensified low-frequency FTC did not affect plant ^{15}N acquisition, whereas high-frequency FTC significantly reduced plant ^{15}N acquisition. Importantly, the impacts of FTC on plant ^{15}N acquisition varied among functional types. Dominant cold-tolerant species (perennial bunch grasses and semi-shrubs) increased ^{15}N acquisition, likely due to earlier root activity, while subordinate species (perennial rhizome grasses and forbs) exhibited reduced acquisition. In conclusion, while intensified FTC did not lead to the loss of winter N sources, it restructures N availability by favoring microbial retention and creating competitive hierarchies among plants in temperate grasslands. The high-frequency FTC-induced shifts in partitioning of winter N resources could

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substantially influence grassland productivity and community structure, highlighting the critical need to integrate winter climate change effects into temperate grassland ecosystem models.

Keywords: freeze-thaw cycle; grassland; N isotope; N dynamic; plant N acquisition; snowmelt; winter

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1 Introduction

Approximately 50 % of terrestrial ecosystems in the Northern Hemisphere experience seasonal snow cover and winter soil freezing (Sommerfeld et al., 1993; IPCC, 2021). Remarkably, soil microorganisms maintain metabolic activity under snowpack and contribute to nutrient mineralization throughout winter (Larsen et al., 2012; Zhang et al., 2011). These winter processes, including soil nitrogen (N) mineralization and microbial N immobilization, constitute a vital nutrient reservoir that supports plant growth across alpine grasslands, temperate grasslands, and boreal forests (Alatalo et al., 2014; Bilbrough et al., 2000; Collins et al., 2017; Edwards and Jefferies, 2010). The springtime release of winter-accumulated N (mainly including NH_4^+, NO_3^-, and dissolved organic N) through freeze-thaw cycles (FTC) synchronizes nutrient availability with plant demand (Kaiser et al., 2011), particularly in N-limited ecosystems where winter N contributions may determine growing season productivity (Schmidt and Lipson, 2004).

Climate warming has emerged as one of the most important global environmental challenges. Evidence shows that climate warming has primarily occurred during winter, with the rate of winter warming exceeding the annual average over the past few decades in China (Zong and Shi, 2020). This trend is expected to intensify, with an anticipated increase in the frequency of extreme warming events (Kreyling et al., 2011; IPCC, 2021). Winter warming might lead to an earlier onset and intensified frequency of freeze-thaw cycles (FTC), potentially altering ecosystem N cycling

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processes (Gao et al., 2018). Consequently, this could affect the availability of winter N sources for plant growth. However, how intensified FTC affect winter N retention remains poorly understood, particularly its subsequent impacts on plant N uptake and ecosystem functioning.

Intensified FTC induces complex shifts in soil N dynamics by simultaneously enhancing N mineralization while disrupting microbial immobilization and ecosystem retention processes. Existing research have demonstrated that intensified FTC can enhance soil N availability in cold regions (Dai et al., 2020; Nie et al., 2024; Teepe and Ludwig, 2004; Wang et al., 2012; Yang et al., 2023). The physical disruption caused by FTC promotes the N release from both soil organic matter and microbial biomass via cell lysis (Koponen et al., 2006; Sawicka et al., 2010; Skogland et al., 1988). However, this FTC-induced N pulse often occurs before plants resume active uptake, leading to substantial N losses through leaching and gaseous emissions (Chen et al., 2021; Elrys et al., 2021; Ji et al., 2024). while microbial mortality reduces microbial N immobilization (Gao et al., 2018), the surviving microbial community exhibits stimulated microbial activity that accelerates nutrient cycling (Fitzhugh et al., 2001; Nie et al., 2024; Sharma et al., 2006; Wang et al., 2024). Notably, a comprehensive meta-analysis by Song et al. (2017) indicated that FTC have no significant effect on microbial biomass N (MBN) across various ecosystems, including forest, shrubland, grassland/meadow, cropland, tundra and wetland ecosystems, suggesting complex compensatory mechanisms in microbial N retention.

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Frequent FTC significantly impact plant-soil N dynamics through multiple pathways. Root damage caused by FTC directly impairs plant N acquisition capacity (Campbell et al., 2014; Song et al., 2017), while simultaneously creating temporal mismatches in N availability. Larsen et al. (2012) utilized ^{15}N tracer reveals that soil microorganisms initially dominate winter N immobilization following snowmelt, with plant functional types exhibiting sequential N uptake patterns: evergreen dwarf shrubs being the first to take up winter N, succeeded by deciduous dwarf shrubs and graminoids in late spring in the alpine ecosystem. This study highlighted a temporal differentiation in the resumption of N uptake among plant functional groups after winter. This temporal niche partitioning is particularly pronounced in temperate regions, where shallower snowpack and more frequent spring FTC create distinct competitive environments compared to alpine systems. Studies in temperate grasslands have shown that perennial bunch grasses present earlier N uptake than perennial rhizome grasses and forbs (Ma et al., 2018, 2020), a phenological advantage that becomes more pronounced under winter warming conditions (Turner and Henry, 2009). These findings collectively highlight how FTC-mediated changes in belowground processes interact with plant functional traits to govern winter N partitioning.

While previous studies have examined winter N cycling in high-altitude and high-latitude regions experiencing rapid warming trends (Alatalo et al., 2014; Bilbrough et al., 2000; Brooks et al., 1996; Edwards and Jefferies, 2010; Miller et al.,

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2007), temperate grasslands remain understudied despite their distinct freeze-thaw regimes. Critically, existing research has predominantly relied on laboratory simulations employing artificial freezing regimes (DeLuca et al., 1992; Teepe and Ludwig, 2004; Yang et al., 2023; Zhang et al., 2024), creating significant gaps regarding the ecological impacts of natural in situ freeze-thaw cycles. Field-based investigations are urgently needed to address two critical questions: (1) how FTC frequency alters winter N retention dynamics, and (2) whether these changes create legacy effects on subsequent growing season productivity and plant community composition in temperate grasslands. This lack of field evidence limits our ability to predict ecosystem responses to climate change.

Temperate grasslands cover nearly 40 % of China's terrestrial ecosystems (Bardgett et al., 2021), and are particularly vulnerable to climate change due to their prolonged near-freezing winter conditions. To understand how intensified FTC affect retention of winter N resources in grasslands, we conducted an in situ $^{15}NH_4{}^{15}NO_3$ tracer experiment across two temperate grassland types. We hypothesize that: (1) intensified FTC would reduce retention of winter N resources through physical disruption of soil aggregates enhancing N mobility, root damage impairing plant N uptake capacity, and microbial cell lysis leading to N leaching and denitrification losses (Fitzhugh et al., 2001; Koponen et al., 2006; Nie et al., 2024; Sawicka et al., 2010; Sharma et al., 2006; Skogland et al., 1988); and (2) intensified FTC would lead to differential utilization of winter N sources among plant species, mediated by interspecific variations in their

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competitive abilities, root system architecture (particularly rooting depth and winter root activity), and temporal niche partitioning in growth phenology (Bilbrough et al., 2000; Hosokawa et al., 2017; Ma et al., 2018, 2020). Specifically, we expected that deep-rooted species with early spring green-up would increase their utilization of winter N sources, while other plants may experience reduced N utilization due to root damage (Campbell et al., 2014; Song et al., 2017).

2 Methods

2.1 Experimental site

We conducted parallel experiments in two contrasting temperate grassland ecosystems: a meadow steppe and a sandy steppe (Table 1; Fig. 1). The meadow steppe was situated at the Hulunber Grassland Ecosystem Observation and Research Station in northeastern China (49°19' N, 120°02' E, 628 m), while the sandy steppe was located at the Ordos Sandy Grassland Ecology Research Station in northern China (39°29' N, 110°11' E, 1290 m).

Both sites have a continental climate. The mean annual precipitation is 420 mm and 310 mm, and the mean annual temperature is -2-1 °C and 6.5 °C in the meadow steppe and the sandy steppe, respectively (http://data.cma.cn/). The non-growing season for the meadow steppe extends from late September to late April of the following year, with a spring freeze-thaw period occurring from late March to late April. In contrast, the non-growing season for the sandy steppe lasts from

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mid-October to late March, with the spring freeze-thaw period occurring from late February to late March. In the meadow steppe, persistent snow cover reached 20-25 cm depth during late winter (January-February), providing consistent thermal insulation. In contrast, the sandy steppe exhibited shallower and more variable snowpack (typically 10 cm depth) due to higher wind redistribution and lower moisture retention. Under natural conditions, the meadow steppe in this study underwent a total of 19 freeze-thaw cycles, while the sandy steppe experienced 21 freeze-thaw cycles in early spring (http://nm.cma.gov.cn/).

The meadow steppe features high plant diversity and fertile soils, while the sandy steppe exhibits lower diversity and nutrient-poor, coarse-textured soils (Table 1). This strategic pairing allows for comprehensive assessment of FTC impacts across varying resource availability and community structures, as evidenced by significant baseline differences in N dynamics between sites. According to the Chinese Soil Classification (GB/T 17296-2009), the predominant soil type in meadow grassland is loam soil, and which in sandy grassland is sandy loam soil. The meadow steppe soil has higher C and N content but slightly lower pH compared to the sandy steppe soil (Table 1). In the meadow steppe, the dominant plant species include perennial bunch grasses like *Stipa baicalensis Roshev*, perennial rhizome grasses such as *Leymus chinensis* (Trin.) Tzvel., as well as perennial forbs including *Carex pediformis* C. A. Mey., which together cover approximately 78% of the site. In the sandy steppe, the dominant plant species are *Cleistogenes squarrosa* (Trin). Keng., *Klasea centauroides* (L). Cass., and

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Hedysarum monglicum Turez, covering about 70 % of the area (Table 1). The complementary strengths of these ecosystems enable robust predictions about grassland responses to changing winter climate regimes.

2.2 Experimental design

In late October 2020, eighteen 3 m × 3 m plots were established at each site, with a 3-meter buffer between neighboring plots. The experiment employed a randomized block design with three treatments and six replicates per site: (1) control (ambient FTC), (2) intensified low-frequency FTC (LFTC; + 6 times), and (3) intensified high-frequency FTC (HFTC; + 12 times). These treatments were designed to simulate projected increases in winter FTC frequency under climate change scenarios.

The treatment levels were based on historical climate data showing approximately 20 natural FTCs typically occur during winter and early spring at both sites (Table 1; https://data.cma.cn/). According to the definition of freeze-thaw cycling, a freeze-thaw cycle is defined as the process in which soil temperature (0-10 cm) rises above 0 ℃ and then subsequent drops below 0 ℃ (Yanai et al., 2007). Therefore, the intensified FTC correspond to total increases in 30 % (+ 6 times) and 60 % (+ 12 times) in the frequency of FTC during winter and spring seasons, respectively.

Within each plot, we established a fixed 1 m × 1 m subplot for ^{15}N tracing. Building upon established ^{15}N tracing approaches (Ma et al. 2020; Bilbrough et al. 2000), we

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applied $^{15}NH_4{}^{15}NO_3$ solution prior to winter soil freezing. A solution containing 24 mg ^{15}N L^{-1} of $^{15}NH_4{}^{15}NO_3$ was injected into 100 holes with a syringe guided by a grid frame (1 m × 1 m), with each hole receiving 2 mL of the labeled solution. The total application per subplot was 200 mL, which is equal to 120 mg ^{15}N m^{-2}. The added ^{15}N was kept within the natural fluctuation range of inorganic N in the soil, approximately 7 %-10 % of background soil inorganic N levels. We injected water into control treatments instead of the ^{15}N tracer, and no significant differences in plant/microbial N concentrations when compared to the ^{15}N treatments. This indicates that the ^{15}N application did not disrupt natural N cycling processes (Ma et al., 2018).

Based on recent 5-year climatic records, our initial FTC treatments were scheduled approximately 15 days prior to the natural spring FTC period (late winter). For the freezing-thaw manipulation, a closed-top tent (3 m length × 3 m width × 2 m height) was installed in each plot during each warming manipulation. The heating tents were constructed with polyester fabric, featuring sealed tops and mesh-sided windows to prevent excessive CO_2 accumulation while maintaining temperature control. Within each tent, we used a propane AirHeater (Mr Heater, USA) to raise soil temperature to 2-3 °C (0-15cm), maintaining this temperature continuously for 8 to 10 hours each time. Continuous temperature logging was performed using 2 temperature detectors per treatment positioned at two critical positions: (a) 5 cm above soil surface (ambient microclimate) and (b) 10 cm soil depth, with data recorded at half-hour intervals throughout the experiment. The temperature was then

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allowed to drop to approximately -2 °C over a period of 4 hours to complete one freeze-thaw cycle. Two intensified FTC regimes were implemented: (i) high-frequency FTC (HFTC) with 12 additional cycles administered every 1-3 days, and (ii) low-frequency FTC (LFTC) with 6 additional cycles every 3-6 days. During the natural freeze-thaw period, all artificial FTC treatments were deliberately conducted when daily mean temperatures remained below -2 °C to avoid interference with natural cycles.

2.3 Sampling and Processing

Samplings were conducted after the freeze-thaw treatments and during the succeeding growing season. In the meadow steppe, samplings were collected on the following dates: 26 March 2021 (early spring); 4 May 2021 (late spring); 23 June 2021 (early summer); 22 July 2021 (late summer); and 26 September 2021 (late autumn). Similarly, in the sandy steppe, samplings were collected on 5 March 2021 (early spring); 29 April 2021 (late spring); 21 June 2021 (early summer); 26 July 2021 (late summer); and 15 October 2021 (late autumn).

For plant samplings, soil blocks (20 cm length × 20 cm width × 20 cm height) containing dominant plant species were carefully excavated and sectioned. Plant roots were washed with distilled water to remove surface ^{15}N, then separated into aboveground and belowground components. All plant materials were oven-dried at 65 °C for 48 hours. For soil samplings, we randomly excavated three labeled core at

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20 cm depth soil (diameter is 3.5 cm) from each plot. We combined three soil core into a composite sample, which was passed through a 2 mm sieve. Within 4 hours of collection, the composite sample was separated into two portions: one for air-drying and soil analysis, and the other stored at -20 °C for microbial analysis.

Soil temperature and moisture at a depth of 10 cm were measured automatically every half hour using HOBO data loggers (H21-USB, Onset Inc., USA) throughout the study period. Soil and plant (aboveground and belowground) dry samples were pulverized using a ball mill. Subsequently, soil samples were sieved through a 100-mesh sieve and plant samples through an 80-mesh sieve. The sieved samples were analyzed for C and N content using an elemental analyzer (Elementar Vario Max CN, Germany). Soil net ammonification and nitrification rates were analyzed using the method of polyvinyl chloride plastic (PVC) core (Raison et al., 1987). A pair of PVC cores was vertically inserted to 20 cm depth soil layer in each plot to incubate the soil without plant uptake. One core was collected as the initial (unincubated) sample to determine the concentrations of NH_4^+-N and NO_3^--N using a flow injection autoanalyzer (Scalar SANplus segmented flow analyzer, Netherlands). The other core was incubated in situ for two weeks within capped cores. After incubation, we analyzed the NH_4^+-N and NO_3^--N in these samples as well. Net ammonification and nitrification rates were estimated based on the changes in NH_4^+-N and NO_3^--N levels between the incubated and initial values. Soil total dissolved organic N (DON) was calculated as total N minus inorganic N (i.e., the sum of NH_4^+-N and NO_3^--N). Soil

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total dissolved organic C (DOC) was calculated as total C minus inorganic C.

The microbial biomass C and N were assessed by the fumigation-extraction method with a total organic C analyzer (TOC multiN/C 3100, Analytik Jena, Germany; Vance et al., 1987). The method calculates microbial biomass C and N by determining the contrast in extractable C or N levels between samples that have been fumigated and those that have not. To prepare the soil extracts, fresh soil samples are moistened to a water retention capacity of 60 %, followed by incubation in the dark at a temperature of 25 °C for a week. After incubation, samples with a moisture content equivalent to 25 g of dry weight were fumigated with chloroform ($CHCl_3$) for a duration of 24 hours. The soil sample was extracted by agitating it with shaking 60ml K_2SO_4 solution for 30min. After filtration, the extractable concentration of organic C or N was determined by elemental analyzer (Elementar Analyzer, Vario MaxCN, Germany). The conversion coefficient is 0.45. The ^{15}N contents in plant (2 mg) and soil subsamples (20 mg) were analyzed with an elemental analyzer coupled with an isotope-ratio mass spectrometer (IRMS, Thermo Finnigan MAT DELTAplus XP, USA). Soil microbial ^{15}N was measured using alkaline persulfate oxidation, followed by a modified diffusion method (Stark and Hart, 1996; Zhou et al., 2003). Soil immobilized ^{15}N was then calculated by subtracting microbial ^{15}N from soil total ^{15}N (Ma et al., 2018).

Soil microbial community structure was determined using the phospholipid fatty acid

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(PLFA) method (Bossio and Scow, 1998). Changes in PLFAs reflect the viable biomass of fungi and bacteria, as well as microbial community structure in situ soils. The fatty acids a13:0, i14:0, i15:0, i16:0, i17:0, a17:0, 16:1ω7c, 17:1ω8c, 18:1ω5c, 18:1ω9t, 17:0cy, and 19:0cy were chosen as representative of the bacterial group. The fatty acids 16:1ω5c, 18:2ω6,9c, and 18:1ω9c were selected to represent the fungal group.

2.4 Statistical Analysis

The ^{15}N acquisition (% of applied ^{15}N) in the shoot and root were calculated as: $[(^{15}N_I - {}^{15}Na) \times \text{biomass}/{}^{15}Nt] \times 100$, where $^{15}N_I$ and ^{15}Na are the ^{15}N concentrations ($g^{15}N\ g^{-1}$ sample) in the labeled and the control samples; biomass is the shoot or root biomass at each sampling time ($g\ m^{-2}$), and ^{15}Nt is the amount of total added ^{15}N tracer ($g\ ^{15}N\ m^{-2}$).

The soil or microbial biomass ^{15}N recovery (% of applied ^{15}N) was calculated as: $[(^{15}N_I - {}^{15}Na) \times V \times BD / {}^{15}Nt] \times 100$, where V represents the soil volume of the 20 cm soil profile ($cm^3\ m^{-2}$), and BD is the bulk density ($g\ cm^{-3}$). Differences in soil, microbial and plant properties, and ^{15}N tracer retention in plants, soil, and microorganisms between the two grasslands were analyzed using One-way ANOVA. Repeated measurement ANOVA was used to analyze the influences of different FTC treatments, sampling times, and grassland types on the measured indicators. Spearman correlation coefficients between variables were calculated using “rcorr”. To assess the

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relative importance of predictors for plant ^{15}N acquisition capacity, a random forest model was constructed using the randomForest and rfPermute packages. Model training utilized 70 % of the dataset for parameter optimization, with the remaining 30 % reserved for model validation. All above mentioned analysis were conducted with SPSS 21.0 software (SPSS for Windows, Chicago, IL, USA) and RStudio 2025.05.0 (Posit Software, Boston, MA, USA), and graphics were plotted using SigmaPlot, 14.0,Origin 14.0 and R Studio.

3 Results

3.1 Soil microclimate

The edaphic conditions, including soil total C content, inorganic N content, and texture, exhibited significant differences between the two temperate grasslands ($p < 0.05$; Table 1). Throughout the winter freezing period, the lowest soil temperatures (0−10cm) were about -23 ℃ in the meadow steppe and -20 ℃ in the sandy steppe, respectively (Fig. 2a, b). In early spring, soil temperatures rose rapidly, accompanied by significant snowmelt. Intensified low-frequency FTCs (LFTC) and high-frequency FTCs (HFTC) enhanced soil moisture by 0.03 $m^3\ m^{-3}$ and 0.05 $m^3\ m^{-3}$, respectively, and raised soil temperatures by 2 ℃ and 3 ℃ during the period. However, neither intensified LFTC nor HFTC had any significant impact on soil moisture or temperature in the subsequent growing season (Fig. 2a, b).

3.2 Soil characteristics

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Throughout the study period, intensified LFTC and HFTC only significantly increased soil NH_4^+-N levels in spring, but did not show any significant effects in the following season ($p < 0.05$, Fig. 3a, b). In the meadow steppe, intensified LFTC and HFTC significantly increased soil NH_4^+-N levels by 25.0 % and 24.0 % in late spring, respectively ($p < 0.05$, Fig. 3a). Additionally, intensified LFTC enhanced net ammonification rates by 44.3 % and 58.6 %, and HFTC increased them by 58.3 % and 50.3 % in early and late spring, respectively ($p < 0.05$, Fig. 3e). In the sandy steppe, LFTC and HFTC increased soil NH_4^+-N by 25.0 % and 23.3 % in late spring ($p < 0.05$, Fig. 3b). Intensified LFTC had no significant impact on net ammonification rates, while HFTC increased net ammonification rates by 16.2 %, 63.3 %, and 37.2 % in early spring, late spring, and early summer, respectively ($p < 0.05$, Fig. 3f). It is important to note that neither LFTC nor HFTC had significant effects on NO_3^--N or net nitrification rates at either site throughout the study period ($p < 0.05$, Fig. 3c, d, g, h).

Intensified LFTC significantly decreased the soil microbial biomass C (MBC) in spring, while the effect of HFTC on microbial biomass N (MBN) persisted to summer ($p < 0.05$, Fig. 4a-d). In the meadow steppe, HFTC decreased MBC by 16.2 % ($p < 0.05$, Fig. 4a). Conversely, both LFTC and HFTC increased MBN by 26.2 % and 26.9 %, respectively ($p < 0.05$, Fig. 4c). In the sandy steppe, HFTC decreased MBC by 11.3% in early spring. Unlike MBC, both LFTC and HFTC increased MBN by 8.5 % and 28.2 %, respectively ($p < 0.05$, Fig. 4b, d).

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3.3 Plant properties

Intensified LFTC did not have significant influences on shoot and root biomass N of the selected plant species during the growing season at either site (Fig. 5a-f). In the meadow steppe, HFTC increased shoot and root biomass N of *Stipa baicalensis* (perennial bunch grass) by 19.7 % and 21.8 % at the end of the growing season, respectively. Conversely, HFTC decreased shoot and root biomass N of *Leymus chinensis* (perennial rhizome grass) by 23.9 % and 16.2 %, and decreased those of *Carex pediformis* (perennial forb) by 22.2 % and 18.0 % ($p < 0.05$, Fig. 5a, c, e). In the sandy steppe, HFTC increased the shoot and root biomass N of *Hedysarum monglicum* (semi-shrub) by 22.6 % and 23.7 %, respectively. However, HFTC decreased those of *Cleistogenes squarrosa* (perennial bunch grass) by 25.3 % and 12.1 %, and those of *Klasea centauroides* (perennial forb) by 23.1 % and 20.3 %. ($p < 0.05$, Fig. 5b, d, f).

3.4 ^{15}N Retention in the soil-microorganism-plant systems

In both grassland types, soil ^{15}N recovery was highest in early spring, followed by a rapid decline from late spring to late summer. This was then followed by a gradual increase in recovery until late autumn (Fig. 6c, d). In contrast, plant ^{15}N acquisition increased steadily throughout the growing season in both grasslands, while microbial ^{15}N recovery exhibited only modest fluctuations over the entire growing season ($p < 0.05$, Fig. 6e-h).

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332 During the early growing season, intensified LFTC had no significant effect on total
333 ^{15}N recovery in soil-microorganism-plant systems, while intensified HFTC
334 significantly increased total ^{15}N recovery (Fig. 6a, b). LFTC did not significantly
335 impact soil ^{5}N recovery, but HFTC significantly increased soil ^{15}N recovery in the two
336 grasslands ($p < 0.05$, Fig. 6c, d). In the meadow steppe, intensified LFTC and HFTC
337 significantly enhanced microbial ^{15}N recovery by 38.0% and 26.6%, respectively, and
338 by 49.5 % and 32.5 % in the sandy steppe ($p < 0.05$, Fig. 6e, f). Intensified LFTC did
339 not significantly impact plant ^{5}N acquisition; in contrast, intensified HFTC
340 significantly decreased plant ^{15}N recovery in the two grasslands ($p < 0.05$, Fig. 6g, h).

341

342 In the meadow steppe, the ^{15}N acquisition in the shoots of *Stipa baicalensis* (perennial
343 bunch grass) and *Carex pediformis* (perennial forb) were comparable, while *Leymus*
344 *chinensis* (perennial rhizome grass) exhibited lower ^{15}N acquisition. In contrast, the
345 highest ^{15}N acquisition in roots was observed in *Leymus chinensis*, followed by *Carex*
346 *pediformis* and *Stipa baicalensis*. In the sandy steppe, both shoot and root ^{15}N
347 acquisition of *Hedysarum monglicum* (semi-shrub) were the highest among the
348 studied species. This was followed by the shoot ^{15}N acquisition of *Cleistogenes*
349 *squarrosa* (perennial bunch grass) and *Klasea centauroides* (perennial forb). Notably,
350 the root ^{15}N acquisition of *Klasea centauroides* was higher than that of *Cleistogenes*
351 *squarrosa*.

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In the meadow steppe, HFTC increased shoot and root ^{15}N acquisition of *Stipa baicalensis* by 5.8 % and 9.3 %, respectively. In contrast, HFTC decreased ^{15}N acquisition in *Leymus chinensis* by 16.4 % and 12.1 %, and in *Carex pediformis* by 4.9 % and 7.8 % ($p < 0.05$, Fig. 7a, c, e). In the sandy steppe, shoot and root ^{15}N acquisition of *Hedysarum monglicum* increased by 3.8 % and 18.4 %, respectively. Conversely, *Cleistogenes squarrosa* experienced decreases of 16.7 % in shoots and 14.4 % in roots, while *Klasea centauroides* showed the largest reductions, with decreases of 16.1 % and 14.1 % in shoot and root ^{15}N acquisition, respectively ($p < 0.05$, Fig. 7b, d, f).

3.5 Controls on plant N acquisition

In meadow steppe, correlation analysis revealed that plant ^{15}N acquisition exhibited the strongest positive correlation with soil temperature, followed by bacterial biomass, soil NO_3^--N levels, soil moisture, soil DOC and fungal biomass under LFTC ($p < 0.05$, Fig. 8a). Negative correlations between net mineralization rates and plant ^{15}N acquisition were observed. Under HFTC, plant ^{15}N acquisition showed the strongest positive correlation with soil temperature, while bacterial biomass, soil NO_3^--N levels, soil dissolved organic C, microbial biomass C, and soil moisture also exhibited significant positive correlations. In contrast, net nitrification rate, soil NH_4^+-N levels, and soil total N content displayed significant negative correlations with plant ^{15}N acquisition ($p < 0.05$, Fig. 8b).

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In sandy steppe, plant ^{15}N acquisition exhibited the strongest positive correlation with soil total C content, followed with microbial biomass N, soil total N content, soil temperature, soil dissolved organic Cand N, and soil NO_3^--N levels Under LFTC ($p < 0.05$, Fig. 8c). Conversely, fungal biomass, microbial biomass C, microbial biomass C-to-N ratio, and bacterial biomass demonstrated the most significant negative correlations with plant ^{15}N acquisition ($p < 0.05$, Fig. 8c). Under HFTC, plant ^{15}N acquisition exhibited the strongest positive correlation with soil total N content, while significant positive correlations were also observed with soil total C content, soil temperature, microbial biomass N, soil dissolved organic C and N. In contrast, the microbial C-to-N ratio, and microbial biomass showed significant negative correlations with plant ^{15}N acquisition ($p < 0.05$, Fig. 8d).

Random forest analysis further identified soil temperature as the primary predictor of plants ^{15}N acquisition capability across all treatments ($p < 0.05$, Fig. 9). In the meadow steppe, dominant predictors shifted from soil total N, bacterial biomass, and net nitrification rate under LFTC to a broader suite of predictors including soil total N, bacterial biomass, soil moisture, soil NH_4^+-N levels, and soil dissolved organic C content under HFTC ($p < 0.05$, Fig. 9a, b). In the sandy steppe, bacterial biomass, fungal biomass, soil NO_3^--N levels, and soil total N were key predictors under LFTC, while soil moisture, soil total N, bacterial biomass, net nitrification rate, and net ammonification rate were important predictors under HFTC ($p < 0.05$, Fig. 9c, d).

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4 Discussion

4.1 Contrasting FTC sensitivity in temperate grasslands

This study investigated how intensified freeze-thaw cycles (FTC) affect dynamics of winter N sources during subsequent growing seasons in two contrasting temperate grasslands, using a $^{15}NH_4^{15}NO_3$ tracer. Contrary to our first hypothesis, intensified FTC significantly enhanced soil net ammonification rates and inorganic N levels, though net nitrification rates remained unaffected (Fig. 3). Our findings are consistent with previous research indicating that intensified FTC significantly enhanced spring soil inorganic N content across diverse ecosystems, including temperate forests, alpine meadows, and wetlands (Dai et al., 2020; Ji et al., 2024; Nie et al., 2024; Teepe and Ludwig, 2004; Wang et al., 2012; Yang et al., 2023). A possible explanation for this phenomenon is that frequent FTC promote the release of DON through the physical disruption of soil aggregates and microbial lysis (Koponen et al., 2006; Sawicka et al., 2010; Skogland et al., 1988). This process likely stimulates microbial activity, accelerating mineralization processes (Fitzhugh et al., 2001; Nie et al., 2024; Sharma et al., 2006). The lack of significant change in NO_3^- levels could be attributed to leaching and minimal variations in nitrification rates (Gao et al., 2018). Notably, substantial retention of soil N and soil microbial biomass N (MBN) (Figure 4, 6) suggests that intensified FTC in early spring did not result in substantial loss of winter N sources, but instead enhanced N availability for plants and soil microbial growth.

The meadow steppe showed greater sensitivity to FTC than the sandy steppe, with

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even increased low-frequency FTC enhancing net ammonification rates (Fig. 3). Following spring thaw, the effects of FTC on ammonification rates and ^{15}N retention gradually diminished. suggesting these perturbations primarily create early-season pulses rather than sustained changes. The differential response in the sandy steppe could reflect its coarser texture, lower organic matter content, and more drought-adapted microbial community that may be inherently more resistant to FTC-induced disturbance (Yanai et al., 2007; Lipson et al., 2000). The contrasting sensitivity between the two grasslands highlights the importance of considering ecosystem-specific characteristics when predicting biogeochemical responses to climate change.

4.2 Intensified FTC alters microbial nutrient-use strategies

Our study found that intensified FTC significantly reduced soil microbial biomass C (MBC) during the early growing season in both grasslands (Fig. 4), consistent with previous observations of microbial lysis under FTC conditions (DeLuca et al., 1992; Walker et al., 2006). This reduction in MBC is mechanistically connected to DOC loss due to intensified FTC (Deng et al., 2023). During snowmelt, soil DOC pool becomes susceptible to leaching, particularly in early spring when plant uptake is minimal, leading to a transient C limitation that further constrains MBC recovery (Lipson et al., 2000; Sullivan et al., 2020). Interestingly, although significant decreases in MBC were observed, microbial biomass N (MBN) presented significant increases in early spring (Fig. 4). This increase in MBN may be attributed to enhanced

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ammonification rates, which allows soil microorganisms to luxuriously utilize soil inorganic N (Christopher et al., 2008; Nielsen et al., 2001; Skogland et al., 1988; Wang et al., 2024; Yu et al., 2011). This decoupled response between MBC and MBN suggests that soil microorganisms can effectively compete for winter-accumulated N even when C becomes limiting, highlighting their adaptive capacity to prioritize N storage under environmental stress (Yu et al., 2011). This also indicates that FTC-induced stress triggers shift in microbial stoichiometry to optimize N retention at the expense of C use efficiency (Schimel and Bennett, 2004), underscoring how winter climate change may fundamentally alter microbial nutrient cycling strategies in temperate grasslands.

4.3 Limited losses of winter N resources under intensified FTC

Our study reveals that intensified FTC did not cause significant losses of winter N resources in temperate grassland ecosystems. We observed that intensified FTC resulted in an increase in total ^{15}N recovery within soil-microorganism-plant systems during the early growing season, though this effect diminished over time and eventually returned to ambient levels (Fig. 6 a, b), indicating that effective ecosystem-level N retention mechanisms. The observed N retention capacity suggests these ecosystems may be more resistant to winter climate change than previously assumed (Han et al., 2018; Song et al., 2017). First, soil ^{15}N recovery remained significantly elevated throughout the entire growing season following FTC, indicating efficient physical protection and chemical stabilization of released N within the soil

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pool (Fig. 6). Second, microbial ^{15}N recovery increased during the early growing season after intensified FTC but gradually returned to ambient levels in the mid-growing season (Fig. 6). This dynamic suggests that soil microorganisms rapidly immobilized winter N resources during early spring, then progressively released it to support subsequent plant growth (Bilbrough et al., 2000; Zheng et al., 2024; Turner and Henry, 2009). Furthermore, the temporal decoupling of microbial N immobilization and plant N uptake may serve as an important stabilizing mechanism of winter N resources, preventing competitive exclusion while fostering mutually beneficial plant-microbe interactions (Ma et al. 2020).

4.4 Divergent plant strategies for ^{15}N acquisition under intensified FTC

Our findings partially support second hypothesis, revealing that high-frequency FTC significantly reduced overall plant ^{15}N acquisition, with contrasting responses observed among different plant functional types (Fig. 7). While dominant species (*S. baicalensis* in the meadow steppe and *H. monglicum* in the sandy steppe) demonstrated enhanced ^{15}N acquisition under intensified high-frequency FTC, other perennial grasses (*L. chinensis* and *C. squarrosa*) and forbs (*C. pediformis* and *K. centauroides*) showed reduced ^{15}N uptake. These effects are likely attributed to phenological differences in N acquisition timing, species-specific root system vulnerability to FTC damage, and variation in competitive abilities under FTC stress (Hosokawa et al., 2017; Reinmann et al., 2019; Song et al., 2017).

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The increased ^{15}N acquisition by dominant species likely reflects their ecological adaptations to cold conditions (Fig. 5). *S.baicalensis*, as a cold-tolerant bunchgrass with early spring phenology (Ma et al., 2018; Wang et al., 2016), and *H.monglicum*, as a deep-rooted legume with nitrogen-fixing capability (Lonati et al., 2015), were able to maintain root activity during freezing periods (Larsen et al., 2012) and effectively compete for winter N sources. This aligns with observations that dominant species can meet N demands during growing seasons through winter root activity (Bilbrough et al., 2000; Miller et al., 2009).

The decreased ^{15}N acquisition in subordinate species (perennial rhizome grasses and forbs) reflects a complex physiological constraints and ecological trade-offs. Their later N uptake phenology (Ma et al., 2018), coupled with greater susceptibility of fine roots to FTC damage (Campbell et al., 2014; Song et al., 2017), limited their ability to absorb winter N sources. First, the delayed N acquisition phenology of these species creates a critical disadvantage. As demonstrated by Ma et al. (2018), perennial rhizome grasses and forbs initiate ^{15}N uptake significantly later than dominant bunch grasses, particularly following freeze-thaw events, missing the early N pulse. Additionally, intensified HFTC induces substantial fine root damage (Campbell et al., 2014; Song et al., 2017), leading to increase mortality rates and constraining their capacity to access winter N sources (Hosokawa et al., 2017; Reinmann et al., 2019). In this study, the significant reduction in root biomass N supports these mechanistic explanations (Fig. 5).

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Second, although rhizome grasses have substantial coverage, they are less competitive than bunch grasses and are more susceptible to environment disturbances, making them vulnerable to damage during FTC period (Walker et al., 2004). Similarly, perennial forbs (*Carex pediformis*) possess slender and creeping roots, which are also prone to damage from FTC (Ye et al., 2017). These findings highlight intensified HFTC may alter competitive hierarchies in grassland ecosystems by favoring cold-adapted dominant species while disadvantaging other functional groups. Such shifts could have important implications for plant community structure and ecosystem functioning under changing winter climate conditions, particularly through potential changes in N cycling dynamics and species composition.

4.5 Future research directions

To advance our understanding of intensified FTC effects on N dynamics in temperate grasslands, future research should prioritize three key directions: first, molecular characterization of cold-adapted microbial communities would elucidate the specific taxa and functional genes responsible for retention of winter N resources during FTC events, providing insights into the microbial mechanisms underpinning ecosystem resilience; second, longer-term studies tracking ^{15}N fate across multiple annual freeze-thaw cycles are needed to assess whether the observed N retention patterns persist over long timescales; finally, comparative investigations across diverse grassland types and climatic gradients would help determine how soil properties,

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vegetation composition, and regional climate modulate ecosystem responses to winter climate change, enabling more accurate predictions of biogeochemical cycling under future climate scenarios.

5 Conclusions

Our study provides novel mechanistic insights into how intensified frequency of freeze-thaw cycles (FTC) regulate the dynamics of winter N sources in temperate grasslands. Three important advanced emerge from our findings: first, the observed decoupling of ammonification and nitrification processes under intensified FTC reveals enhanced retention of winter N resources. Second, the microbial community demonstrates remarkable adaptability to FTC stress, maintaining efficient N immobilization despite carbon limitation, as evidenced by increased microbial biomass N concurrent with decreased biomass C. Most importantly, intensified high-frequency FTC reduced overall plant ^{15}N acquisition, with divergent responses among functional types: dominant cold-tolerant species (perennial bunch grasses and semi-shrubs) maintained higher ^{15}N acquisition through phenological advantages, while subordinate species (perennial rhizome grasses and forbs) showed reduced uptake. These findings indicate that microbial communities serve as resilient buffers against N loss during FTC events, and plant functional traits mediate ecosystem-level responses to changing winter conditions. The demonstrated partitioning patterns of winter N resources challenge current models of grassland N cycling by revealing the importance of winter processes in shaping growing season N availability. Future

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research should focus on quantifying how these FTC-induced N dynamics scale to influence multi-year ecosystem trajectories under climate change scenarios.

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795 **Table 1** Climate, soil, and plant community properties (± Standard Error) in the meadow steppe
796 and the sandy steppe (n = 6).

	Term	Meadow steppe	Sandy steppe
Site information	Location	49°19' N, 120°02' E	39°29' N, 110°11' E
	Soil type	Loam soil	Sandy loam soil
	MAT (℃)	-1.5~1	6.5
	MAP (mm)	420	310
	Elevation (m)	628	1290
	Frequency of spring freeze-thaw cycle (times)	19	21
Soil property	STC (kg m^{-2})	3.98 ± 0.14*	1.00 ± 0.10
	SIN (g m^{-2})	1.79 ± 0.09*	0.86 ± 0.05
	20-2000 μm (%)	63.71 ± 1.58*	48.59 ± 1.98
	2-20 μm (%)	27.23.13 ± 0.63*	36.74 ± 067
	< 2 μm (%)	10.13 ± 0.23*	6.42 ± 0.13
	pH	7.36 ± 0.26	8.57 ± 0.07
	BD	1.37 ± 0.11	1.26 ± 0.10
Plant property	Cover (%)	*Stipa baicalensis* 40 ± 1.26	*Hedysarum monglicum* 35 ± 1.36
		Leymus chinensis 20 ± 0.86	*Cleistogenes squarrosa* 23 ± 0.79
		Carex pediformis 25 ± 0.59	*Klasea centauroides* 12 ± 0.48
The treatment times	HFTC	7 March, 9 March, 10 March, 12 March, 14 March, 15 March, 17 March, 18 March, 20 March, 21 March, 23 March, and 26 March 2021	10 February, 16 February, 18 February, 20 February, 21 February, 23 February, 25 February, 26 February, 28 February, 1 March, 3 March, and 5 March 2021
	LFTC	7 March, 10 March, 14 March, 17 March, 20 March, and 23 March 2021	10 February, 18 February, 21 February, 25 February, 28 February, and 3 March 2021

Significant differences between sites were identified using One-way ANOVAs: *, $p < 0.05$. MAT, mean annual temperature; MAP, mean annual precipitation; STC, soil total C content; SIN, soil inorganic N content; BD, soil bulk density; HFTC, increased high frequency freeze-thaw cycles (12 times); LFTC, increased low frequency freeze-thaw cycles (6 times).

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Figure Legends

Figure 1. Geographical distribution of the transect in a meadow steppe and a sandy steppe in northern China.

Figure 2. Soil temperature (Figure. 2a) and moisture (Figure. 2b) from autumn 2020 to autumn 2021 under intensified low-frequency freeze-thaw cycles (LFTC; 6 times) and high-frequency freeze-thaw cycles (HFTC; 12 times) treatments in a meadow steppe and a sandy steppe in northern China. Shaded vertical bars indicate processing (treatment) period. Vertical lines indicate natural freeze-thaw periods. Nablas indicate sampling times, dates for ^{15}N tracer injection and sampling dates are also shown.

Figure 3. Soil NH_4^+-N, NO_3^--N, net ammoniation and net nitrification rates under intensified low-frequency freeze-thaw cycles (LFTC; 6 times) and high-frequency freeze-thaw cycles (HFTC; 12 times) treatments in a meadow steppe and a sandy steppe in northern China. Vertical bars indicate the standard error (SE) of the means (n = 6). Different lowercase letters indicate statistically significant differences among treatment groups within sampling periods ($p < 0.05$).

Figure 4. Soil microbial biomass C and N under intensified low-frequency freeze-thaw cycles (LFTC; 6 times) and high-frequency freeze-thaw cycles (HFTC; 12 times) treatments in a meadow steppe and a sandy steppe in northern China. Vertical bars indicate the standard error (SE) of the means (n = 6). Different lowercase letters indicate statistically significant differences among sampling periods ($p < 0.05$).

Figure 5. Plant biomass N (shoot and root) under intensified low-frequency freeze-thaw cycles (LFTC; 6 times) and high-frequency freeze-thaw cycles (HFTC; 12 times) treatments in a meadow steppe and a sandy steppe in northern China. Vertical bars indicate the SE of the means (n = 6). Different lowercase letters indicate statistically significant differences among sampling periods ($p < 0.05$).

Figure 6. Dynamics of ^{15}N tracers in soils, microorganisms, and plants under intensified

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Figure 7. Plant ^{15}N acquisition under intensified low-frequency freeze-thaw cycles (LFTC; 6 times) and high-frequency freeze-thaw cycles (HFTC; 12 times) treatments in a meadow steppe and a sandy steppe. Vertical bars indicate the SE of the mean (n = 6). Different lowercase letters indicate statistically significant differences among sampling periods ($p < 0.05$).

Figure 8. Relationships between plant ^{15}N acquisition and environmental predictors under intensified low freeze-thaw cycle (LFTC; 6 times) and high freeze-thaw cycle (HFTC; 12 times) treatments in the meadow steppe and the sandy steppe. DOC represents dissolved organic C content, DON represents dissolved organic N content, F:B denotes the fungal to bacterial biomass ratio, and MBC:MBN indicates the microbial biomass C to N ratio.

Figure 9. Relative importance of environmental predictors for plant ^{15}N acquisition as determined by random forest analysis under intensified low freeze-thaw cycle (LFTC; 6 times) and high freeze-thaw cycle (HFTC; 12 times) treatments in the meadow steppe and the sandy steppe. Predictor importance is expressed as percentage increase in mean squared error (%IncMSE) when each variable is permuted. DOC represents dissolved organic C content, DON represents dissolved organic N content, F:B denotes the fungal to bacterial biomass ratio, and MBC:MBN indicates the microbial biomass C to N ratio.

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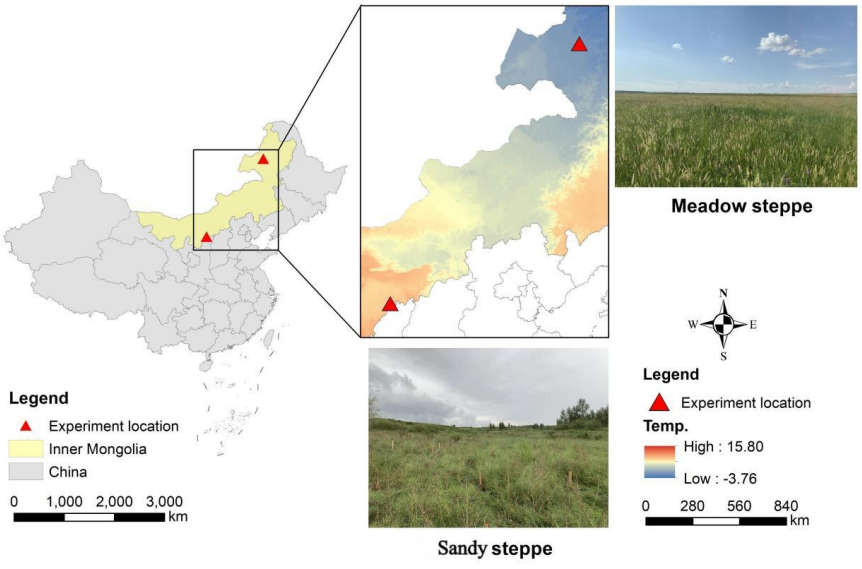

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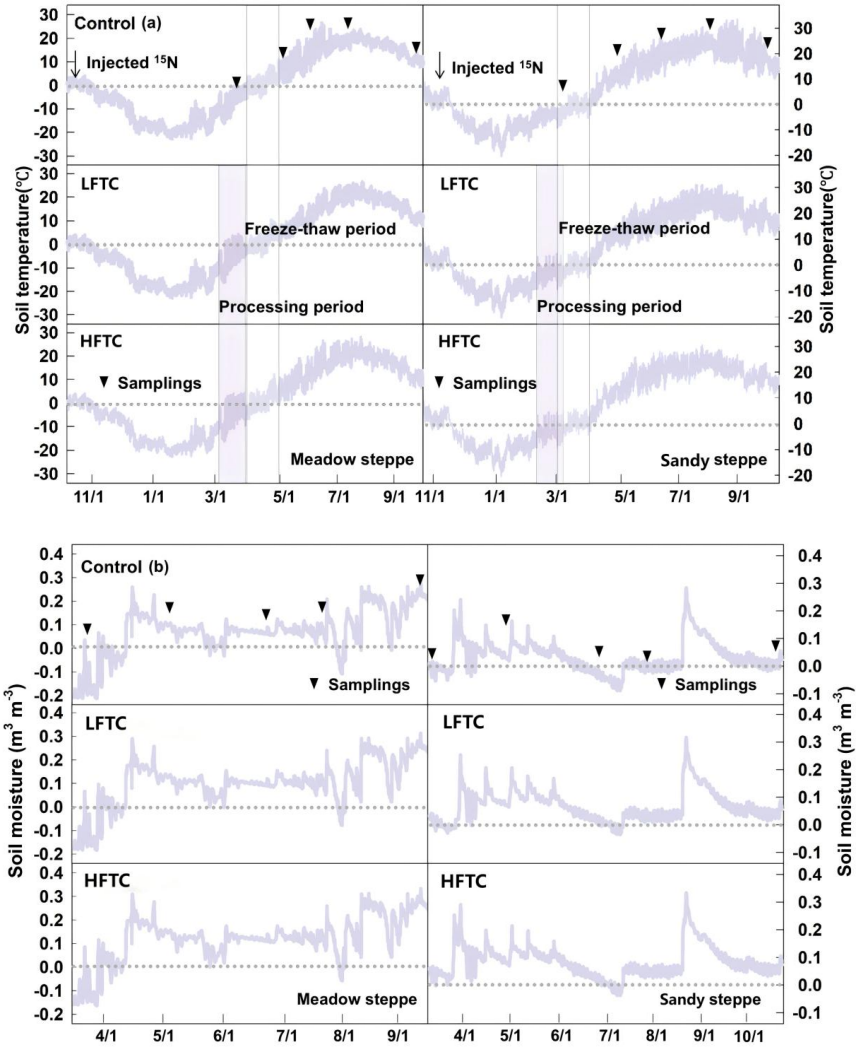

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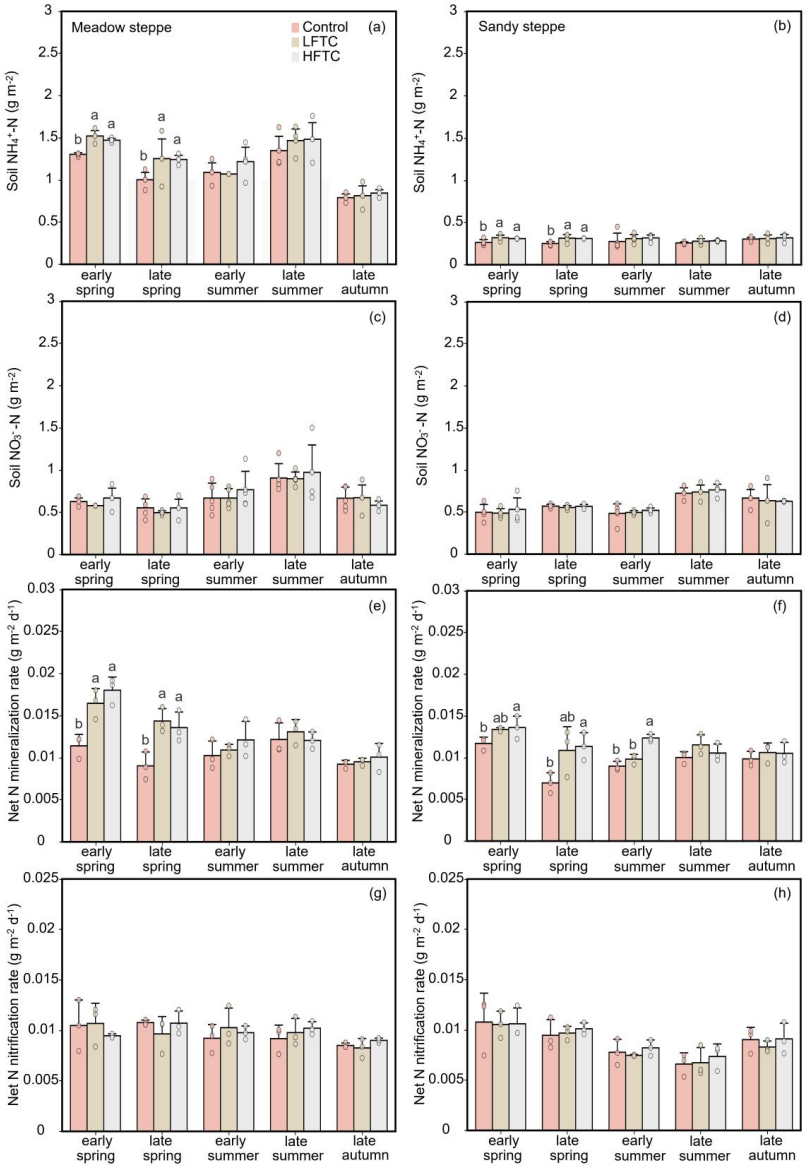

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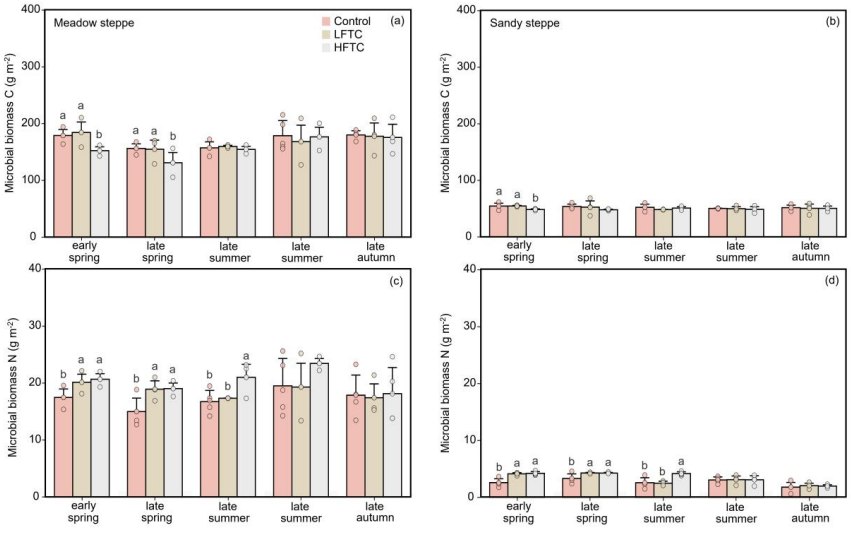

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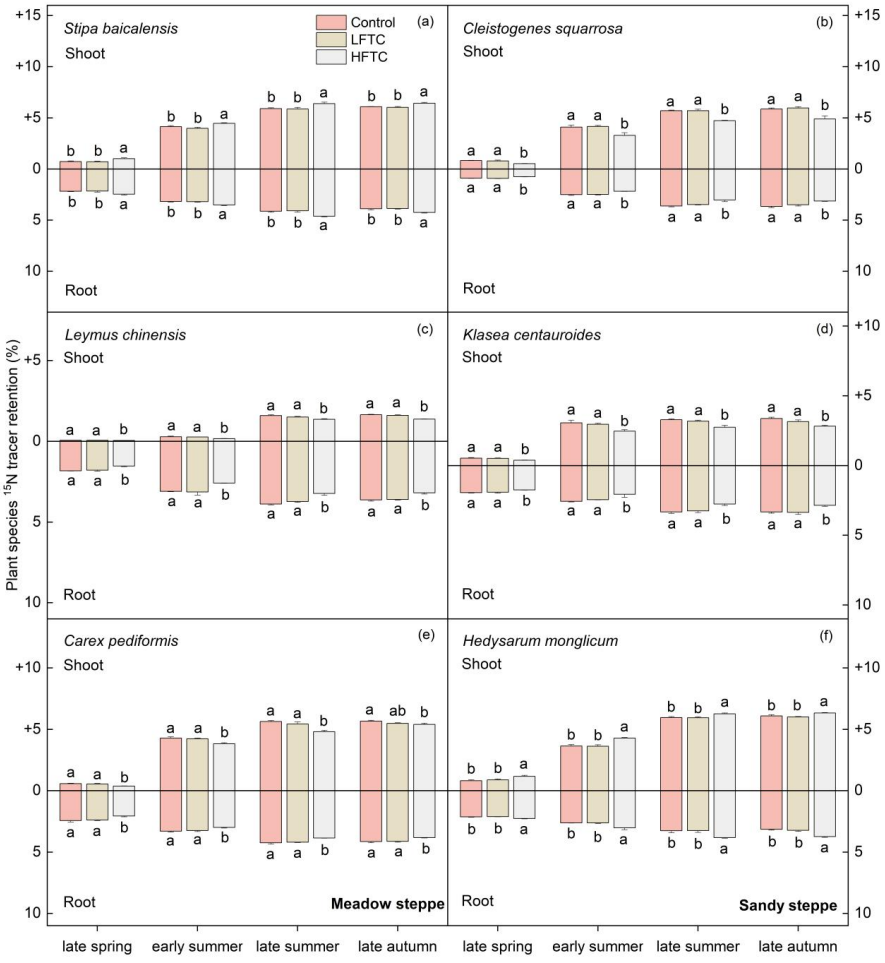

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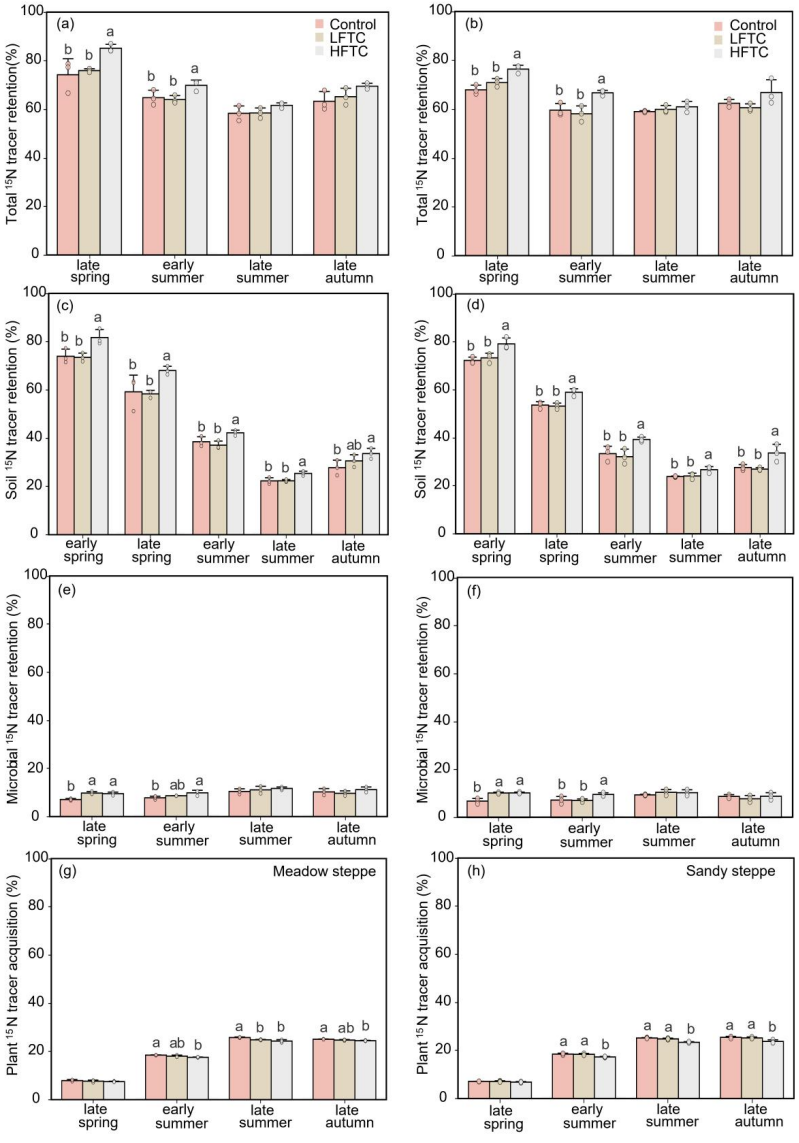

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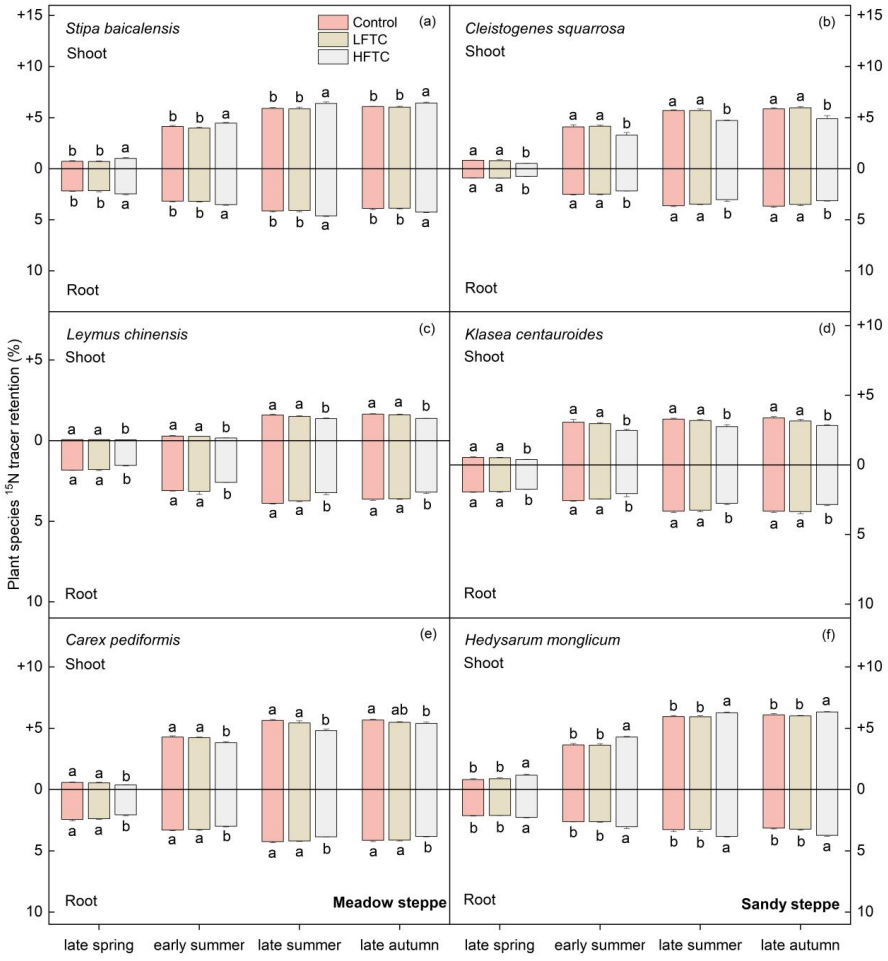

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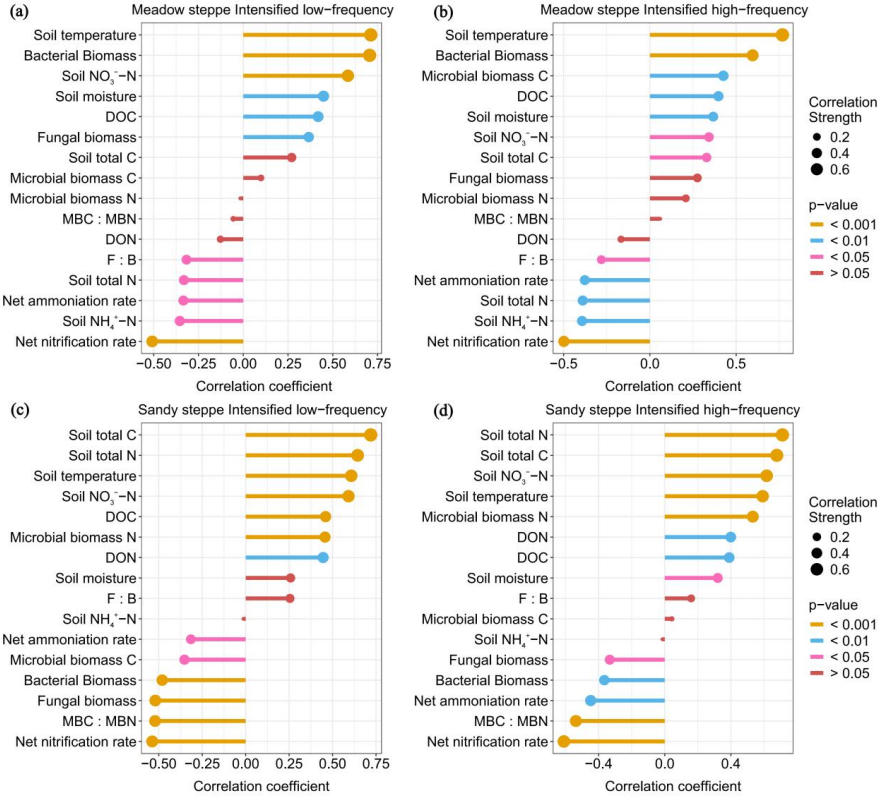

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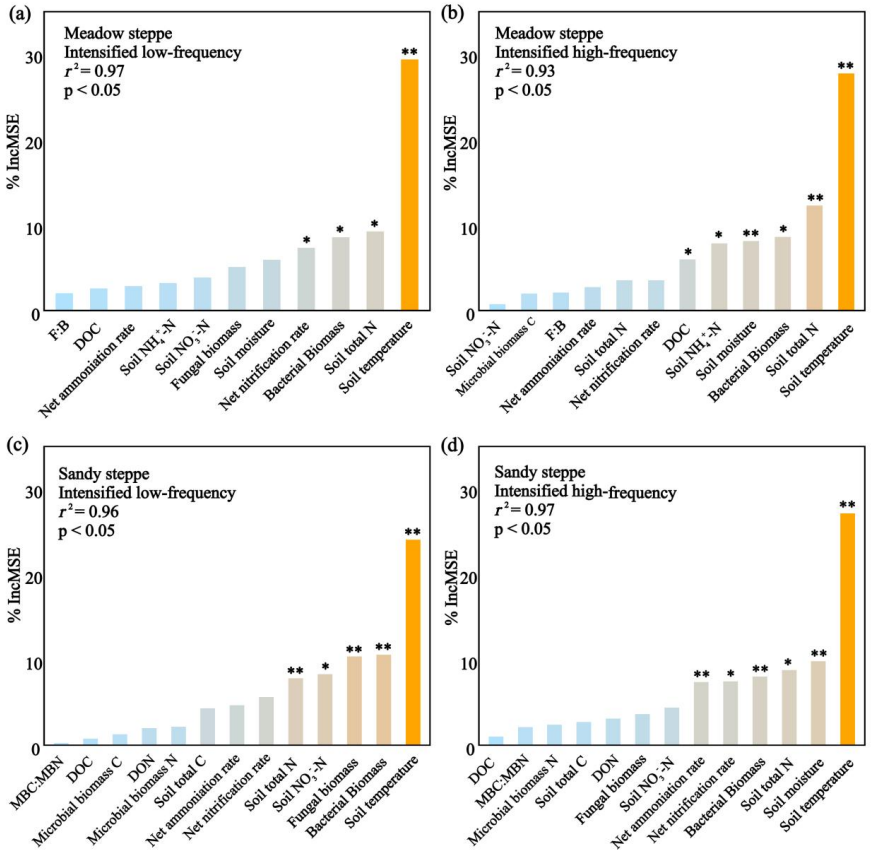

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