

Exploring the overlooked nitrogen transformation pathways for nitrogen loss or retention from the soil scenario: a contemporary and holistic approach towards sustainability

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Exploring the overlooked nitrogen transformation pathways for nitrogen loss or retention from the soil scenario: a contemporary and holistic approach towards sustainability

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Editorial: Exploring the overlooked nitrogen transformation pathways for nitrogen loss or retention from the soil scenario: a contemporary and holistic approach towards sustainability

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KEYWORDS

nitrogen cycle, microbial dynamics, ecosystem management, diazotrophs, sustainable solutions

Editorial on the Research Topic

Exploring the overlooked nitrogen transformation pathways for nitrogen loss or retention from the soil scenario: a contemporary and holistic approach towards sustainability

The nitrogen (N) cycle is not a simple chain of reactions but a dynamic and interconnected web of microbial processes, where countless invisible players collectively determine the fate of N in ecosystems (Figure 1). It is best understood as a modular and evolving network of transformations, continuously shaped by the metabolic diversity and adaptability of microbial communities (1). The trajectory of these transformations, whether N is lost through denitrification or anaerobic ammonium oxidation (ANAMMOX), or retained within the system through dissimilatory nitrate reduction to ammonium (DNRA), is governed largely by the composition, interactions, and functional capacity of the inhabiting microbial guilds (2, 3). What makes N unique among soil elements is not only its abundance but also its major role in plant growth, development, and productivity, directly linking belowground microbial processes with aboveground ecosystem functions (4). Yet, N transformations in soil are anything but uniform. Contrasting aerobic and anaerobic conditions create vastly different biochemical landscapes, where microbes act as unseen engineers, orchestrating redox reactions that regulate nutrient retention, greenhouse gas fluxes, and soil fertility (5, 6). Among the less well-understood microbial pathways is denitrifying anaerobic methane oxidation (DAMO), a process in which *Methyloirabilis* bacteria or *Methanoperedens* archaea use methane as an electron donor to reduce nitrate or nitrite. Despite its profound implications for both the N and carbon cycles, DAMO, along with DNRA and ANAMMOX, remains underrepresented in

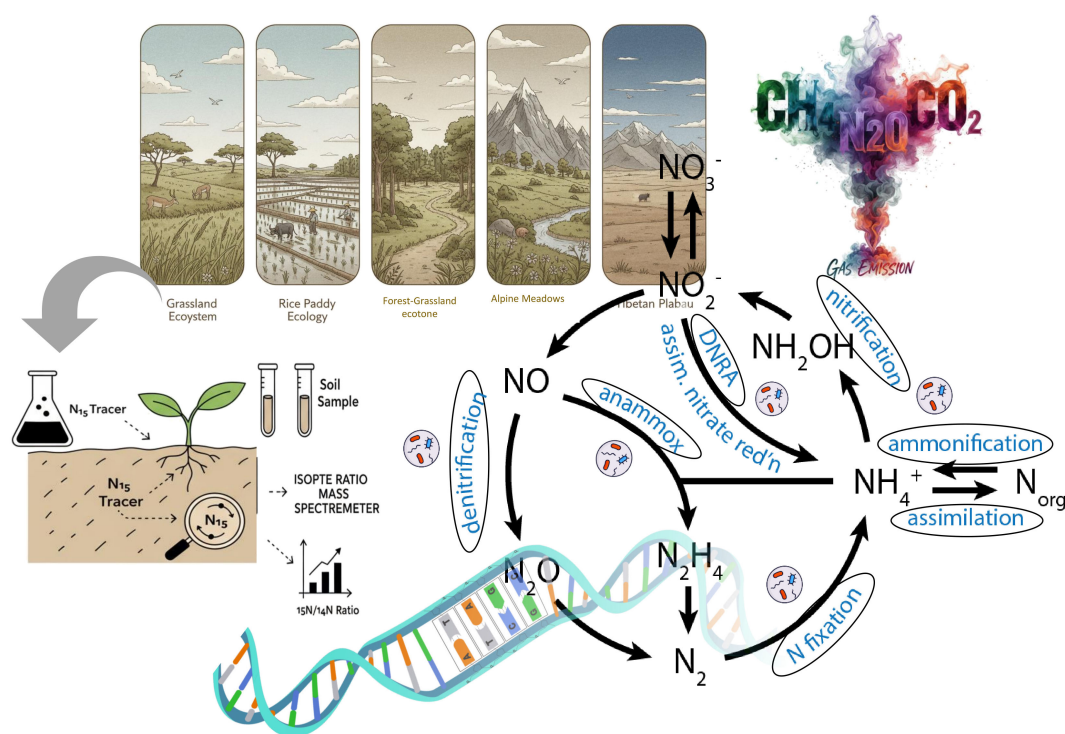


FIGURE 1

Diagram illustrating nitrogen cycling across different ecosystems: grassland, rice paddy, forest-grassland ecotone, alpine meadows, and Tibetan plateau. Processes include nitrification, denitrification, ammonification, and nitrogen fixation. It involves tracing nitrogen-15 isotopes in soil samples to measure isotope ratios. Gas emissions are represented as CH₄, N₂O, and CO₂. The cycle connects soil, plant uptake, and emissions, emphasizing nitrogen transformations and emissions.

models of the global N budget. Their absence from mainstream N research reflects the methodological challenges of detecting and quantifying these processes *in situ*, yet it also signals a frontier brimming with unanswered questions.

This Research Topic was conceived to address precisely this gap by bringing together original research, perspectives, and reviews. It aimed to unravel how microbial communities and the pathways they mediate vary across soils, sediments, wetlands, estuaries, and agricultural landscapes. The contributions highlighted the ecological and functional shifts of microbial populations under aerobic versus anaerobic regimes and revealed the importance of linking taxonomy and phylogeny to function. With the advancement of next-generation sequencing, metagenomics, metatranscriptomics, proteomics, and integrative bioinformatics, researchers are now able to decode these underexplored processes at unprecedented resolution, illuminating genetic markers, metabolic regulation, and ecological outcomes. The exploration of microbial metabolic footprints, as well as the influence of organic and inorganic amendments, opens further opportunities to bridge fundamental microbial ecology with applied soil and crop management. Looking ahead, the potential applications are wide-ranging: from developing microbial inoculants and tailored consortia to employing genome editing tools for improving N use efficiency and soil health. Equally promising is the possibility of mitigating greenhouse gas emissions by

harnessing beneficial plant-microbe-soil interactions, an urgent need under accelerating climate change.

In essence, this editorial initiative envisions the N cycle not as a closed textbook chapter, but as an evolving story in which unseen microbial protagonists continually reveal novel roles, interactions, and pathways. By weaving together overlooked processes, cutting-edge methodologies, and translational opportunities, we aim to inspire a reimagined perspective of the N cycle, one that is grounded in discovery and equally attuned to sustainable solutions. Recent contributions to this Research Topic reaffirm that N dynamics and microbial responses are far more nuanced than simple input-output relationships, being strongly mediated by ecosystem type, management practices, and microbial sensitivities. In forest-grassland ecotones, N additions markedly altered bacterial diversity through pH-driven shifts, while fungal communities remained comparatively stable (Li et al.). In degraded alpine meadows, diazotrophs were found to be more responsive to slope position and microtopography than to N inputs alone (Li et al.). Grazing experiments on the Qinghai-Tibetan Plateau demonstrated that mixed yak-sheep regimes stabilized diazotrophic communities by balancing nutrient return and vegetation dynamics (Sun et al.). Across contrasting grassland soils, N source rather than dose emerged as the dominant driver of microbial restructuring, with ammonium inputs reshaping functional profiles differently in steppe versus shrub systems (Ren

et al.). In agricultural landscapes, management practices offered equally compelling insights. Conservation tillage not only enhanced wheat yields and soil health but also reduced greenhouse gas emissions, highlighting its dual potential for productivity and climate mitigation (Sadiq et al.). Chemical strategies contributed another level of understanding, copper pyrazole was shown to suppress nitrification and denitrification by targeting keystone microbial traits (Wang et al.), while field ^{15}N tracer experiments revealed that nitrification inhibitors promoted the stabilization of fertilizer-derived N into organic pools, sustaining fertility beyond a single growing season (Quan et al.).

Together, these studies show that N cycling is governed not merely by inputs, but by the holistic interplay of soil properties, microbial ecology, and land management. This knowledge provides critical insights for designing strategies that can simultaneously enhance agricultural productivity, safeguard ecosystem resilience, and address the challenges of global environmental change. The research presented here highlights how deeply intertwined microbial communities are with the functioning of the N cycle and its consequences for ecosystem health, productivity, and climate regulation. Yet, the story is far from complete. Future research must move towards integrating overlooked processes such as DNRA, DAMO, and ANAMMOX into global biogeochemical models, while also exploring the ecological trade-offs they create under changing environmental conditions. There is also a pressing need to link molecular-scale discoveries to field-level applications through long-term experiments that evaluate the performance of microbial inoculants, biocovers, or soil amendments under realistic management scenarios. Coupling advanced multi-omics with isotopic tracing and modeling will be key to capturing the true complexity of N transformations in space and time. Ultimately, the challenge and opportunity lie in translating microbial insights into practices that improve nutrient use efficiency, reduce greenhouse gas emissions, and sustain ecosystem resilience in an era of rapid global change.

Author contributions

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Symbiotic diazotrophs in response to yak grazing and Tibetan sheep grazing in Qinghai-Tibetan plateau grassland soils

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Grazing by local livestock is the traditional human practice in Qinghai-Tibetan Plateau grassland, and moderate intensity grazing can maintain high productivity and diversity of alpine grassland. Grazing ecosystems are often nitrogen-limited, but N₂-fixing communities in response to yak grazing and Tibetan sheep grazing in Qinghai-Tibetan Plateau grassland have remained underexplored. In this study, we applied quantitative PCR quantitation and MiSeq sequencing of *nifH* under yak grazing and Tibetan grazing through a manipulated grazing experiment on an alpine grassland. The results showed that the grazing treatments significantly increased the soil ammonium nitrogen (AN) and total phosphorus (TP), but reduced the diazotrophs abundance. Compared with no grazing treatment, the composition of diazotrophs could be maximally maintained when the ratio of yak and Tibetan sheep were 1:2. The foraging strategies of grazing livestock reduced the legumes biomass, and thus reduced the diazotrophs abundance. Data analysis suggested that the direct key factors in regulating diazotrophs are AN and TP, and the changes of these two soil chemical properties were affected by the dung and urine of herbivore assemblages. Overall, these results indicated that the mixed grazing with a ratio of yak to Tibetan sheep as 1:2 can stabilize the soil diazotrophic community, suggesting that MG12 are more reasonable grazing regimes in this region.

KEYWORDS

Tibetan plateau, grazing patterns, *nifH* gene, soil microbes, alpine grassland

1. Introduction

Grasslands cover approximately above 60% of Qinghai-Tibetan Plateau (QTP) (Dong et al., 2020), and grazing by local livestock is the traditional human practice in this area. Natural grasslands are an essential fodder resource for herbivores and prevent the consumption of supplementary feed, contributing to the sustainability of the grazing system (Batalla et al., 2015). Most grasslands are grazed mainly by domestic livestock, from low, moderate, to high grazing intensity (Li et al., 2018). Overgrazing is a key driver which alters plant communities and soil nutrient availability, reducing productivity and sustainability of several ecosystem, especially in historically grazed grasslands (McSherry and Ritchie, 2013; García-Baquero et al., 2020; Alday et al., 2021). Although short-term high grazing intensity may stimulate the increase of soil

organic matter and diversity in forage species composition, overgrazing can result in soil-degradation and loss of the fertile topsoil (Abdalla et al., 2018). Therefore, optimized management practices are required to balance the grassland and the livestock (Dong et al., 2014).

Previous results showed that historical grazing had significant negative legacy effects on the plant biomass, and the strong bottom-up controls of resource addition on soil food webs are mediated by the legacy of grazing intensity (Wang B. et al., 2021). According to the moderate disturbance hypothesis and the grazing optimization hypothesis, moderate grazing intensity generally increases grassland primary productivity (McNaughton, 1993). The peaks of aboveground net primary production (ANPP) at a moderate grazing intensity were consistent with predictions of the grazing optimization hypothesis (Altesor et al., 2005). Therefore, an optimal grazing regimes can promote the regrowth of the grazed plants, and beneficial interactions among the plant, soil, microorganisms and livestock of the degraded grasslands (Dong et al., 2020).

Nitrogen (N) availability predominantly limits grassland ecosystem primary productivity, which is an important factor in affecting grazing practice. Many microbially driven processes in soils can be impacted by land management practices and changes in the plant community (Lindsay et al., 2010). Most of the N_2 -fixing microorganisms carry *nifH*, which encodes one of the components of nitrogenase (Wang Y. et al., 2021). The effects of grazing practice on the abundance of functional genes involved in soil N cycling have been documented in recent years (Ding et al., 2014; Song et al., 2019). Diazotrophs are highly diverse in phylogeny and in a wide distribution in the QTP, the abundance, Shannon diversity, and community composition of soil diazotrophs were significantly correlated with soil moisture (Che et al., 2018), while another study showed that N-fixing communities (*nifH*) were most affected by the soil C:N ratio (Singh et al., 2011). Moreover, the variation in diazotroph community composition has a greater impact on N-fixation rates than did soil characteristics (Hsu and Buckley, 2009), it is inconsistent with a research in a tallgrass prairie used primarily for cattle grazing and agriculture, where they found that abundance of *nifH* genes was not significantly correlated with N_2 -fixation rates (Caton et al., 2018). In addition, there are weakly correlations between grazing and abundances of N functional genes in a grassy woodlands, frequent livestock grazing could lead to a reduction in the biological capacity for nitrogen fixation (Lindsay et al., 2010).

Most researchers had focused on the grazing intensity (McSherry and Ritchie, 2013) and grazing enclosure (grazing or non-grazing) experiment (Ding et al., 2014). However, there are many herbivores, such as yak and Tibetan sheep which as the dominant livestock play a crucial role in alpine grassland ecosystem functions on the QTP (Dong et al., 2014; Yang et al., 2019). Therefore, understanding how the ubiquitous symbiotic diazotrophs are affected by livestock species, especially mixing livestock species grazing patterns may have implications for the optimum model of grazing and sustainable development in an alpine pastoral region. In order to fill this knowledge gap, we collected soil samples from QTP with the mixing livestock species grazing history to assess: (a) How different grazing patterns affect the diazotrophs; and (b) What are the key factors affecting diazotrophs. We hypothesize that the above ground biomass and soil nutrients are affected by the foraging strategies of different livestock. To test this hypothesis, we investigated the abundance, diversity and community

composition of diazotrophs under moderate grazing intensities, and examine the effects of yak grazing, sheep grazing and the mixing ratio of yak to sheep on the community composition of diazotrophs.

2. Materials and methods

2.1. Study sites and experimental design

This research was conducted at the town of Xihai, Haiyan Country (36°44'–37°39' N, 100°23'–101°20' E), Qinghai Province, China, situated in the Qinghai Lake basin. With an average elevation of 3,100m, this region has a typical plateau continental climate. The mean annual temperature and precipitation were 1.4°C and 330–370 mm, respectively. Specifically, the non-growing season in this region is from October to April and is long and cold, with the average temperature of the coldest month being −24.8°C. The growing season is from May to September, with an average temperature of 12.5°C in the hottest months. Moreover, the precipitation also mainly concentrated in the growing season. The soil is a clay loam and the plant community is dominated by *Kobresia humilis* (C. A. Mey. ex Trautv.) Sergiev, *Leymus secalinus* (Georgi)Tzvel., *Elymus nutans* Griseb. Nachr. Ges. Wiss. Gott., *Carex aridula* V. Krecz. and *Potentilla acaulis* L. Sp. Pl.

We set up a grazing experiment with local livestock yak and Tibetan sheep started in 2014 with a completely randomized block design. It had been proved that moderate intensity grazing can maintain higher aboveground biomass and plant diversity (Gao and Carmel, 2020; Liu et al., 2023). Based on moderate intensity grazing, which was represented as the feed intake of livestock for above-ground biomass is about 50–55%, five grazing treatments including single grazing and mixed grazing were set up, which were: single grazing for yaks (YG), single grazing for Tibetan sheep (SG), mixed grazing for yaks and Tibetan sheep at the ratio of 1:2 (MG12), 1:4 (MG14), 1:6 (MG16), respectively, and a control treatment (CK, grazing exclusion) in three blocks, there were 18 plots in total. By adjusting the area of each grazing plot according to the livestock numbers, the grazing intensity of each plot was consistent. A detailed description of the experimental site and design can be found in our previous studies (Yang et al., 2019).

2.2. Sampling and measurement

In August 2019, four sites were randomly selected in each plot for plant community investigation and soil sampling. Aboveground biomass were harvested from a 0.5 m × 0.5 m quadrat in each sampling site. After plant community investigation, soil sampling was conducted in the same quadrat (Yang et al., 2019). Three random soil cores (0–10 cm, 10–20 cm and 20–30 cm) were taken in each quadrats. The samples from each quadrats were collected and mixed to form a composite sample by different depth. After careful removal of the surface litter, earthworms or bird droppings, each sample was mixed and divided into two parts. One part was air-dried for analysis of the soil physicochemical properties, and the other was stored in a freezer at −80°C and then sieved through a 2-mm sieve before analysis of the microbial communities.

The soil total carbon (TC), soil total nitrogen (TN), soil total phosphorus (TP), ammonium nitrogen (AN) and pH were measured following method of Huang (Huang et al., 2019).

2.3. Soil DNA extraction and real-time PCR

Microbial community genomic DNA was extracted using the E.Z.N.A.[®] soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) according to manufacturer's instructions. The DNA extract was checked on 1% agarose gel, and DNA concentration and purity were determined with NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, United States).

The copies of *nifH* gene were quantified using ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The quantification was conducted with universal primer sets for *nifH*, PolF: TGC GAY CCS AAR GCB GAC TC; PolR: ATS GCC ATC ATY TCR CCG GA (Poly et al., 2001; Che et al., 2018). The 10 µL reaction systems contained: 4.4 µL of SYBR Green Mix, 0.3 µL of forward primer (20 µmol L⁻¹), 0.3 µL of reverse primer (20 µmol L⁻¹) and 5 µL of template DNA. The standard curve was constructed using plasmids inserted with the *nifH* gene fragment. The PCR runs started with an initial denaturation at 95°C for 10 min, followed by 40 cycles of 10 s at 95°C, 34 s at 60°C, 15 s at 95°C, 60 s at 60°C and 1 cycle of 30 s at 95°C, 15 s at 60°C. The specificities of PCR products were checked by melting curve analysis.

2.4. MiSeq sequencing and bioinformatics analyses

The PCR amplifications of *nifH* gene was conducted with barcoded universal primers (F: 5'-AAAGGYGGWATCGGYAARTCCACCAC-3'; R: 5'-TTGTTSGCSGCRTACATSGCCATCAT-3'). Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq PE300 platform (Illumina, San Diego, United States) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The raw *nifH* gene sequencing reads were demultiplexed, quality-filtered by fastp version 0.20.0 and merged by FLASH version 1.2.7 (Magoč and Salzberg, 2011). Operational taxonomic units (OTUs) with 97% similarity cutoff were clustered using UPARSE version 7.1, and chimeric sequences were identified and removed (Edgar, 2013). The taxonomy of each OTU representative sequence was analyzed by RDP Classifier version 2.2 against the 16S rRNA database using confidence threshold of 0.7 (Wang et al., 2007).

In total, 898,086 *nifH* gene sequences were obtained for the 54 soil samples, and the total sequence number was reduced to 859,258 after the quality filtering. The qualified sequence number of each sample ranged from 9,808 to 24,478, and thus the sequence number of each sample was rarefied to 9,808 for further analysis. With an identify cutoff of 97%, we obtained 2,421 OTUs. More details of the taxonomic assignment for *nifH* OTUs was conducted similarly as described by (Che et al., 2018). The data were analyzed on the online platform of Majorbio Cloud Platform.¹

¹ www.majorbio.com

2.5. Statistical analyses

One-way ANOVA was used to analyze the effects of grazing on soil properties, abundance of *nifH* genes and OTU numbers, followed by Duncan's new multiple range. Data were tested for normality and homogeneity of variance before analysis using the Shapiro-Wilk test and Levene's Test, respectively. Two-way ANOVA was performed testing the main and interactive effects of grazing and soil depth on soil properties. Mantel tests were performed to assess correlations between diazotrophic taxa and experimental factors. These analyses were performed with the IBM Statistical Package, SPSS version 25.0 (IBM, Armonk, NY, United States), and the histogram plots were plotted by Sigma plot software. Linear regression analyses and structural equation model (SEM) were performed to test the relationships between the OTU numbers and environmental factors using the 'ggplot2' and 'piecewiseSEM' packages in R version 4.1.0.

3. Results

3.1. Soil physiochemical properties with soil depth

Soil properties are shown in Table 1. A two-way ANOVA showed that the grazing treatments significantly affected all soil property parameters ($p < 0.01$), and soil depth effect (0–10 cm, 10–20 cm and 20–30 cm) also influenced these parameters ($p < 0.01$). Grazing treatments and soil depth had an interaction effects on soil ammonium nitrogen content (AN, $p < 0.01$) (Supplementary Table S1). Compared with the no grazing CK, the YG and MG14 treatments significantly reduced the soil pH at 0–10 cm and 10–20 cm, respectively ($p < 0.05$). Soil total carbon (TC) decreased significantly with the increase of soil layer in MG16 treatment ($p < 0.05$). Soil total nitrogen (TN) under grazing treatment did not change significantly in soil layers 0–10 ($p > 0.05$), but with the increase of soil layer, soil total nitrogen decreased significantly ($p < 0.05$). In soil layers 10–20 and 20–30, soil total nitrogen under SG treatment was the highest, and soil total nitrogen under MG16 treatment was the lowest (Table 1). The grazing treatments significantly increased the soil AN ($p < 0.05$), especially in the SG and YG treatments, and the AN are higher in the grazing alone treatment than that in the mixed grazing treatment. Moreover, compared to the CK, the grazing treatments also significantly increased the soil total phosphorus (TP, $p < 0.05$), especially in the SG and YG treatment in soil depth of 0–10 cm (Table 1). In addition, the N/P ratio was lowest in SG treatment, while highest in MG12 treatment ($p < 0.05$).

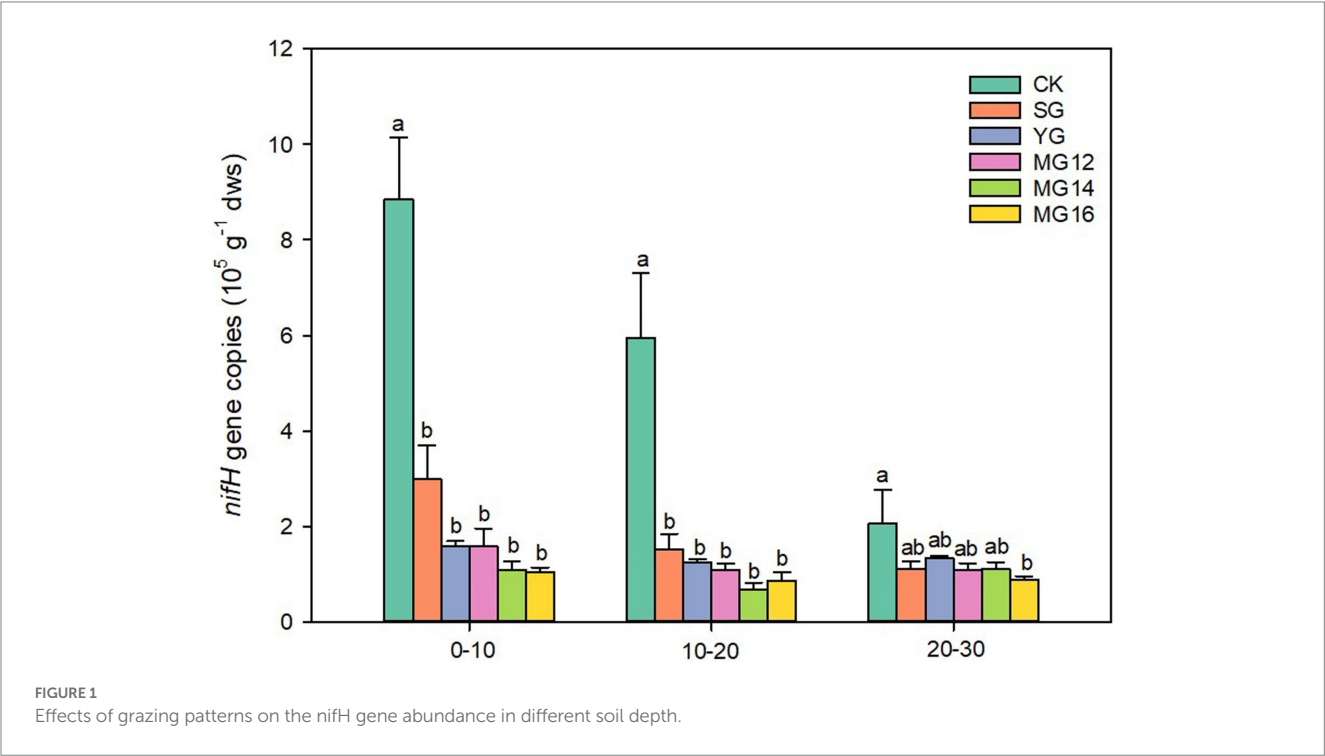
3.2. The *nifH* gene abundance with soil depth

Compared with the CK, both grazing treatments significant decreased the *nifH* gene copy number in all soil depth ($p < 0.05$) (Figure 1). The copy number of *nifH* gene in the CK treatment decreased significantly with the increase of soil layer ($p < 0.05$), whereas no significant change was observed among the other grazing treatment ($p > 0.05$). In the 0–10 cm from the soil surface, the average copy numbers of the six treatments ranged from 0.73×10^5 to 9.19×10^5 gene copies per g dry soil (Figure 1).

TABLE 1 Soil properties in different grazing treatments with soil depth.

Soil properties	Soil depth	CK	SG	YG	MG12	MG14	MG16
pH	0–10	7.84±0.06a A	7.39±0.22ab A	7.18±0.22b B	7.44±0.18ab A	7.08±0.08b B	7.32±0.18ab B
	10–20	7.97±0.04a A	7.63±0.22ab A	7.38±0.05b AB	7.88±0.08ab A	7.61±0.24ab AB	7.87±0.16ab A
	20–30	7.97±0.09a A	7.74±0.07a A	7.9±0.22a A	7.83±0.16a A	7.84±0.15a A	8.16±0.08a A
TC	0–10	37.85±1.66a A	40.64±2.79a A	41.76±0.47a A	41.88±0.75a A	41.81±0.75a A	40.77±0.31a A
	10–20	36.81±1.59a A	39.64±1.31a A	41.3±1.53a A	38.94±1.96a A	39.87±0.68a A	37.72±0.41a B
	20–30	36.4±2.61ab A	38.12±1.48ab A	37.46±1.88ab A	37.15±1.7ab A	40.67±1.33a A	33.09±0.39b C
TN	0–10	3.25±0.13a A	3.49±0.15a A	3.53±0.1a A	3.54±0.06a A	3.56±0.11a A	3.38±0.1a A
	10–20	2.79±0.11ab A	3.17±0.11a A	2.95±0.22ab A	2.85±0.19ab B	2.98±0.09ab B	2.54±0.17b B
	20–30	2.05±0.17ab B	2.56±0.06a B	2.03±0.27ab B	1.98±0.28ab C	2.43±0.2ab C	1.75±0.2b C
AN	0–10	0.91±0.22c A	18.2±1.04a A	15.87±1.36a A	9.71±0.62b A	9.86±0.18b A	9.46±1.34b A
	10–20	0.53±0.06c AB	15.72±1.69a A	13.28±1.34a AB	5.78±0.86b B	6.03±0.28b B	5.28±0.52b B
	20–30	0.28±0.18d B	6.05±0.83b B	10.24±0.45a B	4.18±0.49c B	4.74±0.42bc C	4.97±0.38bc B
TP	0–10	0.48±0.01b A	0.55±0.02a C	0.54±0.01a A	0.51±0.02ab A	0.54±0.02a A	0.52±0.01ab A
	10–20	0.43±0.03b AB	0.52±0.05ab A	0.49±0.00ab B	0.51±0.01ab A	0.58±0.04a A	0.52±0.02ab A
	20–30	0.39±0.01c B	0.54±0.01a A	0.46±0.02b B	0.49±0.03ab A	0.49±0.03ab A	0.5±0.03ab A
N/P	0–10	6.8±0.12ab A	6.36±0.13b A	6.47±0.15ab A	6.97±0.08a A	6.61±0.02ab A	6.5±0.31ab A
	10–20	6.47±0.18a AB	6.28±0.71a A	6.00±0.43ab A	5.56±0.26ab B	5.19±0.32ab B	4.89±0.22b B
	20–30	5.27±0.59a B	4.77±0.17a B	4.37±0.46ab B	4.04±0.37ab C	4.88±0.27a B	3.46±0.23b C
C/N	0–10	11.68±0.75a A	11.64±0.45a B	11.85±0.29a B	11.83±0.17a B	11.75±0.23a B	12.09±0.26a B
	10–20	13.26±1.07ab A	12.51±0.11b B	14.11±0.6ab B	13.73±0.46ab B	13.42±0.40ab B	15.01±0.94a AB
	20–30	18.20±2.96a A	14.91±0.26a A	19.13±2.40a A	19.44±2.29a A	16.96±1.43a A	19.36±2.00a A
C/P	0–10	79.44±5.11a A	74.08±3.24a A	76.62±1.43a A	82.43±1.26a A	77.72±1.30a A	78.39±2.10a A
	10–20	86.04±9.21a A	78.71±9.51a A	84.11±3.07a A	76.24±2.34a A	69.40±3.15a A	73.11±2.66a AB
	20–30	92.36±3.15a A	71.15±3.26bc A	81.57±3.90ab A	76.76±1.58bc A	82.44±5.81ab A	66.02±3.00c B

Lowercase letters in the table indicate significant differences between treatments in the same soil layer, and uppercase letters indicate significant differences between different soil layers of the same treatment.



3.3. OTU richness and relative abundance of diazotrophic taxa

The CK and MG12 treatments had similar OTU numbers, and the YG and MG16 treatments had the lower the number of OTUs ($p < 0.05$) (Figure 2). There were no significant differences among CK, SG, MG12 and MG14 ($p > 0.05$) (Figure 2). OTUs were taxonomically classified into different order across all of the treatments. Figure 3 shows the relative abundances of the most abundant order in soils of the six grazing treatments. The order *Rhodospirillales* was the most abundant, containing 58–81% of the total *nifH* gene sequences in all of the soil samples. Different grazing treatments changed the relative

abundances of these main order (Figure 3). Compared with CK, SG, YG and MG12 had similar dominant communities, while the relative abundances of *Rhodospirillales* decreased and *Nostocales* increased in the MG14 and MG16 treatments.

3.4. Correlation among diazotrophic community structure, soil variables and plant biomass

The Mantel test revealed that the diazotrophic community structures in soil surface were closely correlated with multiple soil

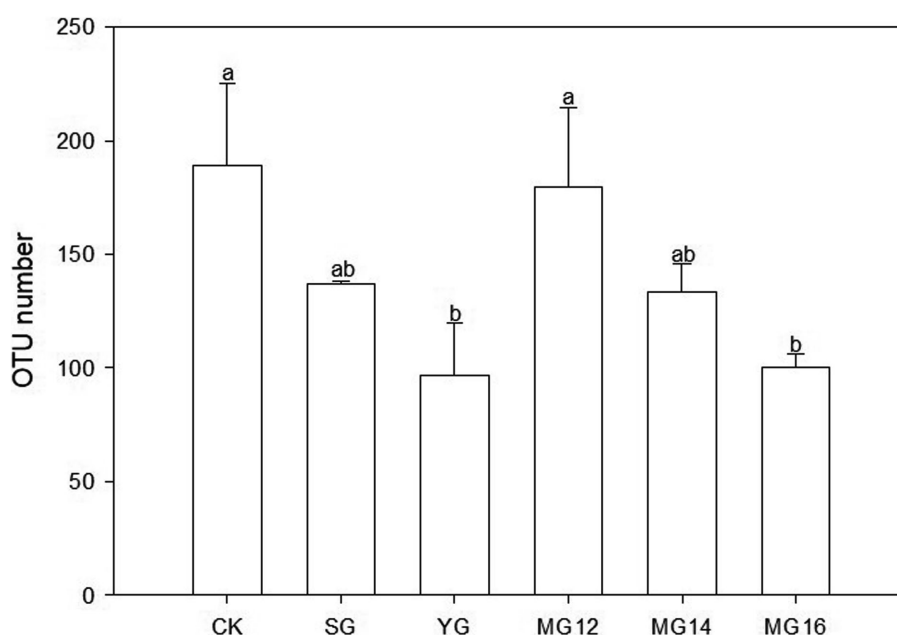


FIGURE 2
Effects of grazing patterns on the OTU numbers in 0–10 cm of soil surface.

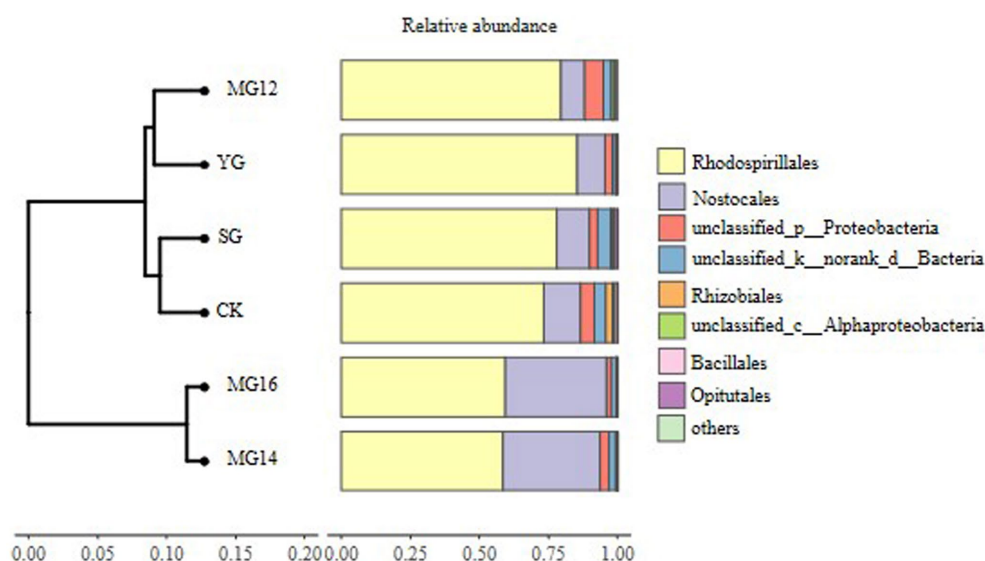


FIGURE 3
Relative abundances of diazotroph community response to different grazing patterns in the soil at the order level.

variables, such as pH, TC, AN and TP in the 0–10 cm of soil ($p<0.05$) (Table 2). then the *nifH* gene copies only had a negative correlation with TP in 10–20 cm and 20–30 cm of soil layers ($p<0.05$). whereas had no significant correlation with aboveground biomass and root biomass ($p>0.05$) (Table 2). However, specifically, *Rhizobiales* and *Bacillales* are closely related to leguminous biomass, and they are related to soil TC and TP, respectively. In addition, *Nostocales* is related to forb biomass, and *Opiritales* is related to aboveground biomass (Figure 4). Based on the linear regression analysis, there are negative correlations between OTUs number, AN and TP in the 0–10 cm of soil ($p<0.05$) (Figure 5).

The effects of soil properties and plant functional group on the structures of diazotrophic communities were further analyzed using the redundancy analysis, based on the selected soil variables, biomass of plant functional group and OTU composition. These soil variables explained 47.5% of the variation, and the first two axes explained 30.43 and 17.07% of the total variation. According to the vectors, the diazotrophic communities of both CK and grazing treatments were associated with higher TP and AN values (Figure 6). In addition, the diazotrophic communities were also associated with leguminous biomass and forbs biomass. These biomass of plant functional group

explained 58.53% of the variation, and the first two axes explained 41.9 and 16.63% of the total variation (Figure 7).

3.5. Key factors driving changes in diazotrophic communities

SEM were used to identify the key drivers on diazotrophic communities. Path analysis indicated that grazing had an impact on diazotrophs through the direct effects on leguminous biomass, AN and TP content. Grazing reduced the biomass of legumes (Supplementary Figure S1), but increased AN and TP content, and legumes were positively correlated with nitrogen-fixing bacteria, so nitrogen-fixing bacteria also decreased under grazing treatment (Figure 8).

4. Discussions

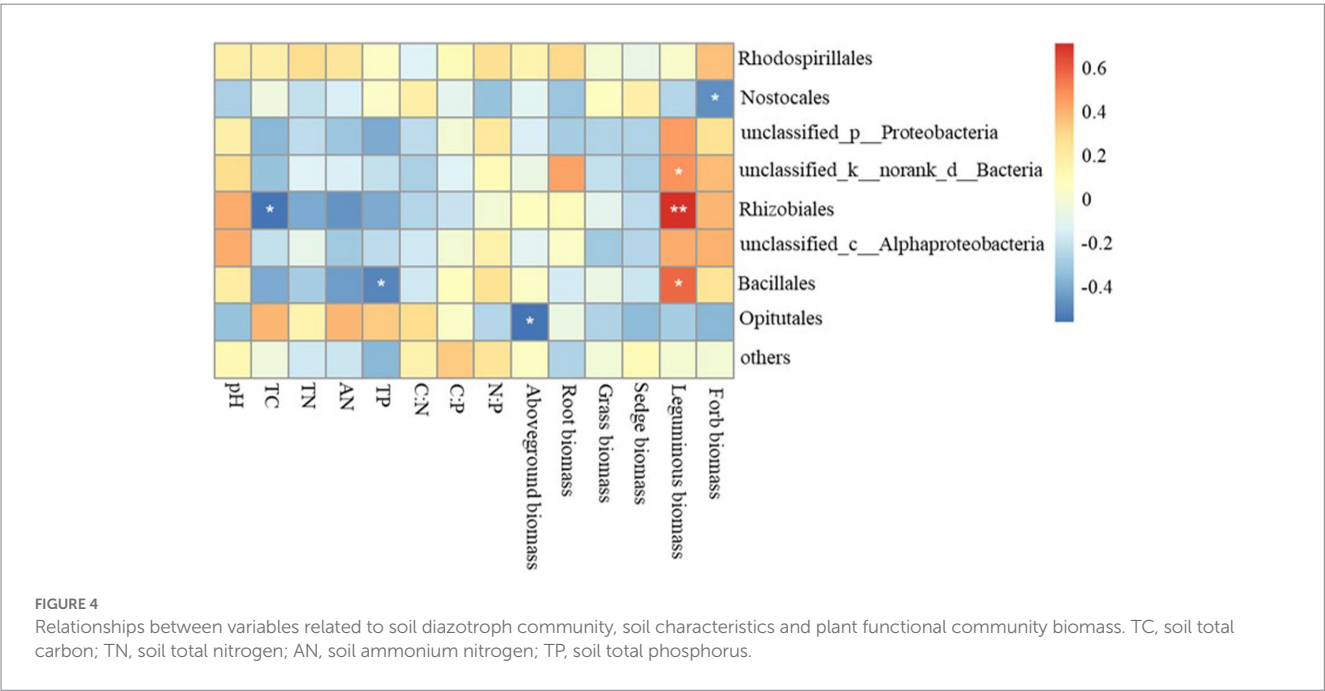
4.1. Diazotrophic abundance

Many microbially driven processes in soils can be impacted by land management practices and changes in the plant community

TABLE 2 Correlations among the *nifH* gene copies, soil properties and plant community biomass.

	pH	TC	TN	AN	TP	C/N	C/P	N/P	Aboveground biomass	Root biomass
<i>nifH</i> gene copies in 0–10	0.602**	−0.664**	−0.417	−0.595**	−0.534*	0.111	−0.134	0.199	0.315	−0.227
<i>nifH</i> gene copies in 10–20	0.349	−0.414	0.021	−0.449	−0.502*	−0.067	0.262	0.460	0.333	0.092
<i>nifH</i> gene copies in 20–30	−0.046	−0.191	0.064	−0.325	−0.557*	−0.223	0.385	0.497*	0.300	0.186

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



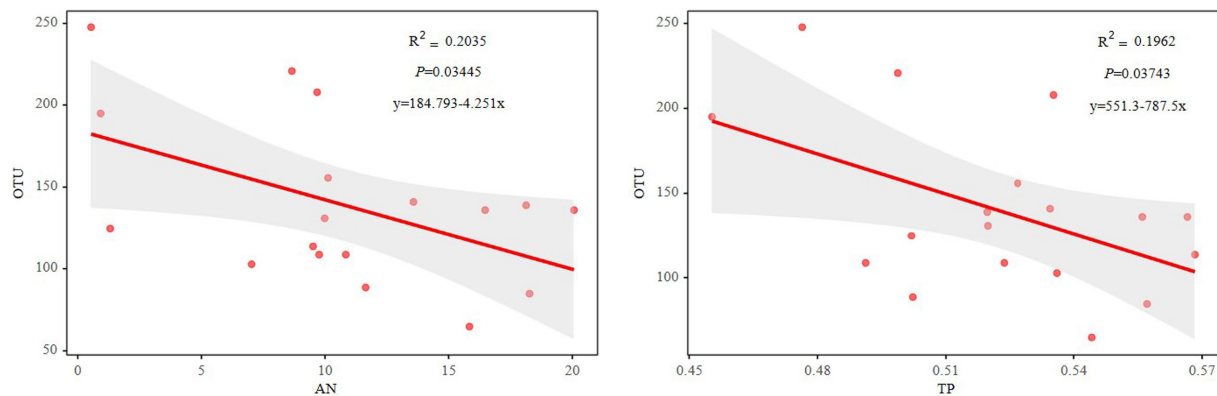


FIGURE 5

Relationships between the OTU numbers and soil AN or TP. AN, soil ammonium nitrogen; TP, soil total phosphorus.

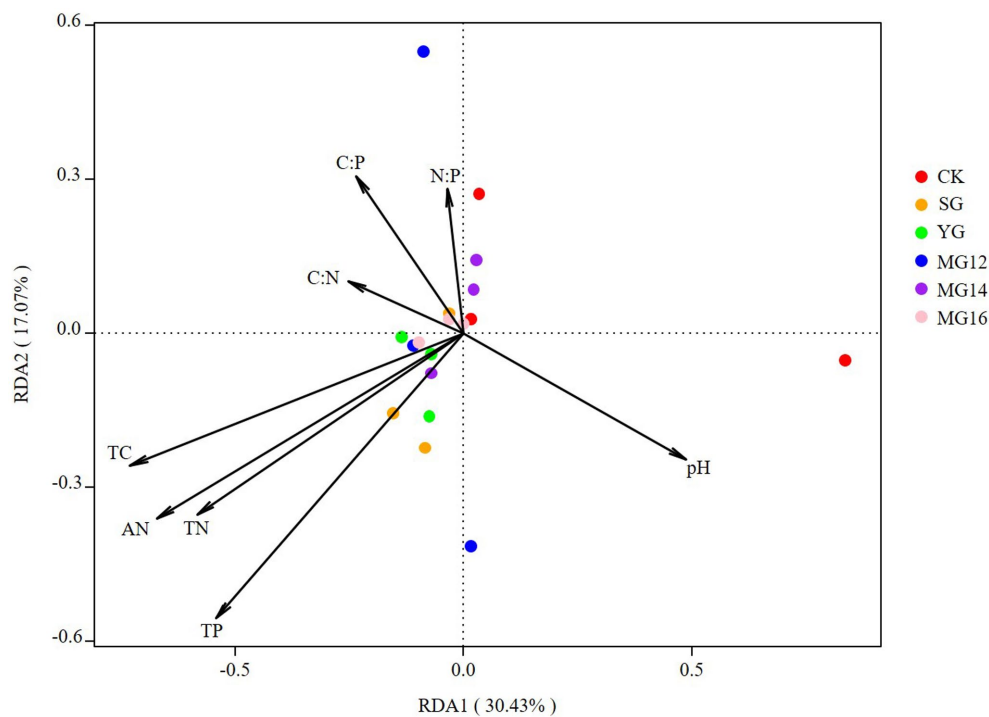


FIGURE 6

Relationships between soil properties and diazotroph community as shown by redundancy analysis (RDA).

(Lindsay et al., 2010; Meyer et al., 2013). Previous studies had described the diversity, composition and structure of the diazotroph community under different systems (Song et al., 2019; Xiao et al., 2020a; Barros et al., 2021). However, little information is available about the changes of diazotrophic community in Qinghai-Tibetan Plateau grassland soils with different grazing patterns. Thus, this study evaluated the influence of grazing patterns on diazotrophic communities in Qinghai-Tibetan Plateau grassland under moderate intensity grazing. The decreased of *nifH* gene copies under all grazing treatments were consistent with previous reports (Yan et al., 2021). In the present study, compared with CK, both singly and mixed grazing

treatment reduced the *nifH* gene copies. This is consistent with Lindsay's findings, they found that there was a trend for higher abundance of *nifH* in sites where grazing was excluded (Lindsay et al., 2010). The significant negative relationship between *nifH* gene abundance and soil physicochemistry (TC, AN and TP) under grazing treatment, suggested that frequent livestock grazing could inhibit dinitrogenase reductase and lead to a reduction in the biological capacity for nitrogen fixation. A large proportion of the nitrogen in dung and urine from livestock can provide high nitrogen inputs in various forms, therefore can reduce the ecosystem dependence on free-living nitrogen fixing organisms (Singh et al., 2011).

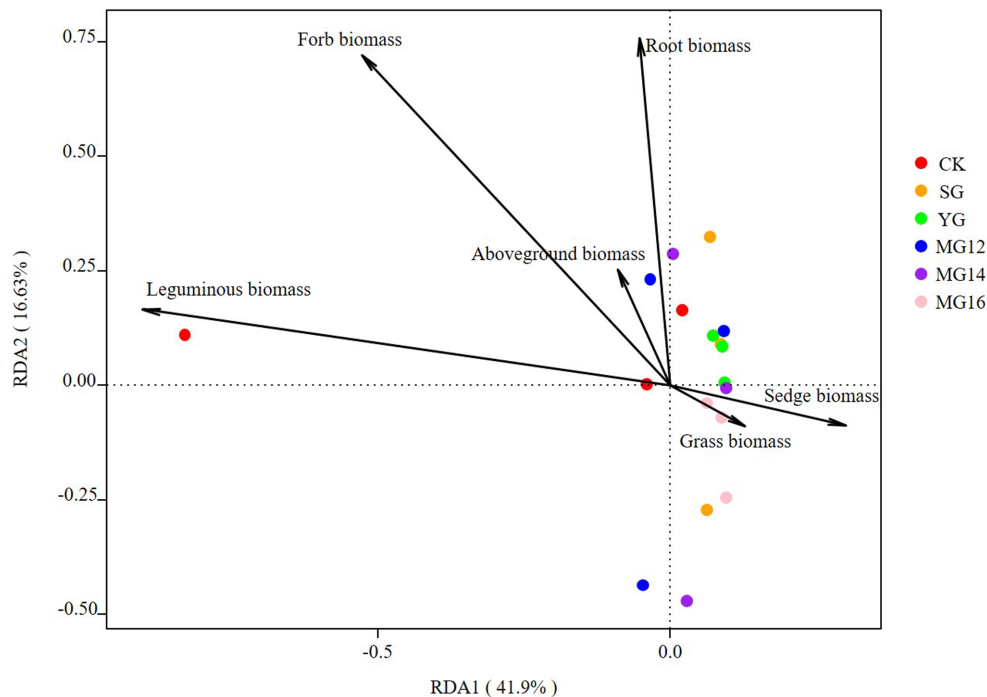


FIGURE 7 Relationships between plant functional community biomass and diazotroph community as shown by redundancy analysis (RDA).

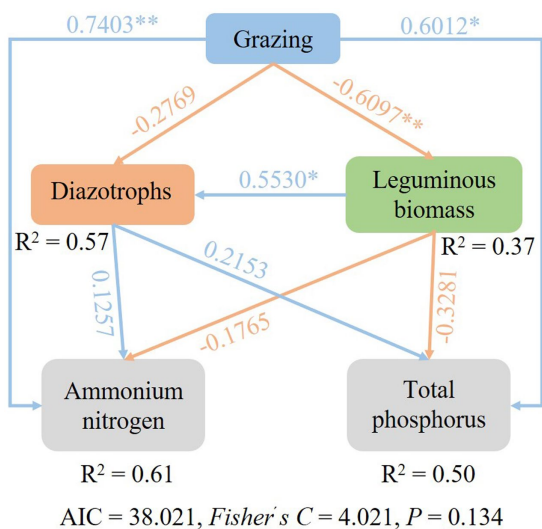


FIGURE 8 Structural equation model (SEM) describing the effects of grazing on soil diazotroph community.

4.2. Compositions and structures of the diazotrophic communities

With regard to the effects of grazing patterns on the diazotrophic community composition, a previous study showed that intensive grazing may increased the relative abundances of *Proteobacteria*, *Bacteroidetes* and *Firmicutes* (Zhang et al., 2023), whereas another study found that grazing exclusion increased the relative abundance of these bacteria (Wang Z. et al., 2021). In our study, YG and MG16 significant

reduced the OTUs number of diazotrophs, and the dominant diazotrophic communities belong to *Proteobacteria*, *Firmicutes*, *Rhizobiales*. The high proportions of *Proteobacteria* in CK and MG12 treatment indicate the mix grazing patterns with ratios of yak to sheep as 1:2 is closed to the natural grassland which without grazing in regulating soil microbial community. Meanwhile, the dominant bacteria had significant correlation with legume biomass suggested the abundance of nitrogen-fixing bacteria in soil is influenced by the foraging strategies of different livestock. Among the *Proteobacteria* and *Firmicutes* phylum, taxa such as *Rhizobiales* and *Bacillales* species are well known for forming root nodules with legumes and conducting symbiotic nitrogen fixation, and varying significantly in abundance among the types of land use (Yan et al., 2021).

4.3. Diazotrophs in response to yak grazing and Tibetan sheep grazing

Both of the *nifH* gene copies and OTUs had negative correlation with soil TP. Although diazotrophic communities were sensitive to phosphorus, a study in karst ecosystems reported that soil P availability played an important role in regulating N_2 fixation by increasing diazotroph diversity (Xiao et al., 2020b), it was inconsistent with our results. Our preliminary work found that the response of soil TP showed a strong dependence on livestock species (Yang et al., 2019), the responses of TP to grazing were significantly positively related to the proportion of sheep in the mixed livestock group. This suggests that changes in N_2 -fixing communities may also be related to the proportion of sheep in mix grazing. In addition, grazing can also increase soil AN content due to the feces and urine of small herbivores have a relative high N content (Liu et al., 2023). Previous studies indicated that high available nitrogen content had a negative effect on soil diazotrophs as the

nitrogenase enzyme is sensitive to ammonia (Che et al., 2018; Han et al., 2019). Moreover, the increased content of TP and AN enriched soil available nutrients and reduced the dependence of plants on nitrogen-fixing bacteria. Consequently, this could explain why *nifH* gene copies decreased in grazing treatments. Livestock grazing in grassy woodlands could potentially alter the nitrogen cycle (Lindsay et al., 2010), and grazing has been found to enhance the activity of soil nitrifying and denitrifying bacteria (Patra et al., 2005). However, herbivory can impose strong limits on diazotroph plant host abundance (Ritchie et al., 1998). Meanwhile, pasture management may affect the bacterial community through change the botanical composition and pasture productivity (Wakelin et al., 2009), and the grass roots can harbor abundant endophytic N₂-fixing microbes (Ritchie and Raina, 2016).

4.4. Key factors driving diazotroph community

The type of land management and land use intensity has been identified as a major driver for microbial performance in soil (Meyer et al., 2013). In this study, grazing treatment had a significantly effects on soil AN. The soil AN and *nifH* were negatively correlated in different grazing patterns, suggesting that aboveground herbivory reduces the capacity for belowground nitrogen fixation (Lindsay et al., 2010). The composition and activities of diazotrophic communities are essential for the functioning of the soil nutrient cycles (Hsu and Buckley, 2009). Due to plants and soils can form a complex mutual feedback relationship, the root biomass and aboveground biomass of legume and herb also play an important role in regulating the diazotrophic communities. Plant communities and soil variables can potentially produce an evident interaction in their influence on the soil organic carbon and nitrogen contents (Yuan and Jiang, 2021). As the most important and common land use of grasslands, livestock grazing and especially over-grazing can alter the composition and productivity of the plant community and can accelerate the loss of soil fertility and the depletion of other resources (Wang B. et al., 2021). In the present study, the herbivore assemblage did not change the diversity of the N₂-fixing communities, but significantly reduced their abundance. Moreover, MG12 and CK had similar OTUs of diazotrophs, which revealed that the composition of N₂-fixing microorganisms could be maximally maintained when yak and Tibetan sheep were 1:2. Based on SEM, the effects of grazing on diazotrophs were achieved by changing leguminous biomass, soil AN and soil TP, respectively.

5. Conclusion

Following the grazing treatment in the alpine meadow, variations of plant communities and soil chemistries influenced the composition of N₂-fixing microbial communities. The results of this study showed that although the grazing treatments significantly reduced the diazotrophs abundance, the OTUs riches of MG12 were closed to grazing exclusion, this suggested that mixing grazing in the ratio of yak to Tibetan sheep 1:2 maintained a relatively stable community structure of diazotrophic communities. Moreover, the effects of grazing on diazotrophs can be seen in two ways: foraging behavior of livestock reduced the legume biomass and simultaneously reduced the abundance of diazotrophs which closely related to legume; the other way is that the feces and urine of livestock increased the soil ammonium nitrogen and

total phosphorus content, thereby inhibiting the abundance of diazotrophs. Based on previous studies in this platform and from a management perspective, MG12 can improve above-ground net primary productivity and is less damaging to diazotrophs. Therefore, under the background of moderate intensity grazing, mixed grazing of yak and Tibetan sheep at a ratio of 1:2 is an optimal choice for the QTP.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: NCBI: SAMN36704862 – SAMN36704915.

Author contributions

SS: Data curation, Funding acquisition, Writing – original draft, YZ: Formal analysis, Writing – original draft. QD: Resources, Writing – review & editing. XY: Methodology, Resources, Writing – review & editing. YL: Data curation, Writing – original draft. WentuL: Investigation, Methodology, Writing – original draft. GS: Investigation, Writing – original draft. WentuL: Investigation, Writing – original draft. CZ: Investigation, Writing – review & editing. YY: Investigation, Writing – original draft.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2023.1257521/full#supplementary-material>

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Different responses of soil fungal and bacterial communities to nitrogen addition in a forest grassland ecotone

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Introduction: Continuous nitrogen deposition increases the nitrogen content of terrestrial ecosystem and affects the geochemical cycle of soil nitrogen. Forest-grassland ecotone is the interface area of forest and grassland and is sensitive to global climate change. However, the structure composition and diversity of soil microbial communities and their relationship with soil environmental factors at increasing nitrogen deposition have not been sufficiently studied in forest-grassland ecotone.

Methods: In this study, experiments were carried out with four nitrogen addition treatments (0 kgN·hm⁻²·a⁻¹, 10 kgN·hm⁻²·a⁻¹, 20 kgN·hm⁻²·a⁻¹ and 40 kgN·hm⁻²·a⁻¹) to simulate nitrogen deposition in a forest-grassland ecotone in northwest Liaoning Province, China. High-throughput sequencing and qPCR technologies were used to analyze the composition, structure, and diversity characteristics of the soil microbial communities under different levels of nitrogen addition.

Results and discussion: The results showed that soil pH decreased significantly at increasing nitrogen concentrations, and the total nitrogen and ammonium nitrogen contents first increased and then decreased, which were significantly higher in the N10 treatment than in other treatments (N:0.32 ~ 0.48 g/kg; NH₄⁺-N: 11.54 ~ 13 mg/kg). With the increase in nitrogen concentration, the net nitrogen mineralization, nitrification, and ammoniation rates decreased. The addition of nitrogen had no significant effect on the diversity and structure of the fungal community, while the diversity of the bacterial community decreased significantly at increasing nitrogen concentrations. Ascomycetes and Actinomycetes were the dominant fungal and bacterial phyla, respectively. The relative abundance of Ascomycetes was negatively correlated with total nitrogen content, while that of Actinomycetes was positively correlated with soil pH. The fungal community diversity was significantly negatively correlated with nitrate nitrogen, while the diversity of the bacterial community was significantly positively correlated with soil pH. No significant differences in the abundance of functional genes related to soil nitrogen transformations under the different treatments were observed. Overall, the distribution pattern and driving factors were different in soil microbial communities in a forest-grassland ecotone in northwest Liaoning. Our study enriches research content related to factors that affect the forest-grassland ecotone.

KEYWORDS

nitrogen addition, soil bacteria, soil fungi, microbial diversity, forest-grassland ecotone

1. Introduction

Since the industrial revolution, atmospheric nitrogen (N) deposition has increased globally due to human activities, such as industrial and agricultural production, fertilizer use, animal husbandry development, and land use change. Atmospheric N content has increased by three- to fivefold in the twentieth century, and the growth rate of global N deposition is expected to reach 1–2 times that of the 2000s by the 2050s (Davidson, 2009). The level of N deposition in China is far higher than the global average. Increased atmospheric N deposition affects both human health and the environment (Eisenlord et al., 2013). Atmospheric N deposition interferes with the N cycle in terrestrial ecosystems by fixating soil carbon (Bai et al., 2010). Excess N deposition leads to soil acidification and changes in plant composition and affects the soil N cycle, thus affecting ecosystem characteristics (BassiriRad, 2015). Therefore, N deposition is a global environmental problem.

Soil microorganisms are the most active components in terrestrial ecosystems. They not only undertake important tasks, such as decomposing plant and animal remains, but also promote the material cycle and energy flow of the ecosystem (Muhammad et al., 2023). The soil microbial structure and diversity are important indicators of soil quality (Morris and Blackwood, 2015). Among the soil microorganisms, bacteria and fungi play an important role in material transformation. Addition of N further changes the structure and diversity of the soil microbial community by changing the original physical and chemical properties of the soil (Hao et al., 2018). Studies have shown that the structure of the soil microbial communities in temperate steppes was considerably changed when supplemental levels of N were greater than $120 \text{ kg N} \cdot \text{hm}^{-2} \cdot \text{a}^{-1}$ (Zeng et al., 2016). Long-term N addition has been shown to improve the soil microbial diversity in Swedish grasslands (Freitag et al., 2005). However, Ling et al. (2017) arrived at the opposite conclusion in their study on the effects of long-term N addition in a temperate steppe in Inner Mongolia, China. Soil microbial richness and diversity indices were negatively correlated with the level of N addition. Similarly, Wang H. et al. (2018) found that addition of N markedly reduced the soil microbial diversity in tropical forest soils in southern China. Yang et al. found that short-term application of N had no substantial effect on the soil microbial community structure (Yang et al., 2015), meanwhile, a previous study showed that short-term nitrogen addition significantly altered soil bacterial community in an alpine steppe on the Tibetan Plateau (Huang et al., 2022). Therefore, the results of nitrogen addition on microorganisms in different ecosystems are inconsistent, so further studies are needed to clarify the effects on microorganisms in our forest grassland ecotone ecosystems.

Different soil microbial groups respond differently to the addition of N. Studies have shown that N addition can affect the abundance of the dominant soil bacterial flora and change the structure of the soil bacterial community (Hongli et al., 2022). Other studies have shown that short-term application of N has no substantial effect on soil bacterial communities (Zhao, 2022); however, treatment with high levels of N lead to considerable changes in the relative abundance of sensitive bacterial communities, resulting in an increase in the abundance of eutrophic bacteria and a decrease in the abundance of hypotrophic groups. Ammonium N is an important source of N for microorganisms, and its content affects the growth of microorganisms sensitive to it. Zhou et al. showed that the increase in the abundance

of Acidomycetes and α -Proteobacteria was related to the increase in ammonium N content. Fungal communities may be more sensitive to N addition than are bacterial communities. Many studies have shown that N addition markedly reduces the richness and diversity of ectomycorrhizal fungi and alters their community composition. High-throughput sequencing has shown that Ascomycota and Zygomycota were the dominant phyla in soil fungi. Input of N affected the relative abundance of fungal community groups. For example, elevated soil N content (due to N input) increases the relative abundance of Ascomycota. Some studies have shown that addition of N leads to soil acidification, which is an important factor that causes changes in the structure of the soil microbial community. However, other studies have shown that the increase in available N content in the soil caused by N deposition leads to changes in the soil microbial community structure rather than soil acidification. Yuan Fang et al. showed that the soil water content and total soil carbon and N (TN) contents affect the composition of the soil microbial community in a meadow steppe. Therefore, the influence of N deposition on soil microbial communities and its driving factors are highly uncertain and require further study.

High N deposition causes changes in the soil N conversion process (Yang et al., 2016), increases the available N content in the soil (Zhang and Han, 2012), and changes the number and composition of N-cycling microorganisms, thus affecting the ecosystem. The main processes involved in soil N conversion include biological N fixation, ammonification, nitrification, and denitrification. A series of transformations after N deposition in the soil cannot be achieved without the participation of related microorganisms. Since N fixation is an energy-consuming process, it is generally believed that the number of N-fixing microorganisms is positively correlated with the content of the organic matter in the soil. The addition of N fertilizers increased the soil organic carbon content and the abundance of Azotobacter, thus promoting the N fixation function of soil microorganisms. Recent studies have shown that the addition of N considerably affects the community structure and abundance of Azotobacter, nitrifying, and denitrifying bacteria (Jorquera et al., 2014). The abundance of Azotobacter and the N-fixing function of soil microorganisms were improved by adding an appropriate amount of N (Orr et al., 2012). However, the addition of high concentrations of inorganic N inhibited the growth of Azotobacter, thus inhibiting the growth of N-fixing microorganisms. Ning et al. (2015) found that N-related functional genes showed different sensitivities to the level of N addition, and the AOB-*amoB* gene of ammonia-oxidizing bacteria was more sensitive to N addition than the AOA-*amoA* gene of ammonia-oxidizing archaea. AOB abundance increased with an increase in N content, but AOA abundance did not change considerably (Shen et al., 2011).

China extends over a vast territory and has rich natural grassland resources, with nearly 400 million hectares of grassland area, accounting for 41% of China's total land area (Zhang et al., 2016). It is the world's second largest grassland country, and the forest-grassland ecotone is located in the transition zone between forest and grassland vegetation types; however, the coexistence of forest and grassland vegetation is characterized by vegetation type. The forest-grassland ecotone in northwest Liaoning is part of the forest-grassland ecotone in northeast China. The forest-grassland ecotone is a typical biodiversity-rich area that is sensitive to climate change, but the overall understanding of the soil microbial communities is relatively limited. In the context of increasing N deposition, it remains unclear

whether the composition and diversity of the soil microbial communities in forest-grassland ecotone have unique response characteristics.

To understand the characteristics and factors that influence the soil microbial communities in the forest steppe ecotone of northwest Liaoning under different N concentrations, we used high-throughput sequencing and quantitative (q) PCR technologies to answer three questions: (1) Do different N concentrations affect the soil physicochemical properties? (2) Do different N concentrations affect the composition of the soil bacterial and fungal communities and the abundance of N-transforming functional microorganisms? (3) What are the potential mechanisms by which environmental factors affect the soil microbial communities in the presence of increased N deposition? Based on previous studies, we hypothesized that: (a) Treatments with different N concentrations would affect the soil physicochemical properties; (b) Notable differences might be observed in the composition of the bacterial and fungal communities and the abundance of functional microorganisms involved in N transformation after treatment with different N concentrations; and (c) Changes in soil TN, pH, and other environmental factors would lead to changes in the soil microbial communities.

2. Materials and methods

2.1. Description of study area

The study area is located in the Zhangwu village of northern Liaoning Province, China (42° 08′–42° 50′ N, 121° 53′–122° 58′ E). This region has a semiarid climate with an average annual precipitation of 450–500 mm, annual pH of 5.98–6.35 and average annual temperature of 6.2–7.2°C. Sixty percent of rainfall occurs between June and August.

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

2.2. Experimental design and sample collection

The experiment was conducted using a one-factor randomized design comprising four treatments. N was applied at rates of 0 (N0), 10 (N10), 20 (N20), and 40 kg N·hm⁻²·a⁻¹ (N40), with five replicates per treatment. Each plot had an area of 2 m² × 2 m², with a 2 m-wide buffer strip separating each plot. The rates of N addition were determined based on global N-deposition levels. N was added in the form of urea (CO(NH₂)₂) three times in May, June, and August, 2016. The amount of fertilizer applied each time was 1/3 of the total amount applied throughout the year (Weigh on a ten-thousandth balance). The fertilizer was dissolved in 6 L of tap water and sprayed evenly in the quadrat using a watering can. For the N0 treatment, the same amount of water was used.

After 4 years of N addition, soil samples were collected in September 2020 at depths of 0–20 cm. Five soil cores from randomly selected locations in each plot were mixed to form a composite sample (Random sampling method). Small stones, roots, and litter were removed from the composite samples, and the soil was divided into two subsamples. One subsample was air-dried and sieved through a 0.25-mm mesh for physicochemical analysis. The other subsample was placed in bags with ice and immediately transferred to a super-cold refrigerator (–80°C) for DNA extraction.

2.3. Soil physicochemical analysis

Soil total nitrogen and total phosphorus contents were measured using SmartChem140 intermittent analyzer (EL III, Elementar, Germany) with indophenol blue colorimetric method and Mo-Sb colorimetric method, respectively. Soil organic carbon content was determined by the K₂Cr₂O₇ oxidation method. Soil pH was determined using the glass electrode method, and conductivity was determined using a conductivity meter. The other portion of the sample soils was stored frozen at –80°C for high-throughput sequencing and determination of ammonium nitrogen and nitrate nitrogen, which were analyzed based on a continuous-flow ion auto-analyzer (SAN++, Skakar, Breda, Holland) after extracting with 2 M KCl. Alkali-hydrolyzed nitrogen was determined by diffusion dish culture method.

2.4. Soil nitrogen mineralization rate

Soil N mineralization was determined using an indoor culture method. Fifteen grams of the screened air-dried soil sample was weighed and placed in a 250 mL plastic wide-mouth bottle. The water content of the soil was adjusted to 20% (mass water content) with deionized water. The bottle was wrapped with polyethylene film, and two holes were tied into the film, and the soil was pre-cultured in an incubator at 25°C for 7 days. After pre-culture, the samples were divided into three groups by adding ammonium sulfate, sodium nitrate solution, and equal weight of deionized water (control group [CK]) to 50 mg N·kg⁻¹ soil (dried weight). Soil moisture in all three groups was adjusted to 40% of the soil water-holding capacity. All culture bottles were re-sealed and incubated in the dark at 25°C for 14 days. The change in soil moisture was determined by weighing, and deionized water was used to replenish moisture. On d 0 and 14 of culture, 75 mL of 2 mol·L⁻¹ KCl solution was added, and the samples were incubated in a constant-temperature oscillator at 25°C for 1 h. The filtrate was treated using a continuous flow analyzer (Auto Analyzer 3; SEAL Analytical, UK) to determine the nitrate and ammonium N concentrations in the soil.

2.5. DNA extraction, Illumina MiSeq high-throughput sequencing, and sequence processing

The Power Soil DNA Isolation Kit (MoBio, USA) was selected to extract genomic DNA from the samples, after which the purity and

TABLE 1 Primers for PCR amplification.

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

concentration of DNA were tested using agarose gel electrophoresis and Nanodrop; The diluted genomic DNA was used as template for PCR using specific primers with Barcode and efficient high fidelity enzymes according to the selection of sequencing regions. We used the 336F:5'-GTACTCCTACGGGAGGCAGCA-3' and 806R:5'-GGACTACHVGGGTWTCTAAT-3' for bacterial amplification primers, and the fungal amplification primer was ITS4 (5'-TCCTCCGCTTA TTGATA TGC-3')/gITS7F (5'-GTGARTCA TCGA RTCTTTG-3'); Library construction was performed using TruSeq® DNA PCR-Free Sample Preparation Kit library construction kit. The constructed libraries were quantified by Qubit and Qpcr. After the libraries were qualified, the v2 sequencing kit (2×250 bp) and Miseq sequencer were used for on-board sequencing.

The obtained raw sequence data were analyzed using the Quantitative Insights into Microbial Ecology (QIIME v.1.8.0) pipeline.¹ Sequences were quality-filtered, denoised, merged, and chimeras were removed using the DADA2 plugin before clustering. Non-chimeric sequences were then 97% reclustered using Vsearch (v2.13.4) to generate representative operational taxonomic unit (OTU) sequences and OTU tables. The PyNAST method was used for sequence alignment. Taxonomy was assigned to amplicon sequence variants (ASVs) using the classify-sklearn Naïve Bayes taxonomy classifier in the feature-classifier plugin against the Greengenes 99% OTUs reference sequences (13_8 release). The alpha-diversity metrics (Chao1 [Chao, 1984], observed species, Shannon [Shannon, 1948a], Simpson [Simpson, 1949], Pielou's evenness [Pielou, 1969], and Good's coverage [Good, 1953]), and beta diversity metrics (Bray-Curtis dissimilarity) were estimated using the diversity plugin, with samples rarefied to sequences.

The DNA sequences in this study have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under accession number PRJNA949941.

2.6. Fluorescence quantitative PCR technique

Quantitative fluorescence PCR was used to determine the abundance of soil microorganisms. The reaction system was as

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

The formulas as follows: $\text{copies} = 10^{\frac{C_t - b}{k}}$

2.7. Calculations and statistical analyses

The following procedure was used to analyze the physical and chemical properties of the soil: first, Microsoft Excel 2019 was used to sort, count, and map the measurement results for each index. SPSS 22.0 (IBM) was used for one-way ANOVA, and the data were expressed as the mean ± standard deviation (SD). The least significant difference (LSD) method was used for multiple comparisons between different treatments ($p < 0.05$).

The formulas for calculating the soil N conversion rate were as follows:

$$\text{NMR} \left(\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1} \right) = \left(\text{Mt} + \text{Nt} \right) - \frac{\text{M0} + \text{N0}}{t}$$

$$\text{NNR} \left(\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1} \right) = \left(\text{Nt} - \text{N0} \right) / t$$

$$\text{NAR} \left(\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1} \right) = \left(\text{Mt} - \text{M0} \right) / t$$

where NMR, NNR, and NAR are the net mineralization, nitrification, and ammoniation rates, respectively; N_0 and N_t are the nitrate N concentrations at the beginning and t days after culture; and M_0 and M_t are the ammonium N concentrations at the beginning and t days after culture.

¹ <https://docs.qiime2.org/2019.4/tutorials/>

2.8. Statistical analyses

One-way analysis of variance (ANOVA) was performed using the software SPSS22.0 (IBM Co, Armonk, NY, USA) to determine the differences of environmental factors among different N treatments. Pearson correlation analysis was performed to explore the correlation between environmental factors and soil microbial diversity. The significance between different treatments was confirmed using Tukey's HSD test at $p < 0.05$.

The "ggplot2" package in R was used to draw box line plots and to visualize the compositional distribution of each sample at the systematic taxonomic level to determine differences in microbial community composition and alpha diversity among different nitrogen addition levels. The structural composition of the microbial communities of the different nitrogen addition levels was visualized using principal coordinate analysis (PCoA) using the "ape" package in R language, which is based on the Bray-Curtis heterogeneity matrix. To examine the effect of nitrogen addition levels on microbial β -diversity, PERMANOVA (Permutational multivariate analysis of variance) analysis was used. The effects of environmental factors on microbial community structure were analyzed by constrained ordinal redundancy analysis (RDA) in CANOCO 5.0 software (Microcomputer Power, Ithaca, NY, USA). The effect of each variable was assessed using an RDA-based Monte Carlo test (999 permutations).

3. Results

3.1. Effects of N addition on soil physicochemical properties

Table 3 shows the addition of N significantly affected TN and NH_4^+ -N contents and the soil pH. The TN content in the N10 treatment was significantly higher than those in the other treatments. The highest pH was recorded in the CK treatment and the lowest in the N40 treatment. However, all values were in the 6–7 range. The NH_4^+ -N content reached a maximum in the N10 treatment and a minimum in the N40 treatment.

As shown in Figure 1, the addition of N had a significant effect only on NNR but not on other rates. NAR, NNR, and NMR first increased and then decreased with increasing N concentrations; the highest values of NMR and NNR were observed in the N20 treatment, while NAR values were the highest in the N10 and N20 treatments.

3.2. Diversity and composition of microbial communities

3.2.1. Relative abundance of dominant phylum

Figure 2 shows the composition of bacterial and fungal communities at the phylum classification level under different N concentrations. Ascomycota, Basidiomycota, and Mortierellomycota had high relative abundances, with average relative abundance ratios of 64.45, 14.79, and 4.4%, respectively. Ascomycetes was the most dominant phylum common to the soil fungal communities, and the relative abundance ratio did not differ significantly under different N treatments. *Actinomyces*, *Proteobacteria*, and *Acidobacteria* were the

TABLE 2 Primers amplification efficiency:

gene	Slope	Y-Inter	R2	Eff%
<i>nifH</i>	−3.2782	39.209	0.9989	101.86
AOA	−3.3755	39.228	0.9984	97.81
AOB	−3.7144	42.123	0.9983	85.86
<i>nirK</i>	−3.4435	42.184	0.9982	95.17

TABLE 3 Soil physical and chemical properties under different nitrogen addition treatments.

Soil chemical properties	Treatments			
	CK	N10	N20	N40
TN(g•kg ^{−1})	0.12 ± 0.02 ^c	0.4 ± 0.08 ^a	0.12 ± 0.01 ^c	0.25 ± 0.08 ^b
TP(g•kg ^{−1})	0.54 ± 0.11	1.19 ± 0.54	0.97 ± 0.42	0.55 ± 0.25
AP(mg•kg ^{−1})	5 ± 2.36	5.35 ± 4.57	5.18 ± 0.93	4.23 ± 0.83
SOC(g•kg ^{−1})	5.22 ± 0.86	5.79 ± 0.7	5.4 ± 1.61	5.16 ± 0.68
pH	6.69 ± 0.06 ^a	6.68 ± 0.04 ^a	6.46 ± 0.15 ^{ab}	6.21 ± 0.25 ^b
EC(S•m ^{−1})	16.86 ± 4.5	18.74 ± 2.75	14.51 ± 0.66	13.87 ± 2.56
NO ₃ [−] -N(mg•kg ^{−1})	0.46 ± 0.02	0.53 ± 0.4	0.59 ± 0.16	0.58 ± 0.11
NH ₄ ⁺ -N(mg•kg ^{−1})	5.11 ± 4.5 ^b	12.27 ± 0.73 ^a	5.81 ± 2.32 ^b	2.65 ± 0.49 ^b
AN(mg•kg ^{−1})	26.25 ± 3.5	33.25 ± 6.31	32.67 ± 4.4	30.33 ± 1.01

TN represents total nitrogen; TP represents total phosphorus; AP represents available phosphorus; SOC represents soil organic carbon; EC represents electrical conductivity; NH_4^+ -N represents ammonium nitrogen; NO_3^- -N represents nitrate nitrogen; AN represents available nitrogen. Different letters in the same column indicate significant difference at the 0.05 level. The same as below.

most abundant bacteria, with average relative abundance ratios of 49.35, 18.06, and 12.68%, respectively. Actinomycetes was the most dominant phylum in the soil bacterial communities, and the relative abundance ratio did not differ significantly under different N treatments. The relative abundance of Acidobacteria was significantly different after treatment with different N concentrations.

Figure 3 shows the N addition significantly increased 2 taxa of the soil bacterial community and increased 3 taxa of the soil fungi community. The significant changes in soil bacterial taxa caused by N addition were mainly related to the *Myxococcales* and *Enterobacteriales*. The significant changes in soil fungi taxa caused by N addition were mainly related to the *Lecanicillium*, *Gibellulopsis*, *Echria* and *Rutstroemiaceae*.

3.2.2. α -Diversity

Figure 4 shows the alpha diversity indices of fungal and bacterial communities. The Chao1 indices of the soil fungal communities under different N additions ranked as follows: N40 < N10 < N20 < CK. The uniformity of the soil fungal community (Pielou's index) ranked as follows: N20 < CK < N10 < N40. The soil fungal community diversity (Shannon's index) ranking order was as follows: N20 < CK < N10 < N40. The soil fungal diversity (alpha diversity) at the four N concentrations showed that, although the fungal community diversity varied with different N treatments, there were no significant differences in the Chao1, observed species, Shannon (1948a,b), and Simpson (1949) indices ($p > 0.05$).

The Chao1 indices of the soil bacterial communities at different N concentrations were in the following order: N40 < N20 < N10 < and

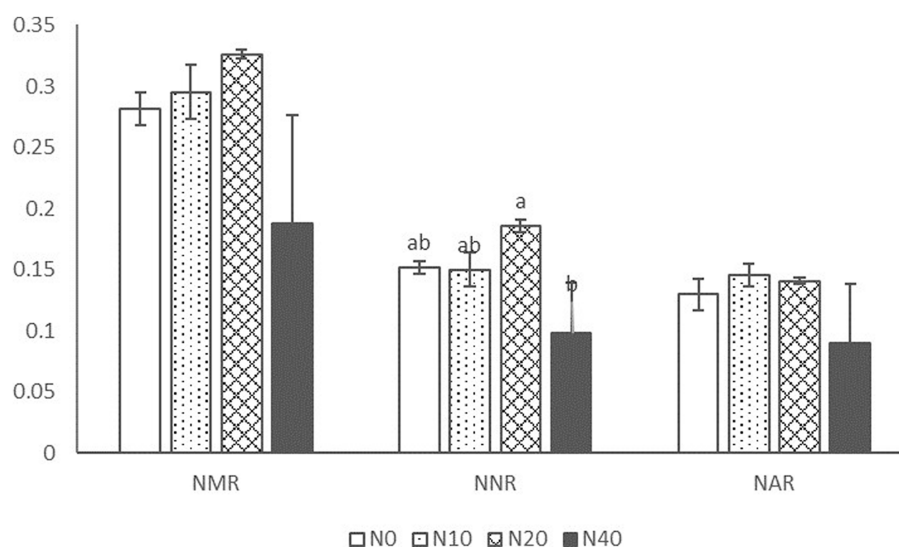


FIGURE 1

NMR, NNR, and NAR, net nitrification, ammonification, and mineralization rates; N0, N10, N20, and N40, application of 0, 10, 20, and 40 kg Nitrogen (N)·hm⁻²·a⁻¹.

CK. The soil bacterial community uniformity (Pielou's index) ranked in the following order: N40 < N20 < N10 < CK. The ranking order of the soil bacterial community diversity (Shannon index) was as follows: N40 < N20 < N10 < CK. The Simpson index was significantly different among treatments ($p < 0.05$), while no significant differences were noted in the other diversity indices.

3.2.3. β -Diversity

The sum of PCo1 and PCo2 explained 31.3 and 27.2% of the variation in the soil fungal and bacterial communities, respectively (Figure 5). All samples were mainly concentrated in four different areas that corresponded to the four different treatments; this result demonstrates that the bacterial and fungal community structures after the different treatments were heterogeneous and had obvious regional characteristics. The distance matrix of fungal is relatively scattered, and most of the distances between the four groups were similar. Under different N treatments, the similarities in the composition, structure, functional characteristics, and other aspects between the two communities was very high, and the range of change was small. In the bacterial community, only the samples from the N10 treatment group were heterogeneous and showed distinct regional characteristics.

PERMANOVA showed that the structural compositions of the fungal and bacterial communities did not differ significantly among the different treatments ($p > 0.05$).

3.2.4. Effects of N addition on abundance of functional genes for soil N transformation

Figure 6 shows the abundance of genes related to soil N transformations. The abundance of the *nifH* gene varied in the range of 2.27×10^6 – 3.15×10^6 copies·g⁻¹ soil. With the initial increase in the N application level, the abundance of *nifH* increased, followed by a decrease after the N20 treatment, and an increase after the N40 treatment. The abundance of *nifH* was the highest under the N10

treatment. The abundance of *nifH* at N10 and N40 was higher than that in the control treatment (N0). The abundance at N20 was lower than that of N0; however, no significant differences were noted among the treatments.

The abundance of the *nirK* gene varied in the range of 3.28×10^8 – 5.23×10^8 copies·g⁻¹ soil. The abundance of *nirK* increased at N10, decreased at N20, and increased at N40. The highest abundance was observed at N40. Overall, the abundance of *nirK* at different treatments was higher than that at N0, but the differences were not significant.

The abundance of the AOA gene varied in the range of 5.12×10^8 – 9.03×10^8 copies·g⁻¹ soil. The AOA gene abundance in the N10 treatment was higher than that in the control, while that in other N treatments was lower than that in the control. The results indicated that the AOA gene abundance decreased with an increase in the N supplementation level. The abundance of the AOB gene varied in the range of 6.37×10^5 – 1.44×10^6 copies·g⁻¹ soil. Compared to the control, the N10 and N40 treatments resulted in higher AOB gene abundance, but the differences were not significant. The copy numbers of the AOB gene in the soil were much lower than those of the AOA gene.

3.2.5. Network diagrams of the soil bacterial and fungal communities

The species in the network diagram of the soil bacterial communities in the northwest Liaoning grassland were more active and formed more associations than those in the fungal community network (Figure 7). As presented in Table 4, the topological parameters of the network showed that the soil bacteria network contained 1913 nodes and 88,416 edges, while the soil fungi network contained only 307 nodes and 813 edges. Importantly, the nodes in the soil bacteria network were more closely connected than those in the soil fungi network. Therefore, the northwest Liaoning grassland is mainly dominated by soil bacteria.

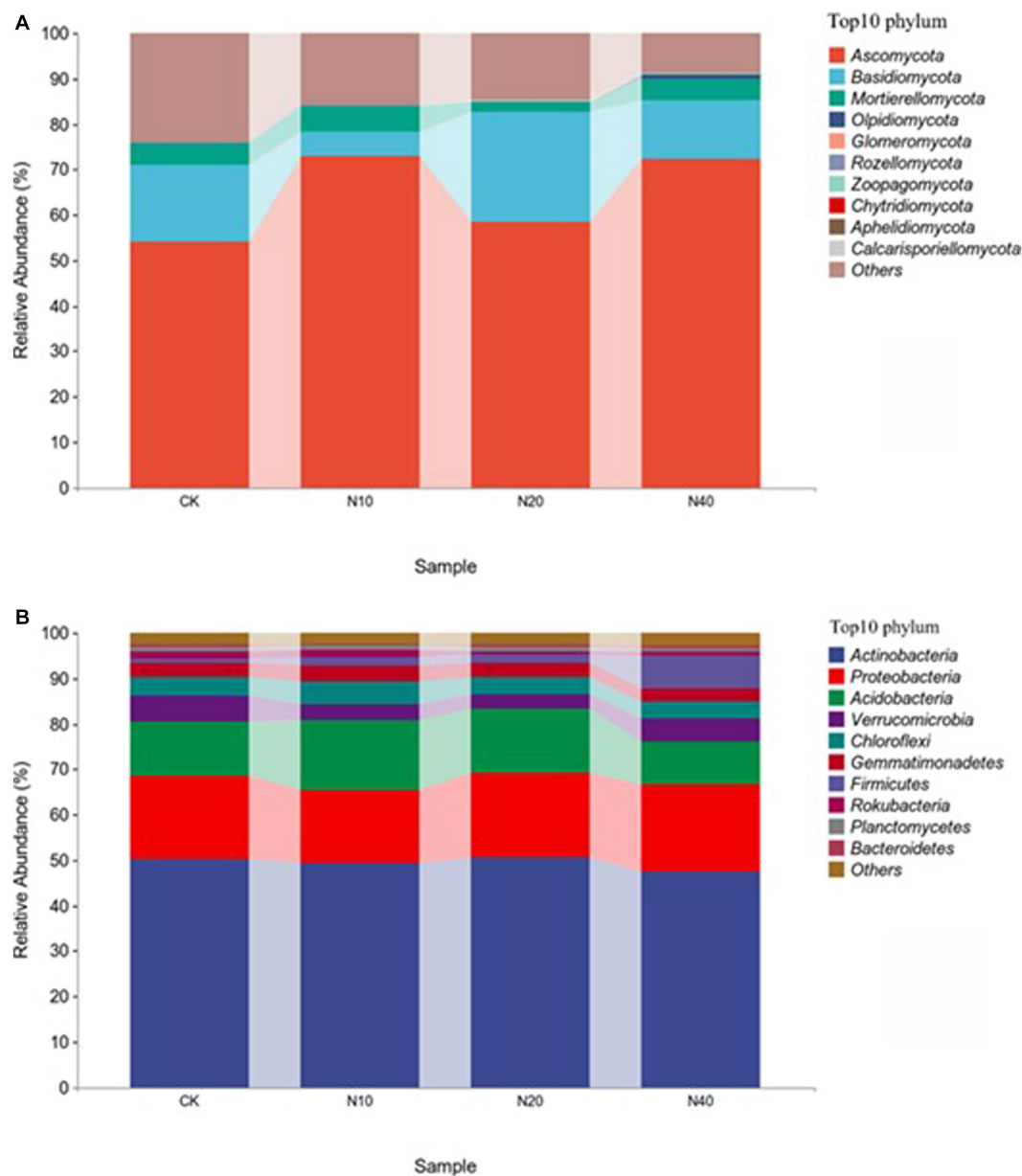


FIGURE 2
Relative abundance of (A) fungal and (B) bacterial communities at the phylum level.

3.3. Correlation among soil variables and microbial community properties

The content of the available phosphorus (AP) in the soil and the Good's coverage index were significantly positively correlated ($p < 0.05$) (Table 5), indicating that fungal coverage increases with increasing soil AP content. The content of the soil organic carbon (SOC) was significantly negatively correlated with the Chao1 and observed species indices ($p < 0.05$), indicating that the number of fungal species in the soil decrease with an increase in SOC content.

The soil pH was positively correlated with the Shannon, Simpson, and Pielou's indices ($p < 0.05$). The soil alpha diversity index was not significantly correlated with TN, AN, $\text{NH}_4^+\text{-N}$, TP, AP, and SOC contents, and EC. These findings suggest that pH affects the structure

of the soil bacterial community. The addition of N changes the soil pH, thus changing the structure of the soil bacterial community.

Figure 8 shows the results of the correlation analysis between microbial communities and environmental parameters. The abundance of Ascomycetes was positively correlated with TN, AN, and $\text{NH}_4^+\text{-N}$ contents and significantly positively correlated with TN content ($p < 0.05$). In contrast, the abundance of Ascomycetes was negatively correlated with TP, $\text{NO}_3^+\text{-N}$, AP, and SOC contents and pH and significantly negatively correlated with AP content ($p < 0.05$). The abundance of Basidiomycetes was negatively correlated with all environmental factors except $\text{NO}_3^+\text{-N}$ content and significantly negatively correlated with TN and AN contents ($p < 0.05$). Zoopagomycota was positively correlated with AP, negatively correlated with other environmental factors, and significantly

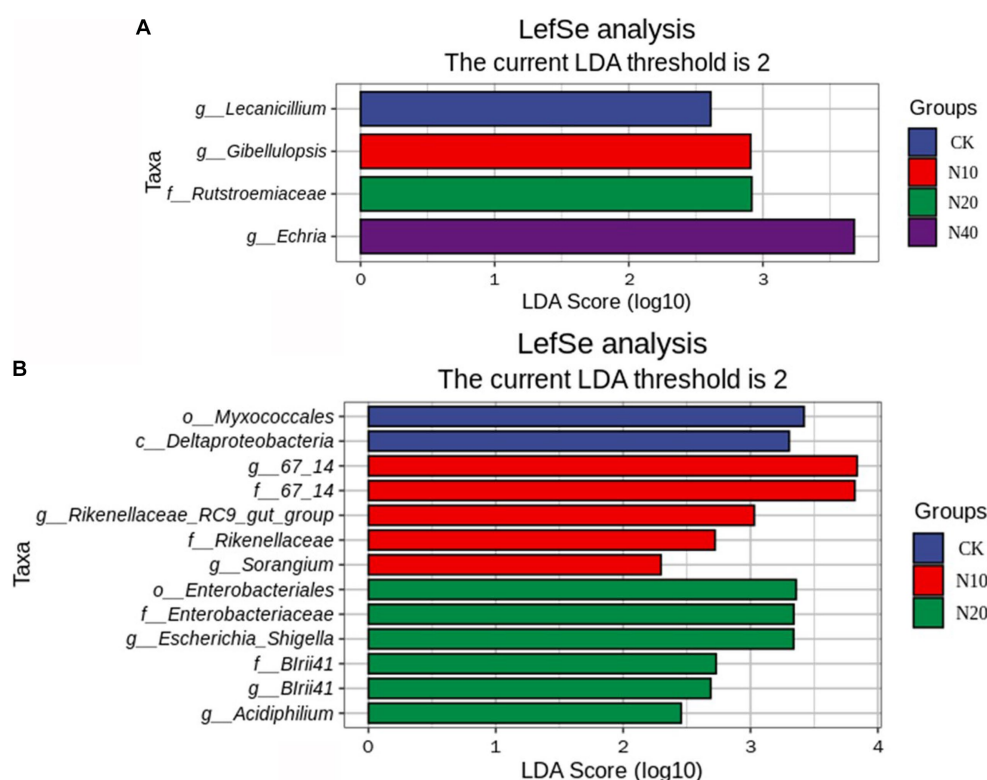


FIGURE 3

Soil fungal (A) and bacterial (B) community with linear discriminant analysis (LDA) scores.

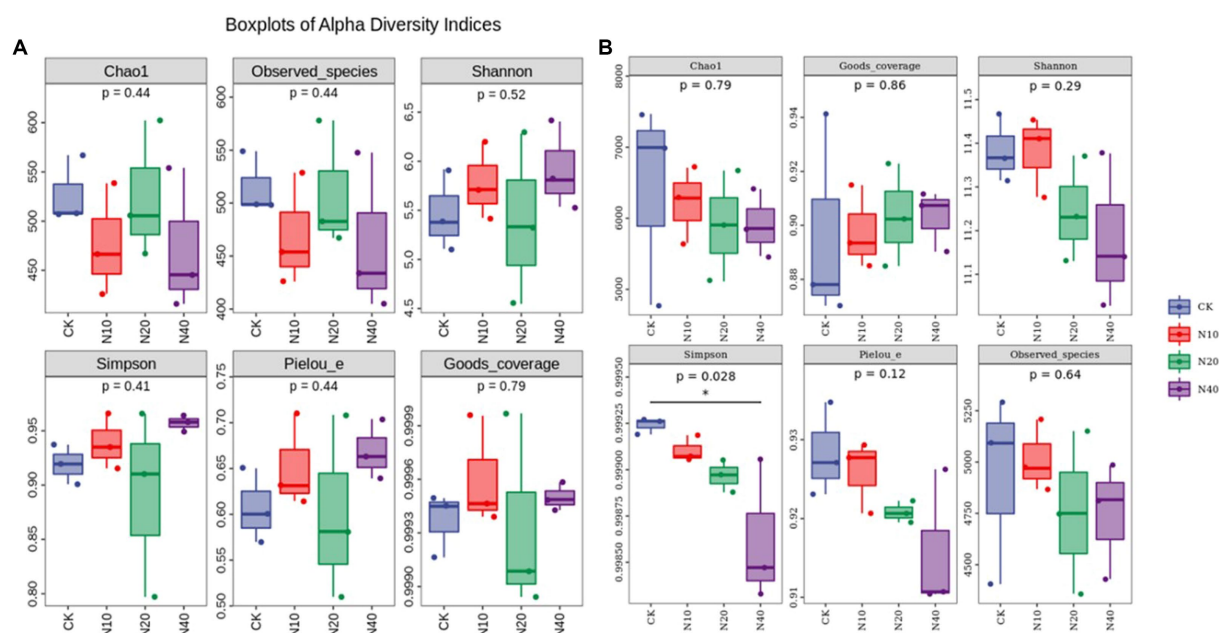


FIGURE 4

Boxplots of alpha diversity indices of (A) fungal and (B) bacterial communities.

negatively correlated with AN ($p < 0.05$). Aphelidiomyceta was significantly positively correlated with the soil pH, positively correlated with TP, AP, and SOC contents and EC, and negatively correlated with other environmental factors.

The genus *Penicillium* was most significantly affected by TN content ($p < 0.05$) and negatively correlated with AP and SOC contents. The abundance of *Trichoderma* was significantly affected by TP content ($p < 0.05$) but negatively correlated with AP content. In the

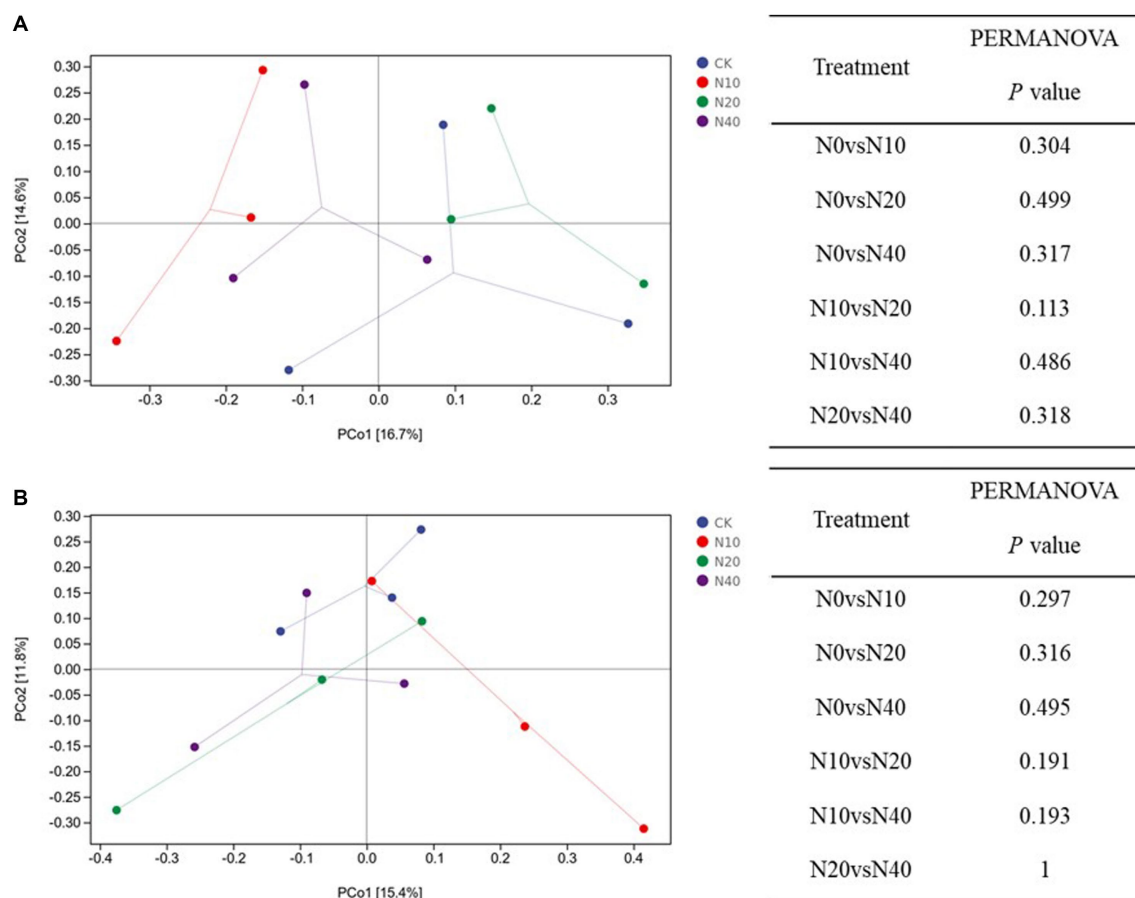


FIGURE 5
PCoA analysis based on Bray-Curtis distances of (A) fungal and (B) bacterial communities.

case of the genus *Geastrum*, we observed significantly positively correlation with the soil pH and EC and significantly negatively correlation with NO_3^- -N content. In this experiment, changes in environmental factors affected the diversity and structural composition of the soil fungal community.

We used RDA to examine the effects of soil properties on the microbial communities. The first and second axes in the RDA of the fungal communities explained 61.43 and 31.45% of total variation, respectively (Figure 9). Basidiomycota were positively correlated with soil SOC, NO_3^- -N, and TP, and negatively correlated with soil AP, AN, TN, NH_4^+ -N, soil pH, and EC. Ascomycota had a positive correlation with soil AN and TN contents and NH_4^+ -N, no correlation with soil EC, and a negative correlation with soil AP, pH, TP, SOC, and NO_3^- -N.

The first and second axis in the RDA analyses of the bacterial communities explained 33.71 and 19.47% of the total variation, respectively. The different N treatments were distributed in all quadrants in the figure, while the CK treatment was mainly concentrated in quadrant IV. RDA analysis of the bacteria showed that soil AN, EC, and soil pH were positively correlated with Actinobacteria. Actinobacteria had a negative correlation with soil SOC, NH_4^+ -N, NO_3^- -N, TN, and AP contents. Proteobacteria had a positive correlation with soil SOC and NO_3^- -N, no correlation with TN, and a negative correlation with soil AN, AP, TP, NH_4^+ -N, pH, and EC. Acidobacteria was positively correlated with soil TP, AP, TN, pH,

NH_4^+ -N, and EC, and negatively correlated with soil SOC, AN, and NO_3^- -N. The NO_3^- -N and TN were significantly correlated with species composition of soil fungal community, The pH and AP were significantly correlated with species composition of soil bacteria community.

The correlation between the gene abundance of soil N-transforming functional microorganisms and soil physical and chemical properties is presented in Table 6. The gene abundance of N-fixing functional genes, denitrification functional genes, and AOB of ammonia-oxidizing bacteria were not affected by environmental factors, while the AOA gene abundance of ammonia-oxidizing archaea showed a significant positive correlation with ammonium N.

4. Discussion

4.1. Effects of N addition on soil properties

In this study, the addition of N had several effects on soil physical and chemical factors. A decreasing trend on the soil pH values was observed with the increase in the supplemented N levels; this effect may be due to the change in ion concentrations caused by N addition, resulting in decreasing pH values at high N concentrations. This is consistent with the results by Jieqiong et al. (2014) on the application

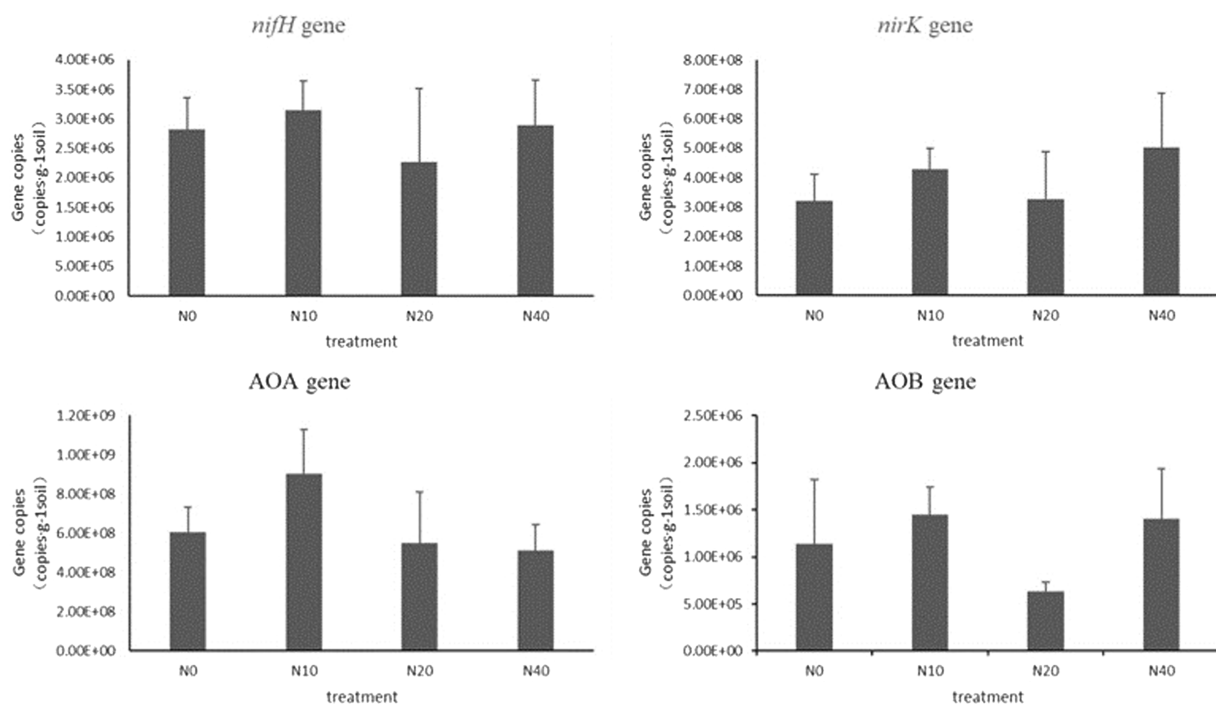


FIGURE 6
Abundances of functional genes for soil nitrogen N transformation.

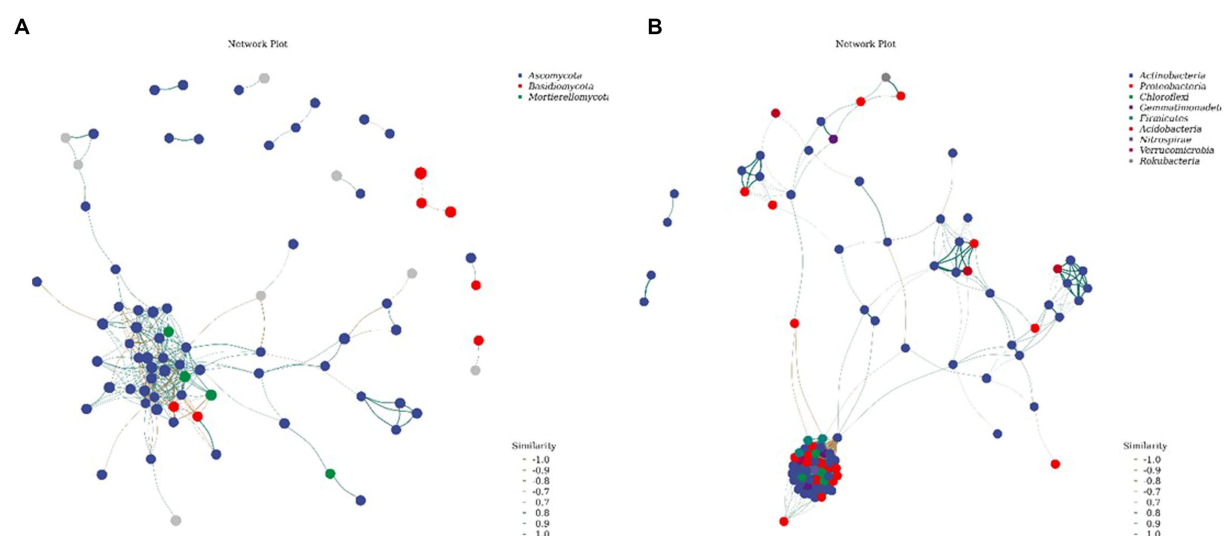


FIGURE 7
Network of (A) fungal and (B) bacterial communities.

of N in a desertification steppe. Compared with the control group, TN content increased significantly only at a low N concentration (N10) because the added N likely has multiple effects on the growth, composition, and function of microorganisms. Excess N inhibits the growth of organisms; therefore, it may be the absorption and utilization by microorganisms and plants that maintains the soil TN content relatively stable. Different N supplementation levels had different effects on the soil chemical properties. A significant increase in TN, TP, and SOC contents was noted under the N10 treatment,

while no significant differences in SOC and nitrate N contents between CK and other N concentrations were identified. In contrast, Liu Xing et al. showed that SOC content increased with an increase in the level of applied N because the increase in N input improved the above-ground productivity of grassland plants as well as the amount of plant litter and root exudates, thus promoting the accumulation of SOC. The discrepancy with our results may be because the effect of short-term N application is not immediately obvious and requires further investigation.

TABLE 4 Network topology parameters of soil bacterial and fungal communities.

	Average nearest neighbor degree	Average path length	Number of vertice	Number of edge	Modularity
Bacterial communities	99.8906	2.417	1913	88,416	0.5766
Fungal communities	6.8056	5.6201	307	813	0.7234

TABLE 5 Correlation analysis between environmental factors and microbial diversity under different nitrogen addition treatments.

		Chao1	Shannon	Simpson	Pielou	Observed	Goods
TN	f	−0.253	0.298	0.298	0.287	−0.236	0.203
(g•kg ^{−1})	b	0.066	0.084	−0.037	−0.058	0.179	−0.105
NO ₃ ⁺ -N	f	−0.229	−0.691*	−0.667*	−0.613*	−0.316	−0.435
(mg•kg ^{−1})	b	−0.213	−0.398	−0.498	−0.381	0.153	−0.031
AN	f	−0.334	0.014	0.014	0.084	−0.341	0.162
(mg•kg ^{−1})	b	0.111	−0.277	−0.217	−0.439	−0.014	−0.22
NH ₄ ⁺ -N	f	−0.167	−0.197	−0.197	−0.12	−0.154	0.168
(mg•kg ^{−1})	b	0.202	0.399	0.366	0.317	0.317	−0.18
TP	f	−0.353	−0.249	−0.249	−0.047	−0.366	0.187
(g•kg ^{−1})	b	−0.329	−0.078	0.087	0.081	−0.205	0.301
AP	f	−0.405	0.127	0.127	0.219	−0.357	0.608*
(mg•kg ^{−1})	b	−0.342	0.188	0.1	0.389	−0.089	0.421
SOC	f	−0.577*	−0.477	−0.477	−0.352	−0.609*	0.125
(g•kg ^{−1})	b	−0.408	−0.325	−0.14	−0.027	−0.474	0.4
pH	f	0.44	0.074	0.074	0.063	0.492	0.092
	b	0.336	0.862**	0.929**	0.802**	0.566	−0.226
EC	f	−0.329	0.178	0.178	0.07	−0.328	0.151
(S•m ^{−1})	b	0.575	0.327	0.168	0.018	0.492	−0.548
NMR	f	−0.287	−0.442	−0.278	−0.331	−0.333	−0.08
	b	0.007	−0.127	−0.177	−0.15	−0.023	0.265

*Represents significant. **Represents very significant. “f” Represents fungi; “b” Represents bacteria.

4.2. Effects of nitrogen addition on microbial diversity and community structure

Ascomycetes and actinomycetes were the common dominant phyla in the fungal communities under different N treatments (Figure 2). This is similar to the composition of the fungal communities in the Hulunbuir sand area, Loess Plateau grassland, and Inner Mongolia desert grassland, but substantially different from their bacterial community structure. This discrepancy may be due to the grassland restoration process in northwest Liaoning. At the same time, the higher the number of connections in the soil microbial community network, the higher its stability and the stronger its ability to inhibit pathogen invasion. In this study, the degree of connectivity of the soil bacterial network was much higher than that of the fungal network (Figure 6), indicating that the bacterial community exhibited strong resistance to invasion. This result suggests that the bacterial community is dominant in the forest steppe ecotone in northwest Liaoning. Bacterial and fungal communities exhibit different sensitivities to N addition (Zhou et al., 2020). Overall, the addition of N had no significant effect on N-transforming microorganisms.

The correlation analysis between environmental factors and N-transforming microorganisms showed that NH₄⁺-N had a significant effect on AOA gene copies. The addition of nitrogen resulted in the change of soil properties in many aspects. With the increase of added nitrogen concentration, NH₄⁺-N content in soil showed a gradual increasing trend, and soil pH value decreased significantly. AOA and AOB showed a trend of first increase and then decrease, and showed the maximum value under N10 treatment. It was common for nitrogen accumulation to lead to their quantity increase. Li also confirmed that both the changes of ammonium and NH₄⁺-N would change the community compositions AOB and AOA (Li and Gu, 2013). In this study, the decrease of AOA and AOB quantity indicated that high concentration of ammonia had an inhibitory effect on ammonia oxidation activity. Whether the decrease of AOA amount caused by the addition of high nitrogen concentration is related to the decrease of ammonia oxidation activity remains to be studied. In our study, the diversity and community structure of microorganisms with nitrogen transformation function were not measured, but only the gene copy number was measured, and the specific changing strains could not be known. Some study showed that nitrogen addition had an impact on diversity and community structure (Wang et al., 2015), so future experiments will focus on it.

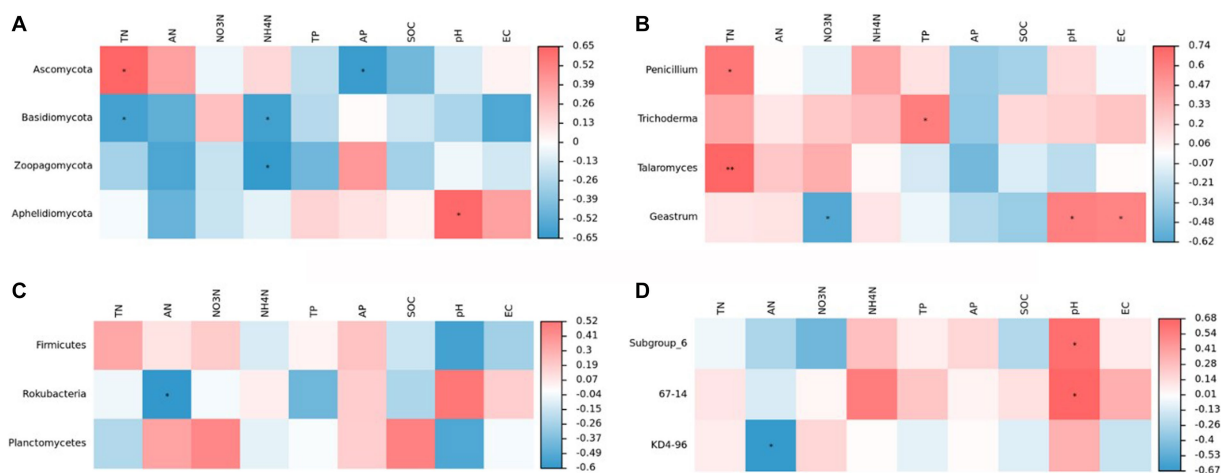


FIGURE 8
Correlation analysis of community composition of soil fungi (A,C) and bacteria (B,D) at phyla and genus levels with soil physicochemical properties.

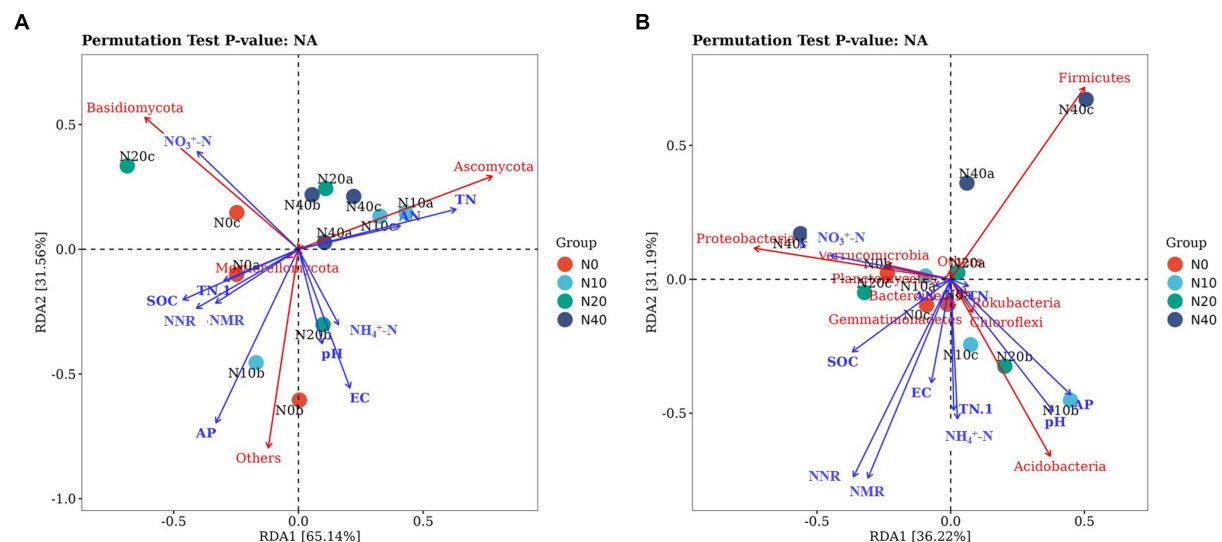


FIGURE 9
Redundancy analysis (RDA) of the relationship between fungal (A) and bacterial (B) communities and environmental factors.

4.3. Changes in soil TN content and soil pH alter the soil microbial community

In the natural environment, the available N may be limited (the diffusion ability of N in soil is hindered), while the addition of exogenous N may alleviate this restriction (Plett et al., 2020). Many studies have been conducted worldwide on the impact of N addition on soil microbial diversity. The general conclusion was that N addition reduced soil microbial diversity in grassland ecosystems (Wang C. et al., 2018). N addition would lead to competition among soil microbial species; thus, those species with high rates of N utilization that rapidly absorb N and grow after N addition inhibit other species (Bobbink and Willems, 1987), resulting in the loss of some soil microbial species, and thus reduced soil microbial diversity (Sui et al., 2015). Overall, the richness and diversity of soil bacteria first decreased and then increased with an increase in N application rate. The richness and diversity of soil bacteria decreased

at medium N application rates and increased at high N application rates; however, the richness and diversity of soil bacteria were lower than those without added N (Li et al., 2020). These results suggest that N addition can reduce the soil microbial diversity in grassland ecosystems. However, some studies have shown that, at different gradients of N addition, the diversity of soil microorganisms increases at low and decreases at high N concentrations. The different effects of N addition on soil microbial communities may be due to the complex response of soil microorganisms to N addition. On the one hand, the addition of an appropriate amount of N alleviates the N limitation in grassland ecosystems and promotes the growth of the above-ground vegetation and the quantity and quality of litter, thus increasing the input of organic matter into the soil. Therefore, abundant resources enable the coexistence of a large number of species. On the other hand, species with a high N utilization rate will rapidly absorb N and grow after N addition, inhibiting the growth of other species; thus, in a high-N

TABLE 6 Correlation analysis between functional genes and physicochemical properties.

	<i>nifH</i>	<i>nirK</i>	AOA	AOB
TN(g•kg ⁻¹)	0.784	0.662	0.786	0.761
NO ₃ ⁺ -N(mg•kg ⁻¹)	-0.431	0.426	-0.31	-0.26
AN(mg•kg ⁻¹)	-0.087	0.292	0.409	-0.08
NH ₄ ⁺ -N(mg•kg ⁻¹)	0.437	-0.074	0.970*	0.239
TP(g•kg ⁻¹)	0.053	-0.077	0.752	-0.087
AP(mg•kg ⁻¹)	-0.085	-0.65	0.669	-0.334
SOC(g•kg ⁻¹)	0.347	-0.007	0.925	0.177
pH	0.26	-0.617	0.688	-0.005
EC(S•m ⁻¹)	0.635	-0.181	0.992	0.408

*Represents significant.

environment, loss of some soil microbial species and reduced soil microbial diversity are expected.

The correlation analysis between soil microbial functional diversity and environmental factors showed that the soil fungal Shannon index was significantly negatively correlated with soil NO₃⁺-N, indicating that the soil N supply affected fungal functional diversity. This effect may be due to the increased NO₃⁺-N content and free hydrogen ions in the soil after N addition, leading to soil acidification. It is generally believed that soil bacterial communities tend to be more uniform at neutral pH conditions, with higher degrees of richness and diversity. The significant positive correlation between the soil bacterial Shannon index and pH values indicates that continuous N addition may cause a decrease in pH values, leading to an overall decrease in the soil microbial functional diversity. Soil microbial growth was affected by soil environmental factors, among which the degree of soil acidity had the most significant effect (Wang et al., 2021). Correlation analysis between the dominant flora and soil chemical factors showed that soil pH values were significantly positively correlated with the relative abundance of Aphelidiomycota but significantly negatively correlated with the relative abundance of Aspergillus. Additionally, soil pH values were positively correlated with the relative abundance of *hexae*, while negatively correlated with the relative abundance of Firmicutes and *Aspergillus*. The results showed that the soil pH was the main factor affecting the soil bacterial community, consistent with previous studies (He et al., 2017). In general, soil microbial diversity is the highest when soil pH is near neutral, and the presence of excess acid or alkali will put the growth of the microbial community under stress, thus reducing microbial diversity. Previous studies have suggested that the ecological selection of microbial communities by soil acidification and the adaptive evolution of different microbial communities under pressure at acidic conditions are key mechanisms that lead to changes in soil microbial communities (Yan et al., 2017). Soil bacteria are more sensitive to the soil environment than fungi.

5. Conclusion

In conclusion, N addition had significant effects on soil TN content, ammonium N content, and pH. N addition had no significant effect on the diversity of fungi and N-transformed microorganisms at the sample site but had a significant effect on the bacterial diversity.

TN and pH were the main causes of changes in the soil microbial community. Our study shows that microbial bacteria dominate the forest steppe ecotone in northwest Liaoning Province, and bacteria can better reflect microbial changes under nitrogen application than fungi. Under the background of relatively limited overall understanding of soil microorganisms in the forest steppe ecotone of northwest Liaoning Province, this study explored the effects of nitrogen addition on soil microbial community structure and diversity and its ecological adaptation mechanism, which not only theoretically enriched the research content of forest steppe ecotone. It is also of great significance for the comprehensive assessment and prediction of the response of forest grassland interlacing to global change, the protection of biodiversity and the maintenance of ecosystem balance. The diversity and community structure of microorganisms related to nitrogen conversion function in the experiment may further help us understand the ecosystem, and future research will focus on this.

Data availability statement

The DNA sequences in this study have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under accession number PRJNA949941.

Author contributions

BR, LB, and JL designed this study. GB, CT, and FY assisted in the field experiment. DL, XM, JY, and MM performed the laboratory analysis. DL and BR wrote the manuscript. All authors reviewed the manuscript and approved for publication.

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Conflict of interest

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

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Glossary

AN	available nitrogen
AOA	ammonia-oxidizing archaea
AOB	ammonia-oxidizing bacteria
AP	available phosphorus
ASVs	amplicon sequence variants
CK	control group
EC	soil electric conductivity
LSD	least significant difference
N	nitrogen
OTUs	operational taxonomic units
qPCR	quantitative PCR
RDA	redundancy analysis
SD	standard deviation
SOC	soil organic carbon
TN	total nitrogen
TP	total phosphorus



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N₂-fixing bacteria are more sensitive to microtopography than nitrogen addition in degraded grassland

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Introduction: Soil bacteria play a crucial role in the terrestrial nitrogen (N) cycle by fixing atmospheric N₂, and this process is influenced by both biotic and abiotic factors. The diversity of N₂-fixing bacteria (NFB) directly reflects the efficiency of soil N fixation, and the diversity of NFB in degraded alpine meadow soil may change with different N fertilizing levels and varied slopes. However, how N addition affects the diversity of NFB in degraded alpine meadows, and whether this influence varies with slope, remain poorly understood.

Methods: We conducted an N addition field experiment at three levels (2, 5, and 10 g N·m⁻²·a⁻¹) to study the effects of N addition on soil NFB diversity on two different slopes in a degraded meadow on the Tibetan Plateau.

Results: There were significant differences in the dominant bacterial species between the two slopes. The Chao1 index, species richness, and beta diversity of NFB did not differ significantly between slopes, but the Shannon index did. Interestingly, N addition had no effect on the diversity of NFB or the abundance of dominant bacteria. However, we did observe a significant change in some low-abundance NFB. The community composition and diversity of NFB were significantly positively correlated with slope and soil physicochemical properties (e.g., total potassium, pH, and total nitrogen).

Conclusions: Our study highlights the variation in NFB communities among different slopes in degraded alpine meadows and their resilience to exogenous N addition. Our results also underscore the importance of considering the effects of micro-topography on soil microbial communities in future studies of alpine ecosystems.

KEYWORDS

nitrogen addition, N₂-fixing bacteria, alpine meadow, slope, Tibetan Plateau

Introduction

Soil nitrogen (N) is a major component of terrestrial ecosystem productivity (Xu et al., 2015). It is mostly fixed by plant growth in the soil, which makes the N supply capacity of soils a determining factor in plant growth (Nannipieri and Eldor, 2009); however, not all soil N can be absorbed by plants. In general, ammonium and nitrate are two effective N forms that can be directly absorbed by plants from the soil under the action of microorganisms (Wang et al., 2020). The microorganisms that can directly use atmospheric N are called N₂-fixing microbes,

and the conversion of N_2 to NH_3 mediated by N_2 -fixing microbes is called biological N fixation, which is the key source of available N in soils (Lindsay et al., 2010; Che et al., 2017). Actually, the N_2 -fixing microbes are composed of prokaryotes, including bacteria and archaea. Among them, N_2 -fixing bacteria (NFB) are characterized by the ferritin *nifH* gene with the catalytic function of nitrogenase (Zehr et al., 2003), which is a genetic marker that has been widely used to examine the community composition of NFB (Kang et al., 2013). Changes in the quantity and diversity of NFB can directly reflect the efficiency of soil N fixation and the normal operation of soil N cycling, thus, they are important indicators of soil functions that have been restored in different types of restored vegetation (Li et al., 2013). It is, therefore, of critical scientific importance to study the community composition and diversity of NFB.

Alpine meadows are a typical grassland ecosystem on the Qinghai-Tibet Plateau (QTP) (Gao et al., 2012). These communities are commonly distributed in highlands and mountainous areas (Zi et al., 2015) and are characterized by a fragile ecological environment and unique biogeochemical processes (Wischniewski et al., 2011). Hence, these areas are more sensitive to human disturbance and global environmental change (Lan, 2004; Wischniewski et al., 2011). In recent years, the alpine meadows on the QTP have suffered serious destruction; for instance, a large area of fine grasslands has been degraded to “*heitutan*” (bare land) (Sun et al., 2013), which is especially common in the Sanjiangyuan area of the central QTP (Chong and Zhang, 2015; Shao et al., 2017). These changes lead to an ecological imbalance in the QTP (Wang et al., 2021; Xu et al., 2021). Thus, effective measures must be taken to prevent the alpine meadow from being degraded.

One common prevention method is *via* N addition to improve soil productivity and maintain an ecological balance in the degraded grassland ecosystems (Wang et al., 2017). N addition promotes plant growth, modifies soil fertility, and induces soil acidification, which further changes soil microbial communities (Verburg et al., 2010; Tian and Niu, 2015; Chen et al., 2017; Han et al., 2017). This is because even small changes in the soil environment may cause a strong response in soil microorganisms that can quickly adapt to the new environment or make countermeasures (Geisseler et al., 2011; Fang et al., 2016). For example, the change in NFB is one of the most important indicators of soil degradation in degraded grasslands (Yan and Chen, 2001; Chen et al., 2020). He et al. (2010) found that exogenous N significantly affected NFB, modifying the soil microbial community composition and then soil N cycles. For instance, N fertilizers changed the composition of soil NFB and promoted the N_2 -fixing function of soil organisms while inhibiting the growth of NFB in grasslands (Orr et al., 2012; Parker, 2013). Numerous studies have been carried out to explore the effects of grazing, enclosure, supplementary sowing, and other management measures on soil properties. Nevertheless, how N addition affects NFB in degraded alpine meadows is still not fully understood. Moreover, due to the variety of terrain types in the Sanjiangyuan region, the structure and diversity of NFB may vary with the slopes of the grassland. However, few studies have explored how NFB change with slope. These knowledge gaps may hamper a better prediction of soil NFB feedback on future climate change.

Here, we conducted an N addition field experiment at three levels on two slopes in a degraded alpine meadow, assuming that N addition will change the community structure of soil NFB and reduce the diversity and that the response of soil NFB to N addition is different on different slopes. Specifically, the following two objectives were explored: (1) How does N addition affect soil NFB on different slopes in a degraded meadow? and (2) What are the main factors affecting NFB?

Methods

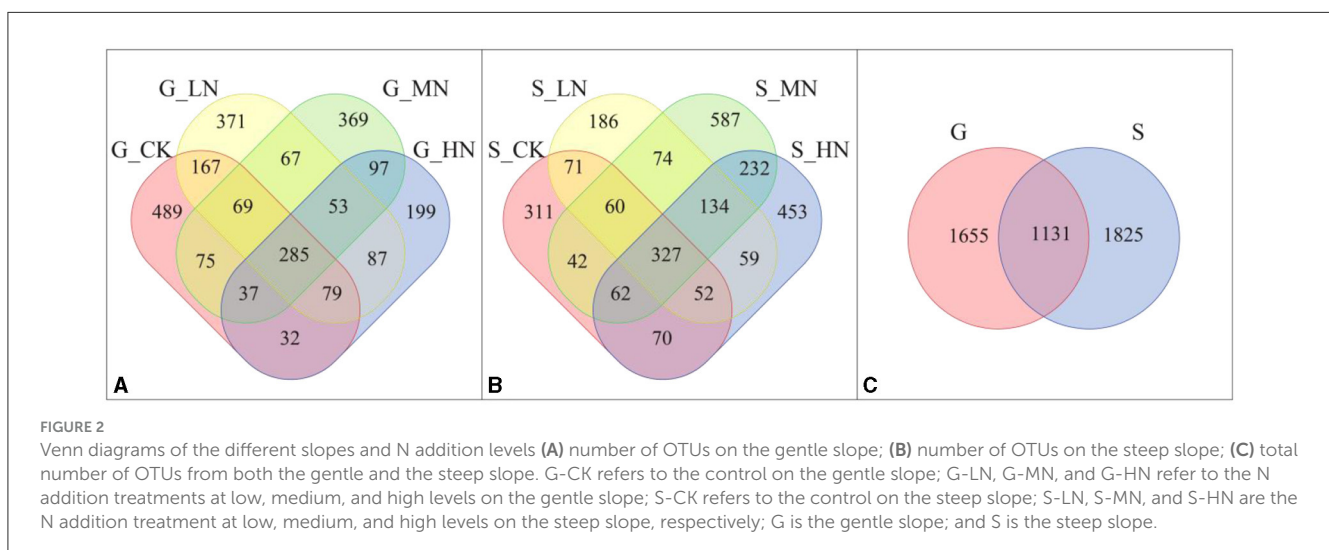
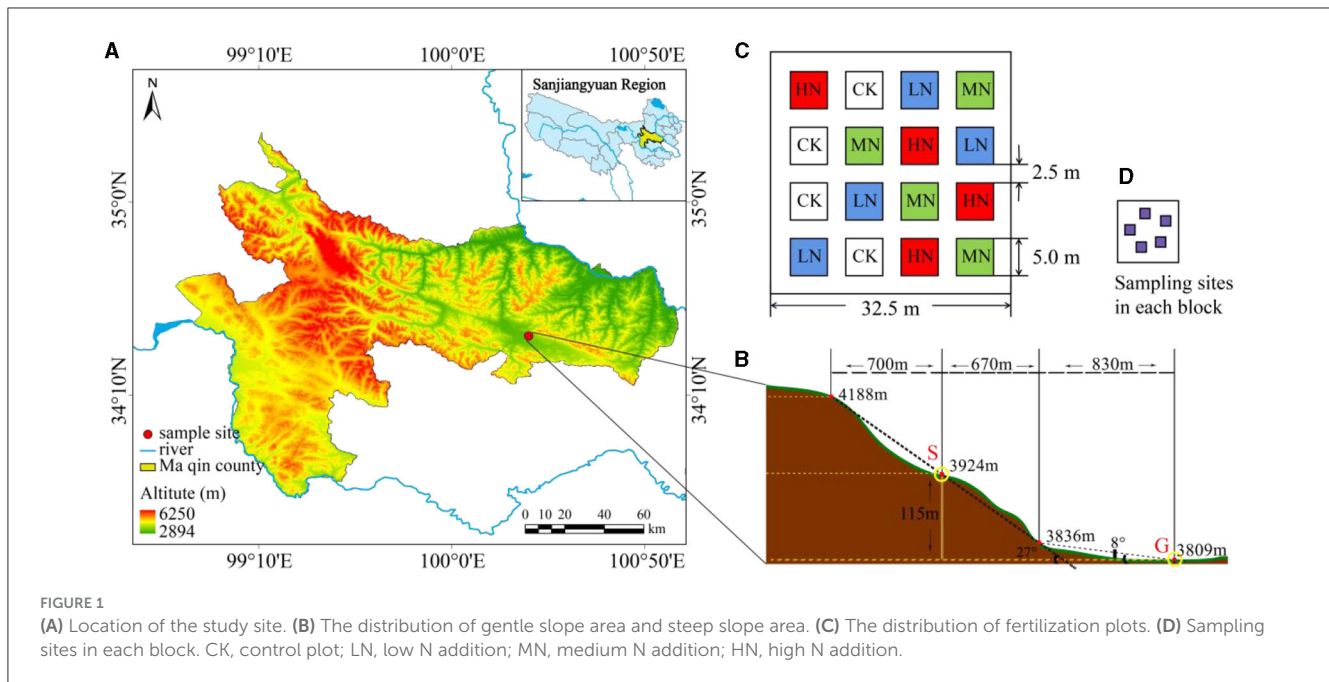
Field experiment description

The study was conducted in Maqin County, Guoluo Tibetan Autonomous Prefecture, Qinghai Province, China. This site is situated at latitude $34^{\circ}25'20.41''$ N, longitude $100^{\circ}19'55.72''$ E (Figure 1A), with an average altitude of 3,768 m. The average annual precipitation and temperature were 528.8 mm and -3.9°C , respectively (Li et al., 2022a). The research site is a moderately degraded alpine grassland (Li et al., 2022b), and the soil type is alpine meadow soil (Zuo and Le, 1980). In addition, the grassland is grazed uncontrollably year-round. The main plant species on different slopes are similar and consist of *Kobresia pygmaea*, *Kobresia humilis*, *Elymus nutans*, *Ligularia virgaurea*, and *Poa pratensis*, among others.

According to the data on dynamic change characteristics of atmospheric N deposition in the alpine meadow ecosystem of the Qilian Mountain on the central QTP (Zhu et al., 2016) and the expectation of a continuous increase of N deposition in the future (Wang et al., 2019), our experiment had a fully randomized block design, with two slopes (G: gentle slope; and S: steep slope), four fertilization levels at 0, 2, 5, and $10\text{ g N}\cdot\text{m}^{-2}\cdot\text{a}^{-1}$ (designated as CK, LN, MN, and HN, respectively), and four replications. In May 2019, $50 \times 50\text{ m}$ degraded alpine meadow plots were selected on two slopes. Both sites have a slope of 12.3° from west to north, but the steep slope has a gradient of 27.0° and the gentle slope's gradient is 8.0° (Figure 1B). There were 16 quadrats ($5 \times 5\text{ m}$) in each slope type, and each fertilization level was randomly allocated, resulting in a total of 32 permutations. The spacing between quadrats was set at 2.5 m (Figure 1C). After the plots were set up, they were fertilized with the N supplement (NH_4NO_3 , grain loading, N content 35%) in dry conditions once a year in May 2019 and 2020.

Soil sample collection and analysis

In August 2020, when herbage was growing vigorously, soil samples were collected from five random cores (top 0–10 cm with 3 cm inner diameter) in differently treated plots (Figure 1D). These five soil samples were pooled to obtain a representative sample per quadrat, resulting in 32 samples in total. The samples were sieved to remove impurities, and a quarter of each soil sample was reserved for microbial detection. The remaining soil samples were separated into two fractions, of which the first was kept at 4°C . The second fraction was air-dried for 2 weeks, ground, and sieved to be used



for determining the soil properties using the same measurement methods and data as described by Li et al. (2022b).

Microbial community analysis

The *nifH* gene fragments were amplified using primers *nifH*-F (AAAGGYGGWATCGGYAARTCCACCAC) and *nifH*-R (TTGTTSGCSGCRTACATSGCCATCAT). For more accurate analysis results, after the original sequencing data were launched, the optimized sequences were obtained through sequence splicing, filtering, and chimera removal to control the data quality. With a 97% similarity using Uparse (Edgar, 2013), the operational taxonomic units (OTUs) used were the Silva (Release128/132; <http://www.arb-silva.de>) database for clustering and annotation (Quast et al., 2012). Diversity analyses were carried out based on these results.

We calculated alpha diversity (Shannon diversity index, Chao1, and observed species number) using the formula developed by Li et al. (2020b). Additionally, the beta diversity of N₂-fixing bacterial communities among samples was analyzed with non-metric multidimensional scaling (NMDS).

Data statistics

The Kruskal test was carried out using the q-value package to analyze the different slopes as well as OTUs and the abundance differences of NFB in relation to N addition treatment for the same slope. If the *P*-value is <0.05, it is regarded as a different OTU or species.

The response of soil alpha diversity to different N application levels on the same slope was examined using a one-way ANOVA with Duncan *post-hoc* test. The Mantel test and Pearson test

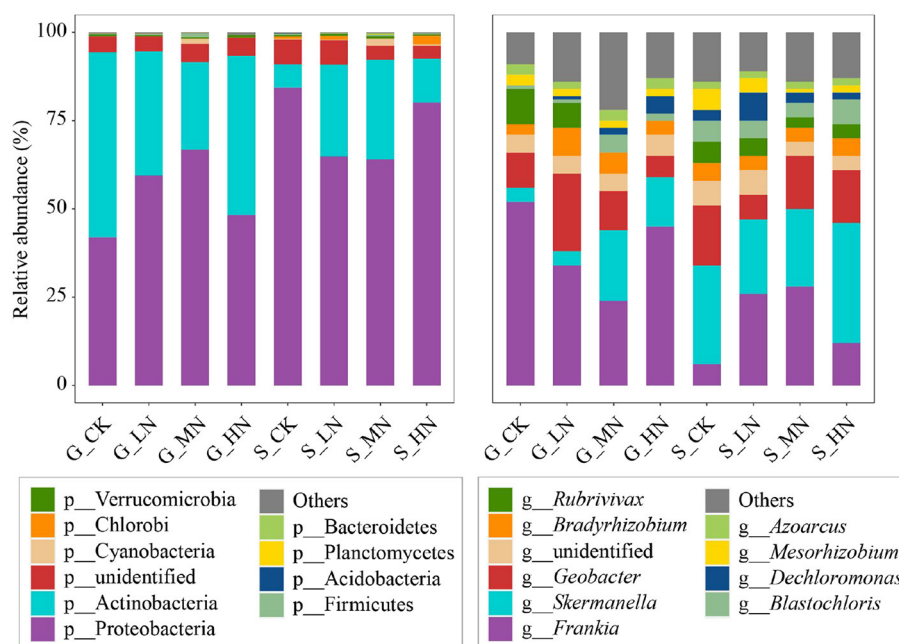


FIGURE 3

Composition of soil N₂-fixing bacteria on the different slopes with different N addition levels at the phylum and the genus levels. The acronyms are described in Figure 2.

were used to evaluate the correlation among all these variables. The different indicators between slopes were analyzed by an independent sample *t*-test ($\alpha = 0.05$).

A NetWork analysis was based on Spearman's test method, which selects the top 20 genus results of all samples for correlation analysis and takes the corresponding phylum as the legend. The calculated results filter out those whose correlation value $|R| < 0.6$ or whose *P*-value is > 0.05 for drawing.

Species composition histogram and Venn diagram were plotted in the R language. All the analyses, including NMDS and network analysis, were conducted with R 4.1.2 using the *vegan* (Oksanen et al., 2013), *ggplot2* (Wilke, 2019), *psych* (Revelle, 2010), *ggcor* (Huang et al., 2020), and *dplyr* (Wang, 2017) packages.

Results

Composition of N₂-fixing bacterial community

Different amounts of OTUs were obtained under different slopes and at different N addition levels (Figure 2). There were 30 and 22 OTUs with significant differences among the different N application levels on the gentle and steep slopes, respectively, and 472 OTUs with significant differences between the two slopes ($P < 0.05$; Supplementary Table S1).

Proteobacteria and Actinobacteria were the dominant phyla of NFB (Figure 3), and their abundance had no significant difference on the same slope to which N was added (Supplementary Table S2). Significant differences existed between both slopes in dominant bacteria, including Proteobacteria,

Actinobacteria, and some NFB of very low abundance (such as Chlorophyta and Bacteroidetes). Actinobacteria were significantly higher on the gentle slope than on the steep slope; in contrast, Proteobacteria on the gentle slope were significantly lower ($P < 0.05$).

The dominant NFB genera identified were *Frankia*, *Skermanella*, and *Geobacter* (Figure 3). The first one belongs to Actinobacteria, and the latter two belong to Proteobacteria. The abundance of the dominant genera of NFB on the same slope and the different N additions were not significant, but significant differences existed in some low-abundance NFB (relative abundance $< 0.5\%$; $P < 0.05$). For example, there were differences in N addition in *Brachybacterium*, *Actinoplanes*, *Bosea*, *Janthinobacterium*, *Plantibacter*, *Alterythrobacter*, and *Curvibacter* on the gentle slope and in *Symploca*, *Achromobacter*, *lusitaniella*, *Gluconacetobacter*, and *Roseomonas* on the steep slope. *Brachybacterium* was selected on the gentle slope; *Symploca* and *Achromobacter* were selected on the steep slope (relative abundance $> 0.1\%$) (Supplementary Table S3). Compared with CK, HN significantly reduced *Brachybacterium* on the gentle slope ($P < 0.05$; Supplementary Table S4). Compared with CK, *Achromobacter* on the steep slope significantly decreased under MN ($P < 0.05$), while *Symploca* significantly increased under HN ($P < 0.05$). There were significant differences between the two slopes in 59 genera, including *Frankia* and *Skermanella* ($P < 0.05$; Supplementary Table S5).

On the gentle slope (Figure 4A), *Paenibacillus* belongs to Firmicutes, and the rest belongs to Proteobacteria. The first part was composed of *Blastochloris*, *Rubrivivax*, *Skermanella*, and *Geobacter*, which were positively correlated. In the second part,

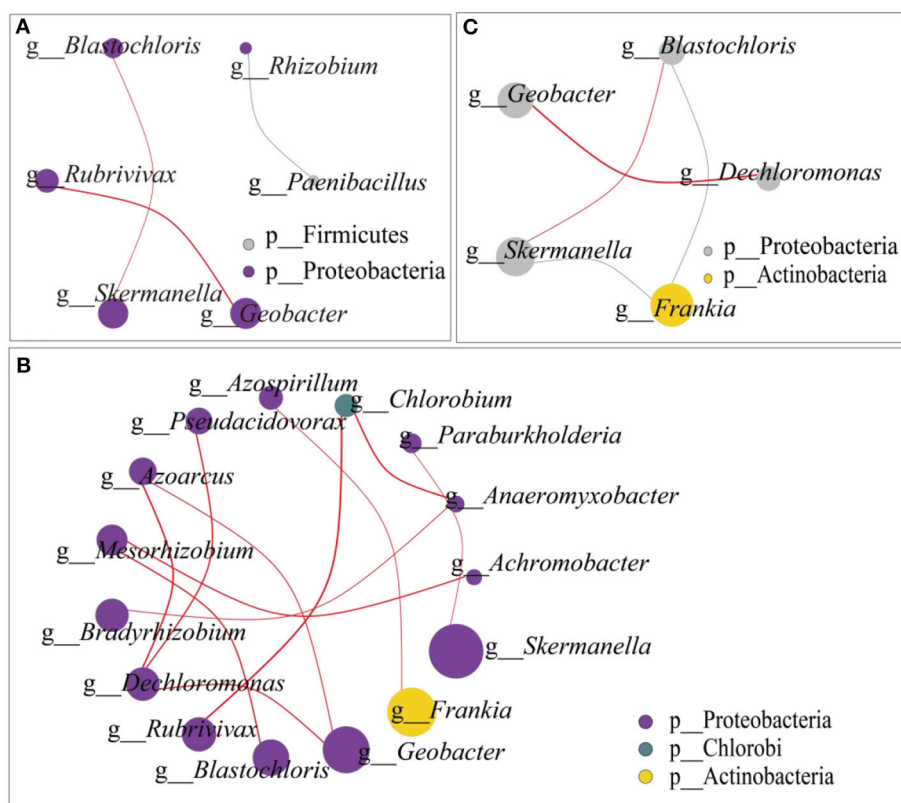


FIGURE 4

Microbial network interaction of N_2 -fixing bacteria (A) microbial network interaction on the gentle slope; (B) microbial network interaction on the steep slopes; (C) microbial network interaction from both the gentle and the steep slopes. The dot size in the graph represents the abundance, and the line thickness represents the correlation. The dot color represents the door to which it belongs, while the red line represents a positive correlation, and the blue line represents a negative correlation.

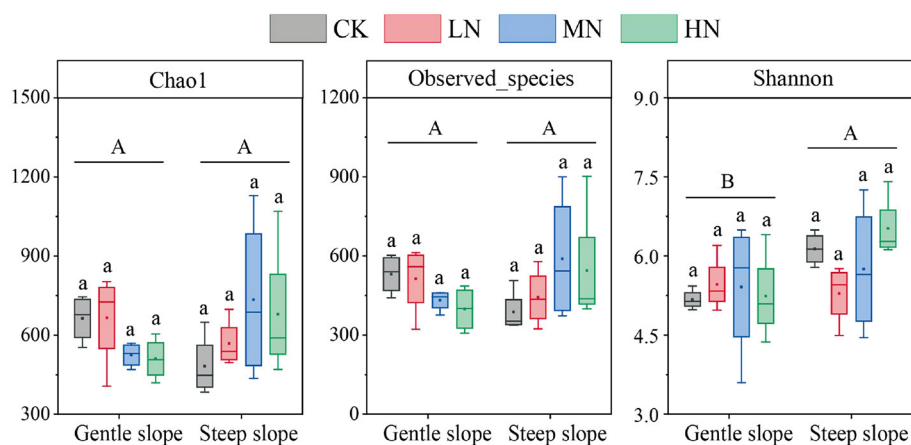


FIGURE 5

Abundance and diversity index of N_2 -fixing bacteria in soil samples. Different lowercase letters represent significant differences among N addition levels in the same slope, and different uppercase letters represent significant differences between the slopes. The acronyms are described in Figure 2.

Rhizobium and *Paenibacillus* were negatively correlated. On the steep slope (Figure 4B), *Chlorobium* belongs to the Chlorobi phylum, *Frankia* belongs to the Actinobacteria phylum, and the other 13 genera belong to the Proteobacteria phylum.

Among them, *Achromobacter*, which showed significant differences among different N addition levels, was positively correlated with *Mesorhizobium*. The network interaction map of the two slopes showed that the dominant genera *Frankia*

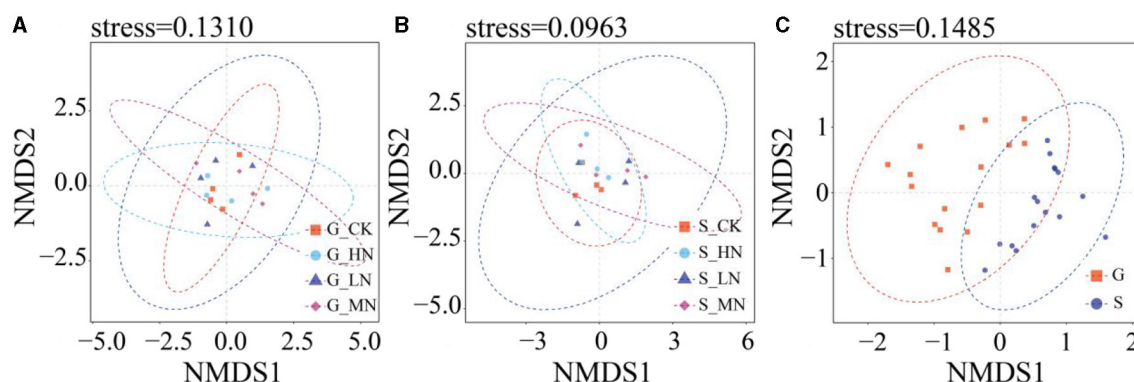


FIGURE 6

NMDS analysis of OTU level (A) groups of N addition on the gentle slope; (B) groups of N addition on the steep slope; (C) groups from both the gentle and the steep slopes.

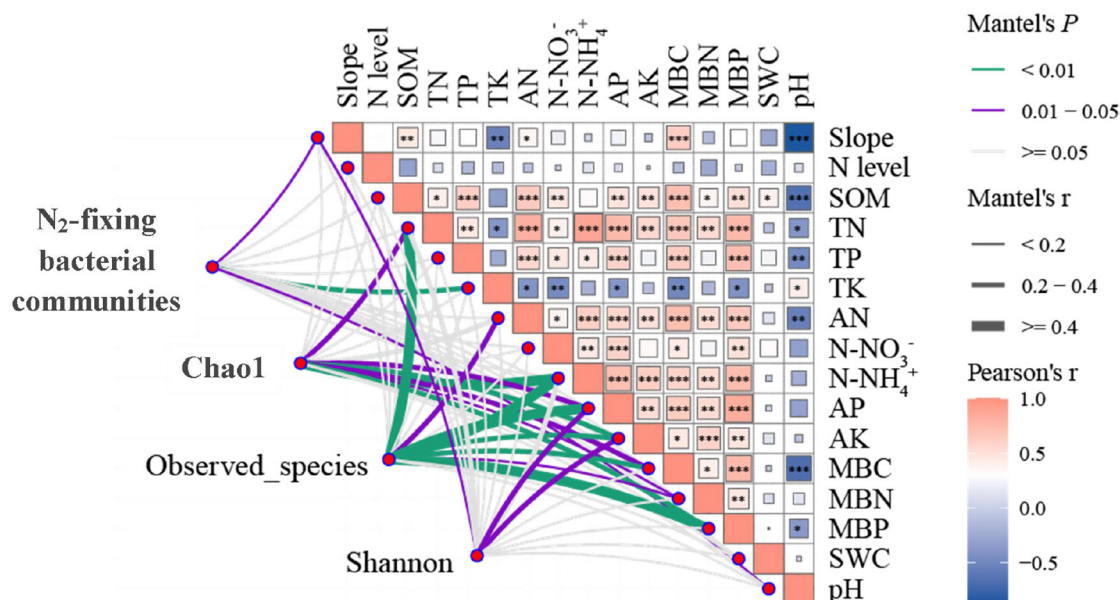


FIGURE 7

Correlation of N_2 -fixing microbial composition and diversity with slope, N addition levels, soil physicochemical properties, and microbial biomass. Mantel edge width corresponds to the Mantel r value, and edge color indicates statistical significance. The color gradient of Pearson correlation coefficient r represents the paired correlation of variables. 27.0° for the steep slope and 8.0° for the gentle slope. N addition levels include CK: 0 g $N \cdot m^{-2}$; LN: 2 g $N \cdot m^{-2}$; MN: 5 g $N \cdot m^{-2}$; and HN: 10 g $N \cdot m^{-2}$. N_2 -fixing microbial community includes N_2 -fixing bacteria phylum with significant differences among different slopes (Chlorobi, Chlorophyta, Actinobacteria, Proteobacteria, Euryarchaeota, and Bacteroidetes). * indicates $0.01 < P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$.

was negatively correlated with *Skermanella* and *Blastochloris* (Figure 4C).

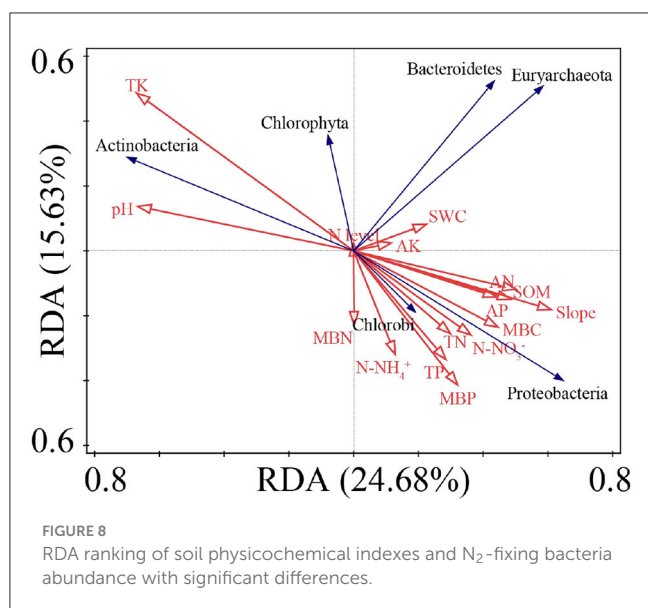
Diversity of soil N_2 -fixing bacteria

On the same slope, the alpha diversity of soil NFB did not differ significantly with N addition levels (Figure 5). The Chao1 and the number of species observed did not differ significantly between the slopes, but the Shannon index did ($P < 0.05$). At the same time, the NMDS showed no differences among N addition levels on the same slope and between the different slopes (Figure 6). There were

significant differences in NFB community diversity in CK between the slopes ($P < 0.05$; Supplementary Table S6).

Relationship between soil properties and N_2 -fixing bacteria

A significantly positive correlation was found among slope, TK, pH, and community abundance of NFB ($P < 0.05$; Figure 7). Furthermore, TN, $N-NH_4^+$, AP, AK, MBC, MBN, MBP, and Chao1 were significantly and positively correlated ($P < 0.05$). A



significantly positive correlation was also found among TN, AN, N-NH_4^+ , AP, AK, MBC, MBN, MBP, and observation species ($P < 0.05$). In addition, a significantly positive correlation was found among slope, AP, AK, and Shannon ($P < 0.05$).

The ranking results of the redundancy analysis (RDA) showed that the first and second ranking axes accounted for 24.68% and 15.63% of the total species variability, respectively (Figure 8). TK, pH, and slope account for 14.9%, 12.0%, and 10.6% of variation in the prime index (Supplementary Table S7), respectively.

Discussion

N addition showed no significant effect on the dominant genera of NFB. Nonetheless, it had a significant effect on the rare genera of NFB; besides, the response of NFB in degraded alpine meadows to N application on different slopes was different. The sensitivity of all NFB phyla to N application was weakened because of the low abundance of genera with significant differences in NFB communities; this indicated that the species specificity of soil NFB under different N addition levels was weak in degraded alpine meadows. At the same time, N addition had no significant effect on the richness and diversity of NFB, regardless of the slopes. This finding totally contravenes that reported by Qin (2021). They reported that the abundance and diversity of NFB changed significantly after N addition such as N addition significantly changed *Azorhizobium* and *Nostoc* (Qin, 2021). Meanwhile, the Shannon index of NFB of $15 \text{ g N} \cdot \text{m}^{-2} \cdot \text{a}^{-1}$ decreased significantly when compared with $1.5 \text{ g N} \cdot \text{m}^{-2} \cdot \text{a}^{-1}$, but the number of observed species and the Chao1 of *nifH* bacteria did not respond to N addition (Qin, 2021). Compared with CK, no significant difference in the Shannon index of NFB existed among 1.5, 3, 5, and $10 \text{ g N} \cdot \text{m}^{-2} \cdot \text{a}^{-1}$ (Qin, 2021). Previous studies had also shown that the long-term application of N fertilizer reduced the competitiveness of soil NFB, which changed their diversity and community structure (Mirza et al., 2014; Wang et al., 2016). Some of these differences may be attributed to different types of grasslands with

different plant community compositions, species characteristics, and external environmental resources. For example, different plant species may promote different absorptions and utilizations of soil N due to their different growth types (Weintraub, 2004) and periods of growth and development. In addition, it should be mentioned that there are also differences in the availability of N sources and in the content of available N in the soil (Hodge et al., 2000). Besides, there may also be differences in the composition of the bacterial population structure containing specific functional genes that lead to transformation into N. Moreover, our results may also depend on the setting of the N addition level and on the duration of the N addition.

As the main topographic factor, slope has an important impact on grassland soil water content, soil erosion degree, and soil thickness, resulting in the differentiation of soil nutrients on different slopes (Zhang, 2019). Many studies have already shown that both the structure and composition of NFB were affected by the physical and chemical environment of the soil (Li et al., 2013). Comparing CK on both slopes, the content of SOM and AN after N addition was significantly higher on the steep slope, while the opposite was true for pH. N addition can also stimulate the change in the composition of NFB on the two slopes. The difference in the dominant phylum bacteria, such as Actinobacteria and Proteobacteria, changed with the slopes. These changes may be related to the natural TK of the gentle and steep slopes. Our study found that there were significant differences in TK between CK on the two slopes and a significant positive correlation between TK and the composition of NFB. We suggest that the difference in the dominant communities of NFB was limited by the total potassium in the soil. This may be because the total potassium content in soil restricts the growth of plants or the physiological metabolism of NFB, such as cell wall synthesis and cell division, which further affects the growth and activities of NFB. In addition, total potassium can also affect the growth environment of soil microorganisms and change the microbial community structure, thus affecting the growth and activities of NFB. Therefore, the response of NFB to an exogenous N addition was not consistent in alpine meadows with different slopes. These results may be more related to the diversity differences of NFB under the natural conditions of gentle and steep slopes: the higher the NFB diversity index, the more stable the grassland soil environment (Zhao et al., 2018). Different levels of N addition will not change the richness and diversity of NFB. The results of our research on fertilization for 1 year showed that, in restoration, the positive effect of short-term exogenous N addition depends on slope, which was more suitable for the restoration of vegetation and soil bacterial diversity on the gentle slope. For the restoration of moderately degraded alpine meadows on the gentle slope, the N addition level should be controlled at $\sim 10 \text{ g} \cdot \text{m}^{-2}$ (Li et al., 2020a,b, 2022b). However, it should be noted that the addition of N on the gentle slope will reduce the content of soil TK and increase the content of AP, while the addition of a high amount of N will reduce the content of soil TK and MBP. It should be noted that the difference in altitude between the two slope plots may also be one of the reasons for the observed differences in experimental results, and this will be further verified in future studies. In addition, the potential N_2 fixation rate (or nitrogenase activity) in the soil will be measured to

further demonstrate the effect of N addition on the soil's N₂-fixing bacterial community.

Conclusion

N addition on the same slope had no significant effect on the richness and diversity of NFB, but significant differences in the richness and diversity of NFB existed between two slopes in a degraded meadow. These differences may be related to differences in the availability of phosphorus and potassium. It is not recommended to take measures of N addition to restore degraded alpine meadows on a steep slope. Our study showed the impact of topographic factors on degraded grasslands, highlighting the need to consider this information to better restore those degraded grasslands.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary material](#), further inquiries can be directed to the corresponding author.

Author contributions

Field experiments were carried out by XL and CL. Data analysis was carried out by CL and YS. The manuscript was prepared by CL, with contributions from XL, GZ, EV, and YS. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2023.1240634/full#supplementary-material>

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Nitrogen-cycling microbial communities respond differently to nitrogen addition under two contrasting grassland soil types

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Introduction: The impact of nitrogen (N) deposition on the soil N-transforming process in grasslands necessitates further investigation into how N input influences the structural composition and diversity of soil N-cycling microbial communities across different grassland types.

Methods: In this study, we selected two types of grassland soils in northwest Liaoning, temperate steppe and warm-temperate shrub, and conducted short-term N addition experiments using organic N, ammonium N, and nitrate N as sources with three concentration gradients to simulate N deposition. Illumina MiSeq sequencing technology was employed to sequence genes associated with N-cycling microbes including N-fixing, ammonia-oxidizing and denitrifying bacteria, and ammonia-oxidizing archaea.

Results and discussion: The results revealed significant alterations in the structural composition and diversity of the N-cycling microbial community due to N addition, but the response of soil microorganisms varied inconsistent among different grassland types. Ammonium transformation rates had a greater impact on soils from temperate steppes while nitrification rates were more influential for soils from warm-temperate shrubs. Furthermore, the influence of the type of N source on soil N-cycling microorganisms outweighed that of its quantity applied. The ammonium type of nitrogen source is considered the most influential driving factor affecting changes in the structure of the microbial community involved in nitrogen transformation, while the amount of low nitrogen applied primarily determines the composition of soil bacterial communities engaged in nitrogen fixation and nitrification. Different groups of N-cycling microorganisms exhibited distinct responses to varying levels of nitrogen addition with a positive correlation observed between their composition, diversity, and environmental factors examined. Overall findings suggest that short-term nitrogen deposition may sustain dominant processes such as soil-N fixation within grasslands over an extended period without causing significant negative effects on northwestern Liaoning's grassland ecosystems within the next decade.

KEYWORDS

nitrogen addition, nitrogen cycling, source and amount of nitrogen application, grassland type, microbial community

1 Introduction

Atmospheric nitrogen (N) deposition is the process through which N precipitates onto terrestrial and marine environments in various forms as a consequence of anthropogenic activities (Du et al., 2014), such as fossil fuel combustion and industrial emissions. One of the primary drivers behind the global decline in terrestrial biodiversity is N deposition (Clark et al., 2013), with numerous studies demonstrating that the frequent addition of N leads to a rapid decline in the diversity of aboveground species (Fang et al., 2012), soil acidification (Tian and Niu, 2015), and alterations in ecosystem functioning (Greaver et al., 2016). According to the analysis based on the published N deposition data, the national average atmospheric N deposition has increased from 11.11 to 13.87 kg ha⁻¹ yr⁻¹ over 30 years, an increase of approximately 25% (Jia et al., 2016). Grasslands, one of the most prevalent vegetation types globally, cover approximately one-third of the earth's land area and play a crucial role in terrestrial ecosystems (Kemp et al., 2013; Merbold et al., 2014). China is a vast country abundant in grassland resources, with the grassland area that accounts for approximately 41.7% of the total land (Shen et al., 2015). Consequently, the impact of N deposition on grassland ecosystems has become a matter of significant concern (Lü et al., 2019). The ecological impacts of N deposition can exhibit significant variations depending on the type of grassland (Qi et al., 2021). Northwest Liaoning, located in central Northeast Asia, possesses abundant grassland resources, primarily utilized for grazing purposes. This region experiences a semi-arid monsoon continental climate characterized by substantial annual and daily temperature fluctuations. Due to inadequate soil management practices and water scarcity issues persisting over the years, the soils demonstrate heightened sensitivity to external environmental changes (Peel et al., 2007; Tong, 2012).

The grassland soil N cycle plays a crucial role in grassland ecosystems, and N deposition significantly affects soil N transformation processes in grasslands (Jiang et al., 2018). Soil microorganisms are important drivers of nutrient cycling and energy flow in ecosystems (Hallin et al., 2009). Previous studies have demonstrated that soil N-cycling microorganisms played a key role in the soil N cycle and influenced the N-cycling processes by participating in N fixation, mineralization, nitrification, denitrification, and anaerobic ammonium oxidation within grassland ecosystems (Harter et al., 2014). For instance, biological N fixation, predominantly mediated by soil N-fixing bacteria, is widely acknowledged as the primary source of N input in grasslands (Cleveland et al., 1999), whereas nitrification processes are attributed to ammonia-oxidizing microorganisms (Jia and Conrad, 2009). Denitrification processes largely depend on denitrifying microorganisms for nitrate or nitrite reduction (Zumft, 1997). The phenomenon of N deposition, which directly affects the soil N cycle, disrupts the indigenous soil microbial community. Studies have shown that the structural composition and diversity of soil microbial communities exhibited distinct responses depending on both the type and quantity of the applied N source (Dambreville et al., 2006; Ren et al., 2022). For example, the abundance of ammonia-oxidizing bacteria increased with nitrate-N fertilization (Wang et al., 2019), and the inorganic N application altered denitrifying bacterial community structure while organic N had no effect on it (Yin et al., 2014). Soil microorganisms exhibit high sensitivity to changes in environmental conditions, leading to alterations in their community structure and

composition for rapid adaptation to environmental shifts, which may result in limitations in certain microbial communities (Victoria et al., 2021; Wang et al., 2021). For example, high pH environments exerted a positive influence on ammonia-oxidizing bacteria while negatively impacting ammonia-oxidizing archaea (Ying et al., 2017). In high-N environments, the abundance of most N-converting functional genes increased, whereas the abundance of *nifH* genes significantly decreased (Silveira et al., 2021). In addition, there existed a significant correlation between soil N conversion rate and N-converting microorganisms, which was profoundly affected by N addition. The rates of soil ammonification, nitrification, and N mineralization are regulated by N-fixing bacteria, ammonia-oxidizing archaea, ammonia-oxidizing bacteria, and denitrifying bacteria (Wang et al., 2017). The addition of N substantially enhanced the soil net N mineralization rate as well as ammonium and nitrification rates (Hu et al., 2021), with changes primarily driven by variations in soil nitrate and ammonium N contents (Yang et al., 2019; Ma et al., 2021).

Although numerous studies have investigated the impact of N application on N-transforming microorganisms in grassland ecosystems, most of these studies have primarily focused on gene copy number levels, with limited attention given to their diversity and structure. In this study, we employed high-throughput sequencing technology to analyze the taxonomic composition and structure characteristics of soil N-transforming microbial communities (N-fixing bacteria, ammonia-oxidizing archaea, ammonia-oxidizing bacteria, and denitrifying bacteria) under varying N sources and levels in two types of grassland soils located in northwestern Liaoning province. Additionally, we integrated these findings with the corresponding environmental factor data to elucidate the influence of N addition on soil microbial communities involved in N transformation in different grassland types. Overall, we proposed three hypotheses: (1) The structural composition and diversity of N-transforming microbial communities in different grassland types in northwest Liaoning are significantly influenced by the source and application amount of N. (2) The response of soils from different grassland types to N addition varies. (3) Any alteration in environmental factors may impose limitations on N-transforming microorganisms.

2 Materials and methods

2.1 Experimental design

The experiment was conducted at the experimental base of Shenyang Agricultural University (41°50'N, 123°34'E). Two types of grassland soils, temperate steppe and warm-temperate shrub, were utilized as a cultivation substrate for the pot test (1.5 kg/pot). The basic information of soil for each grassland type is shown in Table 1. With a test period of 90 days, ice grass (*Agropyron cristatum* (L.) Gaertn.) was cultivated as the dominant species in the temperate steppe, while long manzanita (*Stipa bungeana* Trin.) served as the dominant species in the warm-temperate shrub.

The three types of nitrogen used were organic N (C₂H₅NO₂), ammonium N (NH₄Cl), and nitrate N (Ca(NO₃)₂·4H₂O), with concentration gradients of 0.15 g/kg, 0.30 g/kg, and 0.45 g/kg, respectively (the N applied was pure N). Each gradient of each N fertilizer was replicated four times, and the control group for each

TABLE 1 Basic information regarding soils of different grassland types.

Group	TS ^a	WST ^b
Latitude and Longitude	N 42°48′, E 122°32′	N 42°3′, E 120°5′
TN/(g•kg ⁻¹) ^c	0.12 ± 0.01	0.87 ± 0.01
TP/(g•kg ⁻¹) ^d	0.31 ± 0.02	0.61 ± 0.03
SOC/(g•kg ⁻¹) ^e	1.20 ± 0.14	8.07 ± 0.52
pH	5.74 ± 0.05	7.07 ± 0.12
EC/(S•m ⁻¹) ^f	44.85 ± 7.27	74.75 ± 18.69
AN/(mg•kg ⁻¹) ^g	15.17 ± 5.78	74.08 ± 8.38
NH ₄ ⁺ -N/(mg•kg ⁻¹) ^h	3.17 ± 0.72	3.10 ± 0.05
NO ₃ ⁻ -N/(mg•kg ⁻¹) ⁱ	5.32 ± 1.26	9.93 ± 2.28
SP/(mg•kg ⁻¹) ^j	21.39 ± 1.61	2.81 ± 0.79
ON/(mg•kg ⁻¹) ^k	0.12 ± 0.01	0.87 ± 0.01

^aTS represents temperate steppe.

^bWST represents warm-temperate shrub.

^cTN represents total nitrogen.

^dTP represents total phosphorus.

^eSOC represents soil organic carbon.

^fEC represents electrical conductivity.

^gAN represents alkali-hydrolyzable nitrogen.

^hNH₄⁺-N represents ammonium nitrogen.

ⁱNO₃⁻-N represents nitrate nitrogen.

^jSP represents available phosphorus.

^kON represents organic nitrogen.

The same as below.

grass type had four replicates as well, resulting in a total of 80 pots–40 pots per grass type. The schematic diagram of nitrogen application is shown in [Supplementary Figure S1](#).

2.2 Sample collection and determination

Soil samples were collected on the 10th, 30th, 60th, and 90th days of incubation and were divided into two portions. One portion was stored at 4°C for physicochemical property analyses (soil organic carbon, pH, conductivity, total phosphorus, fast-acting phosphorus, total soil nitrogen, alkaline soluble nitrogen, ammonium nitrogen, nitrate nitrogen, and organic nitrogen) according to [Bao \(2000\)](#). The 90th day remaining soil samples were frozen at –80°C for high-throughput sequencing analyses. The calculation formula for soil nitrogen conversion rate is as follows ([Widdig et al., 2020](#)):

Soil net nitrogen mineralization rate $\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1} = [\text{after cultivation } (\text{NH}_4^+-\text{N} + \text{NO}_3^--\text{N}) - \text{before cultivation } (\text{NH}_4^+-\text{N} + \text{NO}_3^--\text{N})]/\text{day}$,

Soil net nitrogen nitrification rate $\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1} = [\text{NO}_3^--\text{N after cultivation} - \text{NO}_3^--\text{N before cultivation}]/\text{day}$,

Soil net nitrogen ammonification rate $\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1} = [\text{NH}_4^+-\text{N after cultivation} - \text{NH}_4^+-\text{N before cultivation}]/\text{day}$.

2.3 Illumina sequencing

Four typical N-cycling microbial communities were investigated in this study: N-fixing bacteria (*nifH*) of nitrogen fixation, ammonia-oxidizing archaea (AOA), ammonia-oxidizing bacteria (AOB) with ammonia monooxygenase genes (*amoA*), and denitrifying bacteria (*nirK*) of nitrite reduction ([Yin et al., 2014](#)).

Genomic DNA extraction: The Power Soil DNA Isolation Kit (MoBio, United States) was used to extract genomic DNA from the samples, after which the purity and concentration of the DNA were tested using agarose gel electrophoresis and Nanodrop analysis.

PCR amplification: The diluted genomic DNA was used as a template for the polymerase chain reaction (PCR) using specific primers with barcodes and efficient high-fidelity enzymes according to the selection of sequencing regions. Detailed information about the primers and the PCR protocols for the four N-cycling microbial communities could be found in [Supplementary Table S1](#).

High-throughput sequencing library was constructed using the TruSeq® DNA PCR-Free Sample Preparation Kit. The libraries were quantified using Qubit and qPCR. After the libraries were quantified, the v2 sequencing kit (2 × 250 bp) and MiSeq sequencer were used for onboard sequencing at Shanghai Personal Biotechnology Co., Ltd.

2.4 Processing of sequencing data

The initial sequence was acquired using the Quantitative Insights into Microbial Ecology (QIIME v.1.9.1) quality control process to extract high-quality clean tags ([Bolyen et al., 2018](#)),¹ and the sequences were clustered into operational taxonomic units according to 97% pairwise identity using Vsearch (v2.13.4). RDP FrameBot² was used to correct sequencing errors (insertions & deletions) and translate DNA sequences for functional genes. The sequence database referenced in this study was obtained from the National Center for Biotechnology Information (NCBI). Alpha diversity metrics ([Chao, 1984](#)), observed species, ([Shannon, 1948](#); [Simpson, 1949](#)), Pielou's evenness ([Pielou, 1966](#)), and Good's coverage ([Good, 1953](#)) and beta diversity metrics (Bray–Curtis dissimilarity) were estimated using the diversity plugin with samples rarefied to sequences.

The DNA sequences used in this study were obtained from the National Center for Biotechnology Information Sequence Read Archive database under the accession number PRJNA929669.

2.5 Statistical analyses

One-way analysis of variance (ANOVA) was conducted using SPSS software (version 22.0, IBM Corporation, Armonk, NY, United States) to determine the differences in environmental factors and the abundance of soil N-transforming microorganisms among the treatments. Two-factor and multi-factor ANOVAs were conducted on N application, N source, and incubation time to determine whether their interactions had an effect on the study. The Pearson correlation analysis was conducted to explore the correlation between environmental factors and soil N-transformed microbial abundance. Additionally, the analysis examined the correlation with alpha diversity. Significant differences between treatments were confirmed using Tukey's HSD test at $p < 0.05$.

Box plots were drawn using the “ggplot2” package in R, and the compositional distribution of each sample was visualized at the

¹ <https://docs.qiime2.org/2019.4/tutorials/>

² <https://github.com/rdpstaff/Framebot>

phylotaxonomic genus level to determine differences in the N-converted microbial community composition and alpha diversity in different grassland soil types. The structural composition of N-transforming microbial communities in different grassland soil types was visualized using the “ape” package in R using the principal coordinate analysis based on the Bray–Curtis heterogeneity matrix. Analysis of similarities (ANOSIM) was used to investigate the effect of grassland soil types on the β -diversity of N-transforming microorganisms. In addition, the SparCC algorithm was used with the “ggraph” and “igraph” packages to construct network topology index tables to observe differences in different N-cycling microbial communities. Finally, the OmicShare tool was used to produce a network heat map of the environmental factors and the composition and diversity of the N-transforming microbial community.

3 Results

3.1 Nitrogen addition significantly affected soil physicochemical properties in grasslands

As presented in [Supplementary Table S2](#), the application of N resulted in an increase in soil total N, conductivity, alkaline N, ammonium N, nitrate N, and organic N. The magnitude of this effect was positively correlated with the amount of applied N and was most pronounced for ammonium N fertilization. However, no significant changes were observed in soil total P and fast-acting P contents following the application of N; instead, a decrease in the soil total P content was noted. The application of N significantly increased the soil organic carbon content ($p < 0.05$) in the temperate steppe but not in the warm-temperate shrub. Furthermore, while there were no significant effects on soil pH levels following the addition of nitrogen to the temperate steppe, it did have a negative impact on pH levels within the warm-temperate shrub, especially when using ammonium N fertilizers ($p < 0.05$). Additionally, the sampling time significantly affected soil total N, total P, pH, ammonium N, fast-acting P, and organic N ($p < 0.05$), showing an increasing trend followed by the decreasing trend peaking at 30 days. In the temperate steppe, significant or highly significant differences were observed in soil total P, organic carbon, and fast-acting P contents at different incubation times ($p < 0.05$ or $p < 0.01$). In the warm-temperate shrub, there were significant or highly significant differences in soil total N, pH, ammonium N, fast-acting P, and organic N contents ($p < 0.05$ or $p < 0.01$). In addition, with the exception of the soil ammonium N content, significant or highly significant differences ($p < 0.05$, $p < 0.01$) were found in the measured basic physicochemical properties of the different grassland types.

3.2 Nitrogen addition significantly affected the soil N conversion rate in grasslands

The application of N significantly enhanced soil N mineralization and ammonium conversion rates ($p < 0.05$), with the highest rates observed when applying ammonium N. Moreover, increasing the N application resulted in higher soil N mineralization and ammonium conversion rates, which gradually declined with time. In addition, the

rates of N mineralization and ammonification in the soils of the warm-temperate shrub were much higher than those of the temperate steppe ([Figure 1](#)).

The nitrification rates of soils differed significantly across grassland types ($p < 0.05$), with the highest rates observed in soils treated with nitrate-N source type. In the temperate steppe, the soil nitrification rate exhibited an initial increase followed by a decrease, and neither N source types nor N application amount had a significant effect on the rate ($p > 0.05$). However, it showed a time-dependent increase and ultimately demonstrated a positive correlation between the N application rate and soil nitrification rate, and the application of ammonium and nitrate N source significantly increased the soil nitrification rate ($p < 0.05$). In the warm-temperate shrub, the N source types significantly affected the soil nitrification rate ($p < 0.05$), while the nitrate N application led to an increase that gradually declined over time ($p < 0.05$). However, the application of organic N showed an increase followed by a decrease in the soil nitrification rate. The amount of applied N did not have a significant impact on the soil nitrification rate ($p > 0.05$), but its significance increased over time and eventually revealed a positive correlation between the amount of applied N and soil nitrification rate. Additionally, the nitrification rate of soils in the warm-temperate shrub was considerably higher than that observed in the temperate steppe.

3.3 Nitrogen addition significantly affected the composition of soil microbial communities in grasslands

The N-fixing bacterial community was dominated by *Azohydromonas*, *Bradyrhizobium*, and *Azospirillum*, while the ammonia-oxidizing archaea community was mainly composed of *Candidatus Nitrosocosmicus*, *Nitrososphaera*, and *Nitrosopumilus*. *Nitrosospira*, *Nitrosomonas*, and *Nitrosovibrio* were the major genera in the ammonia-oxidizing bacterial community, while *Rhodopseudomonas*, *Mesorhizobium*, and *Bradyrhizobium* were the major genera in the denitrifying bacterial community. Notably, N addition significantly influenced the relative abundance of different genera ($p < 0.05$), with varying effects observed among different grassland types ([Figure 2](#)).

In the temperate steppe, *Azohydromonas*, *Candidatus Nitrosocosmicus*, *Nitrosospira*, and *Rhodopseudomonas* were the dominant genera in the N-fixing bacterial community, ammonia-oxidizing archaea, ammonia-oxidizing bacteria, and denitrifying bacterial communities, with mean relative abundances of 35.76, 42.02, 70.50, and 12.18%, respectively. The application of nitrate source N type significantly increased the relative abundance of *Candidatus Nitrosocosmicus* ($p < 0.05$), and the organic source N type application led to a significant increase in the relative abundance of *Nitrosospira* ($p < 0.05$). Ammonium and nitrate source N type applications caused a significant decrease in the relative abundance of *Rhodopseudomonas* ($p < 0.05$), while the application of organic and nitrate source N was found to significantly reduce the relative abundance of *Bradyrhizobium* ($p < 0.05$). Furthermore, the amount of medium and high N additions resulted in a significant decrease in the relative abundance of *Nitrosovibrio* ($p < 0.05$).

In the warm-temperate shrub, *Bradyrhizobium*, *Nitrososphaera*, *Nitrosospira*, and *Rhodopseudomonas* were identified as the dominant

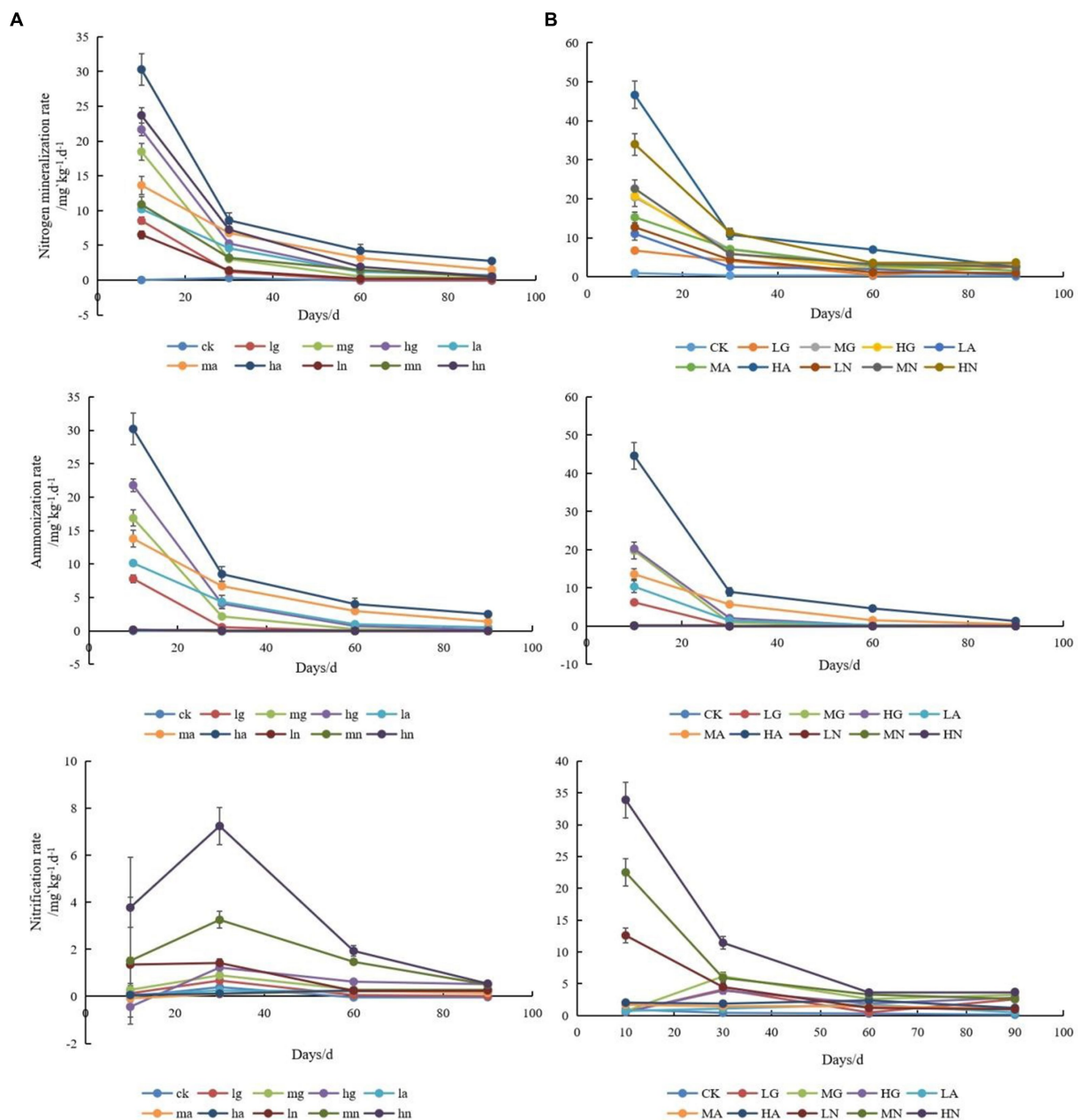


FIGURE 1

Changes in soil N-cycling rates of temperate steppe (A) and warm-temperate shrub (B) grasslands under different treatments at different times. ck, control; lg, low amount of organic N addition; mg, medium amount of organic N addition; hg, high amount of organic N addition; la, low amount of ammonium N addition; ma, medium amount of ammonium N addition; ha, high amount of ammonium N addition; ln, low amount of nitrate N addition; mn, medium amount of nitrate N addition; hn, high amount of nitrate N addition; the lowercase is employed to represent temperate steppe, while the uppercase is employed for warm-temperate shrub. The same as below.

genera in the N-fixing bacterial communities (with average relative abundances of 33.73%), ammonia-oxidizing archaeal communities (5.70%), ammonia-oxidizing bacterial communities (99.03%), and denitrifying bacterial communities (20.85%), respectively. The application of ammonium source N type significantly reduced the relative abundance of *Azospirillum* ($p < 0.05$), while both organic and ammonium source N applications significantly increased the relative abundance of *Candidatus* and *Nitrosocosmicus* ($p < 0.05$). Additionally, the application of organic source N type significantly increased the relative abundance of *Rhodopseudomonas* ($p < 0.05$). Furthermore, the

amount of high N addition resulted in a significant reduction in the relative abundance of *Bradyrhizobium* ($p < 0.05$).

3.4 Microbial community diversity of the two grassland types under different nitrogen application treatments

The alpha diversity of soil N-fixing bacteria, ammonia-oxidizing archaea, ammonia-oxidizing bacteria, and denitrifying bacterial

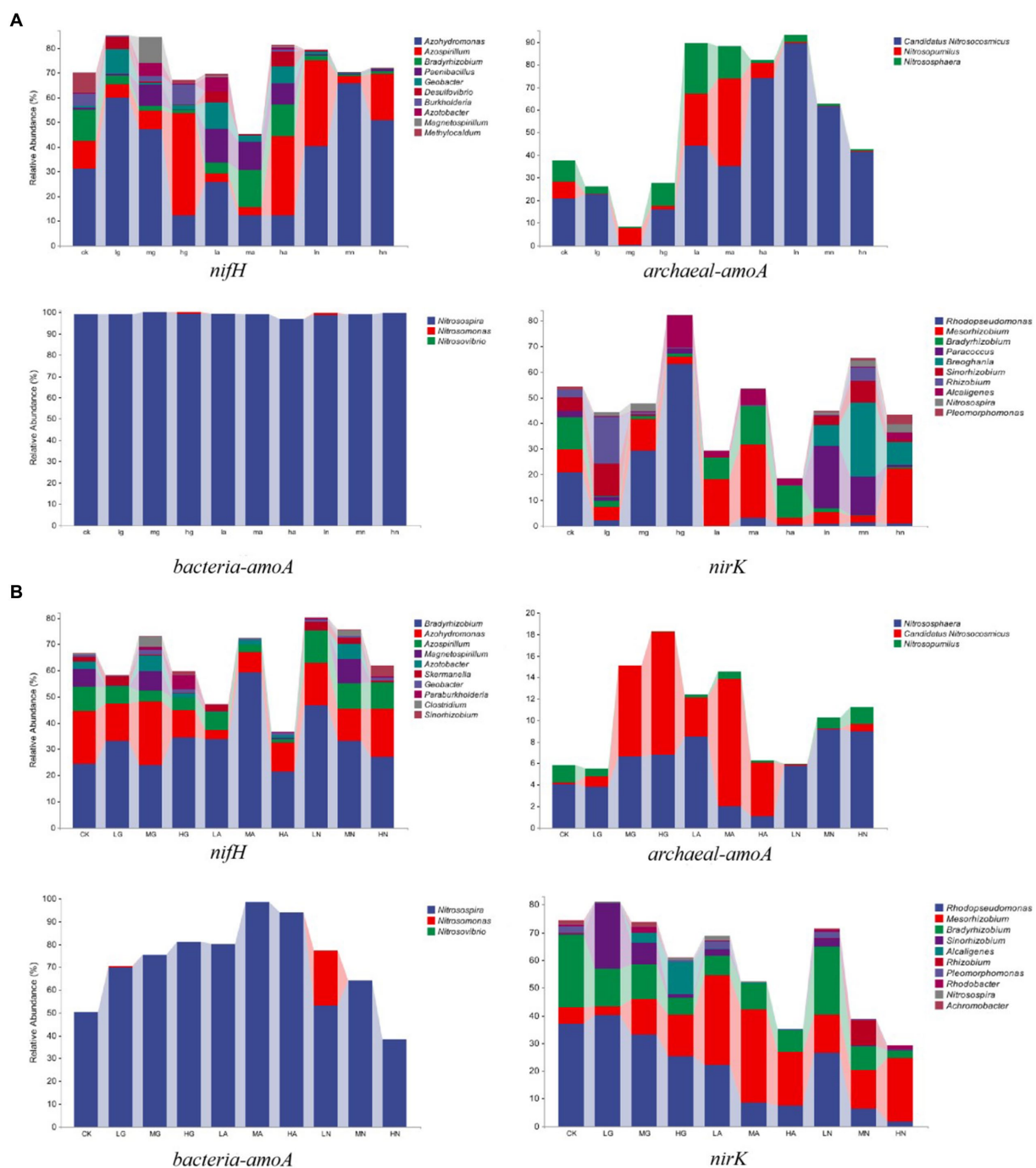


FIGURE 2
Relative abundance of soil N-cycling microbial communities at the genus level in grasslands of temperate steppe (A) and warm-temperate shrub (B).

communities varied among different grassland types and was significantly influenced by different types and amounts of N application treatments ($p < 0.05$, Figure 3).

In the temperate steppe, the alpha diversity of soil N-fixing bacterial communities was significantly influenced by the N source types ($p < 0.05$), while the amount of N applied did not significantly affect their alpha diversity. The Chao1 index showed the highest value when medium organic N was applied and was significantly higher than that of other N treatments ($p < 0.05$). The application of different

N sources generally reduced the alpha diversity of the N-fixing bacterial community; however, only organic N source application increased the species richness within these communities. Neither the N source nor the amount of applied N significantly affected the alpha diversity of the soil ammonia-oxidizing archaeal community. The Chao1 index was the highest with the application of high amounts of ammonium N, which was significantly higher than the other N treatments ($p < 0.05$). The sources of N types significantly affected the alpha diversity of ammonia-oxidizing bacterial communities in the

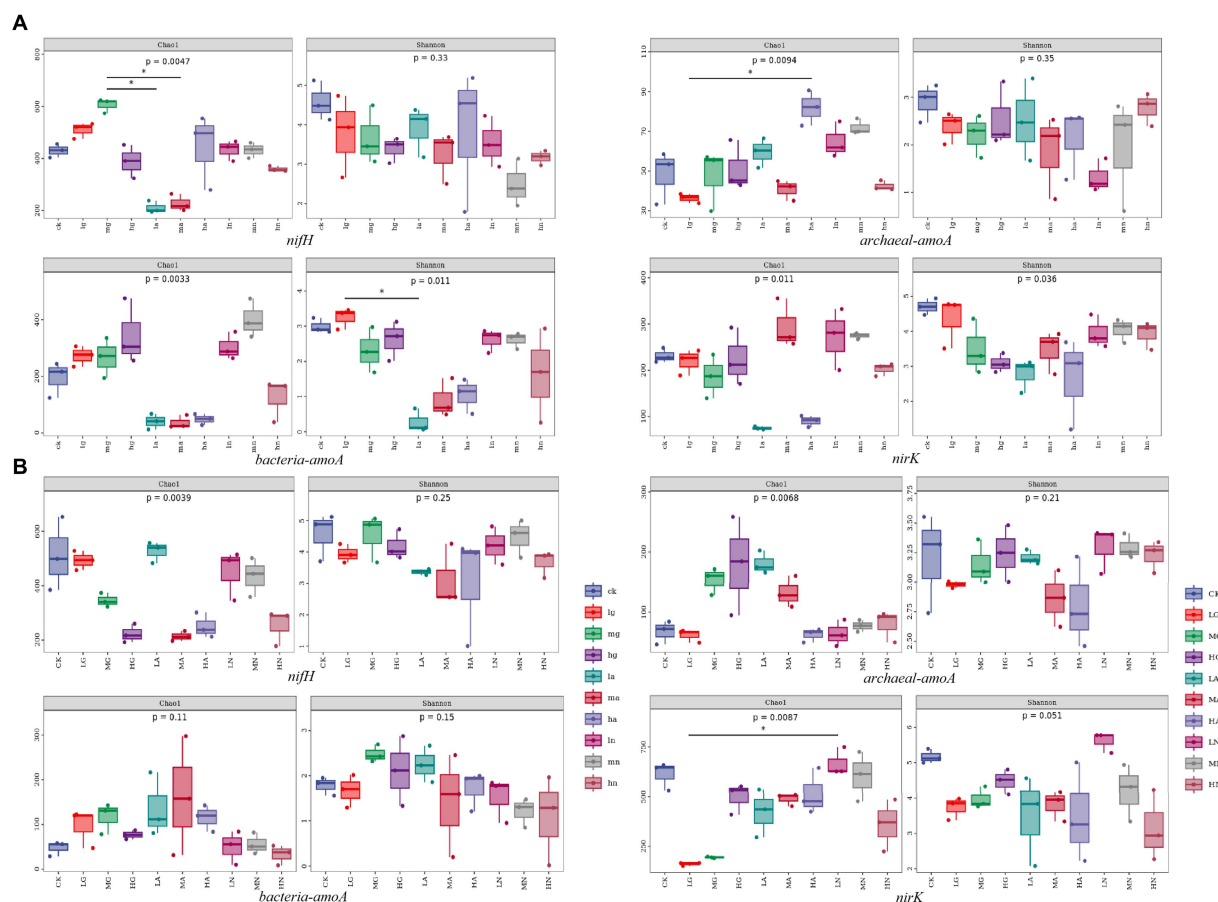


FIGURE 3
Box plot of alpha diversity grouping of soil N-cycling microbial communities in grasslands of temperate steppe (A) and warm-temperate shrub (B).

grassland soils ($p < 0.05$), whereas the amount of applied N did not have a significant effect on their alpha diversity. Among them, the application of ammonium N significantly reduced the alpha diversity of ammonia-oxidizing bacterial communities ($p < 0.05$). The N source types significantly affected the alpha diversity of the denitrifying bacterial community in the grassland soils ($p < 0.05$), whereas the amount of applied N did not have a significant impact on the alpha diversity. The application of ammonium N significantly reduced the diversity of the denitrifying bacterial community ($p < 0.05$).

In contrast, in the warm-temperate shrub, the amount of N application significantly affected the alpha diversity of soil N-fixing bacterial communities in the grasslands ($p < 0.05$), while no significant effect was observed for N source types on the diversity. Almost all treatments resulted in a reduction in the alpha diversity of the N-fixing bacterial community, with high N application showing a significant decrease in species richness and exhibiting a negative correlation with the alpha diversity ($p < 0.05$). The alpha diversity of soil ammonia-oxidizing archaeal communities was not significantly affected by neither the N source nor the amount of applied N. There was no significant effect of the amount of N application on the soil ammonia-oxidizing bacterial community, but the N source types significantly affected the alpha diversity in the grasslands ($p < 0.05$). The N source types significantly affected the alpha diversity of the soil denitrifying bacterial community in the grasslands ($p < 0.05$), whereas there was

no significant effect observed for the amount of N application on the alpha diversity. Organic and ammonium types of N application led to a significant reduction in the alpha diversity of the denitrifying bacterial community ($p < 0.05$).

3.5 Microbial community structure of the two grassland types under different nitrogen application treatments

The beta diversity of N-fixing bacteria, ammonia-oxidizing archaea, ammonia-oxidizing bacteria, and denitrifying bacterial communities was significantly affected by different N application treatments ($p < 0.05$). In the temperate steppe, the N source types had a significant impact on the beta diversity of N-fixing bacteria, ammonia-oxidizing archaea, ammonia-oxidizing bacteria, and denitrifying bacterial communities ($p < 0.05$), whereas the amount of N application only significantly affected the beta diversity of denitrifying bacterial communities ($p < 0.05$). In the warm-temperate shrub, N source types significantly affected the beta diversity of ammonia-oxidizing archaea, ammonia-oxidizing bacteria, and denitrifying bacterial communities ($p < 0.05$), whereas the amount of N application significantly influenced the beta diversity of denitrifying bacterial communities and N-fixing bacteria ($p < 0.05$, Figure 4).

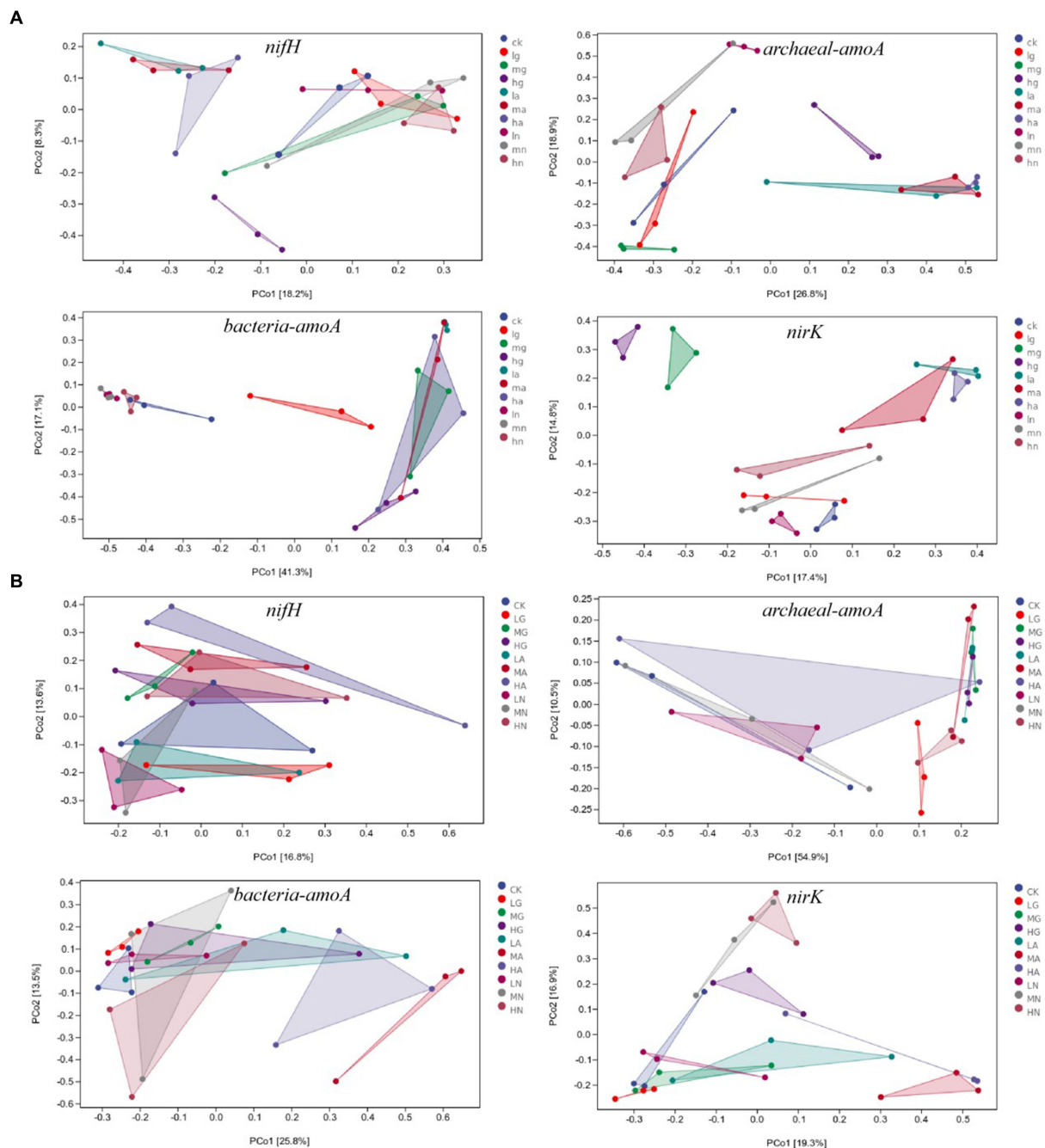


FIGURE 4
PCoA of soil N-cycling microbial communities in grasslands of temperate steppe (A) and warm-temperate shrub (B).

According to the ANOSIM analysis, significant differences were observed in the beta diversity of N-fixing bacterial communities between those without N addition and those with ammonium and nitrate types of N application ($p < 0.05$), as well as between organic and ammonium types of N application in the temperate grassland soils. There were significant differences between the treatments in the beta diversity of ammonia-oxidizing archaeal bacterial communities ($p < 0.05$) with the exception between no N addition and organic N application ($p > 0.05$). Significant differences were observed in the beta diversities of ammonia-oxidizing bacterial communities among different N source types ($p < 0.05$). The beta diversity of denitrifying bacterial communities significantly differed across various N sources

($p < 0.05$), and the amount of N applications with low to medium and high N significantly affected the beta diversity of denitrifying bacterial communities ($p < 0.05$). In the warm-temperate shrub, there were significant differences between the beta diversity of N-fixing bacterial communities with low, medium, and high amounts of N applications ($p < 0.05$). Furthermore, significant differences were found in the beta diversity of ammonia-oxidizing archaeal communities between those without N addition and those with organic and ammonium types of N application, and between those with organic and nitrate types of N application ($p < 0.05$). Significant differences ($p < 0.05$) were identified in the beta diversity of ammonia-oxidizing bacterial communities between those without N addition and those with ammonium and

organic types of N application, as well as between those with ammonium N and nitrate types of N. Finally, significant differences were observed in the beta diversity of denitrifying bacterial communities between those with the application of ammonium type of N and other N sources, between those with organic and nitrate types of N application, and the amount of N applications of low-to-medium and high N significantly affected beta diversity of denitrifying bacterial communities ($p < 0.05$).

3.6 Network characteristics of microbial communities in both grassland types under different nitrogen application treatments

The network topology parameters presented in Table 2 demonstrated that, within the temperate steppe, the symbiotic networks of N-fixing bacteria, ammonia-oxidizing archaea, ammonia-oxidizing bacteria, and denitrifying bacteria consisted of 269, 64, 92, and 122 nodes, respectively, with 834, 393, 1,689, and 2,537 edges, correspondingly. In the warm-temperate shrub, the symbiotic networks of N-fixing bacteria, ammonia-oxidizing archaea, ammonia-oxidizing bacteria, and denitrifying bacteria comprised 271, 116, 56, and 57 nodes, respectively, with 1,104, 1,382, 560, and 551 edges, correspondingly. In addition, the N-fixing bacterial community exhibited the shortest average path length t and the highest levels of the stability and assortativity across all grassland types, indicating its dominance and complexity within this study habitat.

3.7 Correlations between physicochemical properties and soil nitrogen transforming microorganisms

Correlations between physicochemical properties differed among the grassland types. In the temperate steppe, there was a highly significant and positive correlation between the rate of soil N mineralization and the rate of ammonium transformation ($p < 0.01$). Conversely, in the warm-temperate shrub, there was a highly significant and positive correlation between the rate of soil N mineralization and the rate of nitrification ($p < 0.01$). Additionally, in the warm-temperate shrub, there was a stronger correlation between

nitrate and organic N contents compared to that observed for organic carbon content (Figure 5).

The influence of physicochemical properties varied among the grassland types. In the temperate steppe, soil conductivity, alkaline and ammonium N content, N mineralization rate, and ammonification rate were significantly or highly significantly and positively correlated ($p < 0.05$ or $p < 0.01$, respectively) with the species composition of the N-fixing bacterial community. However, no significant correlations were observed between physicochemical properties and the species composition of the other N-transforming microbial communities. Soil organic carbon, pH, ammonium N content, and ammonification rate were significantly or highly significantly and positively correlated with the alpha diversity of the N-fixing bacterial community ($p < 0.05$ or $p < 0.01$, respectively). Soil total N, conductivity, alkaline-solubilized N, ammonium N content, N mineralization rate, and ammonification rate were significantly or highly significantly and positively correlated ($p < 0.05$ or $p < 0.01$) with the alpha diversity of the ammonia-oxidizing archaeal community. Soil total N, total P, organic carbon, pH, conductivity, alkaline N, ammonium N content, ammonification rate, and nitrification rate were significantly and positively correlated with the alpha diversity of ammonia-oxidizing bacterial communities ($p < 0.01$). Soil total N, organic carbon, pH, conductivity, alkaline N, ammonium N content, ammonification rate, and nitrification rate were significantly or highly significantly and positively correlated with the alpha diversity of the denitrifying bacterial community ($p < 0.05$ or $p < 0.01$).

In the warm-temperate shrubs, soil pH was significantly and positively correlated ($p < 0.05$) with the species composition of the N-fixing bacterial community. Soil total N, pH, conductivity, alkaline N, ammonium N content, and ammonification rate were significantly or highly significantly and positively correlated ($p < 0.05$ or $p < 0.01$) with the species composition of the ammonia-oxidizing archaeal community. The soil ammonium N content, N mineralization rate, and nitrification rate were significantly and positively correlated ($p < 0.05$) with the species composition of the ammonia-oxidizing bacterial community. Soil conductivity was significantly correlated with the species composition of the denitrifying bacterial community ($p < 0.01$). Soil organic carbon, pH, conductivity, alkaline N, nitrate content, N mineralization rate, and nitrification rate were significantly or highly significantly and positively correlated with the alpha diversity of the N-fixing bacterial community ($p < 0.05$ or $p < 0.01$). Soil fast-acting P and organic N content were significantly or highly significantly and positively correlated with the alpha diversity of the

TABLE 2 Symbiotic network topology index of soil N transforming microbial communities.

Group	<i>nifH</i>		<i>archaeal-amoA</i>		<i>bacterial-amoA</i>		<i>nirK</i>	
	TS	WST	TS	WST	TS	WST	TS	WST
Average nearest neighbor degree	8.3428	11.2565	14.9508	33.958	42.9504	21.8277	47.8966	21.8418
Average path length	7.4599	5.0908	2.4425	2.0702	1.6046	1.65	1.6792	1.7249
Degree assortativity	0.6565	0.5099	0.2447	0.0164	0.247	0.0316	0.1229	0.2607
Cluster num	9	12	1	1	1	1	1	1
Transitivity	0.6191	0.6308	0.6469	0.5207	0.6123	0.5374	0.53	0.5832
Vertice	269	271	64	116	92	56	122	57
Edge	834	1,104	393	1,382	1,689	560	2,537	551
Modularity	0.7193	0.7678	0.5434	0.3939	0.3561	0.5193	0.5136	0.3313

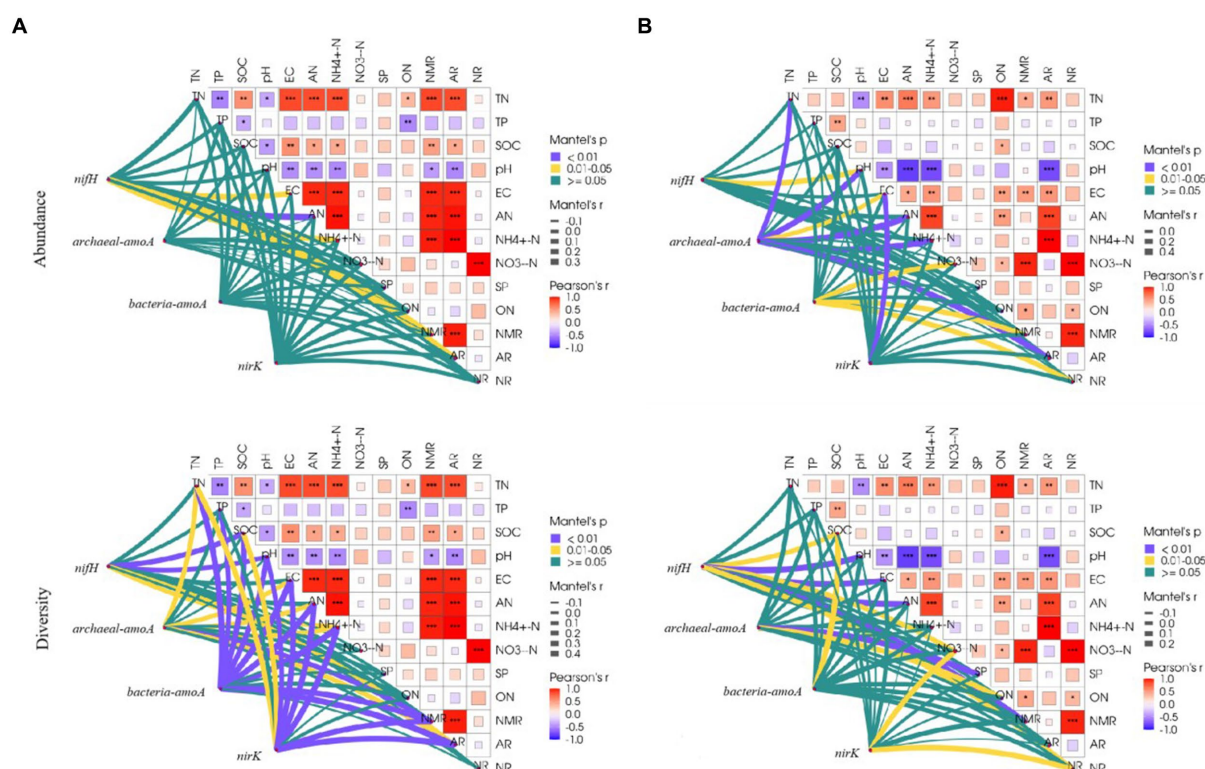


FIGURE 5

Correlation of environmental factors with species composition and alpha diversity of soil N-cycling microbial communities in grasslands of temperate steppe (A) and warm-temperate shrub (B).

ammonia-oxidizing archaeal community ($p < 0.05$ or $p < 0.01$). The soil organic carbon content was significantly and positively correlated ($p < 0.05$) with the alpha diversity of ammonia-oxidizing bacterial communities. The soil nitrate-N content and nitrification rate were significantly and positively correlated with the alpha diversity of the denitrifying bacterial community ($p < 0.05$).

4 Discussion

The addition of N significantly influenced the species composition of soil N-transforming microbial communities in grasslands, with varying effects observed among different grassland types. Previous studies have demonstrated that the environment factors could drive directional shifts in dominant genera of microbial communities (Sovová et al., 2020). In our study, we found distinct dominant genera of N-fixing bacteria and ammonia-oxidizing archaea across different grassland types. This variation may be attributed to the stronger soil nitrification processes occurring in warm-temperate shrubs (Figure 2). In natural ecosystems, soil nitrogen conversion is primarily driven by nitrification during the early growth seasons, while ammonium conversion becomes more prominent toward the end and non-growth seasons (Liu and Ma, 2021). Notably, the end of the growing season occurs later in warm-temperate shrubs compared with temperate steppes (Lee et al., 2002; Hu et al., 2014). In this study, the rate of soil N mineralization in the temperate steppe was significantly and positively correlated with the ammonification rate, while it showed a stronger positive correlation with the nitrification rate in the warm-temperate shrub, demonstrating that the warm-temperate shrub had a stronger nitrification process than the

temperate steppe ($p < 0.01$, Figure 5). In addition, *Azohydromonas* cannot survive anaerobically in environments containing nitrate and nitrite (Nguyen and Kim, 2017), leading to decreased viability and relative abundance in areas with high levels of nitrification. Ammonia-oxidizing archaea primarily derive energy from the oxidation of ammonia, a process that necessitates the presence of oxygen. Nevertheless, certain archaea, including *Nitrososphaera* (Lu and Jia, 2013; Wang et al., 2014), have been observed to thrive in anoxic environments (Kraft et al., 2022). Conversely, warm-temperate shrub-like soils exhibited lower levels of oxygenation (Cook et al., 2013), which adversely impacted genera other than *Nitrososphaera* and consequently leads to their increased relative abundance. Furthermore, numerous studies have shown that types of N sources and the application amount significantly affect the species composition of N-transforming microbial communities (Ma et al., 2018; Xiao et al., 2021). In this study, the relative abundance of the dominant genus within the community of ammonia-oxidizing bacteria was significantly ($p < 0.05$) affected by the amount of N applied, aligning with the findings from Wang's investigation (Wang et al., 2019), where a higher sensitivity toward N input was observed among ammonia-oxidizing bacteria. Moreover, a high amount of N application resulted in a significant decrease in the relative abundance of the dominant genus of denitrifying bacteria ($p < 0.05$, Figure 2), as it effectively reduced potential N_2O emissions (Deng et al., 2022). In contrast, the abundance of the dominant genera of all four N-transforming microorganisms was significantly affected by the types of N source ($p < 0.05$), indicating their distinct adaptations to different forms of N.

The addition of N significantly affected the species diversity of soil N-transforming microbial communities in the grasslands, while the

different types of grasslands were affected differently by N addition. There was an interaction between the types of N source and the amount of N application on the diversity of soil N conversion microbial communities in the grasslands. The diversity of ammonia-oxidizing archaeal communities in temperate steppe soils was significantly affected by N addition ($p < 0.05$), but neither the types of N source nor the amount of N application had a significant individual effect on them. Soil ammonia-oxidizing bacterial communities of warm-temperate shrub-like soils were significantly ($p < 0.05$) affected by the types of N source, but not significantly ($p > 0.05$, Figure 3) influenced by the interaction of the types of N source and the amount of N application. Competition reduces the species diversity of microbial communities (Smith et al., 2018). In this study, the co-occurrence network analysis revealed a low assortativity of ammonia-oxidizing archaea and ammonia-oxidizing bacterial communities in warm-temperate shrub-like soils (Table 2), indicating internal competition within these communities. This finding elucidates the higher diversity of ammonia-oxidizing archaea and ammonia-oxidizing bacterial communities in the temperate steppe compared with the warm-temperate shrub (Figure 3). In addition, a high amount of ammonium source N application significantly enhanced the diversity of soil ammonia-oxidizing archaea species, whereas a low amount of ammonium source N application led to a significant decrease in both abundance and evenness of soil ammonia-oxidizing bacteria in the temperate steppe. Moreover, a low amount of organic source N application resulted in a reduction in the number of soil denitrifying bacterial species in the warm-temperate shrub ($p < 0.05$). These findings suggest that targeted control over the diversity of N-transforming microbial communities can be achieved by adjusting the amount and type of N applied (Fangliang et al., 2013). Furthermore, the diversity of all four N-transforming microbial communities was significantly affected by the types of N source, indicating that different N-transforming microbial communities exhibited distinct adaptations to various forms of N.

The addition of N significantly influenced the community structure of soil N-transforming microorganisms in the grasslands, whereas different types of grasslands exhibited varying responses to N addition. In this study, soil N-fixing bacterial communities dominated with the most complex, stable, and connected symbiotic network (Table 2). This implies that nitrogen addition may result in a long-term concentration of grassland soil nitrogen cycle through nitrogen fixation processes. Almost all the different soil N-transforming microorganisms in the different grassland types were significantly affected by ammonium type of N addition ($p < 0.05$, Figure 4), suggesting that the ammonium type of N source is the most influential factor driving changes in the structure of the N-transforming microbial community. This could be attributed to its strong influence on soil ammonification rates (Figure 1). Previous studies have demonstrated that high soil ammonification rates can enhance the efficiency of ammonium N utilization (Sun et al., 2020). Different forms of nitrogen exert varying effects on the adaptation of microbial communities involved in nitrogen transformation. In the warm-temperate shrub, soil ammonium-oxidizing archaeal communities were significantly ($p < 0.05$) affected by organic and nitrate types of N application, probably due to their susceptibility to environmental factors (Figure 5). Studies have shown that the amount of N applied is the primary determinant of the soil N-fixing bacterial community structure (Coelho et al., 2008). Our findings indicate that low levels of N addition had a significant impact on the structure of soil N-fixing

bacterial communities in the warm-temperate shrub ($p < 0.05$), but no significant effect was observed in the temperate steppe by the amount of N application ($p > 0.05$, Figure 4). The study sites that exhibited a significant correlation with the amount of N application predominantly comprised agricultural lands or soils characterized by high soil nutrient contents. In contrast, soils in the temperate grassland category displayed lower nutrient levels and greater degradation (Supplementary Figure S2), potentially attributable to disparities in the soil-based nutrient conditions. In addition, organic, ammonium, and nitrate types of N and low, medium, and high amounts of N application significantly affected the structure of soil denitrifying bacterial communities in different grassland types ($p < 0.05$). This suggests that denitrifying bacterial communities were primarily impacted by the addition of nitrogen, which is consistent with the finding of Chen et al.'s study, which highlights their heightened responsiveness to environmental disturbances (Chen et al., 2010).

The abundance of microbial communities is affected by external factors and decreases with the decreasing particle size (Chiu et al., 2006). In this study, the grain size of temperate steppe soils was much smaller than that of the warm-temperate shrub (Supplementary Figure S2), which accounts for the lesser impact of environmental factors on the composition of the soil N-fixing bacterial communities in temperate steppe soils (Figure 5). However, the higher rate of soil N conversion in the temperate steppe suggests a faster soil N cycling in the warm-temperate shrub, where nitrification and denitrification processes play a greater role compared with the temperate steppe. This may explain why environmental factors only affect the composition of soil ammonia-oxidizing archaea, ammonia-oxidizing bacteria, and denitrifying bacterial communities in warm-temperate shrubs. Supplementary Table S3 demonstrates that warm-temperate shrub soils exhibit higher stability, indicating their enhanced buffering capacity in protecting microbial cells against environmental fluctuations (Neumann et al., 2013). Furthermore, soil porosity serves as a protective mechanism for preserving the microbial community diversity amidst changing conditions (Xiao et al., 2021). Previous research has revealed an increased occurrence of isolated hydrofilms in soils with larger pores and that soil porosity was greater in sandy soils than in loamy soils (Günel et al., 2018), which may restrict the hydrofilm-mediated protection of the microbial community diversity in temperate grassland-like soils (Chau et al., 2011). This observation also elucidates why the influence of environmental factors on diversity of N-transforming microbial communities is comparatively weaker in warm-temperate shrub-like soils (Figure 5). In addition, the differential response of various N-transforming microorganisms to environmental factors may be attributed to variations in the diversity of soil microorganisms and their distinct reactions to soil minerals. Previous studies have demonstrated that microbial communities with a higher diversity exhibit greater resistance to external influences (Xun et al., 2021), while bacterial, archaeal, and fungal community diversities are differentially affected by mineral composition (Hemkemeyer et al., 2014). The addition of N significantly impacted soil pH, leading to a decrease in pH with higher amounts of N application. It has been reported that an increase in soil pH can create unfavorable conditions for AOA but favorable conditions for AOB (He et al., 2012; Harter et al., 2014). However, the changes in pH resulting from N addition did not significantly affect soil ammonia-oxidizing archaea or ammonia-oxidizing bacterial community diversity in this study, suggesting that alterations in pH due to N deposition do not exert negative effects on the soil microbial community diversity. The application of N in this study was estimated by Dannan Zheng based on

a 2010 study of N deposition in China (Zheng et al., 2014) and data from the National Ecological Data Center.³ The predicted approximate N deposition for 2020 was set as a medium amount of N, with a low amount of N slightly lower than the 2010N deposition and a high amount of N slightly higher than the 2030 predicted N deposition. The results of the study showed that none of the changes in environmental factors due to N addition significantly impacted the composition and diversity of the grassland soil N-transforming microbial community but significantly increased the community abundance and diversity ($p < 0.05$, Figure 5). This may imply that, although short-term N deposition would alter the structural composition and diversity of soil N-transforming microbial communities to some extent, it would not have significant negative effects over the next decade.

5 Conclusion

In this study, we simulated nitrogen deposition through nitrogen addition experiments, employed high-throughput sequencing technology to analyze the impact of nitrogen deposition on the composition, diversity, and structure of soil nitrogen-cycling microbial communities (nitrogen-fixing bacteria, ammonia-oxidizing archaea, ammonia-oxidizing bacteria, and anti-nitrifying bacteria) in different grassland types in northwestern Liaoning Province, and explored the underlying response mechanisms to environmental changes.

The results showed that N addition significantly affected the structural composition and diversity of soil N-transforming microbial communities in grasslands, albeit with varying affecting mechanisms. Moreover, the response of soil N-cycling microbial communities to N addition differed among various grassland types, with the influence of N source types being more pronounced than that of N application amount. The ammonium type of N source is considered to be the most influential driving factor affecting changes in the structure of the N-transforming microbial community, and the amount of low N applied is the primary determinant governing the structure of the soil N-fixing and nitrifying bacterial community. The findings indicate that short-term N deposition would lead to the dominance of the soil N fixation process in grasslands, thereby exerting a positive effect on the grassland ecosystem in northwest Liaoning. Under the background of increasing nitrogen deposition, investigating the characteristics of soil microbial community and its response mechanism to nitrogen addition in different grassland types in northwest Liaoning plays a crucial role in biodiversity conservation, ecosystem balance maintenance, as well as evaluating and predicting the response of diverse grassland types to global change. However, this study has certain limitations due to factors such as the complex environment in northwest Liaoning, differences between pot experiments and the field conditions, and the relatively short duration of the experiment. In future research, expanding the range of N application levels is recommended to determine the threshold values for positive and negative effects on grasslands. Additionally, further investigation into how different types of grasslands respond and adapt to N deposition can provide valuable insights into understanding patterns related to microbial transformations associated with nitrogen.

Data availability statement

The DNA sequences used in this study have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under the accession number PRJNA929669.

Author contributions

BR: Conceptualization, Funding acquisition, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. XM: Conceptualization, Data curation, Investigation, Writing – original draft. DL: Conceptualization, Investigation, Methodology, Writing – original draft. LB: Supervision, Validation, Writing – review & editing. JL: Resources, Supervision, Writing – review & editing. JY: Data curation, Methodology, Writing – original draft. MM: Methodology, Software, Writing – original draft. HL: Investigation, Methodology, Writing – original draft.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2024.1290248/full#supplementary-material>

³ <http://www.nesdc.org.cn/>

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Conservation tillage: a way to improve yield and soil properties and decrease global warming potential in spring wheat agroecosystems

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Climate change is one of the main challenges, and it poses a tough challenge to the agriculture industry globally. Additionally, greenhouse gas (GHG) emissions are the main contributor to climate change; however, croplands are a prominent source of GHG emissions. Yet this complex challenge can be mitigated through climate-smart agricultural practices. Conservation tillage is commonly known to preserve soil and mitigate environmental change by reducing GHG emissions. Nonetheless, there is still a paucity of information on the influences of conservation tillage on wheat yield, soil properties, and GHG flux, particularly in the semi-arid Dingxi belt. Hence, in order to fill this gap, different tillage systems, namely conventional tillage (CT) control, straw incorporation with conventional tillage (CTS), no-tillage (NT), and stubble return with no-tillage (NTS), were laid at Dingxi, Gansu province of China, under a randomized complete block design with three replications to examine their impacts on yield, soil properties, and GHG fluxes. Results depicted that different conservative tillage systems (CTS, NTS, and NT) significantly ($p < 0.05$) increased the plant height, number of spikes per plant, seed number per meter square, root yield, aboveground biomass yield, thousand-grain weight, grain yield, and dry matter yield compared with CT. Moreover, these conservation tillage systems notably improved the soil properties (soil gravimetric water content, water-filled pore space, water storage, porosity, aggregates, saturated hydraulic conductivity, organic carbon, light fraction organic carbon, carbon storage, microbial biomass carbon, total nitrogen, available nitrogen storage, microbial biomass nitrogen, total phosphorous, available phosphorous, total

potassium, available potassium, microbial counts, urease, alkaline phosphatase, invertase, cellulase, and catalase) while decreasing the soil temperature and bulk density over CT. However, CTS, NTS, and NT had non-significant effects on ECE, pH, and stoichiometric properties (C:N ratio, C:P ratio, and N:P ratio). Additionally, conservation-based tillage regimes NTS, NT, and CTS significantly ($p < 0.05$) reduced the emission and net global warming potential of greenhouse gases (carbon dioxide, methane, and nitrous oxide) by 23.44, 19.57, and 16.54%, respectively, and decreased the greenhouse gas intensity by 23.20, 29.96, and 18.72%, respectively, over CT. We conclude that NTS is the best approach to increasing yield, soil and water conservation, resilience, and mitigation of agroecosystem capacity.

KEYWORDS

climate-smart agriculture, carbon sequestration, greenhouse gases, global warming, nutrients, sustainable conservation tillage, yield

1 Introduction

Climate change is one of the main challenges, and it poses a tough challenge to the agriculture industry globally (Feng et al., 2023). Additionally, greenhouse gas (GHG) emissions are the main contributor to environmental change, and croplands are potential sources of major GHG emissions, for instance, carbon dioxide (CO₂), methane (CH₄), and nitrous oxide (N₂O), that lead to increased global warming (Pu et al., 2022; Wu et al., 2022; Li et al., 2023). The agricultural sector, particularly farmlands, mainly contributes almost 12% of GHG emissions in the atmosphere (Raihan and Tuspekova, 2022). The average worldwide temperature will rise by 3.8°C by the end of the 21st century if the GHG concentrations continue to rise to the existing level (IPCC, 2021). Consequently, for achieving carbon reduction or carbon neutrality goals, the decline of emissions of major GHG is very significant to alleviate greenhouse effects in the agroecosystem. In addition, croplands have very low soil organic matter (SOM) due to environmental limitations that limit primary crop productivity (Yuan et al., 2023a). One approach to increasing soil and water conservation and crop yields while reducing greenhouse gas emissions in crop cultivation systems is to adopt climate-smart farming practices known as conservation agriculture (Ma et al., 2021; Roy et al., 2022; Li et al., 2023).

Wheat is lately one of the vigorous cereal crops with universal importance, and it has a massive influence on global food security (Huang et al., 2003). Approximately 24 million hectares' area in China is under cultivation (Li et al., 2019). Climate change or environmental variability seriously affects crop production in the agriculture sector in China, especially in the Northwestern Loess Plateau (Alhassan et al., 2021). Furthermore, traditional or conventional tillage is the dominant crop cultivation practice in wheat cropping, generally plowing two times a year with the removal of crop straw in China, particularly in the Dingxi belt. Nevertheless, this CT practice can cause variations in soil properties (physical, chemical, biochemical, and biological) (Jat et al., 2020; Sadiq et al., 2021a; Yuan et al., 2023a), crop yield (Alhassan et al., 2021; Sadiq et al., 2021b), and greenhouse gas fluxes (Alhassan et al., 2021; Yuan et al., 2022; Feng et al., 2023) and enhance the risk of soil degradation by erosion (Liu et al., 2015; Gao et al., 2019) and global warming by greenhouse gas emissions

(Alhassan et al., 2021; Feng et al., 2023). To decrease land, crop, and environmental degradation under traditional tillage measures, substantial consideration has been paid lately to soil conservation tillage as a maintainable approach for cropland ecosystems (Wulanningtyas et al., 2021; Kristine et al., 2022; Yuan et al., 2022). Soil conservation tillage is an innovative mode of modern farming that might effectively mitigate and solve the negative influences and problems of intensive tillage (Lv et al., 2023).

Conservation tillage system broadly refers to techniques employing no or less soil inversion and a minimum number of tillage operations lacking of any soil inversion and leaving at least 30% stubbles on the surface of the soil, which enhances soil and water conservation. It could be roughly divided into straw incorporation into the field, no-tillage, mulch tillage, or straw-retention with no-tillage system, minimum tillage, ridge tillage, and strip tillage (Yuan et al., 2023a). It is a well-established approach to improving soil physicochemical, microbial, and crop yields, reducing greenhouse gas emissions, mitigating negative influences of conventional tillage and climate change through soil carbon sequestering in agricultural systems, and improving agricultural and environmental sustainability globally (Jat et al., 2021; Yuan et al., 2023b). The worldwide population is exerting a noteworthy burden on land resources due to intensive land cultivation strategies that destroy the soil and environmental quality. Consequently, soil quality must be sustained to certify crop yield and soil and environmental sustainability (Lal, 2005; Pu et al., 2022).

Sustainable crop production in the agricultural industry is highly dependent on the sustainability of the soil system and restricted by soil properties (physical, chemical, and biological) (Wang et al., 2008; Wozniak and Gos, 2014; Indoria et al., 2016). Land management strategies that would fulfill the food demand globally and preserve as well as conserve the previously stressed environmental conditions (Lal, 2005) are significant to sustainable crop production. Conservation tillage practices, for instance, straw-retention or residue mulch and no-tillage systems, are commonly advocated to preserve the soil (Wulanningtyas et al., 2021; Krauss et al., 2022). No-tillage with a crop stubble integration is a more operative approach for enhancing the properties of soil (Yuan et al., 2023a,b), crop yield (Sadiq et al., 2021b), and reduction of global warming (Alhassan et al.,

2021), as well as preserving soil health by increasing the quality of soil (Sadiq et al., 2021a). Nevertheless, the influence of conservation tillage practices on soil properties, crop yield, and global warming mitigation has been intensively discussed due to the extensive contradiction in distinct field research (Zheng et al., 2014). Previous studies have depicted that conservation tillage significantly reduced soil properties (Zhao et al., 2014; Khan et al., 2017), crop yield (Taa et al., 2004), and greenhouse gas emissions (Pu et al., 2022; Salamanca-Fresno et al., 2022; Li et al., 2023). On the contrary, conservation tillage pointedly increased soil properties (Jat et al., 2020; Sadiq et al., 2021a), crop yield (Sadiq et al., 2021b; Yuan et al., 2023a), and greenhouse gas emissions (Dencso et al., 2020). Researchers have also demonstrated insignificant change regarding soil properties (Bayer et al., 2015), yield (Lampurlanes et al., 2002), and greenhouse gas fluxes (Bayer et al., 2015) under conservation agriculture and conventional tillage practice. Consequently, more study on the influence of soil tillage on soil properties, crop yield, and global warming is needed.

The accumulation of GHG in the atmosphere alters the earth's energy balance and participates in the boosted "greenhouse effect." Additionally, GHG emissions have also been implicated in environmental chemistry given their contribution to the depletion of stratospheric ozone (IPCC, 2013). Global warming is chiefly attributed to the raised GHG concentrations in the atmosphere by anthropogenic activities (O'Neill et al., 2021; Wu et al., 2022). Soil management activities and climatic situations determine the croplands' capacity to yield, transport, and consume GHG and accordingly determine the direction and intensity of GHG fluxes in farmlands. Conservation tillage practices have been proposed as a substitute land management technique for CT that can mitigate the agricultural sector's environmental influence through a reduction of GHG emissions (Li et al., 2023). Higher CO₂ emission was stated by Yeboah et al. (2016) and Dencso et al. (2020) under conservation tillage, while Salamanca-Fresno et al. (2022) reported an almost 50% reduction in CO₂ flux in conservation tillage treatment compared with CT. In agroecosystems, CH₄ is also a significant greenhouse gas and acts as a sink or source (Maucieri et al., 2021). The CH₄ uptake improved under conservation tillage in comparison with CT (Alhassan et al., 2021; Pu et al., 2022); however, Maucieri et al. (2021) did not find any significant difference between conservation and conventional agricultural practices. Regarding N₂O flux, Yuan et al. (2022) found a reduction in its emission under conservative tillage systems, whereas Pu et al. (2022) reported the maximum emission of N₂O under conservation agriculture over CT. Moreover, soil physical, chemical, and biological quality indicators greatly influenced GHG fluxes (Alsakaf et al., 2021; Hu et al., 2022). A significant correlation between positive and negative GHG fluxes and soil properties was found by many scholars globally (Yeboah et al., 2016; Feng et al., 2018; Huang et al., 2018; Mei et al., 2018; Alhassan et al., 2021; Shakoor et al., 2021; Li et al., 2023). Greater inconsistency in emissions of major GHG from croplands under divergent land management practices has required further investigation under site-specific and soil conditions.

Agroecosystems' response to diverse soil tillage management systems strongly depends on the local environmental and socioeconomic conditions (Schwilch et al., 2015; Smart SOIL, 2015; Sanz et al., 2017). In this context, broadening the spectrum of scientific research is very essential to cover various environmental (i.e., soil type and climate) and socioeconomic (i.e., funding, market price variations, and type of crop) conditions. In this background, despite the fact that

there are numerous studies available worldwide, there is a lack of studies exploring the effectiveness of different conservation tillage practices toward the improvement of soil physical, chemical, and biological properties and crop yield while reducing greenhouse gas emissions and global warming. Too, few studies exist on some selected soil properties and one or two (carbon and nitrous oxide) major greenhouse gas emissions. Studies on greenhouse gas (carbon, methane, and nitrous oxide) emissions, wheat yield, and soil properties (physical, chemical, and biological) responses to different conservation tillage systems on the Loess Plateau, particularly at the Dingxi Belt, are scarce in spring wheat mono-cropping conditions and abundant.

This research tested the hypothesis that conservation tillage practices CTS, NT, and NTS in spring wheat agroecosystems provide better soil physical, chemical, and biological quality indicators and crop yield and yield-attributing traits and reduce the global warming potential of carbon dioxide, methane, and nitrous oxide compared with conventional tillage system. The overall objective of this research was the exploration of soil and environmental quality-based management practices and parameter identification that are sensitive to disturbance of soil. The specific objectives were (i) to evaluate the influence of conservation tillage systems: stubble incorporation with conventional tillage (CTS), no-tillage (NT) and straw-retention with no-tillage (NTS) in the improvement of numerous soil quality indicators, thereby increasing physical, chemical and biological properties; (ii) to assess the effect of the conservation tillage strategies on spring wheat yield and yield-attributing traits; and (iii) to quantify the impact of the conservation tillage techniques on greenhouse gas emissions, namely carbon dioxide, methane, and nitrous oxide emission and also the response of greenhouse gas fluxes to variations in environmental variables because of different tillage systems.

2 Materials and methods

2.1 Field site description and history of experiment region

The field experiment, installed in 2016, is situated on a Calcaric Cambisol at the Anjiapo catchment of Loess Plateau in Dingxi under the Department of Soil and Water Conservation Administration, Gansu Province, Northwestern China (35°34'53" N, 104°38'30" E), as shown in Figure 1. The study site relief is gently sloped, and the altitude is 2,000 m above sea level. The research county has semi-arid climatic conditions. The type of soil in the study region is a Huangmian sandy loam texture (sand: 60.5%, silt: 24.3%, and clay: 15.2%) according to WRB (IUSS Working Group, 2006), having low soil organic carbon with a slightly alkaline pH (Chinese Soil Taxonomy Cooperative Research Group, 1995).

The average temperatures in this area are −22°C and 35°C in the coolest and warmest months, respectively, and regular frosts in the winter (Xingchen et al., 2019). We have 50 years of continuous climatic data from 1971 to 2020 for this research. The 50-year average annual rainfall from 1971 to 2020 was 400 mm per year, with an irregular distribution. The 50-year average annual radiation is 5,930 MJ m^{−2} with 2,480 h of sunshine per year. This area has 140 days of frost-free period, an average annual evaporation of 1,531 mm, and an annual temperature of 6.9°C from 1971 to 2020. The monthly average of

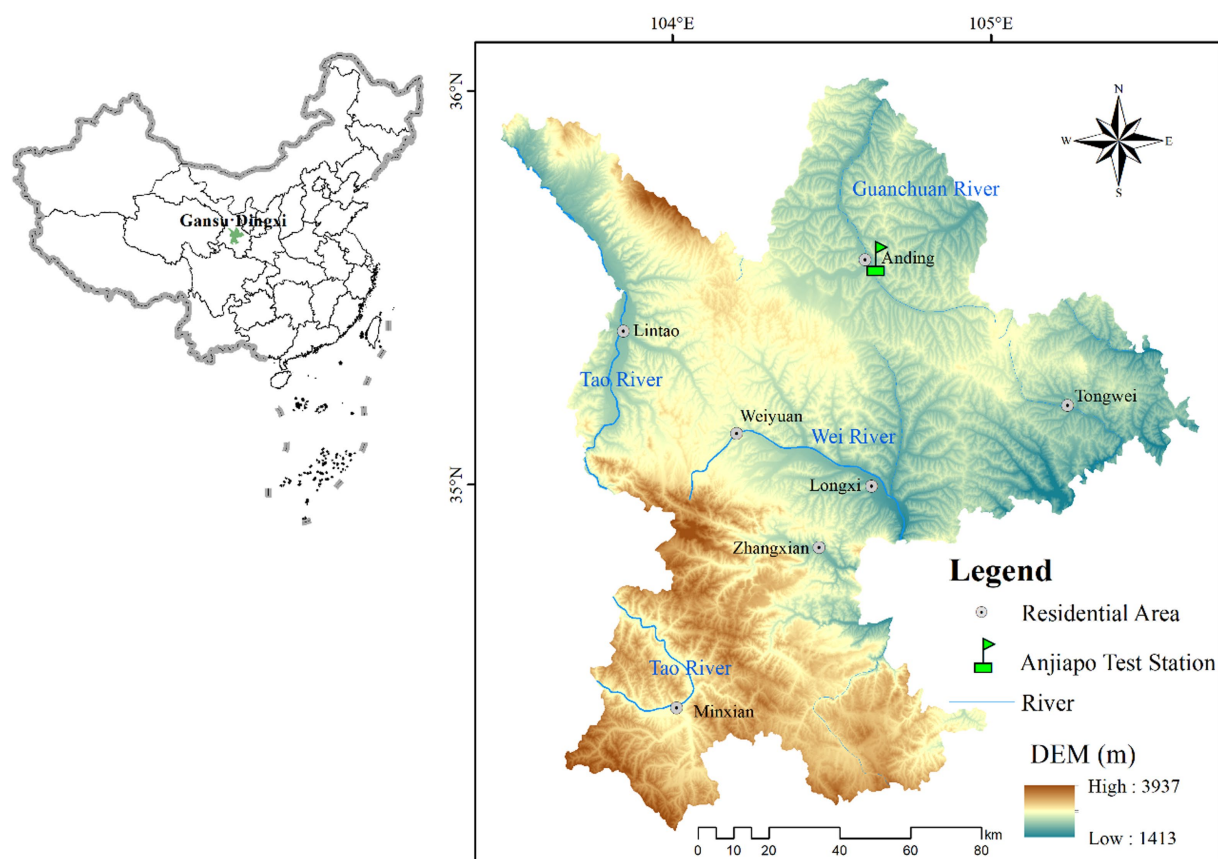


FIGURE 1

The geographical map of the experimental research site in Dingxi County, Gansu Province, China. ArcGIS 10.2 software was applied for production. The basic geographic information data come from the resource and environmental science and data center (<http://www.resdc.cn/>).

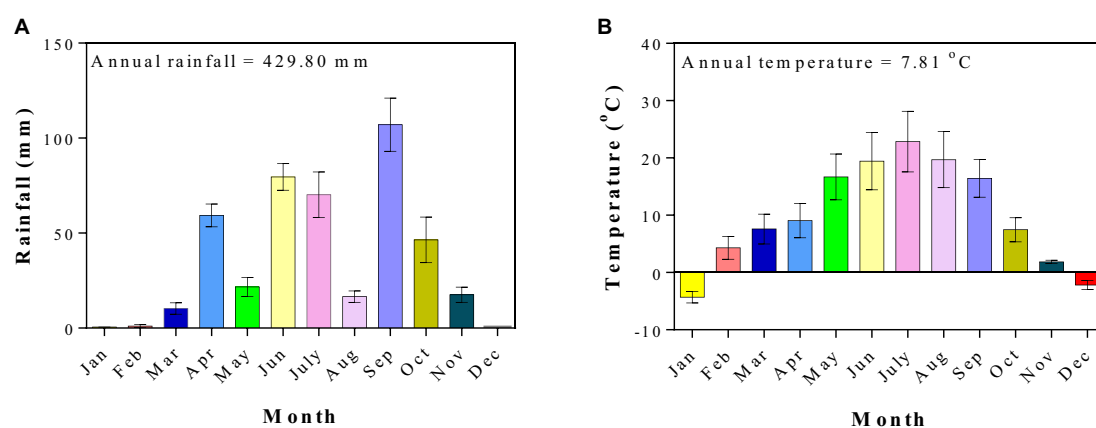


FIGURE 2

Climatic conditions of the study region in 2021. (A) The monthly average rainfall and (B) the monthly average temperature.

rainfall and temperature for the study period in 2021 is presented in Figure 2.

Before the experiment in 2016, the research field area was bare, which was cut and cleared for spring wheat cultivation. Then, spring wheat was introduced and cultivated at different sowing times (early,

normal, and late) under tillage systems. The tillage practices were the same for all spring wheat sowing dates. The Dingxi in Gansu, China, is a research hub and has an extensive wheat cultivation history, and wheat straws were frequently removed prior to the subsequent crop cycle. A comprehensive experiment field site description has been

provided in previous studies (Niu et al., 2016; Xu et al., 2020; Sadiq et al., 2021a).

2.2 Experimental design

This experimental study was conducted in 2021, from March to August. The research comprised four treatments, including one conventional tillage (CT) control and three different conservation tillage regimes, specifically stubble incorporation with conventional tillage (CTS), no-tillage system (NT), and straw-return with no-tillage (NTS). The conservation tillage regimes were compared with conventional tillage control. The experimental treatments were replicated three times in an 8 m × 3 m plot size under randomized complete block design (RCBD), giving a total of 12 individual plots with a total area of 24 m². These conservation tillage regimes have been practiced since 2016, with 5 years of non-stop spring wheat cultivation. At the beginning of the research, soil samples contained an average bulk density (BD) of 1.41 ± 0.02 g cm⁻³, soil porosity (P) 47.05 ± 3.31%, total nitrogen (TN) 0.59 ± 0.02 g kg⁻¹, total phosphorous (TP) 0.43 ± 0.03 g kg⁻¹, total potassium (TK) 18.48 ± 0.01 g kg⁻¹, soil organic carbon (SOC) 5.85 ± 0.34 g kg⁻¹, and pH 8.34 ± 0.05 in 0–15 cm depth (Table 1).

2.3 Tillage systems and crop management practices

The soil tillage management practices compared in this trial were conventional tillage (CT), stubble incorporation with conventionally tilled soil (CTS), no-tillage (NT), and no-till with straw-retention (NTS). Descriptions of these tillage treatments are presented in Table 2. For the CT system, land cultivation was performed three times with moldboard plow in a year at 20 cm, 10 cm, and 5 cm, respectively, and harrowed two times, followed by planting in the absence of stubble. In order to manage CTS, after harvesting the wheat crop, the fields were plowed with moldboard plow, harrowed, and

followed by planting exactly as for the CT practice (three plow passes and harrows two times), but stubble incorporation was done at the time of first plowing. After threshing, all the wheat-straw from the earlier spring wheat crop was returned to the original plot instantly and then incorporated into the field. The spring wheat was planted exactly as in the CT system. In the NT-treated plots, after harvesting the spring wheat, all stubbles were removed and crop planting was performed with a no-tillage crop planter; nevertheless, in the NTS practice, after harvesting the wheat, all stubble was returned to the field and crop sowing was done with a no-tillage crop planter. The wheat-stubble chemical composition is shown in Table 3. The wheat-straw nitrogen, phosphorous, potassium, and carbon were 0.79 ± 0.2%, 0.08 ± 0.01%, 0.49 ± 0.04%, and 39.24 ± 2.8%, respectively. Nitrogen at the rate of (146 kg ha⁻¹) and phosphorous at the rate of (63 kg ha⁻¹) were applied as basal doses in all treatments, counting control in the form of diammonium phosphate and urea. Semi-arid Dingxi in the Loess Plateau zone of China has a suitable concentration of soil potassium (Li et al., 2014), which was satisfactory for encouraging wheat growth and germination; therefore, potassium was not applied in basal fertilization. The spring wheat (cultivar Dingxi 42) crop was sown by hand in mid-March (seed rate was 187.5 kg seeds ha⁻¹) and allowed to grow until late-August (Table 3). The wheat crop was sown using 20 cm row-to-row spacing and a plant density of 400 plant m⁻². In order to control the weeds, the red sun herbicide with glyphosate (30%) was used according to the manufacturer's instructions, and when weeding was required during the growing season, it was done manually.

2.4 Agronomic traits

The agronomic attributes of plant height, spike number per plant, seed m², root yield, aboveground biomass yield, grain yield, 1,000-grain weights, dry matter yield, and harvest index were determined. The wheat plant height was measured using the procedure described by Dokuyucu et al. (2002) and Demuner-Molina et al. (2014).

2.5 Soil measurements

At the spring wheat harvest stage in 2021, five disturbed and undisturbed soil samples were collected with an auger having a diameter of 4 cm from different experimental treatments (CT, CTS, NT, and NTS), including three replications for the determination of soil physicochemical, biochemical, and biological properties. All soil quality indicators were observed at a soil layer of 0–10 cm depth.

The oven-dry method was used for the determination of gravimetric soil water content (SWC) (O'Kelly, 2004), water-filled pore space (WFPS) in percent, and soil water storage (SWS), which was calculated as described by O'Kelly (2004). Soil temperature (ST) was measured using a geothermometer (Lu, 1999). The procedure described by Campbell (1994) was used for soil bulk density (BD) determination with the core sampler method. and soil pore space (P) percent was calculated in accordance with the procedure described by Campbell (1994). The wet-sieved method was used for measurements of soil aggregates (Yang et al., 2018). Auger-hole method using the Guelph Permeameter was used for the determination of saturated soil hydraulic conductivity (Ks) (Reynolds et al., 2008).

TABLE 1 The basic characteristics of 0–15 cm soil depth at the Dingxi research field in 2021.

Soil parameter	Values	Measurement method	References
BD (g cm ⁻³)	1.41 ± 0.02	Core sampler method	Lu (1999)
P (%)	47.05 ± 3.31	(1 - (BD/PD)) × 100 equation	Lu (1999)
TN (g kg ⁻¹)	0.59 ± 0.02	Semimicro-Kjeldahl method	Lu (1999)
TP (g kg ⁻¹)	0.43 ± 0.03	Colorimetric method	Lu (1999)
TK (g kg ⁻¹)	18.48 ± 0.01	Colorimetric method	Lu (1999)
SOC (g kg ⁻¹)	5.85 ± 0.34	Walkley-Black dichromate oxidation	Nelson and Sommers (1983)
pH	8.34 ± 0.05	pH meter	Lu (1999)
Soil textural class	Sandy-loam	Hydrometer method	Bouyoucos (1972)

The abbreviated words stand for BD, bulk density; P, soil porosity; TN, total nitrogen; TP, total phosphorous; TK, total potassium; SOC, soil organic carbon; pH, soil pH; PD, particle density = (2.65 g cm⁻³). Based on USDA soil textural classification.

TABLE 2 Treatment details of tillage regimes and stubble application for spring wheat cultivation tested during the course of this study.

Treatments	Short forms	Description
Conventional tillage + straw harvest	CT	After harvesting the wheat crop, the fields were plowed three times with moldboard plow and harrowed two times followed by planting. The first plowing was done in late-August immediately after harvesting the spring wheat crop, in late-August and late-September, the second and third plowing, respectively, were done. The depths of plow were 20 cm, 10 cm, and 5 cm, respectively. The field harrowing was done before the ground was frozen. This is the typical conventional tillage technique in the Dingxi zone of China. The spring wheat crop was planted with a small seeder drawn by a 13.4 kW (18 HP) tractor and designed by China Agricultural University, letting fertilizers be positioned under the seed rows, followed by concave rubber press wheels in one operation.
Conventional tillage + stubble incorporation	CTS	After harvesting the spring wheat crop, the fields were plowed with moldboard plow, harrowed, and followed by planting exactly as for the conventional tillage practice (3 plow passes and harrows two times) described above, but with stubble incorporated at the time of first plowing. After threshing, all the wheat stubble from the preceding spring wheat crop was returned to the original plot immediately and then incorporated into the field. The spring wheat crop was planted exactly as for the conventional tillage practice.
No-tillage + straw harvest	NT	No-tillage all over the life of the experimental research. The crop stubble was removed from the field and used as feed or fuel. Direct planting with no-till crop planter into 20 cm depth without using any tillage implement.
No-tillage + stubble return	NTS	No-tillage during the experiment life. The field was shielded with the earlier spring wheat crop straw from late-August till the following mid-March. After threshing, all the crop stubble from the previous wheat crop was returned to the original plot immediately. Direct sowing with no-till crop planter into 20 cm deep in the absence of any prior tillage, understanding earlier wheat crop stocks.

CT, conventional tillage is the control for a given study period.

TABLE 3 Wheat-straw properties and crop management practices during the study.

Wheat crop stubble properties						
Nitrogen		Phosphorus		Potassium		Carbon
(%)						
0.79 ± 0.2		0.08 ± 0.01		0.49 ± 0.04		39.24 ± 2.8
Crop management practices during research						
Crop	Crop variety	Plot size	Seed rate (kg ha ⁻¹)	Plant density (plant m ⁻²)	Fertilizer	Weed control (L ha ⁻¹)
Spring wheat	Dingxi 42	24 m ²	187.5	400	Diammonium phosphate (146 kg ha ⁻¹), urea (63 kg ha ⁻¹)	Herbicide (Red sun) with 30% glyphosate

Spring wheat crop was sown in mid-March and harvested in late-August during the study.

The soil organic carbon (SOC) was determined by the standard method of Walkley-Black dichromate oxidation (Nelson and Sommers, 1983), while the density fractionation approach was used for the isolation of light fraction organic carbon (Gregorich and Ellert, 1993), and C and N analyzer (Elementar Vario MACRO cube) was used for its determination. The calculation of soil organic carbon storage (t hm⁻²) was done using the procedure described by Wu et al. (2021). The estimation of microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) was done by the fumigation-extraction method (Vance et al., 1987).

Soil total nitrogen (TN), total phosphorous (TP), total potassium (TK), available nitrogen (AN), available phosphorous (AP), available potassium (AK), electrical conductivity (ECe), and pH were determined by using standard procedures (Lu, 1999). Nitrogen storage was calculated, followed by the equation of Wu et al. (2021). Soil microbial propagules (colony forming units, CFUs) were determined using the enumeration of luminescent colonies on agar media (Li et al., 1996). Soil urease activity was estimated following the procedures by Dick and Burns (2011), soil alkaline phosphates were estimated as described by Zhao and Jiang (1986), soil invertase activity was determined by the method of Frankeberger and Johanson (1983),

soil cellulase activity was estimated following the method of Guan (1986), and soil catalase activity was determined as described by Yan (1988).

2.6 Gas sampling and flux measurement

The procedure of greenhouse gas sampling, such as CO₂, CH₄, and N₂O, was conducted during different crop growth stages of spring wheat in 2021. On the basis of the static dark chamber and gas chromatography method described by Yuesi and Yinghong (2003), gas sampling and flux measurements were done. A stainless-steel base with a collar (50 × 50 × 10 cm) was installed to support sampling chamber placement (50 × 50 × 50 cm) for greenhouse gas sampling in each plot (a total of 12 plots). The samples of air were drawn from the chambers simultaneously, including three replications of each treatment. At five different times (0, 9, 18, 27, and 36 min), respectively, by using 150 mL gas-tight polypropylene syringes, the air samples were drawn and released into 100 mL aluminum foil sampling bags (Shanghai Sunrise Instrument Co. Ltd., Shanghai). Then the samples of gas were analyzed in the laboratory

with a GC system (Echrom GC A90, China) equipped with a flame ionization detector for methane and carbon dioxide and electron capture detector for nitrous oxide analysis. At 250°C temperature and 35 cm³ min⁻¹ H₂ flow rate, the flame ionization detector operates. Methane, carbon dioxide, and nitrous oxide peak areas were analyzed in the Echrom-ChemLab software. Calibrations were done with standard gas obtained from Shanghai Jiliang Standard Reference Gases Co., Ltd., China, before the sample gas analyses. Standard gas concentrations were 2.00 ppmv, 456.00 ppmv, and 0.355 ppmv for methane, carbon dioxide, and nitrous oxide, respectively. In order to obtain the concentration change over sampling time, the concentrations of the sample gas obtained for the five sampling times were plotted against time. Emissions of carbon dioxide in terms of ecosystem respiration, methane, and nitrous oxide fluxes were calculated as described by Wei et al. (2014), followed by equation (1):

$$F = \frac{dC}{dt} \cdot \frac{M}{V_0} \cdot \frac{P}{P_0} \cdot \frac{T}{T_0} \cdot H \quad (1)$$

where dC/dt is the rate of gas concentration change; M is the molar mass of nitrogen or carbon (28 for N₂O and 12 for CO₂ and CH₄); V_0 is standard molar air volume (22.41 mol⁻¹); P is the sampling site air pressure; P_0 is the standard air pressure, T is the chamber air temperature at the sampling time, T_0 is the standard air temperature; and H is the height of the chamber. The cumulative flux of methane, carbon dioxide, and nitrous oxide in kg ha⁻¹ was estimated using the following equation (2):

$$M = \sum \frac{(F_{i+1} + F_i) \times (t_{i+1} - t_i) \times 24}{2 \times 100} \quad (2)$$

where M is the gas cumulative emission during the whole growth period of spring wheat (kg ha⁻¹); F is the gas emission flux (mg/m²h⁻¹); i is the sampling number; t is the sampling time (d).

In order to estimate global warming potential (GWP) of greenhouse gases, the cumulative flux of CO₂ in μmol m⁻²s⁻¹ was converted to mg CO₂-C and typically taken as the reference gas; therefore, methane and nitrous oxide emissions are converted into “CO₂ equivalents” (CO₂-e). The methane GWP is 34 (based on a 100-year time horizon), the carbon dioxide GWP is 1, and the nitrous oxide GWP is 298 (IPCC, 2013). Net global warming potential (GWP) in kg CO₂-eq ha⁻¹ was determined by using equation (3):

$$\text{Net GWP} = \text{CH}_4 \text{ flux} \times 34 + \text{N}_2\text{O flux} \times 298 + \text{Net CO}_2 \text{ flux} \quad (3)$$

Greenhouse gas intensity (GHGI) was determined by using equation (4):

$$\text{GHGI} = \frac{\text{GWP}}{\text{Grain yield}} \quad (4)$$

The sign convention adopted is that positive (+) means emission, whereas negative (−) means absorption.

2.7 Statistical analysis

The experimental data were processed using Excel 2016 (Microsoft Corp., United States). The procedure used to analyze the data obtained from the experiment was one-way factor interaction analysis of variance (ANOVA), suitable for randomized complete block design (RCBD). The linear model procedure of the appropriate computer software program Statistical Package for Social Science (SPSS) window version 25.0 (IBM Corp., Chicago, IL, USA) was used for all statistical analysis. The significant differences among different treatments were separated by Duncan's test at 5% significance level ($p < 0.05$). Data obtained from treatments are displayed as the mean of three replications with standard deviation and computer software Origin 2021 was used for drawing figures. Additionally, in order to explore the multivariate variability introduced by the various tillage practices for soil properties, crop yield, and greenhouse gases, the principle component analysis (PCA) was performed. The Pearson heatmap correlation analysis was used to describe the relationship among the factors.

3 Results and discussion

3.1 Effect of conservation tillage on wheat agronomic traits

Conservation tillage management had an influence on spring wheat agronomic features, including growth, yield, and yield-attributing traits. The NTS practice generated maximum 95.26 ± 3.08 cm plant height, 44.66 ± 5.12 number of spikes per plant, seeds number per meter square 9321.97 ± 623, root yield (RY) 532.88 ± 24 kg ha⁻¹, aboveground biomass yield (ABY) of 5225.31 ± 327 kg ha⁻¹, thousand-grain weight (TSW) 50.95 ± 2.02 g, grain productivity (GY) of 2543.88 ± 275 kg ha⁻¹ and 2681.43 ± 257 kg ha⁻¹ dry matter yield (DMY) while lowest values of all investigated agronomic characters were associated with CT technique. The agronomic parameters of wheat followed the trend of CT > NT > CTS > NTS, except for plant height and spike number per plant, where no-till depicted better performance over CTS. However, CT produced pointedly highest 48.76 ± 1.33% harvest index (HI) compared with other tested conservative tillage treatments (Table 4). This research verified our hypothesis that a conservation tillage technique can improve productivity; it would be a sustainable and reliable agronomical practice in dry regions.

Soil inversion elimination and crop stubble implementation are the most significant practices in the agriculture production system. The data reveal that different conservation tillage systems are suitable for producing spring wheat in the semi-arid Dingxi belt. The significant maximum agronomic trait values of conservative tillage systems indicate the competent use of stubbles (Zhao et al., 2019; Jat et al., 2021; Li et al., 2023), water, and nutrients. The better wheat agronomic performance was due to straw-retention because crop stubble has the potential to retain essential soil nutrients, which led to increased crop agronomic parameters (Han et al., 2020). This is consistent with previous results (Sadiq et al., 2021b), in which they recorded increased crop yield under conservation agricultural practices and associated it with residue retention. The higher wheat yield and yield-attributing traits under NTS, CTS, and NT systems than CT can further be ascribed to increased soil organic matter,

TABLE 4 Wheat agronomic traits as influenced by conservation tillage practices in 2021.

Treatment	Spring wheat agronomic attributes									
	Plant height (cm)	Number of spikes plant ⁻¹	Seed (n m ⁻²)	Root yield (kg ha ⁻¹)	Aboveground biomass yield (kg ha ⁻¹)	Thousand seed weight (g)	Grain yield (kg ha ⁻¹)	Dry matter yield (kg ha ⁻¹)	Harvest index (%)	
CT	81.16±2.05b	31.66±4.04b	7463.14±498b	476.62±13b	3412.74±130b	44.52±1.05b	2200.10±123b	1193.64±211b	65.23±2.36a	
CTS	90.02±3.15ab	34.33±5.04ab	9175.63±545a	520.45±22a	4223.95±363ab	49.58±2.34a	2277.62±232ab	1946.33±230ab	54.25±2.12ab	
NT	92.23±2.52ab	36.66±3.74ab	8902.46±474ab	485.76±16ab	3786.04±284ab	45.95±1.65ab	2219.92±214ab	1578.11±248ab	58.31±1.45ab	
NTS	95.26±3.08a	44.66±5.12a	9321.97±623a	532.88±24a	5225.31±327a	50.95±2.02a	2543.88±275a	2681.43±257a	48.76±1.33b	
<i>p</i> -value	0.024	0.041	0.027	0.033	0.012	0.048	0.039	0.016		0.046

Mean values with the same letter in a column are not significantly different between each treatment at $p < 0.05$ (Duncan's test was performed for mean separation). Values are means \pm SE ($n = 3$). CT: conventional till; CTS: conventional till with stubble incorporation; NT: no-till; NTS: no-till with stubble return. *p*-value in a column indicates the level of probability among treatments.

which improves soil quality and leads to increased crop agronomic parameters (Sadiq et al., 2021a). Maximum crop production under straw-return and residue incorporation and the NT system over CT might also be due to better hydrothermal soil characteristics (Huang et al., 2012; Wang et al., 2017). Our current study results are in agreement with the findings of Alhassan et al. (2021), as they verified that stubble return and residue incorporation and no-till systems enhanced wheat yield compared with CT in a dry region farming system.

3.2 Soil properties as influenced by conservation tillage management practices

Conservation tillage systems have noteworthy impact on soil properties, for instance, soil water content (SWC), water-filled pore space (WFPS), soil water storage (SWS), soil temperature (ST), soil bulk density (BD), soil porosity (P), aggregates, soil saturated hydraulic conductivity (Ks), soil organic carbon (SOC), light fraction organic carbon (LFOC), carbon storage (CS), microbial biomass carbon (MBC), total nitrogen (TN), available nitrogen (AN), nitrogen storage (NS), microbial biomass nitrogen (MBN), total phosphorous (TP), available phosphorous (AP), total potassium (TK), available potassium (AK), microbial counts (MC), urease, alkaline phosphatase, invertase, cellulase, and catalase at a soil depth of 0–10 cm (Figure 3 and Table 5). The concentration of SWC ($p < 0.05$) was maximum ($10.35 \pm 0.64\%$) in the NTS treatment variant with two other CTS and NT conservative tillage treatments, while CT had a minimum ($8.70 \pm 0.40\%$) SWC value. The conservation tillage practices NTS, CTS, and NT increased SWC by 9.35, 9.13, and 9.05%, respectively, over the CT system. The WFPS was highest ($20.80 \pm 0.81\%$) under the NT system, which was followed by NTS and CTS techniques, while the lowest WFPS was associated with CT practice. Additionally, the same trend was observed in the case of SWS, with the highest value in NT and the lowest in CT. The rise in SWC, WFPS, and SWS under CTS and NTS measures may be owing to crop straw-retention, thus decreasing evaporation losses, improving water availability and infiltration rate, and conserving soil water (Yadav et al., 2018; Zhang et al., 2018). Additionally, crop stubble addition to soil increases SOM and thus improves soil water retention capacity. The increase in SWC, WFPS, and SWS under NT practice over the CT system might be due to the earth flip inherent to CT practice, resulting in a big water loss due to evaporation. This water loss is avoided in NT, which lacks the requirement for stubble addition (Liebhard et al., 2022). Parallel findings were found by Yuan et al. (2023a).

The CT had the highest ST, whereas the NTS had the minimum ST values. The temperature moderation influence was observed in conservative tillage treatments as decreased ST by NTS, NT, and CTS systems (Figure 3). This is due to the accumulation of crop straws. The surface of the soil is abstemiously shielded by stubble remnants from prior crops in conservative tillage systems, encouraging the soil to absorb minimum solar radiation (Wang et al., 2009). Furthermore, the CT practice makes the soil more porous, and therefore, the soil under CT possibly has minor thermal conductivity (Sarkar and Singh, 2007). In addition, conservative tillage treatments increased the infiltration rate, which helped water movement toward the bottom (Liebhard et al., 2022) and reduced ST (Yuan et al., 2023a). Similar observations were noted by Sadiq et al. (2021a).

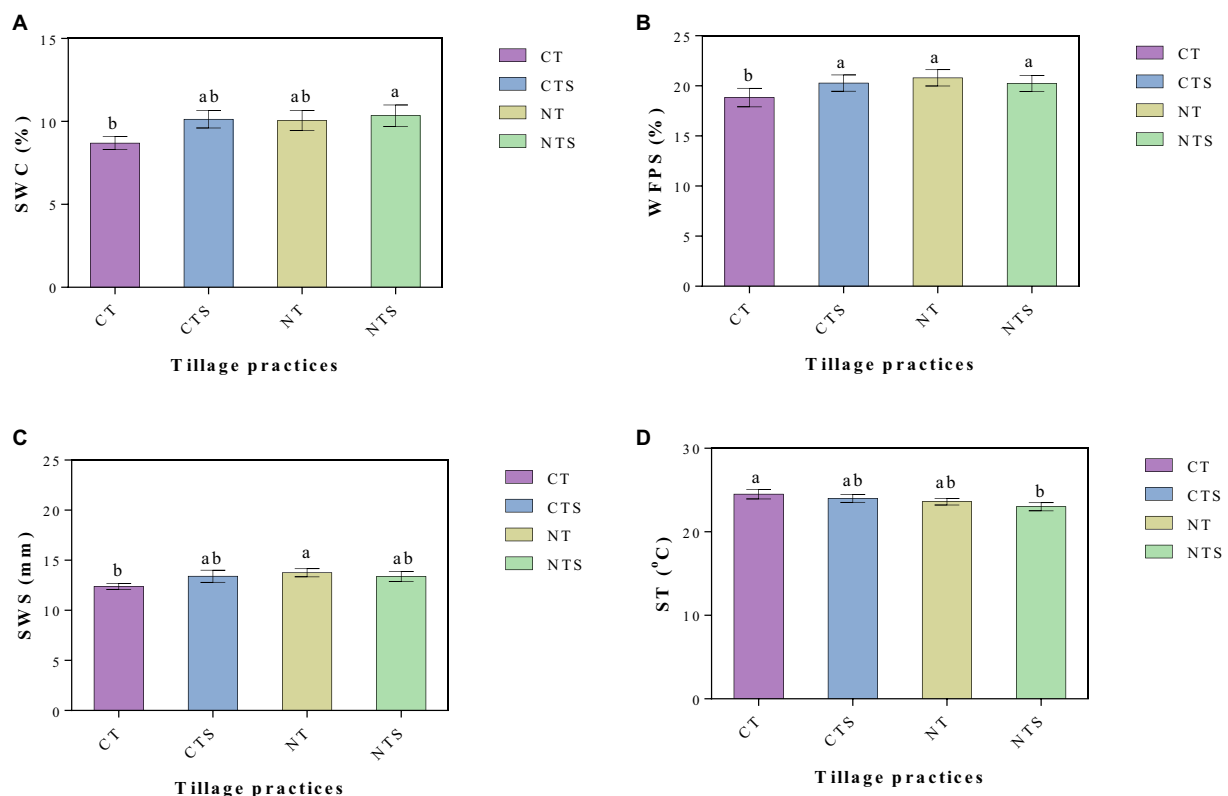


FIGURE 3

Properties of soil under conservative tillage systems in 2021 at 0–10 cm soil depth. (A) The soil gravimetric water content influenced by conservation tillage technique; (B) the water-filled pore spaces under conservation tillage; (C) the soil water storage affected by tillage practices; and (D) the soil temperature affected by tillage measures. Vertical error bars denote the corresponding standard error of mean values; $n = 3$. Significant differences were determined by a Duncan's test.

On average, conservation tillage systems NTS, CTS, and NT reduced BD by 29, 33, and 37%, respectively, over CT. The maximum soil BD under CT was connected to the soil disturbance by tillage implementation, which resulted in soil compactness (Gathala et al., 2011). In addition, due to traffic physical compaction by direct heavy machinery (Jat et al., 2018). In addition, as BD values are connected with soil porosity values, these conservative tillage systems improved the p -values, with the highest under NTS and the lowest recorded in CT. The NTS, CTS, and NT increased the soil porosity because of the organic matter accumulation under conservation tillage, which led to decreased BD and improved p -values (Ordoñez-Morales et al., 2019). Similar results were noted by Khorami et al. (2018), who recorded that conservation tillage decreased the BD and increased pore space over CT.

Almost similar to SWC, NTS attained the highest soil aggregates, followed by CTS, which was statistically on par with NT, while CT linked with the lowest soil aggregates. The CT strongly disturbs the soil due to plowing, which can diminish the aggregate degree and aggregate stability (Yeboah et al., 2018). Higher aggregates in conservation tillage might be due to residue retention (Niu et al., 2016). Similar results are reported in accordance with (Yuan et al., 2023a). Additionally, conservation tillage practices increased the Ks over CT; the highest value of Ks was reported in NTS, which was statistically at par with NT, followed by CTS, while the lowest Ks value was associated with CT. The minimum soil Ks in CT and CTS might be ascribed to the destruction of soil aggregation and reduction of

macro-porosity (Singh et al., 2002). In arid and semi-arid cropping systems, the soil property variations under conservation tillage influences are habitually slow to arise, attributable to the limited production of plant biomass (Mikha et al., 2006).

The SOC, LFOC, CS, and MBC differed significantly ($p < 0.05$) in all tested tillage systems, whereas the non-significant ($p < 0.05$) differences were noted among CTS and NT for SOC and LFOC as well as NT and NTS had also non-significant differences for carbon storage, and in case of MBC, the NTS and CTS are statistically at par (Table 5). The NTS system had the highest values of SOC, LFOC, and carbon storage except for MBC, where CTS was related to higher values, while the CT system had the lowest values. The SOC, LFOC, carbon storage, and MBC contents were low in the CT system because of extensive tillage operations, breakdown of aggregates, and exposure of the soil carbon contents. More values of carbon-associated parameters in CTS, NTS, and NT systems soil were attributable to residue retention and no soil physical disturbance (Chen et al., 2009). In conservative tillage systems, the SOC solid stratification, which was significantly attributable to surface residue retention, is identical to that in other studies (Blanco-Canqui and Lal, 2008; Yuan et al., 2023b). Soil carbon storage is the retention of carbon in the ecosystem and a chief index to measure the gauge and quantity of ecosystem primary productivity. A study by Yeboah et al. (2018) revealed that NTS can significantly increase the soil organic carbon content so as to progress soil carbon storage. This study also found that compared with CT, conservation tillage systems were more helpful in improving soil

TABLE 5 Changes in soil properties under sustainable conservation tillage in 2021 at 0–10 cm soil depth.

Treatment	BD (g cm ⁻³)	P	Aggregates (%)	Ks (mm h ⁻¹)	SOC (g kg ⁻¹)	LFOC (g kg ⁻¹)	Carbon storage (t hm ⁻²)	MBC (mg kg ⁻¹ soil)	TN (g kg ⁻¹)
CT	1.42 ± 0.03a	46.28 ± 1.34b	13.41 ± 1.22b	0.92 ± 0.06b	9.86 ± 0.3b	0.86 ± 0.04b	12.57 ± 0.51b	153.33 ± 14.50b	0.61 ± 0.02b
CTS	1.33 ± 0.05ab	50.06 ± 1.63a	18.13 ± 1.02ab	1.36 ± 0.32ab	11.22 ± 0.7ab	1.38 ± 0.08ab	14.81 ± 0.90ab	247.00 ± 15.56a	0.74 ± 0.03a
NT	1.37 ± 0.05ab	48.17 ± 0.84ab	16.59 ± 1.50ab	1.84 ± 0.10a	11.08 ± 0.6ab	1.08 ± 0.07ab	15.23 ± 1.16a	228.00 ± 12.28ab	0.69 ± 0.05ab
NTS	1.29 ± 0.04b	51.19 ± 1.27a	20.18 ± 2.44a	1.95 ± 0.05a	11.87 ± 0.5a	1.49 ± 0.09a	15.36 ± 0.63a	243.64 ± 7.23a	0.68 ± 0.06ab
<i>p</i> -value	0.025	0.037	0.022	0.035	0.034	0.024	0.041	0.012	0.021
	AN (mgkg ⁻¹)	Nitrogen storage (t hm ⁻²)	MBN (mgkg ⁻¹ soil)	TP (gkg ⁻¹)	AP (mgkg ⁻¹)	TK (gkg ⁻¹)	AK (mgkg ⁻¹)	ECe (dSm ⁻¹)	pH
CT	40.24 ± 1.64b	0.78 ± 0.05b	37 ± 3.5b	0.40 ± 0.03b	13.74 ± 1.63b	17.67 ± 0.01b	221.33 ± 6.02b	0.37 ± 0.01a	8.40 ± 0.02a
CTS	47.40 ± 2.18a	0.92 ± 0.07a	52 ± 6.60a	0.50 ± 0.06a	18.64 ± 2.54ab	18.84 ± 0.02a	272.91 ± 8.12ab	0.41 ± 0.02a	8.37 ± 0.05a
NT	43.21 ± 2.13ab	0.94 ± 0.02a	43 ± 7.21ab	0.47 ± 0.08ab	17.41 ± 1.92ab	18.72 ± 0.03ab	253.84 ± 7.48ab	0.39 ± 0.02a	8.38 ± 0.053a
NTS	46.47 ± 2.26a	0.86 ± 0.04ab	53 ± 6.29a	0.44 ± 0.07ab	19.42 ± 2.39a	18.90 ± 0.02a	292.83 ± 9.84a	0.40 ± 0.03a	8.36 ± 0.06a
<i>p</i> -value	0.030	0.027	0.019	0.045	0.016	0.048	0.018	0.054	0.058
	C:N ratio	C:P ratio	N:P ratio	Microbial counts (10 ⁴ CFUg ⁻¹ soil)	Urease (mg [NH ₃ -N] g ⁻¹ soil d ⁻¹)	Alkaline phosphatase (mg [phenol] g ⁻¹ soil d ⁻¹)	Invertase (mg [glucose] g ⁻¹ soil d ⁻¹)	Cellulase (mg [glucose] g ⁻¹ soil d ⁻¹)	Catalase (ml [0.1molL ⁻¹ KMnO ₄] g ⁻¹ soil h ⁻¹)
CT	16.46 ± 0.77a	26.47 ± 2.10a	1.48 ± 0.19a	111.66 ± 9.52cb	2.47 ± 0.04b	0.75 ± 0.03b	17.56 ± 1.5b	5.53 ± 1.04b	5.09 ± 0.03b
CTS	16.06 ± 1.81a	21.03 ± 2.12a	1.40 ± 0.15a	131.33 ± 17.12ab	2.58 ± 0.09ab	0.85 ± 0.06ab	19.33 ± 2.2ab	7.41 ± 1.72ab	5.20 ± 0.07ab
NT	16.19 ± 1.74a	21.14 ± 4.16a	1.49 ± 0.23a	136.33 ± 12.25ab	2.59 ± 0.08ab	0.90 ± 0.05ab	20.52 ± 1.7ab	7.91 ± 1.24ab	5.22 ± 0.04ab
NTS	17.75 ± 1.02a	19.78 ± 2.63a	1.50 ± 0.10a	146.66 ± 15.41a	2.64 ± 0.07a	0.97 ± 0.04a	23.8 ± 2.4a	9.11 ± 0.98a	5.36 ± 0.06a
<i>p</i> -value	0.062	0.053	0.066	0.022	0.044	0.033	0.039	0.043	0.046

Mean values with the same letter in a column are not significantly different between each treatment at $p < 0.05$ (Duncan's test was performed for mean separation). Values are means ± SE ($n = 3$). CT: conventional till; CTS: conventional till with stubble incorporation; NT: no-till; NTS: no-till with stubble return. *p*-value in a column indicates the level of probability among treatments.

carbon storage. The higher values of MBC appear because of high microbial biomass and soil organic matter contents. Organic stubbles are used as carbon source inputs for the microorganisms' activities, activation with the support of microbial biomass, conservation tillage systems enrich the soil carbon contents (Liu et al., 2017). The role of straw-retention and residue incorporation in increasing carbon elements was also stated by numerous scholars (Zhao et al., 2019; Sadiq et al., 2021a; Yuan et al., 2023b). The conservation tillage system is a straw addition or plowless tillage with a minimum number of tillage operations, and its beneficial impact on soil functions and quality has been extensively identified (Han et al., 2020; Wulanningtyas et al., 2021; Sadiq et al., 2021b).

Conservation tillage practices (NTS, CTS, and NT) significantly improved total and available soil nutrients, namely TN, AN, TP, AP, TK, and AK, compared to CT. In addition, NC and MBN were notably increased under these investigated conservative tillage systems over CT. Higher TN, AN nitrogen storage, and TP were related to CTS but the NTS system had dominant MBN, AP, TK, and AK values, while CT had the lowest values. Beneficial influence of NTS, CTS, and NT on nutrient accumulation and storage might be due to multiple reasons:

- (i) Higher total and available soil nutrients, for instance, nitrogen, phosphorous, and potassium in CTS and NTS, might be because of straw-retention because straw has the potential to add essential nutrients to the soil system (Zhao et al., 2019).
- (ii) Maximum nitrogen accumulation under straw-treated treatments may be attributed to straw microbial biomass and nitrogen immobilization (Huang et al., 2012).
- (iii) Stubble retention and incorporation can decrease leaching and volatilization losses of nitrogen by diminishing the soil temperature and lead to increased nitrogen accumulation (Sadiq et al., 2021a).
- (iv) Highest nitrogen accumulation under conservative tillage treatments (CTS, NT, and NTS) may also be due to better biological activity (Wang et al., 2017).
- (v) Straw can increase organic matter and lead to improved phosphorous solubilization and fixed potassium availability by reducing phosphorous adsorption to mineral surfaces (Celik et al., 2011) and potassium adsorption to clay mineral surfaces (Celik et al., 2011).

Our findings concur with those of Zhao et al. (2019), Han et al. (2020), Sadiq et al. (2021a), Wulanningtyas et al. (2021), Lv et al. (2023), who found that nutrient elements and their storages were higher under conservation tillage than plowed ones.

The ECe, pH, C:N ratio, C:P ratio, and N:P ratio under different conservation tillage measures were inconsistent and non-significant. However, CTS, NT, and NTS showed a trend of increased ECe and decreased pH compared with CT. The dropping of pH under conservative tillage was previously described by numerous scholars (Jat et al., 2018; Sinha et al., 2019). Soil pH reduction in CTS, NT, and NTS conservation-based tillage regimes may be owed to organic acid production during retained stubble decomposition (Sinha et al., 2019). The C:N ratio was lower in CTS and NT than in CT, which might be due to slower decomposition under these systems. Our results are in line with those (Jat et al., 2018), which pointed out the minor values of C:N ratio under conservative tillage over CT. In general, the soil C:P and N:P ratios were highest under CT and NTS treatment, respectively.

These results are in agreement with Feng et al. (2014), who stated that NT practice coupled with straw increases the soil N:P ratio.

Conservation tillage system had a great influence on soil microbial count and enzymatic activities. The highest values of soil microbial count, urease, alkaline phosphatase, invertase, cellulase, and catalase were obtained under NTS treatment, followed by CTS and NT, while the CT system had the lowest values of all these parameters. The conservative tillage systems NTS, CTS, and NT increased microbial count by 31.34, 17.61, and 21.19%, respectively; urease activity by 6.88, 4.45, and 4.85%, respectively; activity of alkaline phosphatase by 29.33, 13.35, and 20%, respectively; invertase activity by 35.53, 10.07, and 16.85%, respectively; cellulase activity by 64.73, 33.99, and 43.03%, respectively; and catalase activity by 5.30, 2.16, and 2.55%, respectively, over CT. The microbial count and enzyme activity depicted the trend of CT < CTS < NT < NTS. The soil microorganisms are a significant portion of the cropland ecosystem and are involved in the decomposition of SOM, nutrient circulation in the soil system, humus formation, and soil fertility (Yu, 2015). Soil microbes' stability is a vital indicator of soil quality, health, and fertility (Yang et al., 2023). Land cultivation methods and cropland management practices affect the soil microbial community. Conservation tillage has the potential to improve the soil microbial community and the activities of soil enzymes (Rakesh et al., 2021). This study depicts that NTS, CTS, and NT raised the microbial community and activities of urease, alkaline phosphatase, catalase, invertase, and cellulase in the soil system to a greater extent than the CT system. This shows that conservative tillage mechanically makes the soil more porous (Yang et al., 2018) and increases the SOM distribution while facilitating the activity of enzymes (Balota et al., 2004). Hence, stubbles coupled with no-tillage (NTS) improved the soil microbial count, urease, alkaline phosphatase, invertase, cellulase, and catalase, which improved crop germination, growth, and yield. Optimal soil physicochemical properties could increase enzyme activity (Wei et al., 2013). In this context, NTS, CTS, and NT treatments were more effective than CT control treatment. Measurements of soil characteristics and variations are crucial for evaluating the influence of soil management techniques (Jat et al., 2021). The improved enzyme activities may be because of augmented carbon sequestration and immobilization of carbon as well as nitrogen during the decomposition of residues, as numerous studies have revealed that the activities of soil enzymes can be influenced by SOC sequestration (He et al., 2020; Pu et al., 2020). Our results are in accordance with Bhattacharya et al. (2020).

Furthermore, the principal component analysis (PCA) in accordance with the Jolliffe cutoff value permits isolating five principal components. In PCA analysis, the observation point made by contact between PC1 and PC2 illustrates the general variance defined by the five chief components. The PCA analysis of 39 variables (namely SWC, WFPS, SWS, ST, BD, P, soil aggregates, Ks, SOC, LFOC, CS, MBC, TN, AN, NS, MBN, TP, AP, TK, AK, ECe, pH, C:N ratio, C:P ratio, N:P ratio, MC, urease, alkaline phosphatase, invertase, cellulase, catalase, plant height, root yield, number of spikes per plant, number of seeds per meter square, grain yield, aboveground biomass yield, dry matter yield, harvest index), PC1 and PC2 were extracted with eigenvalues >1 and explained 65.7% of the total variance. Nevertheless, PC3, PC4, and PC5 do not allow the addition of additional information; that is why they are not involved. The highest PC1 loadings encompassed 50.4% of the total variance, and in PC2, the maximum loadings of 15.3% of the total variance were detected (Figure 4). According to our

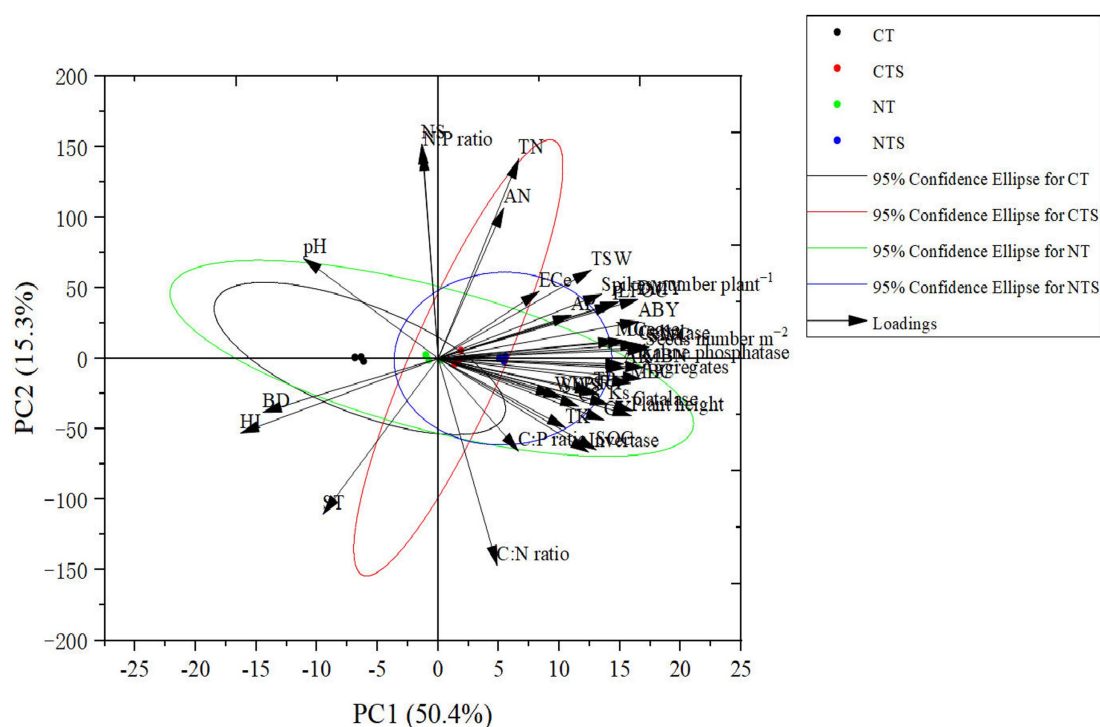


FIGURE 4
Principal component analysis of spring wheat agronomic attributes and soil properties (physical, chemical, and biological).

expectations, the correlation between wheat seed productivity and yield-attributing traits was significantly positive. Moreover, a significant positive correlation was noted between soil hydraulic properties, soil nutrients, and enzymatic activities and the seed yield of spring wheat (Figure 5). This concurs with the results reported by Yuan et al. (2023a).

3.3 Greenhouse gas fluxes and drivers of GHG emissions

Significant variation in GHG (CO_2 , CH_4 , and N_2O) was observed under different tillage regimes (Figure 6). The efflux rates of CO_2 (representing ecosystem respiration) under conservation tillage depicted apparent seasonal variations (Figure 6A). The maximum emissions arising during the summer months peak in the CT system in July, at the grouting spring wheat crop growth stage. The CT and CTS treatments showed the highest ecosystem respiration rates, including all investigated crop growth stages, while the NTS and NT treatments had the lowest CO_2 emission rates, but a significant difference between the different treatments was observed in bloom, grouting, maturity, and harvest stages. The highest CO_2 efflux rates during the summer months correspond with the maximum ST and dry matter accumulation, hence augmented soil respiration (Ussiri et al., 2009). Wu et al. (2011) clarified that an increase in soil temperature from 5°C to 15°C led to a noteworthy increase in the emission of carbon dioxide because of high soil organic matter mineralization and improved soil microbial activity. Lowering of soil CO_2 efflux rates during the growing seasons under conservation tillage was previously reported by many researchers (Alhassan et al.,

2021; Li et al., 2023). Al-Kaisi and Yin (2005) stated a huge 80% reduction in CO_2 efflux rates under the NT compared to the CT system. Average CO_2 efflux rates were higher in tilled soils compared with non-tilled soils. The average CO_2 efflux rates were 180 ± 14.2 , 171 ± 25.8 , 245 ± 8.8 and $260 \pm 15.3 \text{ mg Cm}^{-2} \text{ h}^{-1}$ in NTS, NT, CTS and CT respectively (Figure 4B). A significant positive correlation of CO_2 emission among soil moisture and soil temperature and a negative correlation of CO_2 emission with nitrogen and carbon contents and hydraulic conductivity were observed during the current study (Table 6). The CO_2 efflux rates are habitually controlled by plenty of factors, including carbon dioxide concentration gradient in the environment and the wind speed, soil water, soil temperature, soil medium, and soil physicochemical properties (Raich and Schlesinger, 1992). The tillage effect on these parameters would affect carbon dioxide emissions as well. The CT causes soil disturbance, which increases rates of decomposition because of improved soil microbe activities (Al-Kaisi and Yin, 2005), leading to maximum emissions of CO_2 . Quite the reverse, under NTS and NT treatments, decomposition is slower because of no soil disturbance (Curtin et al., 2000). Conservation tillage practices (CTS, NT, and NTS) might correspondingly improve soil properties, which in turn can decrease soil CO_2 emissions. Maximum SWC under CTS treatment, combined with maximum ST, produced higher cumulative soil CO_2 emissions compared with NT and NTS. The SWC and ST habitually exert a collaborative effect on CO_2 emissions (Bond-Lamberty et al., 2016).

All the tested tillage systems acted as CH_4 sinks during the study period (Figure 6C). Peak absorption arose during the summer months at booting and bloom crop growth stages under the NT system. Seasonal changes were recorded in the sink capacities of

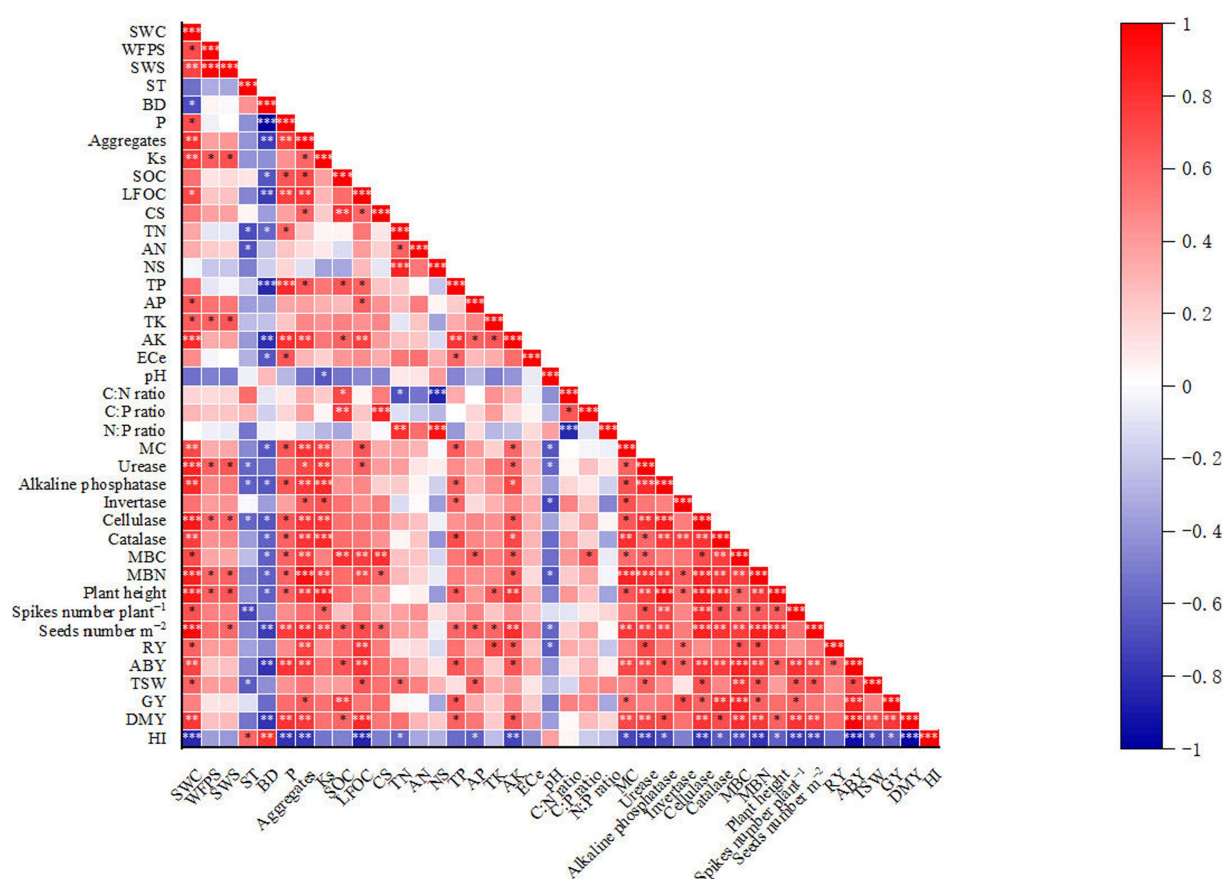


FIGURE 5

Heat map correlation study of wheat agronomic traits and soil physical, chemical, and biological properties. Indicates significance at: * $p < 0.05$, ** $p < 0.010$, and *** $p < 0.0010$. Note: the abbreviated words stand for SWC=soil gravimetric water content; WFPS=water-filled pore space; SWS=soil water storage; ST=soil temperature; BD=soil bulk density; P=soil porosity; Ks=soil saturated hydraulic conductivity; SOC=soil organic carbon; LFOC=light fraction organic carbon; SC=carbon storage; TN=total nitrogen; AN=available nitrogen; NS=nitrogen storage; TP=total phosphorous; AP=available phosphorous; TK=total potassium; AK=available potassium; ECe=soil electrical conductivity; pH=soil pH; MC=soil microbial counts; MBC=microbial biomass carbon; MBN=microbial biomass nitrogen; RY=wheat root yield; ABY=wheat aboveground biomass yield; TSW=thousand seed weight; GY=wheat grain yield; DMY=dry matter yield; HI=harvest index.

different tillage systems. The CT had minimum CH_4 absorption rates during the investigated crop growth stages. A significant difference between different treatments was observed except for emergence and jointing of spring wheat crop growth stages. The maximum CH_4 emissions during the summer months were because of the high SWC status and ST, which may perhaps have boosted the activity of CH_4 -oxidizing bacteria. The analysis of variance showed that all the investigated tillage techniques, namely CT, CTS, NT, and NTS, had a significant influence on average methane emission. In general, our results showed the average absorption rate of CH_4 followed the trend of $\text{CT} < \text{CTS} < \text{NT} < \text{NTS}$ (Figure 4D). Another study done by Yeboah et al. (2016) had comparable consequences. Shen et al. (2018) showed that semi-arid agroecosystems habitually act as sinks of CH_4 as a result of soil aerobic conditions (Schaufler et al., 2010). Similar to the emission of CO_2 , the absorption has a significant positive correlation with ST and SWC and a negative correlation with Ks and carbon and nitrogen elements (Table 6). The results of the current study are consistent with the results recorded by Yeboah et al. (2016) and Wang et al. (2022), who stated that improved SWC status can lead

to high CH_4 uptake owing to the anaerobiosis occurrence and methanogenesis increment. Singh et al. (2010) observed maximum CH_4 emission rates under 13% soil water content in sandy loamy soils, and a parallel situation was detected in this study. Our study depicted that the methane flux was constantly negative, signifying CH_4 uptake by all the tillage systems. The NTS had the highest CH_4 uptake in this study. Under NTS treatment, minor ST might have played a substantial role in high CH_4 uptake. During high ST, the dominant methanogen (Methanosarcinaceae) employs H_2/CO_2 and acetate as precursors of CH_4 producing and produces far maximum CH_4 over the methanogen at lesser temperatures (Methanosaetaceae), which uses solitary acetate as a precursor of CH_4 producing (Ding et al., 2003). Our results are consistent with other appraisals in the Loess Plateau of China, where NT with straw-return was found to be a net sink for atmospheric CH_4 (Alhassan et al., 2021). The greater methane uptake under the NTS system might be because of good soil aeration and less degradation of the soil, which improved the activity of methanotrophs. McLain and Martens (2006) recorded that the activity of methanotrophs was heightened under tolerable gas diffusion and from the microbial

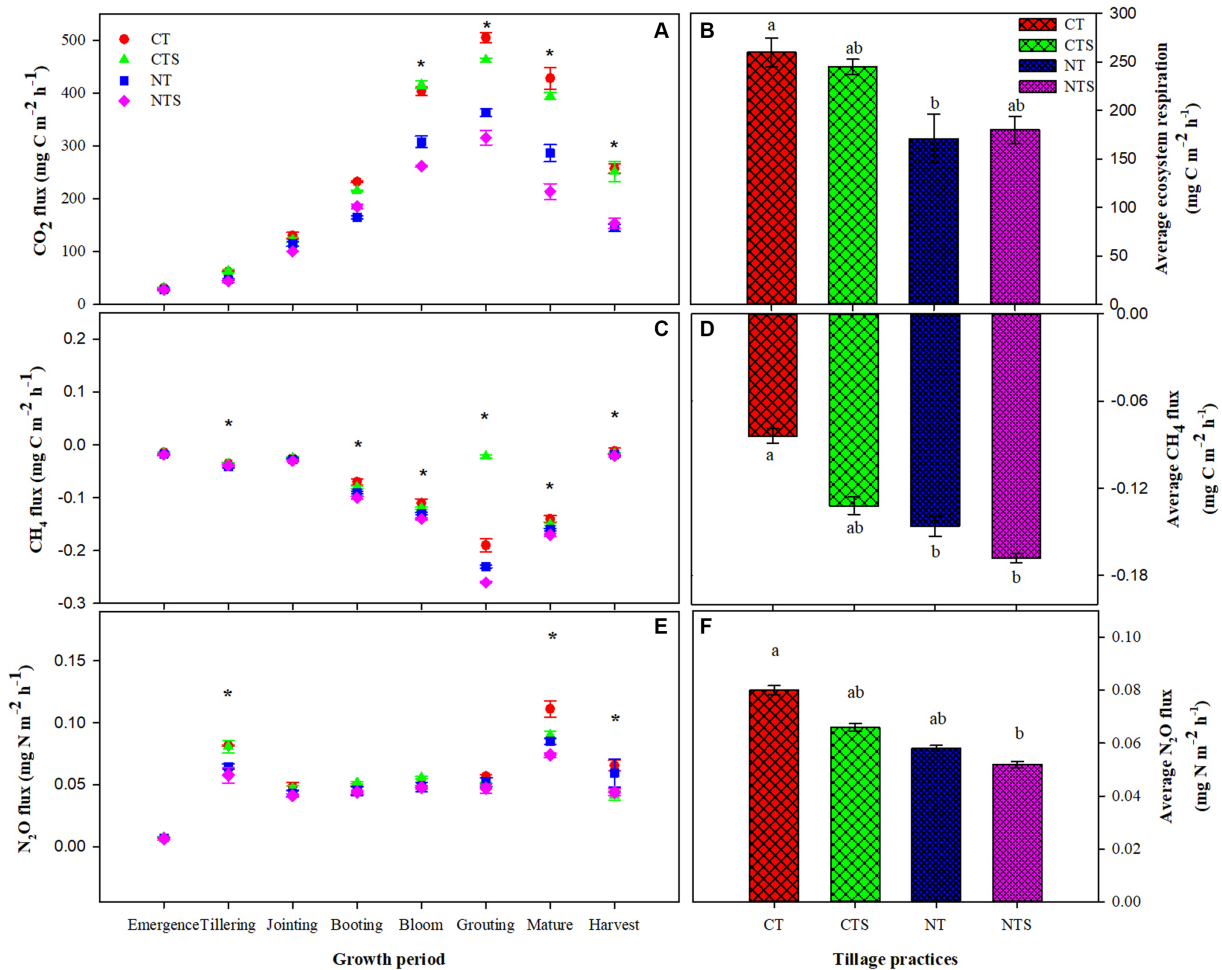


FIGURE 6

Seasonal and average greenhouse gas emissions under tillage systems in spring-wheat agroecosystem in 2021. Error bars represent the corresponding standard error of mean values; $n = 3$. Different lower-case letters and '*' indicate significant differences amongst different treatments at $p < 0.05$ (Duncan's test performed for mean separation). Note: (A,B) is the seasonal and average ecosystem respiration as affected by the tillage treatments; (C,D) is the seasonal and average CH₄ flux under conservation tillage; (E,F) is the seasonal and average N₂O flux under tillage practices.

activity sites. The NTS system inclines to increase soil organic carbon and decrease the bulk density of soil, which might lead to lesser CH₄ emissions risk. Yeboah et al. (2016) stated that the degradation of soil can diminish the soil's ability to oxidize or consume atmospheric methane by as much as 30–90%. However, we proved that the most noteworthy CH₄ uptake control was SWC at the surface soil depth, with ST and Ks brought about by enhanced soil condition or quality and improved SOC. Soil carbon and nitrogen contents are also negatively correlated with CH₄ emission. This obscures the fact that improved soil carbon and nitrogen levels condensed the emission of CH₄.

Our study data showed that peak N₂O emission occurred in August at the final crop maturity growth stage, while the lowest N₂O flux was observed in the spring months. This trend is consistent with the findings stated by Cai et al. (2013) at the identical research site. During most tested crop growth stages, the CT and CTS emit more N₂O compared with the NT and NTS treatments, while at the harvest crop growth stage, NT served as a slight emitter and dominated the CTS system. A significant

difference between different treatments was observed only at tillering, maturity, and harvest crop growth stages (Figure 6E). The N₂O flux pattern depicted that when SWC and ST appeared higher during the summer season, the N₂O emissions were higher. These consequences are similar to earlier studies (Bhattacharyya et al., 2023; Bozal-Leorri et al., 2023; Li et al., 2023). The ST is a significant factor influencing seasonal variations of N₂O flux. Taghizadeh-Toosi et al. (2022) observed that the variation in N₂O flux rate is almost harmonized with the surface temperature of the soil. Zhang et al. (2022) noted that ST increase can diminish the nitrification contribution to N₂O and increase the N₂O amount produced during the denitrification process. The highest N₂O emissions noted during the rainiest months under all tested tillage systems might be credited to the fact that the spell's rainfall levels might be higher than normal on that date, making all tillage systems very wet, so that denitrification situations were not dissimilar among them.

Significantly higher N₂O fluxes were observed under CT treatment compared with NTS, NT, and CTS treatments. The N₂O

TABLE 6 Heat map correlation study of greenhouse gases and soil indicators under tillage systems.

Variables	Greenhouse gases			
	CO ₂	CH ₄	N ₂ O	GWP
SWC	0.95*	0.98*	0.97*	0.96*
WFPS	0.96*	0.97*	0.95*	0.99*
ST	0.97*	0.96*	0.95*	0.98*
BD	0.88	0.84	0.83	0.85
Ks	−0.98*	−0.95*	−0.97*	−0.96*
SOC	−0.86	−0.84	−0.83	−0.85
LFOC	−0.85	−0.82	−0.79	−0.83
TN	−0.75	−0.73	−0.77*	−0.81*
AN	−0.72	−0.70	−0.68	−0.66
MBC	−0.82	−0.76	−0.67	−0.95*
MBN	−0.84	−0.72	−0.78	−0.71

Indicates significance at: * $p < 0.05$, ** $p < 0.010$, and *** $p < 0.0010$. The abbreviated words stand for SWC, soil moisture; WFPS, water-filled pore space; ST, soil temperature; BD, soil bulk density; Ks, soil saturated hydraulic conductivity; SOC, soil organic carbon; LFOC, light fraction organic carbon; TN, total nitrogen; AN, available nitrogen; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; CH₄, methane; CO₂, carbon dioxide; N₂O, nitrous oxide; GWP, global warming potential.

flux rates followed the trend of NTS < NT < CTS < CT (Figure 4F). On average, all investigated tillage systems served as slight N₂O emitters. These results are in accordance with those described by Pokharel and Chang (2021). Quite the reverse, Alhassan et al. (2021) observed maximum N₂O fluxes under the NT system over the CT technique. In this study, different conservation tillage practices, for instance, CTS, NT, and NTS, decreased the emission of N₂O by increasing soil structure, as revealed by bulk density of soil, pore space, soil aggregates, and soil hydraulic conductivity. Higher N₂O emissions under the CT system have been credited to the condensed diffusivity of gas and air-filled pore space (Rabot et al., 2015). Additionally, N₂O flux rates recorded in this experimental research were in the range of N₂O flux rates noted by Yeboah et al. (2016).

A significant positive correlation was noted between ST and N₂O emissions, which is attributed to the microbial respiration augmentation after heating origins oxygen dearth in the soil profile, which generates anaerobic conditions for denitrifying microbes' activity (Castaldi, 2000; Mehnaz et al., 2018), and all together, warming also rises soil denitrification activity (Braker et al., 2010), which led to a rise in N₂O emissions. Additionally, our results also showed positive correlations among SWC and N₂O emissions. This is due to the improvement in SWC that limits the soil oxygen concentration, chiefly the anaerobic soil environment formation, pointedly falling nitrification and increasing denitrification, which in turn led to dominant denitrification to produce N₂O emissions (Pokharel and Chang, 2021). Our results are identical with other scientific reports by Taghizadeh-Toosi et al. (2022), demonstrating the influence of ST and SWC on the emission of soil N₂O.

The correlation study in Table 6 showed a significant negative relationship between N₂O emission and carbon and nitrogen

contents, supporting the (He et al., 2023) that enhancing carbon stocks and nitrogen soil status can decrease the emission of soil N₂O. Consequently, these findings are consistent with earlier results reported by Zhao et al. (2020). High soil bulk density related to the degradation of soil by tillage application can have consequences for soil aeration reduction. Soil degradation reduction, whereas surface soil preservation covered by crop straws, might lead to minor denitrification as well as N₂O emissions risk. Quite the reverse, Li et al. (2005) reported a positive correlation between soil N₂O emissions and soil carbon contents. This is due to the increase in soil carbon contents delivering sufficient sources of nitrogen and a suggestive rise in the heterotrophic microorganism's respiration. All at once, carbon availability in the soil offers electron donors for denitrifying microbes, which endorses the denitrification incidence, in that way increasing emissions of N₂O. Soil nitrogen contents were positively correlated with N₂O emission. Su et al. (2021) stated that improved N₂O production might be a consequence of augmented nitrogen contents and bacterial activities; meanwhile, the activities of microbes are controlled by soil carbon contents. This obscures that soil carbon contents improve the N₂O-producing microbe's activities. This also verifies the results of Guenet et al. (2021), who showed that high carbon contents mostly raise the emission of N₂O. Changes in CO₂, N₂O, and CH₄ emission seemed to be described by variations in soil temperature, moisture content, and saturated hydraulic conductivity in the surface soil layer of 0–10 cm soil depth, with 95% of the data variance described by these environmental variables.

3.4 Global warming potential under conservation tillage system

The GWP of CO₂, CH₄ and N₂O and net global warming potential (GWP) under all investigated tillage practices are shown in (Figure 7). The highest cumulative CO₂ flux was recorded under CT, whereas conservation tillage measures NTS, NT, and CTS pointedly reduced the cumulative CO₂ flux, as the least cumulative CO₂ flux was noted under NT, which was followed by NTS. The global warming potential due to the emissions of CH₄ was significantly lower under NTS, which was followed by NT over CT during the study period. The global warming potential due to the emissions of N₂O was significantly less under NT, which was followed by NTS over CT. The net global warming potential followed the trend of CT > CTS > NT > NTS (Figure 7D). The NTS, NT, and CTS decreased net-GWP by 23.44, 19.57, and 16.54%, respectively, over the CT system. The effect of different tillage practices on the atmospheric radiative forcing and henceforth changing climate can be evaluated by global warming potential determination from the biosphere–atmosphere exchange of numerous greenhouse gases. The noted global warming potential for the current study ranged between 1000 and 1400 kg CO₂-e ha^{−1} which is in the range of those stated by Ma et al. (2013) but superior to those stated by Yeboah et al. (2016). The net-GWP was positively correlated with ST and SWC and negatively correlated with carbon and nitrogen elements and Ks (Table 6). In PCA analysis, we found that soil moisture, soil temperature, bulk density, hydraulic conductivity, carbon, and nitrogen elements explained 96.2, 69.6,

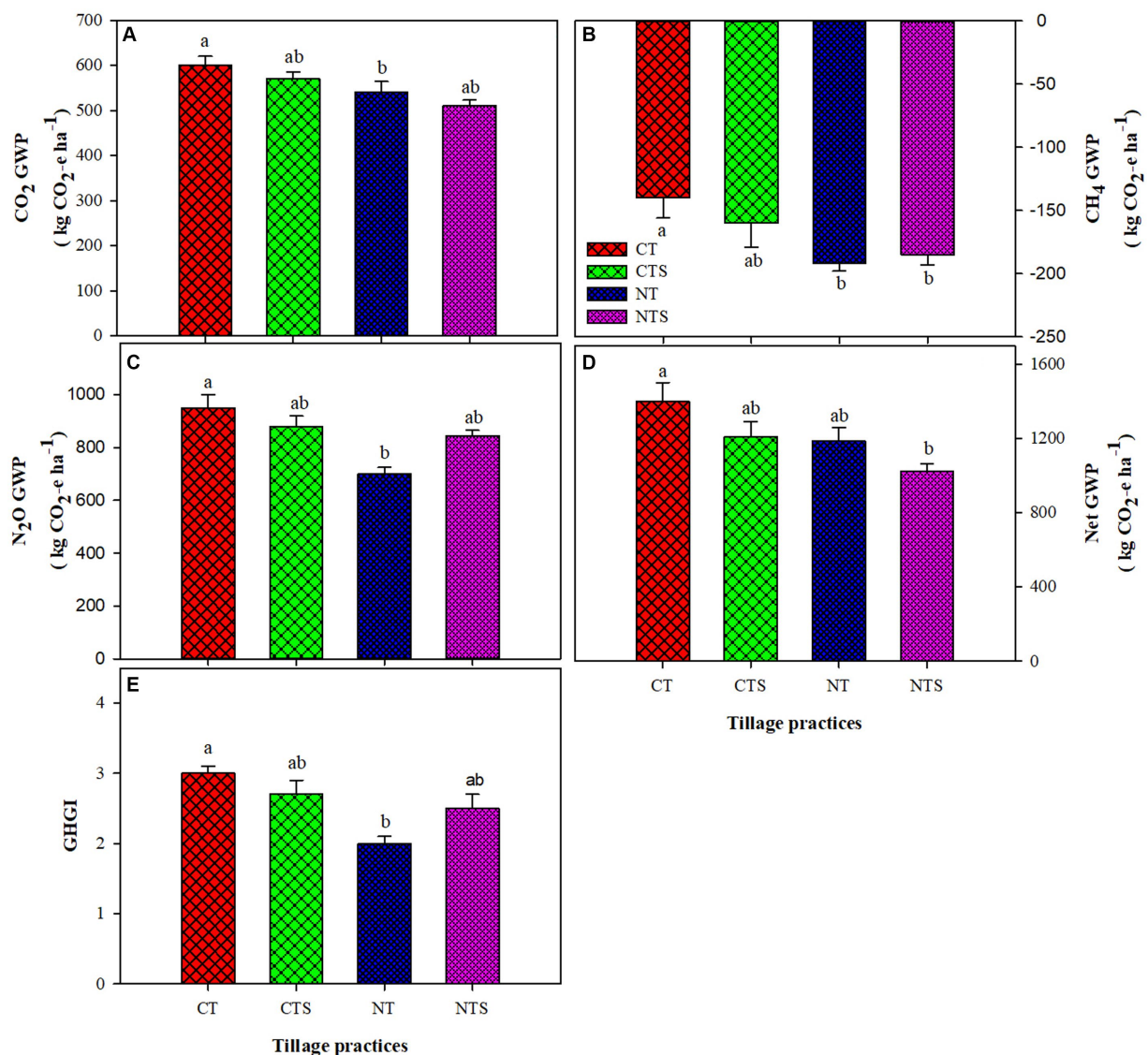


FIGURE 7

Global warming potential (GWP) of CO₂, CH₄ and N₂O and greenhouse gas intensity (GHGI) under tillage practices in spring-wheat agroecosystem in 2021. Error bars represent the corresponding standard error of mean values; $n = 3$. Different lower-case letters and ‘*’ indicate significant differences amongst different treatments at $p < 0.05$ (Duncan’s test performed for mean separation). Note: (A) is the GWP of carbon dioxide as affected by the tillage treatments; (B) is the GWP of CH₄ as influenced by the tillage system; (C) is the GWP of N₂O as affected by the different tillage techniques; (D) is the net-GWP under tillage measures and (E) is the GHGI under tillage practices.

67.8, and 77% of the variations in soil CO₂, CH₄, and N₂O fluxes and GWP, respectively (Figures 8A–D). Compared with CH₄ and N₂O fluxes and GWP, soil CO₂ had a strong relationship with environmental variables, as shown in Figure 8A. Conservation agriculture has the potential to contribute to environmental conservation, though its influence differs depending on management strategies. The higher greenhouse gas intensity (GHGI) was associated with the CT system, while the minimum GHGI value was observed with the NT system, which was followed by the NTS system. The GHGI followed the trend of CT > CTS > NTS > NT. The conservation tillage systems NT, NTS, and CTS decreased GHGI by 29.96, 23.20, and 18.72%, respectively,

compared with CT practice (Figure 7E). The current noted global warming intensity values were higher compared with other studies (Qin et al., 2010; Ma et al., 2013), which is attributable to the overall lower grain yield in this experimental research. Global warming intensity is a crop yield function, and it is directly affected by low grain yield. A significant reduction was observed under NTS and NT practices over CTS and CT systems in case of global warming potential and global warming intensity values. These findings are in line with Alhassan et al. (2021) and Li et al. (2023), who stated that different conservation tillage systems decreased the global warming potential and global warming intensity compared with the CT technique.

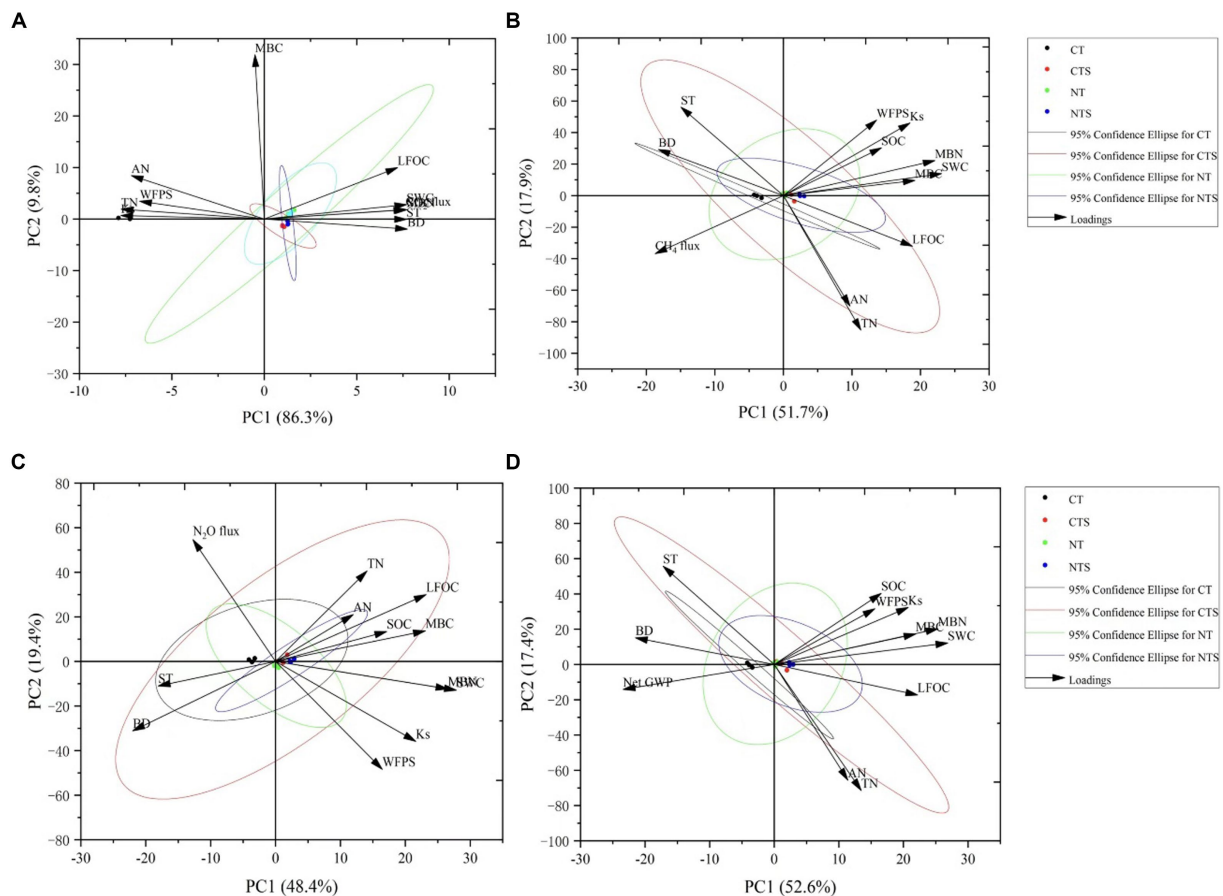


FIGURE 8

Principal component analysis for evaluating the influence of environmental variables on greenhouse gas emissions under conservation tillage practices. (A) The PCA analysis of environmental variables with carbon dioxide emission; (B) the PCA analysis of environmental variables with methane emission; (C) the PCA analysis of environmental variables with nitrous oxide emission; and (D) the PCA analysis of environmental variables with net global warming under different tillage systems.

4 Conclusion

Conservation tillage is a significant climate-smart approach to increasing soil and water conservation, soil carbon sequestration, crop production and reducing greenhouse gas emissions. It is argued to decrease global warming potential in relation to changing climate mitigation. Our study suggests that conservation tillage practices CTS, NT, and NTS have the potential to increase wheat agronomic traits (plant height, number of spikes per plant, seeds number per meter square, root yield, aboveground biomass yield, thousand-grain weight, grain yield and dry matter yield), soil physical properties (gravimetric soil water content, water-filled pore space, water storage, pore space, aggregates, and hydraulic conductivity), chemical properties (soil organic carbon, light fraction organic carbon, carbon storage, total nitrogen, available nitrogen, nitrogen storage, total phosphorous, available phosphorous, total potassium, available potassium), biological properties (soil microbial count, urease, alkaline phosphatase, invertase, cellulase, catalase, microbial biomasses carbon and nitrogen) and decrease carbon, methane and

nitrous oxide emissions in semi-arid sandy loam cropland of Dingxi China in comparison with plowing conventional tillage system. Moreover, NTS and NT significantly reduced the global warming potential of carbon dioxide, methane, and nitrous oxide and their yield-scale global warming potential. The correlation analyses show that crop yield is significantly positively correlated with yield-attributing traits, soil physicochemical properties, and biological properties. The global warming potential due to CO₂, CH₄, and N₂O greenhouse gases had a positive correlation with soil and environmental variables, and the dominant controlled factors were soil moisture and soil temperature. Accordingly, straw-retention with no tilled soil and stubble incorporation with conventionally tilled soil should be recommended and promoted among the smallholder farmer systems in semi-arid zones to raise soil and water conservation, soil and environmental quality, and sustainability. More comprehensive long-term research and different tillage operations are needed regarding carbon sequestration and stocks and genes involved in nitrogen cycling and global warming to produce more powerful data for climate-smart agriculture.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by College of Forestry, Gansu Agricultural University, Lanzhou 730,070, China; khanmahran420@gmail.com (MS). The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because College of Forestry, Gansu Agricultural University, Lanzhou 730,070, China; khanmahran420@gmail.com (MS).

Author contributions

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Conflict of interest

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

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Copper pyrazole addition regulates soil mineral nitrogen turnover by mediating microbial traits

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The huge amount of urea applied has necessitated best-developed practices to slow down the release of nitrogen (N) fertilizer while minimizing nitrate loss. However, the impact of nitrification inhibitors on mineral-N turnover and the associated microbial mechanisms at different stages remains unknown. A 60-day incubation experiment was conducted with four treatments: no fertilizer (CK), urea (U), urea with copper pyrazole (UC), and urea coated with copper pyrazole (SUC), to evaluate the changes about soil ammonia N (NH_4^+ -N) and nitrate N (NO_3^- -N) levels as well as in soil microbial community throughout the whole incubation period. The results showed that copper pyrazole exhibited significantly higher inhibition rates on urease compared to other metal-pyrazole coordination compounds. The soil NH_4^+ -N content peaked on the 10th day and was significantly greater in UC compared to U, while the NO_3^- -N content was significantly greater in U compared to UC on the 60th day. Copper pyrazole mainly decreased the expression of nitrifying (AOB-*amoA*) and denitrifying (*nirK*) genes, impacting the soil microbial community. Co-occurrence network suggested that *Mycobacterium* and *Cronobacter sakazakii*-driven Cluster 4 community potentially affected the nitrification process in the initial phase, converting NH_4^+ -N to NO_3^- -N. *Fusarium*-driven Cluster 3 community likely facilitated the denitrification of NO_3^- -N and caused N loss to the atmosphere in the late stage. The application of copper pyrazole may influence the process of nitrification and denitrification by regulating soil microbial traits (module community and functional genes). Our research indicates that the addition of copper pyrazole alters the community function driven by keystone taxa, altering mineral-N turnover and supporting the use of nitrification inhibitors in sustainable agriculture.

KEYWORDS

nitrification inhibitors, copper pyrazole, soil mineral nitrogen, microbial community, nitrogen-cycling genes

1 Introduction

Nitrogen (N) fertilizer plays a pivotal role in promoting soil productivity and maintaining soil health (Singh and Verma, 2007). Global N fertilizer consumption has exceeded 110 million tons per year and continues to increase (Hu et al., 2023). After they are applied to soil, N-components in fertilizers are transformed into the plant-available forms of ammonium-N ($\text{NH}_4^+\text{-N}$) and nitrate-N ($\text{NO}_3^-\text{-N}$) through nitrification (Subbarao et al., 2006; Adhikari et al., 2021; Hosseini et al., 2023). However, frequent application of N fertilizer may decrease N utilization efficiency, cause soil acidification, and even reduce soil quality and functionality (Chien et al., 2016; Hu et al., 2023). Generally, only slightly over 35% of the urea applied is utilized under field conditions (Al-Juthery et al., 2021), and most of the N is lost in the form of NO_3^- , NH_3 , N_2O , and N_2 , which leads to resource wastage (Al-Juthery et al., 2021; Mahmud et al., 2021; Upadhyay et al., 2023). Therefore, methods to improve N utilization efficiency is currently an urgent research issue worldwide.

Current strategies for reducing soil N loss and enhancing soil N fertilizer use efficiency mainly include the breeding of new plant varieties, optimizing N fertilizer regimes, diversifying crop rotations or intercropping, and using efficient fertilizers with urease and/or nitrification inhibitors (Wing et al., 2018; Woodward et al., 2021; Gao et al., 2022; Liu et al., 2022). Nitrification inhibitors show strong potential for improving N utilization efficiency and increasing crop yields, and the use of these agents are accepted by most farmers (Mahmud et al., 2021; Liu Y. et al., 2014). To the best of our knowledge, pyrazole and its derivatives, as one type of nitrification inhibitors, have a potent inhibitory effect on nitrification (McCarty et al., 1989; Woodward et al., 2021; Jog et al., 2022; Li et al., 2022), which have been widely used in Europe (Gilsanz et al., 2016) and have since been widely used for yield improvement (Woodward et al., 2021). The mechanisms underlying nitrification inhibitor function primarily include direct suppression of nitrification via ammonia monooxygenase (AMO) deactivation, in addition to shifts in the soil microbial community structure (Adhikari et al., 2021; Woodward et al., 2021). Microorganisms, as the main drivers of soil elemental cycling, have received increasing amounts of attention due to their ability to mediate N turnover and have become a hotspot of current research (Adhikari et al., 2021; Gupta et al., 2023). Hence, deciphering the relationship between soil microbial functions and N turnover is particularly crucial for ensuring soil health.

Many authoritative studies suggest that soil microorganisms are involved in the soil N cycle via processes such as biological N fixation, ammonification, nitrification, and denitrification (Liu W. et al., 2014; Woodward et al., 2021). Generally, when urea is applied to soil, a small amount of the $\text{NH}_4^+\text{-N}$ formed can be absorbed and utilized by plants, and most of the $\text{NH}_4^+\text{-N}$ is converted into $\text{NO}_3^-\text{-N}$ by ammonia oxidizer (ammonia oxidizer archaea [AOA] and ammonia oxidizer bacteria [AOB])-driven nitrification (Saud et al., 2022). Subsequently, most $\text{NO}_3^-\text{-N}$ is released as NO_2 and N_2 from the soil due to denitrifying bacteria-driven denitrification (Wrage et al., 2001). Moreover, nitrification inhibitors slow the initial step of nitrification by reducing the abundance and activity of AOB so that more $\text{NH}_4^+\text{-N}$ can be taken up and utilized by plant roots (Topp and Knowles, 1984). In addition, specific microbial species have been indicated to be involved in soil N turnover (Cáceres et al., 2018; Woodward et al., 2021). For example, Li et al. (2018) showed that *Stenotrophomonas*, a

typical denitrifier, was the keystone taxa contributing to N_2O production. Additionally, Van Kessel et al. (2015) reported the enrichment and initial characterization of two *Nitrospira* species that encode AMO necessary for ammonia oxidation via nitrite to nitrate in their genomes. Related studies have shown that nitrification inhibitors can limit the activity of such functional microbes, altering N transformation and enhancing fertilizer use efficiency. Therefore, elucidating the effect of nitrification inhibitor application on microbial functions during different periods is critical.

On the other hand, soil urease activity is a crucial factor influencing N turnover. Urease catalyzes the hydrolysis of urea into NH_3 , thereby reducing the efficiency of urea utilization by plants (Li et al., 2019). Therefore, decreasing urease activity is an important strategy for (further) improving urea utilization efficiency. Research has shown that urease is sensitive to heavy metal ions, which can inhibit urease activity (Hemida et al., 1997; Yang et al., 2006), slowing down the decomposition of N fertilizers and enhancing their utilization efficiency.

Furthermore, some studies have suggested that coated slow-release materials (Naz and Sulaiman, 2016) may be suitable options for nitrification inhibitors. In addition, slow-release material could release urea continuously to ensure the effectiveness of the fertilizer (Naz and Sulaiman, 2016). However, the effects of nitrification inhibitors chelated with heavy metal on soil mineral N content and microbial traits during different periods are poorly understood. Therefore, we conducted a 60-day incubation experiment to (1) determine the dynamics of mineral N content and microbial traits (including N-cycling gene abundance and community) after nitrification inhibitor application and (2) clarify the linkages between mineral N content and microbial traits at different periods. We hypothesized that (i) soil $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ contents as well as microbial N-cycling gene abundance would show distinct responses to different treatments during incubation and (ii) soil $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ turnover were closely related to specific microbial taxa.

2 Materials and methods

2.1 Experimental soil and material preparation

The test soil was obtained from the topsoil (0–20 cm) of a wheat field in Shushan District, Hefei city, Anhui Province, China (31° 90' W, 117° 17' E). The soil samples were air-dried, ground, passed through a sieve of 2 mm and stored at room temperature. The soil chemical properties were as follows: 6.3 pH (W/V, 1:2.5), 7.39 g·kg⁻¹ organic carbon, 0.39 g·kg⁻¹ total phosphorus, and 1.05 g·kg⁻¹ total N.

The metal-pyrazole coordination compounds were prepared using the solvent evaporation method (Das et al., 2010; Zhai, 2020; Li et al., 2022), with $\text{CuCl}_2\cdot 2\text{H}_2\text{O}$, ZnCl_2 , $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$, and CdI_2 as raw materials to form metal-pyrazole coordination compounds. The yield of all metal-pyrazole coordination compounds exceeded 60%, and the specific method can be found in Supplementary Text S1.

Urease activity was assessed using the spectrophotometric method (Fahey et al., 2013). Take 25 μL of urease solution (10 KU/L) in a centrifuge tube and add 25 μL of metal-pyrazole coordination compounds of different concentrations. Place the tubes in a 25°C incubator for 0.5 h. Then add 200 μL of a buffer solution containing

urea and phenol red indicator, mix well, and incubate in a 25°C incubator for another 0.5 h. Measure the absorbance of the samples at a wavelength of 570 nm using a spectrophotometer, and calculate the urease inhibition rate using the following formula:

$$\text{Urease inhibition rate (\%)} = \frac{(A - B)}{A} \times 100\% \quad (1)$$

Where: A is the difference in absorbance before and after the reaction in the sample solution without added urease inhibitor, and B is the difference in absorbance before and after the reaction in the sample solution with added urease inhibitor.

Synthesis of urea coated with copper pyrazole slow-release fertilizer: The speed of the sugar-coating machine (BY-300, Taizhou Jincheng Pharmaceutical Machinery Co., Ltd., China) was adjusted to 45 r/min, the blower was turned on, 250 g of urea was added to the sugar-coating machine, and it was heated at 70°C for 3 min. Then, the heating and blowing were stopped, 11.70 g of copper pyrazole was added to the sugar-coating machine and evenly mixed, and a small amount of NaOH solution (pH=9.5) was sprayed several times until the urea particles did not stick. Next, 112 g of potato starch was added to the sugar-coating machine and mixed evenly, 50 mL of epichlorohydrin and 50 mL of NaOH solution (pH=9.5) were sprayed into the sugar-coating machine several times, and the urea coated with copper pyrazole slow-release fertilizer was obtained after drying with a blower for 30 min. All the above chemical reagents (analytical purity) were purchased from Sinopharm Chemical Reagents Co., Ltd. Shenggong Bioengineering Co., Ltd. (China).

2.2 Experimental design and soil mineral N analysis

Soil N transformation and microbial trait changes were assessed via a destructive incubation experiment. Four treatments were used, namely, control (CK), urea (U), urea and copper pyrazole (UC), and urea coated with copper pyrazole slow-release fertilizer (SUC). Each experiment was conducted in triplicate to ensure the reliability of the results. Three hundred grams of soil was taken from each treatment and placed in a plastic box, and the corresponding materials (386.1 mg of urea, 386.1 mg of urea, 18 mg of copper pyrazole, and 483 mg of urea coated with copper pyrazole slow-release fertilizer) were added to each treatment and mixed homogeneously, followed by 15 parallel samples from each treatment. The mixture was incubated at 25°C for 60 days, after which the soil moisture was adjusted to 60% of the maximum water holding capacity every 7 days during incubation. Thirty grams of the soil from each treatment was collected on Days 0, 4, 10, 30, and 60. One part was kept at −80°C for microbial sequencing, and the other was used for the measurement of soil $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ contents, which were determined by a Kelvin Nitrogen Determination Instrument (K-360, BUCHI BUCHI Laboratory Equipigs Co. Trading Ltd., Switzerland).

2.3 Quantitative PCR

Soil samples at the 10th and 60th days of incubation were selected for microbial analysis, DNA extraction of soil samples was done by

Bemac Biotechnology Co Ltd. (China). The *amoA* gene encoding AMO and the *nirK* gene encoding nitrite reductase and the *nosZ* gene encoding nitrous oxide reductase were analyzed by a real-time fluorescence Quantitative PCR (qPCR) instrument (LightCycler®96, Roche, Switzerland). qPCR used three different primers - *bamoA_1F/bamoA_2R* (AOA-*amoA* and AOB-*amoA*), *Cunir3F/Cunir3R* (*nirK*) and *nosZ-2F/nosZ-2R* (*nosZ*)—to determine the copy numbers of the AOA-*amoA*, AOB-*amoA*, *nirK* and *nosZ* genes in this study (Chen Z. et al., 2021). The 20 µL qPCR mixtures contained 10 µL TB Green Premix Ex Taq polymerase (TaKaRa Bio, China), 0.5 µL each primer, 1 µL template DNA, and 8.5 µL nuclease-free water. Soil DNA was diluted prior to PCR to avoid co-extracted compound inhibition. External plasmid DNA standard curves with 10-fold serial dilutions were utilized to quantify gene copies. The qPCR conditions was initiated at 95°C for 10 min; 40 cycles of 95°C for 30 s, 55°C for 30 s and 68°C for 40 s of extension (AOA-*amoA*, AOB-*amoA*); 40 cycles of 95°C for 60 s, 57°C for 30 s and 72°C for 60 s of extension (*nirK*); 40 cycles of 95°C for 60 s, 56°C for 30 s and 72°C for 60 s of extension (*nosZ*). Melt curve analysis confirmed PCR specificity. qPCR was performed in triplicate, and amplification efficiencies of 95–105% were obtained with r^2 values > 0.99 for all microbial functional genes.

2.4 Amplicon sequencing of the bacterial 16S rRNA and fungal ITS rRNA genes

High-throughput sequencing was performed with the Illumina MiSeq sequencing platform (Illumina Inc.). The primers 341F/806R (5'-CCTAYGGGRBGCASCAG-3'/5'-GGACTACNNGGGTATCTAAT-3') were chosen to amplify the 16S rRNA genes in the V3–V4 hypervariable region (Chen K. H. et al., 2021). A unique 5-bp barcode sequence was added to the forward primers to distinguish the PCR products from different samples. PCR was conducted in a 50-µL reaction mixture containing 27 µL of ddH₂O, 2 µL (5 µM) of each forward/reverse primer, 2.5 µL (10 ng) of template DNA, 5 µL (2.5 mM) of deoxynucleoside triphosphates, 10 µL of 5× Fastpfu buffer, 0.5 µL of bovine serum albumin, and 1 µL of TransStart Fastpfu polymerase (TransGen, Beijing, China). The PCR conditions were 94°C for 5 min; 30 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 30 s of extension; followed by 72°C for 10 min. The reaction products were pooled and purified using the QIAquick PCR purification kit (Qiagen), and they were quantified using a NanoDrop ND-1000 (Thermo Scientific). After the individual quantification step, amplicons were pooled in equal amounts, and paired-end 2,300-bp sequencing was performed using the Illumina MiSeq platform with MiSeq Reagent Kit v3. PCR products from all samples were pooled and purified in equimolar concentrations, and sequencing was performed on an Illumina MiSeq instrument.

The fungal ITS1 region was amplified via polymerase chain reaction (PCR) using the ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC) primers (Ghannoum et al., 2010; Duan et al., 2023). The 50 µL PCR reaction mixture consisted of 1 µL (30 ng) template DNA, 4 µL (1 µM) of each forward and reverse primer, 25 µL PCR Master Mix, and 16 µL ddH₂O. The PCR conditions were 95°C for 5 min; 25 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 40 s of extension; followed by 72°C for 7 min. PCR products from all samples were pooled and purified in equimolar concentrations, and sequencing was performed on an

Illumina MiSeq instrument (Illumina, San Diego, CA, United States). The raw sequence data were processed using the Qualitative Insights into Microbial Ecology (QIIME) pipeline. Poor-quality sequences with lengths less than 200 bp and quality scores less than 20 were discarded, and the chimeras were removed using the UCHIME algorithm (Edgar, 2010). The remaining sequences were assigned to operational taxonomic units (OTUs) with a 97% similarity threshold using UCLUST. Taxonomy was assigned using the UCLUST consensus taxonomic assigner algorithm and the UNITE fungal database (Abarenkov et al., 2010).

2.5 Statistical analysis and network construction

Before statistical analysis, the normality and homogeneity of variance were tested by the Kolmogorov–Smirnov test and Levene's test, respectively. If normality was not met, log or square-root transformation was performed. Analysis of variance (ANOVA) was used via Duncan's test at $p < 0.05$ in IBM SPSS v26.0 to compare significant differences in NH_4^+ -N and NO_3^- -N content and AOA-*amoA*, AOB-*amoA*, *nirK* and *nosZ* gene abundance among the treatments. ANOVA was performed in SPSS ver. 27.0 (SPSS, Inc., Chicago, IL, United States).

Simpson's index was calculated to represent the alpha diversity of soil bacteria and fungi via the R package “vegan.” Changes in the community structure of the soil bacteria and fungi were evaluated using principal coordinate analysis (PCoA), and Adonis analysis was used to test for significant differences in the bacterial and fungal community structures between the treatments. A chord chart was generated by Wekemo Bioincloud¹ to visualize the main phyla and genera of bacteria and fungi. Variation in bacterial and fungal communities was quantified using PCoA Axis 1, and linear regression was used in Origin 2021 to establish associations between soil NH_4^+ -N and NO_3^- -N content and between different network cluster community variations and determine the relationships between specific network clusters and soil mineral N content.

To characterize the patterns of soil microbial interactions during the N cycle, we constructed a co-occurrence network with the R packages “igraph” and “WGCNA.” We constructed microbial networks using bacteria and fungi with relative abundances greater than 0.01%, screened nodes with Pearson's correlation coefficients greater than 0.6 and $p < 0.05$, performed modular analysis based on the connectivity between nodes, visualized the network through Gephi (ver. 0.10), and calculated information on network topological features (Ma et al., 2021). The within-cluster connectivity (Z_i) and among-cluster connectivity (P_i) of different clusters were calculated using MENA² (Ma et al., 2021) and filtered for peripherals ($Z_i \leq 2.5$, $P_i \leq 0.62$), connectors ($Z_i \leq 2.5$, $P_i > 0.62$), cluster hubs ($Z_i > 2.5$, $P_i \leq 0.62$) and network hubs ($Z_i > 2.5$, $P_i > 0.62$) (Deng et al., 2012).

Random forest (RF) analyses were performed to predict the relative importance of keystone taxa to soil NH_4^+ -N and NO_3^- -N

content via the R package “randomForest.” Heatmaps were constructed to reveal the relationships between the relative abundance of NH_4^+ -N and NO_3^- -N in Origin 2021. The enrichment of keystone taxa in the different treatment groups was visualized using bubble plots in Sangerbox³ (Shen et al., 2022).

3 Results

3.1 Synthesis and selection of metal pyrazole coordination compounds

Metal pyrazole coordination compounds were synthesized using cobalt, copper, zinc, and cadmium with pyrazole (Supplementary Figure S1), and their urease inhibition rates were evaluated at a concentration of 100 $\mu\text{mol/L}$. The results indicated that the copper pyrazole exhibited the most potent inhibitory effect on urease, significantly surpassing the other compounds ($p < 0.05$), with inhibition rates ranging from 9.30 to 22.68 times higher than those of the other groups (Supplementary Table S1, Equation 1). Subsequently, we determined the half-maximal inhibitory concentration (IC₅₀) of the copper pyrazole compound on urease, using acetohydroxamic acid as a positive control. The findings (Supplementary Figure S2) revealed that the IC₅₀ of the pyrazole-copper complex was 0.27 $\mu\text{mol/L}$, markedly lower than the 6.30 $\mu\text{mol/L}$ of the positive control ($p < 0.001$), representing only 4.29% of the positive control value. Consequently, we selected the pyrazole-copper complex for further research and analysis.

3.2 Soil mineral N turnover during incubation

Nitrification inhibitors had notable effects on the soil mineral N content during incubation. The UC and SUC treatments changed the contents of NH_4^+ -N and NO_3^- -N during the incubation period (Figure 1). Compared with those in the CK treatment, the contents of NH_4^+ -N and NO_3^- -N in each treatment were significantly greater ($p < 0.05$). The NH_4^+ -N content increased with incubation time, peaked on the 10th day after treatment, and then declined until the end of the incubation. On the 10th day, the UC treatment had the highest NH_4^+ -N content, which was significantly greater than that in the other treatments ($p < 0.05$) followed by U, SUC and CK; on the 60th day, the NO_3^- -N content in each treatment (except for CK) increased gradually with time, and the maximum value was shown on the 60th day. The NO_3^- -N content in the U treatment was significantly greater than that in the other treatments ($p < 0.05$), followed by that in the SUC, UC and CK treatments.

3.3 Soil N-cycling gene abundance and microbial community

The soil microbial traits exhibited different responses to each treatment (Table 1). The UC and SUC treatments significantly

¹ <https://www.bioincloud.tech>

² <http://ieg4.rccc.ou.edu/mena>

³ <http://www.sangerbox.com/tool>

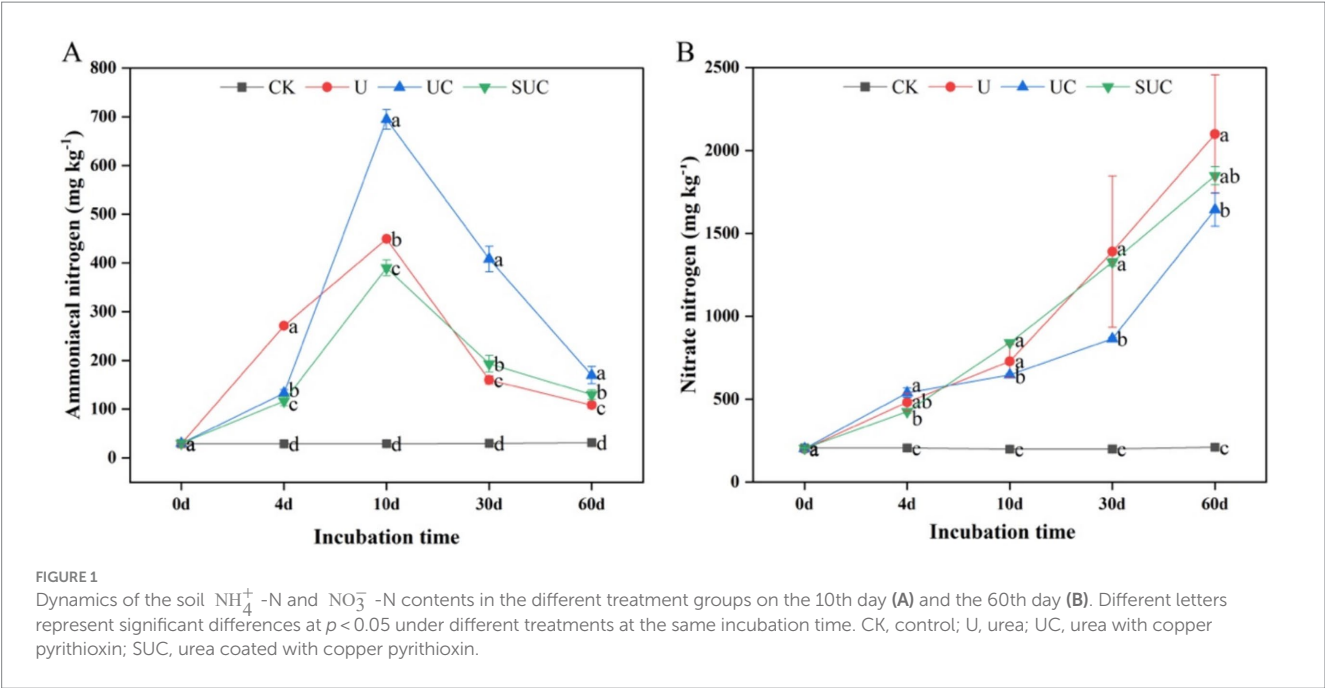


TABLE 1 Abundances of soil N-cycling genes under different treatments during incubation.

Cultivation time	Treatments	AOA-amoA ($\times 10^5$ copies g ⁻¹ dry soil)	AOB-amoA ($\times 10^5$ copies g ⁻¹ dry soil)	nirK ($\times 10^5$ copies g ⁻¹ dry soil)	nosZ ($\times 10^5$ copies g ⁻¹ dry soil)
10th day	CK	9.34 \pm 2.05 a	4.59 \pm 1.22 b	53.57 \pm 10.60 ab	9.52 \pm 1.47 a
	U	7.36 \pm 0.60 ab	39.63 \pm 4.08 a	28.51 \pm 3.24 c	10.70 \pm 0.61 a
	UC	3.40 \pm 1.28 c	1.62 \pm 0.66 b	46.97 \pm 1.49 b	4.53 \pm 1.14 b
	SUC	6.12 \pm 1.10 b	3.15 \pm 0.15 b	62.84 \pm 4.75 a	10.55 \pm 0.31 a
60th day	CK	1.59 \pm 0.04 c	1.53 \pm 0.35 c	68.23 \pm 8.25 a	3.89 \pm 0.95 c
	U	19.52 \pm 2.27 b	132.73 \pm 20.62 a	67.98 \pm 8.83 a	3.94 \pm 0.12 c
	UC	34.99 \pm 1.91 a	85.45 \pm 4.73 b	54.82 \pm 2.82 a	6.25 \pm 0.13 b
	SUC	3.75 \pm 0.41 c	1.89 \pm 1.25 c	31.05 \pm 5.77 b	9.38 \pm 0.34 a

Values are means \pm standard deviation ($n = 3$) and different letters within the same column denote significant differences ($p < 0.05$) under different treatments. CK, Control; U, urea; UC, urea with copper pyrithioxin; SUC, urea coated with copper pyrithioxin.

decreased nitrification and denitrification gene abundance ($p < 0.05$). On the 10th day, compared to those in the U treatment, the UC and SUC treatments significantly decreased the AOA-amoA gene abundance by 53.80 and 16.85%, respectively ($p < 0.05$); UC and SUC significantly decreased the AOB-amoA gene abundance by 95.91 and 92.05%, respectively ($p < 0.05$). The UC treatment decreased the nosZ gene abundance by 57.66%, which was significantly lower than that in the U treatment ($p < 0.05$). Similarly, on the 60th day, the nirK gene abundance was significantly lower (by 19.36 and 54.32%) in the UC ($p > 0.05$) and SUC ($p < 0.05$) groups, respectively, than in the U treatment group. Additionally, on the 60th day, the abundance of nosZ in the UC and SUC treatments was significantly higher than (that) in the U and CK treatments ($p < 0.05$), which is the opposite of what was observed on the 10th day.

Soil microbial diversity and community composition varied among the different treatments (Figures 2A, 3A; Supplementary Table S2). For bacteria, the α -diversities (Simpson's indices) of the UC and SUC treatments were significantly lower than

those of the CK and U treatments on the 10th day ($p < 0.05$), whereas there was no significant difference on the 60th day. There was no significant difference in the fungal Simpson's indices under the different treatments at the 10th and 60th days.

The changes in soil microbial community composition were also visualized by PCoA (Figures 2A, 3A), and Adonis analysis also revealed significant differences in the soil microbial community among the treatments on the 10th and 60th days ($R^2 = 0.39$, $p < 0.01$; $R^2 = 0.50$, $p < 0.001$; Figures 2A, 3A).

3.4 Microbial co-occurrence network

A series of co-occurrence networks were constructed on the 10th and 60th days, and the topological characteristics of the network are shown in Figure 4. The microbial network on the 10th day was divided into four main clusters, and the bacterial population was larger than the fungal population. The correlation between ASVs was mainly

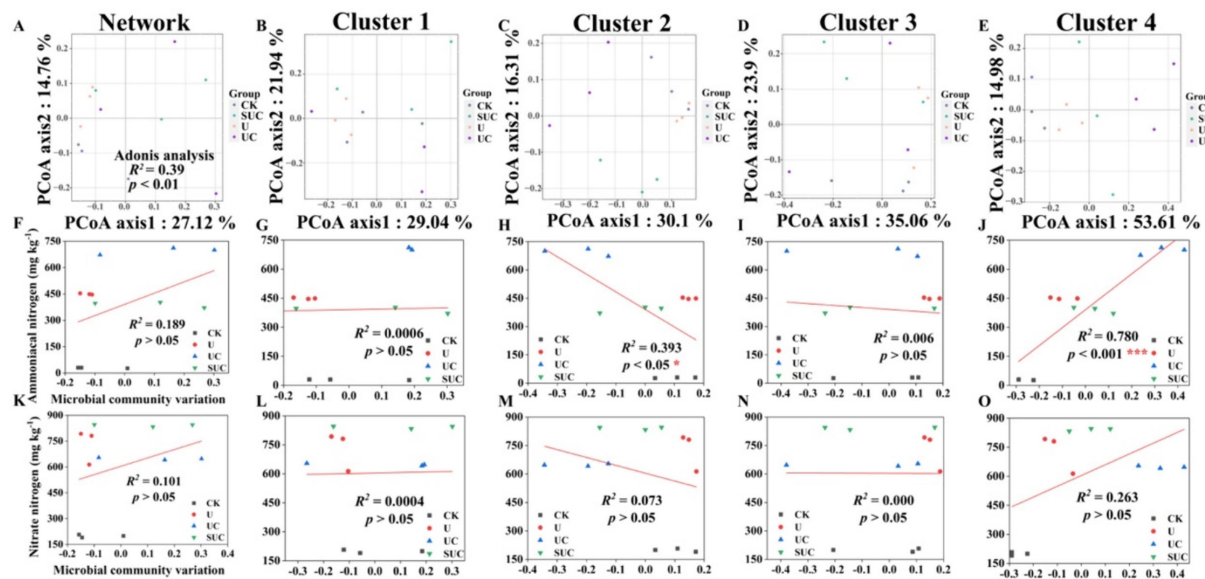


FIGURE 2

PCoA of the community structures of soil microorganisms and linear regression analysis of microbial community variation and NH_4^+ -N and NO_3^- -N contents after the 10th day of cultivation. PCoA analyses of the whole microbial community and the Cluster 1 to Cluster 4 community (A–E); linear regression analyses of the microbial community and NH_4^+ -N content at the cluster scale (F–J); and linear regression analyses of microbial community change and NO_3^- -N content at the cluster scale (K–O). CK, control; U, urea; UC, urea with copper pyriithoxin; SUC, urea coated with copper pyriithoxin.

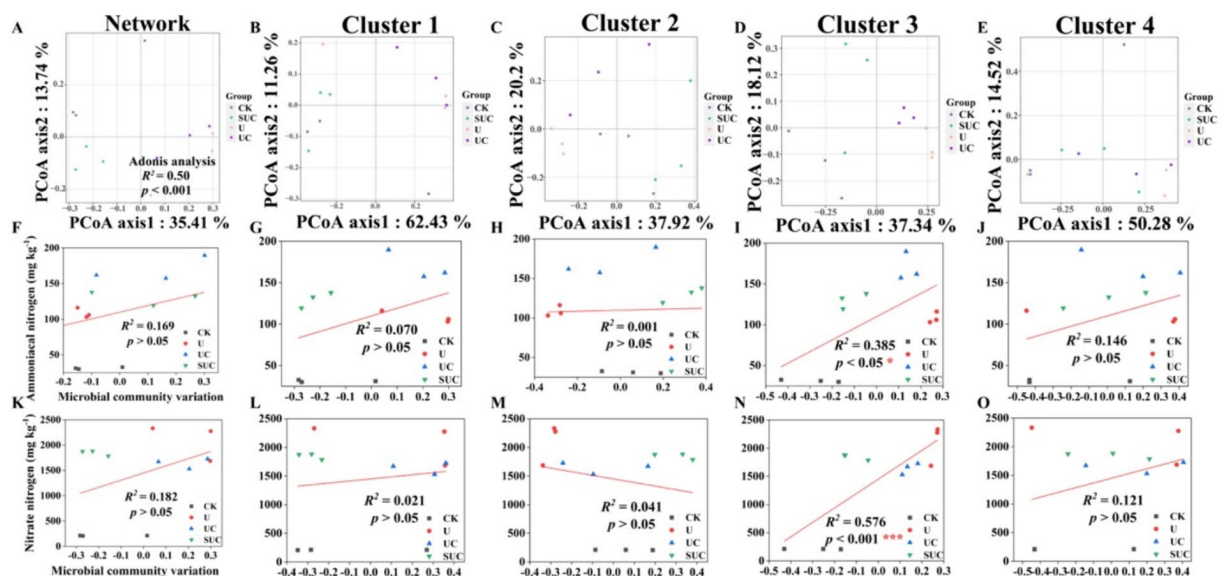


FIGURE 3

PCoA of the community structures of soil microorganisms and linear regression analysis of microbial community variation and NH_4^+ -N and NO_3^- -N contents after the 60th day of cultivation. PCoA analyses of the whole microbial community and the Cluster 1 to Cluster 4 community (A–E); linear regression analyses of the microbial community and NH_4^+ -N content at the cluster scale (F–J); and linear regression analyses of microbial community change and NO_3^- -N content at the cluster scale (K–O). CK, control; U, urea; UC, urea with copper pyriithoxin; SUC, urea coated with copper pyriithoxin.

positive (68.22%), with an average network clustering coefficient of 0.229. On the 60th day, there were four dominant clusters in the microbial network containing predominantly positive correlations (62.80%), with an average network clustering coefficient of 0.444. Overall, the percentage of edges linking bacteria and fungi in the

network was 7.41–28.74% greater than that for fungus-to-fungus connections. The specific network cluster topology features of the above clusters are shown in [Supplementary Figure S3](#).

ZP plots were constructed to identify the topological roles of each node in the network ([Figure 5](#)). On the 10th day, 92 species

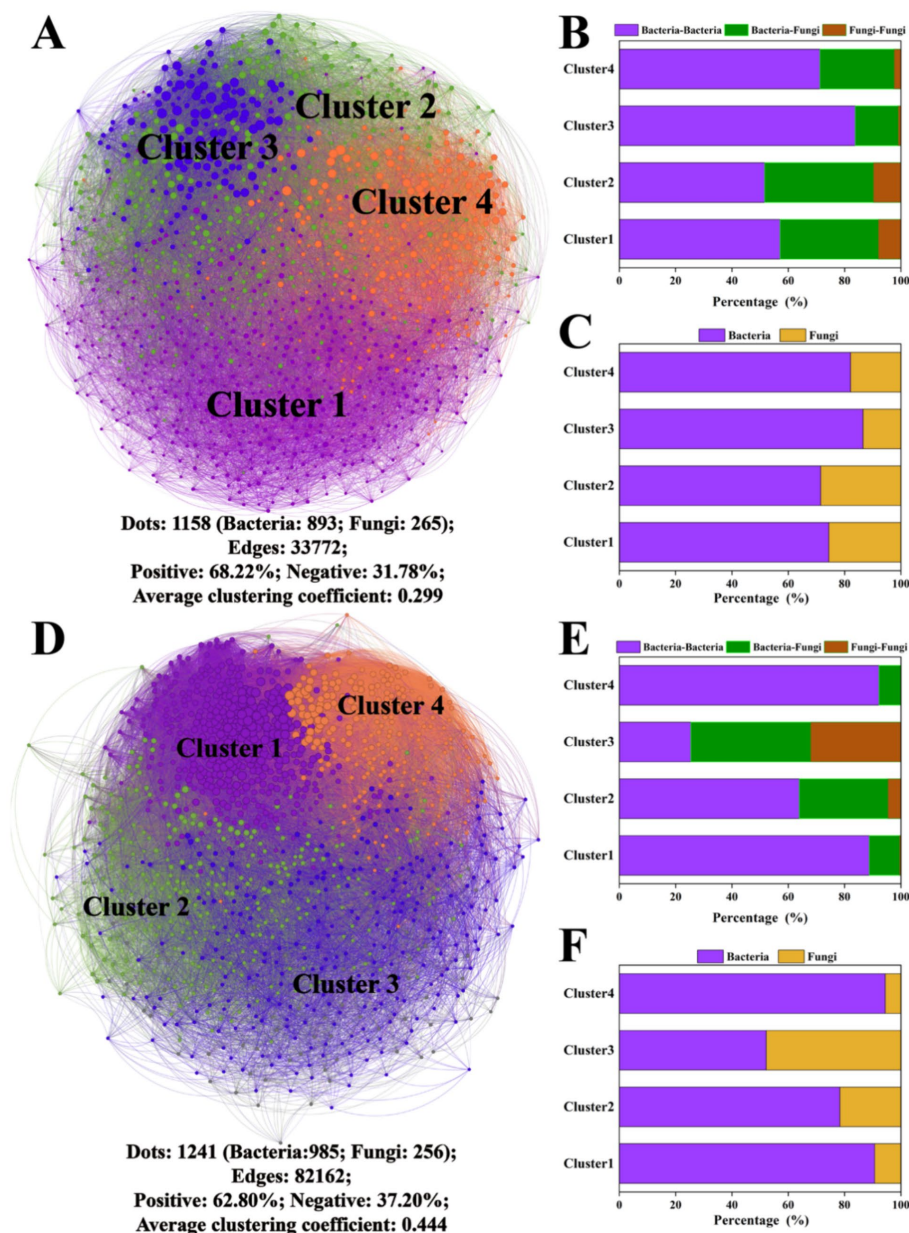


FIGURE 4

Microbial network analysis after the 10th and 60th days of cultivation. Microbial networks on the 10th day (A) and 60th day (D); the proportions of connections between different bacterial and fungal ASVs within the network on the 10th day (B) and 60th day (E); and the proportions of bacterial and fungal ASVs within the network on the 10th day (C) and 60th day (F). The different colors of the nodes in the microbial networks represent different clusters.

were detected as keystone taxa. There were 1, 6 and 85 taxa observed within network hubs, cluster hubs and connectors, respectively (Figure 5A). The distribution of microbial keystone taxa was 16% in Cluster 1, 28% in Cluster 2, 29% in Cluster 3 and 24% in Cluster 4 (Figure 5B). These bacteria belonged to the bacterial phylum Proteobacteria (49%) and the fungal phylum Ascomycota (71%) (Supplementary Table S3). On the 60th day, there were 51 keystone taxa in all the clusters, and 0, 1 and 50 taxa were observed in the network hubs, cluster hubs and connectors, respectively. The distribution of microbial keystone taxa was 49% in Cluster 1 and 37% in Cluster 4 (Figure 5C). The keystone taxa mainly belonged to the bacterial phylum Proteobacteria (58%) and the fungal

phylum Ascomycota (50%) (Supplementary Table S3). The taxonomic information of soil keystone taxa is shown in Supplementary Table S4.

3.5 Relationships between soil microbial traits and mineral N

Soil microbial N-cycling genes may participate in mineral N turnover. A heatmap was generated (Supplementary Figure S4), which shows that the AOA-*amoA* gene abundance was negatively correlated with the NH_4^+N content ($p < 0.001$) on the 10th day, while the

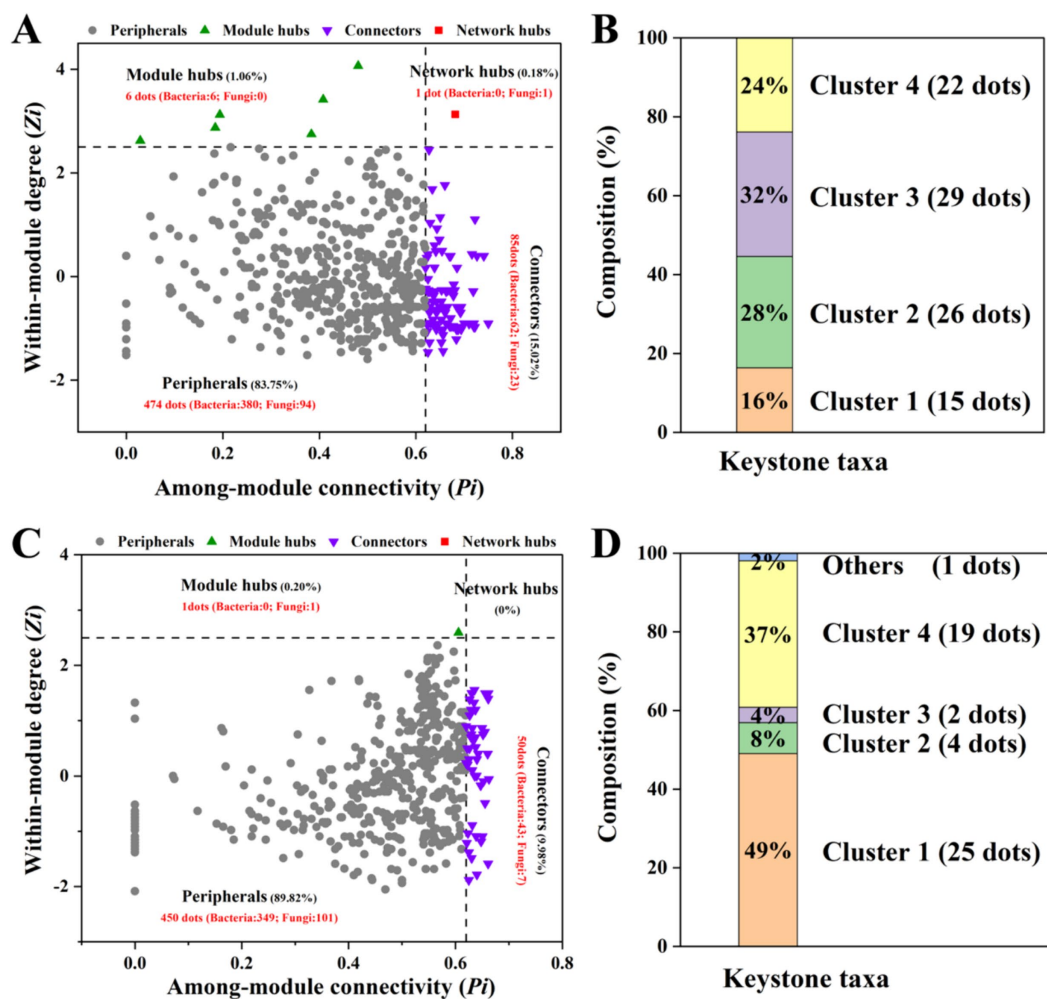


FIGURE 5

Zi-Pi plot showing the keystone taxa in the different microbial networks on the 10th day (A) and 60th day (C). The proportion of keystone taxa in different network clusters on the 10th day (B) and the 60th day (D) of incubation.

AOA-*amoA* gene abundance was positively correlated with the NH_4^+ -N content ($p < 0.01$) on the 60th day. The *NirK* gene abundance was negatively correlated with the NH_4^+ -N content on the 60th day ($p < 0.05$).

Linear regression (Figures 2, 3) revealed that the soil NH_4^+ -N content was significantly correlated with the microbial community variation in Clusters 2 and 4 on the 10th day ($R^2 = 0.393$, $p < 0.05$ for Cluster 2; $R^2 = 0.780$, $p < 0.001$ for Cluster 4). On the 60th day, the soil NH_4^+ -N and NO_3^- -N contents were significantly positively correlated with those in Cluster 3 ($R^2 = 0.385$, $p < 0.05$ for NH_4^+ -N content; $R^2 = 0.576$, $p < 0.001$ for NO_3^- -N content).

It is well known that keystone taxa are crucial drivers of specific cluster communities and affect the function of the whole network. Therefore, it was necessary to clarify the effect of keystone taxa on mineral N turnover during incubation. A series of random forest models (Supplementary Figure S5) were used to determine the relative importance of the selected keystone taxa for the soil mineral N content. We selected 5 bacterial (BASV159, BASV661, BASV129, BASV430, and BASV197) and fungal (FASV59, FASV39, FASV65, FASV69, and FASV4) keystone taxa with the highest relative abundance within Cluster 2 and 4 on the 10th day for further study. A heatmap (Supplementary Figure S6) showed that the relative

abundances of BASV159, BASV661, BASV129, BASV430, FASV59 and FASV39 were significantly negatively correlated with the NH_4^+ -N content on the 10th day. Moreover, the relative abundance of FASV188 was significantly negatively correlated with the NH_4^+ -N content, while the relative abundances of FASV188 and FASV188 were significantly negatively correlated with the NO_3^- -N content on the 60th day.

Furthermore, to visualize the changes in the relative abundance of the selected keystone taxa across treatments, a bubble plot was constructed. Figure 6 shows that, except for FASV69 and BASV197, the relative abundances of the keystone taxa in the CK treatment were greater than those in the other treatments. The relative abundances of BASV129, BASV159, BASV430, and BASV661 in the UC treatment were significantly lower ($p < 0.05$) than those in the U treatment.

4 Discussion

Generally, N leaching or mineralization in agroecosystems results in low N utilization efficiency (Al-Juthery et al., 2021). Nitrification inhibitor application can mediate the soil microbial community and

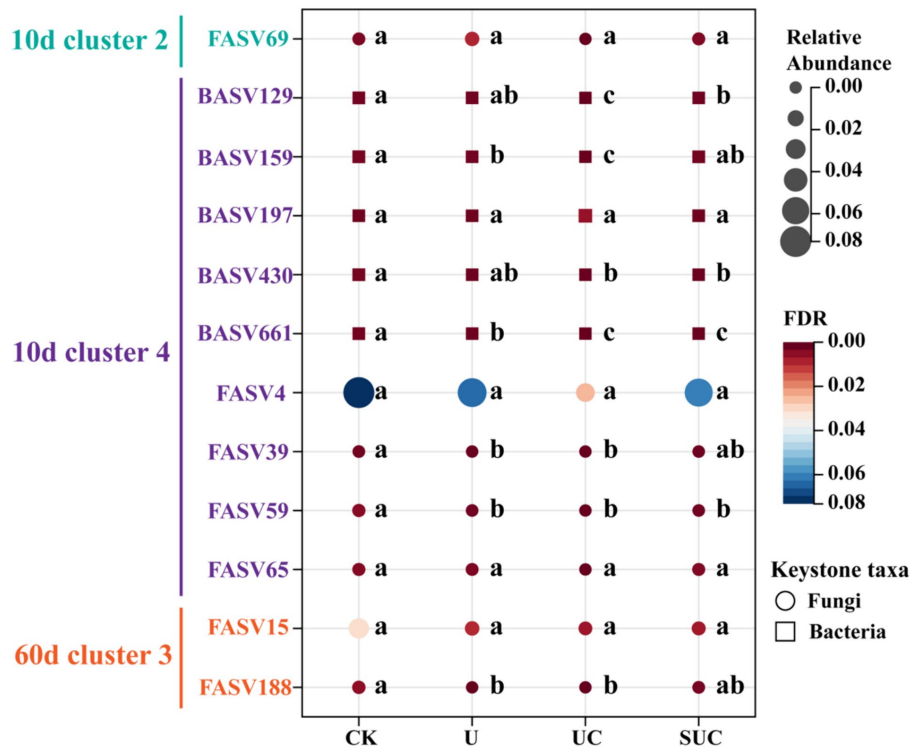


FIGURE 6

Bubble plot of the relative abundance of microbial keystone taxa under different treatments. The different colors and node sizes represent the relative abundance of keystone taxa in each treatment, with circles representing fungal taxa and squares representing bacterial taxa. Different letters in the same row denote significant differences ($p < 0.05$) among the different treatments. CK, control; U, urea; UC, urea with copper pyriothoxin; SUC, urea coated with copper pyriothoxin.

function and thus has become an accepted way to address this issue (Tindaon et al., 2012; Adhikari et al., 2021; Mahmud et al., 2021; Woodward et al., 2021). Additionally, metal ions can inhibit urease activity, thereby slowing down the hydrolysis of urea (Li et al., 2019). The effects of different metal ions on urease are various; Zaborska et al. (2004) found that Cu^{2+} and Zn^{2+} exhibit the strongest inhibitory effects on urease, consistent with our findings. This inhibition may be due to the reaction of metal ions with the enzyme's thiol groups, chelation with the substrate, or interference with the enzyme-substrate complex (Tang et al., 2020). Overall, copper pyrazole effectively inhibits urease activity and nitrification (Li et al., 2022), indicating a promising application for pyrazole-copper in agriculture.

In the present study, the soil mineral N content and microbial traits changed after copper pyrazole application. Our research improves our understanding of the application of copper pyrazole and the microbial mechanisms underlying nitrification and denitrification inhibition by this compound.

4.1 Copper pyrazole affects soil mineral N turnover by inhibiting microbial functional genes

The application of nitrification inhibitors is the key agronomic measure that influences soil mineral N content, which is likely regulated by microbial functions (Liu Y. et al., 2014; Woodward et al., 2021). Nitrification is primarily mediated by the AOA-*amoA* and AOB-*amoA* genes, which are largely regulated by NH_4^+ -N availability (Cáceres et al.,

2018; Chen Z. et al., 2021). Under conditions in which NH_4^+ -N levels are not limiting, AOB-*amoA* may play a more dominant role in nitrification than AOA-*amoA* (Prosser and Nicol, 2012; Ouyang et al., 2017). Consistent with these findings, the present study revealed greater AOB-*amoA* gene abundance in the U treatment (Table 1), indicating that the nitrification process was mainly dominated by AOB-*amoA*. Notably, the abundance of the AOB-*amoA* gene was significantly lower in the UC treatment than in the U treatment during incubation ($p < 0.05$, Table 1). This finding suggested that pyrazole may inhibit the first step of nitrification (NH_4^+ -N to hydroxylamine) mainly by inhibiting the activity of AMO, which is encoded by the *amoA* gene, in AOA and AOB (Wrage et al., 2001; Zhang et al., 2013, 2022). Several studies have shown that Cu has direct inhibitory effects on both the abundance of AOA-*amoA* and AOB-*amoA* genes and further inhibits nitrification (Rijk et al., 2023; Upadhyay et al., 2023). As a result, the NH_4^+ -N content was significantly greater in the UC treatment than in the U treatment ($p < 0.05$; Figure 1), while the NH_4^+ -N content in the SUC treatment was significantly lower than that in the U treatment on the 10th day ($p < 0.05$) and then gradually increased until the end of incubation. This was mainly because of the slow release of N and copper pyrazole from the coated fertilizer, which prolonged the effective treatment time and promoted an increase in the mineral N content (Bröckel and Hahn, 2004; Naz and Sulaiman, 2016). Thus, copper pyrazole retards the transformation of NH_4^+ -N to NO_3^- -N via nitrification by inhibiting AOB-*amoA* gene abundance. Collectively, these findings imply that the chelation complexes formed between pyrazole and copper may significantly inhibit nitrification rates. Therefore, more NH_4^+ -N could be preserved in the soil for longer periods of time, enhancing N utilization efficiency. Meanwhile, on the 10th day,

the AOA-*amoA* abundance in the UC treatment was significantly lower than that in the U treatment ($p < 0.05$), possibly due to the addition of copper pyrazole. The study by Shi et al. (2017) also demonstrated that pyrazole derivatives significantly reduced AOA-*amoA* abundance in the spring, consistent with our findings. Whereas on the 60th day, the abundance of the AOA-*amoA* gene in the UC treatment was significantly higher than in the U and SUC treatments (Table 1). This increase might be due to the different rates of urea hydrolysis, as well as the potential role of Cu^{2+} as a cofactor for the AMO enzyme (Glass and Orphan, 2012), which could be released into the soil over time.

The *NirK* and *nosZ* genes have been proven to be involved in nitrite and nitrous oxide reduction, respectively (Bröckel and Hahn, 2004; Naz and Sulaiman, 2016). The *nirK* gene abundance in SUC and UC was lower than that in U on the 60th day due to the inhibitory effect of Cu on denitrification (Liu et al., 2016). Interestingly, the NO_3^- -N content was significantly greater in the U treatment ($p < 0.05$) than in the SUC and UC treatments on the 60th day, consistent with the differences in *nirK* gene abundance. This is because the presence of copper pyrazole delayed the nitrification process and the associated change from NH_4^+ -N to $-\text{N}$, which is consistent with the findings of Li et al. (2022). Moreover, these results were well supported by the significant negative correlation between *nirK* gene abundance and NH_4^+ -N content in the correlation analysis ($p < 0.05$, Supplementary Figure S4). Additionally, on the 60th day, the abundance of *nosZ* in the UC and SUC treatments was significantly higher than in the U and CK treatments ($p < 0.05$, Table 1). The findings align with the research by Corrochano-Monsalve et al. (2021), which may be due to Cu^{2+} affecting N_2O reductase activity (Sullivan et al., 2013). However, the relative abundance of these genes compared to the CK remains within the same order of magnitude, indicating that the denitrification process may not have been significantly affected. Furthermore, the *nirK* gene abundance in SUC was significantly lower than that in UC ($p < 0.05$), probably due to the slow release of copper pyrazole. Thus, copper pyrazole inhibits the denitrification process by inhibiting the abundance of *nirK* genes, thereby prolonging the retention of NO_3^- -N in soil. This also suggests that the use of coating agents further prolonged the effect of copper pyrazole. In summary, copper pyrazole affects soil mineral N transformation by inhibiting the specific abundance of microbial nitrification and denitrification genes.

4.2 The relationship between keystone taxa and mineral N content supported the effect of copper pyrazole on soil N turnover

Soil microbial communities are essential for maintaining soil health and functionality (Fuhrman, 2009), with keystone taxa playing a crucial role in driving the functions of these communities (Ma et al., 2021). Co-occurrence networks are commonly employed in microbial analysis to identify key species that may significantly influence microbial interactions (Duan et al., 2021). Research has demonstrated that keystone taxa-derived specific clusters influence mineral N turnover (Delgado-Baquerizo et al., 2018; Ma et al., 2021; Wang et al., 2022). Therefore, revealing the function of keystone taxa within microbial clusters is vital for clarifying the microbial mechanisms involved in soil N cycling after copper pyrazole application.

In this study, Cluster 4 was significantly correlated ($p < 0.05$) with the soil NH_4^+ -N content on the 10th day. Additionally, *Mycobacterium* and *Cronobacter sakazakii* within Cluster 4 were selected for further study. Egamberdiyeva (2007) indicated that *Mycobacteria* promoted the growth of plant roots by facilitating N uptake in nutritionally poor environments and thus accelerated N consumption, which is consistent with our results. Ben Taheur et al. (2016) and Cargnin et al. (2023) demonstrated that *Cronobacter sakazakii* could encode a denitrification gene, which could result in potential N loss. In conclusion, the increased relative abundance of *Mycobacterium* and *Cronobacter sakazakii* may stimulate nitrification by regulating microbial Cluster 4, enhancing the transformation of soil NH_4^+ -N to NO_3^- -N on the 10th day.

In the late stage of incubation, the Cluster 3 community was significantly correlated with the soil NH_4^+ -N ($p < 0.05$) and NO_3^- -N ($p < 0.001$) contents. *Fusariumes* were selected as the keystone taxa within Cluster 3 for further study. Subbarao et al. (2006) demonstrated that *Fusarium* propagules were destroyed more rapidly with NH_4^+ -N, particularly following pyrazole application. Moreover, *Fusarium* could markedly contribute to N_2O emissions (Zheng et al., 2020; Shen et al., 2021). In our study, correlation analysis revealed negative associations between *Fusarium* and both NH_4^+ -N and NO_3^- -N content ($p < 0.05$), which supported these conclusions. Therefore, the Cluster 3 community likely facilitates the denitrification of NO_3^- -N to form N_2O .

Notably, the abundance of keystone taxa in Clusters 2 and 4 under copper pyrazole treatment (both UC and SUC) was lower than those in the other treatments on the 10th day (Figure 6). This observation suggested that copper pyrazole may effectively inhibit nitrification by altering the microbial composition and function within these clusters (Gupta et al., 2023), increasing NH_4^+ -N accumulation. Additionally, previous studies have shown that nitrapyrin can inhibit keystone taxa (*Nitrobacter* sp.) mediating the second nitrification step, prolonging N fertilizer availability (Woodward et al., 2021). Consistent with these findings, the results of the present study revealed a reduced abundance of Cluster 3 keystone taxa in UC compared to that in the other treatments on the 60th day (Figure 6), suggesting that copper pyrazole may also limit the denitrification of NO_3^- -N into N_2O . Further investigations are needed to experimentally verify that the above keystone species play an important role in the N transformation. Overall, copper pyrazole addition can change the soil N turnover process by altering the relative abundance of keystone taxa. Therefore, the construction of co-occurrence network further supports the role of copper pyrazole in improving the N fertilizer availability from another perspective.

5 Conclusion

Overall, copper pyrazole affects soil mineral N transformation by decreasing the abundance of nitrification and denitrification genes and mediating the functions of the soil microbial community. Copper pyrazole exhibited significantly higher inhibition rates on urease compared to other metal-pyrazole coordination compounds, and inhibited the activities of nitrification genes (especially of AOB-*amoA*) and denitrification genes (especially *nirK*), slowing nitrification and denitrification processes, and promoting the accumulation of NH_4^+ -N and NO_3^- -N during incubation.

Moreover, specific taxa play pivotal roles in soil mineral N turnover. In the initial phase, copper pyrazole potentially decreased the relative abundance of *Mycobacterium* and *Cronobacter sakazakii*,

suppressing nitrification and retarding NH_4^+ -N conversion to NO_3^- -N. In the late stage, copper pyrazole possibly limited the propagation of *Fusarium*, thereby inhibiting NO_3^- -N denitrification to N_2O . This study established the dynamic relationships among copper pyrazole, soil microbial traits and mineral N transformation, providing a scientific and theoretical basis for the application of novel inhibitors.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material. Raw sequence data obtained in this study have been deposited in the NCBI repository, accession number PRJNA1159739 (<https://www.ncbi.nlm.nih.gov/sra/PRJNA1159739>).

Author contributions

YW: Writing – original draft, Formal analysis, Writing – review & editing. WZ: Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. XZ: Writing – review & editing, Methodology, Data curation, Conceptualization. MC: Writing – review & editing, Supervision, Conceptualization. ZN: Writing – review & editing, Validation, Data curation. MZ: Writing – review & editing, Visualization, Data curation. JL: Writing – review & editing, Investigation, Funding acquisition. YD: Writing – review & editing, Funding acquisition. LW: Writing – review & editing, Supervision, Funding acquisition.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2024.1433816/full#supplementary-material>

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Nitrification inhibitor promotes fertilizer N stabilization in soil as organic forms during a growing season of maize: a field ^{15}N tracer study

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There is limited knowledge regarding the impact of nitrification inhibitors (NIs) and straw application on fertilizer N retention and in-season release. We conducted a trial to study the transformation of ^{15}N -labeled urea in soils during the growing season of maize. To facilitate multiple destructive samplings throughout the season, we utilized a larger plot (25 m²) and a lower abundance ^{15}N -fertilizer (1.193%) than usual. Soil extractable mineral N, mineral fixed ammonium, and organic N (ON) recovered $20 \pm 21\%$ (mean \pm standard deviation), $6 \pm 5\%$, and $25 \pm 6\%$ of the applied fertilizer N across three sampling stages of the growing season. On average, the bioavailability of fertilizer N in extractable mineral form was four times higher than that of mineral fixed ammonium. In contrast, fertilizer-derived ON represented a relatively stable N pool, maintaining high content throughout the growing period and becoming the major form (82%–93%) in the pool of total soil ^{15}N at the physiological maturity stage of maize. Moreover, the co-application of nitrapyrin (a type of NI) significantly promoted fertilizer N storage in the ON form while the effect of straw was not significant. In conclusion, the NI-induced promotion of fertilizer-derived ON likely plays a critical role in storing fertilizer N for subsequent cultivations, rather than providing N nutrients for crop uptake during the current season.

KEYWORDS

soil N transformation, microbial N assimilation, fertilizer N fate, immobilization and remineralization, straw returning

1 Introduction

Fertilizer nitrogen (N) applied to agricultural soil can be rapidly converted into mineral N forms (ammonium and nitrate), which increases the soil N supply to crops and contributes to yield improvements (1). However, the prematurely accumulated mineral N in the topsoil is easily lost to the environment, failing to synchronize the soil N supply with root N absorption (2, 3). Two measures to conserve fertilizer N in soils are the application of nitrification inhibitors (NIs) and the incorporation of straw. These strategies aim to transform fertilizer-derived mineral N into stable N pools during the early stage of crop growth, ensuring a gradual release that meets the crop's N demand during the middle and late stages (4, 5). However, due to a lack of experimental methods, it remains unclear whether and to what extent these measures can enhance the release of early-conserved fertilizer N during the middle and late stages of crop growth (6).

The ^{15}N tracer technique is the primary tool used to trace the transformation and fate of fertilizer N in soil-crop systems. Over the last four decades, many studies utilizing this technique have explored the effects of various management practices on fertilizer N fate (7, 8). However, due to limitations in the detection accuracy of ^{15}N , most experiments required the use of high abundance ^{15}N -fertilizer, which increased costs. To reduce expenses, researchers have limited the size of plots (e.g., $<2\text{ m}^2$). As a result, most sampling occurred only at harvest, as small plot sizes restricted the frequency of destructive sampling (9). However, with advancements in testing technology over the past decade, the detection accuracy of ^{15}N has significantly improved (10, 11), allowing for the use of lower abundance ^{15}N -fertilizer and enabling larger plot sizes.

We conducted a tracer study using lower abundance ^{15}N -fertilizer and a larger plot size to monitor the seasonal dynamics of fertilizer-derived N in soil under different nutrient management practices (i.e., NI and straw application). Our goal was to compare the bioavailability of various soil N constituents to root uptake during one growing season of maize. We hypothesize that these practices can enhance the transformation of fertilizer N into fixed ammonium and organic N pools, which can be released for crop absorption and utilization during the middle or late stages of maize growth.

2 Materials and methods

2.1 Experimental design

The field ^{15}N tracer trial was conducted in a suburban area of Gongzhuling City, Northeast China ($43^{\circ}30'\text{N}$, $124^{\circ}48'\text{E}$). The site features a semi-humid continental monsoon climate. From 2011 to 2020, the average annual precipitation and temperature at the study site were 666 mm and 6.8°C , respectively. Maize, the region's primary cereal crop, has been cultivated annually for decades without any rotation with other crops. The soil in the area is classified as a Mollisol according to the US soil taxonomy and is typical black soil in China. Two days prior to the trial, soil samples were collected from a depth of 0–20 cm to assess soil characteristics.

The background information of the soil: pH, 6.19; total carbon, 19.1 g/kg; total N, 1.52 g/kg; sand, 15%; silt, 52%; clay, 33%.

Five treatments were established: 1) 100%N, 200 kg urea N/ha; 2) 100%N+S (straw), 200 kg urea N/ha and 2400 kg dry straw/ha; 3) 80% N, 160 kg urea N/ha; 4) 80%N+NI, 160 kg urea N/ha and nitrification inhibitor (Nitrapyrin, $\text{C}_6\text{H}_3\text{C}_{14}\text{N}$, 1.6 kg/ha); 5) 80%N+NI+S, 160 kg urea N/ha, 1.6 kg Nitrapyrin/ha and 2400 kg dry straw/ha. Each treatment consisted of three plots (replicates). To allow for multiple samplings within the growing season, each plot had an area of 25 m^2 , and all areas were labeled by ^{15}N fertilizer. We arranged a trial for ridge-furrow cultivation similar to the management practices used by local farmers, as described by Quan et al. (9). Specifically, all fertilizers (including ^{15}N -urea), NI, and maize straws were placed on a ridge 5 cm below the ground before sowing, all on the same day (May 6). No topdressing was performed during the growing period of maize. The abundance of applied ^{15}N -urea was 1.193%. After fertilization, a hand-powered hole-drilling machine was used for sowing on the ridge. The local farmers' agronomic practices were followed during the maize growth period.

The maize variety used in this study was *Xianyu 335*, with a planting density of 70000 plants per hectare. The row and plant spacings were set at 60 cm and 20 cm, respectively. Other agronomic practices during the maize growth period adhered to local farmers' procedures. Irrigation and fertilization were not conducted during the growth period. A nearby meteorological station recorded daily mean air temperature and precipitation throughout the trial period (Figure 1).

2.2 Soil and plant sampling

Soil samples were collected three times after fertilization and sowing, on days 42, 82, and 152 (Figure 1). At these three time points, maize growth stages were recorded as V6, VT, and R6, corresponding to the six-leaf, tasseling, and physiological maturity stages, respectively. To account for the uneven distribution of fertilizers in the soil, we used a frame that covered both the ridge and furrow to assist with sampling. In all cases, soil within the frame was excavated from a depth of 0–10 cm, mixed thoroughly by hand on plastic sheeting, and a portion was set aside for sampling.

Plant samples were collected exclusively during the maize harvest. All aboveground plant material within the plot was harvested to quantify the mass of both the straw and maize cobs. Additionally, three maize plants were randomly selected and divided into four parts: stem, leaf, cob, and grain. Each part was weighed separately to determine their relative proportions. Fresh samples were chopped into pieces smaller than 3 cm. Portions of these samples were transported to the laboratory, where they were dried in an oven at 70°C . This process determined their water content and facilitated the calculation of the dry weight of each part.

2.3 Chemical and isotope analysis

Subsamples of fresh soil were extracted with 2 M potassium chloride, shaken for one hour, and then filtered through filter paper

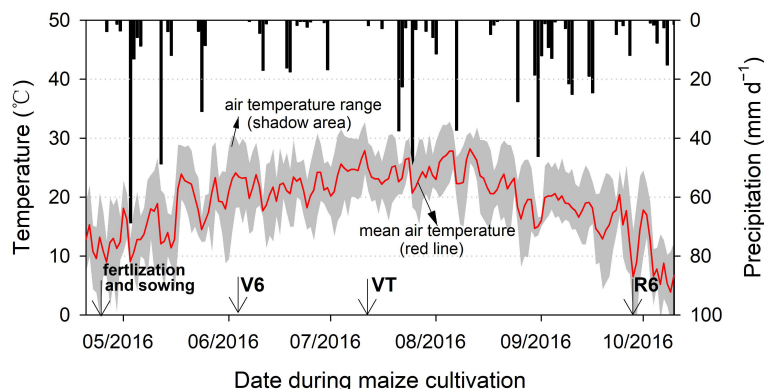


FIGURE 1
Average daily air temperature and precipitation throughout the experiment.

to measure the mineral N ($\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$) concentrations. Subsequently, $^{15}\text{N}\text{-NH}_4^+$ and $^{15}\text{N}\text{-NO}_3^-$ abundances in the extracts were measured using the hypobromite oxidation/hydroxylamine reduction method (10) and the modified azide method (11). Dry soil and plant samples were pulverized and finely ground to analyze the total nitrogen (TN) concentration and ^{15}N abundance using an elemental analyzer and a stable isotope ratio mass spectrometer (EA-IRMS). Mineral fixed $\text{NH}_4^+\text{-N}$ concentration and its ^{15}N abundance in residual soils after extraction were determined by the EA-IRMS after organic N was removed by excessive alkaline KOBr solution (12). The fertilizer-derived organic N (ON), mainly soil microbial biomass or necromass N or other non-biologically synthesized organic matter, was calculated by subtracting fertilizer-derived $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$ and mineral fixed $\text{NH}_4^+\text{-N}$ from soil fertilizer-derived TN based on the mass-balance principle (13). In fertilizer-derived ON pool, extractable organic N (EON) was considered ignorable owing to its low concentration and ^{15}N recovery in our previous studies (14).

2.4 Statistical analysis

Statistical analysis in this study was performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance was used to test differences among the five treatments, and multiple comparisons were performed using the least significant difference (LSD) test with a 95% confidence interval.

3 Results and discussion

Over the course of growth from the V6 to R6 stage, the ^{15}N recoveries of the extractable ammonium (NH_4^+) and nitrate (NO_3^-) pools decreased from 21%–52% to 0.1%–4% and from 9%–18% to 1%–4%, respectively, primarily due to soil turnover, crop uptake, and environmental losses (Figure 2). Compared with conventional fertilization (100%N and 80%N), the application of NI (80%N+NI and 80%N+NI+S) slowed the nitrification process, increased the

residence time of fertilizer-derived extractable NH_4^+ , enhanced root- or soil microbe-mediated N immobilization, and decreased the accumulation of highly mobile NO_3^- (15, 16). As a result, NI application can encourage fertilizer N retention and may enhance subsequent soil N supply during the middle and late growth stages (17). However, this study suggests that the remineralization ability of newly formed fertilizer-derived organic N (ON) was lower than expected. For treatments without NI addition, ^{15}N recoveries as soil ON decreased by 8%–27% as the growth stage progressed from V6 to R6 ($P>0.05$). In contrast, for treatments with NI addition, ^{15}N recoveries as soil ON were maintained and even increased at the VT and R6 stages of growth (Figure 2). The stable ^{15}N recovery in fertilizer-derived ON pool is likely due to its resistant components, as well as the physicochemical protection by minerals and aggregates (18).

When fertilizer N was converted to ON through microbial immobilization or anabolism, its availability decreased not only in the current season but also in subsequent seasons (3, 19). For example, Smith and Chalk (8) conducted a global meta-analysis using published data from *in situ* ^{15}N tracer trials and found that the legacy N utilization by crops decreased significantly compared to the first season. The low legacy N utilization indicates that the newly retained ON may require a long period before it is released back into the soil, and the related mechanism remains to be further explored. In recent years, an increasing number of studies suggest that soil N assimilated by microorganisms is an important process of soil N stabilization (20, 21). The “microbial carbon pump” theory of Liang et al. (22), and the “mineral carbon pump” theory of Xiao et al. (23) both provide explanations for this phenomenon.

Since our experiment was conducted over only one season, the responses of grain yield and maize N uptake to NI and straw application were not significant. However, the application of NI (80%N+NI and 80%N+NI+S) significantly reduced the proportion of maize N derived from fertilizer (%Ndff) and the recovery of ^{15}N in aboveground biomass (Figure 3). Compared to treatments without NI addition, soil ^{15}N -nitrate availability decreased significantly in the NI addition treatments (Figure 2). Considering that maize is a nitrate-preferring plant, the decreases in %Ndff and crop ^{15}N recovery under NI treatments are understandable. These results are consistent with the findings of Ma et al. (21) from their

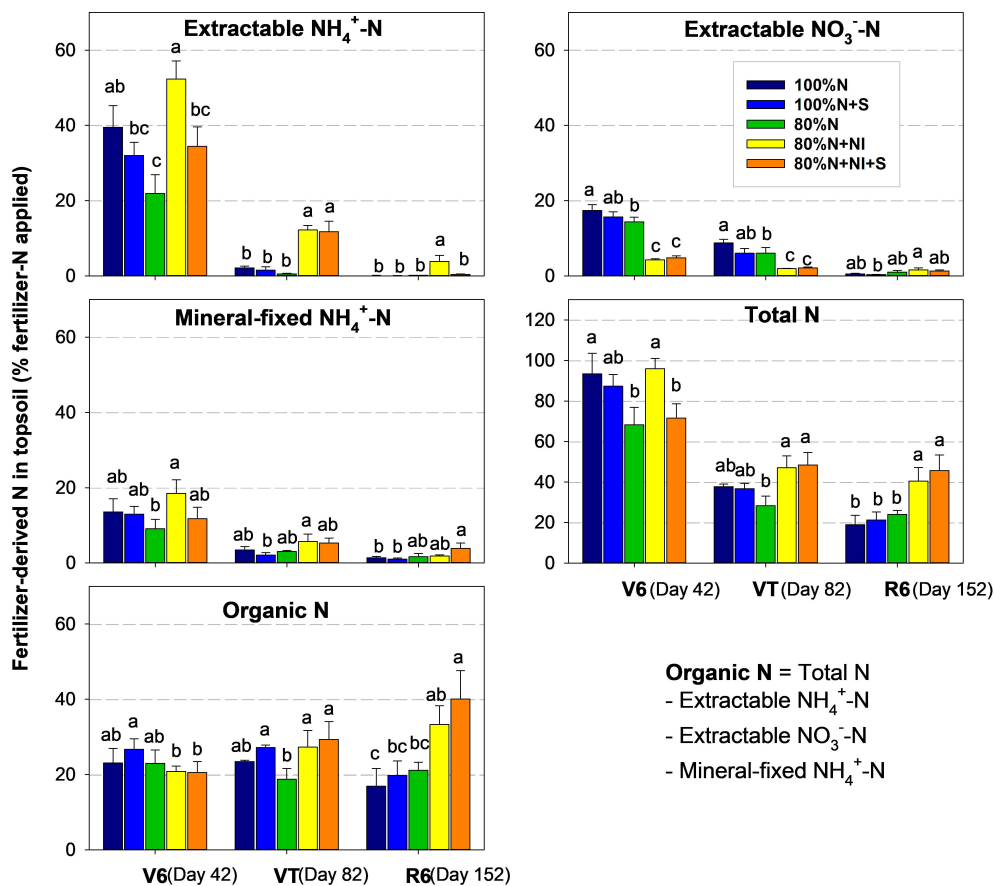


FIGURE 2

Fertilizer N components in topsoil (0–10 cm) during the maize growing season. The y-axis represents the proportion of different soil N components derived from the applied fertilizer N. Organic N (ON) was calculated as the difference between total N and mineral N (extractable NH_4^+ -N + extractable NO_3^- -N + mineral fixed NH_4^+ -N). The recovery of ^{15}N was determined using the formula: ^{15}N recovery = (N content in a soil component \times %N)/fertilizer N rate. The %N indicates the proportion of fertilizer N in the corresponding soil N component and was calculated based on the ^{15}N abundances of both the soil and the fertilizer. Specifically, %N = (soil ^{15}N abundance in the ^{15}N -fertilized plot - 0.3663%)/(fertilizer ^{15}N abundance - 0.3663%). The fertilizer ^{15}N abundance was 1.193%. Error bars represent standard errors (n = 3). Different lowercase letters above the columns in the same cluster, within the same sampling stage indicate significant differences (LSD, $P < 0.05$).

pot experiments, which indicated that the addition of NI promoted the retention of fertilizer-derived N and its subsequent release, primarily as mineral fixed NH_4^+ -N rather than ON, regardless of whether crop straw was added. Under field conditions, even without the addition of exogenous organic material, crop roots can also provide carbon sources for microbial N immobilization (24). A previous study confirmed our results, finding that the %Ndff of crop N uptake decreased with NI addition, while the fertilizer-derived hydrolyzable N in the soil increased (25). Therefore, we speculate the function of NI application likely shifts from solely regulating fertilizer N release to simultaneously promoting microbial N immobilization, thereby increasing the residence time of fertilizer N and reducing its losses (26).

In our study, the recovery of ^{15}N in the TN pool decreased with the growth stage due to root N uptake and gaseous or hydrologic losses (Figure 2). The two mineral components—fertilizer-derived extractable mineral N and fertilizer-derived mineral fixed NH_4^+ -N, exhibited strong positive relationships with fertilizer-derived TN during the V6 and VT stages, with slopes of 0.81 and 0.20, respectively (Figure 4). This suggests that mineral fixed NH_4^+

plays a critical role in buffering and supplying fertilizer N during the maize growing season (17). However, its bioavailability was only approximately a quarter of that of the extractable mineral ^{15}N , although it was significantly higher than ^{15}N -ON during the maize growing season.

With the rapid depletion of soil extractable mineral N, the release of fertilizer-derived ON and mineral fixed NH_4^+ has become critical for soil N supply during the middle and late growth stages (27). In this study, the ^{15}N recovery in the ON pool showed a stable or minimal reduction trend during the three stages of maize growth (V6, VT, R6) (Figures 2, 4), indicating that the in-season N supply capacity of fertilizer-derived ON is limited (21). An earlier ^{15}N tracer study by Clay et al. (25) also observed the relatively stable nature of fertilizer-derived soil ON at three sampling times during maize growth. In summary, the addition of NIs promotes the preservation of fertilizer N in the form of soil ON, which could improve crop yields. However, we know little about the underlying mechanisms. Future research needs to explore the extent to which soil preserved fertilizer-derived ON can be released and how efficiently it can provide N to crops in the long term.

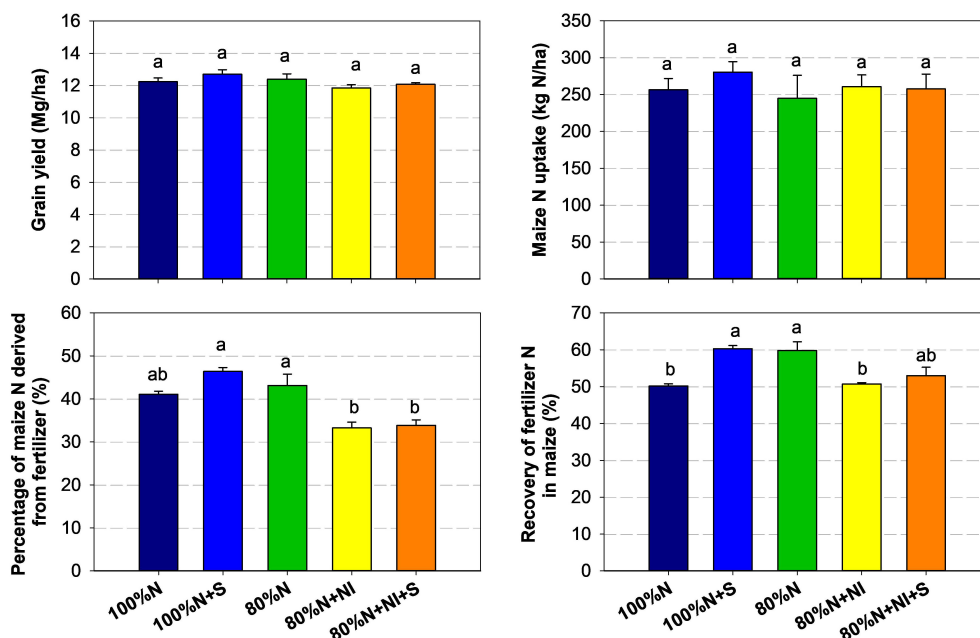


FIGURE 3

Grain yield, maize N uptake, proportions of maize N derived from fertilizer (N%), and fertilizer ^{15}N recovery at R6 stage of the maize growing season. Maize N uptake includes four organs: stem, leaf, cob, and grain. The recovery of ^{15}N was determined using the formula: ^{15}N recovery = (N content in a plant organ \times %N)/fertilizer N rate. The %N indicates the proportion of fertilizer N in the corresponding organ and was calculated based on the ^{15}N abundances of both the plant and the fertilizer. Specifically, %N = (plant ^{15}N abundance in the ^{15}N -fertilized plot - 0.3663%)/(fertilizer ^{15}N abundance - 0.3663%). The fertilizer ^{15}N abundance was 1.193%. Error bars in the figure are standard errors (n = 3). Different lowercase letters above the columns indicate significant differences (LSD, P < 0.05).

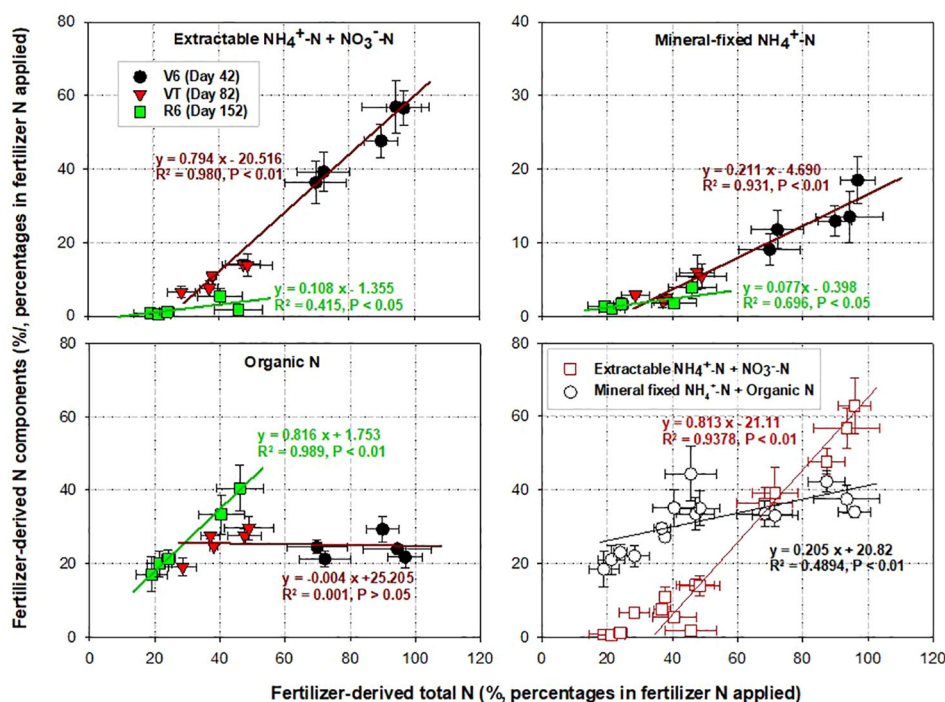


FIGURE 4

Pearson correlation analysis showing the relationships between fertilizer-derived TN and its components: extractable mineral N ($\text{NH}_4^+-\text{N} + \text{NO}_3^--\text{N}$), mineral fixed NH_4^+-N , and organic N (ON). Organic N is calculated as the difference between soil TN and soil mineral N. In the figure, error bars represent standard errors (n = 3).

4 Conclusions

Our findings demonstrate that the newly retained fertilizer-derived ON during the early growth stage of maize serves as a relatively stable N reservoir. The addition of NI is an effective strategy for stabilizing the applied fertilizer N, primarily in the form of ON. However, the release of ON was unsuccessful during the middle and late growth stages of maize. This suggests that the long-term N supply could be enhanced by regulating the conversion of N fertilizer from mineral N pools to ON pools.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

ZQ: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. SL: Data curation, Formal analysis, Investigation, Methodology, Resources, Writing – review & editing. DL: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. CL: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. ZX: Formal analysis, Investigation, Methodology, Writing – review & editing. XC: Conceptualization, Methodology, Supervision, Validation, Visualization, Writing – review & editing. YF: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Resources, Supervision, Visualization, Writing – review & editing.

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Conflict of interest

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

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