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Excessive nitrogen addition accelerates N assimilation and P utilization by enhancing organic carbon decomposition in a Tibetan alpine steppe

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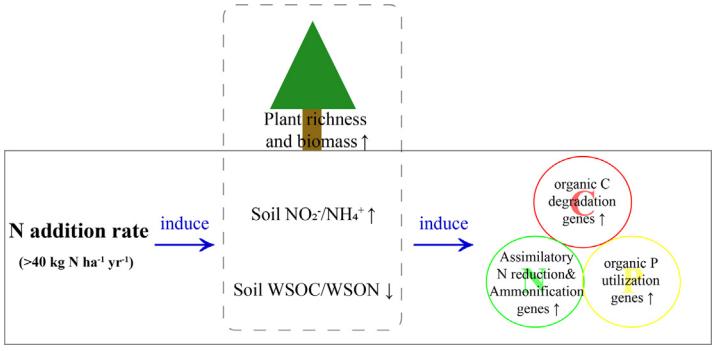
HIGHLIGHTS

- A nitrogen addition rate of 40 kg N ha^{-1} yr^{-1} was the critical value for soil microbial functional genes in the studied site.
- Nitrogen addition exceeding 40 kg N ha^{-1} yr^{-1} enhanced the degradation of soil labile organic carbon.
- Plant richness, soil NO_2^-/NH_4^+ and WSOC/WSON were significantly correlated with the abundance of microbial functional genes.
- The enhanced degradation of soil labile organic carbon drove changes in soil nitrogen assimilation and phosphorus utilization.

GRAPHICAL ABSTRACT



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ABSTRACT

High amounts of deposited nitrogen (N) dramatically influence the stability and functions of alpine ecosystems by changing soil microbial community functions, but the mechanism is still unclear. To investigate the impacts of increased N deposition on microbial community functions, a 2-year multilevel N addition (0, 10, 20, 40, 80 and 160 kg N ha^{-1} $year^{-1}$) field experiment was set up in an alpine steppe on the Tibetan Plateau. Soil microbial functional genes (GeoChip 4.6), together with soil enzyme activity, soil organic compounds and environmental variables, were used to explore the response of microbial community functions to N additions. The results showed that the N addition rate of 40 kg N ha^{-1} $year^{-1}$ was the critical value for soil microbial functional genes in this alpine steppe. A small amount of added N (≤40 kg N ha^{-1} $year^{-1}$) had no significant effects on the abundance of microbial functional genes, while high amounts of added N (>40 kg N ha^{-1} $year^{-1}$) significantly increased the abundance of soil organic carbon degradation genes. Additionally, the abundance of microbial functional genes associated with NH_4^+, including ammonification, N fixation and assimilatory nitrate reduction pathways, was significantly increased under high N additions. Further, high N additions also increased soil organic

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Nitrogen cycle
Phosphorus cycle

phosphorus utilization, which was indicated by the increase in the abundance of *phytase* genes and alkaline phosphatase activity. Plant richness, soil NO_2^-/NH_4^+ and WSOC/WSON were significantly correlated with the abundance of microbial functional genes, which drove the changes in microbial community functions under N additions. These findings help us to predict that increased N deposition in the future may alter soil microbial functional structure, which will lead to changes in microbially-mediated biogeochemical dynamics in alpine steppes on the Tibetan Plateau and will have extraordinary impacts on microbial C, N and P cycles.

1. Introduction

Nitrogen (N) deposition has increased substantially since the middle of the 20th century due to agricultural fertilization and the combustion of fossil fuels (Galloway et al., 2004, 2008). The large amount of N deposition has approached or even exceeded the tolerance threshold of plants, which seriously affects the stability and functions of terrestrial ecosystems (Capek et al., 2018; Schleuss et al., 2019; Xie et al., 2018). These ecosystem functions affected by N deposition include nitrification, N mineralization, carbon (C) sequestration, soil organic matter (SOM) decomposition, and greenhouse gas emissions (Y. Liu et al., 2013; X. Liu et al., 2013; Zou et al., 2020). By 2030, worldwide N deposition is predicted to increase by 50% to 100% compared with the N deposition level in 2000 (Y. Liu et al., 2013; X. Liu et al., 2013; Reay et al., 2008), which will have a strong impact on the degradation of soil organic C and then affect soil N and phosphorus (P) cycles in terrestrial ecosystems. It is well known that soil microorganisms are the main participants in nutrient cycling in terrestrial ecosystems (Capek et al., 2018; Falkowski et al., 2008), but the specific mechanism by which soil microorganisms respond to increased N deposition is poorly understood.

The response of microbial functional genes to N addition has received great attention in recent years (Li et al., 2020; Xie et al., 2018; Zou et al., 2020). N addition usually decreases the abundance of the N-fixation gene (*nif*H) and nitrification gene (A*mo*A) (Tang et al., 2016; Tian et al., 2019; Wang et al., 2018). In contrast, the abundance of denitrification genes, including *nar*G, *nir*K, *nir*S, and *nos*Z, is significantly increased under N addition (Tang et al., 2016; Tian et al., 2019). N deposition has significant effects on the soil C:N:P ratio, and the stoichiometric deviations between the substrate and microbial community caused by the change in the soil C:N:P ratio under N deposition may further alter the microbial processes of C, N and P cycles in terrestrial ecosystems. Allison et al. (2008) reported that increased N deposition strengthened microbial activities related to soil cellulose degradation but inhibited microbial activities associated with soil protein and chitin decomposition. In addition, a recent study has shown that increased N fertilization could aggravate the effect of P limitation on plant biomass production (Capek et al., 2018). Many studies highlight that soil C, N, and P cycles are tightly coupled in terrestrial ecosystems (Bracken et al., 2015; Chen et al., 2018; Yang and Luo, 2011), but little is known about how these processes interact with each other under N addition.

In general, the responses of microbial communities to N deposition in terrestrial ecosystems vary with N addition duration and quantity (Treseder, 2008). Guo et al. (2017) found that soil bacteria may be seriously limited by soil available C but can become the dominant decomposer of complex P compounds after 3 years of N addition, particularly under high N addition. Li et al. (2020) recently confirmed that when the N deposition rate was lower than a certain threshold, the abundance of N-cycling genes would increase, and a rate higher than this threshold would decrease their abundance. In addition, soil organic C decomposition and greenhouse gas emissions also showed similar N addition dosage effects. For example, a recent study reported that low N addition contributed to the accumulation of labile-fraction soil organic matter, while high N addition promoted the degradation of labile-fraction soil organic matter (Chen et al., 2020). Geng et al. (2017) also observed that N addition at a low rate of 10 kg N ha^{-1} $year^{-1}$ significantly stimulated soil CO_2 emissions and CH_4 uptake in a temperate forest, whereas a high N addition rate (140 kg N ha^{-1} $year^{-1}$) significantly inhibited soil CO_2 emissions and CH_4 uptake. Some studies reported that these different N addition dosage effects on microorganisms were mainly induced by changes in plant species composition and primary productivity under N additions (Liu and Greaver, 2010; Lange et al., 2015; Ramirez et al., 2010). Other studies have shown that soil acidification was caused by excessive N addition, which is also a main reason for changes in microbial communities under increased N (Treseder, 2008; Rousk et al., 2009). Although it is known that decreases in available soil C and pH play important roles in changes in microbial communities under N addition, the main factors that affect the microbial functional genes in alpine grasslands are still unclear.

Alpine ecosystems are particularly susceptible to N deposition due to their poor quality soil and low biological buffering capacity (Bowman et al., 2008). Atmospheric N deposition over the Tibetan Plateau ranges from 8.7 to 13.8 kg N ha^{-1} $year^{-1}$ (Lue and Tian, 2007) and has increased dramatically in the last several decades (Jia et al., 2014). At present, increased N deposition has led to soil acidification in some parts of the Tibetan Plateau (Yang et al., 2012) and has significantly affected plant biomass and diversity (Y. Liu et al., 2013; X. Liu et al., 2013; Zong et al., 2015). However, the response of microbial communities and their functions to increased N deposition on the Tibetan Plateau remains unclear, and such uncertainty limits our ability to predict the effects of future climate changes on nutrient cycling in alpine ecosystems. Previous research has demonstrated that plants in alpine ecosystems have a critical value for N addition (Y. Liu et al., 2013; X. Liu et al., 2013; Zong et al., 2015). Based on this, we hypothesize that microorganisms in alpine ecosystems also have a critical value for N addition, and above and below this critical value, microorganisms may have opposite responses to N addition. To test our hypothesis, we conducted a multilevel N addition (0, 10, 20, 40, 80, and 160 kg N ha^{-1} $year^{-1}$) field experiment in an alpine steppe on the Tibetan Plateau. Soil microbial functional genes (GeoChip 4.6), combined with soil enzyme activity, soil organic compounds and environmental variables, were used to explore the mechanism of microbial community functions in response to multiple levels of N addition.

2. Materials and methods

2.1. Site description and soil sampling

The experimental site is located at Nam Co Station, a multisphere observation and research station, on the central Tibetan Plateau (30°46.4′ N, 90°59.31′ E, 4730 m above sea level). The annual mean air temperature is −0.6 °C, and the annual precipitation is 415 mm, which is much lower than the annual pan evaporation (1726 mm). The monthly mean temperature ranges from −9 °C in January to 10 °C in July. More than 70% of the annual precipitation occurs in the growing season (May to September) (Y. Yang et al., 2014; K. Yang et al., 2014). The dominant vegetation at the research site includes *Kobresia pygmaea*, *Stipa purpurea*, *Androsace tapete*, and *Leontopodium pusillum*.

Three homogenous plots were randomly assigned as replicates in the studied alpine steppe in May 2009. In each plot, six subplots (~13 m^2) were arranged in a circle as six N addition levels, namely, 0, 10, 20, 40, 80 and 160 kg N ha^{-1} $year^{-1}$ (N0, N10, N20, N40, N80 and N160), with a 2 m buffer zone between adjacent subplots (Fig. S1).

There were 18 subplots in total. The subplots with the N addition level of 0 kg N ha^{-1} $year^{-1}$ were regarded as the control treatment. Fertilizer was supplied as NH_4NO_3, which was sprayed onto the subplots using a sprayer during the first week of each month from May to September in 2010 and 2011. After two years treatment, soil samples were collected at a depth of 0–10 cm in August. To minimize spatial heterogeneity, three cores were randomly collected in each plot and subsequently combined as a sample. Each soil sample (~500 g) was sieved through a 2-mm mesh to remove stones and roots and was stored in a refrigerator at −80 °C for microbiological analyses and 4 °C for soil physiochemical analyses. The vegetation species richness, coverage and average height were determined according to the descriptions in a previous study (Zhao et al., 2011). The vegetation was mowed, dried at 75 °C for 48 h and weighted as aboveground biomass (AGB). The measurement variables and their related methods/techniques are summarized in Table S1.

2.2. Soil property measurements

Soil pH was measured with a pH-meter (soil:water (w/v) = 1:2.5). Air-dried soil was used to measure total organic carbon (TOC) using a TOC analyzer (TOC-VCPH, Shimadzu, Japan). Total nitrogen (TN) and total phosphorus (TP) were determined using the $CuSO_4$ Se powder diffusion method and the NaOH fusion-Mo colorimetric method, respectively. To measure the concentrations of soil NH_4^+-N, NO_3^--N and NO_2^--N, 10 g fresh soil samples were suspended in 40 mL 0.05 M K_2SO_4 extracts and shaken for 30 min at 120 r/min. Subsequently, the suspension was centrifuged for 5 min at 600 r/min and filtered with a 0.45 μm filter (Organic membrane filter, Solarbio, USA). Supernatants were determined by an autoanalyzer (Auto Analyzer 3, Bran Luebbe, Germany). Water-soluble organic carbon (WSOC) and water-soluble organic nitrogen (WSON) were extracted by adding 2.5 g fresh soil to 25 mL of pure water, shaking for 1 h, filtering the mixture with a filter (Bragazza et al., 2006), and then determining the supernatant using a Shimadzu TOC-TN analyzer (Shimadzu, Japan).

2.3. Soil organic compound measurements

Soil organic compounds were characterized by pyrolysis-gas chromatography/tandem-mass spectrometry (Py-GC–MS/MS). Specific experimental methods and calculation methods refer to Chen et al. (2020), and the original data of soil organic compounds were obtained from Chen et al. (2020). In this study, we further divided these soil organic compounds from abovementioned article into two categories, labile organic C (including alkyl compounds and polysaccharides) and recalcitrant organic C (including lignin, phenols, chitin, N compounds, aromatics and polyaromatics) (Almendros et al., 1996; Buurman et al., 2007; Gupta et al., 2007; Lorenz et al., 2007), for subsequent calculation.

2.4. Phospholipid fatty acid (PLFA) and soil enzyme activity assays

Microbial PLFAs were extracted from lyophilized soil using the single-phase Bligh and Dyer method with minor modifications (White et al., 1979). The specific experimental operation can be found in Si et al. (2015). All PLFA samples were analyzed by capillary gas chromatography using a Hewlett-Packard 7890A GC with a flame ionization detector. The total biomass was estimated from the summed concentrations of the following PLFAs: i14:0, i15:0, a15:0, C15:1, i16:0, 16:1w9c, 16: w7c, 16:1w5c, 16:1w3c, i17:0, a17:0, C17:1, cy17:0, C18:1, i19:0 and cy19:0, 18:1w9c and 18:2w6,9 (Federle et al., 1986; Frostegard et al., 1993; Tunlid et al., 1989).

The potential activities of extracellular enzymes, including hydrolases (invertase, β-glucosidase, urease, and phosphatase), and oxidases (phenol oxidase and peroxidase), were measured on the basis of previous research (Guan, 1986). Briefly, β-glucosidase activity was detected by incubating 1 g of soil with 1 mL of 5 mM *p*-nitrophenyl-β-glucopyranoside at 37 °C for 1 h (Eivazi and Tabatabai, 1988). Invertase activity was detected by incubating 1 g of soil mixed with 15 mL 2 M acetate buffer and 15 mL 1.2% sucrose substrate solution at 50 °C for 3 h (Gopal et al., 2007). Phenol oxidase and peroxidase were determined by incubating 1 g of soil with 10 mL of 1% pyrogallic acid at 30 °C for 2 h (Allison and Jastrow, 2006). Urease activity was detected by incubating 1 g of soil at 37 °C for 2 h with a 10% urea solution as the substrate (Kandeler and Gerber, 1988). Alkaline phosphatase activity was detected by the p-nitrophenol released from a mixture of 1 g of soil, 0.2 mL of toluene, 4 mL of buffer solution (pH 11.0) and 1 mL of *p*-nitrophenyl phosphate, which was incubated at 37 °C for 1 h (Tabatabai, 1982). All enzyme activities were analyzed using a colorimetric method with a spectrophotometer. The measuring wavelengths were as follows: polyphenol oxidase: 490 nm, peroxidase: 294 nm, β–glucosidase: 400 nm, invertase: 508 nm, urease: 578 nm, and alkaline phosphatase: 430 nm.

2.5. GeoChip 4.6 experiments

Soil metagenomic DNA was extracted from 0.5 g of fresh soil samples with a soil DNA extraction kit (FastDNA® SPIN Kit for Soil, Catalog#6560-200), and the extracted DNA was sent to the IEG (Institute for Environmental Genomics, Department of Botany and Microbiology) Laboratory at the University of Oklahoma to complete the GeoChip test. The basic steps were as follows: The Templiphi kit (GE Healthcare, Piscataway, NJ) was used to amplify the whole DNA genome, which was labeled with fluorescent Cy-5. The labeled DNA was purified by a QIAquick kit (Qiagen, Valencia, CA, USA) and then dried under vacuum at 45 °C for 40 min for chip hybridization. The MAUI hybrid workstation (BioMicro, Salt Lake City, UT, USA) was used in this study. After hybridization, the chip was scanned by a NimbleGen Ms200 scanner (Roche, Madison, WI, USA), and the image was transformed into digital information by ImaGene 6.0 software.

The original gene data were transformed into effective data after online quality control by the IEG website (http://ieg.ou.edu/microarray/). The steps were as follows: 1. Remove low-quality points. 2. Remove points with noise ratios less than 2 (signal-to-noise ratio, SNR). 3. If a point only appears in one repeated sample, remove this point. The above data were further standardized such that the total gene abundance of each sample was the same, and then the log values of each probe were calculated. The data were used for microbial ecological analysis. The number of gene probes was counted as the richness index of functional genes. Microbial functional gene names and the full names of corresponding enzymes are listed in Table S3.

2.6. Data analyses

The processed GeoChip data were analyzed with statistical methods as follows: 1. Alpha diversities of the whole community, C cycling, N cycling and P cycling were assessed by calculating the richness, Shannon and Simpson index values; 2. detrended correspondence analysis (DCA) and permutational multivariate analysis of variance (PERMANOVA) were used to analyze the microbial functional community structure under N additions; 3. linear regression and nonlinear regression were used to further analyze the responses of microbial functional genes to N additions (the calculated data were the difference between each treatment and the control); 4. canonical correspondence analysis (CCA), the Mantel test and multiple ordinary least squares (OLS) regression were used to analyze the environmental factors driving the changes in the microbial community structure under N additions. The above analyses (1–4) were conducted with R software (version 2.12.1, https://www.r-project.org/); 5. Pearson correlation was used to study the relationships among each microbial functional gene; 6. A path model was constructed to explore the relationships between microbial functional genes involved in C, N and P cycling under N additions; 7. The differences in alpha diversities, the abundance of functional genes, and environmental factors were tested using one-way

analysis of variance (ANOVA) followed by Duncan's new multiple range test. The above analyses (5–7) were performed with SPSS 19.0 statistical software (IBM Corporation, Armonk, NY, USA).

3. Results

3.1. Effects of N additions on soil properties and plants

As shown in Table S4, soil pH decreased significantly only at N160 compared with N0, but it was still higher than 7. N additions had no significant effects on soil TOC, TN and TP but had significant effects on their ratios. With increasing N addition, the soil TOC/TN ratio decreased, and the soil TN/TP ratio reached its highest value at N160. Compared with the control, WSOC and WSOC/WOSN significantly decreased when the N addition rate was ≥40 kg N ha^{-1} $year^{-1}$, and WSON significantly increased at N160. With an increase in N addition, the concentration of soil NH_4^+ showed no significant change, but the concentration of soil NO_3^-, NO_2^-, and NO_2^-/NH_4^+ significantly decreased at N160 compared with N0. Both aboveground biomass and species richness of plants increased with increasing N addition and reached their highest values at N160 ($p < 0.05$).

3.2. Effects of N additions on microbial biomass and functional genes

N addition significantly decreased microbial biomass, but there was no significant difference in the microbial biomass at N addition rates from N20 to N160 (Fig. 1a). Compared with the control, microbial diversity (Shannon index) decreased when the N addition was ≥40 kg N ha^{-1} $year^{-1}$ and reached the lowest value at N160 (Fig. 1a). The richness and diversity (Shannon and Simpson indexes) of total functional genes showed no significant difference when the N addition rate was <40 kg N ha^{-1} $year^{-1}$, whereas these indexes decreased when the N addition rate was ≥40 kg N ha^{-1} $year^{-1}$ ($p < 0.05$) (Table S5). In addition, the responses of the richness and diversity of C-cycle and N-cycle genes to N additions were similar to those of total functional genes (Table S5). The DCA ordination plot of total functional genes showed a significant difference between the low (≤40 kg N ha^{-1} $year^{-1}$) and high (>40 kg N ha^{-1} $year^{-1}$) N addition treatments (Fig. 1b), which was further confirmed by the PERMANOVA results ($p < 0.05$) (Fig. S2).

3.3. Effects of N additions on the abundance of genes mediating the C, N, and P cycles

First, the abundance of genes involved in the C cycle increased from N0 to N80 and decreased from N80 to N160 (Fig. S3a). Although there was no significant difference in the abundance of genes involved in C sequestration among N addition treatments ($p > 0.05$) (Fig. S3b), the abundance of RuBisCO and *pcc* significantly decreased at N160 compared with N0 ($p < 0.05$) (Fig. 2a). In addition, there were no significant differences in the abundances of the methane cycling genes, *mcrA* and *pmoA* in each N treatment (Figs. S3c, 2b); only the abundance of *mmoX* significantly increased at N80 and N160 compared with N0 (Fig. 2b).

The abundance of organic carbon degradation genes increased with increasing N addition (Fig. S3d). At the same time, there were significant positive correlations between the abundance of labile organic C degradation genes (including starch and hemicellulose degradation genes) and the N addition rate, as well as between the abundance of recalcitrant organic C degradation genes (including cellulose, chitin, and lignin degradation genes) and the N addition rate (Fig. S4a and b). In detail, the abundance of starch degradation genes (mainly *amyA*, *nplT*, *pulA* and *glucoamylase*) and hemicellulose degradation genes (mainly *ara* and *mannanase*) increased with increasing N addition (Figs. S4c and 2c, S4d and 2d), which was consistent with the response of β-glucosidase and invertase activities to N additions (Fig. S5a and b). In addition, the abundance of chitin degradation genes (mainly *acetylglucosaminidase*) was positively correlated with the N addition rate ($p < 0.05$) (Figs. S4e and 2e), while the abundance of cellulose degradation genes had no significant correlation with the N addition rate, which might be because there was a positive correlation between the abundance of *CDH* and the N addition rate but a significant negative correlation between the abundance of endoglucanase and the N addition rate ($p < 0.05$) (Figs. S4g and 2g). The abundance of lignin degradation genes (mainly *glx* and *phenol oxidase*) increased from N0 to N80 and decreased from N80 to N160 ($p < 0.05$) (Figs. S4f and 2f), which was consistent with the responses of phenol oxidase and peroxidase activities to the N addition rate (Fig. S5c and d). The recalcitrant/labile organic C degradation gene ratio was negatively correlated with the N addition rate, plant richness and soil NO_2^-/NH_4^+ but positively correlated with WSOC/WSON ($p < 0.05$) (Fig. 3a–d). At the same time, the molar mass of labile organic compounds (including alkyl compounds and polysaccharides) and recalcitrant organic compounds (including lignin, phenols, chitin, N compounds, aromatics and polyaromatics) were both negatively correlated with the N addition rate (Fig. 3e–f).

Second, the abundance of genes involved in the N cycle increased from N0 to N80 and decreased from N80 to N160 (Fig. S6a). The abundance of ammonification genes (mainly *ureC*), N assimilatory genes (mainly *NiR* and *nirA*) and N fixation genes (mainly *nifH*) was significantly positively correlated with the N addition rate (Figs. S6b and 4a, S6d and 4b, S6h and 4c). However, the abundance of anammox genes (*hzo*), nitrification genes (*hao*) and N dissimilatory genes had no significant correlation with the N addition rate (Figs. S6c, S6e, S6g, 4c). For N dissimilatory genes, there was a negative correlation between the

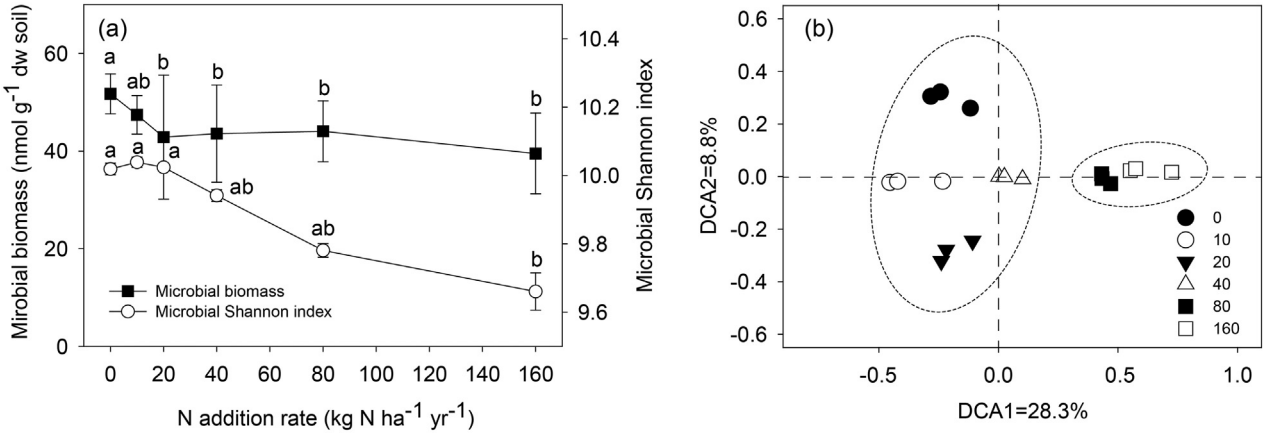

Fig. 1. Effects of nitrogen addition on microbial biomass and the Shannon index (a) and detrended correspondence analysis (DCA) of GeoChip data (b). Bars show the mean ± s.e. Significant ($p < 0.05$) differences among N addition rates are indicated by letters above the bars.

abundance of *napA* and the N addition rate, and a positive correlation between the abundance of *nrfA* and the N addition rate (Fig. 4d). In addition, there was a significant negative correlation between the abundance of denitrification genes and the N addition rate, and the abundances of *nirK*, *norB* and *nosZ* all decreased with increasing N addition (Figs. S6f, 4e).

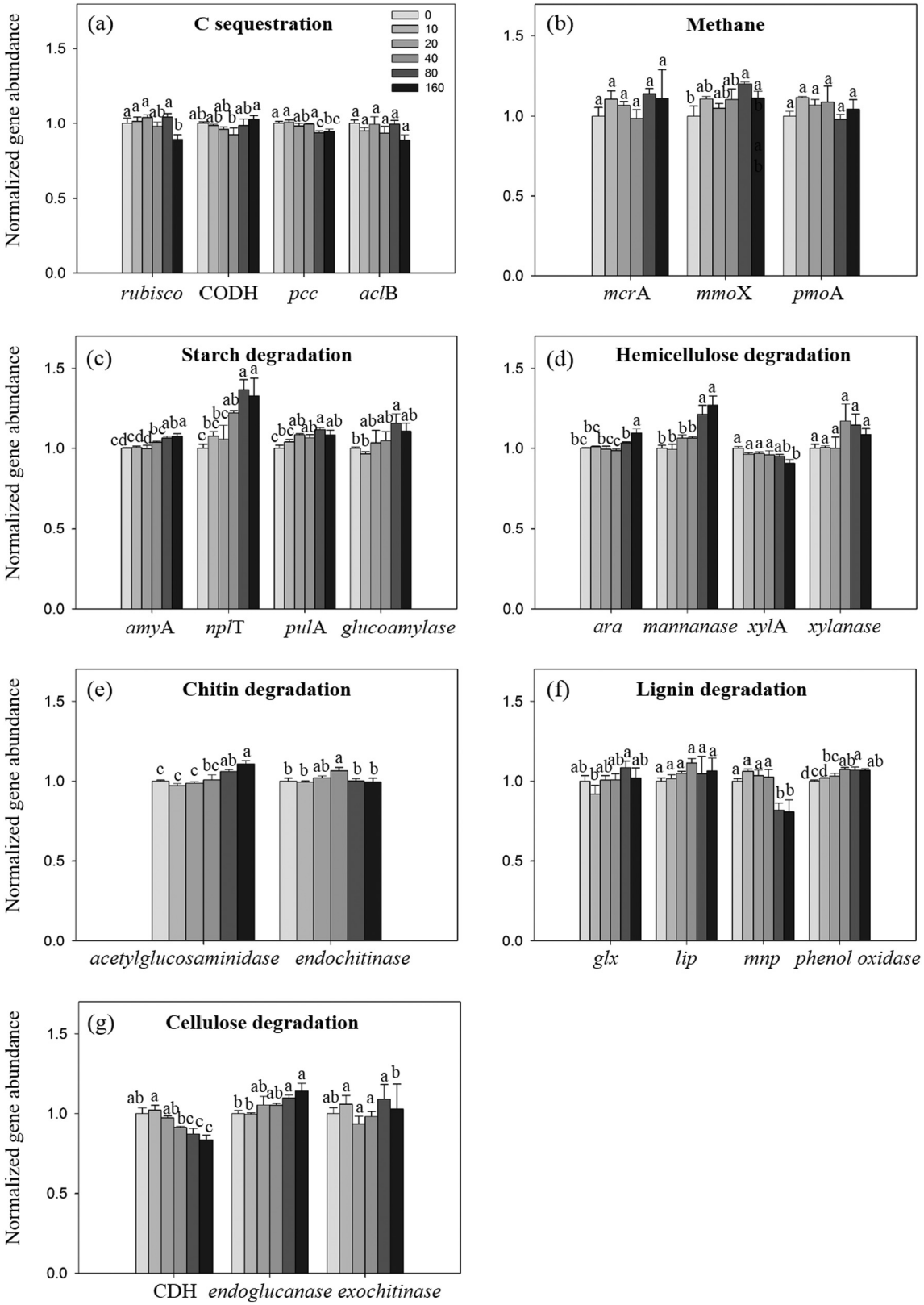

Fig. 2. The abundance (normalized relative to the control) of C-cycling gene families under N addition treatments. (a) Carbon sequestration genes; (b) methane genes; (c) starch degradation genes; (d) hemicellulose degradation genes; (e) chitin degradation genes; (f) lignin degradation genes; (g) cellulose degradation genes. Different lowercase letters indicate a significant difference ($p < 0.05$) according to ANOVA.

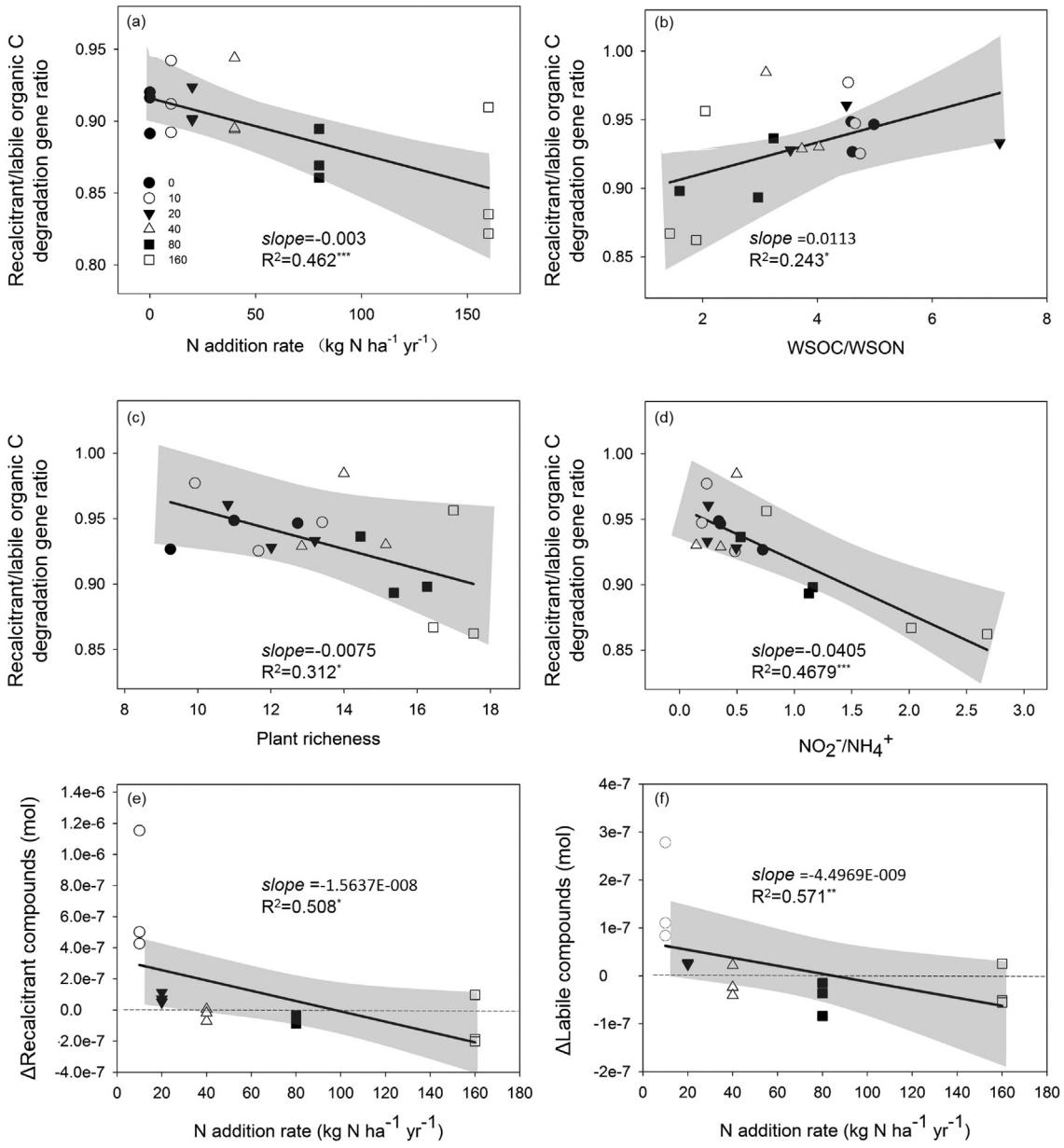

Fig. 3. Correlations between carbon degradation genes and the N addition rate and environmental variables. (a) Correlation between recalcitrant/labile organic C degradation gene abundance and the N addition rate; (b) correlation between recalcitrant/labile organic C degradation gene abundance and WSOC/WSON; (c) correlation between recalcitrant/labile organic C degradation gene abundance and plant richness; (d) correlation between recalcitrant/labile organic C degradation gene abundance and NO_2^-/NH_4^+; (e) molar mass of recalcitrant organic compounds (normalized relative to the control) and the N addition rate; (f) molar mass of labile organic compounds (normalized relative to the control) and the N addition rate. Recalcitrant organic C degradation related genes include: *endoglucanase*, CDH, *exoglucanase*, *acetylglucosaminidase*, *endochitinase*, *phenol oxidase*, *mnp*, *glx*, and *lip*; labile organic C degradation related genes include: *amy*A, *pul*A, *npl*T, *glucoamylase*, *ara*, *mannanase*, *xylanase*, and *xyl*A. The differences (Δ) in soil organic compounds were calculated as the differences between each N addition treatment and the control. Significance levels ($p < 0.05$) are indicated by * asterisks; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Third, three genes, i.e., *phytase*, *ppk* and *pps*, were used to indicate the response of soil P to N additions. There was a significant positive correlation between the abundance of *phytase* and the N addition rate, a significant negative correlation between the abundance of *ppx* and the N addition rate, and no significant correlation between the abundance of *ppk* and the N addition rate (Fig. 5). The response of *phytase* to N additions was the same as the response of alkaline phosphatase activities to N additions (Fig. S5f).

3.4. Relationships between environmental variables and microbial functional genes

The CCA results showed that the soil pH, TP, NO_2^-/NH_4^+, NO_3^-, TOC/TN ratio, TN/TP ratio, WSOC/WSON and plant richness (constrained 62.18%, $p = 0.003$, Table S6) shaped microbial functional potentials under N additions (Fig. S8). The Mantel test and partial Mantel test demonstrated similar results: soil NO_2^-/NH_4^+, WSOC/WSON and plant richness were the predominant factors that influenced microbial functional potential under N additions (Table 1). Multiple ordinary least squares (OLS) regression analysis showed that the soil NO_2^-/NH_4^+ ratio exhibited the greatest importance (27.35%) among these factors, followed by plant richness (PR) (17.08%), soil NO_3^- (12.46%) and WSOC/WSON (10.95%).

Pearson's correlation analysis showed that the abundance of C fixation and labile C degradation genes was significantly correlated with the abundance of ammonification, assimilatory N reduction, denitrification, and N fixation genes. At the same time, the abundance of both labile and recalcitrant organic C degradation genes was

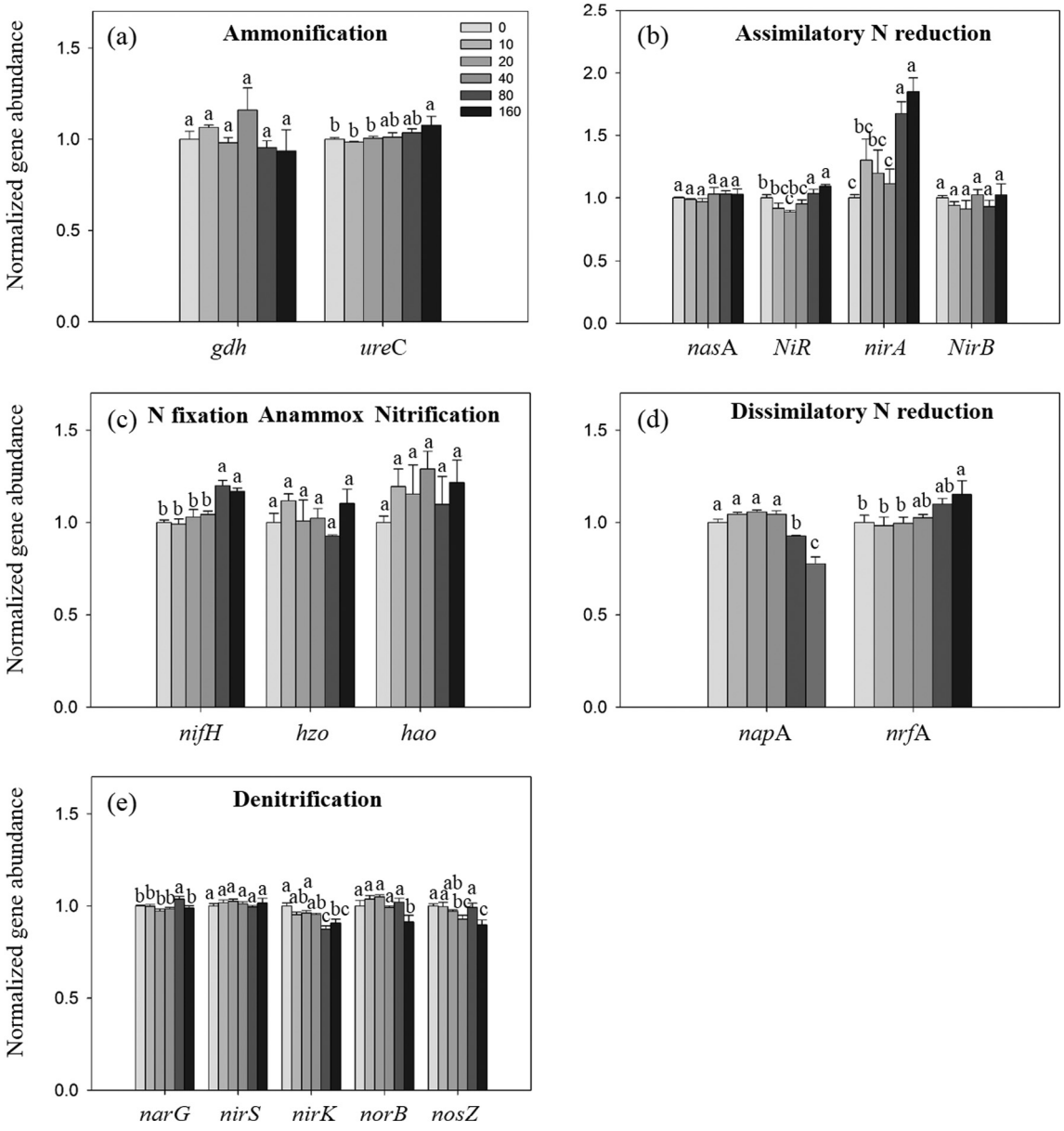

Fig. 4. The abundance (normalized relative to the control) of N-cycling genes under N addition treatments. All data are presented as the mean ± s.e. calculated from biological triplicates. (a) Ammonification genes; (b) assimilatory N reduction genes; (c) nitrogen fixation, anammox and nitrification genes; (d) dissimilatory N reduction genes; (e) denitrification genes. Different lowercase letters (a, b and c) indicate a significant difference ($p < 0.05$) according to ANOVA.

significantly correlated with the abundance of P utilization genes (Fig. S9).

Structural equation modelling (SEM) was used to explore the structural relationships among microbial functional genes, enzymatic activities, and environmental factors. The SEM showed that the N addition rate was significantly positively correlated with plant richness and negatively correlated with WSOC/WSON. In addition, plant richness and soil NO_2^-/NH_4^+ were also significantly correlated with the abundance of soil labile C degradation genes. At the same time, a positive influence was found for NO_2^-/NH_4^+ with respect to ammonification gene abundance, and a negative influence was found for WSOC/WSON with respect to P utilization gene abundance. Among microbial functional genes, soil recalcitrant C degradation gene abundance was significantly positively correlated with ammonification and P utilization gene abundance, and soil labile C degradation gene abundance was significantly positively correlated with assimilatory nitrate reduction gene abundance (Fig. S10).

4. Discussion

Our results showed that the influence of N additions on microbial community functions was dose-dependent; that is, the diversity and richness of microbial functional genes remained unchanged when the N addition rate was ≤40 kg N ha^{-1} $year^{-1}$, whereas they significantly decreased when the N addition rate was >40 kg N ha^{-1} $year^{-1}$ (Table S5, Figs. 1 and S2). The results suggested that the N addition rate of 40 kg N ha^{-1} $year^{-1}$ was the critical N loading value for soil microbial functional genes in this studied alpine steppe, which is consistent with the critical N loading value of plants in this area (Y. Liu et al., 2013; X. Liu et al., 2013). Compared with other studies, the critical N loading value of plants and soil microbes determined in this study was lower than the values of 105 and 91.7 kg N ha^{-1} $year^{-1}$ for plants in temperate grasslands (Bai et al., 2010) and semiarid grasslands (Chen et al., 2016), respectively. However, our result was close to the values (46 and 50 kg N ha^{-1} $year^{-1}$) determined in alpine grasslands (Bowman

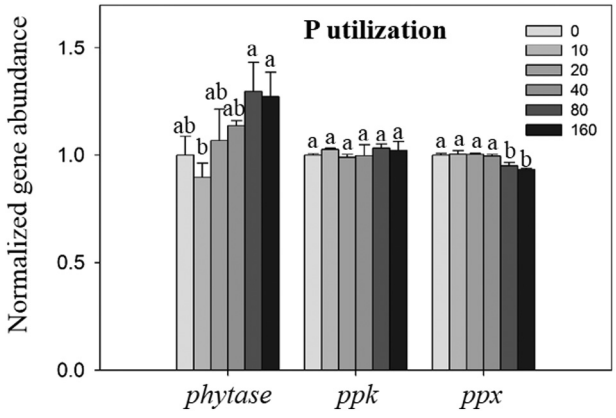

Fig. 5. The abundance (normalized relative to the control) of P utilization related genes under N addition treatments. All data are presented as the mean ± s.e. calculated from biological triplicates. Different lowercase letters (a, b and c) indicate a significant difference ($p < 0.05$) according to ANOVA.

et al., 2012) and semihumid alpine steppe meadows (Zong et al., 2015). This indicated that the critical N load of the alpine steppe was lower than that of low-altitude grasslands, which might be because the alpine region is usually limited by N because of the low temperature and low rainfall (Y. Liu et al., 2013; X. Liu et al., 2013).

In this study, increased N addition significantly enhanced the degradation of labile and recalcitrant organic C in labile-fraction soil organic matter, and labile organic C was more sensitive to N additions (Figs. 2, 3, S4 and S5). This is because appropriate N addition can alleviate N limitation in plants, thus promoting plant growth and enhancing rhizosphere secretions and C input (Jing et al., 2019). Subsequently, the increased available organic C can provide additional substrates for soil microorganisms and can consequently enhance microbial C acquisition and utilization (Li et al., 2017). Our results revealed that N addition increased the abundance of *amyA*, *nplT*, *pulA*, *glucoamylase*, *ara*, and *mannanase* (Fig. 2c and d), suggesting an increase in labile organic C (starch, hemicellulose) degradation capacities. Similarly, the increase in the abundance of *acetylglucosaminidase*, *phenol oxidase*, and *glx* under N additions also indicated the enhancement of recalcitrant organic C (cellulose, chitin, and lignin) degradation capacities (Fig. 2e, f and g). Schleuss et al. (2019) recently reported that in semiarid grassland soil, the activities of labile C degrading enzymes (including β-glucosidase and *N*-acetylglucosaminidase) increased with N increasing addition, and microbes invested more energy in C acquisition under a high inorganic N load, which is consistent with our results. In addition, our results showed that N addition mainly promoted the degradation of chitin, while the degradation of cellulose remained stable under N additions, which might be related to differences in root exudates and litter composition (Li et al., 2017).

N addition rate above 40 kg N ha^{-1} $year^{-1}$ significantly increased the abundance of genes associated with ammonification, N fixation and assimilatory nitrate reduction pathways but decreased those involved in denitrification pathways (Figs. 4 and S6). Ammonification, N fixation and assimilatory nitrate reduction usually indicate an increase in soil NH_4^+ production. However, the concentration of NH_4^+ in the soil remained stable under each N addition treatment (Table S4). This may be due to the high volatility of NH_4^+ in alkaline soils and its loss in the form of gases (Y. Liu et al., 2013; X. Liu et al., 2013). As reported in previous studies, the denitrification pathway would be strengthened in the case of a large amount of nitrate input (Y. Liu et al., 2013; X. Liu et al., 2013; Zhu et al., 2015). However, in this study, NO_3^- accumulation and denitrification capacity decreased with increasing N addition (Table S4, Figs. 4 and S6). In general, the accumulation of NO_3^- is due to less consumption than production. In this studied alpine steppe soil, the absorption and utilization of N by plants tended to be saturated when the N addition rate was >40 kg N ha^{-1} $year^{-1}$ (Y. Liu et al., 2013; X. Liu et al., 2013). Simultaneously, the abundance of genes involved in denitrification decreased when the N addition rate was >40 kg N ha^{-1} $year^{-1}$, while the abundance of genes related to nitrification remained unchanged under each N treatment in our study. Therefore, we believed that the accumulation of NO_3^- in soil was caused by excessive N addition and saturated N absorption and utilization. Previous studies have reported that *nar*G, *nir*K, and *nir*S, involved in the first two denitrification steps, were readily promoted by an increase in the N content, while *nor*B and *nos*Z were not sensitive to N addition (Chen et al., 2012; Tang et al., 2016). However, we found that the abundance of *nir*K, *nor*B and *nos*Z significantly decreased when the N addition rate was >40 kg N ha^{-1} $year^{-1}$, while *nar*G significantly increased only at N80 (Fig. 4). This might be because NO_3^- is the first substrate of denitrification, and the change in its concentration also had a significant impact on *nar*G expression, which is involved in the first step of denitrification (Kuypers et al., 2018). The next step was the denitrification reduction sequence, and the substrate (NO_2^-) is used for both dissimilatory and assimilatory purposes (Kuypers et al., 2018). Combined with the significant increase in the abundance of *nrf*A and *nir*A, we speculated that the enhanced N assimilation led to a decrease in the substrate (NO_2^-) concentration and eventually resulted in a reduction in the abundance of *nir*K, *nor*B and *nos*Z.

In addition, altered plant richness and species under N additions can influence the quantity and quality of soil resources, which usually results in an increase in plant-derived C input into soil (Y. Yang et al., 2014; K. Yang et al., 2014). According to the concept of consumer-driven nutrient recycling, the soil C and N cycles are mainly controlled by microbial element demand and supply. An increase in available C could stimulate the degradation of labile and recalcitrant soil organic C. To maintain a stable cellular C:N:P ratio, soil microorganisms usually promote N acquisition and assimilation. When the N addition rate was >40 kg N ha^{-1} $year^{-1}$, the abundance of *ure*C significantly increased, indicating that when soil N was saturated, the microorganisms tended to promote soil N mineralization according to their C:N:P stoichiometry, which might be due to N additions promoting plant roots to release more organic substrate into the soil. In this study, the abundance of *nif*H significantly increased when the N addition rate was >40 kg N ha^{-1} $year^{-1}$, which is inconsistent with other studies in which N fertilization had no significant effect on its abundance (Berthrong et al., 2014; Zheng et al., 2017). This may be due to the difficulty of NH_4^+ preservation in this alpine soil, resulting in the continuous demand for microbial N fixation. In addition, a recent study has shown that soil moisture, organic C, available P, and inorganic N contents played key roles in determining soil diazotroph distribution in Tibetan grassland soils (Chen et al.,

Table 1
Mantel and partial-Mantel test of GeoChip data with environmental attributes.

Environmental variables	Mantel		Partial-Mantel	
	r	*p*	r	*p*
pH	0.342	**0.020**	0.118	0.243
TOC	0.005	0.426	−0.113	0.798
TN	0.092	0.192	−0.139	0.879
TP	−0.157	0.892	−0.113	0.814
TOC/TN	0.170	0.074	0.155	0.095
TN/TP	0.022	0.377	−0.189	0.968
WSOC	−0.024	0.555	−0.095	0.746
WSON	0.464	**0.004**	0.155	0.171
WSOC/WSON	0.475	**0.002**	0.267	**0.047**
NO_2^-	0.472	**0.005**	0.159	0.178
NO_3^-	0.621	**0.002**	**0.420**	**0.006**
NH_4^+	0.043	0.355	−0.050	0.535
NO_2^-/NH_4^+	0.630	**0.002**	0.538	**0.001**
AGB	0.273	**0.040**	0.037	0.370
PR	0.467	**0.001**	0.355	**0.004**

Note: TOC: total organic carbon; TN: total nitrogen; TP: total phosphor; WSOC: water-soluble organic carbon; WSON: water-soluble organic nitrogen; AGB: aboveground biomass; PR: plant richness. Significant differences ($p < 0.05$) are indicated in bold.

2018). Therefore, the increase in soil organic C may further promote the growth of diazotrophs, thus improving the N-fixing potential.

In this study, the high abundance and activity of *phytase* and *ppx* indicated that a large amount of N addition could promote phosphatase production and organic P degradation (Figs. 5, S5h and S6). Previous studies have shown that high levels of exogenous N input can induce P limitation (Vitousek et al., 2010), accelerate P turnover and increase extracellular phosphatase expression (Olander and Vitousek, 2000; Treseder and Vitousek, 2001). Phosphatase availability is usually low because organic phosphoric acid is often tightly combined with multifarious soil components after it is released from plant residues or manure (Lung and Lim, 2006). Under the condition of high N in soil, the enhanced ability of organic C degradation causes the complex organic matter to degrade further, releasing more organic phosphoric acid and ultimately improving the utilization rate of organic P by soil microorganisms.

Our results showed that the abundance of organic C degradation genes was significantly correlated with the abundance of N-cycle genes (Fig. S9), which is consistent with previous studies, indicating that soil C and N cycles are tightly coupled in alpine ecosystems (Y. Yang et al., 2014; K. Yang et al., 2014; Yang and Luo, 2011). At the same time, the linkage of soil C and N cycles under N addition was also proven by the SEM results in this study (Fig. S10). Furthermore, we found that N additions simulated the degradation of labile and recalcitrant organic C in the soil labile fraction. The enhanced recalcitrant organic C degradation further promoted N ammonification and organic P degradation, and increased labile organic C degradation stimulated the assimilatory nitrate reduction (Fig. S10). In this study, the change in plant richness under N additions played an important role in affecting the abundance of genes involved in organic C degradation (Figs. S8b and S10), which might be due to the altered plant richness and species under N addition, which usually influence the quantity and quality of soil resources (Y. Yang et al., 2014; K. Yang et al., 2014). As a previous study showed, gross ecosystem productivity (GEP) increased when the N additions rate was >40 kg N ha^{-1} $year^{-1}$ in this study site (Liu, 2014), which could lead to an increase in the input of plant-derived C into the soil. Subsequently, the increase in plant C input stimulated microbial C degradation and utilization (Figs. 3 and S4) and ultimately affected the microbial C:N:P ratio. In addition, the enhanced degradation of soil organic matter from plants increased the availability of organic N and P in soil (Treseder and Vitousek, 2001), which further promoted ammonification and organic phosphorus utilization. In summary, our findings emphasized that increased plant-derived C input induced by high N addition (>40 kg N ha^{-1} $year^{-1}$) promoted the degradation of organic C by soil microorganisms and then increased the abundance of functional genes involved in soil N and P cycling to maintain the C:N:P ratio in microbial cells.

5. Conclusion

In this study, we found that N40 was the critical N load value for microbial functional genes in a Tibetan alpine steppe; that is, the abundance of microbial functional genes had no significant change when the N addition rate was ≤N40 but significantly changed above the N40 level. In addition, under high N additions (>N40), vegetation productivity increased significantly, which increased in plant-derived C input and then enhanced the ability of microbes to degrade soil organic C. To maintain a stable C:N:P ratio, microorganisms further enhanced ammonification, assimilatory nitrate reduction, N fixation and organic P degradation to obtain more available N and P. Our study revealed the microbial molecular mechanism of the C, N and P cycles in an alpine steppe under N addition, providing a worthwhile theoretical basis for further predictions of the responses of alpine ecosystems to future increased N deposition.

CRediT authorship contribution statement

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Xu Ri: Conceptualization, Methodology
Jizhong Zhou: Methodology
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Gengxin Zhang: Conceptualization, Methodology, Editing, Supervision.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

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