Effects of Nitrogen Addition on Nitrogen Transformation and Related Functional Gene Abundance in Pinus sylvestris var. mongolica Plantations (Postprint)

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Date: 2022-04-29T22:11:46+00:00

Abstract

Soil nitrogen (N) availability is an important factor affecting soil microbial community structure and soil nitrogen cycling; however, the effects of N addition on soil N transformation and N functional genes (NFGs) expression in Mongolian pine plantations remain poorly understood. To explore the effects of N addition on nitrogen transformation in Mongolian pine plantations and its underlying mechanisms, this study conducted a 2-year N addition experiment in a Mongolian pine plantation at Saihanba Qiancengban Forest Farm, with four N addition levels of 0, 1, 5, and 10 gN m-2 · year-1, designated as N0, N1, N5, and N10, respectively. Using the functional gene microarray GeoChip 5.0 system and laboratory soil incubation methods, the responses of soil NFGs to N addition and their effects on nitrogen transformation processes were investigated. The results showed that: (1) Compared with N0, medium and low N addition treatments (N1, N5) promoted the relative abundances of genes related to ammonification (ureC, nirA, nrfA), nitrification (amoA), and denitrification (norB), while high N treatment (N10) inhibited the expression of all NFGs. (2) Correlation analysis indicated that the promoting effects of N1 and N5 were significantly correlated with soil organic carbon (SOC), nitrate nitrogen (NO3-N), and microbial biomass carbon (MBC), while N10 treatment significantly reduced the relative abundances of NFGs in all nitrogen transformation processes, and this negative effect was associated with decreased dissolved organic carbon (DOC) and MBC contents. (3) Similar to the trend observed in nitrogen transformation gene abundances, N1 and N5 treatments significantly increased net N nitrification, net N mineralization, and N2O emission rates, whereas the promoting effect of N10 was not significant, indicating that there exists a threshold for the promoting effect of N addition on nitrogen transformation. (4) Multiple regression analysis further demonstrated that amoA-AOB and MBC were key

factors affecting net N nitrification, ureC, nirK, and MBC were key factors influencing net nitrogen mineralization, and narG and nirS were key factors affecting N2O emissions. In summary, N addition can enhance nitrogen transformation and increase the relative abundances of some specific enzyme functional genes in Mongolian pine plantations; however, there exists a threshold for N addition levels. When applying 10 g N m-2 · year-1, nitrogen transformation is inhibited, and 5gN m-2 · year-1 is the optimal level for promoting soil N transformation in Mongolian pine plantations.

Full Text

Title and Authors

Effects of Nitrogen Addition on Nitrogen Transformation and Related Functional Gene Abundance in *Pinus sylvestris* var. *mongolica* Plantations

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Abstract: Soil nitrogen (N) availability is a critical factor influencing soil microbial community structure and N cycling, yet little is known about how N addition affects soil N transformation and N functional gene (NFG) expression in *Pinus sylvestris* var. *mongolica* plantations. To explore these effects and underlying mechanisms, we conducted a two-year N addition experiment in a *Pinus sylvestris* var. *mongolica* plantation at Saihanba Mechanical Forest Farm, applying four N addition levels: 0, 1, 5, and 10 g N m^{-2} $year^{-1}$ (designated N0, N1, N5, and N10). Using the GeoChip 5.0 functional gene microarray system and laboratory soil incubation, we examined soil NFG responses to N addition and their influence on N transformation processes. Results showed: (1) Compared with N0, moderate N addition (N1, N5) promoted the relative abundance of genes related to ammonification (ureC, nirA, nrfA), nitrification (amoA), and denitrification (norB), while high N treatment (N10) inhibited expression of all NFGs. (2) Correlation analysis revealed that the promotional effects of N1 and N5 were significantly correlated with soil organic carbon (SOC), nitrate nitrogen (NO_3^--N), and microbial biomass carbon (MBC), whereas N10 significantly reduced relative abundance of NFGs for all N transformation processes, an effect associated with decreased dissolved organic carbon (DOC) and MBC content. (3) Consistent with NFG abundance patterns, N1 and N5 treatments significantly increased net N nitrification, net N mineralization, and N_2O emission rates, but N10 showed no significant promotion, indicating a threshold effect for N addition on N transformation. (4) Multiple regression analysis further identified amoA-AOB and MBC as key factors affecting net N nitrification, ureC, nirK, and MBC as key factors influencing net N mineralization, and narG, nirS as key factors controlling N_2O emissions. In conclusion, N addition can enhance

N transformation and increase relative abundance of specific enzyme functional genes in *Pinus sylvestris* var. *mongolica* plantations, but a threshold exists. At 10 g N m^{-2} $year^{-1}$, N transformation was inhibited, while 5 g N m^{-2} $year^{-1}$ represented the optimal level for promoting soil N transformation.

Keywords: nitrogen addition, nitrogen functional genes (NFGs), net nitrogen transformation, N_2O emissions

Introduction

Soil nitrogen (N) availability is a crucial factor affecting plant growth and soil biogeochemistry in terrestrial ecosystems (Ju, 2014). Global N deposition is altering soil N availability and influencing ecosystem N cycling (Liao et al., 2021; Wu et al., 2019), including N mineralization, nitrification, and N_2O emissions, which may lead to environmental problems such as N supply-demand imbalance, non-point source pollution, and greenhouse gas emissions (Li et al., 2021). Soil microorganisms are considered the primary drivers of N cycling processes, yet the response of core microbial processes in N cycling to N deposition and their relationship with N transformation remain poorly understood.

Nitrogen functional genes (NFGs) directly encode key enzymes that influence N cycling processes and serve as direct indicators of N biotransformation function (Liao et al., 2019). Previous NFG research has focused primarily on agricultural ecosystems and subtropical forest systems. In agricultural ecosystems, nitrogen fertilization has shown no significant effect on N fixation gene (nifH) abundance but increased relative abundance of nitrification genes (amoA) and denitrification genes (nirK, nirS, nosZ), primarily regulated by N form and soil pH (Ouyang et al., 2018). In subtropical forest soils, N addition decreased abundance of N fixation genes (nifH) and nitrification genes (amoA) while increasing denitrification gene abundance (Tian et al., 2019). Compared with subtropical forests and agricultural soils, cold-temperate forest soils in northern regions are characterized by relatively poor nutrient status, low N input, low precipitation, and large temperature fluctuations (Tian et al., 2019), which may result in different microbial community structures and differential effects on N transformation. However, few studies have reported on the effects of N addition on NFGs in cold-temperate forest soils.

In recent decades, scholars have conducted extensive research on N transformation in forest ecosystems, revealing preliminary mechanisms by which abiotic and biotic factors drive specific N transformation processes (i.e., nitrification, mineralization, and N_2O emissions) under N addition (Chelsea et al., 2016). Among all NFGs, the amoA gene affecting the rate-limiting step of nitrification, the narG gene affecting the first step of denitrification, and nir genes affecting rate-limiting steps are considered hub factors controlling N transformation (Ouyang et al., 2018; Chelsea et al., 2016). Soil available phosphorus, pH, and dissolved N have been identified as primary factors influencing NFG abundance (Wang et al., 2017), indicating that soil properties and microbial function play

important roles in N transformation. Exogenous N addition can directly affect soil properties and microbial community structure, exerting profound influences on N transformation processes (Wang et al., 2017). However, the extent to which these factors affect N transformation processes and the dominant factors influencing N transformation remain unclear. Therefore, critical questions remain: Can N addition mediate soil NFG expression to affect related N transformation processes? What are the relationships among soil N transformation processes, NFGs, and soil properties? These questions severely constrain our understanding of soil N transformation and its influencing mechanisms in *Pinus sylvestris* var. *mongolica* plantations. This study addresses these gaps by examining a *Pinus sylvestris* var. *mongolica* plantation in Saihanba, Hebei Province, using the GeoChip 5.0 functional gene microarray system and laboratory soil incubation. By analyzing effects of different N addition levels on soil properties, NFGs, and N transformation parameters, we explore the relative contributions of NFGs and soil properties to N transformation under different N addition levels and identify key factors affecting related N transformation processes, providing a theoretical basis for N fertilizer management in *Pinus sylvestris* var. *mongolica* plantations.

Materials and Methods

Study Area

The study area is located at the intersection of the Greater Khingan Mountains and the northern Hebei mountainous region, within the QianCengBan Forest Farm of Saihanba Mechanical Forest Farm in Weichang Manchu-Mongolian Autonomous County, northern Hebei Province (17°39 42 E, 42°35 45 N). Established in 1962, the forest farm encompasses 20,029.8 ha of artificial forest, representing a major artificial *Pinus sylvestris* var. *mongolica* population in China, with stand density of 1,300–1,600 trees per hectare. The plantation undergoes harvesting and replanting every 8–10 years. The region features a temperate continental semi-arid and semi-humid climate with long, cold winters and short, dry, windy spring and autumn seasons. The average background N deposition level is 3.34 g N m^{-2} $year^{-1}$. The experimental site has an elevation of 1,432 m, mean annual precipitation of 454.2 mm, mean annual sunshine duration of 2,368.8 h, mean annual evaporation of 1,244.9 mm, mean annual relative humidity of 75.3%, and mean annual temperature of -1.4 °C. The soil type is primarily aeolian sandy soil derived from aeolian, residual, and accumulative parent materials. Understory shrubs and herbs are sparse, dominated by *Thalictrum petaloideum*, *Potentilla longifolia*, *Sanguisorba officinalis*, and *Carex lanceolata*.

Experimental Design

In March 2018, we selected 12-year-old trees and established four N treatments with four replicates each, totaling 16 plots of 6 m × 6 m, separated by 4 m buffer zones. Nitrogen was applied as ammonium nitrate (NH_4NO_3) solution at four levels: 0 (N0), 1 (N1), 5 (N5), and 10 (N10) g N m^{-2} $year^{-1}$. Compared

with the natural N deposition rate (3.34 g N m^{-2} $year^{-1}$), N1, N5, and N10 represented low, moderate, and high N deposition levels, respectively. For each application, the corresponding NH_4NO_3 was dissolved in 10 L distilled water and applied every two months from June 2018 to June 2020, totaling 12 applications. Applications occurred at approximately 10:30 AM at the beginning of each month, ensuring no rainfall for 15 hours before and after application. The N0 treatment received equal volumes of distilled water with identical timing and method.

Soil Sampling and Processing

On June 20, 2020 (a sunny day), surface soil samples (0–20 cm) were collected using a stainless steel soil auger (20 cm length, 6 cm diameter) after removing surface litter. Five random sampling points were composited into one sample per plot and stored in sterile polyethylene bags. Samples were immediately placed in foam boxes with dry ice and transported to the laboratory. After removing residual roots, litter, and gravel, each sample was divided into three portions: one stored at 4 °C for microbial biomass and dissolved nutrient measurements and incubation experiments; another air-dried for basic physicochemical analysis; and the final portion immediately stored at -80 °C for NFG relative abundance analysis using the GeoChip 5.0 functional gene microarray system.

Soil Physicochemical Analysis

Soil physicochemical properties were determined following Bao (2000). Soil pH was measured by weighing 10.00 g air-dried soil into a 50 mL beaker, adding distilled water (water:soil = 2.5:1), intermittently stirring for 30 min, settling for 15 min, and measuring with an Ohaus ST3100/F pH meter. Soil organic carbon (SOC) was determined by weighing 0.5000 g air-dried soil (passed through 0.149 mm sieve) into a 150 mL Erlenmeyer flask, adding 0.10 g powdered $AgNO_3$, then precisely adding 5 mL each of 0.8 mol · L^{-1} potassium dichromate ($K_2Cr_2O_7$) solution and concentrated sulfuric acid, covering with a small funnel, heating in a 230 °C electric sand bath for 15 min, and titrating with 0.1 mol · L^{-1} ferrous sulfate solution. Total nitrogen (TN) was measured by weighing 0.5000 g air-dried soil into a Kjeldahl flask, moistening with distilled water, adding 8 mL concentrated sulfuric acid, digesting in a fume hood until light blue, and analyzing with an automatic Kjeldahl nitrogen analyzer (KDN-520, Hangzhou Lvbo Instrument Co., Ltd.). Total phosphorus (TP) was determined by weighing 0.25 g air-dried soil into a nickel crucible, adding 3 drops of anhydrous ethanol, covering with 2 g NaOH, heating in a muffle furnace at 400 °C for 15 min, washing with 75 °C distilled water, transferring to a 100 mL volumetric flask, taking 5 mL digested mother solution to a 50 mL volumetric flask, diluting to 30 mL with distilled water, adding 3 drops of dinitrophenol and 5 mL molybdenum-antimony anti-color reagent, and measuring at 700 nm with a UV spectrophotometer (T-6M, Shanghai Feile Instrument Co., Ltd.). Available phosphorus (AP) was measured by weighing 0.25 g air-dried soil into a 200

mL capped plastic bottle, extracting with 50 mL of 0.5 mol · L^{-1} $NaHCO_3$ at constant temperature (25 °C) for 30 min, filtering with phosphorus-free filter paper, adding 5 mL ammonium molybdate solution and 1 drop of stannous chloride reagent to 5 mL filtrate, developing color for 15 min, and measuring at 680 nm with a UV spectrophotometer. Ammonium nitrogen (NH_4^+-N) and nitrate nitrogen (NO_3^--N) were extracted by weighing 5.00 g air-dried soil into a centrifuge tube, adding 25 mL of 2 mol · L^{-1} KCl, shaking (120 r · min^{-1}) for 2 h, centrifuging at 8,000 r · min^{-1} for 15 min, filtering, and analyzing two aliquots of filtrate—one with HCl-EDTA buffer for NO_3^--N and another with phenol-EDTA buffer for NH_4^+-N—using a continuous flow analyzer (SmartChem 200, AMS/Alliance, Italy). Dissolved organic carbon (DOC) was determined by weighing 20.0 g air-dried soil into a 150 mL capped plastic bottle, adding 60 mL of 5 g · L^{-1} sodium metaphosphate, reciprocally shaking at 120 r · min^{-1} for 18 h, drying the suspension at 60 °C for 48 h, and analyzing with a TOC auto-analyzer (TOC-LCPH, Shimadzu, Japan). Total dissolved nitrogen (TDN) was measured by weighing 5.00 g air-dried soil into a 50 mL capped plastic bottle, adding 25 mL of 1 mol · L^{-1} potassium chloride solution, reciprocally shaking at 180 r · min^{-1} for 1 h, settling for 10 min, filtering into a 50 mL volumetric flask, oxidizing 8 mL filtrate with 10 mL potassium persulfate oxidant for 15 min, and analyzing with a continuous flow auto-analyzer. Dissolved organic nitrogen (DON) was calculated as TDN minus (NH_4^+-N + NO_3^--N) (Ji et al., 2020). Microbial biomass carbon (MBC) and nitrogen (MBN) were determined by fumigation-extraction: 70.0 g fresh soil at 40% field capacity was placed in an 80 mL beaker inside a vacuum desiccator with a beaker of chlorform, evacuated to -0.07 MPa until chloroform boiled vigorously for 5 min, repeated until complete fumigation, then transferred to a plastic bottle, extracted with 100 mL of 0.5 mol · L^{-1} potassium sulfate solution by shaking (25 °C, 300 r · min^{-1}) for 30 min, filtered, and analyzed with a TOC auto-analyzer—one 10 mL aliquot with 10 mL sodium hexametaphosphate solution for MBC, and another digested with 5 mL concentrated sulfuric acid for 3 h for MBN.

Measurement of Net N Transformation and N_2O Emission Rates

Net N transformation was divided into net nitrification rate (Rn) and net mineralization rate (Rm), both determined through 14-day soil incubation. Briefly, 40.00 g soil was placed in a 500 mL polyethylene bottle (8 cm height, 6 cm diameter) at 60% water-holding capacity, pre-incubated in a 25 °C constant-temperature dark incubator for 6 days, then formally incubated for 14 days under the same conditions. Soil samples were taken on days 1, 7, and 14 to determine inorganic N concentrations. Rn and Rm (mg N kg^{-1} d^{-1}) were calculated following Wang (2021): Rn = (post-incubation NO_3^--N - pre-incubation NO_3^--N) / incubation time; Rm = (post-incubation inorganic N (NH_4^+-N + NO_3^--N) - pre-incubation inorganic N) / incubation time.

N_2O emission rates were measured in parallel incubation experiments. All polyethylene bottles were covered with sterile breathable organic film to prevent

moisture loss while maintaining aeration. N_2O emission rates were measured on days 1, 3, 5, 7, and 14 using a Picarro G2508 gas concentration analyzer (Picarro G2208 Environment, Picarro Inc., CA, USA) (Ma et al., 2015). Bottles were sealed for 12 h before measurement, and N_2O emission rates (g kg^{-1} d^{-1}) were calculated from concentration changes during the 12 h sealed period.

Soil Microbial NFG Analysis

The GeoChip 5.0 functional microarray system was used for comprehensive analysis of microbial NFG sequences. This high-throughput platform contains 57,000 oligonucleotide probes covering over 144,000 gene sequences in 373 gene families, detecting broader gene types than qPCR and enabling analysis of diverse microbial functions and biogeochemical processes (Lu et al., 2018).

For each treatment, 0.50 g soil was thawed and DNA extracted using a PowerSoil kit (MoBio, Carlsbad, USA) with three parallel samples per site. DNA purity and quantity were assessed with a spectrophotometer and microplate reader system (FLUOstar OPTIMA, BMG Labtech, Jena, Germany). Qualified DNA samples were fluorescently labeled, hybridized to chips, and scanned (Li et al., 2017), with three replicates per microarray experiment.

Detailed procedures: DNA was labeled with Cy-3 fluorescent dye using random primers, purified with a QIA purification kit (QIAGEN QUICK Purification Kit, Roche NimbleGen Inc, USA), and dried in a rotary evaporator (Savant SVC200, Thermo Savant, Holbrook, NY, USA) at 45 °C for 45 min. Labeled DNA was dissolved in 120 L hybridization buffer (40% formamide, 0.1% SDS, 10 g unlabeled DNA, 2×SSC), denatured at 90 °C for 5 min, held at 50 °C for 30 min, and hybridized on a MAUI platform at 40 °C for 16 h. Hybridized chips were scanned at 633 nm using a NimbleGen MS200 microarray scanner (Roche NimbleGen, Inc., Madison, WI, USA). Images were converted, extracted, and normalized using Imagene 6.0 software. Normalization included inter-array normalization and false-positive removal, adjusting total signal intensity to the highest sample level in each batch, removing low-quality spots with signal-to-noise ratio < 2.0, normalizing signal values, and converting to natural logarithms (Zhang et al., 2017). All NFG analyses were performed by Shanghai Personal Biotechnology Co., Ltd.

Data Analysis

One-way ANOVA and Tukey' s multiple comparison tests (IBM SPSS 22.0) evaluated N addition effects on soil properties, gene relative abundance, and N transformation rates. Pearson correlation analysis examined relationships among soil properties, NFG abundance, and N transformation rates. Figures were prepared using Origin 8.5. All results are presented as means ± standard deviation (Mean ± SD) of four replicates.

Results

Effects of N Addition on Soil Physicochemical Properties

Two years of N addition significantly altered several soil physicochemical properties (Fig. 1). Soil NO_3^--N and DON concentrations increased with N addition level, reaching maximum values under N10 that were significantly greater than N0 ($P < 0.05$). Soil NH_4^+-N content showed a decreasing trend with increasing N addition, though differences among treatments were not significant. SOC, MBC, and C/N ratio exhibited initial increases followed by decreases, with maximum values under N5. DOC content also decreased with increasing N addition, with the highest value under N1. Other soil properties (MBN, TN, TP, AP, pH) showed minimal variation among treatments with no significant differences.

Effects of N Addition on NFG Relative Abundance

N addition significantly altered the relative abundance of several NFGs (Fig. 2). Among individual genes, N fixation gene (nifH) and denitrification gene (narG) showed the highest abundance values, accounting for 16.69–18.03% and 17.42–18.98% of total abundance, respectively. Compared with N0, N1 significantly increased relative abundance of amoA-AOA and amoA-AOB genes (encoding ammonia monooxygenase) involved in nitrification, norB gene (encoding nitrite reductase) involved in denitrification, nirA gene (encoding nitrate reductase) involved in assimilatory N reduction, and nrfA gene (encoding nitrate reductase coenzyme II) involved in dissimilatory N reduction. N5 significantly increased relative abundance of ureC gene (encoding urease) involved in ammonification, amoA-AOB, norB, nirA, and nrfA. In N10 treatment, all NFGs except hzsa gene (encoding glutamate synthase) involved in ammonia oxidation showed significantly decreased relative abundance.

Effects of N Addition on Total NFG Abundance by N Transformation Process

Total relative abundance of NFGs for each N transformation process revealed that denitrification, N fixation, and ammonification had the greatest microbial influence, collectively accounting for over 73.7% of total NFG abundance (Fig. 3). Across the seven N transformation processes (denitrification, N fixation, ammonification, anammox, nitrification, assimilatory N reduction, and dissimilatory N reduction), all processes except anammox showed significantly lower abundance under N10 compared with other treatments, following the trend N5 > N1 > N0 > N10, with no significant differences between N0 and N1.

Effects of N Addition on Net N Nitrification, Net N Mineralization, and N_2O Emission Rates

Two years of N addition significantly increased net N nitrification, net N mineralization, and N_2O emission rates under N1 and N5 compared with N0, reaching

maximum values under N5 and declining under N10, which showed no significant difference from N0 (Table 1). Compared with N0, N addition increased net N nitrification, mineralization, and N_2O emission rates by 18.62–57.41%, 10.95–49.88%, and 1.69–28.55%, respectively.

Correlation Analysis Between NFG Abundance and Soil Factors

Correlation analysis among 12 soil physicochemical indicators and NFG abundance for seven N transformation processes showed that TN, TP, NO_3^--N, and DOC were significantly correlated with NFG abundance for certain processes, while other soil factors showed no clear relationships (Table 2). TN was significantly positively correlated with nitrification, assimilatory N reduction, and dissimilatory N reduction processes. TP showed significant positive correlation with dissimilatory N reduction NFG abundance. DOC was significantly positively correlated with N fixation, ammonification, denitrification, assimilatory N reduction, and dissimilatory N reduction, while NO_3^--N was significantly positively correlated with NFG abundance for all processes except anammox.

Correlation Analysis of Net N Transformation Rates with NFGs and Soil Properties

Net nitrification rate (Rn), net mineralization rate (Rm), and N_2O emission rate (Re) were closely correlated with relative abundance of specific NFGs and soil properties (Table 3). Rn showed significant positive correlations with ureC, amoA-AOB, nirA, nrfA, SOC, NO_3^--N, and MBC, with particularly strong correlations with MBC and SOC. Rm was significantly positively correlated with NFGs involved in N cycling (ureC, gdh, nifH, amoA-AOB, narG, nirK, norB, nirA, nrfA) and SOC and MBC, with stronger correlations with MBC and nrfA. Re showed highly significant correlations with all nitrification and denitrification genes except amoA-AOA and hao, and highly significant positive correlations with SOC and MBC, particularly with amoA-AOB and narG.

Regression Analysis of Net N Transformation and N_2O Emission Rates

Stepwise multiple regression analysis identified amoA-AOB relative abundance and soil MBC content as dominant factors affecting net N nitrification rate (Rn), with $R^2 = 0.64$ ($P < 0.001$) (Table 4). UreC and nirK relative abundance and soil MBC content were dominant factors affecting net N mineralization rate (Rm), with $R^2 = 0.75$ ($P < 0.001$). NarG and nirS relative abundance were dominant factors affecting N_2O emission rate (Re), with $R^2 = 0.69$ ($P < 0.001$).

Discussion

Appropriate N addition can alleviate N limitation in ecosystems, providing substrates and energy for microbial growth and basal metabolism, thereby stimulating soil nutrient availability and enhancing microbial functional activity (Li

et al., 2017). Our study site in Saihanba has been confirmed to have low N reserves (Ren, 2012), suggesting N addition may influence substrate availability. Different N levels had differential effects: SOC, MBC, and DOC showed initial increases followed by decreases with increasing N addition, suggesting that excessive N addition may cause soil N saturation, affecting nutrient allocation and indicating a threshold for N addition effects. During the two-year experiment, soil pH ranged from 7.62 to 7.76 with no significant differences among treatments and no significant correlation with NFG relative abundance (Table 2), suggesting weak pH effects on NFGs in this study. This may be due to high soil buffering capacity (Jiang et al., 2019) or co-effects with other microorganisms such as arbuscular mycorrhizal hyphae (Cao et al., 2021). Additionally, no significant differences were observed among treatments for TN, TP, AP, and MBN, possibly because the two-year N addition period was too short to cause comprehensive changes in soil fertility (Ouyang et al., 2016).

Using the GeoChip 5.0 microarray system, we explored relative abundance of key genes involved in N transformation processes. Moderate N addition (N1, N5) showed promotional effects on all NFGs, particularly those related to ammonification, nitrification, and denitrification, while high N addition (N10) significantly reduced relative abundance of all NFGs.

Among ammonification-related genes, ureC relative abundance significantly increased under N1 and N5, peaking under N5 and declining significantly under N10, while glutamine synthesis gene (gdh) and anammox gene (hzsa) abundance remained unchanged, reflecting a trend toward mineralization as N approached saturation threshold (Zhang et al., 2019). Additionally, nifH showed no significant increase under N1 and N5, consistent with previous findings that N fertilization does not affect nifH relative abundance (Berthrong et al., 2014). This may be because available N (NH_4^+-N) obtained from the environment eliminates the need for additional energy metabolism investment (Zheng et al., 2017).

For nitrification-related genes, N addition (N1, N5) significantly increased relative abundance of amoA-AOA and amoA-AOB, consistent with findings in temperate forests by Szukics (2012), suggesting regional climate is not the primary driver of nitrification. Nitrification gene abundance was significantly positively correlated with soil TN and NO_3^--N (Table 2), indicating that N addition-induced increases in soil N content can enhance nitrification gene abundance. Notably, amoA-AOA abundance was consistently higher than amoA-AOB across all treatments. Previous studies indicate amoA-AOB is mediated by functional microorganisms with ribosomes, showing higher relative abundance in nutrient-rich environments or under moderate N addition, while amoA-AOA has higher affinity for ammonium and better tolerance for N-limited or N-saturated conditions (Ouyang et al., 2019). Therefore, amoA-AOA dominance in abundance but greater sensitivity of amoA-AOB to N addition in our study region mirrors observations in agricultural soils (Song et al., 2020).

For denitrification functional genes, N1 and N5 significantly increased norB rel-

ative abundance while other denitrification genes remained largely unchanged, differing from previous forest system studies showing that narG, nirK, and nirS abundance increased substantially with N addition while norB and nosZ were insensitive (Tang et al., 2016). This discrepancy may relate to the sequential steps of denitrification: nitrate is the initial substrate, and its concentration significantly affects narG expression; as denitrification proceeds, nitrate concentration decreases due to plant uptake or gaseous N loss (Liu et al., 2020), potentially weakening N addition effects on norB and nosZ (Chen et al., 2012). Additionally, denitrification gene relative abundance was significantly positively correlated with NO_3^--N and DOC (Table 2), consistent with previous findings that narG and napA relative abundance positively correlate with SOC and NO_3^--N content (Wang et al., 2017), likely because denitrifying microorganisms are mostly heterotrophic and thus more dependent on organic matter.

Soil N transformation processes, primarily nitrification, mineralization, and N_2O emission, are important indicators of N supply and loss (Lin et al., 2020). Our study found that moderate and low N addition significantly increased net nitrification rate (Rn), net mineralization rate (Rm), and N_2O emission rate (Re), but these metrics declined under high N treatment (N10). Rn and Rm are direct indicators of net changes in soil inorganic N pools, reflecting interactions among organic matter decomposition, mineralization, nitrification, and immobilization (Chen et al., 2012). Rn was significantly correlated with core genes of related N transformation processes (Table 3). Stepwise regression analysis identified amoA-AOB relative abundance and MBC content as key factors affecting Rn, with correlation analysis showing significant relationships between amoA-AOB and Rn, suggesting amoA-AOB may be the primary gene driving soil nitrification in semi-arid plantation ecosystems (Zhang et al., 2019; Tang et al., 2019). Similarly, stepwise regression identified ureC, nirK abundance, and MBC content as important parameters affecting Rm, indicating Rm is closely related to organic N mineralization and denitrification processes. Re was significantly correlated with NFGs involved in nitrification and denitrification (Table 3), with denitrification pathway genes narG and nirS closely related to Re, indicating denitrification is the primary pathway for N_2O release in temperate forest soils. The significant positive correlation between narG, nirS and Re suggests nirS abundance can serve as an effective parameter for estimating Re emissions (Flaa et al., 2019).

Conclusion

This study demonstrates that moderate and low N addition levels (1, 5 g N m^{-2} $year^{-1}$) had no significant effect on total relative abundance of N functional genes (NFGs) but increased relative abundance of specific genes involved in ammonification, nitrification, and denitrification. High N addition treatment (N10) significantly reduced relative abundance of NFGs for all N transformation processes. These effects were primarily associated with changes in soil C and N nutrient indicators (SOC, NO_3^--N, DON) rather than pH. The promo-

tional effects were significantly correlated with soil SOC, NO_3^--N, and MBC, while the negative effects of N10 were associated with reduced DOC and MBC content. Consistent with NFG abundance patterns, N1 and N5 treatments significantly increased net N nitrification, net N mineralization, and N_2O emission rates, but N10 showed no significant promotion, indicating a threshold effect. Stepwise multiple regression analysis identified amoA-AOB relative abundance and MBC content as key predictors of net N nitrification rate; ureC, nirK relative abundance and MBC content as key predictors of net N mineralization rate; and narG, nirS relative abundance as key predictors of N_2O emission rate. These results provide a theoretical basis for N fertilizer management in *Pinus sylvestris* var. *mongolica* plantations.

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Note: Figure translations are in progress. See original paper for figures.