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RESEARCH ARTICLE

Decreased soil N_2O and N_2 emissions during the succession of subtropical forests

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Abstract

Background and aims Natural forest succession may modify soil nitrogen (N) cycling and N gas emissions. However, little is known about how this ecological succession modulates soil N_2O and N_2 emissions. We focused on three typical succession chronsequences of subtropical forests: the early stage of an *Alnus nepalensis* forest (~ 60 years), the intermediate stage of a *Populus bonatii* forest (~ 100 years), and the late stage of an *evergreen broad-leaved* forest (> 300 years).

Methods The acetylene inhibition technique and molecular method were used to investigate the changing patterns of soil N_2O and N_2 emissions, as well as the key abiotic and biotic factors that regulate gas emissions.

Results The highest rates of soil N_2O and N_2 emissions were observed in the early-successional stage, which were 10–21 times and 6–12 times higher than those of the intermediate and late stages, respectively. This stimulation in the early stage was mainly related to the pure stands of N-fixing trees, thus amplifying soil inorganic N pools and providing additional substrates for nitrification- and denitrification- driven N_2O. Although N_2O emissions under denitrifying conditions were 2–131 times higher than those under nitrifying conditions, N_2 was the dominant N gas loss

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in subtropical forests. Changes in *nirK*-denitrifier abundance with forest succession were closely related to N_2O emissions.
Conclusion Our findings suggest that variations in soil active nitrogen pools and *nirK* abundance associated with subtropical forest succession could reduce N_2O and N_2 emissions, thus resulting in positive feedbacks for climate change mitigation.

Highlights

- The highest N_2O and N_2 emissions were observed in early-successional stage of *Alnus* forest.
- Enhanced N_2O and N_2 emissions by 6-21-fold in the early stage compared with the intermediate and late stages were observed.
- N_2O emission could be explained by changes in the nitrate pool and *nirK* abundance.
- N_2 rather than N_2O was the dominant gaseous N loss under denitrifying conditions.

Keywords Forest succession · N_2O and N_2 · Chronosequence · Denitrification · N-fixing trees · Subtropical forest

Introduction

Nitrous oxide (N_2O) is the third largest greenhouse gas globally, which can deplete the ozone layer and possesses a longer atmospheric lifespan than carbon dioxide (CO_2). On a 100-year scale, the single-molecule global warming potential of N_2O is approximately 300 times higher than that of CO_2 (Zhong et al. 2023). Therefore, accumulation of N_2O may have a destructive impact on a large time scale, and the increase in N_2O emissions has attracted global concern.

Soil is the largest nitrogen pool in the terrestrial ecosystem, storing approximately 136 Pg of nitrogen and 75% of N_2O emissions originate from soil (Kuypers et al. 2018; Oertel et al. 2016). According to the Food and Agriculture Organization's Global Forest Resources Assessment 2020, forests account for 31% of the total land area, and play a crucial role in mitigating global warming and mediating atmospheric N_2O concentration as a source or sink of N_2O (Zhang et al. 2019, 2023). For forests in different climate zones, subtropical forests have been identified as the largest natural source of N_2O in the world (Christensen and Rousk 2024; Xu and Cai 2007; Zhao et al. 2007). With the disturbance of humans on primary forests, the area of global secondary forests is increasing, and forest succession has become one of the focuses of research on terrestrial ecosystems (Liu et al. 2023a). The succession of plant communities holds significant importance in forest ecosystems, and understanding the process of succession is an important foundation for forest ecosystem restoration (Qu et al. 2020). Forest succession is driven by the interaction between above- and belowunderground parts (Chai et al. 2019). Alterations in plant community composition and diversity, along with variations in the quantity and quality of litter input, can trigger cascading effects on the soil carbon and nitrogen cycles, as well as microbial activity and other related processes, such as N gas fluxes (Banning et al. 2011; Cline and Zak 2015; Smith et al. 2015). These aspects form the heterogeneity of the forest environment at different succession stages, thus leading to different microbial succession patterns and functions. In turn, these variations affect the transformation of soil carbon and nitrogen and the loss of gaseous nitrogen. Although previous studies have suggested that succession can lead to changes in soil greenhouse gas emissions (Chabrerie et al. 2003; Jia et al. 2005), the current understanding of the changed patterns in soil N_2O and dinitrogen (N_2) emissions during succession is very limited.

Soil N_2O emissions are affected by multiple factors. The substrate concentrations (NH_4^+-N and NO_3^--N), N_2O source pathways (nitrification and denitrification), and N_2O-associated microorganisms are the three direct determinants of soil N_2O emissions (Butterbach-Bahl et al. 2013; Deng et al. 2019). On the other hand, the availability of oxygen (O_2) represents a critical regulatory factor. O_2 simultaneously controls nitrification and denitrification processes at the microbial cell level through enzyme synthesis and activity, thus determining the distribution of the final gas products N_2O and N_2 (Burgin and Groffman 2012; Chen et al. 2015). At present, the intricate processes underlying N_2O emissions, driven by microbial action and the complex interplay with soil and various environmental factors, remain enigmatic. Few quantitative studies have examined the impact of

O_2 on soil N_2O and N_2 emissions, particularly in the process of subtropical forest succession, thus greatly limits our understanding of the contribution of soil nitrification and denitrification to N_2O production and consumption during the natural succession of subtropical forests.

The net flux of N_2O at the soil atmosphere interface is the result of the dynamic balance between N_2O production and consumption processes in the soil (equal to N_2O production minus N_2O consumption, also known as net negative flux or net negative emissions when less than zero, indicating the existence of N_2O consumption processes) (Yang et al. 2022). The main mechanisms for producing N_2O include nitrification and denitrification, and the amount of N_2O produced by other biological or abiotic processes is minimal (Chapuis-Lardy et al. 2006). However, there is still little research on the mechanism of N_2O consumption in subtropical forest soils. Denitrification is a microbial driven procedure of nitrate allochthonous reduction, and the intermediate product of N_2O can only be consumed by the final step of denitrification, that is, the reduction of N_2O to N_2 by N_2O reductase. Denitrifying enzymes coded by the corresponding genes are often selected as functional markers for the analysis of microbial communities in various habitats such as lakes and soils. In turn, the study of denitrification functional genes contributes to the design of new primers and probes, as well as the development of new molecular ecology methods. For example, recent studies have demonstrated the existence of two distinct branches of the denitrification functional gene *nosZ*, namely branch I (Clade I) and branch II (Clade II) (Sanford et al. 2012). This newly discovered branch provides new possibilities for the consumption of N_2O by soil microorganisms and the discovery and application of *nosZ*-strains in ecosystems (Domeignoz-Horta et al. 2018; Shan et al. 2021).

Due to limitations in experimental conditions and methods, as well as the widespread distribution of denitrification genes, early research overlooked the important regulatory role of the population composition and diversity of denitrification microorganisms in the denitrification process. In fact, the presence of denitrification genes does not necessarily mean that denitrification genes are necessarily expressed in the environment (Chen et al. 2018b). Therefore, the different response mechanisms of denitrification microorganisms to changes in environmental conditions, such as oxygen and substrate variations, are of great significance for understanding the denitrification process and its influencing factors at different stages of forest succession. Therefore, the objectives of this study were (i) to investigate the impacts of subtropical forest succession on soil N_2O and N_2 emissions under varying oxygen conditions and (ii) to understand the relationship between functional microbial abundance and soil N_2O and N_2 emissions, as well as the dynamic shifts of soil carbon and nitrogen substrate. Based on previous findings that N_2O emissions decreased and N_2 emissions increased in subtropical forests under anaerobic conditions (Tang et al. 2018) and that the *nosZ*-denitrification gene could be used as a functional marker to reflect N_2O emissions (Chen et al. 2015), we hypothesize that: (i) with forest succession, nitrogen is no longer a limiting factor for plants and there will be more N substrate for microbes and thus, soil N_2O emissions will increase; (ii) acidic subtropical forest soil is more conducive to the production of denitrification intermediates, with N_2O being the major end gaseous product.

Materials and methods

Study area

The study area was located in the Ailao Mountain Nature Reserve (24°32′N, 101°01′E; 2450 m elevation), which is the junction of the Yunnan-Guizhou Plateau, Hengduan Mountains and Qinghai-Tibet Plateau. The natural area preserves the largest original Zhongshan wet broadleaved forest in the same-latitude subtropical region of the world, which is a hotspot area for biodiversity protection (Song et al. 2017). The average annual precipitation at this location is 1947 mm, with an average annual temperature of 11.3 °C and an average temperature of 15.3 °C (Liu et al. 2023b).

Due to deforestation, fire and the construction of reservoirs in the 1960s, some of the primary forests were destroyed, and later a variety of secondary forests were formed. Among them, *Alnus nepalensis* and *Populus bonatii* are the pioneer species in different succession processes. To examine these types, we selected three typical succession forests for this study. The details are as follows:

The *Alnus nepalensis* forest (early stage) is considered to be in the early stage of succession after disturbance, with a recovery time of approximately 60 years, and distributed in small patches on the margins of the primary forest (Fig. S1). The natural succession of *Alnus nepalensis* forest is usually in the form of pure woody stands. The shrub and herb layer consists of *Rhododen-dron delavayi*, *Lyonia ovalifolia*, and *Di-chroa febrifuga*. The forest canopy of this community is sparse, with a canopy density of 80% (Li et al. 2013). *Alnus nepalensis* is an N-fixing tree distributed in India, Bhutan, Nepal, and southwestern China. Due to its effective nitrogen fixing ability, it is widely used in forests operated in India. As a local tree species, *Alnus nepalensis* has been applied in managed forests or as a pioneer tree in natural forest succession in southwestern China.

The *Populus bonatii* forest (intermediate stage) is formed in the intermediate stage of forest succession. Its recovery time is longer than that of the *Alnus nepalensis* forest, which is approximately 100 years, and it occurs mainly in small patches in the region's low hills and slopes. (Fig. S1). The arborous layer is 15–20 m high with 80%-85% canopy density. In the arborous layer, Yunnan poplar accounts for an advantage, combined with *L. hancei*, *Terns-troemia gymnanthera*, and *Mucoraceae*. Few species can be found in the shrub layer (Li et al. 2013). In many areas, there were a small number of species, such as *Lithocarpus xylocarpus*, *Castanopsis rufescens*, and *Schima noronhae*, indicating that the community was moving towards the direction of *evergreen broad-leaved* forest.

The *Evergreen broad-leaved* forest (late stage) is the largest and the best protected primary forest in the area. Being in its stage, this forest has surpassed 300 years in age and is in an advanced stage of succession. Moreover, this area accounts for approximately 77.9% of the region (Fig. S1). The arboreal layer is 20–25 m high with 95% canopy density. *Lithocarpus xylocarpus*, *Castanopsis rufescens*, and *Schima noronhae* are the main species in this area (Li et al. 2013).

The soil of these three forest succession types is mountain yellow brown soil, which is a common soil type in Ailao Mountain. Its basic characteristics are that organic matter and cation exchange are significantly higher than that of the same type of soil in the horizontal zone, and it is acidic (pH = 4.2). For the soil texture, the soil is a loamy Lixisol or Ustalf Alfisol (by USDA Soil Taxonomy), originating from weakly metamorphosed marine sediments (Qiao et al. 2014).

Study design

In May 2021, soil samples were collected from three forest succession stages at Ailao Mountain (Fig. S1). Plant roots, animals, and debris were removed, and the soil was sieved through a 5 mm mesh and subsequently stored at 4 °C. A disturbance-free incubation experiment was conducted on the basis of growing season temperature of 15.3 °C. Three forest succession stages (early, intermediate, and late) and three oxide conditions with different oxygen conditions (aerobic, anaerobic, and anaerobic + 10% acetylene) were combined in pairs for the study. Acetylene (C_2H_2) with a volume concentration of 10% can inhibit the activity of nitrous oxide reductase in soil and block the reduction of N_2O to N_2. To ensure the consistency of atmospheric pressure inside and outside of the flask, a needle was used concurrently to release gas pressure with the filling gas was injected into the flask. A total of nine treatments were performed with eight replicates per treatment. Four parallel replicates are used for gas determination and the other four are used for physicochemical analysis without interference (Chen et al. 2015). Preculture was performed to stabilize soil metabolism at a temperature of 15.3 °C. Measure the original moisture content of the soil sample, and then measure the moisture content at 100% water holding capacity (WHC). The soils from the three forest moistures levels were adjusted to 50% WHC to eliminate the impact of moisture differences. The mass of water required is then calculated based on the target WHC (50%). Next, 50 μg N 25 g^{-1} soildw (NH_4NO_3) and 5 μmol C g^{-1} soildw (glucose) as substrate to provide carbon and nitrogen sources were added to the water for dissolution and evenly addition to the soil. In addition, a 250 mL culture bottle was filled with 25 g of soil (dry weight). Afterwards, the bottles for anaerobic and anaerobic + 10% C_2H_2 treatments were vacuum-pumped and replaced with 99.999% high-purity N_2. Among them, 25 mL N_2 in anaerobic + 10% C_2H_2 treatment bottles was replaced by 25 mL high purity C_2H_2.

During the incubation period, 25 mL air samples from the bottles were collected by syringe at 0, 1, 3, 7, 11, 14, 17, and 21 days. The N_2O concentration of the air samples was immediately assessed using gas chromatography (Shimadzu, Japan, GC-2014, with ECD). After collecting gas samples, the bottles were opened for 20 mins to assure the release of accumulated gases. Subsequently, the aforementioned procedure was repeated after sealing the bottles. Soil samples were collected at 0, 7, 14, and 21 days from each parallel bottle. Part of the soil sample was stored at 4 °C to investigate soil physicochemical properties, while the other part was stored at -80 °C for DNA extraction.

Analysis of soil physicochemical properties

The Soil water content was measured by subjecting fresh soil to oven-drying for 24 h (105 °C). For each parallel sample, 8 g of soil was extracted using 40 mL of 0.5 mol L^{-1} K_2SO_4 solution. The resulting extracts were utilized to determine the soil physicochemical properties. Among them, NH_4^+-N, NO_3^--N, and NO_2^--N were determined by ultraviolet spectrophotometry, and the dissolved organic carbon (DOC) concentration was determined by a TOC analyzer (TOC-L CPN; Shimadzu). Detailed information can be found in Dannenmann et al. (2009).

PCR amplification and paired-end sequencing of the 16S rRNA gene amplificon

Prokaryotic communities in different forest succession soils were studied using the V5-V7 hypervariable regions of 16S rRNA gene. The products were amplified with primer pairs 799F and 1193R by T100 Thermal Cycler PCR thermocycler (BIO-RAD, USA) (Caporaso et al. 2011). Then, Paired-end sequencing of the amplification products was performed using the Illumina PE300 platform (Illumina, San Diego, USA). The PE reads obtained by Illumina sequencing was quality filtered with fastq (0.19.6), while merged according to the overlap relationship between paired-end reads (Chen et al. 2018a). Then the high-quality sequences were de-noised using DADA2 (Callahan et al. 2016). DADA2 de-noised sequences are usually referred to as amplicon sequence variants (ASVs). PICRUSt2 was used to predict representative ASV sequences (Douglas et al. 2020). After the analysis, the absolute abundance of the related genes was converted to the relative abundance and then was carried out to facilitate subsequent analysis.

N_2O emissions and N transformation

N_2O emissions equation:

$$F = \rho \times V \times \frac{dc}{dt} \times \frac{1}{m,} \times \frac{P}{P_0} \times \frac{T_0}{T}$$

F represents the gas flux (μg N_2O kg^{-1} soil d^{-1}); ρ represents the density of N_2O in the standard state (1.977 kg L^{-1}); V represents the volume of the bottles following the withdrawal of 25 mL of gas (mL); $\frac{dc}{dt}$ represents the change in N_2O concentration collected per unit time; m, represents the dry soil weight (g); $\frac{P}{P_0}$ represents the ratio of the air pressure of the sample point to the standard atmospheric pressure; $\frac{T_0}{T}$ represents the ratio of absolute temperatures (273.15 K) at the start and sampling time (273.15 + t [°C] K).

Accumulated N_2O emissions equation:

$$E = \sum_{i=1}^{n} \frac{F_i + F_{i+1}}{2} \times \left(t_{i+1} - t_i\right).$$

E represents the accumulated N_2O emissions (μg N_2O kg^{-1}); the rate of N_2O emissions is represented by F (μg N_2O g^{-1} d^{-1}); i represents the i^{th} measurement; t_{i+1}—t_i represents the time interval.

Soil denitrification rate:

$$R_{denitrification} = \frac{N_2O - N_i + N_2 - N_i}{i}.$$

N_2O-N_i and N_2-N_i are the N_2O and N_2 emissions on day i of incubation, respectively; where, N_2 emissions is equal to N_2O emissions under anaerobic + 10% C_2H_2 conditions minus N_2O emissions under anaerobic conditions.

Statistical analysis

R 4.0.3, SPSS 27 (SPSS Inc, Chicago, USA) and Origin 2021 (OriginLab Corp., Northampton, MA, USA) were used for all statistical analysis and visualization. One-factor and multifactor ANOVAs were used to investigate the impacts of different forest successional stages, oxygen conditions, and incubation times on soil N_2O emissions, soil physicochemical

properties, and denitrification gene abundances. Differences between treatments were compared by the LSD test at $P<0.05$, following the homogeneity of the variance test. Meanwhile, Spearman correlations were employed to examine the intricate relationships among soil N gas emissions, soil physicochemical properties, and functional genes. Bray–Curtis distance was used in Mantel test.

Results

Potential N_2O and N_2 emissions

Forest succession and oxygen conditions significantly influenced soil N_2O emissions (Fig. 1, Table 2). Under aerobic conditions, the N_2O emissions of the early forest stage were 10.6 and 19.2 times significantly higher than those of the intermediate and late stages, respectively ($P<0.05$) (Fig. 1d). In addition, N_2O emissions from the intermediate and late stages did not exhibit a significant difference (-0.54 ± 0.06 and -0.57 ± 0.16 μg kg^{-1} dry soil for the intermediate and late stages, respectively, as shown in Fig. 1d. A similar pattern of N_2O and N_2 emissions was found under anaerobic conditions. The N_2O emissions of the early forest stage were the highest, which were 10.3 and 20.5 times higher than those of the intermediate and late stages, respectively (Fig. 1d). The N_2 emissions of the early forest stage were 11.1 and 6.1 times higher than those of the intermediate and late stages, respectively (Fig. 1d).

For different oxygen conditions, N_2O emissions from the anaerobic treatment were 2.7–131 times higher than those from the aerobic treatment (Fig. 1). Meanwhile, under anaerobic conditions, N_2 rather than N_2O was the dominant N gas product during the incubation (Fig. 2a, b). In particular, the emissions of

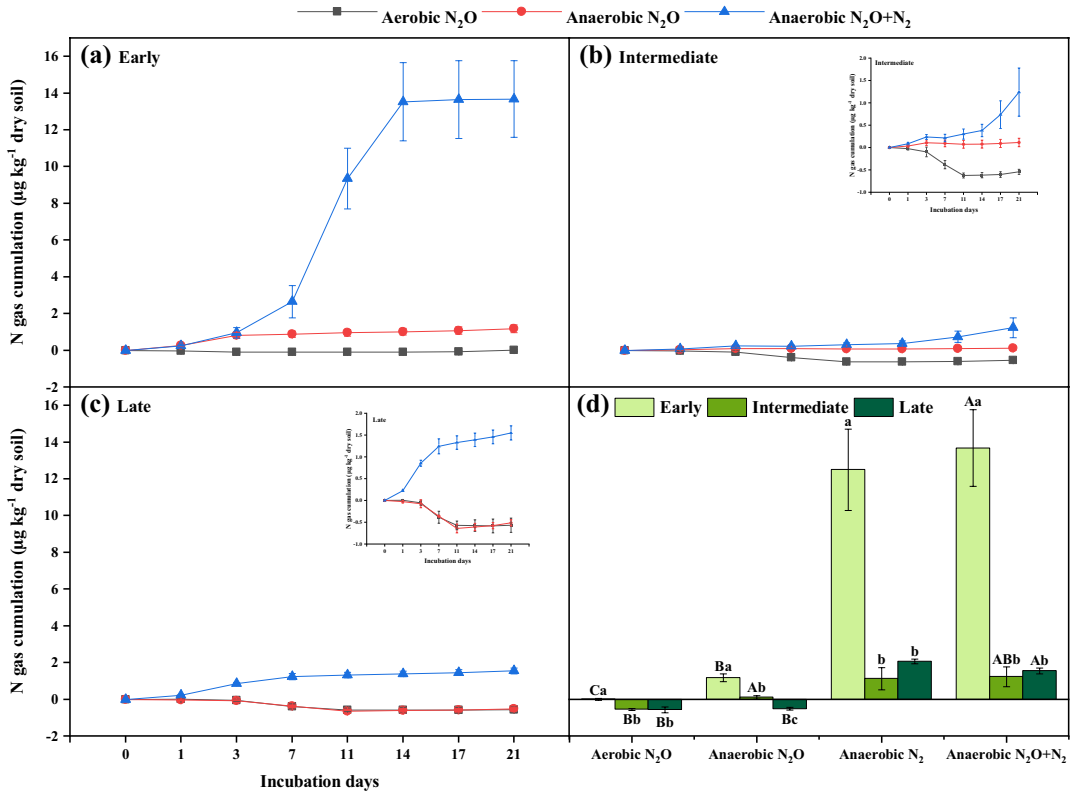

Fig. 1 Variations in accumulated N_2O and N_2 emissions under different forest succession (**a–c**) and oxygen conditions. Note: Values are means ± SEs ($n=4$). **d** represents the accumulated emissions of N gas during 21 days of incubation under different conditions. Uppercase letters represent differences for identical succession stages, and lowercase letters represent differences for identical oxygen conditions

N_2 were 9–31 times higher than those of N_2O at the end of incubation (Fig. 1d). Furthermore, with the succession of the forest, the ratio of anaerobic N_2O to aerobic N_2O gradually decreased, which manifested as the ratio of the early, intermediate, and late stages at 130.9, 12.5, and 2.7, respectively. The ratio of N_2 to N_2O was similar in the early and intermediate stages of forest succession (the early and late stages were 10.7 and 9.9, respectively) and significantly increased to 30.8 in the late stage (Fig. 1). The variations of $N_2O/(N_2O+N_2)$ showed a similar result, that is during the incubation process, there were dynamic changes of $N_2O/(N_2O+N_2)$, and overall, the ratio of $N_2O/(N_2O+N_2)$ in early stage and middle stage was significantly higher than that of late successional stage (Fig. 2a, b).

Soil denitrification rate

During forest succession, the early forest stage showed a significantly higher denitrification rate than the intermediate and late stages (Fig. 2c, d). The observed trend in the early stage exhibited an initial increase followed by a subsequent decrease during the incubation period, thus reaching its peak value of 0.97 μg N kg^{-1} d^{-1} on the 14th day of incubation (Fig. 2c). For the intermediate and late stages, the denitrification rate showed a similar trend. However, the value was much lower, with peak values of 0.08 ± 0.02 and 0.28 ± 0.02 μg N kg^{-1} d^{-1} for the intermediate and late stages, respectively (Fig. 2c). The mean denitrification rate of the early forest stage was 12 and 4 times higher than that of the intermediate and late stages, respectively (Fig. 2d).

Changes in soil physicochemical properties

The in situ soil physicochemical properties of the three successional stages were measured firstly, and the results showed that soil nitrogen content including the NH_4^+-N, NO_3^--N and total nitrogen (TN) in the early stage of forest succession were significantly higher than those in the middle and late stages of forest succession, whereas the soil organic

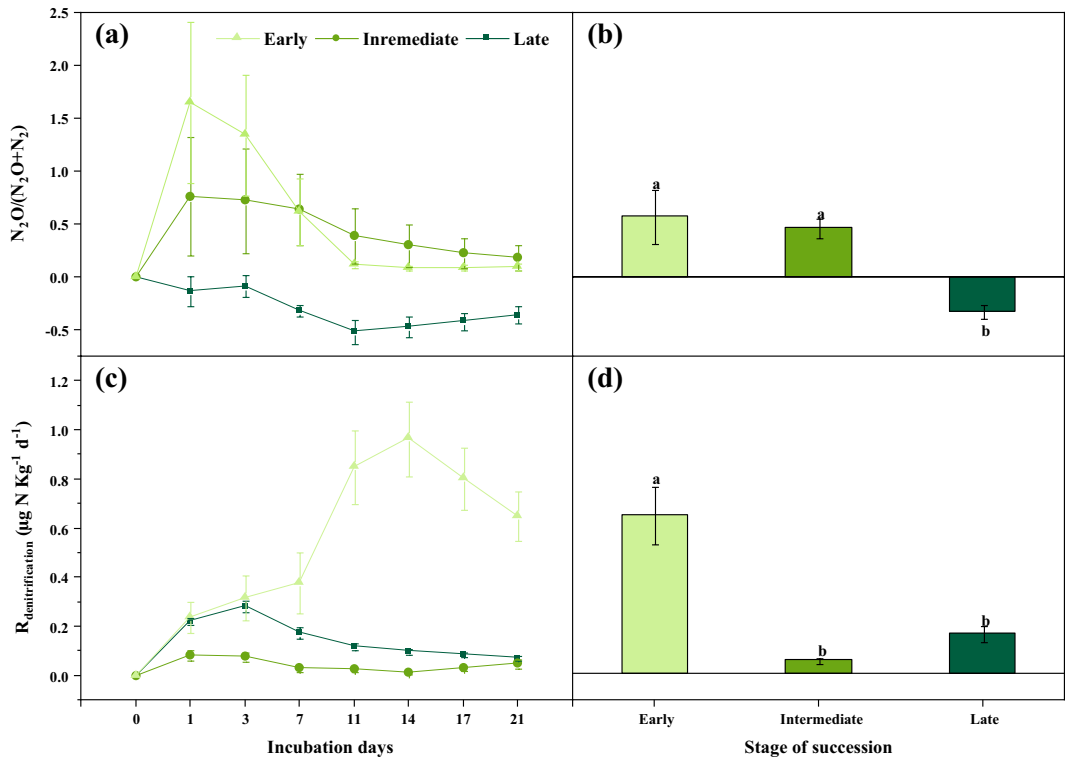

Fig. 2 Variations in the $N_2O/(N_2O+N_2)$ ratio (**a** and **b**) and soil denitrification rate (**c** and **d**) under forest succession. Note: **b** and **d** represent the mean denitrification rate and $N_2O/(N_2O+N_2)$ ratio under different forest successions, respectively

carbon (SOC) content was significantly lower than that in the late stage of succession (Table 1).

At the beginning of the incubation experiment, the same amount of carbon and nitrogen substrates were added and the dynamic changes were monitored. The soil NH_4^+-N concentration increased with time for the three forest succession stages (Fig. 3d, e, f). Compared with the initial value at the beginning of incubation, the NH_4^+-N concentration increased by 15.4%, 30.3%, and 13.7% for the early, intermediate, and late stages, respectively. For the soil NO_3^--N concentration, the changes were not significant under aerobic conditions throughout across the incubation (Fig. 3j, h, i). Under anaerobic conditions, during the whole incubation period, NO_3^--N showed a pronounced decrease after incubation, with declines of 17%, 32.9%-58%, and 1.8%-11% for the early, intermediate, and late forest stages, respectively (Fig. 3j, h, i). The pattern of NO_2^--N variation showed a similar trend to the change in NH_4^+-N. In addition, the NO_2^--N concentration increased by 67.3%, 47.9%, and 49.9% in the early, intermediate, and late stages, respectively, at the end of incubation (Fig. 3g, k, l). No significant change occurred in the DOC concentration under aerobic and anaerobic conditions without the addition of C_2H_2. However, a significant increase of 55%-85% occurred across forest succession under anaerobic + 10% C_2H_2 conditions (Fig. 3a, b, c). Multivariate analysis showed that forest succession and oxygen conditions had significant impacts on soil substrate variations during the incubation period (Table 2).

Relative abundance of the related nitrogen cycling genes in forest succession

Based on the results of gas experiments, the N_2O and N_2 emissions under denitrification conditions are absolutely dominant than that of nitrification conditions. Therefore, we focused on the denitrifier community and their relative abundance variations during the forest succession. In general, the relative abundance of most denitrifying genes (*napA*, *narG*, *narH*, and *nirK*) was significantly higher in the early succession of *Alnus nepalensis* forest soil than that in the middle and late succession stages (Fig. 4a-d). In contrast, the relative abundance of *nosZ* gene was significantly lower than in the middle and late succession stages (Fig. 4f).

Relationships between N gas and denitrifying genes and substrate concentrations

We examined the strength of the correlation between N gas emissions and their related biotic and abiotic factors, which were shown by heat maps (Fig. 5). The results of Mantel test showed that *nirK* rather than *nirS* composition, was closely related to the N gas emissions and substrate contents of mineral N and DOC (Fig. 5). Further analysis with denitrifying gene abundance indicated that the anaerobic N_2O and N_2 emissions were positively correlated with the *nirK* abundance but negatively correlated with the *nosZ* abundance (Fig. 5). In terms of soil physicochemical properties, the N gas emission was positively correlated with the concentration variations of NO_3^--N and NH_4^+-N ($r > 0.7$, $p < 0.01$, Fig. 5). In addition, pathways of N_2O emissions in forest succession under different oxygen conditions was analyzed by the piecewise SEM, and the results showed that under anaerobic conditions, changes in substrate NO_3^--N and NH_4^+-N concentration explained 89% of the variation in net N_2O emissions (Fig. S2). Meanwhile, changes in NO_3^--N concentration explained 87% of the gross N_2O emissions ($N_2O + N_2$) (Fig. S2c). Under aerobic conditions, forest succession and *nosZ* gene abundance accounted for

Table 1 Means ± SEs of the important factors in different forest succession stages at the field condition

	Early	Intermediate	Late
pH	3.67 ± 0.02b	4.18 ± 0.14a	3.54 ± 0.19b
NO_3^--N (mg kg^{-1})	26.21 ± 1.53a	3.74 ± 1.61b	4.91 ± 1.68b
NO_2^--N (mg kg^{-1})	0.16 ± 0.02b	0.11 ± 0.02b	0.34 ± 0.07a
NH_4^+-N (mg kg^{-1})	170.34 ± 9.16a	59.76 ± 13.53b	75.31 ± 23.88b
TN (g kg^{-1})	17.76 ± 1.98a	4.81 ± 1.05b	9.99 ± 2.47b
DOC (mg kg^{-1})	560.43 ± 38.13a	730.27 ± 105.39a	627.35 ± 80.84a
SOC (g kg^{-1})	287.42 ± 18.62b	210.71 ± 8.66c	404.66 ± 23.11a

Lowercase letters denote significant differences between the three forests ($P < 0.05$)

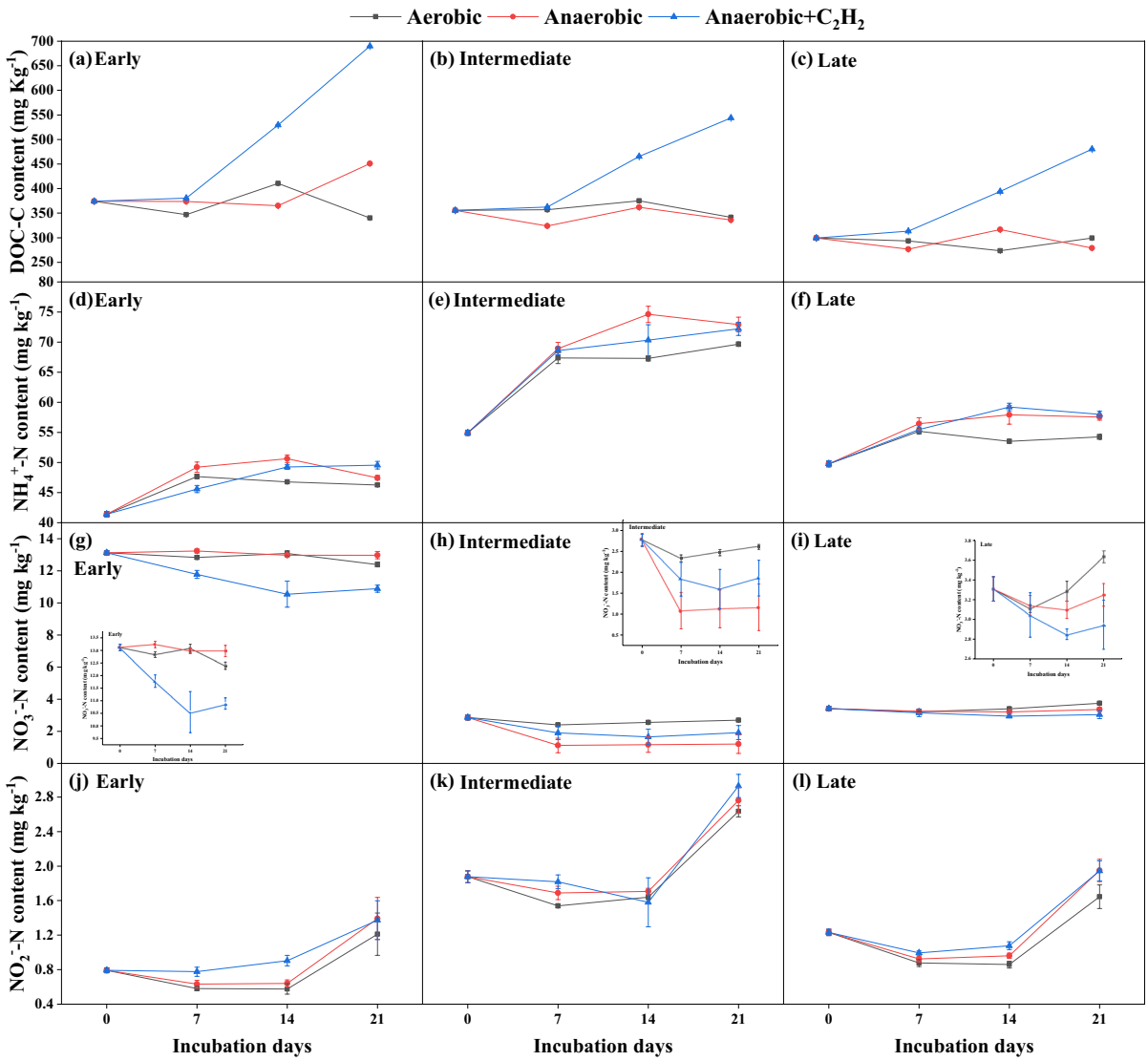

Fig. 3 Variations in soil physicochemical properties (DOC-C, NH_4^+-N, NO_3^--N, NO_2^--N) under forest succession and oxygen conditions (**a–l**, each property is shown separately according to forest succession). Note: Values are the means ± SEs ($n = 4$). Gray represents aerobic conditions, red represents anaerobic conditions, and blue represents anaerobic + 10% C_2H_2

79% of the variation in net N_2O emissions (Fig. S2a). Overall, the availability of inorganic nitrate substrate and the denitrifying genes of *nirK* and *nosZ* were identified as the primary factors that influenced soil N_2O emissions during forest succession.

Discussion

Our study found that the N_2O emissions from the early succession stage were 10.3–20.5-fold higher than those in the intermediate and late stages of forest succession. Similar results were observed for N_2, i.e., the N_2 emissions of the early stage of *Alnus nepalensis* monoculture was 6.1–11.1-fold higher than that in the intermediate and late stages. These results suggested that the early forest succession of *Alnus nepalensis* might be the peak period of N gas emissions. With forest succession, soil N_2O and N_2 emissions were significantly reduced after *Alnus nepalensis* was replaced. Notably, *Alnus nepalensis* was the N-fixing tree of the *Alnus* genus, and *Alnus nepalensis* existed

Table 2 Multivariate ANOVA analysis investigating the impacts of forest succession (Early, Intermediate, Late), oxygen conditions (aerobic, anaerobic, and anaerobic + 10% C_2H_2), incubation time on N_2O emissions, soil physicochemical properties and genes abundance, mineralization rate and denitrification rate

	Forest succession	O_2 condition	Time	Forest succession × time	O_2 condition × time	Forest succession × O_2 condition
N_2O	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
NH_4^+-N	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	0.149
NO_3^--N	**< 0.001**	**< 0.001**	**< 0.001**	**0.021**	**< 0.001**	**< 0.001**
NO_2^--N	**0.002**	**< 0.001**	**< 0.001**	0.459	**0.002**	0.979
DOC	**< 0.001**	**< 0.001**	**0.004**	**0.002**	**0.040**	0.057
nirK abunance	0.803	**< 0.001**	0.692	0.803	0.077	0.691
nirS abunance	0.548	**< 0.001**	0.952	0.548	**0.023**	0.555
nosZ-I abunance	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.021**
Mineralization	0.143	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	0.982
Denitrification	**< 0.001**	-	0.012	**< 0.001**	-	-

It aims to investigate the interactions among these factors to gain deeper insights into combined impacts. Significant difference at $P < 0.05$ level was shown in bold

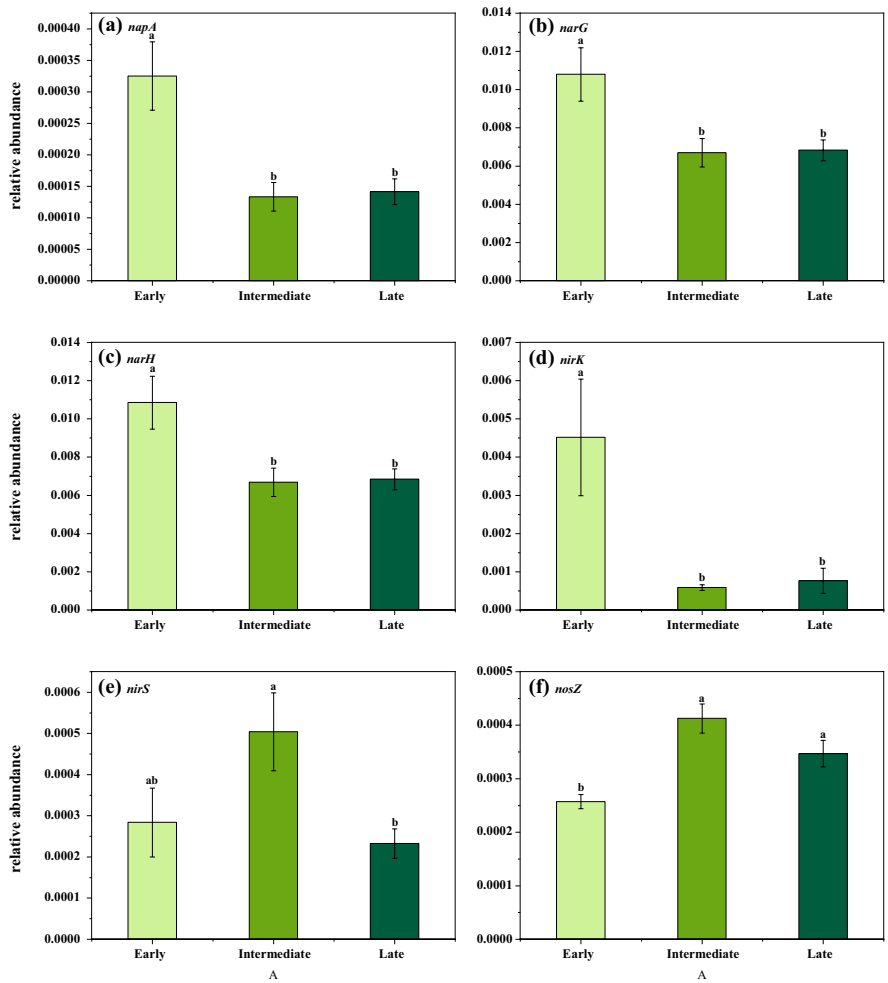

Fig. 4 Effects of forest succession on relative abundance of nitrogen cycling gene (**a–f**: *napA, narG, narrH, nirK, nirS, nosZ*). Note: Values are means ± SEs ($n = 4$)

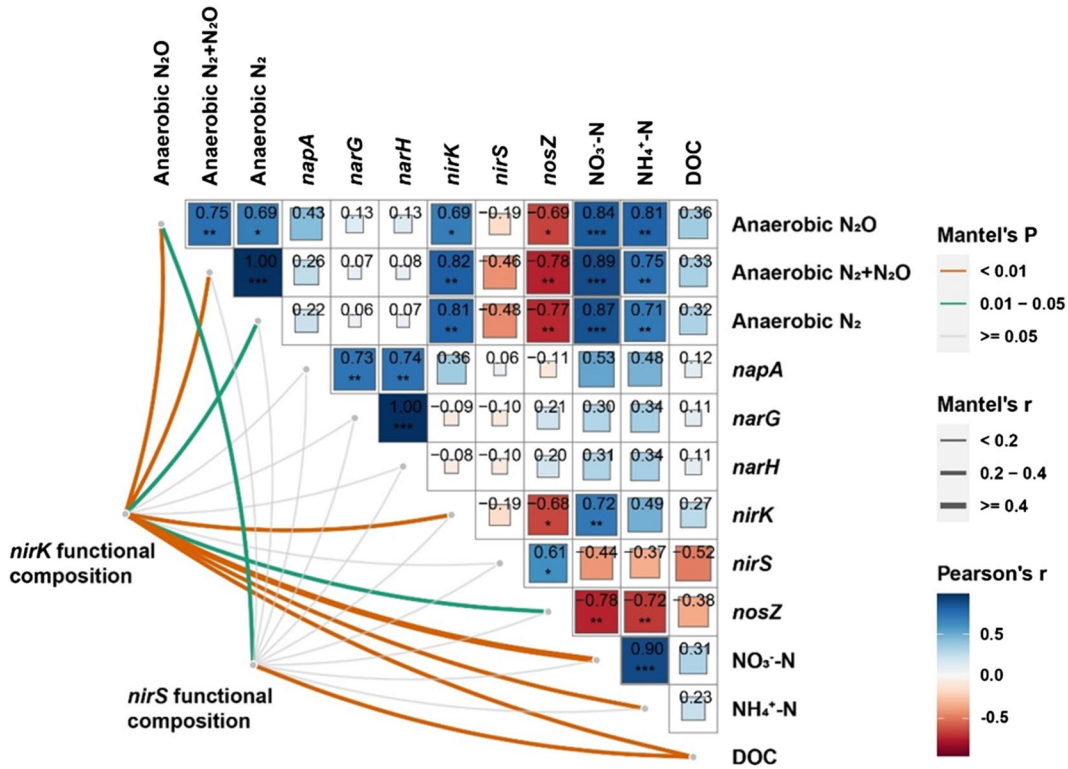

Fig. 5 Relationships between gas emissions and their related factors and the denitrifying gene functional composition. Note: Pairwise comparisons of gas emissions and their related factors were displayed with a color gradient, denoting Spearman's correlation coefficients. Functional community composition, based on biochemical KEGG modules, was assessed for its relation to each factor using Mantel test

almost exclusively in the early stages of natural succession. In addition to being at the forefront of natural forest succession in the subtropics, it is also one of the most widely planted trees in managed forests in Yunnan Province (He et al. 2013). It is considered an important ecological restoration plant due to its high nitrogen fixation rate and soil improvement effect (Joshi and Garkoti 2021, 2023). Our research results are consistent with most previous studies, which observed that compared with nonfixing trees, N-fixing trees significantly increased soil N_2O emissions. Recently, Kou-Giesbrecht and Menge (2021) conducted a systematic meta-analysis that showed a 2.17-fold increase in N_2O emissions from forests with N-fixing trees compared with forests with nonfixing trees. This effect was not significantly different between natural and managed N-fixing forests. N-fixing forests mainly promote N_2O emissions by increasing the soil nitrogen pool. Specifically, N-fixing trees mainly enhance the soil nitrogen pool through three pathways: root exudates (Batterman et al. 2013); free-living N-fixing microorganisms in the soil (Haney and Long 2010); and the degradation of leaf litter, roots, and nodules (Adams et al. 2016). Our measurement of soil inorganic nitrogen and total nitrogen as well as the structural equation models support this point (Table 1, Fig. 5, Fig. S2). In the surface soil of the *Alnus nepalensis* forest, the contents of ammonium, nitrate, and total nitrogen were significantly higher than those in the soil in the intermediate and late stages of succession (Table 1), thus providing more substrates for nitrification and denitrification and promoting N_2O emissions in the *Alnus nepalensis* forest. The results of correlation heat map also showed that NO_3^--N and NH_4^+-N have significant positive effects on N_2O and N_2 emissions (Fig. 5). The study by Joshi and Garkoti (2021, 2023) found that the nitrogen content in the leaves of the *Alnus* species is higher than that of other plants. The degradation of leaves and litter provides large amounts of nitrogen to the soil,

which can improve soil fertility. Similarly, our previous study found that compared with the intermediate and late stages of succession, the humus layer of *Alnus nepalensis* contains more inorganic nitrogen and SOC, which also provides more carbon sources for heterotrophic denitrification (Liu et al. 2023b).

In addition to the positive effects on the soil nitrogen pool, N-fixing tree forests can significantly increase the soil carbon pool potentially with active carbon components (Binkley 2005; Levy-Varon et al. 2019). These carbon sources, such as DOC, provide sufficient energy and electron donors for denitrification microorganisms. This is consistent with our findings (Figs. 1 and 2). The N_2O emissions under anaerobic conditions were approximately 50-fold (2.7–131-fold) higher than those under aerobic conditions. This ratio significantly decreased with forest succession, i.e., the ratios in the early, intermediate, and late stages of succession were 131, 12.5, and 2.7, respectively. These results indicated that denitrification was likely the main pathway for N_2O emissions in subtropical forests. Moreover, the contribution of denitrification to N_2O emissions was highest in the early stage, which was consistent with our results (Fig. 2d). Additionally, litter is one of the main inputs of carbon in soil. Most of the plant branches and leaves are distributed in the surface soil, and the decomposed humus accumulates in the surface soil. After leaching and other processes, the carbon-containing compounds enter the soil and are utilized by microorganisms. Compared with the *Populus bonatii* forest in the intermediate stage and the evergreen broad-leaved forest with dominant population of *Quercus* in the later stage, the litter of *Alnus nepalensis* is more easily decomposed (Feckler et al. 2023), thus providing more available carbon sources for denitrifying microorganisms to produce N_2O.

Given that denitrification contributed significantly to N_2O and N_2 emissions, we speculated that the abundance of denitrification genes (*nirK*, *nirS*, and *nosZ*) may have a strong positive correlation with N gas emissions. However, our results were not entirely consistent with this assumption. *nirK* abundance was positively correlated with N gas emissions, but *nosZ* was negatively correlated with them. Studies by Tang et al. (2018) and many others (Dandie et al. 2011; Dieng et al. 2015; Henderson et al. 2010; Miller et al. 2008) also found that the abundance of denitrification genes was decoupled from N_2O emissions. The contribution of fungi to N_2O emissions in forest soil may be more pronounced than that of bacteria. In addition, the genetic markers used in this study and most studies only target denitrifying bacteria. Therefore, they cannot represent all the number or activity of denitrifying microbes. Further research on the microbial production of N_2O and N_2 in subtropical forest soils should pay additional attention to the contribution of fungi. Although studies have suggested that the presence of N-fixing trees can stimulate the production of N_2O in the soil, the substrate of nitrogenase is not only N_2 but also other substances including N_2O (Vieten et al. 2008). Nitrogenase can reduce N_2O to N_2 to maintain nitrogen balance in the ecosystem and mitigate the greenhouse effect (OHara and Daniel 1985; Sameshima-Saito et al. 2004). Therefore, the number of N-fixing bacteria and the expression level of *nifH* genes may also affect N_2O and N_2 emissions (Kaneko et al. 2002; Wen et al. 2016), which also needs to be considered in future observations.

During the incubation period, a continuous decline in the ratio of $N_2O/(N_2O+N_2)$ was observed, which finally reached a level below 0.5. This result indicates that N_2 was the dominant gaseous N from subtropical forests (Fig. 6), which was in contrast to previous studies that showed acidic soil (pH = 4.2) favors the formation of N_2O intermediates over the final product of N_2 (Koehler et al. 2009; Zhang et al. 2021). This outcome may be related to the increase in soil DOC availability. Moreover, previous studies have shown that a higher soil DOC/NO_3^--N ratio promotes denitrification populations and soil nitrate-to-denitrification microsite diffusion rates (Saggar et al. 2012). More available carbon (C) promotes the growth of soil denitrifiers and increases the activity of soil nitrous oxide reductase, thereby reducing the $N_2O/(N_2O+N_2)$ ratio (Clough et al. 1998; Gillam et al. 2008). Furthermore, soil denitrifying bacteria primarily rely on soil NO_3^--N as the main electron acceptor; a positive relationship exists between the NO_3^--N content and the denitrification rate (Senbayram et al. 2012; Zaman et al. 2007). Higher $N_2O/(N_2O+N_2)$ ratios have been noted when soil nitrate levels reach high levels (80 mg N kg^{-1}), which may be due to inhibition of nitrous oxide reductase activity, the enzyme responsible for reducing nitrous oxide to N_2 (Senbayram et al. 2019). In contrast, the NO_3^--N concentration in this study did not change significantly

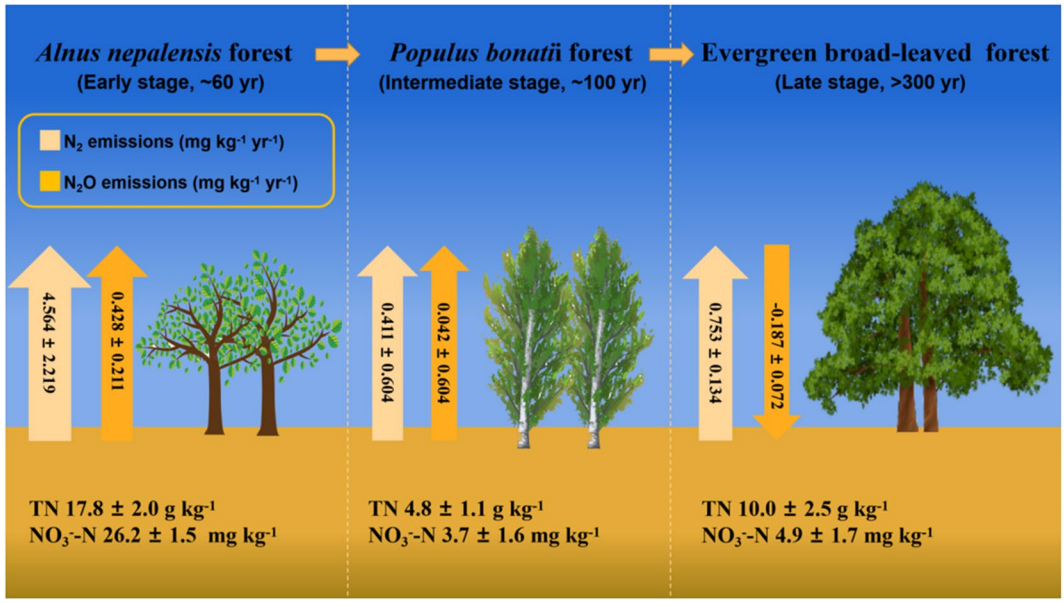

Fig. 6 Scheme of N_2O and N_2 emissions with different forest succession stages. Note: The thickness of the arrows is representative of the respective N_2O and N_2 emissions rates

under aerobic conditions and showed a decreasing trend under anaerobic conditions. This outcome indicates that N_2O reductase was not inhibited and that N_2O was converted to N_2, thus resulting in a lower ratio of $N_2O/(N_2O+N_2)$. In addition, when the supply of organic carbon substrates as reductants exceeded the supply of oxidants, such as the availability of nitrate, complete denitrification occurred, with N_2 as the main product (Yang et al. 2022).

Deviation in AIT technology for denitrification rate determination primarily results from two factors: incomplete inhibition of nitrous oxide reductase and suppressed nitrification (Yeomans and Beauchamp 1978; Yu et al. 2010). Regarding the incomplete inhibition of nitrous oxide reductase, three main reasons are identified: (i) incomplete diffusion of acetylene into soil particles. We addressed this problem by gently shaking the bottles to ensure uniform soil distribution in each repeated operation. (ii) consumption of acetylene by heterotrophic soil microorganisms. Studies indicate that acetylene is consumed primarily under aerobic conditions over short durations, with anaerobic conditions showing acetylene consumption only over extended periods (Culbertson et al. 1981; Qin et al. 2013). The experiment's short duration for each air extraction interval falls within the former circumstance. (iii) acetylene exhibits insensitivity towards certain denitrifying bacteria. Research findings suggest that incubation with 10% acetylene leads to complete or near-complete inhibition of N_2O reduction in specific bacteria such as *Pseudomonas perfectomarinus*, *Pseudomonas aeruginosa*, and Micrococcus denitrificans (Yoshinari and Knowles 1976). Considering that *Pseudomonas chlororaphis* is the dominant organism in nitrogen-rich environments and remains unaffected, the method retains some applicability. Moreover, both the inhibition of nitrification and incomplete suppression of N_2O are highly time-dependent, and the linear distribution of N_2O is the strong evidence of the effectiveness of AIT (Groffman et al. 2006), thus a linear trend of N_2O emission (Fig. 1) obtained by this study supporting the validity of the methods and results.

Conclusion

Our study revealed that subtropical forest succession had a significant impact on soil N_2O and N_2 emissions. Generally, soil N_2O and N_2 emissions in the early stage of the *Alnus nepalensis* forest were one order of magnitude higher than those in the

intermediate and late stages of succession. This outcome is mainly related to the N-fixation of the *Alnus nepalensis* forest. The pure stands of N-fixing trees pronouncedly stimulated the availability of soil nitrate, ammonium and DOC, thus providing more substrates for nitrification- and denitrification-driven N_2O emissions. Furthermore, N_2O produced under denitrifying conditions was the main pathway for N_2O emissions from subtropical forests, i.e., denitrifying N_2O emissions were 2–131-fold higher than those nitrifying emissions, and the contribution of denitrification to N_2O emissions decreased sharply with succession. These findings were mainly attributed to the higher nitrate pools as well as the larger *nirK*-denitrifier abundance in the early stages of succession. In addition, the emissions of N_2 were 10–31-fold higher than those of N_2O, thus indicating that N_2 was the dominant N gas loss in subtropical forests under denitrifying conditions. N-fixing trees are pioneers in natural succession or the main tree species applied in managed forests worldwide. Hence, our research revealed that their planting areas may have high potential gaseous N losses, especially N_2O emissions, which can weaken the carbon sequestration of N-fixing trees and exert a positive effect on greenhouse gas emissions. With forest succession, the loss of gaseous N will be significantly reduced after N-fixing trees are gradually replaced, thus indicating that the natural succession of subtropical forests is beneficial for alleviating global N_2O emissions.

Authors contribution CZ conceived the ideas and designed the study. YMY conducted the experiments, data analysis and led the writing of the first draft of the manuscript. LP and LZY contributed to revising the manuscript. All authors contributed to the drafts and gave final approval for publication.

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Declarations

Competing interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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