Microbial adaptation to long-term N supply prevents large responses in N dynamics and N losses of a subtropical forest

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Abstract

Atmospherically-deposited nitrogen (N) can stimulate complex soil N metabolisms and accumulations over time. Whether long-term (decadal) N deposition effects on soil N transformations and functional microbes differ from the short-term (annual) effects have rarely been assessed. Here we conducted a laboratory 15N tracing study with soil samples from a short-term (one year) N addition site and a long-term (12 years) site in a subtropical forest. The effects of simulated N deposition on soil N2O emissions, N transformation rates and microbial nitrifying and denitrifying genes were determined. Our results showed that: (1) long-term N addition did not change soil N2O fluxes significantly in comparison to the short-term N addition. Denitrification, heterotrophic nitrification and autotrophic nitrification contributed 53%, 23% and 18% to total N2O emissions, respectively. (2) Autotrophic nitrification was the dominant N transformation process, except for the high-N treatment at the long-term site. The magnitude of soil N transformation rates was significantly different among N addition treatments but not between short- and long-term N addition sites. However, long-term N addition changed the responses of specific N transformation rates to N addition markedly, especially for the rates of nitrification, organic N mineralization to NH4+, NO3- immobilization and dissimilatory NO3- reduction to NH4+ (DNRA). (3) Responses of ammonia oxidizing archaea and bacteria (AOA and AOB) were stronger than denitrifying N2O-producers (nirK) and denitrifying N2O-reducers (nosZ) at the long-term site compared to the short-term site. (4) The close correlations among N2O flux, functional genes and soil properties observed at the short-term site was weakened at the long-term site, posing a decreased risk for N losses in the acid subtropical forest soils. There is evidence for an adaptation of functional microbial communities to the prevailing soil conditions and in response to long-term natural and anthropogenic N depositions.

Full Text

Preamble

Microbial adaptation to long-term N supply prevents large responses in N dynamics and N losses of a subtropical forest

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Abstract

Atmospherically-deposited nitrogen (N) can stimulate complex soil N metabolisms and accumulations over time. However, whether long-term (decadal) N deposition effects on soil N transformations and functional microbes differ from short-term (annual) effects has rarely been assessed. Here we conducted a laboratory ^{15}N tracing study with soil samples from a short-term (one year) N addition site and a long-term (12 years) site in a subtropical forest to determine the effects of simulated N deposition on soil N_2O emissions, N transformation rates, and microbial nitrifying and denitrifying genes. Our results showed that: (1) long-term N addition did not change soil N_2O fluxes significantly compared to short-term N addition. Denitrification, heterotrophic nitrification, and autotrophic nitrification contributed 53%, 23%, and 18% to total N_2O emissions, respectively. (2) Autotrophic nitrification was the dominant N transformation process, except for the high-N treatment at the long-term site. The magnitude of soil N transformation rates differed significantly among N addition treatments but not between short- and long-term N addition sites. However, long-term N addition markedly changed the responses of specific N transformation rates to N addition, especially for nitrification, organic N mineralization to NH_4^+, NO_3^- immobilization, and dissimilatory NO_3^- reduction to NH_4^+ (DNRA). (3) Responses of ammonia oxidizing archaea and bacteria (AOA and AOB) were stronger than those of denitrifying N_2O-producers (nirK) and denitrifying N_2O-reducers (nosZ) at the long-term site compared to the short-term site. (4) The close correlations among N_2O

flux, functional genes, and soil properties observed at the short-term site were weakened at the long-term site, suggesting a decreased risk for N losses in these acid subtropical forest soils. These findings provide evidence for adaptation of functional microbial communities to prevailing soil conditions in response to long-term natural and anthropogenic N depositions.

Keywords: Short- vs. long-term N deposition, ^{15}N tracing model, Gross N transformation, Net mineral N production, N_2O flux, Microbial functional gene

1. Introduction

Nitrous oxide (N_2O) is an important greenhouse gas with stronger radiative forcing than CO_2 (Matson et al., 2002; Wang et al., 2014a). Terrestrial ecosystems contribute approximately 57% of global N_2O emissions (Werner et al., 2007), with tropical and subtropical forests representing the largest natural terrestrial source of N_2O globally (Werner et al., 2007; Cheng et al., 2014). In recent years, tropical and subtropical regions have experienced severe atmospheric N deposition due to enhanced atmospheric reactive N (Nr) from anthropogenic N fertilization (MacDonald et al., 2002; Herrmann et al., 2005; Du et al., 2014; Zhu et al., 2015), biomass burning, and fossil fuel combustion (Boy et al., 2008; Chen et al., 2010). The deposited inorganic and organic N subsequently participates in soil internal N turnover (Avrahami et al., 2002; Bai et al., 2014; Deppe et al., 2017), likely affecting forest soil N_2O emissions (Galloway et al., 2004; Galloway et al., 2008; Vet et al., 2014). However, the relationship between increased N deposition and soil N transformation/N_2O emission in subtropical and tropical forests remains poorly understood due to complex interactions among climate, forest type, soil properties, microbial communities, and land-use history (Chatskikh et al., 2005; Alm et al., 2007; Werner et al., 2007; Zhang et al., 2008; Chen et al., 2014).

Although abiotic processes such as chemodenitrification and chemical decomposition of hydroxylamine may contribute to soil N_2O production (Williams et al., 1992; Bremner, 1997; Butterbach-Bahl et al., 2013), microbial nitrification and denitrification account for over 70% of global N_2O emissions (Avrahami et al., 2002; Braker and Conrad, 2011; Butterbach-Bahl et al., 2013). Different soil microorganism groups participate in N transformation processes that produce N_2O (Levy-Booth et al., 2014). For example, ammonia (NH_3) oxidation, the first step of autotrophic nitrification catalyzed by the amoA-encoded ammonia monooxygenase of ammonia oxidizing archaea and bacteria (AOA and AOB), represents an important rate-limiting step for N_2O production (Szukics et al., 2010; Isobe et al., 2012; Long et al., 2012; Wertz et al., 2012; Wang et al., 2014b; Tang et al., 2016). Denitrification—the reduction of nitrate (NO_3^-) to N_2O and further N_2O to N_2, catalyzed by several enzymes such as narG-encoded nitrate reductase, nirK/nirS-encoded nitrite reductase, norB-encoded nitric oxide reductase, and nosZ-encoded nitrous oxide reductase—is also essential for N_2O formation and consumption in soils (Freedman et al., 2013; Jones et al., 2013; Levy-Booth et al., 2014; Orellana et al., 2014; Yu et al., 2014; Domeignoz-Horta et al., 2017).

Quantifying soil nitrifiers and denitrifiers, particularly nitrifying N_2O-producers (AOA-amoA, AOB-amoA), denitrifying N_2O-producers (nirK/nirS), and denitrifying N_2O-reducers (nosZ and the newly identified N_2O-reducing clade), may facilitate understanding of N_2O production and consumption in soils (Isobe et al., 2012; Yu et al., 2014; Zhang et al., 2014; Domeignoz-Horta et al., 2015; Tang et al., 2016; Domeignoz-Horta et al., 2017; Hallin et al., 2017). Moreover, various environmental factors such as soil moisture, redox potential, pH, and nutrient availability affect soil nitrifiers and denitrifiers (Bárta et al., 2010; Hu et al., 2013; Zhang et al., 2014; Zhang et al., 2015; Tang et al., 2016). Although numerous studies have examined relationships among soil properties, functional microbes, and N_2O fluxes (Fang et al., 2008a; Yu et al., 2014; Zhang et al., 2014; Faeflen et al., 2016; Chen et al., 2017), our understanding of microbial-regulated N_2O emission responses to elevated N deposition in subtropical forest soils remains limited (Avrahami et al., 2002; Zhong et al., 2015; Cui et al., 2016; Gao et al., 2016).

Soil N_2O production and emission occur in diverse soil N pools via different transformation processes, including N_2O from the nitrate nitrogen (NO_3^--N) pool via denitrification, from the ammonium nitrogen (NH_4^+-N) pool via autotrophic nitrification, and from the organic nitrogen (Norg) pool via heterotrophic nitrification (Müller et al., 1998; Zhang et al., 2011). Quantifying contributions from these N_2O production pathways is essential for understanding the fate of atmospherically-deposited inorganic and organic N (Fang et al., 2008b). Since production and consumption of NH_4^+ and NO_3^- are tightly associated with mineralization of organic N to NH_4^+ and NO_3^-, immobilization of NH_4^+ and NO_3^- to organic N, and dissimilatory NO_3^- reduction to NH_4^+ (DNRA), quantifying contributions from these processes is also crucial for uncovering the fate of atmospherically deposited N. Over the last decade, advanced microbial and stable isotope methods have improved understanding of internal N transformations and underlying microbial drivers. The development of ^{15}N tracing techniques allows quantification of specific N processes by differentially labeling soil N pools (e.g., $^{15}NH_4^+$ or $^{15}NO_3^-$) (Hart et al., 1994). Moreover, atmospherically-deposited N stimulates complex soil N metabolisms and accumulations over time (Fang et al., 2009; Wang et al., 2015; Deppe et al., 2017).

Long-term N deposition effects likely induce rate and microbial responses that differ from short-term effects. While some previous studies have addressed temporal effects of N deposition on forest soil N transformations, they mainly focused on seasonal comparisons (Breuer et al., 2000; Kiese et al., 2003; Zhu et al., 2013a). Less attention has been paid to differentiating short-term (a few years) versus long-term (over a decade) N deposition effects (Isobe et al., 2012; Gao et al., 2016; Gurmesa et al., 2016).

Subtropical forests in south China suffer from high atmospheric N deposition (Mo et al., 2006; Zhu et al., 2013a; Zhu et al., 2015) with hitherto unknown effects on ecological processes. The Dinghushan Long-term Nitrogen Research (DHSLTNR) study (Lu et al., 2008; Liu et al., 2011) is one of the longest-running

N deposition projects in China, having received 12 years of simulated N addition. This provides a unique research facility for time-scale comparisons.

To compare long- versus short-term effects of N deposition on soil N transformations and associated functional microbes, we established a short-term N addition site adjacent to the long-term site. The similar hydrothermal conditions at the two sites allow comparison of N_2O emission, N transformation, and gene regulation differences over time at different N deposition levels. Our specific objectives were to: (1) quantify the relative contribution of N_2O production pathways in subtropical forest soils, (2) characterize the responsive behaviors of various N transformation processes to simulated N deposition, (3) assess the role of functional microbes in regulating N_2O emission and the fate of deposited N, and (4) compare long- versus short-term N addition effects.

2.1 Site description and sample collection

The simulated short-term and long-term N deposition sites are located in the Dinghushan Biosphere Reserve (DHSBR) (23°10 N, 112°10 E) in Guangdong Province, south China. The DHSBR covers 1155 ha, with a humid monsoon climate characterized by 1927 mm mean annual precipitation. Approximately 75% of precipitation occurs from March to August, creating distinct wet-dry seasonality. The mean annual temperature is 21.0 °C, with minimum and maximum monthly averages of 12.6 °C in January and 28.0 °C in July (Mo et al., 2006). The reserve has experienced naturally high rates of atmospheric N deposition (20-50 kg N ha^{-1} a^{-1}), with total wet N deposition reaching 34.4 kg N ha^{-1} a^{-1} compared to the average value of 29 kg N ha^{-1} a^{-1} (as inorganic N in bulk precipitation) in the 1990s (Fang et al., 2008b; Lu et al., 2013; Gurmesa et al., 2016). Both study sites are located in a monsoon evergreen broadleaf forest that has been well protected from human activities for the past 400 years (Mo et al., 2006). The soil is Ferrasols according to the World Reference Base for Soil Resources (WRB) classification system (FAO, 1998; Chen et al., 2005), with depth ranging from 30 to 70 cm (Mo et al., 2006; Fang et al., 2009). Major tree species include *Castanopsis chinensis*, *Schima superba*, *Gironniera subaequalis*, *Syzygium acuminatissimum*, and *Aporusa yunnanensis* (Mo et al., 2006).

The field N addition experiment at the long-term site (L site) began in July 2003 by spraying ammonium nitrate (NH_4NO_3) solution monthly (Mo et al., 2006). Four N-addition gradient treatments were established with applications of 0, 50, 100, and 150 kg N ha^{-1} a^{-1} as NH_4NO_3, abbreviated as control (LC), low N (LL), medium N (LM), and high N (LH), respectively. Three replicate plots (each 10 m × 20 m) were established for each N treatment (12 plots total), with a 10 m buffer strip between adjacent plots. For each plot, the required NH_4NO_3 amount was dissolved in 20 L water and sprayed evenly below the canopy using a backpack sprayer (application rate of 0.1 L m^{-2} was low to avoid liquid application effects) (Mo et al., 2006; Lu et al., 2013).

The short-term site (S site) received N depositions from September 2014 onwards

at 0, 35, 70, and 105 kg N ha^{-1} a^{-1} for control (SC), low N (SL), medium N (SM), and high N (SH), respectively. Three replicates (each 15 m × 15 m, with 10 m wide buffer strips) under each N treatment formed 12 plots total. NH_4NO_3 fertilizer was dissolved in 30 L water and sprayed evenly to N-plots (application rate of 0.1 L m^{-2}). At soil sampling in July 2015, the long-term and short-term sites had received simulated N deposition for approximately 12 years and one year, respectively.

Twenty-four soil samples (2 sites × 4 N treatments × 3 replicates) were collected from surface soil (0-20 cm) after litter removal in July 2015. Six soil cores were randomly selected in each replicate plot and mixed into a composite sample. All soil samples were sieved (2 mm mesh) and split into three subsamples: one stored at 4 °C for incubation studies, one at -80 °C for functional gene analyses, and one used directly or air-dried for soil physicochemical property determination, including soil water content (WC), water holding capacity (WHC), pH, ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), total organic carbon (TOC), total nitrogen (total-N), microbial biomass carbon (MBC), and nitrogen (MBN).

2.2 ^{15}N-tracing experiment

A paired ^{15}N tracing experiment was conducted on collected soil samples with two ^{15}N treatments: $^{15}NH_4NO_3$ and $NH_4{}^{15}NO_3$, both at 10 atom% ^{15}N enrichment. For all samples from both sites, 240-250 ml flasks (2 ^{15}N treatments × 2 sites × 4 N treatments × 3 replicates × 5 sampling points) were prepared. Each flask contained fresh soil equivalent to 20 g dry weight and was covered with a sealing membrane with three pinholes to allow gas exchange while preventing moisture loss. Soils were pre-incubated overnight in the dark at 20 °C and 40% WHC. After pre-incubation, 2 ml of $^{15}NH_4NO_3$ or $NH_4{}^{15}NO_3$ solution was evenly added to the soil at 20 mg N kg^{-1} soil (oven-dried weight), and labeled soils were incubated in the dark at 20 °C and 60% WHC for 336 h (14 d).

Gas samples were collected from flask headspaces at 0.5 h (0 d), 24 h (1 d), 72 h (3 d), 168 h (7 d), and 336 h (14 d) after label addition according to Zhang et al. (2011). Two 30-ml gas samples were taken from each flask headspace with a gastight syringe (30 mL, Ping An, China) and transferred into pre-evacuated vials (12 mL, Jing Yang, China). One sample was used to determine N_2O concentration, and the other for analysis of ^{15}N enrichment in N_2O. After gas collection, soil samples were extracted with 2M KCl solution at a 1:5 soil:extractant ratio. Following KCl extraction, soils were washed with distilled water to remove inorganic nitrogen, then filtered and dried for analysis of ^{15}N enrichment in organic N.

2.3 Determination of soil properties, N_2O fluxes and pathways, N transformation rates, and gene abundances

2.3.1 Soil properties

Soil NH_4^+-N was measured using the Alpha-Naphthol Blue spectrophotometer method after KCl extraction, and NO_3^--N was measured using the Dual Wavelength Spectrophotometric method. Soil MBC and MBN were measured using the chloroform-fumigation-extraction method (Vance et al., 1987). Soil pH was measured in a soil/water mixture at a 1:2.5 (m:v) ratio using a portable pH detector (F-71G, LAQUA, HORIBA, Japan). Soil TOC was measured using the H_2SO_4-$K_2Cr_2O_7$ heating method. Total-N content was analyzed after H_2SO_4 digestion using both the Alpha-Naphthol Blue spectrophotometer method and the Mo-Sb Anti-spectrophotometer method (Liu, 1996).

2.3.2 N concentrations and ^{15}N enrichments in inorganic-N, organic-N, and N_2O

The ^{15}N enrichment of soil inorganic N (NH_4^+ and NO_3^-) in KCl extracts was measured by an isotope ratio mass spectrometer (IRMS, Europa Scientific Integra, Crewe, UK). $^{15}NH_4^+$ and $^{15}NO_3^-$ were first separately transferred to $^{15}NH_3$ and then converted to $(^{15}NH_4)_2SO_4$ (Wang et al., 2015), while concentrations were measured by 0.005 M H_2SO_4 titration using 2% H_3BO_3 solution as a color indicator. Briefly, the KCl extract containing $^{15}NH_4^+$ and $^{15}NO_3^-$ was steam-distilled with magnesium oxide (MgO) to convert NH_4^+ to liberated NH_3, and the same extract was distilled again with Devarda' s alloy to transfer NO_3^- to liberated NH_3 (Feast and Dennis, 1996). The liberated NH_3 from both distillations was separately trapped using boric acid solution for concentration measurement. The trapped N was then acidified with H_2SO_4 solution to convert to $(NH_4)_2SO_4$, dried at 80 °C, and analyzed for ^{15}N enrichment. After KCl extraction and filtration, remaining soils were washed four times with distilled water, dried at 60 °C, and analyzed for organic-N concentration and ^{15}N enrichment. For gas samples, N_2O concentration was measured using an Agilent 7890A gas chromatograph (Agilent Technologies, Inc, USA), and ^{15}N enrichment in N_2O was measured using a mass spectrometer (Finnigan MAT 253).

2.3.3 Calculation of N_2O fluxes and pathway contributions

N_2O flux at each sampling point was calculated according to standard methods (Yu et al., 2014; Xie et al., 2015) based on N_2O concentration changes over time during incubation. Based on ^{15}N enrichment (atom% excess) in N_2O, NH_4^+-N, NO_3^--N, and organic-N pools in the paired ^{15}N tracing treatments, we calculated contributions from different N_2O emission fractions. We assumed N_2O originated from NH_4^+-N, NO_3^--N, and organic N pools via autotrophic nitrification, denitrification, and heterotrophic nitrification, respectively. Contributions from these three pathways were calculated according to established

equations (Rütting et al., 2010; Zhang et al., 2011) using the back-solver method in Excel (Microsoft, Inc.).

To understand ^{15}N losses from labeled inorganic N pools as N_2O, we calculated the percentage of ^{15}N enrichment (atom% excess) in N_2O relative to ^{15}N enrichment in the NH_4^+-N pool ($^{15}N_2O/^{15}NH_4^+$%) in the $^{15}NH_4^+$-labeled treatment, and the percentage relative to the NO_3^--N pool ($^{15}N_2O/^{15}NO_3^-$%) in the $^{15}NO_3^-$-labeled treatment for each sampling point.

2.3.4 Quantification of N transformation rates

Gross N transformation rates were quantified using a ^{15}N tracing model (Müller et al., 2007) [FIGURE:S1]. The model considered ten transformation processes related to organic and inorganic N turnover: (1) MNrec, mineralization of recalcitrant organic N to NH_4^+; (2) INH_4-Nrec, immobilization of NH_4^+ to recalcitrant organic N; (3) MNlab, mineralization of labile organic N to NH_4^+; (4) INH_4-Nlab, immobilization of NH_4^+ to labile organic N; (5) ONrec, oxidation of recalcitrant organic N to NO_3^- (heterotrophic nitrification); (6) INO_3, immobilization of NO_3^- to recalcitrant organic N; (7) ONH_4, oxidation of NH_4^+ to NO_3^- (autotrophic nitrification); (8) DNO_3, dissimilatory NO_3^- reduction to NH_4^+ (DNRA); (9) ANH_4, adsorption of NH_4^+ on cation exchange sites; (10) RHN_4a, release of adsorbed NH_4^+. The model calculated these rates based on concentration and ^{15}N enrichment values (average $\pm$ standard deviations) of inorganic and organic N from the two distinct ^{15}N-labeled treatments during the experimental period. N transformation rates for all eight N addition treatments at both sites were calculated over the 14 d period and expressed in mg N kg^{-1} soil day^{-1}.

Based on the ^{15}N tracing model results, we calculated net NH_4^+ production rates ($NETNH_4prod.$) and net NO_3^- production rates ($NETNO_3prod.$).

2.3.5 Soil DNA extraction and functional gene abundance quantification

DNA was extracted from 0.25 g fresh soil (before incubation) using a PowerSoil® DNA Isolation kit (ANBIOSCI TECH LTD) following manufacturer instructions. Extracted DNA quantity and quality were checked using a Nanodrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). AOB-amoA and AOA-amoA were selected to represent nitrifying communities, while nirK and nosZ represented denitrifying communities. Primers for the four genes are listed in Table S1 based on previous studies (Rich et al., 2003; Levy-Booth et al., 2014).

Quantitative PCR was performed with an ABI 7500 CFX96 Optical Real-Time Detection System (Bio-Rad Laboratories, Inc., Hercules, CA) using SYBR green kits. The 20 L reaction mixture contained 10 L SYBR Premix Ex Taq (Tli RNaseH Plus, Takara Biotechnology, Japan), 0.4 L forward primer, 0.4 L reverse primer, 0.4 L Rox Reference Dye II (Takara Biotechnology, Japan), 2 L

DNA template, and 6.8 L ddH_2O. Standard curves were obtained by serially diluting (10^{-2} - 10^{-9} copies l^{-1}) quantified plasmid DNA containing AOB-amoA, AOA-amoA, nirK, or nosZ gene fragments. Standard plasmids were prepared from extracted DNA samples using the same primers. PCR amplification efficiency and R^2 values were 103.0% and 0.997 for AOB-amoA, 101.0% and 0.982 for AOA-amoA, 99.8% and 0.980 for nirK, and 95.3% and 0.994 for nosZ. Gene reaction programs are listed in Table S2 based on previous studies (Rich et al., 2003; Levy-Booth et al., 2014).

2.4 Statistical analyses

One-way ANOVA followed by least significant difference (LSD) tests were used to test significant differences in soil properties, N_2O emission rates, gross N transformation rates, and functional gene abundances among N addition levels at each site. Two-way ANOVA was performed to compare N_2O fluxes, N transformation rates, and gene abundances between short- and long-term sites, focusing on two pairs of N addition levels: short-term (SL: 35 kg N ha^{-1} a^{-1}; SH: 105 kg N ha^{-1} a^{-1}) and long-term (LL: 50 kg N ha^{-1} a^{-1}; LM: 100 kg N ha^{-1} a^{-1}). Data normality was tested before ANOVAs, with logarithmic transformation applied when necessary. To quantitatively compare N addition effects between sites, we calculated the relative change percentage (%) of soil N_2O fluxes, N transformation rates, and gene abundances under N addition treatments relative to controls. Pearson correlation analyses (SPSS 17.0, SPSS Inc., Chicago, USA) were conducted to detect relationships among soil properties, gene abundances, and N_2O fluxes for both sites. Statistically significant differences were analyzed at $P < 0.05$ unless otherwise stated.

3.1 Background soil chemical properties at the short- and long-term sites

Soils from both sites were strongly acidic, with average pH of 3.8 at the long-term site and 3.9 at the short-term site ($p < 0.05$, Table S3). Soil NO_3^- concentration was significantly higher than NH_4^+-N concentration at both sites, though the short-term site had relatively lower NO_3^--N content compared to the long-term site. Long-term site soils also had relatively higher TOC content ($p < 0.05$) and higher total-N content than short-term site soils (Table S3). No significant difference in C/N ratio was found among the four N treatments within each site or between the two sites ($p > 0.05$, Table S3).

3.2 N concentrations and ^{15}N enrichments in inorganic-N and N_2O

During the 14-day tracing incubation, soil NH_4^+ concentration decreased on average by 7.7% and 15.5% across the four N treatment levels for short- and long-term sites, respectively, while soil NO_3^- concentration increased on average by 25.0% and 10.2% for short- and long-term sites, respectively (Fig. S2a, b, d, e).

A decline in ^{15}N enrichment of the NH_4^+-N pool was observed when ammonium was labeled (Fig. S2c), indicating natural abundance or low-abundance NH_4^+ entered this pool. A similar declining trend was observed in ^{15}N enrichment of the NO_3^--N pool when nitrate was labeled (Fig. S2f), showing natural or low-abundance NO_3^- entered the pool. Furthermore, ^{15}N enrichment of the NO_3^--N pool increased in the ammonium-labeled treatment (Fig. S2c), indicating conversion of labeled NH_4^+ to NO_3^- (e.g., ammonia oxidation). Modeled and observed data agreed well, with R^2 generally exceeding 0.92.

The ^{15}N enrichment in N_2O ranged from 0.01 to 0.53 atom% excess during incubation, showing an initial increase followed by a decreasing trend toward the end of incubation (14 d) (Fig. 1a [Figure 1: see original paper], c). N_2O emitted from nitrate-labeled soils showed significantly higher ^{15}N enrichment than N_2O from ammonium-labeled soils during incubation ($p < 0.05$, Fig. 1a, c). A significantly higher $^{15}N_2O/^{15}NO_3^-$% was found compared to $^{15}N_2O/^{15}NH_4^+$ throughout the incubation (Fig. 1b, d).

3.3 N_2O fluxes and production pathways

With increasing N addition level, soil average N_2O fluxes from low or medium N treatments increased during incubation, while N_2O fluxes from high N treatments decreased at both sites (Fig. 2a [Figure 2: see original paper]). The N_2O flux in SL, SM, and SH was 42.3% higher, 2.7% higher, and 11.6% lower than the control (SC) at the short-term site, while the N_2O flux in LL, LM, and LH was 35.7%, 378.5%, and 106.7% higher than the control (LC) at the long-term site. Except for the significantly higher flux in LM, N_2O fluxes were not significantly different among N treatments or between sites ($p > 0.05$).

Regarding N_2O emission pathways, denitrification (d) contributed more than 40% to soil N_2O production, though the trend within increasing N levels differed between sites (short-term: SC<SH<SL<SM; long-term: LL<LM<LC<LH) (Fig. 2b). Heterotrophic (h) and autotrophic nitrification (a) contributions ranged from 17.7-36.8% and 14.3-23.2%, respectively. However, the two nitrification fractions differed between short- versus long-term sites within increasing N levels (h: short-term SM<SC<SL<SH, long-term LC<LH<LM<LL; a: short-term SL<SH<SM<SC, long-term LM<LL<LH<LC) (Fig. 2b). Furthermore, the contribution of different N_2O emission fractions shifted from dominant heterotrophic nitrification (N_2Oh) to denitrification (N_2Od) toward the study' s end, while autotrophic nitrification (N_2Oa) contribution increased in the early stage (0-7 d) of incubation (Fig. 2c). Average contributions were 53% (d), 28% (h), and 18% (a) during incubation, showing overall denitrification dominance in these subtropical forest soils (Fig. 2c).

3.4 Gross and net N transformation rates

Gross autotrophic nitrification rate (ONH_4) was highest among all ten N transformation rates under almost all N treatments, followed by gross mineralization

rates of recalcitrant and labile organic N to NH_4^+ (MNrec and MNlab) (Table S4). Gross heterotrophic nitrification rate (ONrec) was much lower than gross autotrophic nitrification rate in these subtropical forest soils (Table S4). Total gross nitrification rate (ONH_4 + ONrec) ranged from 0.08 to 0.19 mg kg^{-1} d^{-1}, with ONH_4 contributing over 99% to total NO_3^- production.

Except for SC and LH treatments, soil gross NO_3^- immobilization rate (INO_3) was higher than gross NH_4^+ immobilization rates (INH_4-Nrec, INH_4-Nlab) and gross DNRA rates (DNO_3) under most N treatments (Table S4), indicating NO_3^- was more easily trapped in the organic N pool than the NH_4^+-N pool. Net NO_3^- production rate ($NETNO_3$prod.) was much higher than net NH_4^+ production rate, while LH had the highest net NH_4^+ loss but lowest net NO_3^- production among all eight N treatments. $NETNO_3$prod. also differed significantly among the four N-treatments at both short- and long-term sites ($p < 0.05$). All ten gross N transformation rates differed statistically with respect to N treatments ($p < 0.05$, Table S4). However, two-way ANOVA results showed long-term N addition did not significantly change N transformation rates compared to the short-term site ($p > 0.05$).

The response direction of gross organic N mineralization rates (MNlab and MNrec) to N addition was opposite between short- and long-term sites, as was the gross heterotrophic nitrification rate (ONrec) (Fig. 3a [Figure 3: see original paper], b). Gross mineralization rate of labile organic N to NH_4^+ (MNlab) was 82%, 144%, and 58% higher in SL, SM, and SH than SC at the short-term site, but 44%, 20%, and 47% lower in LL, LM, and LH than LC at the long-term site (Fig. 3a). In contrast, gross mineralization rate of recalcitrant organic N to NH_4^+ (MNrec) was 49%, 16%, and 15% lower in SL, SM, and SH than SC at the short-term site, but 51%, 85%, and 128% higher in LL, LM, and LH than LC at the long-term site (Fig. 3b). The large stimulation effect of N addition on gross NO_3^- immobilization (INO_3) and DNRA (DNO_3) rates at the short-term site decreased or became inhibitory at the long-term site (Fig. 3d, f). Compared with the short-term site, the high N treatment at the long-term site induced the largest inhibition effect on gross autotrophic nitrification rate (ONH_4) and net NO_3^- production rate ($NETNO_3$prod) (Fig. 3c, g).

3.5 Functional gene abundance

AOA-amoA was most abundant, followed by nirK, nosZ, and AOB-amoA, with AOB-amoA being least abundant at both sites (Fig. 4a, b, d, e). The ratio between denitrifying and nitrifying gene abundance ((nirK + nosZ)/(AOB-amoA + AOA-amoA)) was mostly less than 1.0 at both sites (Fig. 4c [Figure 4: see original paper]), indicating nitrifiers were more abundant than denitrifiers. The nosZ/nirK ratio was mostly less than 1.0 at both sites (Fig. 4f), indicating N_2O-producers were less abundant than N_2O-reducers. Although AOA-amoA, nirK, and the (nirK + nosZ)/(AOB-amoA + AOA-amoA) ratio responded significantly to N addition within each site ($p < 0.01$) (Fig. 4b, c, d), all assessed functional gene abundances showed no significant difference between the two

sites ($p > 0.05$).

Similar stimulation effects of N addition on nirK abundance were observed at both N addition sites (Fig. 4d, inset). Except for the high N treatments, the direction of nosZ abundance, (nirK + nosZ)/(AOB-amoA + AOA-amoA), and nosZ/nirK ratios was also consistent (Fig. 4c, e, f, insets), indicating stimulation by low and medium N additions. However, except for high N treatments, the direction of AOB-amoA abundance response to N addition was opposite between sites (Fig. 4a, inset). AOA-amoA abundance was 26.5% lower, 72.0% higher, and 127.3% higher in SL, SM, and SH than SC at the short-term site, but 46.8% lower, 8.0% higher, and 27.7% lower in LL, LM, and LH than LC at the long-term site (Fig. 4b, inset), indicating that under high N treatment, AOA-amoA abundance was stimulated at the short-term site but suppressed at the long-term site.

3.6 Correlations among soil properties, functional genes, and N_2O fluxes

Correlation relationships among soil properties, functional gene abundance, and N_2O fluxes differed between short- and long-term sites (Fig. 5 [Figure 5: see original paper]). At the short-term site, soil N_2O flux was positively correlated with nosZ abundance ($p < 0.05$). The abundance of nosZ was also positively correlated with both nirK ($p < 0.05$) and AOB-amoA abundances ($p < 0.01$), implying possible interactions between nitrifying and denitrifying groups in regulating N_2O emission. AOA-amoA abundance was negatively correlated with soil pH ($p < 0.01$) but positively with soil C/N ratio ($p < 0.05$) (Fig. 5). The abundance of AOB-amoA and nosZ was positively correlated with soil NH_4^+-N, total N, and C/N ratio ($p < 0.05$) (Fig. 5), indicating nitrifying N_2O-producers and denitrifying N_2O-reducers were linked to substrate availability in the acid soil environment. Positive correlations were also found between the (nirK + nosZ)/(AOB-amoA + AOA-amoA) ratio and soil NH_4^+-N, and between the nosZ/nirK ratio and TOC (Fig. S3a), further indicating that nitrifier and denitrifier composition could be affected by soil substrate availability.

At the long-term N addition site, positive correlations were only found between N_2O flux and AOA-amoA abundance ($p < 0.05$), and between AOB-amoA abundance and pH ($p < 0.05$) (Fig. 5). In general, correlations among soil properties, functional gene abundance, and N_2O fluxes were tighter at the short-term than at the long-term site.

4.1 Responses of N_2O fluxes and production pathways to short- and long-term N additions

The stimulation effect of N addition on N_2O emission rates was evident only in low and medium N treatments at both sites (Fig. 2a). The N_2O production-consumption balance during denitrification in forest soil (Xu et al., 2012; Zhang et al., 2014) may have changed: low and medium N additions favored N_2O

production over N_2O consumption, though this effect weakened under high N addition. The dominance of N_2O production over consumption is also reflected by more abundant N_2O-producers (nirK) than N_2O-reducers (nosZ) (Fig. 4d, e, f), implying higher nirK than nosZ activity in these subtropical forest soils (Zhang et al., 2014). However, in high N treatments, N_2O emissions were reduced at the short-term site but still stimulated at the long-term site compared to controls (Fig. 2a), contrasting with results from a montane forest with 3-4 years versus 11-12 years of N addition (Corre et al., 2014). Possible reasons include that soil microbes responsible for N_2O emissions were more sensitive to short-term, higher N additions but gradually adapted over longer periods, causing the stimulation effect at high N to subside.

Although long-term N addition did not induce significant changes in total N_2O emission, the contribution of various emission pathways changed, with higher denitrification contribution (53%) and lower heterotrophic (28%) and autotrophic nitrification contributions (18%) (Fig. 2b). Denitrification remained the dominant pathway for N_2O emissions in this subtropical forest soil, consistent with Zhang et al. (2011). The $^{15}N_2O/^{15}NH_4^+$% and $^{15}N_2O/^{15}NO_3^-$% were not significantly different among increasing N levels or between short- and long-term sites (Fig. 1b, d), contrary to previous findings that increasing ammonium concentrations support higher nitrification contributions to N_2O production (Müller et al., 1998; Avrahami et al., 2002). Possible reasons include that topsoils from this subtropical forest were naturally N-saturated in control treatments (SC and LC) (Gurmesa et al., 2016), causing non-recognizable responses of nitrification or denitrification contributions to additional NH_4^+ or NO_3^-.

Combining our observed N rate dynamics, we suspect added NH_4^+ in the forest soil was quickly transformed to NO_3^- and subsequently denitrified to gaseous N (N_2O). This coupled nitrification-denitrification process is prevalent when autotrophic nitrification rates are high and may have contributed to the low contribution of nitrification-related N_2O (Müller et al., 2014). This process may also explain the significantly lower ^{15}N enrichment in N_2O compared to higher ^{15}N enrichments in NH_4^+ and NO_3^- in the subtropical forest soil (Fig. 1a, c; Fig. S2c). We separately labeled the NH_4^+-N and NO_3^--N pools in the paired ^{15}N tracing incubation. The ^{15}N enrichment in NO_3^- was higher than in N_2O in the $^{15}NH_4^+$-labeled treatment, and ^{15}N enrichment in NH_4^+ was also higher than in N_2O in the $^{15}NO_3^-$-labeled treatment (Fig. S2). This further implies transformation from NH_4^+ to NO_3^- was more prevalent than transformation from inorganic N to N_2O. Autotrophic nitrification contributed only 18% to total N_2O flux despite its dominance among the ten N transformation processes, supporting this reasoning. This may indicate that fast autotrophic nitrification-induced N_2O production also stimulated further N_2O reduction to N_2, possibly via nitrifier-denitrification (Müller et al., 2014). Measurement of N_2 and its ^{15}N enrichment would further elucidate this process.

Heterotrophic nitrification contributed considerably (28%) to N_2O fluxes while

contributing only a tiny fraction (1%) to total NO_3^- production (Table S4, Fig. 2c). This could be explained by the "hole-in-the-pipe"model and N_2O gas leakage characteristics in subtropical forest soils: higher autotrophic nitrification rates induced a larger "hole" for N_2O leakage, while lower heterotrophic nitrification rates induced a smaller "hole," thus maintaining comparability between the two pathway contributions (Firestone and Davidson, 1989; Zhang et al., 2014).

4.2 Responses of soil N transformation rates to short- and long-term N additions

Except for the high N treatment at the long-term site, subtropical forest soils receiving both natural (SC, LC) and anthropogenic N depositions showed higher gross autotrophic nitrification and mineralization rates than other N transformation rates (Table S4). According to Cheng et al. (2014), relatively fast transformations from NH_4^+ to NO_3^- and from organic N to NH_4^+ might explain natural N enrichment in humid subtropical forest soils. Moreover, more dominant autotrophic nitrification than mineralization processes (except for the high N treatment at the long-term site) explains the higher NO_3^- than NH_4^+ concentrations in forest soil (Table S3). The particularly higher gross NH_4^+ immobilization rates (INH_4-Nrec, INH_4-Nlab), higher gross mineralization rates (MNrec), but lower gross autotrophic nitrification rates (ONH_4) in the long-term high N treatment (LH) (Table S3) suggest that soil mineralization-immobilization turnover (MIT) became more dominant than NH_4^+-NO_3^- turnover (i.e., autotrophic nitrification) after long-term anthropogenic N addition. Additionally, significant differences in soil N transformation rates among N treatments at both sites (Table S4) strengthen the importance of N transformation responses for N enrichment, N loss, and soil acidification in subtropical forest ecosystems (Gao et al., 2015).

However, N transformation rates did not differ significantly between short- and long-term sites (Table S4). Previous results revealed that soil N transformation rates are affected by multiple factors, including interacting N transformation processes (nitrification-mineralization, mineralization-immobilization), soil microbes (microbial activity and functional abundances), and soil properties (C/N ratio, TOC, and pH) (Bengtsson et al., 2003; Zhu et al., 2013b; Gao et al., 2015; Chen et al., 2017). The insignificant change in soil N transformation rates at the long-term site might reflect that, although long-term anthropogenic N addition induced significant changes in soil N transformation rates, the interacting soil microbes that drive these processes have adapted to current soil conditions. The combined effects of diverse soil abiotic and biotic factors on N transformations were weakened, thus preventing large responses of N transformations to prolonged N addition.

Our results showed that rate responses of specific N transformation processes, primarily associated with the fate of NH_4^+ and NO_3^-, differed between short- and long-term sites (Fig. 3). Previous studies revealed that soil organic matter fractions are linked to different functional microbes with varying responses to

N deposition (Frey et al., 2004; Cusack et al., 2011). Therefore, quantifying gross mineralization rates of labile organic N (MNlab) and recalcitrant organic N (MNrec) facilitates understanding of fast (labile organic N) and slow (recalcitrant organic N) turnover of soil organic N pools (Gao et al., 2016). In our study, MNlab was stimulated by short-term N addition but inhibited by long-term N addition, while MNrec showed the opposite pattern (Fig. 3a, b insets), demonstrating different response directions not only between labile and recalcitrant organic N forms but also between short- and long-term N addition treatments.

Possible reasons include that short-term N addition limited biomass and activity of soil fungi participating in recalcitrant organic N mineralization, indirectly inhibiting MNrec (Frey et al., 2004; Schimel and Bennett, 2004). Additionally, N addition may enhance transformation of soil labile organic matter to recalcitrant organic matter (Maaroufi et al., 2015), potentially explaining stimulated MNlab but inhibited MNrec by short-term N addition. The stimulation effect of long-term N additions on MNrec might reflect alleviated microbial N limitation after long-term N addition, increasing microbial activity for organic matter decomposition (Koranda et al., 2014; Gao et al., 2016). The inhibited MNlab at the long-term site might represent a downregulation response to microbial upregulation of MNrec, while opposite responses in MNrec and MNlab under N additions could result from changed stabilization of soil organic matter into recalcitrant compounds and altered microbial extracellular enzyme activities that conduct production and decomposition of soil organic compounds (Sinsabaugh et al., 2005; Janssens et al., 2010; Gao et al., 2016).

The response of soil gross autotrophic nitrification rate was most significant, except for the high N treatment at the long-term site (Fig. 3c inset), indicating that at the highest N addition rate (150 kg N ha^{-1} a^{-1}), soil conditions changed most substantially, contributing much less to nitrifying N_2O emission. This special change in the long-term high N treatment was also reflected by the lowest net NO_3^- production rate and highest NH_4^+ immobilization rate compared to other N treatments (Table S4). Soil gross heterotrophic nitrification rate (ONrec) was stimulated by short-term N addition but inhibited by long-term N addition (Fig. 3d), implying that N_2O and NO_3^- production via heterotrophic nitrification tended to increase at the short-term site but decrease at the long-term site. The more positive responses of ONrec, INO_3, and DNO_3 at the short-term than long-term site prevent higher loss risk of soil NO_3^- through gaseous N emission (denitrification) (Wang et al., 2015).

4.3 Regulations of soil nitrifiers and denitrifiers in N_2O emissions and their abiotic controllers

Previous studies exhibited higher abundance of ammonia-oxidizing archaea (AOA) in the same subtropical forest, consistent with our findings (Isobe et al., 2012). Several studies have highlighted a predominant role of AOA in nitrification (Gubry-Rangin et al., 2010; Verhamme et al., 2011; Zhang et al., 2012; Faeflen et al., 2016). The dominant autotrophic nitrification in our

results (Table S4) might be explained by more abundant AOA than AOB in the strong acidic soil (Zhang et al., 2012; Faeflen et al., 2016). To better define the relative importance of AOA and AOB in nitrification processes, future exploration of archaeal and bacterial ammonia oxidizer communities is needed (Gubry-Rangin et al., 2010).

Functions of nirK and nosZ in relation to denitrification processes differ: nirK plays roles in N_2O production while nosZ plays roles in N_2O reduction or consumption (Zhang et al., 2014; Domeignoz-Horta et al., 2017). Our results revealed slightly higher nirK than nosZ abundance (Fig. 4d, e, f), potentially indicating N_2O production exceeds N_2O reduction, contributing to N_2O emission from forest soil. This aligns with previous findings that the studied subtropical forest soil acts as a natural N_2O source (Fang et al., 2008a; Tang et al., 2006). However, NO as another denitrification intermediate cannot be neglected when considering gene regulation functions, since nirK plays essential roles in the second denitrification step (nitrite reduction to NO) (Levy-Booth et al., 2014). Additionally, nirS as another molecular marker gene for denitrifying bacteria in this step requires further understanding (Prieme et al., 2002; Bárta et al., 2010).

The nosZ/nirK ratio in most N treatments was less than 1 (Fig. 4f), yielding an average value of 0.68 (data not shown) without considering N addition effects. This dramatically contrasts with Zhang et al. (2014), who found an average nirK/nosZ ratio of 0.7 in subtropical soils. Possible reasons include that the strong acidic, highly weathered soil in our subtropical forest contributes to higher redox potential, supporting more active nirK than nosZ communities (Qafoku et al., 2004; Xu and Cai, 2007; Zhang et al., 2014).

Compared with the short-term N site, prolonged N addition at the long-term site did not induce significant changes in functional gene abundances but markedly changed responses of specific functional gene abundances to N addition treatments (Fig. 4). This highlights the greater importance of functional gene response patterns to N addition levels than to N addition duration. Possible reasons for insignificant gene abundance changes under long-term N additions include that soil nitrifier and denitrifier activities are strongly affected by soil abiotic conditions, including pH, redox potential, N availability, and other nutrients (Avrahami et al., 2002; Bárta et al., 2010; Hu et al., 2013; Yu et al., 2014; Zhang et al., 2014). Thus, comprehensive effects of soil abiotic factors may have eliminated potentially significant responses of functional groups with prolonged N addition.

We observed significant responses of AOA-amoA and nirK abundances but not AOB-amoA or nosZ abundances to N addition treatments at both sites (Fig. 4), suggesting different response sensitivities of soil functional microbial communities to N addition (Avrahami et al., 2002; Li and Gu, 2013). Significant positive correlations between AOB-amoA and nosZ abundances, and between nirK and nosZ abundances at the short-term site (Fig. 5) indicate combined gene regulation of N_2O emissions from both nitrification and denitrification pathways

(Zhang et al., 2014; Tang et al., 2016; Domeignoz-Horta et al., 2017). However, this close relationship linking both nitrifying and denitrifying functional groups was not found at the long-term site, possibly because short-term N addition induced more significant changes in soil properties that were weakened by long-term N addition. This is supported by more significant correlations between soil properties (pH, C/N ratio, total N, and NH_4^+) and functional gene abundances at the short-term than long-term site (Fig. 5).

The positive correlation between nosZ abundance and N_2O flux at the short-term N addition site appears contradictory, possibly because the N_2O flux we calculated was a sum of three N_2O emission fractions associated with autotrophic nitrification, heterotrophic nitrification, and denitrification pathways (Zhang et al., 2011). At the long-term site, we only found a significant negative correlation between pH and AOB-amoA abundance and a significant positive correlation between AOA-amoA abundance and N_2O flux (Fig. 5), implying a relatively looser relationship among soil properties, gene abundance, and N_2O fluxes. Previous studies suggested that soil properties and microbial communities may distinctly play dominant roles in explaining lower and higher N_2O emission rates, respectively (Domeignoz-Horta et al., 2017). This leads us to speculate that deepened acidification in long-term site soil induced not only community changes but also ecological niche distinctions between the two ammonia oxidizers (AOA and AOB) (Nicol et al., 2008; Hu et al., 2013; Remy et al., 2018). Moreover, changed soil conditions under long-term N addition reduced the close relationship between AOA and other variables in this acid soil (Hu et al., 2013), finally inducing the positive correlation between pH and AOB-amoA abundance. In brief, changed soil and climatic conditions (rainfall, moisture, temperature, etc.) affected microbial activities and gene abundance related to N_2O production and further reduction to N_2, which is influenced by soil acidity (Liu et al., 2014). Thus, relationships among soil properties, gene abundance, and N_2O fluxes at the short-term N addition site are much tighter than at the long-term site where microbial communities have adapted to prevailing conditions.

Our results showed changed functional gene regulation of subtropical forest N_2O emission in response to prolonged N addition duration. However, understanding soil nitrifying and denitrifying functional group changes from only gene abundance remains limited, since gene function operation is reflected by corresponding gene transcripts (Xu et al., 2012; Wu et al., 2017). To better elucidate the importance of internal mechanisms of soil microbial functional groups in regulating N_2O emissions, future studies of corresponding RNA transcripts and their relationships with N_2O production (Liu et al., 2014; Liu et al., 2010; Hassan et al., 2016) are needed. Moreover, comprehensive exploration of soil microbial responses to N deposition from microbial structure, gene abundance, and transcription levels should be conducted (Li and Gu, 2013).

4.4 N_2O productions in relation to N additions, N transformations, and functional gene regulations

Consistent with previous results (Zhang et al., 2011), heterotrophic nitrification contribution was lower than denitrification but higher than autotrophic nitrification to total N_2O fluxes (Fig. 2b). However, the higher heterotrophic than autotrophic nitrification contribution is not reflected by either the significantly higher gross autotrophic nitrification rates or the most abundant AOA-amoA (Table S4, Fig. 4b), while the dominant denitrification contribution is also inconsistent with relatively less abundant denitrifiers (nirK + nosZ) than nitrifiers (AOA-amoA + AOB-amoA) in the forest soil (Fig. 4c). Based on the conceptual "hole-in-the-pipe" (HIP) model of N_2O emission and its modifications (Firestone and Davidson, 1989; Zhang et al., 2014; Zhang et al., 2015), we speculate that the more abundant AOA contributed to higher autotrophic nitrification rates but also triggered a larger N_2O leaking "hole" from this pathway' s "pipe." The lower heterotrophic nitrification rate induced a smaller "hole" for N_2O leakage, thus maintaining considerable N_2O production via this pathway. Denitrification serves as an essential pathway for NO_3^- removal from this subtropical forest (Fang et al., 2015), but higher nirK than nosZ abundance potentially induces more N_2O production than reduction, finally causing denitrification' s highest contribution to N_2O emission (Fig. 2b).

Our study revealed changed response patterns of specific N transformation rates to long-term N addition (Fig. 3), directing changes in transformation fates of NH_4^+ and NO_3^-, including NH_4^+ production via mineralization of labile and recalcitrant organic N and NH_4^+ consumption via autotrophic nitrification, NO_3^- production via autotrophic and heterotrophic nitrification, and NO_3^- consumption via immobilization and DNRA, respectively, with prolonged N addition. The different responses of net NO_3^- production to increased N addition at the two sites (Fig. 3g) further reflect changed NO_3^- retention potential in subtropical forest soil with prolonged N addition duration. Our study revealed that soil functional genes, especially the two nitrifying functional genes (AOB-amoA, AOA-amoA), changed more in response to prolonged N addition (Fig. 4). However, it remains controversial to conclude that insignificant N_2O change under long-term N addition represents a combined response pattern of the four studied genes. N_2O emission rate reflects a balance among regulations by nitrifying N_2O-producers (AOB-amoA, AOA-amoA), denitrifying N_2O-producers (nirK/nirS), and denitrifying N_2O-reducers (nosZ). Therefore, full understanding requires consideration of other important functional groups not included in this study, including nirS for N_2O production from NO and the newly identified N_2O-reducing clade for N_2O reduction to N_2 (Levy-Booth et al., 2014; Zhang et al., 2014; Tang et al., 2016; Domeignoz-Horta et al., 2017). Correspondingly, NO as an intermediate and N_2 as a final product in both nitrification and denitrification processes cannot be neglected in future studies due to their close linkage with N_2O production and consumption (Zhang et al., 2014; Zhang et al., 2015; Tang et al., 2016).

In this study, we simulated N deposition by adding equal amounts of NH_4^+ and NO_3^- (as NH_4NO_3) to subtropical forest soils. Natural atmospheric N deposition typically shows unequal contributions from these inorganic N forms, with an approximate 60/40 ratio (NH_4^+/NO_3^-) (Fang et al., 2011; Fang et al., 2015). A study of two subtropical masson pine forests in south China even found a 70/30 ratio (Chen et al., 2004), providing different transformation capacities for deposited NH_4^+ and NO_3^-. The studied subtropical forest in DHSBR has received atmospheric N deposition exceeding 30 kg N ha^{-1} a^{-1} since 1990, so long-term natural N accumulation in forest soils might explain the significant net NO_3^- production in our findings, although leaching and denitrification have been shown to be main fates of produced NO_3^- in this forest (Fang et al., 2009; Fang et al., 2015). Although high autotrophic nitrification rates were measured in almost all N addition treatments at both sites, we did not find correspondingly high ^{15}N enrichment in N_2O. One reason could be high conversion rates of N_2O to N_2, so determination of ^{15}N enrichment in N_2 should be considered in future studies.

Despite obvious changes in specific soil N transformation rates and gene abundances but not N_2O fluxes in response to long-term N addition, we found looser relationships among soil properties, functional gene abundance, and N_2O fluxes at the long-term site compared to the short-term site based on correlation analysis. We argue that functional microbial community adaptations occur in response to prevailing soil conditions and high natural ambient N depositions. This aligns with responses of microbial community structure, diversity, and gene expression to changing biotic and abiotic factors (Avrahami et al., 2002; Smith et al., 2010; Németh et al., 2014), while N deposition effects prevail over other influencing factors. This would also explain changes in correlations between functional gene abundance and N_2O flux with prolonged N addition duration (Fig. 5). However, to further elucidate this, shifts in microbial activity rather than just gene expression would likely be more indicative.

5. Conclusion

In this study, we compared responses of N_2O emissions, N transformations, and gene regulation traits between two subtropical evergreen broadleaf forest sites that received either one year (short-term) or 12 years (long-term) of simulated N deposition. Our results revealed that long-term N addition did not induce significant changes in N_2O fluxes, soil N transformation rates, or functional gene abundances. However, compared with short-term N addition: (i) long-term N addition changed responses of specific N transformation rates, especially those related to NH_4^+ and NO_3^- fate (mineralization, nitrification, NO_3^- immobilization, and DNRA); (ii) long-term N addition induced greater changes in responses of soil nitrifying N_2O-producers (AOA-amoA, AOB-amoA) to N treatments than in responses of denitrifying N_2O-producers (nirK) or N_2O-reducers (nosZ); (iii) long-term N addition weakened the close correlations among soil properties, functional gene abundance, and N_2O fluxes observed at the short-term site. The

observed differences in N dynamics and N_2O emission traits at the two simulated N deposition sites cannot be solely explained by soil microbial nitrifying and denitrifying functional genes. Soil microenvironment conditions (pH, substrate availability, etc.) and their transition effects from short- to long-term N deposition are key to understanding microbial functional gene expressions and associated N dynamics (including N_2O). We argue that microbial regulation adaptations exist in response to prevailing soil conditions and long-term natural and anthropogenic N depositions.

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Figure Legends

Fig. 1. The ^{15}N enrichment in N_2O during the paired ^{15}N tracing incubation (a, c), and $^{15}N_2O/^{15}NH_4^+$ ratios in the ammonium-labeled incubation (b) and $^{15}N_2O/^{15}NO_3^-$ ratios in the nitrate-labeled incubation (d). Symbols (points and lines) in light green and orange denote short-term and long-term N addition treatments, respectively. SC, SL, SM, and SH denote control, low-N, medium-N, and high-N treatments at the short-term site. LC, LL, LM, and LH denote control, low-N, medium-N, and high-N treatments at the long-term site. , , indicate significant differences between $^{15}NH_4^+$-labeled and $^{15}NO_3^-$-labeled treatments at $p < 0.05$, $p < 0.01$, and $p < 0.001$ levels, respectively.

Fig. 2. Soil N_2O average fluxes (a), specific pathway fractions of emitted N_2O in each N treatment (b), and average pathway contributions over the 14-day incubation (c). SC, SL, SM, and SH denote control, low-N, medium-N, and high-N treatments at the short-term site. LC, LL, LM, and LH denote control, low-N, medium-N, and high-N treatments at the long-term site. The "*" above light green bars in panel a indicates significant differences ($p < 0.05$) between the corresponding N treatment and other treatments. Bars in light green, moderate green, and dark green in panel b denote contributions from heterotrophic nitrification (h), autotrophic nitrification (a), and denitrification (d), respectively. Areas in light gray, moderate gray, and dark gray in panel c denote contributions from heterotrophic nitrification (N_2Oh), autotrophic nitrification (N_2Oa), and denitrification (N_2Od), respectively, during incubation.

Fig. 3. The ^{15}N tracing model (Müller et al., 2007) and relative change percentages of soil gross/net N transformation rates in N addition treatments compared to controls at the two sites (insets a-g). White squares and black arrows indicate soil N pools and N transformation processes, respectively. NH_4^+: ammonium; NH_4^+_{ads}: adsorbed NH_4^+; Nlab: labile organic N; NO_3^-: nitrate; Nrec: recalcitrant organic N. MNrec: mineralization of recalcitrant organic nitrogen to NH_4^+; INH_4-Nrec: immobilization of NH_4^+ to recalcitrant organic nitrogen; MNlab: mineralization of labile organic nitrogen; INH_4-Nlab: immobilization of NH_4^+ to labile organic nitrogen; ONrec: oxidation of recalcitrant organic nitrogen to NO_3^-; INO_3: immobilization of NO_3^- to recalcitrant organic N; ONH_4: oxidation of NH_4^+ to NO_3^-; DNO_3: dissimilatory NO_3^- reduction to NH_4^+; ANH_4: adsorption of NH_4^+ on cation exchange sites; RNH_4a: release of NH_4^+ from cation exchange sites; $NETNO_3prod$: net NO_3^- production. Insets a-g show relative change percentages of MNlab, MNrec, ONH_4, DNO_3, ONrec, INO_3, and $NETNO_3prod$, respectively. Bars in light green and orange denote short- and long-term site results, respectively. L, M, and H on the x-axis denote low-N, medium-N, and high-N treatments, respectively. Y-axis values denote relative change percentages (%) of individual N transformation rates in N addition treatments compared to control.

Fig. 4. Abundance (copy numbers g^{-1} dry soil) and abundance ratios of functional genes (AOB-amoA, AOA-amoA, nirK, and nosZ) under different N

treatments at the two sites. Bars in light green and orange denote short-term and long-term N addition treatments, respectively. Insets show relative change percentages of gene abundance or abundance ratios in N-addition treatments compared to control. L, M, and H on the x-axis denote low-N, medium-N, and high-N treatments, respectively. Y-axis values denote relative change percentages (%) compared to control. D and N indicate two-way ANOVA results: D shows significance between N addition durations (short- and long-term), and N (N level) shows significance among N treatments. Only significant differences are shown.

Fig. 5. Pearson correlation analysis results of soil properties, gene abundances, and N_2O fluxes at short- and long-term sites. N_2O production pathways considered (heterotrophic nitrification, autotrophic nitrification, and denitrification) and roles of studied functional genes (AOA-amoA, AOB-amoA, nirK, nosZ) in regulating N_2O emissions are shown. White squares and ellipses denote N pools and gaseous N forms, respectively. Black arrows denote N transformation directions. The cloud ellipse denotes N_2O fluxes. Green ellipses denote functional genes associated with N transformation processes, with ellipse size indicating abundance magnitude (larger = higher abundance). Dark-yellow squares denote soil properties significantly correlated with gene abundances. Light-green and orange lines denote significant correlations at short- and long-term sites, respectively. The "-" sign indicates negative correlation. Insignificant correlations are not shown.

Supplementary Information

Table S1. Primer information of selected soil functional genes.

Gene	Primers	Primer sequence (5' -3')	Location	Length	References
AOB-amoA	amoA 1FamoA 2R	5' GGGGTTTC-TACTGGTGGT 3' 5' CCCCTCKGSAAAGC-CTTCTTC 3'		500bp	Rich et al., 2003; Levy-Booth et al., 2014
AOA-amoA	CrenamoA 23FCre-namoA 616R	5' ATGGTCTGGCT-WAGACG 3' 5' GCCATCCATCTG-TATGTCCA 3'		620bp	Levy-Booth et al., 2014
nirK	F560-589R906-935	5' -GGGCATGAACGGCGCGCTCATGGTGCTGCC-3' 5' -CGGGTTGGCGAACTTGCCGGTGGTCCAGAC-3'		376bp	Levy-Booth et al., 2014

Gene	Primers	Primer sequence (5’ –3’)	Location	Length	References
nosZ	nosZ-FnosZ-R	5’ -CGCTGTTCITCGACAGYCAG-3’ 5’ -ATGTGCAKIGCRTGGCAGAA-3’		700bp	Rich et al., 2003

Table S2. Reaction programs of quantitative PCR for selected functional genes.

Primers	Thermal cycling conditions	References
amoA 1F/amoA 2R	95° 30’ ‘-40(95° 15’ ‘-53° 15’ ‘-72° 40’’)	Rich et al., 2003; Levy-Booth et al., 2014
CrenamoA23F/CrenamoA616R	95° 30’ ‘-40(95° 5’ ‘-53° 34’ ‘-72° 60’’)	Levy-Booth et al., 2014
nirK F560-589/R906-935	95° 30’ ‘-40(95° 5’ ‘-65° 34’ ‘-72° 60’’)	Levy-Booth et al., 2014
nosZ-F/nosZ-R	95° 30’ ‘-40(95° 5’ ‘-56° 34’ ‘-72° 40’’)	Rich et al., 2003

Table S3. Soil physiochemical properties (average ± standard error, n = 3) before tracing incubation. SC, SL, SM, and SH indicate control, low-N, medium-N, and high-N treatments at the short-term site. LC, LL, LM, and LH indicate control, low-N, medium-N, and high-N treatments at the long-term site. Same lowercase letters following values indicate insignificant differences ($p > 0.05$) among N treatments within each site.

Treatment	N-addition (kg N ha^{-1} a^{-1})	pH	NH_4^+-N (mg kg^{-1})	NO_3^--N (mg kg^{-1})	TOC (g kg^{-1})	Total N (g kg^{-1})	C/N ratio
SC	0	3.90(0.03)a	4.0(2.2)a	7.1(1.7)a	25.5(3.3)b	1.9(0.1)a	13.6(0.5)a
SL	35	3.90(0.07)a	6.7(3.5)a	7.5(0.7)a	25.8(4.7)b	1.7(0.4)a	15.3(0.6)a
SM	70	3.84(0.02)a	6.0(2.2)a	7.0(1.9)a	25.7(1.7)b	2.0(0.5)a	13.3(1.4)a
SH	105	3.80(3.02)a	3.0(2.2)a	9.6(0.8)a	27.6(2.9)ab	1.9(0.3)a	14.7(0.6)a
LC	0	3.88(0.03)a	5.9(3.5)a	7.1(3.1)a	26.8(2.2)ab	2.0(0.1)a	13.5(0.3)a
LL	50	3.82(0.04)a	6.0(4.9)a	8.1(4.5)a	28.8(8.5)ab	2.0(0.6)a	14.3(0.1)a
LM	100	3.71(0.02)a	4.0(2.9)a	9.3(1.6)a	35.6(8.2)a	2.4(0.4)a	14.7(1.0)a
LH	150	3.67(0.07)a	7.5(3.1)a	10.0(2.8)a	31.2(0.2)ab	2.3(0.2)a	13.8(0.7)a

Table S4. Gross N transformation rates (average ± standard deviation) during incubation of 8 N-treatments. Kinetics: 0 = zero-order, 1 = first-order; LSDS or LSDL: least significant difference when comparing any two means in short-term or long-term site at p = 0.05. Rates in mg N kg^{-1} d^{-1}. N rates: MNrec (mineralization of recalcitrant organic N to NH_4^+), INH_4-Nrec (immobilization of NH_4^+ to recalcitrant organic N), MNlab (mineralization of labile organic N), INH_4-Nlab (immobilization of NH_4^+ to labile organic N), ONrec (oxidation of recalcitrant organic N to NO_3^-), INO_3 (immobilization of NO_3^- to recalcitrant organic N), ONH_4 (oxidation of NH_4^+ to NO_3^-), DNO_3 (dissimilatory NO_3^- reduction to NH_4^+), ANH_4 (adsorption of NH_4^+ on cation exchange sites), RNH_4a (release of NH_4^+ from cation exchange sites). $NETNH_4prod$: net NH_4^+ production; $NETNO_3prod$: net NO_3^- production.

Fig. S1. ^{15}N tracing model (Müller et al., 2007). NH_4^+: ammonium; NH_4^+_{ads}: adsorbed NH_4^+; Nlab: labile organic N; NO_3^-: nitrate; Nrec: recalcitrant organic N. MNrec: mineralization of recalcitrant organic nitrogen to NH_4^+; INH_4-Nrec: immobilization of NH_4^+ to recalcitrant organic nitrogen; MNlab: mineralization of labile organic nitrogen; INH_4-Nlab: immobilization of NH_4^+ to labile organic nitrogen; ONrec: oxidation of recalcitrant organic nitrogen to NO_3^-; INO_3: immobilization of NO_3^- to recalcitrant organic N; ONH_4: oxidation of NH_4^+ to NO_3^-; DNO_3: dissimilatory NO_3^- reduction to NH_4^+; ANH_4: adsorption of NH_4^+ on cation exchange sites; RNH_4a: release of NH_4^+ from cation exchange sites.

Fig. S2. Measured (scatters, average ± standard deviation, n = 3) and modeled (lines) N concentration (a, b, d, e) and ^{15}N enrichment (c, f) in inorganic-N of the eight N treatments during incubation. Symbols (points and lines) in green and orange indicate short-term and long-term N deposition treatments, respectively. SC, SL, SM, and SH indicate control, low-N, medium-N, and high-N treatments at the short-term site. LC, LL, LM, and LH indicate control, low-N, medium-N, and high-N treatments at the long-term site.

Fig. S3. Pearson correlation analysis results of soil properties, N_2O fluxes, N_2O emission proportions from ammonium ($^{15}N_2O/^{15}NH_4^+$) and nitrate pools ($^{15}N_2O/^{15}NO_3^-$), gene abundances, denitrification/nitrification ((nirK + nosZ)/(AOA-amoA + AOB-amoA)) and nosZ/nirK gene ratios at short-term (a) and long-term (b) sites. N_2Oa, N_2Oh, and N_2Od indicate N_2O fractions from autotrophic nitrification (a), heterotrophic nitrification (h), and denitrification (d) pathways, respectively. Red, blue, and yellow squares indicate NH_4^+-N and organic-N pools (including ^{15}N labeled components from original isotopic addition solutions or N transformation). Dark yellow and light green ovals indicate soil properties and gene indicators (gene abundance and relative ratio), respectively. Overlaps between adjacent indicators indicate significant correlations. Symbol sizes have no meaning. All mentioned correlations are positively significant unless otherwise stated.

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