N_2O emission from a subtropical forest is dominantly regulated by soil denitrifiers under exogenous N enrichment and seasonal precipitation distribution change

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Key Points:

- Soil N_2O efflux of the forest was significantly influenced by only the NP treatment in the dry season.
- Soil *nirK* and *nosZ* denitrifiers played more dominant roles than soil nitrifiers (AOB, AOA, NOB) in regulating N_2O emissions.
- Precipitation change was a more dominant factor than nitrogen deposition elevation in influencing soil denitrifiers and N_2O emission.

Abstract

Nitrogen-rich tropical/subtropical forest soil acts as a terrestrial source of nitrous oxide (N_2O) emissions, a greenhouse gas commonly affected by soil nitrogen availability and soil moisture. However, in tropical and subtropical regions experiencing both elevated nitrogen deposition and altered precipitation regimes, it is unclear whether nitrogen deposition and precipitation regimes have interactive effects on forest soil N_2O emissions and what roles N_2O-associated nitrifiers/denitrifiers play in these interactions. We conducted a two-year field study in a subtropical evergreen broadleaf forest in southern China by applying four treatments: nitrogen addition (N), seasonal precipitation distribution change (P), both nitrogen addition and seasonal precipitation distribution change (NP) and a control (C). The Results showed that N_2O efflux from the forest soil was significantly greater in the wet season than in the dry season, but was promoted by the NP treatment only in the dry season. Soil moisture and pH decreased in the P and N treatments, respectively. The abundances of the nitrifying gene AOA-*amoA* and denitrifying gene *nosZ* in the wet season and the abundance of the denitrifying gene *nirK* in the dry season differed significantly among the four treatments. A structural equation model showed that precipitation change was more important than nitrogen addition in affecting soil properties (e.g. moisture and pH) and N_2O-associated nitrifiers/denitrifiers, while soil *nirK*- and *nosZ*-denitrifiers were the dominant functional microbes in regulating N_2O emissions. The results support predictions of future nitrogen losses (N_2O) in subtropical forests in the context of interactions between elevated nitrogen deposition and altered precipitation regimes.

Plain Language Summary

In this study, we examined the interactive effects of nitrogen (N) deposition increases and precipitation regime changes on soil nitrous oxide (N_2O) emissions from a subtropical forest and the underlying changes in soil functional microbial groups. Soil pH rather than soil available N was significantly affected by simulated N deposition, while soil moisture was significantly affected by simulated precipitation changes. Soil N_2O emissions were greater in the wet season than in the dry season but were enhanced by the interaction of simulated N deposition increases and precipitation changes only in the dry season. Moreover, the abundance of soil nitrifying and denitrifying functional genes responded differently to the interaction of N deposition increases and precipitation changes. Structural equation modelling results indicated that precipitation change was more important than increased N deposition in affecting N_2O-associated nitrifiers and denitrifiers, while soil *nirK*- and *nosZ*-type denitrifiers were the dominant functional microbial groups in regulating N_2O emissions.

1 Introduction

As the largest nitrogen (N) pool in terrestrial ecosystems, soil acts not only as a carrier for various forms of N (e.g. ammonium N, nitrate N, and organic N) for the growth of plants but also as a source or a sink for main greenhouse gases such as nitrous oxide (N_2O) (Oertel et al., 2016). However, soil N dynamics have been disrupted by a series of global/regional environmental changes in recent decades, such as increases in N deposition and changes in precipitation regimes (Dore, 2005; Kanakidou et al., 2016). N deposition and precipitation changes have been demonstrated to induce changes in soil N availability and water availability,

respectively (Chen et al., 2017; Cheng et al., 2019), inducing changes in N_2O emissions by influencing the community, abundance and activity of N_2O-associated nitrifying and denitrifying functional microbes in soil (Avrahami et al., 2002; Levy-Booth et al., 2014). Moreover, atmospheric N deposition is generally closely linked to precipitation because of coupling between the nitrogen and water cycles, which is particularly pronounced in subtropical/tropical regions where a significant wet-dry seasonality exists and wet N deposition depends strongly on precipitation. For instance, southern China experiences not only a high amount of atmospheric N deposition but also changes in precipitation patterns (Zhou et al., 2011; Yu et al., 2019). The total natural N deposition rate of this region is over 35 kg N ha^{-1} yr^{-1} (Zhu et al., 2015), and a transition has occurred from the traditional reduced N (NH_x) deposition dominance to nearly equal NH_x and oxidized N (NO_y) in wet deposition, particularly in recent decades (Yu et al., 2019). The annual precipitation in this region has changed little over the past 60 yr (1950-2009), but incidences of heavy rain events have increased in the wet season and chances of drought have increased in the dry season (Zhou et al., 2011). Due to these new trends and coupled changes in N and water dynamics, it is necessary to predict the interactive effects of atmospheric N deposition increases and changes in seasonal precipitation distribution on soil N_2O emissions in this region. Notably, subtropical/tropical forests are commonly considered the largest natural terrestrial sources of N_2O globally (Werner et al., 2007; Cheng et al., 2014). Therefore, the seasonal wet-dry variations in forest N_2O emissions due to the typical subtropical monsoon climate (Tang et al., 2006) may become more pronounced under the changing seasonal distribution of precipitation, and more complex interactions might occur between N deposition and precipitation changes. However, to date, few studies have been focused on solving this problem, and the underlying mechanisms are still poorly understood.

N_2O is produced mainly via microbial nitrification and denitrification processes and is reduced via microbial denitrification (Conrad, 1996; Levy-Booth et al., 2014). The N_2O-associating steps of these two biological processes are catalysed by enzymes of different microbial groups, and each enzyme is encoded by specific functional genes (Levy-Booth et al., 2014). According to previous studies, soil nitrifiers and denitrifiers show different sensitivities to changes in soil N availability (Avrahami et al., 2002), water availability (Szukics et al., 2010) or other soil abiotic factors such as pH (Nicol et al., 2008; Giles et al., 2012), that regulate the final emission rates of N_2O (Zhang et al., 2021; Chen et al., 2022). For instance, ammonia-oxidizing archaea (AOA) are generally more abundant than ammonia-oxidizing bacteria (AOB) in many acidified forest soils but are less responsive to N deposition than are AOB (Isobe et al., 2012; Carey et al., 2016; Han et al., 2018). Moreover, the ammonia monooxygenase-encoding gene *amoA* of AOA and the nitrous oxide reductase-encoding gene *nosZ* were reported to play roles in regulating nitrification rates and N_2O emissions in response to seasonal precipitation changes (Chen et al., 2017). Soil *nirK* (nitrite reductase encoding gene)-denitrifiers were demonstrated to be sensitive to moisture changes, while AOA responded negatively, but AOB were more stable than AOA in responding to soil moisture increases (Szukics et al., 2010; Wang et al., 2017). AOA-*amoA* abundance was suggested to be more sensitive than AOB-*amoA* abundance to pH changes in acidic soils (Cuhel et al., 2010), while *nosZ*-encoding N_2O reductase was shown to be influenced by pH (Giles et al., 2012), further indicating that soil nitrifiers and denitrifiers play different roles in N_2O emissions from acidified soils caused by N deposition (Liu et al., 2011). As a consequence, (i) forest soil N_2O emissions are often enhanced by N deposition or N addition because of the increase in N substrates (NH_4^+-N, NO_3^--N) for nitrification or denitrification (Huang et al., 2013; Bai et al., 2014); (ii) N_2O emissions are inhibited by

increased soil moisture because of the increase in soil anoxic conditions and the reduction of N_2O to N_2 (Szukics et al., 2010; Cheng et al., 2014); and (iii) N_2O emissions, as a response to soil N or water condition changes, are a result of changes in soil nitrifiers/denitrifiers and corresponding nitrification/denitrification processes (Szukics et al., 2010). Notably, although nitrite-oxidizing bacteria (NOB) only recently have been identified, the participation of NOB in nitrification determines whether the fixed N (nitrite) remains in ecosystems or is lost to the atmosphere (Daims et al., 2016; Daims and Wagner, 2018). Thus, quantifying the abundance of the nitrite oxidoreductase-encoding gene *nxrB* (Pester et al., 2014) in soil will facilitate an understanding of the role of NOB in nitrification and nitrification-based N_2O production. Overall, soil nitrogen and moisture changes, arising from N deposition increases and seasonal precipitation distribution changes, respectively, may induce more complex interactive effects on soil nitrifiers/denitrifiers and thereby forest soil N_2O emissions (Wang et al., 2017), but these effects are still poorly understood, particularly in forest ecosystems.

The key objectives of this study were to reveal the responses of N_2O emissions to the interaction between N deposition increases and precipitation changes and the underlying microbial regulatory mechanisms in a subtropical broadleaf forest in southern China. The effects of increases in N deposition were determined monthly by N addition. The effects of seasonal precipitation distribution changes were simulated by artificially excluding precipitation in the dry season and increasing precipitation in the wet season. We hypothesized that (1) N_2O emission is stimulated by N addition, (2) N_2O emission is stimulated by precipitation reduction in the dry season but is inhibited by precipitation increase in the wet season, (3) N_2O emission is synergistically stimulated by the N addition-precipitation change interaction in the dry season but is slightly inhibited by that in the wet season, and 4) the roles of soil nitrifiers/denitrifiers in N_2O emissions are different among experimental treatments and between wet and dry seasons. The results provide comprehensive insights for understanding forest soil N losses under future environmental changes.

2 Materials and Methods

2.1 Study site

The field experimental site is located at the Heshan National Field Research Station of Forest Ecosystem, Chinese Academy of Sciences (112°54′ E， 22°41′ N), Heshan city, Guangdong Province of China (Fig. S1). The forest where the study site is located is a subtropical broadleaf mixed forest. The dominant tree species of the forest are *Schima superba* and *Michelia macclurei*. Dominant species of the shrub layer are *Psychotria asiatica*, *Melicope pteleifolia* and *Ilex asprella*. The herb layer is dominated by *Lophatherum gracile*, *Blechnum orientale* and *Adiantum flabellulatum*. The region where the forest belongs to has a typical subtropical climate, with the whole year being divided into a wet season (from April to September) and a dry season (from October to March). The average annual precipitation is 1700 mm. The average annual atmospheric temperature is 21.7 ℃. The highest monthly average temperature is 29.2 ℃ (July) while the lowest monthly average temperature is 12.6 ℃ (January) (Wang et al., 2009). The forest soil belongs to typical laterite (or Oxisols in the USDA soil taxonomy), developed from sandstone and has a high leaching potential (Chen et al., 2017). The top layer (0-10 cm) of the forest soil has a pH value of 3.9, ammonium N of 1.4 mg kg^{-1}, nitrate N of 2.3 mg kg^{-1}, total N of 0.2% and total phosphorous of 0.02%. The total inorganic N

deposition amount of this zone is 47.2 kg N ha^{-1} yr^{-1}, with the ratio of ammonium and nitrate deposition rates being nearly 1.0 (Huang et al., 2015).

2.2 Experiment design

The field experiment simulating N deposition elevation and precipitation regime alteration started in October 2018. Four experimental treatments were set at the site: N addition (N), seasonal precipitation distribution change (P), interaction of N addition and precipitation change (NP) and control (C). The N addition level is 100 kg N ha^{-1} yr^{-1}, which is doubled based on the value of the natural inorganic N deposition (Huang et al., 2015), to simulate the N deposition elevation. The precipitation change treatment was set as drier in the dry season and wetter in the wet season, but the total annual precipitation did not change. The dry-season precipitation reduction and the wet-season precipitation increase was done by excluding throughfall and adding water in the two distinct seasons, respectively.

Four replicated plots, each 12 m × 12 m, are set up for each experimental treatment. A 3 m wide buffer strip is set to separate adjacent plots (Fig. S1). For each plot of the N and NP treatments, starting in October 2018 and at the beginning of each month, 342.86 g ammonium nitrate (NH_4NO_3) is dissolved in 20 L water and sprayed evenly under canopy (equals to an increase of 1.7 mm precipitation in each year). As a control, each plot of the C and P treatments receive the same volume of water to avoid the effect of extra water addition. For the P and NP treatments, 11 or 12 pieces of transparent plastic film (length: 12 m, width: 0.5-1 m, total width: 8 m) are fixed on the top of each plot. The supporting system of the plastic film consists of 16 vertical galvanized steel tubes (φ = 10 cm, 3 m length and ~ 0.6 m depth in soil) and 8 horizontal stainless steel frames (12 m length). The films cover 67% of the total area of each plot when they are fully expanded. Several water sprayers with a water pipe are fixed on top of each frame, to guarantee an even water addition in the wet season. The throughfall exclusion occurs from mid-September to mid-April (dry season) of each year by fully expanding the plastic film. After that, all films are hanged beside the frame for each plot totally accepting natural precipitation. During this period, a same volume water that equals to the excluded throughfall in the previous dry season is added to the plot for several times (each time 50-55 mm). The added water is from a pond near the site after filtration. The pH and other properties of the pond water are near to natural precipitation (Chen et al., 2017).

During the first year of precipitation change (September^{16th} 2018 - September^{15th} 2019), the natural precipitation was 528.0 mm in the dry season and 1691.5 mm in the wet season. The throughfall that excluded in the dry season was 302.0 mm. Water that equaled to the amount of the excluded throughfall was added 6 times (June^{16th} 2019, July^{8th} 2019, July^{19th} 2019, August^{10th} 2019 August^{24th} 2019 and September^{8th} 2019, each time 50.3 mm) in the wet season. During the second year of precipitation change (September^{16th} 2019 - September^{15th} 2020), the natural precipitation was 332.1 mm in the dry season and 1222.5 mm in the wet season, while the excluded throughfall in the dry season of this year was 185.0 mm. Water that equaled to the amount of the excluded throughfall was added 3 times (June^{12th} 2020, July^{8th} 2020 and August^{10th} 2020, each time 61.55 mm) in the wet season.

2.3 Gas sampling and analysis

The N_2O emission flux of the forest soil was measured using the closed chamber method (Hutchinson and Mosier, 1981). The closed chamber consists of a PVC-made main body (φ = 25

cm, H = 35 cm) and a PVC-made base ($\varphi_{\text{out-ring}}$ = 33 cm, $H_{\text{out-ring}}$ = 11 cm; $\varphi_{\text{inner-ring}}$ = 25 cm, $H_{\text{inner-ring}}$ = 8 cm). A little fan is fixed inside the main body and linked to an external battery, to mix the gas of the chamber evenly when collecting gases. Two bases were randomly installed in each plot in 2012 and placed permanently. The corresponding main body of the closed chamber was placed nearby each base.

Starting in October 2018, gas samples were collected monthly at the middle of each month. The gas collection time of each sampling day is limited in 9:00-11:00 am, since the gas emission rate during this time period was proven to approximately equal to the average rate the whole day (Kessavalou et al., 1998; Tang et al., 2006). Before sample collection, the bottom of the chamber body is placed between the two rings of the base, while the gap between the inner and outer rings of the base is filled with water for creating a sealing environment. The fans of the chamber are also opened to mix the gas inside. Gas samples are taken from the sampling pot on top of the chamber at 0 min, 10 min, 20 min and 30 min using a plastic syringe (100 ml, Pingan, China). The sample of each time point is immediately transferred to a pre-evacuated air bag (0.2 L, Shanghaieler LTD, ELCR, Shanghai, China) for next-step measurement. During the time period of gas collection, we recorded the atmospheric pressure and temperature using an aneroid barometer (Type DYM3, Fengyang, Tianjin, China), as well as soil moisture and temperature using a TDR soil water measurement system (Type TRIME-PICO, Aozuo, Beijing, China). The atmospheric temperature and precipitation are recorded by Heshan National Field Research Station of Forest Ecosystem, Chinese Academy of Sciences.

The gas samples were analyzed using the electron capture detector (ECD) of a gas chromatography (Type 7890A, Agilent, Santa Clara, USA) within one week of collection. The N_2O emission flux is calculated as follows:

$$F = \rho \times \frac{V}{A} \times \frac{P}{P_0} \times \frac{T_0}{T} \times \frac{dC_1}{dt} \quad (1)$$

where, ρ: the N_2O density in the standard condition (g L^{-1}); V: the gas volume in the closed chamber (m^3); A: the chamber coverage area (m^2); P: the atmosphere pressure (Pa) of the sampling site; P_0: the standard atmosphere pressure in the standard condition (Pa); T_0: the absolute temperature in the standard condition; T: the absolute temperature of the sampling time; dC_1/dt: the liner slope of gas concentration changes within time (ppb h^{-1}). The N_2O flux is expressed in the unit of μg N m^{-2} h^{-1}.

2.4 Soil sampling and analysis

Starting in October 2018, soil samples were collected monthly at the middle of each month (on the same day of the gas collection). Six soil cores were randomly selected in each plot. After litter removal, the surface soil (0-20cm) of each soil core were collected using a corer (φ = 4 cm). The surface soil that collected from all the six cores were then fully mixed into a composite sample. Therefore, a total of 16 soil samples were collected in each month. After taken back and sieved through a 2-mm mesh, each soil sample was divided into three subsamples and stored in different conditions for future laboratory analyses.

The first subsample was naturally dried, then directly used or used after more carefully sieved for the determination of soil pH and total nitrogen (total N). Soil pH was determined using a portable pH detector (F-71G, LAQUA, HORIBA, Japan) by mixing soil and water in a

ratio of 1:2.5 (m: v). Soil total N was determined by the Alpha-Naphthol Blue-spectrophotometer method after sulfuric acid heating.

The second subsample was stored at 4 °C for the determination of soil water content (SWC), ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N) and microbial biomass nitrogen (MBN). SWC was determined using the oven-drying method. The ammonium N and nitrate N were determined by using the flow injector (Type QC8000, Lachat, USA) after the fresh soil was extracted by a 2 M KCl solution at a ratio of 1:5 (m: v). MBN were determined using the chloroform-fumigation-extraction method (Vance et al., 1987).

The third subsample was stored at -80 °C for quantification of functional genes. Soil total DNA was extracted from 0.25 g soil using a PowerSoil® DNA Isolation kit (MoBio, Anbiosci Tech Ltd, USA) by following the manufacture's protocol. The extracted DNA was first tested using a Nanodrophotometer (Thermo NanoDrop™ One, Thermo Scientific, USA) and agarose gel electrophoresis and then stored at -20 °C for further analyses.

2.6 Quantification of nitrifying and denitrifying functional genes

Five enzyme encoding functional genes of soil nitrifiers and denitrifiers were chosen in this study, including 1) the *amoA* gene of ammonia-oxidizing bacteria (AOB-*amoA*), 2) the *amoA* gene of ammonia-oxidizing archaea (AOA-*amoA*), 3) the *nxrB* gene of nitrite-oxidizing bacteria (NOB), 4) the nitrite reductase gene *nirK* and 5) the nitrous oxide reductase gene *nosZ*. The primer information of each functional gene is listed in Table S1.

The abundance of functional genes was quantified by absolute real-time polymerase chain reactions (PCR). The PCR reaction was carried out in a 384-well microplate (Labtide, Greystone Biosciences, USA) with a LightCycler® 480II real-time fluorescent quantitative PCR system (Roche, Switzerland). SYBR green was used as the detection system. Moreover, the volume of the reaction mixture was 20 μl, including 10.4 μl SYBR Green Premix Ex Taq™ II (TaKaRa, Japan), 0.4 μl of 10 μM forward primer (0.2 μM in the final reaction system), 0.4 μl of 10 μM reverse primer (0.2 μM in the final reaction system), 2.0 μl DNA template and 6.8 μl double distilled water. The standard plasmid of each functional gene, i.e. the plasmid that contains the fragment of AOB-*amoA*, AOA-*amoA*, *nxrB*, *nirK* or *nosZ*, was prepared from the extracted DNA samples using same primers as above. The standard plasmid was then gradually diluted into a serial plasmid solutions (10^2-10^{10} copies μl^{-1}) to obtain the standard curve of the target gene. The PCR reaction programs are listed in Table S2. Three technical replicates of all the DNA samples and standard curves were designed when performing the qPCR amplifications. The PCR amplification efficiency and the R^2 for the functional genes was in a range of 80.8-118.5% and 0.900-9.994, respectively.

2.7 Statistical analysis

Comparisons were conducted mainly among the four experimental treatments (C, N, P, NP) and between the wet and dry seasons. During the two-year investigation, the average soil properties, functional gene abundances and N_2O effluxes of the dry season and the wet season were calculated. Two-way ANOVA analyses followed by least significant difference (LSD) testes were used to examine the difference of soil properties, functional gene abundances and N_2O effluxes among the four treatments and between the two seasons. All data were assessed for normality (Kolmogorov-Smirnov test) and quality of variances (Levène test) before analyses. Data that did not fit normality or quality of variances were logarithmically transformed for

further analyses. The statistically significant difference was analyzed at $p < 0.05$ level. All statistical analyses were performed in SPSS 20.0 (SPSS Inc. Chicago, USA) and Sigmaplot 12.5 (Systat Software Inc.).

A principal component analysis (PCA) was performed separately in each treatment, to test the relationship of the soil properties, functional gene abundances and N_2O effluxes. A structural equation model (SEM) was constructed to explore the effects of N addition and precipitation change on soil properties, nitrifying/denitrifying functional gene abundances and N_2O effluxes (Fig. S2). Briefly, the conceptual model includes variables of mainly three aspects: 1) soil properties, including soil water content (SWC), soil pH, ammonium N (NH_4^+-N) and nitrate N (NO_3^--N). 2) Abundances of nitrifying and denitrifying functional genes, including AOB-*amoA*, AOA-*amoA* and *nxrB* for nitrification, and *nirK* and *nosZ* for denitrification. (3) Soil N_2O effluxes. Moreover, different relationships were established among the soil, microbial and N_2O indicators based on previous studies. The conceptual model was aimed to test that under the N addition and precipitation change treatments, how changes in soil water and soil inorganic N conditions induce responses in soil nitrifiers and denitrifiers and further influencing the microbial regulations in N_2O emission. Based on the model running results, we got the standard regression coefficient and significant level of each relationship, and the squared multiple correlation (R^2) of each variable in the model. The SEM analyses were conducted by AMOS 21.0 (SPSS Inc., Chicago, IL, USA). The final model analysis results were illustrated and expressed schematically.

3 Results

3.1 Soil properties

During the two-year period, the soil water content (SWC) of the study site ranged from 14% to 33%, with significantly greater values in the wet season than in the dry season ($p < 0.001$) (Fig. 1a, e). The soil pH ranged from 3.6 to 4.0, with a relatively low value in the first wet season of the experimental treatments (Fig.1b). The soil ammonium N and nitrate N concentrations ranged from 0.4 mg kg^{-1} to 8.4 mg kg^{-1} and from 0.7 mg kg^{-1} to 15.9 mg kg^{-1}, respectively, but fluctuated weakly with time, expect for an increase in March 2019 and June 2020 (Fig. 1c, d). Moreover, soil nitrate N was generally greater than ammonium N, indicating the potential for nitrification in the forest soil. According to two-way ANOVA, there were significant differences among the four treatments in terms of SWC, pH and nitrate N ($p < 0.05$). The SWC decreased in the experimental treatments and was greatest in the C treatment but lowest in the P treatment ($p < 0.05$) in both seasons (Fig. 1e). In contrast, soil pH was significantly lower in the N treatment ($p < 0.05$) but greater in the P and NP treatments in both seasons than in the C treatment, with the lowest value in the N treatment and the greatest value in the P treatment (Fig. 1f). Compared with the C treatment, the N treatment increased the soil ammonium N by 18.3% and 10.6% in the dry season and wet season, respectively ($p > 0.05$), while the N treatment increased the soil nitrate N by 13.9% and 10.6% in the dry season and wet season, respectively ($p > 0.05$). In contrast, the P treatment decreased the soil ammonium N by 14.5% and 2.7% in the dry season and wet season, respectively ($p > 0.05$) (Fig. 1g, h), while the P treatment decreased the soil nitrate N by 33.8% and 20.9% in the dry season and wet season, respectively ($p > 0.05$) (Fig. 1g, h). Except for the nitrate N content in the wet season, the contents of all four soil indicators in the NP treatment were between those in the N and P treatments, showing no significant difference from those in the C treatment ($p > 0.05$) (Fig.1 e-h).

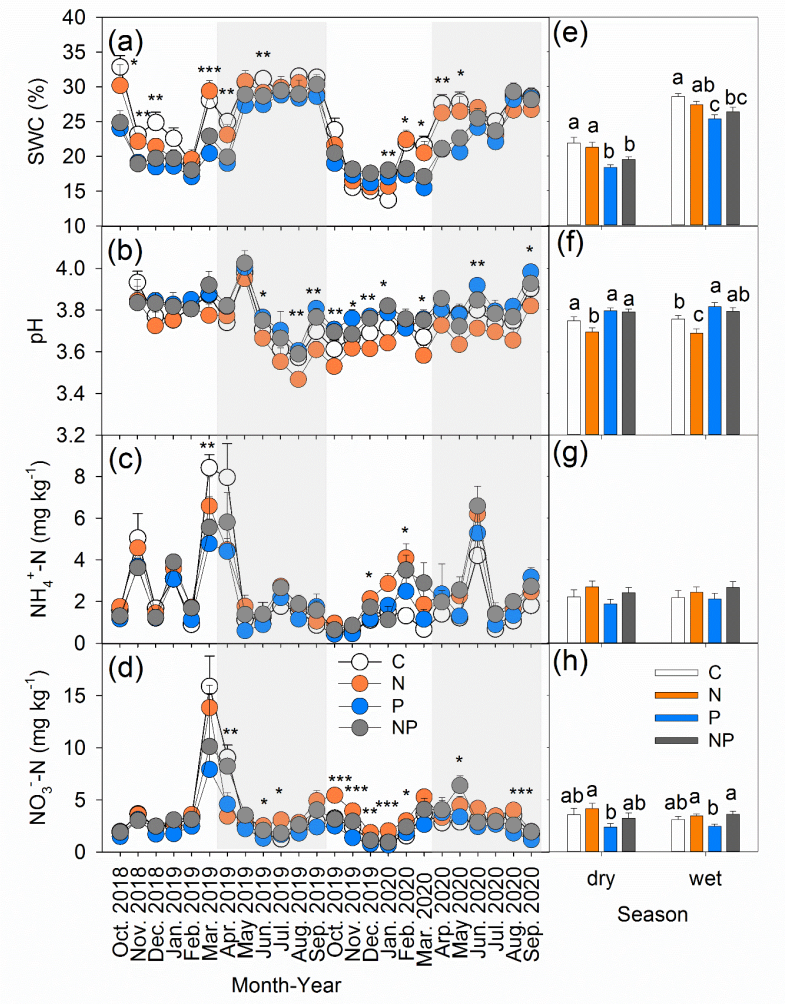


Figure 1 Soil (0-20 cm) physiochemical property (average ± standard error, n = 4) of each month (a-d) and of wet/dry season (e-h). C, N, P and NP indicate the treatment control, N addition, precipitation change, and interaction of N addition and precipitation change. Gray shades and white regions indicate wet seasons and dry seasons, respectively. Follows are the same. Asterisks in (a) - (d) indicate that the difference is significant ($p < 0.05$) among the four treatments (One-way ANOVA), while *, ** and *** indicate the difference is significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$ level, respectively. Same letters in (e)-(h) indicate that the difference between any two treatments of a specific season is nonsignificant ($p > 0.05$) (One-way ANOVA). Follows are the same.

3.2 N_2O efflux

The N_2O efflux varied over the months and showed significant wet-dry seasonality, with an increasing trend in the wet season in comparison to the previous dry season ($p < 0.001$) (Fig. 2). The average N_2O efflux of the C treatment was 22.3 ± 4.5 μg N m^{-2} h^{-1} in the dry season and 55.4 ± 9.9 μg N m^{-2} h^{-1} in the wet season (Fig. 2b). Compared with the C treatment, N_2O efflux in the N treatment increased nonsignificantly by 31.8% ($p > 0.05$) in the dry season but decreased nonsignificantly by 21.7% in the wet season ($p > 0.05$). Compared with the C treatment, the N_2O efflux in the P treatment decreased nonsignificantly by 14.7% and 3.3%,

respectively, in the dry season and wet season ($p > 0.05$). In contrast, the N_2O efflux in the NP treatment increased significantly (77.4%, $p < 0.05$) and nonsignificantly (20.4%, $p > 0.05$) in the dry season and wet season, respectively (Fig. 2b). Therefore, the soil N_2O efflux of the forest was significantly influenced by only the NP treatment in the dry season.

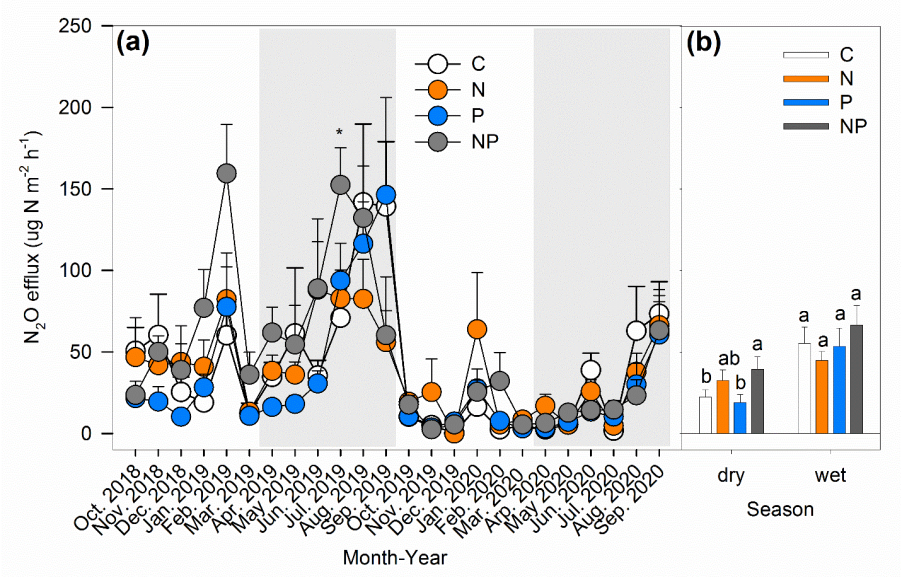


Figure 2 Monthly dynamics of N_2O effluxes (a) and average N_2O effluxes during the wet season and the dry season (b). Significance levels are indicated by * at $p < 0.05$ level. Same letters in (b) indicate that the difference between different treatments is nonsignificant ($p < 0.05$) (One-way ANOVA).

3.3 Abundances of soil nitrifying and denitrifying functional genes

AOB-*amoA* was most abundant in the soil, followed by *nxrB*, *nirK*, *nosZ* and AOA-*amoA* (Fig. S3, Fig. 3). The AOB-*amoA* abundance and *nxrB* abundance accounted for approximately 68.5% and 28.5%, respectively, while the abundances of AOA-*amoA*, *nirK* and *nosZ* accounted for approximately 3% of the total abundance of all five genes. Compared with the dry season soil, the wet season soil showed a significant increase in AOA-*amoA* abundance ($p < 0.001$), significant decreases in *nxrB* and *nirK* abundances ($p < 0.001$), and nonsignificant decreases in AOB-*amoA* and *nirK* abundances ($p > 0.05$) (Fig. 3a, c, d). Compared with those in the C treatment, the AOA-*amoA* and *nosZ* abundances in the N treatment soil decreased significantly in the wet season, and the *nirK* abundance in the N treatment soil decreased significantly in the dry season ($p < 0.05$) (Fig. 3a, d, e), indicating an inhibitory effect of N addition. Moreover, the soils in the N treatment had the lowest abundances of AOA-*amoA*, *nirK* and *nosZ*, followed by the soils in the NP and P treatments, but there were no significant differences among the C, P and NP treatments ($p > 0.05$) (Fig. 3a, d, e). According to two-way ANOVA, the *nirK* abundance significantly differed among the four treatments ($p < 0.05$) (Fig. 3d).

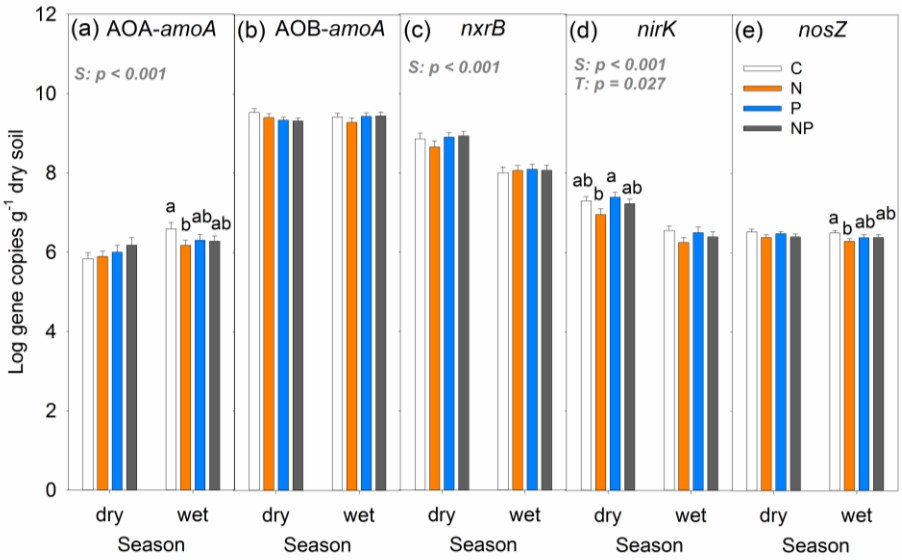


Figure 3 Abundances (average ± standard error) of nitrifying and denitrifying functional genes in the dry season and wet season. S and T indicate the significance of differences between seasons and among treatments, respectively, according to two-way ANOVA. Same letters indicate that the difference between any two treatments of a specific season is nonsignificant ($p > 0.05$) (One-way ANOVA).

3.4 Relationships among soil properties, abundance of soil nitrifiers/nitrifiers and N_2O efflux

The PCA results showed different patterns among the four treatments (C, N, P, and NP). The first two PCs explained 67.8%, 64.1%, 64.5% and 59.4% of the total variability in the C, N, P, and NP soils, respectively (Fig. 4). Generally, the functional gene abundances of all four treatment soils had positive PC1 values, while the soil properties had positive PC2 values expect for SWC in the NP soil. Moreover, pH showed close relationships with the AOA-*amoA* abundance in the C, N and P soils and with the AOA-*amoA*, *nxrB* and *nirK* abundances in the NP soil. The N_2O efflux showed close relationships with SWC in the C, P and NP soils, but did not show close relationships with any soil property indicators in the N soil. In contrast, the N_2O efflux did not show a close relationship with the abundances of soil nitrifying and denitrifying functional genes in each treatment (Fig. 4).

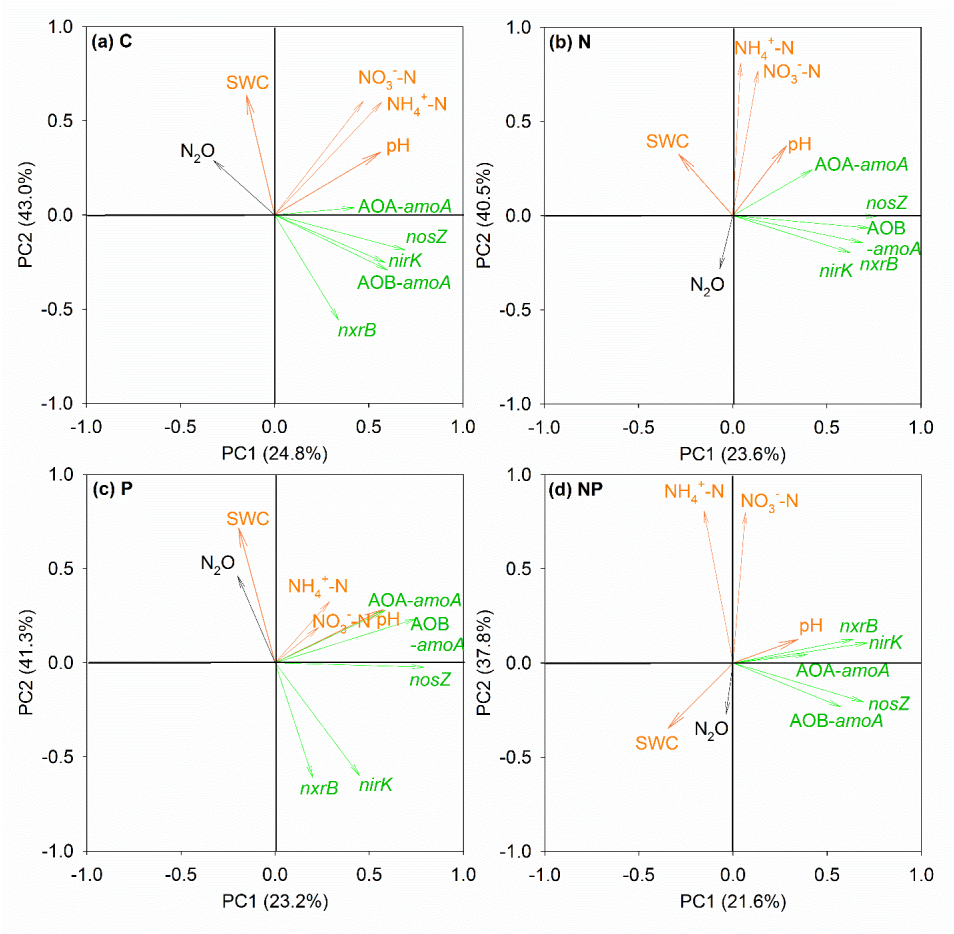
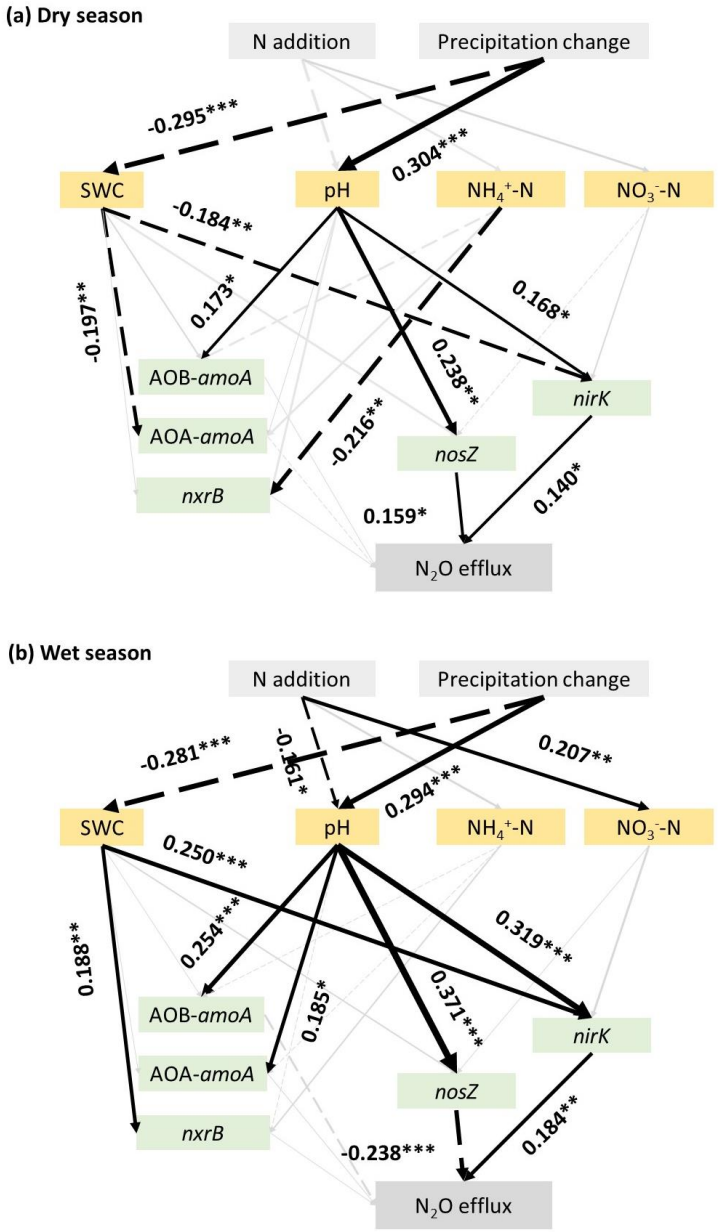


Figure 4 Principal component analysis (PCA) illustrating the relationships among soil properties, functional gene abundances and N_2O effluxes. Arrows in orange, green and black indicate the factor loadings of soil property, nitrifying/denitrifying functional gene abundance and N_2O efflux, respectively, on the PC1/PC2 axes.

The SEM results showed that the effects of N addition and precipitation change on soil properties, nitrifying/denitrifying functional gene abundance and N_2O efflux differed between the wet and dry seasons. In the dry season, there were no significant relationships between N addition and soil properties (Fig. 5a). In contrast, precipitation change (precipitation decrease) was negatively related to SWC (r = -0.295, $p < 0.001$) but had a positive relationship with pH (r = 0.304, $p < 0.001$). SWC was negatively related to the AOA-*amoA* abundance (r = -0.197, p = 0.007) and *nirK* abundance (r = -0.184, p = 0.009). In contrast, pH showed significant positive relationships with the abundances of AOB-*amoA* (r = 0.173, p = 0.019), *nosZ* (r = 0.238, p = 0.001) and *nirK* (r = 0.168, p = 0.02). Both the *nirK* and *nosZ* abundances showed significant positive relationships with the N_2O efflux (r = 0.140, p =0.048; r = 0.159, p = 0.025). Except for a negative relationship between soil ammonium N and *nxrB* abundance (r = -0.126, p = 0.002), there were no significant relationships between soil inorganic N and nitrifying/denitrifying functional gene abundances (Fig. 5a).

The soil in the wet season showed relationships similar to those in the dry season, that is, negative relationships between precipitation change (precipitation increase) and SWC (r = -0.281,

410 $p < 0.001$), between precipitation change and pH (r = 0.294, $p < 0.001$), between pH and the
411 abundances of AOB-*amoA* (r = 0.254, $p < 0.001$), *nosZ* (r = 0.371, $p < 0.001$) and *nirK* (r = 0.319,
412 $p < 0.001$), and between *nirK* abundance and N_2O efflux (r = 0.184, p = 0.008). In contrast, *nosZ*
413 abundance showed a negative relationship with N_2O efflux (r = -0.238, $p < 0.001$). Moreover, N
414 addition had a negative relationship with pH (r = -0.161, $p = 0.018$) but a positive relationship
415 with soil nitrate N (r = 0.207, $p = 0.004$), while pH had a positive relationship with AOA-*amoA*
416 abundance (r = 0.185, $p = 0.013$) (Fig. 5b).



417

418 **Figure 5** Structural equation model simulating the effects of N addition and precipitation change
419 on soil property, functional gene abundance and N_2O efflux. Squares in light gray, yellow, light
420 green and middle gray indicate the indicators of N addition/precipitation change, soil property,
421 functional gene abundance and N_2O efflux, respectively. (1) Arrows in black indicate the
422 relationship is significant ($p < 0.05$), while arrows in gray indicate the relationships is

nonsignificant ($p > 0.05$). (2) Solid black arrows indicate the relationship is positive, while dotted black arrows indicate the relationship is negative, according to the model results. (3) Numbers on adjacent to each arrow indicate the standard regression index of corresponding relationship, while numbers of nonsignificant relationships were not shown in this figure. *, **, and *** indicate that the relationship is significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$ level, respectively. (4) The arrows are of different thickness, the higher the absolute value of the standard regression value, the thicker the arrow.

4 Discussion

4.1 Effects of N addition and seasonal changes in the distribution of precipitation on N_2O emissions

(1) Unlike the first hypothesis and previous studies in subtropical forests showing that N addition promoted N_2O emissions (Zhang et al., 2008; Nie et al., 2019), in this study, forest soil N_2O effluxes were not significantly stimulated in the dry season but inhibited in the wet season by N addition (Fig. 2). However, this is in line with the findings of a study showing the response of N_2O emissions to a gradient of N addition in a subtropical forest of southern China (Han et al., 2019). Moreover, soil ammonium N and nitrate N were nonsignificantly stimulated by N addition in either season (Fig. 1g, h). Therefore, we speculated that in the dry season, the increased N_2O emissions under N addition were a result of increased substrate (NH_4^+-N, NO_3^--N) availability for nitrification and denitrification, while the decreased N_2O effluxes in the wet season were influenced by soil factors (e.g., soil moisture) other than soil inorganic N availability. (2) The precipitation change treatment resulted in relatively lower soil N_2O effluxes and significantly lower soil moisture in both seasons (Fig. 1 a, e; Fig. 2). This finding did not support the second hypothesis. It was unexpected that in the wet season, soil moisture in the surface layer (0–20 cm) was inhibited by increased precipitation. Possible reasons for this finding might be that there was high natural rainfall in the wet season, which induced high moisture levels in the forest soil. The water holding capacity of the surface soil might have been exceeded under simulated precipitation, and the extra water was lost via leaching to deeper soil layers (20–40 cm and 40-60 cm). This was supported by the findings that the soil water content in the soil in the P treatment was higher than that in the C treatment (data not shown). Thus, the inhibition of N_2O emissions in the wet season may have occurred due to the decreased soil nitrifier and denitrifier abundances under the precipitation change treatment (Fig. 3a, c, d). (3) The N_2O effluxes were significantly stimulated by the NP treatment in the dry season but were nonsignificantly affected in the wet season (Fig. 2b). This is partly consistent with the third hypothesis, suggesting that the added N may stimulate soil N usage and N loss, while precipitation changes may cause less changes in N_2O production and gaseous emissions. To confirm this speculation, a field ^{15}N labelling method for tracing the transformation and fate of added N (Gurmesa et al., 2016) must be employed in future studies.

4.2 Effects of N addition and seasonal precipitation distribution changes on the abundances of soil nitrifiers and denitrifiers

The AOB-*amoA* gene outnumbered the AOA-*amoA* gene in the studied forest soil (Fig. 3), which contrasts with previous findings showing that AOA are generally more dominant than AOB in acidic forest soils (Isobe et al., 2012) but the finding is in line with the study of Petersen (2012) in a black spruce forest. Moreover, the *nxrB* gene was less abundant than AOB-*amoA* but

much more abundant than AOA-*amoA* (Fig. 3a-c), implying a greater potential for N_2O production than NO_3^- production during autotrophic nitrification. The *nirK* gene was more abundant than the *nosZ* gene (Fig. 3d-e), showing the potential for more N_2O production than N_2O reduction during denitrification. Notably, the *nirK* gene was less abundant than the AOB-*amoA* gene, which contrasts with findings of Yin et al. (2023) in four highly acidic soils. Compared with the dry season soil, the wet season soil showed significantly greater AOA-*amoA* abundance, but the AOB-*amoA* abundance did not change markedly, while the trends in AOA-*amoA* with season were similar with those of SWC (Fig. 1e), showing that AOA may be more sensitive than AOB to soil moisture changes. However, this finding is inconsistent with the results of Wang et al. (2017), who reported that unlike AOA, AOB responded positively to soil moisture increases. In contrast, the *nxrB* abundance decreased significantly in the wet season, implying that higher soil moisture may not benefit NOB community or further facilitate the production of N_2O. This might be one reason why significantly greater N_2O effluxes were observed in the wet season than in the dry season (Fig. 2b). The *nirK* abundance decreased significantly but the *nosZ* abundance varied less from the dry to wet season, showing that higher soil moisture may limit the production of N_2O via the denitrification pathway.

Our results showed that the three experimental treatments (N, P, and NP) did not induce significant changes in the abundance of AOB-*amoA* or *nxrB* in either season (Fig. 3b, c), indicating that the soil AOB and NOB communities were not sensitive to either N addition or precipitation increase/decrease. This is reflected in the generally similar PCA patterns of functional genes in the soils of the four treatments (Fig. 4). Moreover, this finding is inconsistent with previous findings showing that AOB abundance increased by both N supply and soil moisture increase (Wang et al., 2017). Possible reasons for this difference might be that AOB were dominant in the studied forest soil and were adapted to the soil conditions (Petersen et al., 2012) induced by long-term high N input via N deposition and to the high precipitation in the subtropical region. Thus, short-term (2 yr) exogenous N input or increasing/decreasing seasonal precipitation did not change the AOB community much. On the other hand, the *nxrB* abundance decreased significantly from the dry to wet season but did not change significantly with increasing precipitation, implying that the soil moisture change induced by natural wet-dry seasonal variation was more influential than that induced by the precipitation change treatment, although the soil moisture content decreased significantly under this treatment (Fig. 1e). However, more evidence is required to confirm this speculation. AOA-*amoA* was nonsignificantly stimulated in the dry season but significantly inhibited in the wet season by N addition (Fig. 3a), implying that increased NH_4^+ supply (Fig. 1g) may stimulate AOA in the dry season, but this was not the dominant factor influencing the AOA community in the wet season. The abundance of soil denitrifiers, especially *nosZ* denitrifiers, was inhibited by N addition (Fig. 3d, e), which conflicts with the observation that the NO_3^- content increased but is consistent with the decreased pH of the soil (Fig. 1h, e), implying that pH may play more a essential role than substrate (NO_3^-) availability in soil denitrifier groups (Bárta et al., 2010). Although not significant, the *nirK* and *nosZ* abundances were generally reduced by the three experimental treatments in both seasons (Fig. 3d, e), showing that soil denitrifiers were sensitive to soil N and water changes to some extent.

4.3 Effects of soil abiotic and biotic factors on N_2O emissions

Soil N_2O efflux has been proven to be closely linked to many soil abiotic factors such as moisture (Zhang et al., 2008; Cheng et al., 2014), pH (Cuhel et al., 2010) and N availability

(Huang et al., 2014; Zhang et al., 2014). This is partly reflected in the PCA results of the four treated soils in present study (Fig. 4). Other than the soils of the N addition treatment (N), the soils in the treatments (C, P and NP), especially those in the P treatment, exhibited close relationships between N_2O efflux and soil moisture (Fig. 4a, c, d). This implies that soil moisture was more important in influencing forest soil N_2O emissions than pH and inorganic N. According to the PCA, no close relationships existed between the abundances of nitrifiers/denitrifiers and N_2O effluxes in the soils of all four treatments (Fig. 4), implying that the final emission of N_2O was not determined by single pathway of nitrification or denitrification. On the other hand, all four treated soils showed close relationships between pH and AOA-*amoA* abundance, again demonstrating the high sensitivity of the AOA community in the acidic forest soil (Isobe et al., 2012). Moreover, the close relationship between pH and the abundance of AOA-*amoA*, *nxrB* and *nirK* in the NP soil further increase the impact of the interaction of N addition and precipitation changes on the microbial community.

4.4 Role of soil functional microbes in the production and emission of N_2O under elevated N deposition and seasonal precipitation distribution changes

We conducted pathway analyses by combining indicators of soil properties, nitrifying/denitrifying functional microbes and N_2O emissions (Fig. 5). Nitrogen addition had negative and positive impacts on soil pH and nitrate N, respectively, but only in the wet season (Fig. 5b). This implies that the increase in precipitation and increase in soil moisture during the wet season may accelerate soil acidification and autotrophic nitrification, which is reflected in the relatively high AOA-*amoA* abundance during this season (Fig. 4a). This was also supported by the results of Nie et al. (2019), who reported that in a tropical forest, the rate of net nitrification was greater in the wet season than dry season under N addition. However, to confirm this speculation, the nitrification rate of the forest soil further must be quantified.

The SEM showed that compared with N addition, precipitation change induced more significant impacts on soil nitrifier/denitrifier abundances and N_2O emissions in both seasons (Fig. 5). This reveals that microbial regulation of soil N_2O emissions is more dependent on soil water changes than on soil inorganic N availability changes, which is also supported by the PCA (Fig. 4). For dry season soil, a decrease in precipitation had negative impacts on SWC, and a change in SWC had negative impacts on the abundances of AOA-*amoA* and *nirK*, while a change in *nirK* abundance had positive impacts on N_2O efflux (Fig. 5a). This could have occurred because a decrease in precipitation in the dry season led to lower soil moisture levels, further inhibiting the growth of soil AOA and *nirK* denitrifiers. The change in AOA-*amoA* abundance did not significantly change N_2O production via the autotrophic nitrification pathway, but the decrease in *nirK* abundance contributed to a significant decrease in N_2O production via the denitrification pathway. This may be one of the reasons for the significantly lower N_2O effluxes in the soil in the dry season than in the wet season (Fig. 2b). On the other hand, we found that a decrease in precipitation had positive impacts on pH, and pH had positive impacts on the abundances of AOB-*amoA*, *nirK* and *nosZ*, while the abundances of the two denitrifying functional genes further had positive impacts on N_2O efflux (Fig. 5a). This might have occurred because the decrease in precipitation alleviated soil acidification (Fig. 1f), further facilitating the growth of soil AOB and *nirK*/*nosZ* denitrifiers, while pH had a more significant impact on *nosZ* denitrifiers than on *nirK* denitrifiers (Fig. 5a), which further facilitated N_2O reduction ($N_2O \rightarrow N_2$) rather than N_2O production via the denitrification pathway. This may be another reason for the lower N_2O effluxes observed in the dry season (Fig. 2b). (2) The soil in the wet season

556 showed similar negative and positive impacts on the SWC and pH under precipitation increase,
557 respectively, as those in the dry season (Fig. 5). A possible reason for this might be that the
558 natural precipitation during the wet season kept the soil moisture level high (Fig. 1e), and the
559 water added during this season may have leached more to the deeper soil layer. SWC had
560 positive impacts on *nxrB* and *nirK* abundances, indicating that the growth of NOB and *nirK*
561 denitrifiers may be limited by high soil moisture levels and simulated precipitation in the wet
562 season. In the wet season, pH had positive impacts on *nirK* and *nosZ* abundances similar to that
563 in the dry season, but the *nirK* abundance had positive impacts, while the *nosZ* abundance had
564 negative impacts on N_2O efflux (Fig. 5b). Considering that *nirK* abundance decreased but *nosZ*
565 abundance changed little from the dry to wet season (Fig. 3d, e), we speculate that in the wet
566 season, the N_2O production of the denitrification pathway may be limited because of the
567 decreased *nirK* copy numbers in soil; however, the N_2O reduction was also limited, thus
568 alleviating N_2O consumption and further facilitating N_2O emission from the soil. This may be
569 one of the reasons for the high N_2O effluxes in the wet season (Fig. 2b).

570 Unlike soil moisture and pH, soil ammonium N and nitrate N did not significantly impact
571 the abundances of nitrifiers or denitrifiers in either season, even though nitrate N was positively
572 affected by N addition in the wet season (Fig. 1, Fig. 5). This indicates that soil inorganic N
573 availability was not a predominant factor regulating the abundance of soil nitrifiers and
574 denitrifiers, which is inconsistent with previous findings (Carey et al., 2016; Cheng et al., 2019).
575 However, this may have occurred because the studied forest soil was N-saturated after a high
576 level of long-term natural N deposition (Huang et al., 2015). On the other hand, no relationships
577 were detected between pH and *nxrB* abundance in either season, which is not in line with the
578 results of Li et al. (2019), who reported that *nxrB* abundance was significantly affected by pH.
579 One reason for this difference might be that the soil of the forest in this study is acidic, and the
580 variation in pH was relatively low (3.6-4.0) in comparison to the pH range gradient (4.8-7.0) in
581 the study by Li et al. (2019).

582 Generally, the SEM revealed that for the forest soils with interactions between N addition
583 and seasonal precipitation distribution changes, (1) changes in precipitation modified forest soil
584 physicochemical properties more than N addition and thus played more essential roles in
585 influencing soil nitrifier/denitrifier abundances and final N_2O emissions; (2) pH was most
586 sensitive to changes in precipitation change and N addition, followed by soil moisture, and
587 induced significant effects on soil nitrifiers and denitrifiers; (3) soil *nirK* and *nosZ* denitrifiers
588 played more dominant roles than soil nitrifiers (AOB, AOA, NOB) in regulating N_2O emissions;
589 and (4) N_2O emissions during the dry and wet seasons differed in terms of the potential to
590 regulate soil functional microbes due to the different responses of the microbes to seasonal
591 changes and the experimental treatments.

592 **5 Conclusions**

593 In this study, we tested whether N addition and changes in seasonal precipitation
594 distribution had individual and interactive effects on subtropical forest soil N_2O emissions and
595 investigated the underlying microbial mechanisms involved. We showed that soil N_2O emissions
596 were significantly greater in the wet season than in the dry season but were promoted by
597 interactions between N addition and precipitation change only during the dry season.
598 Precipitation change was more influential than N addition in affecting soil properties, especially
599 pH and moisture, while N addition and the interaction between N addition and precipitation

change did not induce significant changes in soil ammonium N and nitrate N contents. The structural equation model results showed that soil pH played a more essential role than soil moisture in regulating soil nitrifiers and denitrifiers and soil N_2O efflux. Soil nitrifiers, especially AOB-*amoA* and *nxrB*, were much more abundant than soil denitrifiers in the studied subtropical forest soil. However, under the N addition and precipitation change treatments, soil denitrifiers were more important in regulating N_2O emissions in both seasons, but there was a higher abundance of *nirK* than *nosZ* in the dry season soil, which contributed to N_2O production and reduction, but the decreased *nirK* abundance and the insubstantial change in *nosZ* abundance in the wet season soil limited the reduction of N_2O after production, contributing to more soil gaseous loss in the form of N_2O. In the future studies, soil nitrification rates and other N transformation rates should be determined to confirm the role of soil functional microbes in responding to soil N and water level changes and in regulating emissions of N_2O.

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5 Data availability statement

Data of N_2O efflux, soil property, soil nitrifying and denitrifying functional gene abundance are available in Bolin Centre Database https://doi.org/10.17043/21ki6u-1 (Han et al., 2024).

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