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# Effects of long-term increased N deposition on tropical montane forest soil $N_2$ and $N_2O$ emissions

**Running head:** Soil $N_2$ and $N_2O$ emissions from two tropical forests

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## Abstract

Nitrogen (N) deposition is projected to substantially increase in the tropics over the coming decades, which is expected to lead to enhanced N saturation and gaseous N emissions from tropical forests (via NO, $N_2O$, and $N_2$). However, it is unclear how N deposition in tropical forests influences both the magnitude of gaseous loss of nitrogen and its partitioning into the $N_2$ and $N_2O$ loss mechanisms. Here, for the first time, we employed the acetylene inhibition technique and the $^{15}N$-nitrate labeling method to quantify $N_2$ and $N_2O$ emission rates for long-term experimentally N-enriched treatments in primary and secondary tropical montane forest. We found that during laboratory incubation under aerobic conditions long-term increased N addition of up to 100 kg N $ha^{-1}$ $yr^{-1}$ at Jianfengling forest, China, did not cause a significant increase in either $N_2O$ or $N_2$ emissions, or $N_2O/N_2$. However, under anaerobic conditions, $N_2O$ emissions decreased and $N_2$ emissions increased with increasing N addition in the secondary forest. These changes may be attributed to substantially greater $N_2O$ reduction to $N_2$ during denitrification, further supported by the decreased $N_2O/N_2$ ratio with increasing N addition. No such effects were observed in the primary forest. In both forests, N addition decreased the contribution of denitrification while increasing the contribution of co-denitrification and heterotrophic nitrification to $N_2O$ production. Denitrification was the predominant pathway to $N_2$ production (98-100%) and its contribution was unaffected by N addition. Despite the changes in the contributions of denitrification to $N_2O$ gas emissions, we detected no change in the abundance of genes associated with denitrification. Our results indicate that the effects of N deposition on gaseous N loss were ecosystem-specific in tropical forests and that, while the mechanisms for these

different responses are not yet clear, the microbial processes responsible for the production of N gases are sensitive to N inputs.

**Keywords:** nitrogen deposition, tropical montane forests, nitrous oxide emission, dinitrogen emission, denitrification, denitrification genes

## 1. Introduction

Anthropogenic nitrogen (N) deposition is increasing due to fossil fuel combustion, industrialization, cultivation of N-fixing crops, and application of N fertilizers. Elevated N deposition can directly alter N cycling in forest ecosystems and is expected to enhance N gas loss from soils along with N leaching (Hall & Matson, 1999; Schlesinger, 2009; Corre et al., 2010). Nitrous oxide ($N_2O$) and dinitrogen gas ($N_2$) are the main forms of gaseous N losses. Elevated $N_2O$ gas loss can deplete stratospheric ozone and contribute to global warming, and so are likely to drive increases in temperature increases and a significant shift in the amount and distribution of precipitation (Aber & Melillo, 1989; Aber et al., 1998; Gundersen et al., 1998; Schlesinger, 2009; Greaver et al., 2016).

The increases in nitrogen deposition in the tropics are projected to be among the highest globally in the coming decades (Galloway et al., 2008; Cusack et al., 2016). Tropical forests play a crucial role in regulating regional and global climate dynamics and may show significant responses to elevated N deposition (Matson et al., 1999; Zhou et al., 2013). To understand the effects of elevated N deposition on tropical forests, several N addition experiments have been performed across the world (Hall & Matson, 1999, 2003; Cusack et al., 2009, 2011; Corre et al., 2010, 2014; Zhu et al., 2015). However, research on gaseous N loss dynamics in response to N addition in

tropical forest is still limited and key questions remain unresolved. Studies on the effects of N addition on N loss from soils have focused on N-oxide ($NO_x$ and $N_2O$) fluxes, especially $N_2O$ (Hall & Matson, 1999, 2003; Koehler et al., 2009; Martinson et al., 2013; Müller et al., 2015). Some studies report that increased N addition significantly enhances $N_2O$ loss (Hall & Matson, 1999, 2003; Silver et al., 2005; Corre et al, 2010, 2014; Martinson et al., 2013; Wang et al., 2014; Chen et al., 2016), yet several others find no effect or even a decreasing trend (Venterea et al., 2003; Morse et al., 2015; Müller et al., 2015). No increase of $N_2O$ emission is speculated to be due to an increase in the capacity of soil $N_2O$ reduction to $N_2$ induced by N addition (Müller et al., 2015), but this remains to be verified. Recently, some reports have suggested that the main contributor of gaseous N emissions is $N_2$ instead of $N_2O$ (Houlton et al., 2006; Bai & Houlton, 2009; Fang et al., 2015); however, to our knowledge, it remains unclear how soil $N_2$ gas loss responds to N deposition in tropical forests. Measuring small fluxes of $N_2$ from soil in natural terrestrial ecosystems is very difficult due to the large pool of background atmospheric $N_2$ (nearly 78%).

Gaseous N emissions can be produced by many microbial processes, e.g., nitrification, denitrification, co-denitrification, anammox, and dissimilatory nitrate reduction to ammonium (DNRA) (Butterbach-Bahl et al., 2013). The description of microbial nitrification and denitrification as a source of N gas emissions is a simplification because while these two processes account for the majority of soil gaseous N loss (Houlton et al., 2006, Butterbach-Bahl et al., 2013, Fang et al., 2015) others are also important. Notably, co-denitrification (Spott & Stange, 2011) and anammox (Xi et al., 2016) also contribute to soil N gas loss under anaerobic conditions. Co-denitrification produces $N_2O$ and $N_2$ by consuming $NO_2^-$ combined

with other N compounds (Spott & Stange, 2011), and anammox reduces $NO_2^-$ and oxidizes ammonium to $N_2$ (Dalsgaard et al., 2003). Recent studies have shown that co-denitrification and anammox both contribute to $N_2$ emissions in some grassland and temperate forest ecosystems (Selbie et al., 2015; Xi et al., 2016). However, it is still unclear whether these two processes contribute to $N_2$ emission in the tropics. Under increasing N deposition, microbial processes related to soil gaseous N emissions may shift, but the research on how their responses to increased N deposition remains limited.

Nitrogen deposition in China has been increasing and is projected to continue increasing over the coming decades (Liu et al., 2013). The increased N deposition may affect plant growth or net primary production at ecosystem scales, increase soil nutrient availability and alter disturbance regimes, such as increasing N gas emissions (Cusack et al., 2016). To evaluate the effects of elevated N addition on tropical montane forests, in 2010 a long-term N addition experiment was set up in primary and secondary tropical montane rainforests in Jianfengling, Hainan Island, China, a site with low background atmospheric N deposition (Wang et al. 2018 Forest Ecology and Management). After six years of N addition treatments - typically thought to be sufficient time to change the N cycle and microbial community in tropical forests (Cusack et al., 2016) -, we incubated forest soils and measured $N_2O$ and $N_2$ emission rates using the acetylene inhibition technique (AIT) and the $^{15}N$ labeling method (Yang et al., 2012, 2014; Sgouridis et al., 2016; Xi et al., 2016).

The aims of this study were: 1) to determine $N_2O$ and $N_2$ emission rates and their response to elevated N in the two study forests; 2) to quantify the contributions of individual microbial processes to $N_2O$ and $N_2$ emissions, and their responses to elevated soil N; and 3) to examine if the abundance of microbial genes associated

with denitrification changed after long-term N addition. We hypothesized that long-term N addition would enhance soil $N_2O$ and $N_2$ emissions due to increased N availability. Since long-term N deposition would decrease soil pH in tropical ecosystems (Lu et al., 2014), we expected that, in the Jianfengling forests, the 6-year N addition would lead to soil acidification, which in turn would increase the proportion of $N_2O$ in gaseous N losses because reduced pH inhibits $N_2O$ reductase (Simek & Cooper, 2002; Cheng et al., 2015). We also expected that long-term N addition would change microbial processes of $N_2O$ and $N_2$ production, as well as their associated gene abundance.

## 2. Materials and methods

### 2.1 Site description and long-term experimental design

This study was conducted in Jianfengling (JFL) National Natural Reserve (18°23'–18°50' N, 108°36'–109°05' E), in southwest Hainan Island, China. JFL National Reserve has an area of 470 $km^2$, 150 $km^2$ of which is covered by montane rainforests (Chen et al., 2010). The natural distribution of montane rainforests is from 800 to 1000 m above sea level. The study site has a marked seasonal shift between wet (May–October) and dry (November–April) seasons, with an average annual precipitation of 2449 mm (approximately 80–90% falls during the wet season) and a mean annual temperature of 19.8°C (Chen et al., 2010). The ambient wet deposition is 6.1 kg N $ha^{-1}$ $yr^{-1}$ (Wang et al., 2014, 2018). Soil is predominantly lateritic yellow (Zhou et al., 2017), with a bulk density of 1.1 $g/cm^3$. There are two main forest types: primary forest and secondary forest. The primary forest is dominated by long-lived tree species such as Castanopsis patelliformis, Lithocarpus fenzelianus, and Livistona saribus, while the secondary forest consists of naturally regenerated taxa such as

Castanopsis fissa, Sapium discolor, C. tonkinesis, Syzygium tephrodes, and Schefflera octophylla (Xu et al., 2009; Zhou et al., 2017). The topography in each forest type is relatively homogeneous, with slopes ranging from 0° to 5° and from 10° to 15° for primary forest and secondary forest, respectively (Zhou, 2013).

In September 2010, to simulate the effects of atmospheric N deposition on the ecosystem N cycle, two N addition experiments were established as a randomized block with four treatment levels (three N addition levels and one control) and three replicates for each treatment in two adjacent primary and secondary forest blocks. The blocks were more than 100 m from each other and within each, four 20 m × 20 m plots were established, each surrounded by a 10-m wide buffer strip. Four treatments, low N addition (25 kg N $ha^{-1}$ $yr^{-1}$), medium N addition (50 kg N $ha^{-1}$ $yr^{-1}$), high N addition (100 kg N $ha^{-1}$ $yr^{-1}$), and control (no N addition), were assigned randomly to the four plots within each block. The added N was in the form of $NH_4NO_3$. Since September 2010, for each N application, a designated amount of $NH_4NO_3$ was dissolved in 100 L groundwater and applied monthly to corresponding plots using a sprayer near the soil surface. The same amount of groundwater (100 L) was applied to each control plot. More information about N fertilization at the site can be found in Du et al (2014).

## 2.2 Soil sampling

To analyze the seasonal dynamics of N gaseous emissions, soil was sampled in the wet season (June 30$^{th}$, 2016), early dry season (November 30$^{th}$, 2015) and late dry season (March 8$^{th}$, 2016). Before sampling, each plot was divided into two 10 m × 20 m subplots. Soil samples were collected at least one week after the most recent fertilization in subplots from six randomly chosen soil cores (10 cm depth of mineral

soil, 5 cm core inner diameter). In total, 48 soil samples (2 subplots × 4 treatments × 3 replicates × 2 forest types) were collected from both primary and secondary forests in each season. Soil samples were stored in a sterile plastic bag, sealed, and covered with ice. In the laboratory, after roots, litter, worms, and other visible items were removed, the samples were passed through a 2-mm sieve. Soils collected in the late dry season and wet season were stored at 4°C and analyzed within a week, and those from the early dry season were stored at −20°C before analysis due to the instruments being unavailable. Before analysis, each sample was divided into two sub-samples, one of which was used for soil physico-chemical analysis and the other for soil incubation.

2.3 Analysis of soil physical and chemical properties

Soil ammonium ($NH_4^+$) and nitrate ($NO_3^-$) concentrations and extractable dissolved organic carbon (DOC) were determined using fresh soils. Before soil isotope labeling incubation, fresh sieved soils from each sample were extracted with 2 M KCl (soil: extract = 1:4 on a weight basis). Ammonium ($NH_4^+$) and nitrate ($NO_3^-$) concentrations in the extracts were measured colorimetrically using an auto discrete analyzer (Smartchem 200). Soil DOC concentration was measured on an OI Analytical Model 700 TOC analyzer (Sanderman & Amundson, 2009). Soil pH was determined in a 1:2.5 mixture of soil:deionized water with a pH meter equipped with a glass electrode. Total carbon (TC) and total nitrogen (TN) concentrations were determined by a vario micro elemental analyzer (Elementar Analysen Systeme, GmbH, Germany). The soil gravimetric water content (GWC) was calculated by weight loss after oven drying for 24 h at 105°C.

2.4 Aerobic incubation

Soils collected in the late dry season and wet season were delivered to the Stable Isotope Ecology Laboratory in the Institute of Applied Ecology, CAS. Then, approximately 8 g fresh soil from each sample was placed into 20-mL glass vials (Chromacol, 125 × 20-CV-P210). Vials were sealed tightly with gray butyl septa (Chromacol, 20-B3P, No.1132012634) and aluminum crimp seals (ANPEL Scientific Instrument (Shanghai) Co. Ltd., 6G390150). To set up water-saturated conditions, we established a watered treatment with 2 ml water addition. Thus, each soil sample was subjected to one of four treatments: no water and no $C_2H_2$ addition (0 mL water + 0% $C_2H_2$ in the headspace); no water but 20% $C_2H_2$ addition (0 mL water + 20% $C_2H_2$ v/v); 2 mL water and no $C_2H_2$ addition (2 mL water + 0% $C_2H_2$ v/v); and 2 mL water and 20% $C_2H_2$ addition (2 mL water + 20% $C_2H_2$ v/v). We used $C_2H_2$ to inhibit $N_2O$ reductase; therefore, the gases from the sample with $C_2H_2$ treatment indicated the total production of $N_2$ and $N_2O$. The vials were shaken gently to ensure that the bulk density of the soil in vials, which was confirmed by calculating the volumes of 8 soil samples in each vial, was similar to that in the field, followed by incubation in the dark at 21°C for 24 hours (Xi et al., 2016). Incubation was terminated by injecting 0.5 mL of 7 M $ZnCl_2$ solution; then, 2 mL sterile deionized water was added to the vials with no water addition. Finally, the headspace gas of each vial was sampled for $N_2O$ and $CO_2$ concentration analysis (see below).

## 2.5 Anaerobic incubation

For soil samples collected in the early dry season and wet season, we conducted anaerobic slurry incubation experiments to measure the emission rates of $N_2O$ and $N_2$. Four specimens of approximately 8 g of fresh soil were taken from each sample and placed into 20-mL glass vials; then, 2 mL $N_2$-purged sterile deionized water was

added to the vials to generate slurries. Vials were immediately sealed tightly with gray butyl septa (same above) and aluminum crimp seals. All vials were vacuumed and flushed with ultrahigh purity $N_2$ (100 mL $min^{-1}$) for 3 minutes. Then, vials were shaken gently and slurries were incubated in the dark at 21°C for 60 h to minimize background $NO_3^-$ concentrations (Xi et al., 2016).

After pre-incubation, each vial was again vacuumed and flushed with ultrahigh purity $N_2$. Then, each vial of every soil sample underwent one of the following four treatments: analysis of $NO_3^-$ concentration after pre-incubation; isotope labeling incubation with $K^{15}NO_3$ addition; $K^{14}NO_3$ addition without $C_2H_2$; and $K^{14}NO_3$ with 20% $C_2H_2$ addition. An ultrahigh purity $N_2$-purged stock solution (0.5 mL) of $^{15}N$-labeled ($K^{15}NO_3$, 99.19 atom%) or un-labeled $KNO_3$ was injected to achieve final concentrations of 10 μg $^{15}N$ $g^{-1}$ fresh soil and 10 μg $^{14}N$ $g^{-1}$ fresh soil (as $KNO_3$) for the $^{15}N$ labeling (Yang et al., 2014) and $C_2H_2$ inhibition treatments respectively. For the treatment of $K^{14}NO_3$ with 20% $C_2H_2$ addition, 20% highly purified $N_2$ was replaced with $C_2H_2$ in each vial. Then, all vials were shaken gently to homogenize the solution. Slurries were incubated in the dark at 21°C for 24 h. Incubation was terminated by injecting 0.5 mL of 7 M $ZnCl_2$ solution, and the headspace gas of each vial was sampled for analyzing the isotopes of $N_2O$ and $N_2$ and the concentrations of $N_2O$ and $CO_2$ (see below).

### 2.6 $N_2O$ production measurement

After incubation, for $^{15}N$ labeling experiments, 0.5-ml gas samples were taken with gas-tight syringes to analyze the $^{15}N$ abundance of $N_2$. After that, 20 ml of high purity $N_2$ was injected into the vials, and mixed gas samples (20 ml) were taken from the headspace with gas-tight syringes and transferred to exetainers (Labco, UK) that

were evacuated before use. Then, the mixed gases were used to determine $N_2O$ and $CO_2$ concentrations using a gas chromatograph (GC-2014, Shimadzu, Japan). $CO_2$ production rates were similar in $C_2H_2$-amended and un-amended vials (data not provided), indicating that soil respiration (microbial respiration) was not affected by 20% $C_2H_2$ amendment.

Concentrations of $^{15}N$ in $N_2O$ were measured by a trace-gas preconcentrator (TG) coupled with a continuous flow isotope ratio mass spectrometer (IRMS; Isoprime 100 Isoprime Ltd, UK). The m/z 44, 45, and 46 beams enabled calculation of molecular ratios of $^{45}R$ ($^{45}N_2O/^{44}N_2O$) and $^{46}R$ ($^{46}N_2O/^{44}N_2O$) for $N_2O$. As we added relatively large quantities of $^{15}N$-$NO_3^-$ (10 ug $^{15}N$ $g^{-1}$ soil) and pre-incubated soils for 60 h to consume the original $NO_3^-$, the $^{15}N$ enrichment of the source pool was high (typically $\geq$ 0.9), leading to non-random $^{15}N$ distribution in $N_2O$. Hence, both m/z 45 and 46 were used to determine $^{15}N$ enrichment of $N_2O$ using the following equation (1) (Stevens et al., 1993; Stevens et al., 1997).

$$\text{Atom\% } ^{15}\text{N-N}_2\text{O} = 100(^{45}R+2 \times {}^{46}R - {}^{17}R - 2 \times {}^{18}R)/(2+2 \times {}^{45}R+2 \times {}^{46}R) \quad (1)$$

where $^{45}R$ = 45/44 and $^{46}R$ = 46/44 ratios reported by IRMS. $^{17}R = 3.8861 \times 10^{-4}$ and $^{18}R = 2.0947 \times 10^{-3}$ (Kaiser et al., 2003).

Then, the mole fractions of $^{45}N_2O$ ($f^{45}$) and $^{46}N_2O$ ($f^{46}$) in sample $N_2O$ were calculated using the following equation (2):

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Production rates of $^{45}N_2O$ ($P_{45}$) and $^{46}N_2O$ ($P_{46}$) in the vials over the incubation period were calculated using the molecular fractions of $f^{45}$ and $f^{46}$ using equation (3):

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where $F_{N2O}$ is the $N_2O$ production within each vial according to the measured change in $N_2O$ concentration during incubation, t and 0 are the incubation time and time zero, respectively, and $M_{soil}$ is the dry soil mass in the incubation vials (g).

During anaerobic incubation, there are three pathways of $N_2O$ production: denitrification ($D_{N2O}$), co-denitrification ($C_{N2O}$), and heterotrophic nitrification ($H_{N2O}$). We assumed that there was no autotrophic nitrification, because incubation was strictly anaerobic and no oxygen was available for ammonium oxidation. According to the $^{15}N$ pairing principle (Thamdrup & Dalsgaard, 2002), denitrification produces $^{44}N_2O$ ($D_{44}$), $^{45}N_2O$ ($D_{45}$), and $^{46}N_2O$ ($D_{46}$); co-denitrification produces $^{44}N_2O$ ($C_{44}$) and $^{45}N_2O$ ($C_{45}$); and heterotrophic nitrification produces only $^{44}N_2O$ ($H_{44}$). We assumed that: (1) in natural soil, the $^{15}N$ abundance is 0 at%; (2) the additional $^{15}N$ source is homogeneously distributed within the study area and does not have a negative effect on microbial processes; (3) all $^{15}N_2O$ comes from $^{15}NO_3^-$ added during the experiment; and (4) contributions of $^{14}N^{14}N^{17}O$ and $^{14}N^{14}N^{18}O$ to $^{45}N_2O$ and $^{46}N_2O$ are minor and negligible. Then, the following hold:

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Thus, equations (4)–(8) allow calculation of $N_2O$ production through heterotrophic

nitrification, co-denitrification, and denitrification pathways.

2.7 $N_2$ production measurement

For $N_2$, according to $^{29}R$ ($^{29}N_2/^{28}N_2$) and $^{30}R$ ($^{30}N_2/^{28}N_2$) ratios measured by IRMS, the molar fractions of $^{29}N_2$ and $^{30}N_2$ are calculated using equation 9 (Yang et al., 2014):

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Assuming that vial headspace $N_2$ concentration did not change during the 24-h incubation, the mass of $N_2$ ($M_{total}$) in the vial headspace is calculated using equation 10 (Yang et al., 2014):

**Error! Reference source not found.** (10)

Production rates of $^{29}N_2$ ($P_{29}$) and $^{30}N_2$ ($P_{30}$) in the vials can be calculated using the following equations (Xi et al., 2016):

**Error! Reference source not found.** (11)

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In the $^{15}NO_3^-$ anaerobic incubation experiment, $^{30}N_2$ is only produced by denitrification, and $^{29}N_2$ and $^{28}N_2$ are from denitrification, anammox, and co-denitrification contributions. We separate $N_2$ production rates from denitrification and from anammox plus co-denitrification. More detailed calculations are provided in Xi et al., 2016.

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where $D_{30}$ and $D_{29}$ are the productions of $N_2$ through denitrification as $^{30}N_2$ and $^{29}N_2$,

respectively, and $F_n$ is the fraction of $^{15}N$ in $NO_3^-$. The rate of $N_2$ contributed by anammox plus co-denitrification can be calculated by equation (13):

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and the total $N_2$ emission rate ($N_{2\text{-total}}$) can be calculated by equation (14):

$$N_{2\text{-total}} = D_{total} + AC_{total} \quad (14)$$

2.8 Quantification of gene abundance

The abundance of reductase genes is an essential microbial factor that regulates N gas emissions during denitrification (Cavigelli & Robertson, 2000). The nir (Nitrite Reductase encoding) genes (nirS and nirK) and nosZ gene are of particular interest because they mark the crucial first and last gas-formation and transformation steps in the process. The nir genes regulate the transformation of nitrite ($NO_2^-$) to N-gas emissions from soil (Lennon & Houlton, 2016), while the nosZ gene regulates how $N_2O$ is reduced to $N_2$ (Liu et al., 2013). The responses of denitrifying genes to N addition may directly help us understand gaseous N emission rate dynamics during denitrification. Thus, soils sampled in the wet season (June 30$^{th}$, 2016) were used to quantify the abundance of functional genes involved in denitrification, including nitrite reductase (nirK and nirS), and nitrous oxide reductase (nosZ) genes. For quantification of target genes, standards of known amounts of template DNA gene copies were created. A gene fragment cloned from a soil sample using the TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA) was selected to create the standard curve. Duplicate standard curves were obtained using tenfold serial dilutions (from $10^7$ to $10^1$ copies) of recombinant plasmids containing cloned nosZ, nirK, and nirS. Reactions were performed in a Mastercycler ep realplex (Eppendorf, Germany) in

triplicate, based on the fluorescence intensity of SYBR green dye.

2.9 Statistical analysis

Statistical analyses were performed using SPSS (Version 19.0; SPSS Inc., Chicago, IL, U.S.A). One-way ANOVA with least squares distance (LSD), using an $\alpha$ of 0.05, was conducted to determine the differences in all variables among N treatments for each forest.

## 3. Results

3.1 Effects of N addition on soil properties

After 6 years of N addition, the soil DOC content, total C, total N, C/N ratio, and $NH_4^+$ concentration did not differ significantly among the four treatments in either the primary or secondary forest (Table 1). The soil DOC content ranged from 0.2 to 1.3 g $kg^{-1}$ dry soil. Soil total N and total C varied from 0.15 to 0.22% and from 1.92 to 2.80%, respectively. The ratio of C/N ranged from 11.6 to 13.5. The $NH_4^+$ concentration ranged between 0.3 and 4.3 mg of N $kg^{-1}$ dry soil, except for soils sampled in the early dry season, which had especially high concentrations, varying from 31.0 to 44.1 mg of N $kg^{-1}$ dry soil. The $NO_3^-$ concentration was between 1.0 and 19.1 mg of N $kg^{-1}$ dry soil, depending on the sampling season, and increased with N addition (Table 1). Soil pH was 0.1 to 0.2 pH units lower in some N-addition treatments compared to the control for some sampling seasons and showed a decreasing trend with increasing N additions (Table 1).

3.2 Nitrogen gas loss under aerobic conditions

Soil $N_2O$ and $N_2$ emissions did not vary significantly with N addition, whether

for dry season or wet season, for the primary or secondary forest, or for soils with and without water addition (Fig. 1 a,b,d,e; Table 1, 2). We also found no significant change in the ratio of $N_2O/(N_2O+N_2)$. However, water addition itself increased soil $N_2O$ and $N_2$ emission rates very strongly - by 47 to 1400 times, and 46 to 816 times, respectively (Fig. 1).

3.3 Nitrogen gas loss under anaerobic conditions

In the primary forest, soil $N_2O$ emission determined by both the AIT and the $^{15}N$ labeling method showed no evident change with increasing N addition in both seasons ($P < 0.05$) (Fig. 2 a). The emission rates of $N_2O$ ranged from 0.8 to 4.0 nmol N $g^{-1}$ dry soil $h^{-1}$ and from 0.5 to 2.8 nmol N $g^{-1}$ dry soil $h^{-1}$ for the two measurement methods, respectively. The change in $N_2$ emission with elevated N addition was similar to that for $N_2O$ (Fig. 2 b), except that it showed a decreasing trend with increasing N addition in the dry season when measured by the $^{15}N$ labeling method ($P < 0.05$) (Fig. 2 b). Soil $N_2$ emission rates determined by the AIT (ranged from 5.1 to 5.9 nmol N $g^{-1}$ dry soil $h^{-1}$) were significantly lower than those measured by the $^{15}N$ labeling method (ranged from 8.0 to 19.9 nmol N $g^{-1}$ dry soil $h^{-1}$) ($P < 0.05$). The ratio of $N_2O/(N_2O+N_2)$ did not change markedly after N addition, with values ranging from 0.12 to 0.44 and from 0.04 to 0.27 when determined by AIT and $^{15}N$ labeling methods, respectively (Table 3).

In contrast to the primary forest, the secondary forest showed a significant decreasing trend of $N_2O$ emissions but a significant increasing trend of $N_2$ emissions after N addition. This was observed in both seasons with both the AIT and $^{15}N$ labeling methods ($P < 0.05$) (Fig. 2 d, e). As a result, the ratio of $N_2O/(N_2O+N_2)$ exhibited a significant decreasing trend with elevated N addition in both seasons ($P <$

0.05) (Table 3).

### 3.4 Microbial pathways of $N_2O$ and $N_2$ production under anaerobic conditions

In the primary forest, the $N_2O$ produced by denitrification significantly decreased with increasing N addition (Table 4), by up to 65% in the high N addition treatment compared to the control (Table S2). In contrast, $N_2O$ production by co-denitrification and heterotrophic nitrification was insensitive to N addition (Table 4, Table S2). Consequently, the contribution of denitrification to $N_2O$ emission significantly decreased with increasing N addition level ($P < 0.05$), e.g., from higher than 55% in the control to 31% in the high N treatment (Table S2).

In the secondary forest, the $N_2O$ produced by three processes was depressed by N addition (Table 4), and denitrification was more sensitive to N addition compared with the other two processes. For example, in the wet season, rates of $N_2O$ produced by denitrification were 1.77 nmol N $g^{-1}$ dry soil $h^{-1}$ in the control and 0.44 nmol N $g^{-1}$ dry soil $h^{-1}$ in the high N addition treatment, while respective $N_2O$ production rates due to co-denitrification were 0.54 nmol N $g^{-1}$ dry soil $h^{-1}$ and 0.21 nmol N $g^{-1}$ dry soil $h^{-1}$ (Table 4). As a result, this different sensitivity of the three processes to N addition resulted in a decreasing importance of denitrification to $N_2O$ production in response to N addition, while the contributions of co-denitrification and heterotrophic nitrification increased (Table S2).

Denitrification contributed more than 98% of total $N_2$ emissions, and co-denitrification plus anammox produced less than 2% of that among the four N addition treatments (Table S2). The contributions of denitrification and co-denitrification plus anammox to $N_2$ emission did not change with elevated N addition in both seasons or in the primary or secondary forest (P between 0.05 and

0.939) (Table 4).

3.5 Denitrifier gene abundance

The abundance of three denitrification genes in forest soils examined in this study (nirS, nirK, and nosZ) were not altered by increased N addition, with the exception of nosZ in the primary forest soil (Fig. 3).

## 4. Discussion

4.1 Evaluations of the two methods in determining gaseous nitrogen productions

The acetylene inhibition technique (AIT) is a rather simple method to determine $N_2$ losses from incubated soils since acetylene at high concentrations (>10%, v/v) in the headspace of culture vials can inhibit the microbial reduction of $N_2O$ to $N_2$ (Felber et al., 2012). However, this method has some limitations in determining the $N_2$ gas production rate. First, acetylene may not completely block the reduction of $N_2O$ to $N_2$, which could underestimate the $N_2$ emission rate and may affect the result of the response patterns of $N_2$ production to increased N additions (Fig. 1, 2). Second, acetylene inhibits autotrophic nitrification at low concentration (0.1%, v/v) and reduces $NO_3^-$ available for denitrification. This is one of the reasons that the determined $N_2$ emission rates were negligible or negative under aerobic conditions in the present study (Fig. 1 b, e), and this also indicates that $N_2O$ was mainly produced by nitrification under aerobic conditions. In addition, this technique is incapable of separating contributions of microbial processes to $N_2O$ or $N_2$ production. For example, autotrophic nitrification, nitrifier denitrification and coupled nitrification denitrification could not be differentiated from nitrification using the method in the present study.

Compared with the AIT, the $^{15}N$ labeling method holds much promise as a more reliable technique but requires the addition of an $^{15}N$-labeled tracer to understand the roles of microbial processes. However, there are also some drawbacks in determining gaseous N productions via this method, which is based on some assumptions (see 2.6 Section). If any assumption is wrong, for instance, the added substrate is not homogeneously distributed in the soil, the production rates of $N_2O$ and $N_2$ could be underestimated. Although there are some strengths and limitations of the AIT and $^{15}N$ labeling methods in determining N gas emissions, the results of N gas emissions determined by these two methods are broadly accepted (Groffman et al., 2006).

4.2 Comparison with field studies

In situ soil $N_2O$ emission rates were monitored from 2013 to 2014 for the study forests using the static chamber technique. The results show that the mean rates over the monitoring period were 0.04, 0.1, 0.04 and -0.02 mg $N_2O$ $m^{-2}$ $h^{-1}$ for the control, low-N, medium-N and high-N in the primary forest and 0.04, 0.05, -0.7 and -0.3 mg $N_2O$ $m^{-2}$ $h^{-1}$ in the secondary forest, respectively (Peng et al., unpublished data). These results suggest that N addition decreased soil $N_2O$ emission rates. This decrease is consistent with the observation of laboratory incubation for the secondary forest under anaerobic conditions in the present study (Fig. 2), suggesting that increased $N_2O$ reduction to $N_2$ is probably one of mechanisms for reduced soil $N_2O$ emission rates observed in the field. The experimental design in the present study allows us to reveal the mechanism of reduced $N_2O$ emission with increasing N addition level (see below).

4.3 Effects of N addition on soil gaseous N emission rates

We expected that long-term N addition over six years should have enhanced soil $N_2O$ and $N_2$ productions due to increased N availability. However, under aerobic conditions, we did not found any dramatic increase in gaseous N emission in our laboratory incubation, though our results showed a slight increase in the secondary forest with field water moisture content. When soils were incubated with extra water (water-saturated), but with the headspace filled with air, we found no increase in $N_2O$ production in the N addition treatments relative to the control in the secondary forest, although $N_2O$ production rates were substantially increased after water addition (Fig. 1). Under anaerobic conditions, we even observed a significant decrease in $N_2O$ production due to increased $N_2O$ reduction to $N_2$, but only in the secondary forest (see more below), and the effect was more pronounced with an increase in the N addition level (Fig. 2). This result implies that the decreased in situ $N_2O$ emission may be caused by increased $N_2O$ reduction to $N_2$. In the primary forest, we found no increase in $N_2O$ or $N_2$ in all incubation experiments. These results demonstrate that the soil gas N loss response to long-term N addition was dependent on the forest type or succession stage.

The difference in the responses of N gas emissions to N addition may be mainly due to the varying N status among tropical rainforests, but it remains to be further explored. When a forest is N-limited, N addition can supply more substrates for N gas production by increasing N availability within the ecosystem, accelerating N cycle processes, and enhancing the mineralization capacity of soil N additions (Corre et al., 2010; Hall & Matson, 1999). It has been reported that $N_2O$ emission increased markedly after N additions to forests with low nitrogen availability in Panama and Hawai'i (Corre et al., 2010; Hall & Matson, 1999). However, when a forest has high N availability, the excess substrates for N gas production may not be effectively used

(Hall & Matson, 1999). In the primary forest of this study, no significant increase in N gaseous emission could be attributed to any existing N limitation in this forest (Jiang, 2016). Moreover, besides N availability within an ecosystem, surface runoff and/or leaching in soil may also partially affect soil gaseous N emission. Due to the sandy soil texture and steep erosive slopes, tropical montane forests are usually leaky ecosystems (Corre et al., 2010; Chapin et al., 2011), and the added N in the field may rapidly runoff or be leached out from the ecosystems immediately after intensive precipitation events.

### 4.4 Effects of N addition on ratios of $N_2O/(N_2O+N_2)$

Incubated under aerobic conditions, the ratios of $N_2O/(N_2+N_2O)$ in our study ranged from 0.63 to 1 (Table 2), suggesting that $N_2O$ is the main N species emitted from the study forests under such conditions. However, under anaerobic conditions, the ratios decreased to 0.07 to 0.26 (Table 3), indicating that $N_2$ is the most important N species (in terms of quantity) under those conditions. Previous studies, e.g., by Houlton et al (2006) and Fang et al (2015), who used the $^{15}N$ natural abundance isotope method, showed that $N_2$ was a more important N species than $N_2O$ in terms of gaseous N losses for the studied tropical forests.

It has been suggested that N addition acidifies soil and reduces soil pH (Lu et al., 2014, Tian and Niu et al., 2015). As a consequence, N addition is likely to inhibit the reductase of $N_2O$ to $N_2$, leading to an increase in the ratio of $N_2O/(N_2O+N_2)$ with increasing N addition. This has been confirmed in a lowland tropical forest of Panama, where $N_2O$ to $N_2$ reduction and soil pH significantly decreased after about 10 years of N addition (Koehler et al., 2012). However, our results showed that the ratio of $N_2O/(N_2O+N_2)$ did not increase significantly and even decreased after long-term N

addition in the secondary forest soil when incubated anaerobically (Table 3). This may be partly because there was no significant increase in soil acidity (Table 1), but additionally, N addition promoted denitrification and thus accelerated the reduction of $N_2O$ to $N_2$. Our result is consistent with the report of Müller et al. (2015), who also found that long-term N addition in tropical montane rainforests of southern Ecuador might promote the reduction of $N_2O$ to $N_2$, inhibiting soil $N_2O$ emission increases following N addition.

4.5 Contribution of microbial pathways to soil N gas emissions

Soil $N_2O$ emission is regulated by multiple microbial processes, such as autotrophic nitrification, heterotrophic nitrification, co-denitrification, and denitrification. Of these, $N_2O$ was predominantly produced by autotrophic nitrification under aerobic conditions (Fig. 1 a, d). Additionally, microbial processes were also greatly influenced by soil moisture, which affects $N_2O$ emission. In this study, we found that $N_2O$ emission increased significantly following water addition (Fig. 1 a, d). Water addition promoted nitrification (Stark & Firestone, 1995) and nitrifier denitrification (Zhu et al., 2013), which in turn significantly increased $N_2O$ emission. Moreover, water addition also resulted in the reduction of soil air content and enhanced denitrification, which may increase the emission of the denitrification by-product ($N_2O$) (Klemedtsson et al., 1988).

Under anaerobic conditions, our results show that $N_2O$ gas emission was mainly affected by denitrification and was less affected by the co-denitrification and heterotrophic nitrification (Table 4). We cannot explain why these processes responded differently to N addition, but this indicates that the microbes that perform co-denitrification and heterotrophic nitrification are less sensitive to N addition than

565 are the denitrifiers. We also note that there are other processes that can produce $N_2O$,
566 for instance, nitrifier denitrification, coupled nitrification-denitrification, and DNRA.
567 However, in the present study, due to the design of the laboratory incubation, we
568 cannot quantify the contribution of those processes to $N_2O$ emission. The combined
569 $^{15}N$ labeling and $^{18}O$ labeling method will be helpful to solve this issue (Kool et al.,
570 2010; Zhu et al., 2013).

571 Our results suggest that nitrogen addition altered the contribution of microbial
572 processes to $N_2O$ emissions, not only $N_2O$ production rates (Table 4). However, the
573 response magnitude was different between the two forests. In the primary forest, only
574 denitrification was sensitive to N addition, while in the secondary forest, all three
575 processes were sensitive, and denitrification was the most sensitive. At the present
576 time, the understanding of $N_2O$ production by heterotrophic nitrification and
577 co-denitrification is still limited, calling for more research. It is not clear why these
578 two forests responded to N addition differently.

579 The present study is the second one that has partitioned microbial processes to
580 N2 production for forest soils anywhere, to the best of our knowledge, and the first for
581 the tropics. Our work shows that $N_2$ gas emission from the tropical montane
582 rainforests was mainly affected by denitrification and was much less affected by
583 anammox and co-denitrification (from 0% to 0.9%). Indeed, the combined
584 contribution of anammox and co-denitrification observed in these two tropical forests
585 is smaller than that reported by Xi et al. (2016) for a temperate forest in northeastern
586 China. Finally, our results show that the effects of N deposition on gaseous N loss
587 vary even within tropical forests, and, while the mechanisms for these different
588 responses are not yet clear, the microbial processes responsible for the production of
589 N gases are indeed sensitive to N inputs.

590

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784 **Table 1** Soil physical and chemical characteristics (0–10 cm) of different nitrogen addition treatments in primary forest (PF) and secondary

785 forest (SF) soils with samples acquired at different seasonal stages.

| Forest type | Sampling season | N treatment | GWC (%) | pH ($H_2O$) | TC (%) | TN (%) | C/N | $N\text{-}NH_4^+$ (mg $kg^{-1}$) | $N\text{-}NO_3^-$ (mg $kg^{-1}$) | DOC (g/kg) |
|---|---|---|---|---|---|---|---|---|---|---|
| PF | Early dry season† | Control | 26.51±1.76 | 4.50±0.06 | 1.92±0.18 | 0.15±0.01 | 12.8±0.3 | 32.3±2.9 | 7.2±1.5 | 0.3±0.0 |
| | | Low-N | 28.10±2.77 | 4.47±0.04 | 2.13±0.18 | 0.17±0.01 | 12.4±0.2 | 34.0±3.1 | 7.5±2.0 | 0.3±0.1 |
| | | Medium-N | 27.63±3.16 | 4.35±0.06 | 2.16±0.26 | 0.17±0.02 | 13.0±0.4 | 31.0±1.8 | 8.9±2.0 | 0.3±0.1 |
| | | High-N | 28.87±4.97 | 4.35±0.09 | 2.10±0.36 | 0.17±0.03 | 12.9±0.4 | 32.1±4.6 | 10.1±2.6 | 0.3±0.1 |
| | Late dry season | Control | 28.21±3.34 | - | - | - | - | 2.8±0.7 | $8.9±1.5^{a}$ | 0.4±0.1 |
| | | Low-N | 30.60±4.12 | - | - | - | - | 3.4±1.2 | $11.0±3.0^{ab}$ | 0.3±0.1 |
| | | Medium-N | 25.92±2.83 | - | - | - | - | 2.9±0.6 | $12.0±2.5^{ab}$ | 0.3±0.0 |
| | | High-N | 29.47±5.22 | - | - | - | - | 3.4±0.7 | $19.1±5.2^{b}$ | 0.2±0.0 |
| | Wet season | Control | 32.32±1.50 | $4.23±0.06^{ab}$ | 2.12±0.19 | 0.17±0.01 | $12.4±0.3^{ab}$ | 0.4±0.1 | $1.1±0.21^{a}$ | 1.3±0.2 |
| | | Low-N | 33.71±2.94 | $4.29±0.10^{a}$ | 2.14±0.14 | 0.19±0.01 | $11.6±0.2^{a}$ | 0.7±0.2 | $1.3±0.2^{ab}$ | 1.0±0.1 |
| | | Medium-N | 34.04±2.58 | $4.08±0.06^{ab}$ | 2.35±0.14 | 0.19±0.01 | $12.1±0.3^{ab}$ | 0.5±0.2 | $1.5±0.2^{ab}$ | 1.0±0.1 |
| | | High-N | 32.32±1.50 | $4.05±0.07^{b}$ | 2.38±0.25 | 0.19±0.02 | $12.5±0.3^{b}$ | 0.5±0.1 | $1.9±0.3^{b}$ | 1.0±0.1 |
| SF | Early dry season† | Control | 25.82±1.49 | 4.40±0.07 | $2.64±0.16^{ab}$ | $0.20±0.03^{ab}$ | 13.5±0.3 | $35.6±2.9^{ab}$ | $4.9±1.3^{a}$ | 0.9±0.2 |
| | | Low-N | 22.93±0.72 | 4.41±0.03 | $2.25±0.10^{a}$ | $0.17±0.01^{a}$ | 13.2±0.4 | $31.7±1.6^{a}$ | $7.2±0.5^{ab}$ | 1.0±0.3 |
| | | Medium-N | 26.73±2.10 | 4.35±0.03 | $2.55±0.20^{ab}$ | $0.19±0.01^{ab}$ | 13.2±0.4 | $39.8±3.6^{ab}$ | $7.6±1.2^{b}$ | 0.9±0.2 |
| | | High-N | 27.84±2.43 | 4.28±0.08 | $2.77±0.19^{b}$ | $0.21±0.02^{b}$ | 13.5±0.1 | $44.1±5.7^{b}$ | $7.7±0.3^{b}$ | 1.1±0.2 |
| | Late dry | Control | 26.57±1.39 | - | - | - | - | 2.3±0.6 | $9.8±1.0^{a}$ | 0.3±0.0 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| season | Low-N | 24.59±0.63 | - | - | - | - | 2.3±0.8 | 9.2±0.5[a] | 0.3±0.1 |
| | Medium-N | 26.45±1.76 | - | - | - | - | 3.6±0.6 | 11.9±0.8[a] | 0.3±0.0 |
| | High-N | 28.35±2.73 | - | - | - | - | 4.3±0.8 | 16.5±2.0[b] | 0.4±0.1 |
| Wet season | Control | 33.36±1.80 | 3.95±0.06 | 2.30±0.15[a] | 0.19±0.01[ab] | 12.4±0.2 | 0.3±0.1 | 1.0±0.1[a] | 1.2±0.1 |
| | Low-N | 31.08±0.86 | 3.91±0.07 | 2.13±0.10[a] | 0.17±0.01[a] | 12.2±0.2 | 0.8±0.6 | 1.4±0.3[ab] | 1.1±0.1 |
| | Medium-N | 35.26±2.32 | 3.94±0.07 | 2.52±0.20[ab] | 0.20±0.01[ab] | 12.6±0.5 | 0.8±0.2 | 1.3±0.2[ab] | 1.1±0.0 |
| | High-N | 34.69±2.40 | 3.86±0.08 | 2.80±0.17[b] | 0.22±0.01[b] | 13.0±0.2 | 0.8±0.2 | 1.9±0.3[b] | 1.0±0.1 |

GWC = gravimetric water content (water gravity (g)/dry soil mass (g)); TC = total carbon; TN = total nitrogen; C/N = ratio of carbon to nitrogen; DOC = dissolved organic carbon (g $kg^{-1}$).

Data are the mean ± 1 SE. Different letters denote significant differences (ANOVA, $P < 0.05$) between treatments in different forest types sampled at different times. TC, TN, pH, and C/N were not measured in soils collected on March 8th, 2016.

Control: 0 kg N $ha^{-1}$ $year^{-1}$; Low-N: 25 kg N $ha^{-1}$ $year^{-1}$; Medium-N: 50 kg N $ha^{-1}$ $year^{-1}$, and High-N: 100 kg N $ha^{-1}$ $year^{-1}$.

† Soils sampled in the early dry season were stored at −20°C for one month before analysis.

**Table 2** Ratios of $N_2O/(N_2O+N_2)$ measured by the acetylene inhibition technique (AIT) under aerobic conditions for soils with water addition in the primary forest (PF) and secondary forest (SF).

| Forest type | N treatments | Sampling season | |
|---|---|---|---|
| | | Late dry season | Wet season |
| PF | Control | 0.72±0.06 | 0.79±0.04 |
| | Low-N | 0.82±0.13 | 0.72±0.04 |
| | Medium-N | 0.71±0.05 | 0.69±0.06 |
| | High-N | 0.63±0.13 | 0.77±0.05 |
| SF | Control | 0.79±0.05 | 0.63±0.02 |
| | Low-N | 0.71±0.07 | 0.54±0.08 |
| | Medium-N | 0.83±0.06 | 0.54±0.03 |
| | High-N | 0.84±0.07 | 0.65±0.04 |

Control: 0 kg N $ha^{-1}$ $year^{-1}$; Low-N: 25 kg N $ha^{-1}$ $year^{-1}$; Medium-N: 50 kg N $ha^{-1}$ $year^{-1}$ and High-N: 100 kg N $ha^{-1}$ $year^{-1}$. Ratios under low soil water conditions are not provided due to the detection of negative $N_2$ emission rates. Data are the mean ± 1 SE, and no significant difference was found among any N addition levels in both forests using ANOVA.

800 **Table 3** Ratios of $N_2O/(N_2O+N_2)$ measured by the $^{15}N$ labeling method and acetylene
801 inhibition technique (AIT) in soil from the primary forest (PF) and secondary forest
802 (SF) under anaerobic conditions.

| Forest type | N treatments | Early dry season | | Wet season | |
|---|---|---|---|---|---|
| | | $^{15}N$ labeling | AIT | $^{15}N$ labeling | AIT |
| PF | Control | 0.07±0.02 | 0.22±0.05 | 0.26±0.08 | 0.44±0.02 |
| | Low-N | 0.04±0.02 | 0.19±0.07 | 0.27±0.08 | 0.42±0.12 |
| | Medium-N | 0.04±0.02 | 0.12±0.03 | 0.18±0.02 | 0.41±0.02 |
| | High-N | 0.06±0.04 | 0.17±0.08 | 0.16±0.03 | 0.40±0.01 |
| SF | Control | 0.14±0.06[a] | 0.30±0.15[a] | 0.22±0.03[a] | 0.34±0.05[a] |
| | Low-N | 0.03±0.01[b] | 0.02±0.01[b] | 0.10±0.03[a] | 0.36±0.05[a] |
| | Medium-N | 0.002±0.001[b] | 0.009±0.004[b] | 0.11±0.03[ab] | 0.23±0.05[b] |
| | High-N | 0.001±0.001[b] | 0.006±0.002[b] | 0.06±0.02[b] | 0.15±0.03[b] |

803 Control: 0 kg N $ha^{-1}$ $year^{-1}$; Low-N: 25 kg N $ha^{-1}$ $year^{-1}$; Medium-N: 50 kg N $ha^{-1}$
804 $year^{-1}$ and High-N: 100 kg N $ha^{-1}$ $year^{-1}$. Data are the mean ± 1 SE. Different letters
805 denote significant differences (ANOVA, $P < 0.05$) among the four N addition
806 treatments.

807 **Table 4** $N_2O$ emission rates from denitrification, co-denitrification, and heterotrophic nitrification, and $N_2$ emission rates from denitrification and

808 co-denitrification plus anammox under anaerobic conditions in the primary forest (PF) and secondary forest (SF).

| Forest type | Sampling season | N treatments | $N_2O^{\#}$ (n mol N $g^{-1}$ dry soil $h^{-1}$) | | | $N_2^{※}$ (n mol N $g^{-1}$ dry soil $h^{-1}$) | |
|---|---|---|---|---|---|---|---|
| | | | $D_{N2O}$ | $C_{N2O}$ | $H_{N2O}$ | $D_{N2}$ | $CA_{N2}$ |
| PF | Early dry season | Control | 0.71±0.37[a] | 0.54±0.43 | 0.11±0.08 | 19.94±1.79 | 0.00±0.00 |
| | | Low-N | 0.34±0.20[ab] | 0.40±0.20 | 0.06±0.01 | 18.42±1.27 | 0.00±0.00 |
| | | Medium-N | 0.24±0.11[b] | 0.24±0.08 | 0.05±0.01 | 18.33±2.53 | 0.60±0.29 |
| | | High-N | 0.25±0.14[b] | 0.47±0.27 | 0.16±0.10 | 14.34±1.28 | 0.04±0.04 |
| | Wet season | Control | 1.64±0.42[a] | 0.98±0.45 | 0.23±0.07 | 7.88±1.61 | 0.08±0.04 |
| | | Low-N | 1.51±0.35[a] | 0.75±0.29 | 0.41±0.11 | 7.91±1.24 | 0.15±0.02 |
| | | Medium-N | 1.14±0.09[ab] | 0.97±0.13 | 0.25±0.02 | 11.37±1.24 | 0.08±0.04 |
| | | High-N | 0.61±0.15[b] | 1.03±0.29 | 0.36±0.04 | 10.84±1.43 | 0.20±0.07 |
| SF | Early dry season | Control | 0.90±0.35[a] | 1.05±0.45[a] | 0.10±0.02[a] | 19.89±4.64 | 0.04±0.04 |
| | | Low-N | 0.25±0.09[b] | 0.27±0.09[b] | 0.05±0.02[b] | 20.26±1.32 | 0.03±0.03 |
| | | Medium-N | 0.02±0.01[b] | 0.02±0.00[b] | 0.01±0.00[b] | 25.67±2.33 | 0.07±0.04 |
| | | High-N | 0.01±0.01[b] | 0.01±0.00[b] | 0.01±0.00[b] | 26.81±2.07 | 0.04±0.04 |
| | Wet season | Control | 1.77±0.24[a] | 0.54±0.08[a] | 0.81±0.16[a] | 11.46±1.01[a] | 0.07±0.03[a] |
| | | Low-N | 0.69±0.16[b] | 0.42±0.15[ab] | 0.41±0.09[b] | 15.34±1.36[b] | 0.21±0.05[b] |
| | | Medium-N | 0.81±0.18[b] | 0.40±0.10[ab] | 0.64±0.13[ab] | 16.22±1.41[b] | 0.23±0.02[b] |

| | | | | | | |
|---|---|---|---|---|---|---|
| | High-N | 0.44±0.20$^{b}$ | 0.21±0.08$^{b}$ | 0.41±0.12$^{b}$ | 15.48±1.03$^{b}$ | 0.19±0.06$^{ab}$ |

Data are the mean ± 1 SE. Different letters denote significant differences ($P < 0.05$) among the four N addition treatments.

$^{\#}D_{N2O}$, $C_{N2O}$, and $H_{N2O}$ are the $N_2O$ emission rates produced by denitrification, co-denitrification, and heterotrophic nitrification, respectively.

$^{※}D_{N2}$, and $CA_{N2}$ represent contributions of denitrification and co-denitrification plus anammox to $N_2$ emission rates, respectively.

## Legends for figures

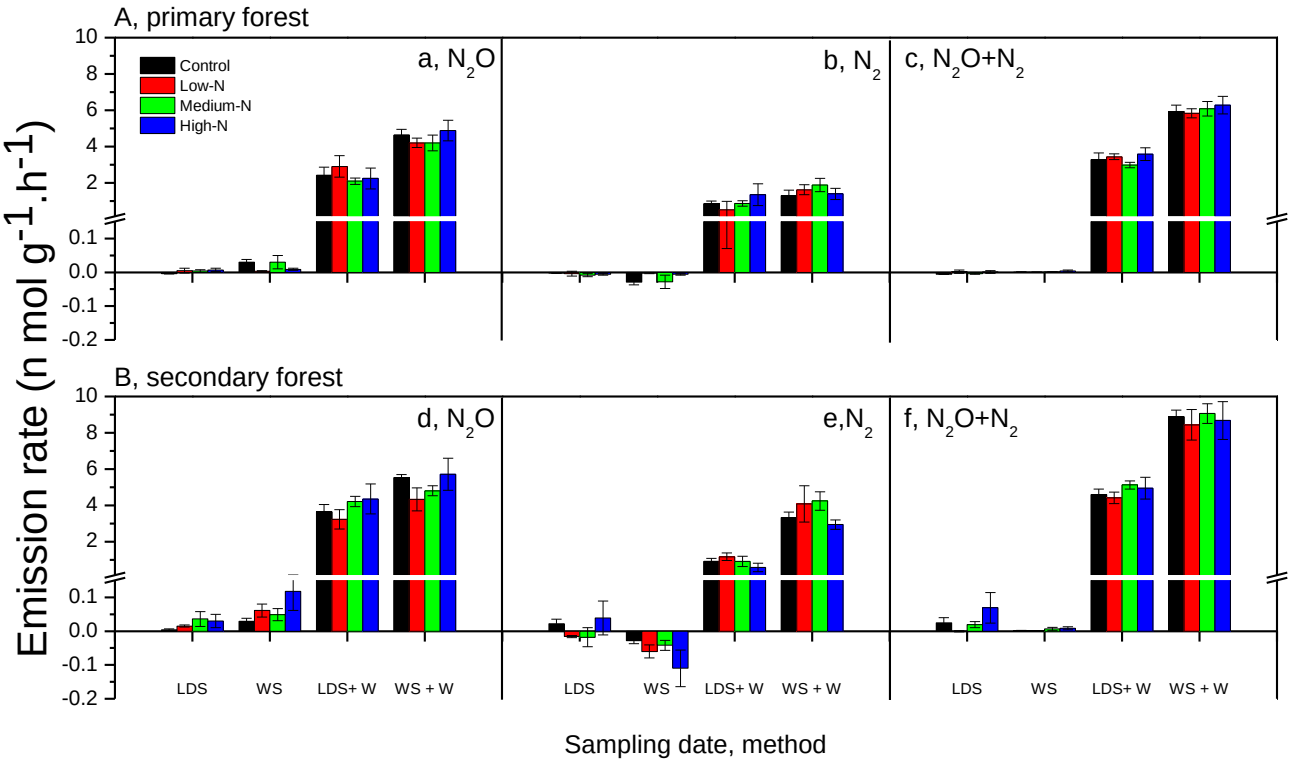
**Fig. 1** Nitrogen emission rates for 0–10 cm deep mineral soil in the primary forest (A) and secondary forest (B) under aerobic incubation conditions. (a) and (d) $N_2O$ (incubated without 20% $C_2H_2$); (b) and (e) $N_2$ ($N_2O$ emission rate amended with 20% $C_2H_2$ minus $N_2O$ without 20% $C_2H_2$); and (c) and (f) total gas ($N_2O$ + $N_2$, incubated with 20% $C_2H_2$). Soils were sampled in the late dry and wet seasons and were incubated for 24 h either with or without the addition of 2 mL of water. Values (±1 SE) are the means of six measurements (3 plots × 2 sample replications) in control, low-N, medium-N, and high-N treatment plots. No significant differences in N gas emissions were found among the control, low-N, medium-N, and high-N treatments for any sampling date or water addition treatment. Abbreviations: LDS=late dry season, WS=wet season, LDS+W= late dry season + water, WS+W= wet season + water.

**Fig. 2** Nitrogen emission rates for the 0–10 cm deep mineral soil in the primary forest (A) and secondary forest (B) determined by AIT and $^{15}N$ labeling methods under anaerobic incubation. (a) and (d) $N_2O$; (b) and (e) $N_2$ (with AIT treatment, $N_2$ emission rates were calculated through $N_2O$ emission rates from soil with 20% $C_2H_2$ treatment minus $N_2O$ emission rates from soils without $C_2H_2$ additions); and (c) and (f) total gas ($N_2O$ + $N_2$). Soils sampled in wet and early dry seasons were amended with 10 μg $^{14}N$ $g^{-1}$ fresh soil for AIT and 10 μg $^{15}N$ $g^{-1}$ fresh soil for the $^{15}N$ labeling method after 60 h pre-incubation under anaerobic conditions. Values are the means (±1 SE) of six measurements (3 plots × 2 sample replications) in the control, low-N, medium-N, and high-N treatment plots. Different letters indicate significant differences in nitrogen gas emissions among the control, low-N, medium-N, and

high-N treatments for each sampling date and method at $P < 0.05$. Abbreviations: EDS=late dry season, WS=wet season, 15N=$^{15}$N labelling.

**Fig. 3** Abundance of microbial nirS, nirK, and nosZ genes in the primary forest (A) and secondary forest (B) soils in the wet season under the control, low-N, medium-N, and high-N addition treatments, expressed as the number of gene copies $g^{-1}$ dry soil. The different letters above the bars indicate significant differences among the four N addition treatments at $P < 0.05$.

844 **Fig. 1**

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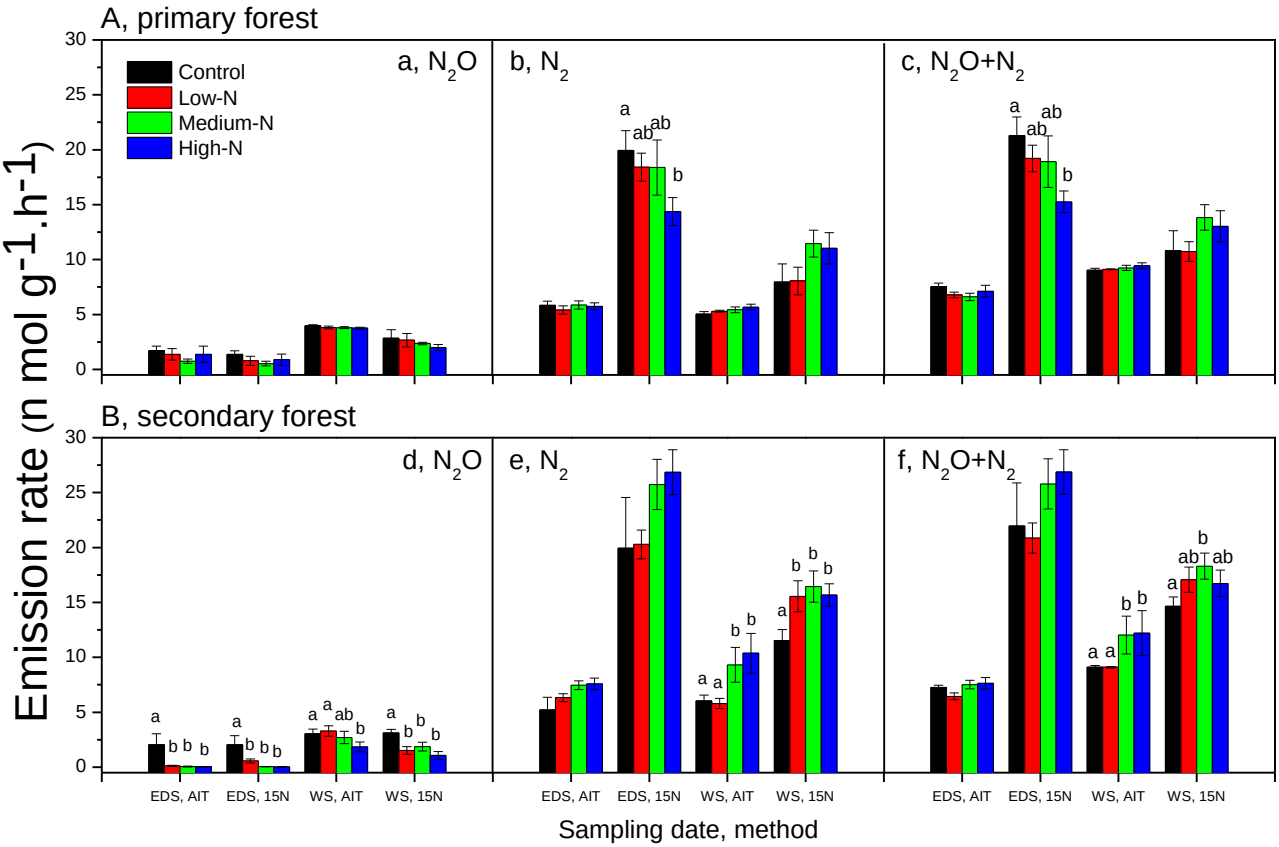
847 **Fig. 2**

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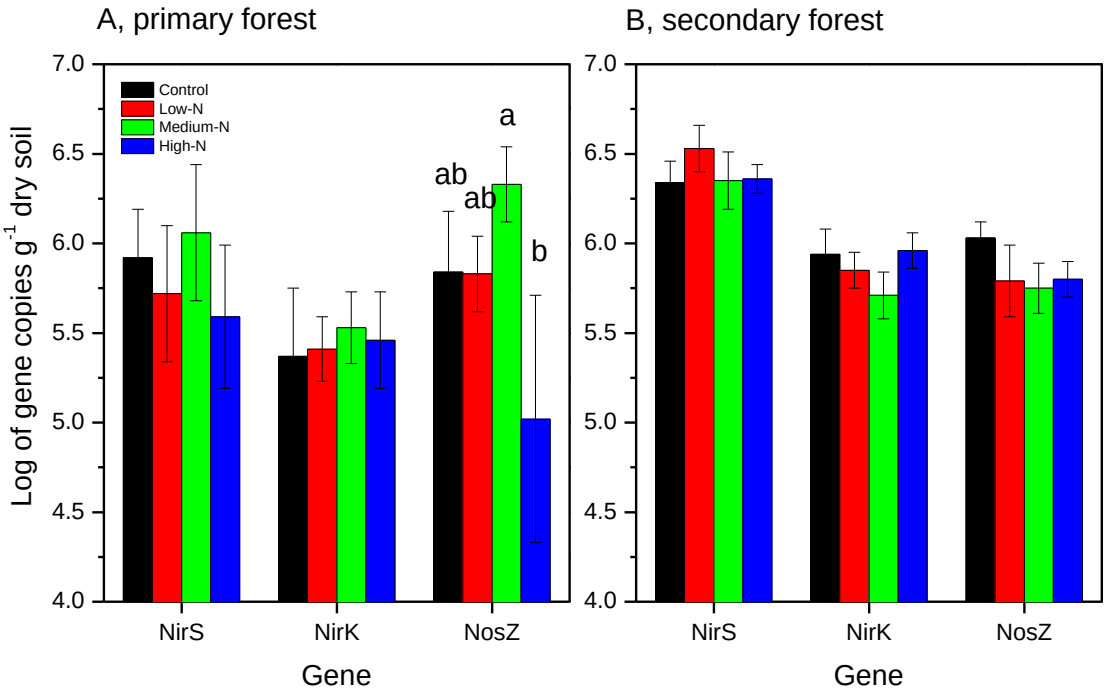
851 **Fig. 3**

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