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Tang, W, Chen, D, Phillips, OL orcid.org/0000-0002-8993-6168 et al. (10 more authors) (2018) Effects of long-term increased N deposition on tropical montane forest soil N_2 and N_2O emissions. Soil Biology and Biochemistry, 126. pp. 194-203. ISSN 0038-0717

https://doi.org/10.1016/j.soilbio.2018.08.027

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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Type of paper: Primary research article

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 30th, 2016), early dry season (November 30th, 2015) and late dry season (March 8th, 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):

Error! Reference source not found. (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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Error! Reference source not found. (8)

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.

Error! Reference source not found., **Error! Reference source not found.**; (12)

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

340 respectively, and F_n is the fraction of ^{15}N in NO_3^-. The rate of N_2 contributed by
341 anammox plus co-denitrification can be calculated by equation (13):
342 **Error! Reference source not found.**, **Error! Reference source not found.**
343 (13),
344 and the total N_2 emission rate ($N_{2\text{-total}}$) can be calculated by equation (14):
345 $$N_{2\text{-total}} = D_{\text{total}} + AC_{\text{total}} \quad (14)$$
346
347 *2.8 Quantification of gene abundance*
348 The abundance of reductase genes is an essential microbial factor that regulates
349 N gas emissions during denitrification (Cavigelli & Robertson, 2000). The *nir* (Nitrite
350 Reductase encoding) genes (*nirS* and *nirK*) and *nosZ* gene are of particular interest
351 because they mark the crucial first and last gas-formation and transformation steps in
352 the process. The *nir* genes regulate the transformation of nitrite (NO_2^-) to N-gas
353 emissions from soil (Lennon & Houlton, 2016), while the *nosZ* gene regulates how
354 N_2O is reduced to N_2 (Liu *et al.*, 2013). The responses of denitrifying genes to N
355 addition may directly help us understand gaseous N emission rate dynamics during
356 denitrification. Thus, soils sampled in the wet season (June 30th, 2016) were used to
357 quantify the abundance of functional genes involved in denitrification, including
358 nitrite reductase (*nirK* and *nirS*), and nitrous oxide reductase (*nosZ*) genes. For
359 quantification of target genes, standards of known amounts of template DNA gene
360 copies were created. A gene fragment cloned from a soil sample using the TOPO TA
361 cloning vector (Invitrogen, Carlsbad, CA, USA) was selected to create the standard
362 curve. Duplicate standard curves were obtained using tenfold serial dilutions (from
363 10^7 to 10^1 copies) of recombinant plasmids containing cloned *nosZ*, *nirK*, and *nirS*.
364 Reactions were performed in a Mastercycler ep realplex (Eppendorf, Germany) in

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

366

367 *2.9 Statistical analysis*

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

372

373 **3. Results**

374 *3.1 Effects of N addition on soil properties*

375 After 6 years of N addition, the soil DOC content, total C, total N, C/N ratio, and
376 NH_4^+ concentration did not differ significantly among the four treatments in either the
377 primary or secondary forest (Table 1). The soil DOC content ranged from 0.2 to 1.3 g
378 kg^{-1} dry soil. Soil total N and total C varied from 0.15 to 0.22% and from 1.92 to
379 2.80%, respectively. The ratio of C/N ranged from 11.6 to 13.5. The NH_4^+
380 concentration ranged between 0.3 and 4.3 mg of N kg^{-1} dry soil, except for soils
381 sampled in the early dry season, which had especially high concentrations, varying
382 from 31.0 to 44.1 mg of N kg^{-1} dry soil. The NO_3^- concentration was between 1.0 and
383 19.1 mg of N kg^{-1} dry soil, depending on the sampling season, and increased with N
384 addition (Table 1). Soil pH was 0.1 to 0.2 pH units lower in some N-addition
385 treatments compared to the control for some sampling seasons and showed a
386 decreasing trend with increasing N additions (Table 1).

387

388 *3.2 Nitrogen gas loss under aerobic conditions*

389 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 (*nir*S, *nir*K, and *nos*Z) were not altered by increased N addition, with the exception of *nos*Z 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

591 **Acknowledgements**

592 This work was financially supported by the National Key Research and Development
593 Program of China (No. 2016YFA0600800), Fundamental Research Funds for the
594 Central Non-profit Research Institution of CAF (Nos. CAFYBB2016ZD002,
595 RITFYWZX201401, RITFYWZX201404), the Strategic Priority Research Program
596 of Chinese Academy of Sciences (No. XDB15020200), the National Natural Science
597 Foundation of China (Nos. 31422009, 41773094, 31400422 and 31370464), and the
598 Key Research Program of Frontier Sciences of Chinese Academic of Sciences (No.
599 QYZDB-SSW-DQC002). OLP acknowledges the support of the ERC Advanced Grant
600 291585 (T-FORCES) and a Royal Society-Wolfson Research Merit Award. The work
601 was also jointly supported by the Jianfengling National Key Field Station. We thank
602 Sarah A. Batterman at University of Leeds and James A. Hogan at Florida
603 International University for their constructive comments on an earlier version of the
604 manuscript. We also thank Ruming Peng and other colleagues in Jianfengling
605 National Field Station for their assistance in field work, and Xiaoming Fang, Ang
606 Wang, Lu Cheng, Xianfeng Wang, Ying Tu, Dongwei Liu, Linlin Song, and Zhengjie
607 Li for their skillful assistance in the laboratory. The authors declare no conflict of
608 interests.

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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

786 GWC = gravimetric water content (water gravity (g)/dry soil mass (g)); TC = total carbon; TN = total nitrogen; C/N = ratio of carbon to nitrogen;

787 DOC = dissolved organic carbon (g kg^{-1}).

788 Data are the mean ± 1 SE. Different letters denote significant differences (ANOVA, $P < 0.05$) between treatments in different forest types

789 sampled at different times. TC, TN, pH, and C/N were not measured in soils collected on March 8th, 2016.

790 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} $year^{-1}$.

791 † 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.

812 **Legends for figures**

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

824

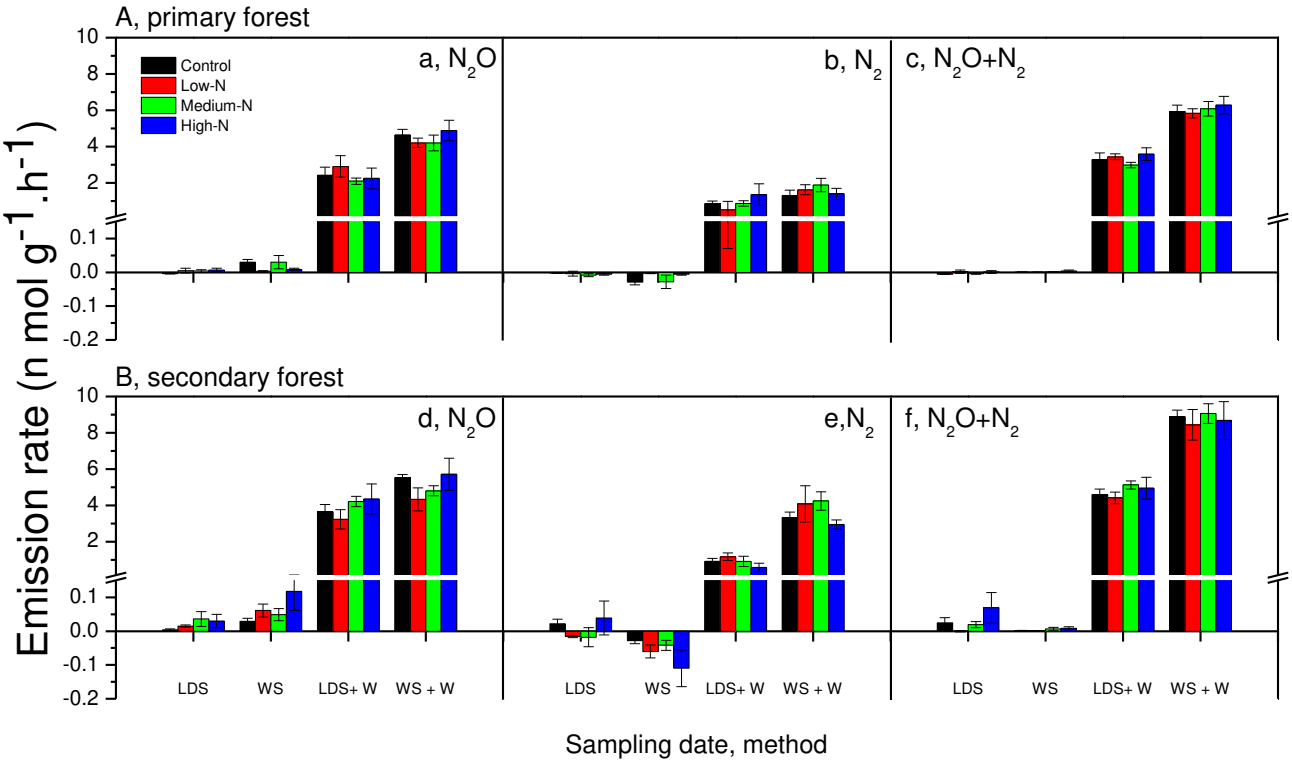
825 **Fig. 2** Nitrogen emission rates for the 0–10 cm deep mineral soil in the primary
826 forest (A) and secondary forest (B) determined by AIT and ^{15}N labeling methods
827 under anaerobic incubation. (a) and (d) N_2O; (b) and (e) N_2 (with AIT treatment, N_2
828 emission rates were calculated through N_2O emission rates from soil with 20% C_2H_2
829 treatment minus N_2O emission rates from soils without C_2H_2 additions); and (c) and (f)
830 total gas (N_2O + N_2). Soils sampled in wet and early dry seasons were amended with
831 10 μg ^{14}N g^{-1} fresh soil for AIT and 10 μg ^{15}N g^{-1} fresh soil for the ^{15}N labeling
832 method after 60 h pre-incubation under anaerobic conditions. Values are the means
833 (±1 SE) of six measurements (3 plots × 2 sample replications) in the control, low-N,
834 medium-N, and high-N treatment plots. Different letters indicate significant
835 differences in nitrogen gas emissions among the control, low-N, medium-N, and

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

838

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

844 **Fig. 1**



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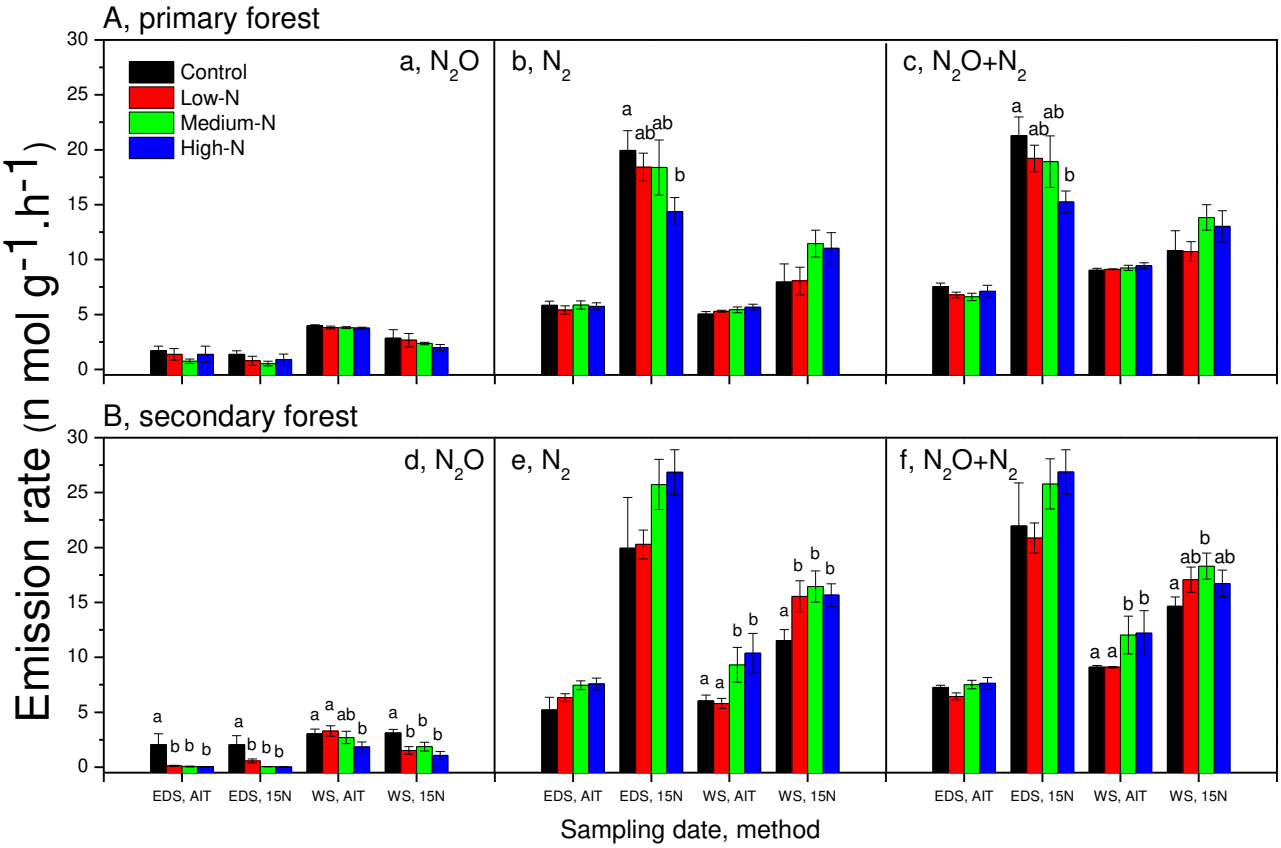
847 **Fig. 2**



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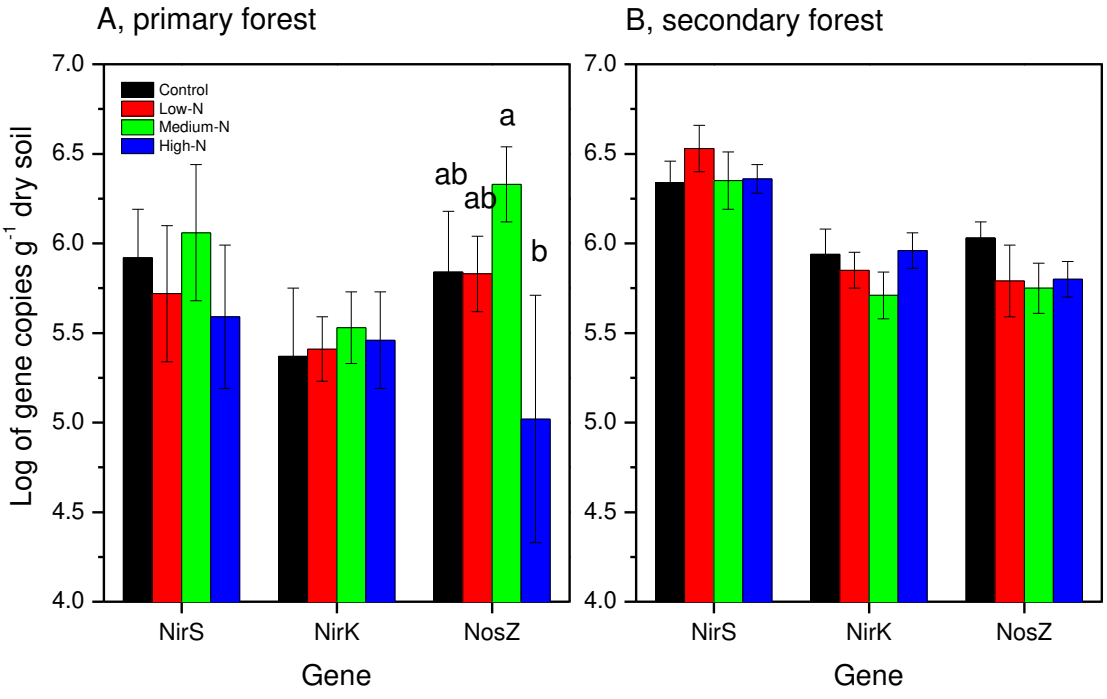
851 **Fig. 3**



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