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

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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. While the mechanisms for these different responses are not yet clear, our results indicate that the effects of N deposition on gaseous N loss were ecosystem-specific in tropical forests and that the microbial processes responsible for the production of N gases are sensitive to N inputs.

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 and 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 and Melillo, 1989; Aber

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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 and 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 and 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 and 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 and 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 and 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 and 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., 2013a,b). 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). 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 and 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., 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 (2013).

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 and 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 h (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 min. 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 pre-concentrator (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 μg ^{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 ≥ 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, 1997).

$$\text{Atom\% } ^{15}N\text{-}N_2O = 100(^{45}R + 2 \times {}^{46}R – {}^{17}R - 2 \times {}^{18}R)/(2 + 2 \times {}^{45}R + 2 \times {}^{46}R) \quad (1)$$

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):

$$f^{45} = \frac{^{45}N_2O}{^{44}N_2O + {}^{45}N_2O + {}^{46}N_2O} = \frac{\frac{^{45}N_2O}{^{44}N_2O}}{1 + \frac{^{45}N_2O}{^{44}N_2O} + \frac{^{46}N_2O}{^{45}N_2O}} = \frac{^{45}R}{1 + {}^{45}R + {}^{46}R}$$

$$f^{46} = \frac{^{46}N_2O}{^{44}N_2O + {}^{45}N_2O + {}^{46}N_2O} = \frac{\frac{^{46}N_2O}{^{44}N_2O}}{1 + \frac{^{45}N_2O}{^{44}N_2O} + \frac{^{46}N_2O}{^{44}N_2O}} = \frac{^{46}R}{1 + {}^{46}R + {}^{46}R} \quad (2)$$

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):

$$P_{45} = \{F_{N2O} \times [((f^{45})_t - (f^{45})_0)]\}/(t \times M_{soil})$$
$$P_{46} = \{F_{N2O} \times [((f^{46})_t - (f^{46})_0)]\}/(t \times M_{soil}) \quad (3)$$

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 and 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:

$$\begin{aligned} D_{N2O} &= D_{44} + D_{45} + D_{46}, \\ D_{46} &= D_{N2O} \times F_n^2, \\ D_{45} &= D_{N2O} \times 2 \times F_n \times (1 - F_n), \\ D_{44} &= D_{N2O} \times (1 - F_n)^2, \end{aligned} \tag{4}$$

$$\begin{aligned} C_{N2O} &= C_{44} + C_{45}, \\ C_{44} &= C_{N2O} \times (1 - F_n), \\ C_{45} &= C_{N2O} \times F_n \end{aligned} \tag{5}$$

$$H_{N2O} = H_{44} \tag{6}$$

$$\begin{aligned} P_{44} &= D_{44} + C_{44} + H_{44}, \\ P_{45} &= D_{45} + C_{45}, \\ P_{46} &= D_{46}. \end{aligned} \tag{7}$$

$$F_{N2O} = D_{N2O} + C_{N2O} + H_{N2O} \tag{8}$$

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):

$$\begin{aligned} f^{29} &= \frac{^{29}N_2}{^{28}N_2 + {}^{29}N_2 + {}^{30}N_2} = \frac{\frac{^{29}N_2}{^{28}N_2}}{1 + \frac{^{29}N_2}{^{28}N_2} + \frac{^{30}N_2}{^{28}N_2}} = \frac{29R}{1 + 29\,R + 30R} \\ f^{30} &= \frac{^{30}N_2}{^{28}N_2 + {}^{29}N_2 + {}^{30}N_2} = \frac{\frac{^{30}N_2}{^{28}N_2}}{1 + \frac{^{29}N_2}{^{28}N_2} + \frac{^{30}N_2}{^{28}N_2}} = \frac{30R}{1 + 29\,R + 30R} \end{aligned} \tag{9}$$

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):

$$M_{\text{total}} = \text{Density of } N_2 \times \text{Volume of headspace} \tag{10}$$

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):

$$\begin{aligned} P_{29} &= \{M_{\text{total}} \times [(f^{29})_t - (f^{29})_0]\}/(t \times M_{\text{soil}}) \\ P_{30} &= \{M_{\text{total}} \times [(f^{30})_t - (f^{30})_0]\}/(t \times M_{\text{soil}}) \end{aligned} \tag{11}$$

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).

$$\begin{aligned} D_{\text{total}} &= D_{30} \times F_n^{-2} \\ D_{30} &= P_{30} \\ D_{29} &= P_{30} \times 2 \times (1 - F_n) \times F_n^{-1} \end{aligned} \tag{12}$$

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):

$$AC_{29} = P_{29} - D_{29},\ AC_{\text{total}} = AC_{29} \times F_n^{-1} \tag{13}$$

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

$$N_{2\text{-total}} = D_{\text{total}} + AC_{\text{total}} \tag{14}$$

2.8. Quantification of gene abundance

The abundance of reductase genes is an essential microbial factor that regulates N gas emissions during denitrification (Cavigelli and 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 and Houlton, 2016), while the *nosZ* gene regulates how N_2O is reduced to N_2 (Liu et al., 2013a,b). 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 30th, 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 α 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 0.1–0.2 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).

Table 1
Soil physical and chemical characteristics (0–10 cm) of different nitrogen addition treatments in primary forest (PF) and secondary 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-NH_4^+ (mg kg^{-1})	N-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 season	Control	26.57 ± 1.39	–	–	–	–	2.3 ± 0.6	9.8 ± 1.0^{a}	0.3 ± 0.0
		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.

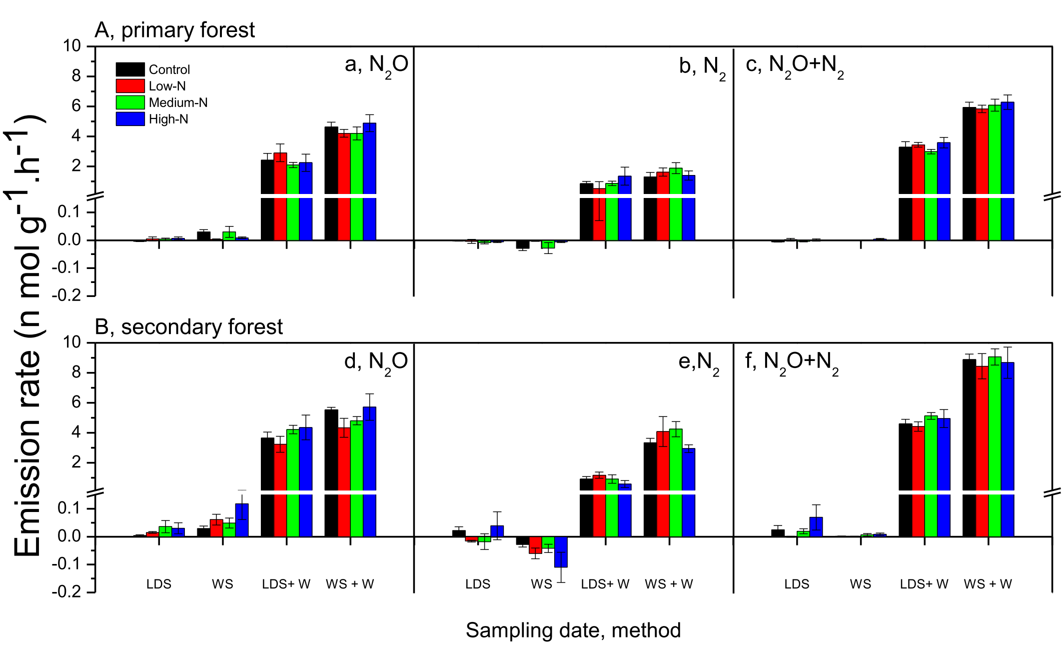

Fig. 1. Nitrogen emission rates for 0–10 cm deep mineral soil in the primary forest (A) and secondary forest (B) under aerobic incubation conditions. (a) and (d) N_2O (incubated without 20% C_2H_2); (b) and (e) N_2 (N_2O emission rate amended with 20% C_2H_2 minus N_2O without 20% C_2H_2); and (c) and (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.

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.

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; Tables 1 and 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 47–1400 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

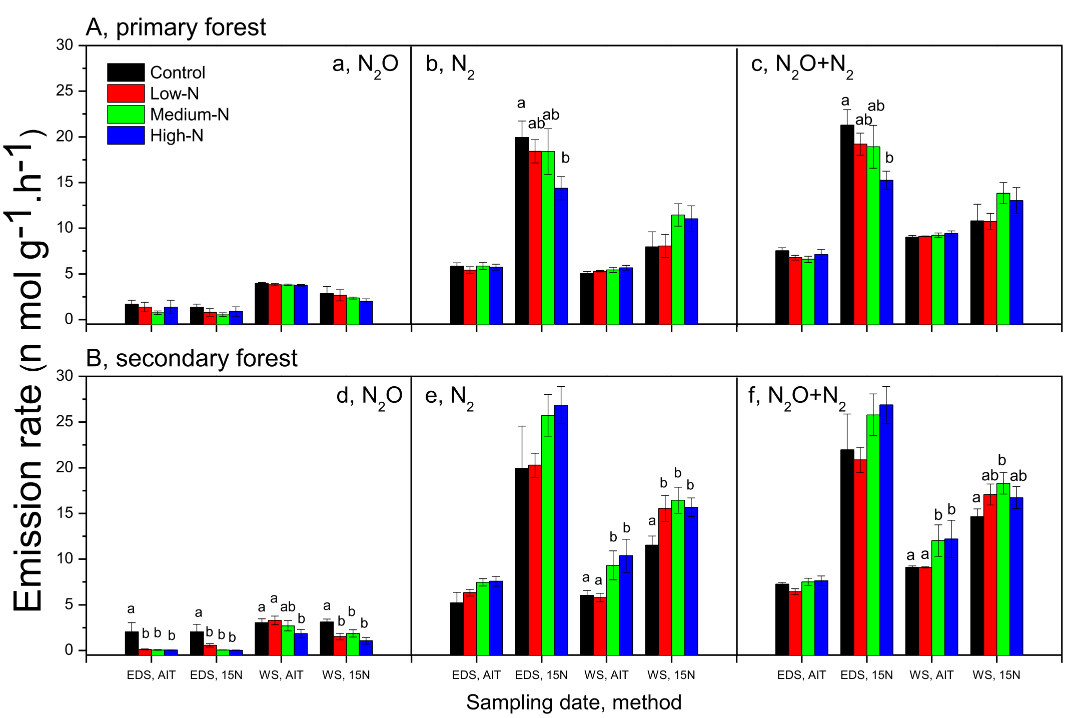

Fig. 2. Nitrogen emission rates for the 0–10 cm deep mineral soil in the primary forest (A) and secondary forest (B) determined by AIT and ^{15}N labeling methods under anaerobic incubation. (a) and (d) N_2O; (b) and (e) N_2 (with AIT treatment, N_2 emission rates were calculated through N_2O emission 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.

Table 3
Ratios of $N_2O/(N_2O + N_2)$ measured by the ^{15}N labeling method and acetylene inhibition technique (AIT) in soil from the primary forest (PF) and secondary forest (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}

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

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 (Figs. 1 and 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,

Table 4
N_2O emission rates from denitrification, co-denitrification, and heterotrophic nitrification, and N_2 emission rates from denitrification and 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.

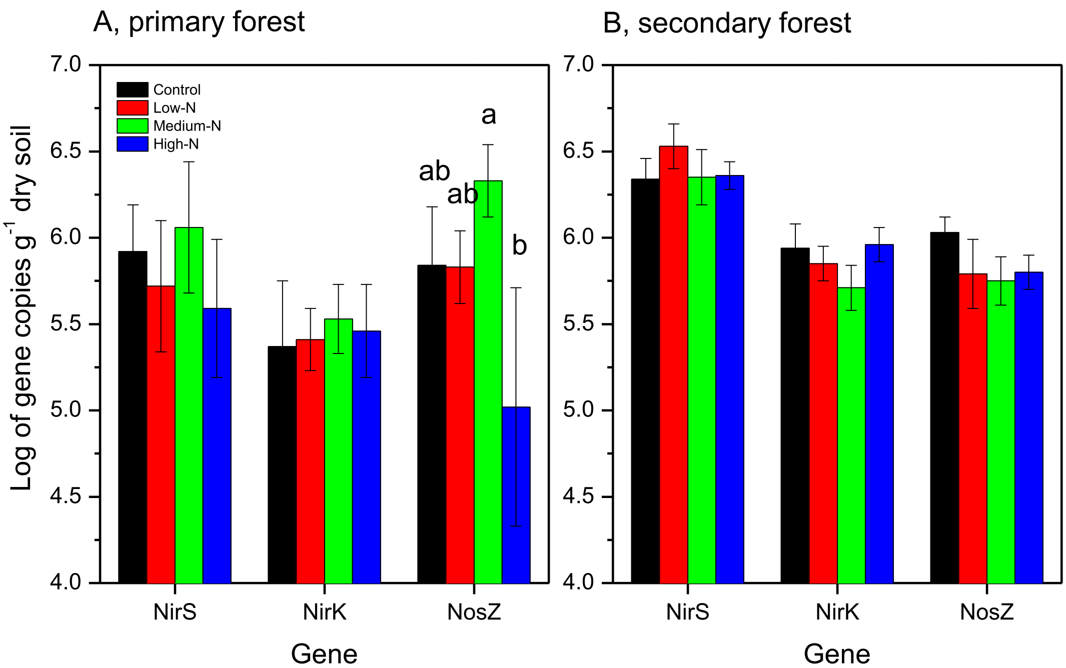

Fig. 3. Abundance of microbial *nirS*, *nirK*, and *nosZ* genes in the primary forest (A) and secondary forest (B) soils in the wet season under the control, low-N, medium-N, and high-N addition treatments, expressed as the number of gene copies g^{-1} dry soil. The different letters above the bars indicate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

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 and 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 and Matson, 1999). However, when a forest has high N availability, the excess substrates for N gas production may not be effectively used (Hall and 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, 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., 2009). 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 substantially following water addition (Fig. 1 a, d). Water addition promoted nitrification (Stark and 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 produced by denitrification and was affected by N addition (Table 4). In contrast, the N_2O production rates of co-denitrification and heterotrophic nitrification are less sensitive to N addition than those produced by denitrificaition (Table 4). We also note that there are other processes that can produce N_2O, for instance, nitrifier denitrification, coupled nitrification-denitrification, and DNRA. However, in the present study, due to the design of the laboratory incubation, we cannot quantify the contribution of those processes to N_2O emission. The combined ^{15}N labeling and ^{18}O labeling method will be helpful to solve this issue (Kool et al., 2010; Zhu et al., 2013).

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

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

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.soilbio.2018.08.027.

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