UNIVERSITY OF LEEDS

This is a repository copy of *Effects of long-term increased N deposition on tropical montane forest soil N₂ and N₂O emissions*.

White Rose Research Online URL for this paper:
<http://eprints.whiterose.ac.uk/137643/>

Version: Accepted Version

Article:

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 N₂ and N₂O emissions. *Soil Biology and Biochemistry*, 126. pp. 194-203. ISSN 0038-0717

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

Crown Copyright © 2018 Published by Elsevier Ltd. All rights reserved. Licensed under the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Reuse

This article is distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs (CC BY-NC-ND) licence. This licence only allows you to download this work and share it with others as long as you credit the authors, but you can't change the article in any way or use it commercially. More information and the full terms of the licence here: <https://creativecommons.org/licenses/>

Takedown

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

eprints@whiterose.ac.uk
<https://eprints.whiterose.ac.uk/>

Effects of long-term increased N deposition on tropical montane forest soil N₂ and N₂O emissions

Running head: Soil N₂ and N₂O emissions from two tropical forests

Authors: Wenguang Tang^{1, 2}, Dexiang Chen^{1*}, Oliver L. Phillips³, Xian Liu⁴, Zhang Zhou¹, Yide Li¹, Dan Xi⁴, Feifei Zhu^{2, 7}, Jingyun Fang⁵, Limei Zhang⁶, Mingxian Lin¹, Jianhui Wu¹, and Yunting Fang^{2, 7*}

Affiliations:

¹Jianfengling National Key Field Observation and Research Station for Forest Ecosystem, Research Institute of Tropical Forestry, Chinese Academy of Forestry, Guangzhou 510520, China

²CAS Key Laboratory of Forest Ecology and Management, Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang, 110016, China

³School of Geography, University of Leeds, Leeds LS2 9JT, UK

⁴College of Forestry, Fujian Agriculture and Forestry University, Fuzhou 350002

⁵Department of Ecology, College of Urban and Environmental Sciences, and Key Laboratory for Earth Surface Processes of the Ministry of Education, Peking University, Beijing, 100871, China

⁶State Key Laboratory of Urban and Regional Ecology, Research Centre for
Eco-environmental Sciences, Chinese Academy of Sciences, Beijing, 100085, China

⁷Qingyuan Forest CERN, the Chinese Academy of Sciences, Shenyang, Liaoning
110014, China

***Corresponding author:**

Dexiang Chen, Research Institute of Tropical Forestry, Chinese Academy of Forestry,
Guangzhou 510520, China. E-mail:

Yunting Fang, Institute of Applied Ecology, Chinese Academy of Science, 72 Wenhua
Road, Shenyang, China 110016. Tel: +86-24-83970302. E-mail:

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, N₂O, and N₂). However, it is unclear how N deposition in tropical forests influences both the magnitude of gaseous loss of nitrogen and its partitioning into the N₂ and N₂O loss mechanisms. Here, for the first time, we employed the acetylene inhibition technique and the ¹⁵N-nitrate labeling method to quantify N₂ and N₂O 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 N₂O or N₂ emissions, or N₂O/N₂. However, under anaerobic conditions, N₂O emissions decreased and N₂ emissions increased with increasing N addition in the secondary forest. These changes may be attributed to substantially greater N₂O reduction to N₂ during denitrification, further supported by the decreased N₂O/N₂ 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 N₂O production. Denitrification was the predominant pathway to N₂ production (98-100%) and its contribution was unaffected by N addition. Despite the changes in the contributions of denitrification to N₂O 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 (N₂O) and dinitrogen gas (N₂) are the main forms of gaseous N losses. Elevated N₂O 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 N₂O and N₂ 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 N₂O in gaseous N losses because reduced pH inhibits N₂O reductase (Simek & Cooper, 2002; Cheng et al., 2015). We also expected that long-term N addition would change microbial processes of N₂O and N₂ 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 km², 150 km² 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/cm³. 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 NH₄NO₃. Since September 2010, for each N application, a designated amount of NH₄NO₃ 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 $\times$ 4 treatments $\times$ 3 replicates $\times$ 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 C₂H₂ addition (0 mL water + 0% C₂H₂ in the headspace); no water but 20% C₂H₂ addition (0 mL water + 20% C₂H₂ v/v); 2 mL water and no C₂H₂ addition (2 mL water + 0% C₂H₂ v/v); and 2 mL water and 20% C₂H₂ addition (2 mL water + 20% C₂H₂ v/v). We used C₂H₂ to inhibit N₂O reductase; therefore, the gases from the sample with C₂H₂ treatment indicated the total production of N₂ and N₂O. 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 ZnCl₂ 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 N₂O and CO₂ 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 N₂O and N₂. Four specimens of approximately 8 g of fresh soil were taken from each sample and placed into 20-mL glass vials; then, 2 mL N₂-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 N₂ (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 NO₃⁻ concentrations (Xi et al., 2016).

After pre-incubation, each vial was again vacuumed and flushed with ultrahigh purity N₂. Then, each vial of every soil sample underwent one of the following four treatments: analysis of NO₃⁻ concentration after pre-incubation; isotope labeling incubation with K¹⁵NO₃ addition; K¹⁴NO₃ addition without C₂H₂; and K¹⁴NO₃ with 20% C₂H₂ addition. An ultrahigh purity N₂-purged stock solution (0.5 mL) of ¹⁵N-labeled (K¹⁵NO₃, 99.19 atom%) or un-labeled KNO₃ was injected to achieve final concentrations of 10 µg ¹⁵N g⁻¹ fresh soil and 10 µg ¹⁴N g⁻¹ fresh soil (as KNO₃) for the ¹⁵N labeling (Yang et al., 2014) and C₂H₂ inhibition treatments respectively. For the treatment of K¹⁴NO₃ with 20% C₂H₂ addition, 20% highly purified N₂ was replaced with C₂H₂ 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 ZnCl₂ solution, and the headspace gas of each vial was sampled for analyzing the isotopes of N₂O and N₂ and the concentrations of N₂O and CO₂ (see below).

2.6 N₂O production measurement

After incubation, for ¹⁵N labeling experiments, 0.5-ml gas samples were taken with gas-tight syringes to analyze the ¹⁵N abundance of N₂. After that, 20 ml of high purity N₂ 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 N₂O and CO₂ concentrations using a gas chromatograph (GC-2014, Shimadzu, Japan). CO₂ production rates were similar in C₂H₂-amended and un-amended vials (data not provided), indicating that soil respiration (microbial respiration) was not affected by 20% C₂H₂ amendment.

Concentrations of ¹⁵N in N₂O 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 ⁴⁵R (⁴⁵N₂O/⁴⁴N₂O) and ⁴⁶R (⁴⁶N₂O/⁴⁴N₂O) for N₂O. As we added relatively large quantities of ¹⁵N-NO₃⁻ (10 ug ¹⁵N g⁻¹ soil) and pre-incubated soils for 60 h to consume the original NO₃⁻, the ¹⁵N enrichment of the source pool was high (typically ≥ 0.9), leading to non-random ¹⁵N distribution in N₂O. Hence, both m/z 45 and 46 were used to determine ¹⁵N enrichment of N₂O using the following equation (1) (Stevens et al., 1993; Stevens et al., 1997).

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

where ⁴⁵R = 45/44 and ⁴⁶R = 46/44 ratios reported by IRMS. ¹⁷R = 3.8861 × 10⁻⁴ and ¹⁸R = 2.0947 × 10⁻³ (Kaiser et al., 2003).

Then, the mole fractions of ⁴⁵N₂O (f⁴⁵) and ⁴⁶N₂O (f⁴⁶) in sample N₂O were calculated using the following equation (2):

$$\text{Error! Reference source not found.} \quad (2)$$

$$\text{Error! Reference source not found.}$$

Production rates of ⁴⁵N₂O (P₄₅) and ⁴⁶N₂O (P₄₆) in the vials over the incubation period were calculated using the molecular fractions of f⁴⁵ and f⁴⁶ using equation (3):

$$\text{Error! Reference source not found.} \quad (3)$$

$$\text{Error! Reference source not found.}$$

where F_{N_2O} 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_{N_2O}), co-denitrification (C_{N_2O}), and heterotrophic nitrification (H_{N_2O}). 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:

Error! Reference source not found., Error! Reference source not found., Error! Reference source not found., Error! Reference source not found. Error! Reference source not found. Error! Reference source not found.

Error! Reference source not found., Error! Reference source not found., Error! Reference source not found. Error! Reference source not found. Error! Reference source not found.

Error! Reference source not found. (6)

Error! Reference source not found., Error! Reference source not found., Error! Reference source not found. (7)

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 N₂ production measurement

For N₂, according to ²⁹R (²⁹N₂/²⁸N₂) and ³⁰R (³⁰N₂/²⁸N₂) ratios measured by IRMS, the molar fractions of ²⁹N₂ and ³⁰N₂ are calculated using equation 9 (Yang et al., 2014):

Error! Reference source not found. (9)

Error! Reference source not found.

Assuming that vial headspace N₂ concentration did not change during the 24-h incubation, the mass of N₂ (M_{total}) in the vial headspace is calculated using equation 10 (Yang et al., 2014):

Error! Reference source not found. (10)

Production rates of ²⁹N₂ (P₂₉) and ³⁰N₂ (P₃₀) in the vials can be calculated using the following equations (Xi et al., 2016):

Error! Reference source not found. (11)

Error! Reference source not found.

In the ¹⁵NO₃⁻ anaerobic incubation experiment, ³⁰N₂ is only produced by denitrification, and ²⁹N₂ and ²⁸N₂ are from denitrification, anammox, and co-denitrification contributions. We separate N₂ 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)

Error! Reference source not found.

where D₃₀ and D₂₉ are the productions of N₂ through denitrification as ³⁰N₂ and ²⁹N₂,

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):

Error! Reference source not found., Error! Reference source not found.

(13),

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

$$\text{N}_{2\text{-total}} = \text{D}_{\text{total}} + \text{AC}_{\text{total}} \quad (14)$$

2.8 Quantification of gene abundance

The abundance of reductase genes is an essential microbial factor that regulates N gas emissions during denitrification (Cavigelli & Robertson, 2000). The nir (Nitrite Reductase encoding) genes (nirS and nirK) and nosZ gene are of particular interest because they mark the crucial first and last gas-formation and transformation steps in the process. The nir genes regulate the transformation of nitrite (NO_2^-) to N-gas emissions from soil (Lennon & Houlton, 2016), while the nosZ gene regulates how N_2O is reduced to N_2 (Liu et al., 2013). The responses of denitrifying genes to N addition may directly help us understand gaseous N emission rate dynamics during denitrification. Thus, soils sampled in the wet season (June 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 to 0.2 pH units lower in some N-addition treatments compared to the control for some sampling seasons and showed a decreasing trend with increasing N additions (Table 1).

3.2 Nitrogen gas loss under aerobic conditions

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

for dry season or wet season, for the primary or secondary forest, or for soils with and without water addition (Fig. 1 a,b,d,e; Table 1, 2). We also found no significant change in the ratio of $\text{N}_2\text{O}/(\text{N}_2\text{O}+\text{N}_2)$. 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 $\text{N}_2\text{O}/(\text{N}_2\text{O}+\text{N}_2)$ 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 $\text{N}_2\text{O}/(\text{N}_2\text{O}+\text{N}_2)$ exhibited a significant decreasing trend with elevated N addition in both seasons ($P <$

0.05) (Table 3).

3.4 Microbial pathways of N₂O and N₂ production under anaerobic conditions

In the primary forest, the N₂O 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, N₂O production by co-denitrification and heterotrophic nitrification was insensitive to N addition (Table 4, Table S2). Consequently, the contribution of denitrification to N₂O 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 N₂O 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 N₂O 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 N₂O 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 N₂O 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 N₂ 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 N₂ emission did not change with elevated N addition in both seasons or in the primary or secondary forest (P between 0.05 and

0.939) (Table 4).

3.5 Denitrifier gene abundance

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

4. Discussion

4.1 Evaluations of the two methods in determining gaseous nitrogen productions

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

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

4.2 Comparison with field studies

In situ soil N_2O emission rates were monitored from 2013 to 2014 for the study forests using the static chamber technique. The results show that the mean rates over the monitoring period were 0.04, 0.1, 0.04 and -0.02 $\text{mg N}_2\text{O m}^{-2} \text{h}^{-1}$ for the control, low-N, medium-N and high-N in the primary forest and 0.04, 0.05, -0.7 and -0.3 $\text{mg N}_2\text{O m}^{-2} \text{h}^{-1}$ 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 N₂O and N₂ productions due to increased N availability. However, under aerobic conditions, we did not find 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 N₂O production in the N addition treatments relative to the control in the secondary forest, although N₂O production rates were substantially increased after water addition (Fig. 1). Under anaerobic conditions, we even observed a significant decrease in N₂O production due to increased N₂O reduction to N₂, 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 N₂O emission may be caused by increased N₂O reduction to N₂. In the primary forest, we found no increase in N₂O or N₂ 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 N₂O 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 $\text{N}_2\text{O}/(\text{N}_2\text{O}+\text{N}_2)$

Incubated under aerobic conditions, the ratios of $\text{N}_2\text{O}/(\text{N}_2+\text{N}_2\text{O})$ 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 $\text{N}_2\text{O}/(\text{N}_2\text{O}+\text{N}_2)$ 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 $\text{N}_2\text{O}/(\text{N}_2\text{O}+\text{N}_2)$ 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

are the denitrifiers. We also note that there are other processes that can produce N₂O, 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 N₂O emission. The combined ¹⁵N labeling and ¹⁸O 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 N₂O emissions, not only N₂O 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 N₂O production by heterotrophic nitrification and co-denitrification is still limited, calling for more research. It is not clear why these two forests responded to N addition differently.

The present study is the second one that has partitioned microbial processes to N₂ production for forest soils anywhere, to the best of our knowledge, and the first for the tropics. Our work shows that N₂ 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.

Acknowledgements

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

References

- Aber J, Mcdowell W, Nadelhoffer K et al. (1998) Nitrogen Saturation in Temperate Forest Ecosystems: Hypotheses revisited. *BioScience*, **48**, 7-10.
- Aber JD, Melillo JM (1989) Nitrogen Saturation in Northern Forest Ecosystems. *BioScience*, **39**, 378-386.

-
- 615 Bai E, Houlton BZ (2009) Coupled isotopic and process-based modeling of gaseous
616 nitrogen losses from tropical rain forests. *Global Biogeochemical Cycles*, **23**,
617 269-277.
- 618 Butterbach-Bahl K, Baggs EM, Dannenmann M, Kiese R, Zechmeister-Boltenstern S
619 (2013) Nitrous oxide emissions from soils: how well do we understand the
620 processes and their controls? *Philosophical Transactions of the Royal Society*
621 *B: Biological Sciences*, **368**, 20130122.
- 622 Cavigelli MA, Robertson GP (2000) The functional significance of denitrifier
623 community composition in a terrestrial ecosystem. *Ecology*, **81**, 1402-1414.
- 624 Chapin FS III, Matson PA, and Vitousek P (2011) *Principles of terrestrial ecosystem*
625 *ecology*, 2nd edn. New York: Springer Science & Business Media.
- 626 Chen DX, Li YD, Liu HP et al. (2010) Biomass and carbon dynamics of a tropical
627 mountain rain forest in China. *Science China Life Sciences*, **53**, 798-810.
- 628 Chen H, Gurmesa GA, Zhang W et al. (2016) Nitrogen saturation in humid tropical
629 forests after 6 years of nitrogen and phosphorus addition: hypothesis testing.
630 *Functional Ecology*, **17**, 59-73.
- 631 Cheng Y, Zhang JB, Wang J, Cai ZC, Wang SQ (2015) Soil pH is a good predictor of
632 the dominating N₂O production processes under aerobic conditions. *Journal of*
633 *Plant Nutrition & Soil Science*, **178**, 370-373.
- 634 Corre MD, Sueta JP, Veldkamp E (2014) Nitrogen-oxide emissions from tropical
635 forest soils exposed to elevated nitrogen input strongly interact with rainfall
636 quantity and seasonality. *Biogeochemistry*, **118**, 103-120.
- 637 Corre MD, Veldkamp E, Arnold J, Wright SJ (2010) Impact of elevated N input on
638 soil N cycling and losses in old-growth lowland and montane forests in
639 Panama. *Ecology*, **91**, 1715-1729.

-
- 640 Cusack DF, Silver W, McDowell WH (2009) Biological Nitrogen Fixation in Two
641 Tropical Forests: Ecosystem-Level Patterns and Effects of Nitrogen
642 Fertilization. *Ecosystems*, **12**, 1299-1315.
- 643 Cusack DF, Silver WL, Torn MS, Burton SD, Firestone MK (2011) Changes in
644 microbial community characteristics and soil organic matter with nitrogen
645 additions in two tropical forests. *Ecology*, **92**, 621.
- 646 Cusack DF, Karpman J, Ashdown D et al. (2016) Global change effects on humid
647 tropical forests: Evidence for biogeochemical and biodiversity shifts at an
648 ecosystem scale. *Reviews of Geophysics*, **54**, 523-610.
- 649 Dalsgaard T, Canfield DE, Petersen J, Thamdrup B, Acuña González J (2003) N₂
650 production by the anammox reaction in the anoxic water column of Golfo
651 Dulce, Costa Rica. *Nature*, **422**, 606.
- 652 Du E, Zhou Z, Li P et al. (2013) NEECF: a project of nutrient enrichment experiments
653 in China's forests. *Journal of Plant Ecology*, **6**, 428-435.
- 654 Fang Y, Koba K, Makabe A et al. (2015) Microbial denitrification dominates nitrate
655 losses from forest ecosystems. *Proceedings of the National Academy of*
656 *Sciences*, **112**, 1470-1474.
- 657 Felber R, Conen F, Flechard CR, Neftel A (2012) Theoretical and practical limitations
658 of the acetylene inhibition technique to determine total denitrification losses.
659 *Biogeosciences*, **9**, 4125-4138.
- 660 Galloway JN, Townsend AR, Erisman JW et al. (2008) Transformation of the nitrogen
661 cycle: recent trends, questions, and potential solutions. *Science*, **320**, 889-892.
- 662 Greaver TL, Clark CM, Compton JE et al. (2016) Key ecological responses to
663 nitrogen are altered by climate change. *Nature Climate Change*, **6**, 836.
- 664 Groffman PM, Altabet MA, Böhlke JK et al. (2006) Methods for measuring

denitrification: Diverse approaches to a difficult problem. *Ecological Applications*, **16**, 2091-2122

Gundersen P, Emmett BA, Kjonaas OJ et al. (1998) Impacts of nitrogen deposition on nitrogen cycling: a synthesis. *Forest Ecology & Management*, **101**, 37-55.

Hall SJ, Matson PA (1999) Nitrogen oxide emissions after nitrogen additions in tropical forests. *Nature*, **400**, 152-155.

Hall SJ, Matson PA (2003) Nutrient status of tropical rain forests influences soil N dynamics after N additions. *Ecological Monographs*, **73**, 107-129.

Houlton BZ, Sigman DM, Hedin LO (2006) Isotopic evidence for large gaseous nitrogen losses from tropical rainforests. *Proceedings of the National Academy of Sciences*, **103**, 8745-8750.

Jiang L (2016) Effects of nitrogen and phosphorus fertilization on carbon cycling of tropical mountain rainforests in Hainan Island, China. Ph.D. Thesis, Peking University, Beijing.

Kaiser J, Röckmann T, Brenninkmeijer CaM (2003) Complete and accurate mass spectrometric isotope analysis of tropospheric nitrous oxide. *Journal of Geophysical Research Atmospheres*, **108**, 1191-1198.

Klemedtsson L, Svensson BH, Rosswall T (1988) Relationships between soil moisture content and nitrous oxide production during nitrification and denitrification. *Biology and Fertility of Soils*, **6**, 106-111.

Koehler B, Corre MD, Veldkamp E, Wullaert H, Wright SJ (2009) Immediate and long-term nitrogen oxide emissions from tropical forest soils exposed to elevated nitrogen input. *Global Change Biology*, **15**, 2049-2066.

Kool DM, Wrage N, Zechmeister-Boltenstern S et al. (2010) Nitrifier denitrification can be a source of N₂O from soil: a revised approach to the dual-isotope

labelling method. *European Journal of Soil Science*, **61**(5), 759-772.

Lennon EFE, Houlton BZ (2016) Coupled molecular and isotopic evidence for denitrifier controls over terrestrial nitrogen availability. *Isme Journal*, **11**, 727-740.

Liu X, Chen CR, Wang WJ et al. (2013) Soil environmental factors rather than denitrification gene abundance control N₂O fluxes in a wet sclerophyll forest with different burning frequency. *Soil Biology & Biochemistry*, **57**, 292-300

Liu X, Zhang Y, Han W et al. (2013) Enhanced nitrogen deposition over China. *Nature*, **494**, 459.

Lu X, Mao Q, Gilliam FS, Luo Y, Mo J (2014) Nitrogen deposition contributes to soil acidification in tropical ecosystems. *Global Change Biology*, **20**, 3790-3801.

Matson PA, McDowell WH, Townsend AR, Vitousek PM (1999) The globalization of N deposition: ecosystem consequences in tropical environments. *Biogeochemistry*, **46**, 67-83

Müller AK, Matson AL, Corre MD, Veldkamp E (2015) Soil N₂O fluxes along an elevation gradient of tropical montane forests under experimental nitrogen and phosphorus addition. *Frontiers in Earth Science*, **3**:66. doi: 10.3389/feart.2015.00066

Martinson GO, Corre MD, Veldkamp E (2013) Responses of nitrous oxide fluxes and soil nitrogen cycling to nutrient additions in montane forests along an elevation gradient in southern Ecuador. *Biogeochemistry*, **112**, 625-636.

Morse JL, Durán J, Beall F, Enanga EM, Creed IF, Fernandez I, Groffman PM (2015) Soil denitrification fluxes from three northeastern North American forests across a range of nitrogen deposition. *Oecologia*, **177**, 17-27.

Sanderman J, Amundson R (2009) A comparative study of dissolved organic carbon

715 transport and stabilization in California forest and grassland soils.
 716 Biogeochemistry, **92**, 41-59.

717 Schlesinger WH (2009) On the fate of anthropogenic nitrogen. Proceedings of the
 718 National Academy of Sciences, **106**, 203-208.

719 Selbie DR, Lanigan GJ, Laughlin RJ et al. (2015) Confirmation of co-denitrification
 720 in grazed grassland. Scientific Reports, **5**.

721 Simek, M., Cooper JE (2002) The influence of soil pH on denitrification: progress
 722 towards the understanding of this interaction over the last 50 years. European
 723 Journal of Soil Science, **53**, 345-354.

724 Sgouridis F, Stott A, Ullah S (2016) Application of the ¹⁵N gas-flux method for
 725 measuring in situ N₂ and N₂O fluxes due to denitrification in natural and
 726 semi-natural terrestrial ecosystems and comparison with the acetylene
 727 inhibition technique. Biogeosciences, **13**, 1821-1835.

728 Silver WL, Thompson AW, Reich A, Ewel JJ, Firestone MK (2005) Nitrogen cycling
 729 in tropical plantation forests: potential controls on nitrogen retention.
 730 Ecological Applications, **15**, 1604-1614.

731 Spott O, Stange CF (2011) Formation of hybrid N₂O in a suspended soil due to
 732 co-denitrification of NH₂OH. Journal of Plant Nutrition and Soil Science, **174**,
 733 554-567.

734 Stark JM, Firestone MK (1995) Mechanisms for soil moisture effects on activity of
 735 nitrifying bacteria. Applied & Environmental Microbiology, **61**, 218-221.

736 Stevens RJ, Laughlin RJ, Atkins GJ, Prosser SJ (1993) Automated Determination of
 737 Nitrogen-15-Labeled Dinitrogen and Nitrous Oxide by Mass Spectrometry.
 738 Soil Science Society of America Journal, **57**, 981-988.

739 Stevens RJ, Laughlin RJ, Burns LC, Arah JRM, Hood RC (1997) Measuring the

740 contributions of nitrification and denitrification to the flux of nitrous oxide
 741 from soil. *Soil Biology & Biochemistry*, **29**, 139-151.

742 Thamdrup B, Dalsgaard T (2002) Production of N₂ through anaerobic ammonium
 743 oxidation coupled to nitrate reduction in marine sediments. *Applied and*
 744 *Environmental Microbiology*, **68**, 1312-1318.

745 Tian D, Niu S (2015) A global analysis of soil acidification caused by nitrogen
 746 addition. *Environmental Research Letters*, **10**, 024019.

747 Venterea RT, Groffman PM, Verchot LV, Magill AH, Aber JD, Steudler PA (2003)
 748 Nitrogen oxide gas emissions from temperate forest soils receiving long-term
 749 nitrogen inputs. *Global Change Biology*, **9**, 346-357.

750 Wang A, Fang YT, Chen DX, et al. (2014) Variations in nitrogen-15 natural
 751 abundance of plant and soil systems in four remote tropical rainforests,
 752 southern China. *Oecologia*, **174**, 567-580.

753 Wang F, Li J, Wang X, Zhang W, Zou B, Neher DA, Li Z (2014) Nitrogen and
 754 phosphorus addition impact soil N₂O emission in a secondary tropical forest of
 755 South China. *Scientific Reports*, **4**, 5615.

756 Xi D, Bai R, Zhang L, Fang Y (2016) Contribution of anammox to nitrogen removal
 757 in two temperate forest soils. *Applied & Environmental Microbiology*, **82**,
 758 4602-4612

759 Xu H, Li Y.D, Luo TS, Lin MX, Chen DX, Mo JH, Luo W, Hong XJ, Jiang ZL 2009.
 760 Community structure characteristics of tropical montane rain forests with
 761 different regeneration types in Jianfengling. *Scientia Silvae Sinicae* **45**, 14–20
 762 (in Chinese).

763 Yang WH, Weber KA, Silver WL (2012) Nitrogen loss from soil through anaerobic
 764 ammonium oxidation coupled to iron reduction. *Nature Geoscience*, **5**,

765 538-541.

766 Yang WH, Mcdowell AC, Brooks PD, Silver WL (2014) New high precision approach
767 for measuring ^{15}N - N_2 gas fluxes from terrestrial ecosystems. *Soil Biology &*
768 *Biochemistry*, **69**, 234-241.

769 Zhou X, Fu Y, Zhou L, Li B, Luo Y (2013) An imperative need for global change
770 research in tropical forests. *Tree Physiology*, **33**, 903.

771 Zhou Z (2013) Effects of nitrogen and phosphorus fertilization on carbon cycling of
772 tropical mountain rainforests in Hainan Island, China. Ph.D. Thesis, Peking
773 University, Beijing.

774 Zhou Z, Ouyang Y, Li Y, Qiu Z, Moran M (2017) Estimating impact of rainfall change
775 on hydrological processes in Jianfengling rainforest watershed, China using
776 BASINS-HSPF-CAT modeling system. *Ecological Engineering*, **105**, 87-94.

777 Zhu X, Burger M, Doane TA, et al. (2013) Ammonia oxidation pathways and nitrifier
778 denitrification are significant sources of N_2O and NO under low oxygen
779 availability. *Proceedings of the National Academy of Sciences*, **110**,
780 6328-6333.

781 Zhu X, Zhang W, Chen H, Mo J (2015) Impacts of nitrogen deposition on soil
782 nitrogen cycle in forest ecosystems: A review. *Acta Ecologica Sinica*, **35**,
783 35-43.

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 ₂ O)	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	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
	Control	33.36±1.80	3.95±0.06	2.30±0.15 ^a	0.19±0.01 ^{ab}	12.4±0.2	0.3±0.1	1.0±0.1 ^a	1.2±0.1
	Low-N	31.08±0.86	3.91±0.07	2.13±0.10 ^a	0.17±0.01 ^a	12.2±0.2	0.8±0.6	1.4±0.3 ^{ab}	1.1±0.1
	Medium-N	35.26±2.32	3.94±0.07	2.52±0.20 ^{ab}	0.20±0.01 ^{ab}	12.6±0.5	0.8±0.2	1.3±0.2 ^{ab}	1.1±0.0
	High-N	34.69±2.40	3.86±0.08	2.80±0.17 ^b	0.22±0.01 ^b	13.0±0.2	0.8±0.2	1.9±0.3 ^b	1.0±0.1

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

DOC = dissolved organic carbon (g kg⁻¹).

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

Control: 0 kg N ha⁻¹ year⁻¹; Low-N: 25 kg N ha⁻¹ year⁻¹; Medium-N: 50 kg N ha⁻¹ year⁻¹, and High-N: 100 kg N ha⁻¹ year⁻¹.

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

Table 2 Ratios of $\text{N}_2\text{O}/(\text{N}_2\text{O}+\text{N}_2)$ 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 N₂ emission rates. Data are the mean ± 1 SE, and no significant difference was found among any N addition levels in both forests using ANOVA.

Table 3 Ratios of $\text{N}_2\text{O}/(\text{N}_2\text{O}+\text{N}_2)$ 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.

807 **Table 4** N₂O emission rates from denitrification, co-denitrification, and heterotrophic nitrification, and N₂ 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 ₂ O [#] (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}	C _{AN2}
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}
--	--------	------------------------	------------------------	------------------------	-------------------------	-------------------------

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

810 [#]D_{N2O}, C_{N2O}, and H_{N2O} are the N₂O emission rates produced by denitrification, co-denitrification, and heterotrophic nitrification, respectively.

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

Legends for figures

Fig. 1 Nitrogen emission rates for 0–10 cm deep mineral soil in the primary forest (A) and secondary forest (B) under aerobic incubation conditions. (a) and (d) N_2O (incubated without 20% C_2H_2); (b) and (e) N_2 (N_2O emission rate amended with 20% C_2H_2 minus N_2O without 20% C_2H_2); and (c) and (f) total gas ($\text{N}_2\text{O} + \text{N}_2$, 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 $\times$ 2 sample replications) in control, low-N, medium-N, and high-N treatment plots. No significant differences in N gas emissions were found among the control, low-N, medium-N, and high-N treatments for any sampling date or water addition treatment. Abbreviations: LDS=late dry season, WS=wet season, LDS+W= late dry season + water, WS+W= wet season + water.

Fig. 2 Nitrogen emission rates for the 0–10 cm deep mineral soil in the primary forest (A) and secondary forest (B) determined by AIT and ^{15}N labeling methods under anaerobic incubation. (a) and (d) N_2O ; (b) and (e) N_2 (with AIT treatment, N_2 emission rates were calculated through N_2O emission rates from soil with 20% C_2H_2 treatment minus N_2O emission rates from soils without C_2H_2 additions); and (c) and (f) total gas ($\text{N}_2\text{O} + \text{N}_2$). Soils sampled in wet and early dry seasons were amended with $10 \mu\text{g } ^{14}\text{N g}^{-1}$ fresh soil for AIT and $10 \mu\text{g } ^{15}\text{N g}^{-1}$ 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 $\times$ 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

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

845

846

Fig. 2

850

851 **Fig. 3**

852