



# Soil N<sub>2</sub>O fluxes along an elevation gradient of tropical montane forests under experimental nitrogen and phosphorus addition

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Nutrient deposition to tropical forests is increasing, which could affect soil fluxes of nitrous oxide (N<sub>2</sub>O), a powerful greenhouse gas. We assessed the effects of 35–56 months of moderate nitrogen (N) and phosphorus (P) additions on soil N<sub>2</sub>O fluxes and net soil N-cycling rates, and quantified the relative contributions of nitrification and denitrification to N<sub>2</sub>O fluxes. In 2008, a nutrient manipulation experiment was established along an elevation gradient (1000, 2000, and 3000 m) of montane forests in southern Ecuador. Treatments included control, N, P, and N+P addition (with additions of 50 kg N ha<sup>-1</sup> yr<sup>-1</sup> and 10 kg P ha<sup>-1</sup> yr<sup>-1</sup>). Nitrous oxide fluxes were measured using static, vented chambers and N cycling was determined using the buried bag method. Measurements showed that denitrification was the main N<sub>2</sub>O source at all elevations, but that annual N<sub>2</sub>O emissions from control plots were low, and decreased along the elevation gradient (0.57 ± 0.26–0.05 ± 0.04 kg N<sub>2</sub>O-N ha<sup>-1</sup> yr<sup>-1</sup>). We attributed the low fluxes to our sites' conservative soil N cycling as well as gaseous N losses possibly being dominated by N<sub>2</sub>. Contrary to the first 21 months of the experiment, N addition did not affect N<sub>2</sub>O fluxes during the 35–56 month period, possibly due to low soil moisture contents during this time. With P addition, N<sub>2</sub>O fluxes and mineral N concentrations decreased during Months 35–56, presumably because plant P limitations were alleviated, increasing plant N uptake. Nitrogen plus phosphorus addition showed similar trends to N addition, but less pronounced given the counteracting effects of P addition. The combined results from this study (Months 1–21 and 35–56) showed that effects of N and P addition on soil N<sub>2</sub>O fluxes were not linear with time of exposure, highlighting the importance of long-term studies.

**Keywords:** denitrification, nitrification, net nitrogen cycling, nitrous oxide emissions, nitrogen addition, phosphorus addition, <sup>15</sup>N tracing

## INTRODUCTION

Nitrous oxide (N<sub>2</sub>O) is both a potent greenhouse gas and a dominant ozone-depleting substance (Denman et al., 2007). Soil emissions are the largest natural (i.e., non-anthropogenic) source of N<sub>2</sub>O (Ehhalt et al., 2001), which is mainly produced and consumed by the microbial processes of nitrification and denitrification (Chapuis-Lardy et al., 2007). While nitrification is an obligate

aerobic process, which depends on ammonium (NH<sub>4</sub><sup>+</sup>) and/or organic nitrogen (N) as substrates, denitrification is an anoxic process which is controlled mainly by the soil aeration/oxygen status (i.e., soil water content), nitrate (NO<sub>3</sub><sup>-</sup>) availability, microbially-available organic carbon, and soil pH (Firestone and Davidson, 1989). Thus, soil N availability and water content play a crucial role in controlling the amounts and relative ratios of N-oxide fluxes from soils.

Given the controlling factors for N<sub>2</sub>O flux, moist tropical forests with high natural N availability have the potential to be strong N<sub>2</sub>O sources. Indeed, tropical forest soils currently account for 30% of global N<sub>2</sub>O emissions from unmanaged soils (Ehhalt et al., 2001). However, since atmospheric N deposition from biomass burning, fertilizer use, and industrialization is rapidly increasing (Hietz et al., 2011), it is possible that N<sub>2</sub>O emissions in tropical forests may increase (Koehler et al., 2009; Martinson et al., 2013). In temperate forest ecosystems, elevated N deposition has been shown to increase soil N availability and accelerate soil N cycling, causing substantial N losses in the form of N<sub>2</sub>O emissions (e.g., Butterbach-Bahl et al., 1998; Gundersen et al., 1998). In a meta-analysis of N enrichment effects on greenhouse gas fluxes, Liu and Graever (2009) showed that the N<sub>2</sub>O response to elevated N input could even be stronger in tropical forests than in other ecosystems. These authors suggested that the strength of the tropical forest response to N enrichment may be due to the phosphorus (P) limitation (rather than N limitation) of many tropical soils.

Tropical montane forests (TMFs) make up over 11% of the world's tropical forests. They occur across large elevation gradients and contain a large variety of environmental conditions (FAO, 1993; Bubb et al., 2004). TMFs usually exhibit "conservative" soil N cycling (similar rates of mineral N production and consumption), which results in low N losses. This is in contrast to tropical lowland forest soils, which typically have a more "leaky" soil N cycle; they often have relatively high soil N cycling rates (Vitousek and Matson, 1988; Corre et al., 2010), N-oxide (NO, N<sub>2</sub>O) fluxes (e.g., Matson and Vitousek, 1987; Keller et al., 2005; Purbopuspito et al., 2006; Koehler et al., 2009), and NO<sub>3</sub><sup>-</sup> leaching (e.g., Hedin et al., 2003; Dechert et al., 2005; Schwendenmann and Veldkamp, 2005). However, N addition experiments in TMFs of Hawaii (Hall and Matson, 2003) and Panama (Koehler et al., 2009; Corre et al., 2014) have shown increases in soil mineral N production - especially nitrification rates - and N-oxide fluxes in as little as 1–2 years after the onset of N addition. In the Panamanian TMF, soil N<sub>2</sub>O emissions during the third and fourth year of N addition reached levels as high as those from the lowland forest, which had already experienced 11–12 years of N addition (Corre et al., 2014). Therefore, there is a strong potential for TMF soil to become a significant N<sub>2</sub>O source when subjected to chronic N input.

Tropical South America is also experiencing increases in atmospheric P deposition, mainly due to biomass burning (Mahowald et al., 2005). As mentioned above, soil P limitation may affect the response of soil N cycling (and therefore N<sub>2</sub>O

flux) to N enrichment (Liu and Graever, 2009). Hall and Matson (1999), found that N addition to a Hawaiian tropical forest soil resulted in higher inorganic N concentrations and N<sub>2</sub>O emissions in P-limited soils as compared to N-limited soils. However, field studies with P addition have shown varying results. In a plantation of N-fixing trees in Indonesia, a one-time application of 100 kg P ha<sup>-1</sup> followed by 2 years of measurements, exhibited decreased soil N contents and N<sub>2</sub>O emissions, with increased plant N uptake (Mori et al., 2013). In contrast, Wang et al. (2014) showed that the interaction between N and P addition could result in increased N<sub>2</sub>O emissions in a tropical lowland forest in Southern China. Phosphorus is known to limit plant growth in TMFs (Tanner et al., 1998; Homeier et al., 2012), but the effect of elevated P deposition on soil N cycling in these areas has been little studied.

Since responses of ecosystem processes to chronic nutrient addition may be non-linear with time (e.g., Aber et al., 1998), quantifying changes in N-oxide fluxes and their controlling factors need to be conducted in long-term and large-scale nutrient manipulation experiments. Here, we report the changes in soil N<sub>2</sub>O fluxes, contributions of nitrification and denitrification to N<sub>2</sub>O fluxes, and net rates of soil N cycling (an index of plant-available N) during Months 35–56 of moderate N, P, and combined N+P addition (with additions of 50 kg N ha<sup>-1</sup> yr<sup>-1</sup> and 10 kg P ha<sup>-1</sup> yr<sup>-1</sup>), along an elevation gradient (1000, 2000, and 3000 m) of TMFs in southern Ecuador.

Martinson et al. (2013) reported the effects of nutrient manipulation on soil N<sub>2</sub>O fluxes from Months 1–21 of this experiment. Along the elevation gradient, they observed higher soil N<sub>2</sub>O emissions from soils with N and N+P addition, with soils at 1000 m responding more rapidly than the higher elevations. They also observed slight increases in net nitrification at 2000 and 3000 m (as compared to the previously-undetectable net nitrification activity) with N and N+P additions. However, they did not detect any effect of P addition on soil N<sub>2</sub>O fluxes or net soil N cycling rates at any elevation.

The objectives of the present study were to (1) determine the cumulative effect of 35–56 months of moderate nutrient additions on soil N<sub>2</sub>O fluxes and net soil N cycling rates, and (2) assess the contributions of nitrification and denitrification to soil N<sub>2</sub>O fluxes. By using an elevation gradient, we were also able to assess whether N<sub>2</sub>O fluxes and/or the microbial processes responsible for N<sub>2</sub>O production changed with the different environmental conditions associated with elevation (Supplementary Table 1). In response to nutrient addition, we hypothesized that soil N<sub>2</sub>O fluxes, together with net soil N cycling rates, would further increase as N and N+P additions continued, whereas the moderate P addition might begin to alleviate P limitation, enabling more N uptake and therefore decreasing soil N<sub>2</sub>O emissions.

## MATERIALS AND METHODS

### Study Area

The study area was located in the Andes of southern Ecuador, in the provinces of Loja and Zamora Chinchipe. The experiment

**Abbreviations:** HIP, Hole-in-the-pipe; IRMS, Isotope ratio mass spectrometry; TMF, Tropical montane forest; WFPS, Water-filled pore space.

was conducted within and close to Podocarpus National Park, in three old-growth forest sites spanning an elevation gradient from 1000 to 3000 m above sea level (Supplementary Table 1; Homeier et al., 2012; Martinson et al., 2013). Forest types across the elevation gradient ranged from premontane at “1000 m” (990–1100 m), to lower montane at “2000 m” (1950–2100 m) and upper montane at “3000 m” (2900–3050 m) (Homeier et al., 2012). At 1000 m, sandy Cambisol soil (covered by only a thin layer of decomposing leaves) had developed from deeply weathered granitic rock (Litherland et al., 1994). At 2000 and 3000 m, loamy textured Cambisol and Histosol soil, respectively, had developed from metamorphic schists (Litherland et al., 1994); these soils were covered by 10–40 cm of thick organic layers (Supplementary Table 1). The study area displayed only slight seasonal variability (Emck, 2007). Mean annual air temperature decreased with elevation from 19.4°C at 1000 m, 15.4°C at 2000 m to 9.4°C at 3000 m. Mean annual precipitation ranged from 1950 mm yr<sup>-1</sup> (2000 m) to 4500 mm yr<sup>-1</sup> (3000 m), with intermediate rainfall of 2230 mm yr<sup>-1</sup> at 1000 m (Moser et al., 2007). Ambient bulk and dry deposition of N and P in the study region ranged between 14 and 45 kg N ha<sup>-1</sup> yr<sup>-1</sup> and 0.4 and 4.9 kg P ha<sup>-1</sup> yr<sup>-1</sup>, with an increasing tendency for deposition from 1998 to 2010 (Boy et al., 2008; Homeier et al., 2012) and thereafter (personal communication, W. Wilcke). Due to data gaps, we were not able to get detailed information on monthly rainfall during our measurement period. We attribute differences between the three forest sites to the combination of climatic, vegetation, and soil factors associated with the different elevations. However, the elevations themselves were not replicated, so our results may not represent these elevations in other TMF areas.

## Experimental Design

At each elevation, we established nutrient manipulation experiments (NUMEX) with 16 plots (20 × 20 m each) equally distributed to four blocks. The four blocks served as replicates and included minimal topographic differences (50–100 m) within each elevation. Each block consisted of four plots: N, P, and N+P additions, and untreated controls; these plots were separated by at least 10 m (Homeier et al., 2013; Martinson et al., 2013). Fertilization started in 2008 with two equal applications per year (February/March and August/September), with the exception of 2010 when there was a 4-month delay of the second fertilization. Fertilizers were applied in solid form at rates of 50 kg N ha<sup>-1</sup> yr<sup>-1</sup> of urea (CH<sub>4</sub>N<sub>2</sub>O) and 10 kg P ha<sup>-1</sup> yr<sup>-1</sup> of sodium hydrogen phosphate (NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O and NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, with analytic grade quality).

## Soil N<sub>2</sub>O Flux, Temperature, Moisture and Mineral N Concentrations

Measurements of soil N<sub>2</sub>O flux, temperature, moisture, and mineral N concentrations followed the same procedure described in detail by Martinson et al. (2013). Measurements were performed monthly from Month 35 to Month 56 of the experiment (with January 2008 defined as Month 1) in three out of the four blocks, with a minimum distance of 2 m to plot borders for the nutrient-addition plots. In each plot, measurements were conducted at four locations that were laid

out in a stratified random pattern (Martinson et al., 2013); after Month 37, we added one additional location per plot as part of a small scale manipulation study, to measure soil trace gas fluxes. Since we were interested in long-term effects of nutrient deposition rather than the transitory peaks of N<sub>2</sub>O that occur after N addition, we only included measurements that were taken at least 3 weeks after fertilization. This timespan was chosen based on a study from our working group in Panama, where mineral N concentrations and N<sub>2</sub>O emissions peaked within 3 weeks following N application in a TMF (Koehler et al., 2009). As shown by Wullaert et al. (2010), we could assume that minimal leaching occurred during those 3 weeks; therefore, the N<sub>2</sub>O measured throughout the rest of the year can be considered a realistic long-term response to increasing soil nutrients.

Soil N<sub>2</sub>O fluxes were measured using static vented chambers with permanently installed round polyvinyl chloride chamber bases (area 0.04 m<sup>2</sup>, height 0.15 m, ~0.03 m inserted into the soil) and polyethylene chamber hoods with a Luer lock sampling port and a vent for pressure equilibrium (0.16 m height of chamber cover, 0.03 m overlapping width with chamber base for tight cover, and 12 L total volume). Gas samples were taken at 2, 14, 26, and 38 min or at 3, 13, 23, and 33 min after chamber closure and stored in pre-evacuated glass containers (60 ml vials until Month 40 and 12 ml Exetainers<sup>®</sup> afterwards). Gas samples were either analyzed in Ecuador or in Germany, after shipping as over-pressured samples in Labco Exetainers<sup>®</sup> (Labco Limited, UK). We have tested these Exetainers<sup>®</sup> for their quality during extended sample storage and aircraft transport (see also Glatzel and Well, 2008). Gas samples were analyzed using gas chromatographs (Shimadzu GC-14B, Duisburg, Germany for samples analyzed in Ecuador and GC 6000 Vega Series 2, Carlo Erba Instruments, Milan, Italy for samples analyzed in Germany; both of these and the standard gases are owned by our group and were calibrated regularly) equipped with an electron capture detector and autosamplers. Nitrous oxide concentrations were determined from the comparison of integrated peak areas from samples to three or four standard gases (ranging from 350 to 3000 ppb; Deuste Steininger GmbH, Mühlhausen, Germany). Fluxes of N<sub>2</sub>O, expressed as N<sub>2</sub>O-N flux per area (μg N m<sup>-2</sup> h<sup>-1</sup>), were calculated from the linear increase of N<sub>2</sub>O concentration in the chamber headspace over time, corrected for the air pressure and temperature measured at the time of field sampling. Annual soil N<sub>2</sub>O fluxes were approximated by applying the trapezoid rule on time intervals between measured flux rates. Annual rates combine measurements from Months 1–21 (using data from Martinson et al., 2013) to Months 35–56 (this study), assuming constant flux rates per day.

Soil temperature was measured parallel to gas sampling at a 0.05-m depth close to each of the four chamber bases per plot using a GTH 175/Pt-E digital precision thermometer (Greisinger electronics GmbH, Regenstauf, Germany). Soil moisture and mineral N concentrations were determined parallel to gas sampling for each plot from pooled soil samples of the top 5 cm of soil, consisting of four samples taken within 1 m of each chamber. Soil moisture was determined by oven-drying subsamples at 105° C for at least 24 h and was expressed as percentage of water-filled pore space (WFPS) using the

measured bulk densities in the top 5 cm of soil (reported by Martinson et al., 2013) and particle densities of 2.65 g cm<sup>-3</sup> for mineral soil at 1000 m and 1.4 g cm<sup>-3</sup> for organic layers at 2000 and 3000 m (Linn and Doran, 1984; Breuer et al., 2002). Soil extraction for mineral N concentration determination was carried out *in-situ* in order to avoid alterations of mineral N concentrations due to storage after field sampling (Arnold et al., 2008). A subsample of soil was added into a prepared extraction bottle with 150 ml of 0.5 mol l<sup>-1</sup> K<sub>2</sub>SO<sub>4</sub> solution. After returning to the laboratory on the same day, samples were shaken (1 h), filtered and kept frozen until lab analyses were conducted. Analysis of mineral NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> concentration was done at the University of Goettingen, using continuous flow injection colorimetry (Cenco/Skalar Instruments, Breda, Netherlands); NH<sub>4</sub><sup>+</sup> was analyzed by the Berthelot reaction method (Skalar Method 155-000) and NO<sub>3</sub><sup>-</sup> by the copper-cadmium reduction method with NH<sub>4</sub>Cl buffer but without ethylenediamine tetraacetic acid (Skalar Method 461-000).

## Net Soil N Cycling Rates: Ammonification and Nitrification

Net rates of soil N cycling were determined three times, in Months 38 and 48 (just over 1 month and about 4 months following fertilization) and in Month 52 (about 2 months following fertilization), using the *in-situ* buried bag method (Hart et al., 1994). Two pairs of intact soil cores were taken from the top 5 cm of mineral soil (at 1000 m) or organic layer (at 2000 and 3000 m) in each plot of all four blocks. One soil core of each pair was extracted immediately in the field with 0.5 mol l<sup>-1</sup> K<sub>2</sub>SO<sub>4</sub> (as described above); the other soil core was put in a plastic bag, inserted back into the soil to incubate for 10 days and afterwards extracted. Net soil N cycling rates of each sampling pair were calculated by subtracting the initial soil mineral N concentrations from mineral N concentrations of incubated soils. Net ammonification is the difference in NH<sub>4</sub><sup>+</sup> concentrations and net nitrification is the difference in the NO<sub>3</sub><sup>-</sup> concentrations.

## <sup>15</sup>N Tracing to <sup>15</sup>N<sub>2</sub>O: Contributions of Nitrification and Denitrification to Soil N<sub>2</sub>O Flux

Short-term tracing from <sup>15</sup>NH<sub>4</sub><sup>+</sup> or <sup>15</sup>NO<sub>3</sub><sup>-</sup> to <sup>15</sup>N<sub>2</sub>O was used to determine the contributions of nitrification and denitrification to soil N<sub>2</sub>O fluxes; we used the same method applied in a montane forest in Panama (Corre et al., 2014). Tracing was conducted in all four replicate plots of the control and N-addition treatments at all three elevations and was carried out in Month 43 and Month 49, 4, and 5 months after the last fertilization. In each of the four control or N-addition plots, two additional chamber bases (same dimensions and material as described above) were installed >10 m apart, at least 3 weeks prior to sampling. In N-addition plots, the bases were >2 m from plot edges. For the second sampling, the chamber bases were located close to the previous chambers of the same <sup>15</sup>N source but always upslope to prevent any possible influences from previously applied <sup>15</sup>N. Each of the two chambers in a plot was labeled separately with either <sup>15</sup>NO<sub>3</sub><sup>-</sup> or <sup>15</sup>NH<sub>4</sub><sup>+</sup>.

The amounts of added <sup>15</sup>N (either NH<sub>4</sub><sup>+</sup> or NO<sub>3</sub><sup>-</sup>) were calculated based on the extant soil mineral N levels in the control and N-addition plots such that the added <sup>15</sup>N would be at most 50% of the native levels and the volume of solution would not increase the soil moisture contents. In each plot, 0.52 mg <sup>15</sup>N-KNO<sub>3</sub> in 50 ml distilled water was applied to the soil within one chamber (0.04 m<sup>2</sup> area) and 13.29 mg of <sup>15</sup>N-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in 50 ml distilled water was applied to the soil within the other chamber. Half of the amount of the <sup>15</sup>N solution was injected about 25 cm deep with a side-port needle at several points inside the chamber. The other half was sprayed with a hand sprayer onto the surface of the soil after removal of the leaf litter layer (which was returned afterwards). Transparent plastic covers (0.9 × 0.6 m) were put 0.5 m above the chamber bases 1–2 days before labeling to prevent immediate leaching losses of the applied <sup>15</sup>N tracers in case of rainfall. These applied amounts of <sup>15</sup>NO<sub>3</sub><sup>-</sup> and <sup>15</sup>NH<sub>4</sub><sup>+</sup> were the same for all plots and represented 3–30% and 7–46% of the native NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> levels, respectively, in the top 5 cm of soil across all control and N-addition plots.

Thirty minutes after <sup>15</sup>N application, gas samples were taken with a syringe at 2, 17, and 32 min following chamber closure and stored as overpressured samples in 100 ml pre-evacuated glass vials with butyl rubber septa. These glass vials were tested as leak proof in an earlier study (Corre et al., 2014). Gas samples were analyzed for N<sub>2</sub>O concentrations using the same gas chromatograph described above and <sup>15</sup>N<sub>2</sub>O was determined using isotope ratio mass spectrometry (IRMS; Finnigan Delta<sup>Plus</sup> XP, Thermo Electron Corporation, Bremen, Germany). Following the third of the three gas samples per chamber, we took a soil sample of the top 5 cm in the center of each chamber base to determine soil moisture and mineral N concentrations following the procedures described above. Additionally, <sup>15</sup>N from NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> was determined by the <sup>15</sup>N diffusion procedures described in detail by Corre and Lamersdorf (2004) and analyzed using IRMS (Delta C, Finnigan MAT, Bremen, Germany). Contributions of nitrification and denitrification to soil N<sub>2</sub>O fluxes were calculated following the same calculations given by Corre et al. (2014).

## Statistical Analysis

Data were checked for normality and homoscedasticity, and we used either square root or logarithmic transformation (adding a constant value if the dataset included negative values) for data with non-normal distribution and unequal variance. If after transformation the data were still non-normally distributed we used non-parametric statistical tests.

The influence of soil factors (moisture, temperature, mineral N concentrations) on soil N<sub>2</sub>O fluxes was assessed using Pearson's correlation tests: first, across the elevation gradient considering the control plots only to assess which of these soil factors control N<sub>2</sub>O fluxes under ambient nutrient conditions, and second for each elevation considering all treatment plots to determine if changes in these soil factors due to nutrient amendment influence changes in N<sub>2</sub>O fluxes. These analyses were conducted for the measurements from Month 35–56 on the treatment means (average of three replicate plots) on each sampling day.

Effects of elevation and nutrient addition on time series data (soil N<sub>2</sub>O flux, temperature, WFPS, mineral N concentration and net N-cycling rates) were assessed using linear mixed effects (LME) models (Piepho et al., 2004; Crawley, 2012). Analyses were based on plot means (the average of four or five chamber measurements for N<sub>2</sub>O and two measurements for net soil N cycling) with three replicate plots (for all parameters) or four replicate plots (for soil N-cycling rates). Elevation effects were assessed for control plots only and nutrient-addition effects were assessed separately for each of the three elevations. Elevation or treatments were considered fixed effects whereas sampling month and plot (as spatial replication) were included as random effects. The following structures were included in the LME model if this improved the relative goodness of the model fit based on the Akaike information criterion: (1) a first-order temporal autoregressive process accounting for decreasing correlation of measurements with increasing time difference (Zuur et al., 2009) and (2) a variance function varIdent to model heteroscedasticity of residual variances (Crawley, 2012). The significance of the fixed effects was then determined by analysis of variance and stepwise model simplification.

For the short-term <sup>15</sup>N tracing method of N<sub>2</sub>O sources, we first assessed the effects of added <sup>15</sup>N solution on soil parameters (mineral N concentrations, WFPS, NO<sub>3</sub><sup>-</sup>/NH<sub>4</sub><sup>+</sup> ratio) and soil N<sub>2</sub>O fluxes for each measurement campaign, elevation and treatment. We compared <sup>15</sup>NH<sub>4</sub><sup>+</sup> - with <sup>15</sup>NO<sub>3</sub><sup>-</sup> -labeled chambers and both with reference (without <sup>15</sup>N) chambers that were measured in the nearest sampling months, using Paired *T*-tests. Second, we tested the differences in relative contributions of nitrification and denitrification to N<sub>2</sub>O fluxes between years for

each elevation and treatment, between elevations for the control plots only and between treatments for each elevation, using either *T*-tests (independent and paired) or a Mann–Whitney *U*-test.

The significance level was defined at  $P \leq 0.05$  and mean values in the text are given with  $\pm$  standard error (SE). Statistical analyses were conducted using R 2.14.0 (R Development Core Team, 2012).

## RESULTS

### Effects of Elevation on Soil Temperature, Wfps, and Net N Cycling under Ambient Environmental Conditions

From Month 35 to 56, soil temperature and WFPS in the top 5 cm of soil in control plots differed between elevations (Table 1) but showed no clear seasonal pattern at any elevation. Soil temperature decreased with increasing elevation ( $P < 0.001$ ), while WFPS was highest at 2000 m, followed by 3000 m and then 1000 m ( $P < 0.001$ ).

Net ammonification rates in control plots did not differ across the elevation gradient ( $P = 0.126$ ; Table 2), which was caused by the large spatial variability (i.e., large SE), but net nitrification rates were larger at 1000 m than at 2000 and 3000 m ( $P < 0.001$ ; Table 2). At all elevations, the dominant form of mineral N in the top 5 cm of soil was NH<sub>4</sub><sup>+</sup> (Table 2). Soil NH<sub>4</sub><sup>+</sup> was higher at 1000 and 2000 m compared to 3000 m ( $P < 0.001$ ), and did not vary markedly between the measurement periods. Similar to net nitrification, NO<sub>3</sub><sup>-</sup> decreased with increasing elevation ( $P < 0.001$ ; Table 2), and larger NO<sub>3</sub><sup>-</sup> concentrations at 1000 m

**TABLE 1 | Mean ( $\pm$ SE,  $n = 3$ ) soil temperature, water-filled pore space (WFPS) and N<sub>2</sub>O fluxes in tropical montane forests along a 1000-m to 3000-m elevation gradient.**

Elevation (m)	Treatment	Soil temperature (°C)	WFPS (%)	Soil N <sub>2</sub> O fluxes (hourly) from Months 35–56 ( $\mu\text{g N m}^{-2} \text{h}^{-1}$ )	Soil N <sub>2</sub> O fluxes (annual) from Months 35–56 ( $\text{kg N ha}^{-1} \text{yr}^{-1}$ )	Soil N <sub>2</sub> O fluxes (annual) from Months 1–56 ( $\text{kg N ha}^{-1} \text{yr}^{-1}$ )
1000	Control	18.43 $\pm$ 0.10 <sup>A</sup>	43.35 $\pm$ 3.87 <sup>C</sup>	6.40 $\pm$ 3.17 <sup>A,ab</sup>	0.57 $\pm$ 0.26	0.50 $\pm$ 0.16
	Nitrogen (N)	18.50 $\pm$ 0.04	45.26 $\pm$ 6.91	7.50 $\pm$ 1.20 <sup>a</sup>	0.64 $\pm$ 0.08	0.71 $\pm$ 0.17
	Phosphorus (P)	18.60 $\pm$ 0.09	51.41 $\pm$ 9.69	3.63 $\pm$ 1.31 <sup>b</sup>	0.33 $\pm$ 0.13	0.43 $\pm$ 0.02
	N + P	18.66 $\pm$ 0.07	54.06 $\pm$ 6.68	6.73 $\pm$ 0.67 <sup>a</sup>	0.59 $\pm$ 0.06	0.92 $\pm$ 0.15
2000	Control	14.67 $\pm$ 0.28 <sup>B</sup>	71.12 $\pm$ 4.21 <sup>A</sup>	2.05 $\pm$ 0.64 <sup>B,ab</sup>	0.17 $\pm$ 0.06	0.16 $\pm$ 0.09
	N	14.77 $\pm$ 0.19	71.64 $\pm$ 2.00	2.99 $\pm$ 0.42 <sup>a</sup>	0.27 $\pm$ 0.04	0.31 $\pm$ 0.04
	P	14.81 $\pm$ 0.17	72.88 $\pm$ 2.41	1.09 $\pm$ 0.40 <sup>b</sup>	0.09 $\pm$ 0.03	0.09 $\pm$ 0.06
	N + P	14.64 $\pm$ 0.18	69.00 $\pm$ 1.76	2.98 $\pm$ 0.51 <sup>a</sup>	0.25 $\pm$ 0.05	0.48 $\pm$ 0.02
3000	Control	9.80 $\pm$ 0.26 <sup>C,b</sup>	58.58 $\pm$ 0.82 <sup>B,a</sup>	0.47 $\pm$ 0.62 <sup>B</sup>	0.05 $\pm$ 0.04	-0.08 $\pm$ 0.01
	N	9.61 $\pm$ 0.26 <sup>b</sup>	57.00 $\pm$ 2.25 <sup>a</sup>	1.00 $\pm$ 0.21	0.10 $\pm$ 0.02	0.20 $\pm$ 0.04
	P	10.03 $\pm$ 0.26 <sup>a</sup>	50.34 $\pm$ 2.13 <sup>ab</sup>	0.78 $\pm$ 0.75	0.07 $\pm$ 0.05	-0.02 $\pm$ 0.13
	N + P	9.74 $\pm$ 0.10 <sup>b</sup>	44.61 $\pm$ 6.91 <sup>b</sup>	1.40 $\pm$ 0.33	0.12 $\pm$ 0.02	0.26 $\pm$ 0.07

Measurements of temperature, WFPS and hourly N<sub>2</sub>O flux occurred between November 2010 and August 2012 (Months 35–56 of nutrient addition). Values given for annual N<sub>2</sub>O fluxes also show the combined measurements from Months 1 to 21 (Martinson et al., 2013) and Months 35 to 56 (this study). For soil temperature, WFPS and hourly soil N<sub>2</sub>O fluxes, means followed by different capital letters indicate significant differences across the elevation gradient for the control plots, and means followed by small letters indicate significant differences among treatments within each elevation (linear mixed effects model at  $P \leq 0.05$ ). Annual soil N<sub>2</sub>O fluxes were approximated by applying the trapezoid rule on time intervals between measured flux rates, assuming constant flux rates per day (Note: hourly and annual fluxes do not include any transitory peaks that may have occurred directly following nutrient addition, as measurements always occurred at least 3 weeks after fertilization).

**TABLE 2 | Mean (±SE) net soil N cycling rates ( $n = 4$ ) and soil mineral N concentrations ( $n = 3$ ) in the top 5 cm of tropical montane forest soils along a 1000-m to 3000-m elevation gradient.**

Elevation (m)	Treatment	Net soil N cycling rates (mg N m <sup>-2</sup> d <sup>-1</sup> )		Soil mineral N concentration (mg N m <sup>-2</sup> )	
		Ammonification	Nitrification	NH <sub>4</sub> <sup>+</sup>	NO <sub>3</sub> <sup>-</sup>
1000	Control	15.24 ± 5.59 <sup>A,a</sup>	35.48 ± 6.82 <sup>A</sup>	335.79 ± 31.38 <sup>A</sup>	43.41 ± 26.01 <sup>A,c</sup>
	Nitrogen (N)	3.02 ± 1.73 <sup>ab</sup>	42.93 ± 1.83	308.96 ± 25.59	76.07 ± 15.40 <sup>a</sup>
	Phosphorus (P)	19.71 ± 8.45 <sup>a</sup>	52.95 ± 7.96	317.02 ± 15.62	19.44 ± 8.33 <sup>d</sup>
	N+P	-3.76 ± 2.23 <sup>b</sup>	38.56 ± 3.28	328.13 ± 55.86	58.60 ± 7.88 <sup>b</sup>
2000	Control	1.25 ± 5.64 <sup>A,b</sup>	0.25 ± 0.29 <sup>B,b</sup>	359.96 ± 17.12 <sup>A,c</sup>	9.59 ± 2.33 <sup>B,b</sup>
	N	44.97 ± 19.37 <sup>a</sup>	4.39 ± 3.02 <sup>a</sup>	745.60 ± 49.82 <sup>a</sup>	44.08 ± 14.81 <sup>a</sup>
	P	1.58 ± 4.12 <sup>b</sup>	0.34 ± 0.30 <sup>b</sup>	324.51 ± 20.85 <sup>c</sup>	8.78 ± 2.33 <sup>b</sup>
	N+P	19.04 ± 6.97 <sup>a</sup>	8.39 ± 2.45 <sup>a</sup>	563.17 ± 57.76 <sup>b</sup>	60.49 ± 6.46 <sup>a</sup>
3000	Control	-0.04 ± 1.93 <sup>A,c</sup>	-0.03 ± 0.09 <sup>B,b</sup>	217.95 ± 10.71 <sup>B,b</sup>	3.77 ± 0.83 <sup>C,c</sup>
	N	21.31 ± 5.48 <sup>a</sup>	1.24 ± 0.71 <sup>a</sup>	504.82 ± 99.84 <sup>a</sup>	22.25 ± 1.11 <sup>a</sup>
	P	5.69 ± 3.79 <sup>bc</sup>	0.08 ± 0.11 <sup>b</sup>	159.51 ± 9.94 <sup>c</sup>	2.53 ± 0.77 <sup>d</sup>
	N+P	14.57 ± 5.83 <sup>ab</sup>	1.76 ± 1.36 <sup>a</sup>	248.07 ± 64.80 <sup>b</sup>	6.77 ± 1.36 <sup>b</sup>

Soil mineral N concentrations were measured monthly from Months 35 to 56 of nutrient addition. Net N-cycling rates were measured three times (Months 38, 48, and 52 of nutrient addition). Means followed by different capital letters indicate significant differences across the elevation gradient for the control plots and means with superscript small letters indicate significant differences among treatments for each elevation (linear mixed effects model at  $P \leq 0.05$ ).

during Month 38, Months 47–48, and Months 55–56, and at 2000 m during Month 47, coincided with months of large litterfall (Homeier et al., unpublished data on litterfall).

## Effects of Elevation on Soil N<sub>2</sub>O Fluxes under Ambient Environmental Conditions

Soil N<sub>2</sub>O fluxes in control plots decreased with increasing elevation; hourly fluxes at 1000 m were more than three times larger than at 2000 m and more than 13 times larger than at 3000 m (Table 1). Temporal variability of N<sub>2</sub>O fluxes from the control plots was largest at 1000 m, but there was no clear seasonal trend (Figure 1).

In the control plots at each elevation, addition of <sup>15</sup>N solutions for the short-term <sup>15</sup>N tracing of N<sub>2</sub>O did not affect soil N<sub>2</sub>O fluxes, WFPS or mineral N concentrations ( $P \geq 0.060$ ) as compared to the reference (without <sup>15</sup>N) chambers, except at 2000 m during the first measurement campaign, where addition of <sup>15</sup>NH<sub>4</sub><sup>+</sup> solution increased soil NH<sub>4</sub><sup>+</sup> concentrations ( $P < 0.009$ ). The relative contributions of nitrification and denitrification to N<sub>2</sub>O fluxes did not differ between the two measurement campaigns ( $P \geq 0.500$ ), and hence we report the means (±SE,  $n = 4$ ) of these two periods. Denitrification dominated N<sub>2</sub>O fluxes in control plots along the elevation gradient with contributions of  $67 \pm 26\%$  at 1000 m,  $100 \pm 0\%$  at 2000 m and  $98 \pm 3\%$  at 3000 m (Figure 2). There was a larger contribution of nitrification to N<sub>2</sub>O fluxes at 1000 m than at 2000 m ( $P = 0.029$ ). The amounts of <sup>15</sup>N<sub>2</sub>O emitted during 30 min of chamber closure were very small: up to 0.003% of soil <sup>15</sup>NH<sub>4</sub><sup>+</sup> and up to 0.755% of soil <sup>15</sup>NO<sub>3</sub><sup>-</sup> in the top 5 cm of soil across the elevation gradient.

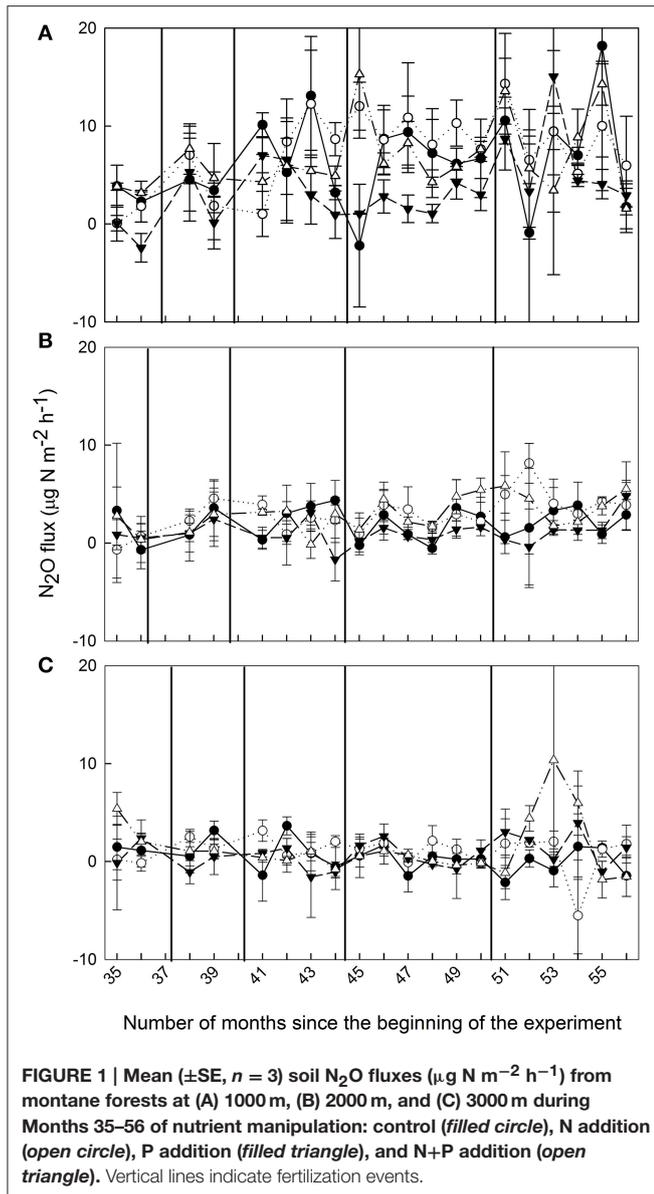
Across the elevation gradient, soil N<sub>2</sub>O fluxes from control plots were positively correlated with soil temperature and NO<sub>3</sub><sup>-</sup> (Table 3). Soil temperature and soil NO<sub>3</sub><sup>-</sup> concentration

were negatively correlated with WFPS. Soil temperature was positively correlated with soil NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> concentrations. Soil NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> concentrations were also positively correlated. Correlation tests performed for each elevation revealed significant correlations at 1000 m only: positive correlations between soil N<sub>2</sub>O fluxes and WFPS ( $r^2 = 0.59$ ;  $P = 0.004$ ;  $n = 22$ ) and between soil temperature and NH<sub>4</sub><sup>+</sup> concentrations ( $r^2 = 0.52$ ;  $P = 0.013$ ;  $n = 22$ ).

## Effects of Nutrient Addition on Soil Temperature, WFPS, and Net N Cycling Across an Elevation Gradient

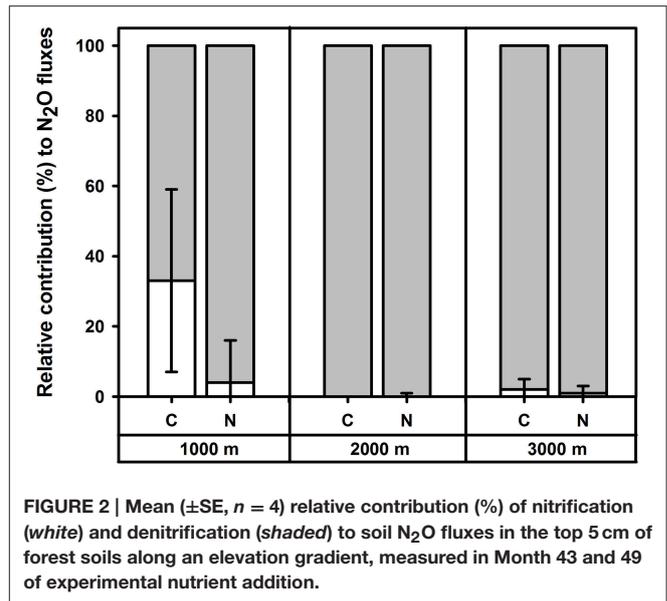
Soil temperature and WFPS measured between Month 35 and 56 differed between treatments only at 3000 m ( $P < 0.001$ ; Table 1). Soil temperature was higher in P plots compared to all other treatments ( $P = 0.006$ – $0.033$ ) whereas WFPS was lower in N+P plots compared to control ( $P = 0.013$ ) and N plots ( $P = 0.026$ ).

Soil mineral N concentrations measured monthly between Month 35 and 56 were also influenced by nutrient addition (Table 2). At 1000 m, NH<sub>4</sub><sup>+</sup> concentrations did not differ between treatments ( $P = 0.601$ ) whereas NO<sub>3</sub><sup>-</sup> concentrations decreased in the order of N, N+P, control, and P plots ( $P \leq 0.017$ ; Table 2). At 2000 m, NH<sub>4</sub><sup>+</sup> concentrations in N and N+P plots were larger than in control plots ( $P < 0.005$ ) with concentrations in N plots being larger than in N+P plots ( $P = 0.007$ ). Soil NO<sub>3</sub><sup>-</sup> concentrations displayed similar differences between treatments as that of NH<sub>4</sub><sup>+</sup> (Table 2). At 3000 m, NH<sub>4</sub><sup>+</sup> concentrations were higher in N plots and lower in P plots compared to control and N+P plots ( $P < 0.001$ ) whereas NO<sub>3</sub><sup>-</sup> concentrations displayed the same treatment differences described for 1000 m with descending concentrations in the order of N, N+P, control, and P plots ( $P < 0.001$ ; Table 2).



**FIGURE 1 |** Mean ( $\pm$ SE,  $n = 3$ ) soil N<sub>2</sub>O fluxes ( $\mu\text{g N m}^{-2} \text{h}^{-1}$ ) from montane forests at (A) 1000 m, (B) 2000 m, and (C) 3000 m during Months 35–56 of nutrient manipulation: control (filled circle), N addition (open circle), P addition (filled triangle), and N+P addition (open triangle). Vertical lines indicate fertilization events.

Nutrient addition affected net soil N cycling rates across all elevations (Table 2). At 1000 m, net ammonification rates decreased in N+P plots ( $P = 0.017$ ) compared to control and P plots ( $P = 0.004$ ) whereas the N plots showed intermediate rates (Table 2). There was no treatment difference detected for net nitrification rates ( $P = 0.357$ ). At 2000 m, net ammonification and nitrification rates increased in N and N+P plots compared to control and P plots ( $P = 0.001$ – $0.033$ ; Table 2). At 3000 m, net ammonification rates increased in N ( $P = 0.001$ ) and N+P plots ( $P = 0.007$ ) compared to control plots, and P plots did not differ from the control ( $P = 0.196$ ; Table 2). Furthermore, net ammonification rates in N plots were higher than in P plots ( $P = 0.029$ ). Net nitrification rates increased in N ( $P = 0.011$ ) and N+P plots ( $P = 0.005$ ) whereas P plots were comparable with the control ( $P = 0.536$ ).



**FIGURE 2 |** Mean ( $\pm$ SE,  $n = 4$ ) relative contribution (%) of nitrification (white) and denitrification (shaded) to soil N<sub>2</sub>O fluxes in the top 5 cm of forest soils along an elevation gradient, measured in Month 43 and 49 of experimental nutrient addition.

### Effects of Nutrient Addition on Soil N<sub>2</sub>O Fluxes Across an Elevation Gradient

At 1000 m, over the entire measurement period, soil N<sub>2</sub>O fluxes from nutrient-amended plots were not different from control plots ( $P = 0.059$ – $0.146$ ; Figure 1A, Table 1). The P plots, however, had lower soil N<sub>2</sub>O fluxes compared to N ( $P = 0.001$ ) and N+P plots ( $P = 0.004$ ) (Table 1). At 2000 m, nutrient additions had the same effects as at 1000 m; N<sub>2</sub>O fluxes from nutrient-amended plots were not significantly different than control plots ( $P = 0.119$ – $0.128$ ), but P plots had lower soil N<sub>2</sub>O fluxes compared to N and N+P plots (both  $P = 0.002$ ; Figure 1B, Table 1). At 3000 m, there were no detectable treatment differences in soil N<sub>2</sub>O fluxes ( $P = 0.391$ – $0.651$ ; Figure 1C, Table 1).

For the short-term <sup>15</sup>N tracing method of N<sub>2</sub>O sources, addition of <sup>15</sup>N solutions did not affect soil N<sub>2</sub>O fluxes, WFPS or mineral N concentrations ( $P \geq 0.062$ ) as compared to the reference chambers in each N plot at each elevation. Relative contributions of nitrification and denitrification to soil N<sub>2</sub>O fluxes did not differ between the two measurement campaigns for each treatment ( $P \geq 0.500$ ) and hence we reported the average values of these two measurements. We did not detect a significant difference in the sources of N<sub>2</sub>O fluxes between control and N plots at any elevation ( $P = 0.625$ ) and mean ( $\pm$ SE,  $n = 4$ ) contributions of denitrification to N<sub>2</sub>O fluxes in N plots were  $96 \pm 12\%$  at 1000 m,  $100 \pm 1\%$  at 2000 m and  $99 \pm 2\%$  at 3000 m (Figure 2). The amounts of <sup>15</sup>N<sub>2</sub>O emitted during 30 min of chamber closure were maximally 0.004% of soil <sup>15</sup>NH<sub>4</sub><sup>+</sup> and 0.065% of soil <sup>15</sup>NO<sub>3</sub><sup>-</sup> in the top 5 cm across the elevation gradient.

Across all treatments plots for each elevation, correlations of soil N<sub>2</sub>O fluxes with soil temperature, WFPS and mineral N varied (Table 4). At 1000 m, N<sub>2</sub>O fluxes were positively correlated with WFPS. At 2000 m, there was a positive correlation of N<sub>2</sub>O fluxes with WFPS, NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> concentrations. At

**TABLE 3 | Pearson correlation coefficients for monthly average ( $n = 66$ ) soil N<sub>2</sub>O flux ( $\mu\text{g N m}^{-2} \text{h}^{-1}$ ), soil temperature ( $^{\circ}\text{C}$ ), water-filled pore space (WFPS; %), and mineral N concentrations ( $\text{mg N m}^{-2}$ ) in control plots of forests across an elevation gradient.**

	Soil temperature	WFPS	NH <sub>4</sub> <sup>+</sup>	NO <sub>3</sub> <sup>-</sup>
Soil N <sub>2</sub> O flux	0.51*	-0.13	0.21	0.36*
Soil temperature		-0.40*	0.50*	0.67*
WFPS			0.05	-0.41*
NH <sub>4</sub> <sup>+</sup>				0.34*

\*  $P < 0.01$ .

**TABLE 4 | Pearson correlation coefficients for monthly average ( $n = 80$ ) soil N<sub>2</sub>O flux ( $\mu\text{g N m}^{-2} \text{h}^{-1}$ ), soil temperature ( $^{\circ}\text{C}$ ), water-filled pore space (WFPS; %), and mineral N concentrations ( $\text{mg N m}^{-2}$ ) of all treatment plots of forests along an elevation gradient.**

Elevation (m)		Soil temperature	WFPS	NH <sub>4</sub> <sup>+</sup>	NO <sub>3</sub> <sup>-</sup>
1000	Soil N <sub>2</sub> O flux	-0.21	0.25*	0.02	0.12
	Soil temperature		-0.29**	0.49**	0.15
	WFPS			-0.16	-0.24**
	NH <sub>4</sub> <sup>+</sup>				0.36**
2000	Soil N <sub>2</sub> O flux	0.09	0.32**	0.21*	0.10**
	Soil temperature		-0.16	0.03	-0.04
	WFPS			0.01	-0.10
	NH <sub>4</sub> <sup>+</sup>				0.58**
3000	Soil N <sub>2</sub> O flux	0.10	-0.04	0.09	0.03
	Soil temperature		-0.01	0.07	0.03
	WFPS			0.33**	0.08
	NH <sub>4</sub> <sup>+</sup>				0.56**

\*  $P < 0.05$ , \*\*  $P < 0.01$ .

3000 m, we did not detect any significant correlation between N<sub>2</sub>O fluxes and these controlling soil factors.

## DISCUSSION

### Effects of Elevation on Soil N<sub>2</sub>O Fluxes and Controlling Factors under Ambient Environmental Conditions

Annual soil N<sub>2</sub>O fluxes from control plots across the elevation gradient (Table 1) were lower than those from other TMFs at comparable elevations that reported *in-situ*, year-round measurements, e.g., in Indonesia (0.29–1.11 kg N<sub>2</sub>O-N ha<sup>-1</sup> yr<sup>-1</sup>; Purbopuspito et al., 2006), Panama (1.13 kg N<sub>2</sub>O-N ha<sup>-1</sup> yr<sup>-1</sup>; Koehler et al., 2009), Brazil (0.96 kg N<sub>2</sub>O-N ha<sup>-1</sup> yr<sup>-1</sup>; Sousa Neto et al., 2011), and Peru (0.44–2.23 kg N<sub>2</sub>O-N ha<sup>-1</sup> yr<sup>-1</sup>; Teh et al., 2014). Our hourly N<sub>2</sub>O fluxes (Table 1) were, however, within the range of others reported from our general study area (Wolf et al., 2011) as well as those previously measured from our same control sites (-0.03 to 0.24 kg N<sub>2</sub>O-N ha<sup>-1</sup>

yr<sup>-1</sup> in 2008 and -0.34 to 0.48 kg N<sub>2</sub>O-N ha<sup>-1</sup> yr<sup>-1</sup> in 2009; Martinson et al., 2013).

According to the hole-in-the-pipe (HIP) model, the amount of gaseous N loss from soils is primarily controlled by soil N availability, which is commonly measured using the soil N cycling rate (Firestone and Davidson, 1989; Davidson et al., 2000). We compared the net soil N cycling rates from our control plots (Table 2) with published data from other old-growth TMFs that used *in-situ* incubation of intact soil cores. Along our elevation gradient, net nitrification in the top 5 cm of soil was highest at 1000 m, likely reflecting the lower organic C and WFPS at 1000 m as compared to the higher elevations (Martinson et al., 2013). However, our net N cycling rates from 1000 m were lower than values reported for TMFs on Andosol soils located between 700 and 1500 m in northwestern Ecuador and Costa Rica (Arnold, 2008; Arnold et al., 2009). Similarly, net nitrification rates in the top 5 cm of the organic layer in an Andosol soil at 1200 m in Panama (Koehler et al., 2009) were more than 10 times higher than values from the same depth of organic layers in our Cambisol soil at 2000 m and our Histosol soil at 3000 m. A separate study carried out at our study sites showed that gross rates of mineral N production (N mineralization and nitrification) in our control plots were low and closely coupled with microbial N immobilization (Baldos et al., 2015), which is typical for conservative soil N cycling and supports our low net soil N cycling rates. Therefore, it is not surprising that the N<sub>2</sub>O losses from our study sites were very low, with mean daily N<sub>2</sub>O fluxes (Table 1) accounting for only 0.02–0.06% of gross N mineralization rates (used as an index of soil available N, ranging from 60 ± 10 mg N m<sup>-2</sup> d<sup>-1</sup> at 3000 m over 191 ± 53 mg N m<sup>-2</sup> d<sup>-1</sup> at 2000 m to 235 ± 30 mg N m<sup>-2</sup> d<sup>-1</sup> at 1000 m in the top 5 cm of soil; Baldos et al., 2015). This was comparable with the 0.06% N<sub>2</sub>O loss in proportion to gross N mineralization rate in the top 5 cm of soil reported for a TMF in Panama (Corre et al., 2014).

The second level of control on gaseous N losses from soils in the HIP model is the soil aeration status, usually represented by the soil WFPS, which influences the relative contributions of nitrification and denitrification to gaseous N losses. Denitrification is proposed to become the dominant source of N<sub>2</sub>O fluxes above a threshold value of 60% WFPS (Davidson et al., 2000) and to become the only N<sub>2</sub>O source at WFPS >70% (Davidson, 1991; Macheferf and Dise, 2004; Bateman and Baggs, 2005). Although, WFPS in the top 5 cm of soil only surpassed these threshold values at 2000 m (Table 1), the <sup>15</sup>N tracing method showed that denitrification was the dominant source of N<sub>2</sub>O at 1000 m and the only N<sub>2</sub>O source at 2000 and 3000 m. However, in previous studies, it has been shown that WFPS threshold values can vary substantially depending on soil texture; for example, in acid brown earth (Cambisol) with 48% sand in Northern Ireland, 60–80% of N<sub>2</sub>O was derived from denitrification at 40% WFPS (Stevens et al., 1997). This is comparable to our results from the sandy loam mineral soil (Cambisol) at 1000 m. At 2000 and 3000 m, the 59–71% WFPS in the top 5 cm of the organic layer should theoretically have included some nitrification-derived N<sub>2</sub>O fluxes. However, these organic layers had very high gravimetric soil moisture contents

(on average, 3.4 and 4.9 g g<sup>-1</sup>) due to the high water holding capacity of the organic matter (Hudson, 1994). To illustrate this: approximately 27–30 kg H<sub>2</sub>O m<sup>-2</sup> was stored in the top 5 cm organic layer, which was much more than the approximately 15 kg H<sub>2</sub>O m<sup>-2</sup> stored in the top 5 cm of mineral soil at 1000 m. Such high gravimetric water contents in organic layers can create plenty of anaerobic microsites in which denitrification can occur despite the relatively low total WFPS. Indeed, the positive correlations between N<sub>2</sub>O flux and NO<sub>3</sub><sup>-</sup> (Table 3) also supported our results from the <sup>15</sup>N tracing method that denitrification was the dominant N<sub>2</sub>O source. Thus, our findings illustrate that in contrast to mineral soils, different threshold values of WFPS should be used for organic layers in estimating limits of the relative importance of nitrification and denitrification as N<sub>2</sub>O sources.

### N-addition Effects on Soil N<sub>2</sub>O Fluxes

In partial confirmation of our hypothesis, NO<sub>3</sub><sup>-</sup> concentrations (at all elevations), as well as net ammonification (at 2000 and 3000 m), net nitrification (at 2000 and 3000 m) and NH<sub>4</sub><sup>+</sup> concentrations (at 2000 m), were all higher in N-amended (i.e., N and N+P) plots as compared to control plots (Table 2). These observed increases were supported by the increased gross rates of N mineralization and nitrification and decreases in microbial immobilization of NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup>, which were measured in the third and fourth year of nutrient manipulation at our sites (Baldos et al., 2015). However, in contrast to our hypothesis, these increases in net and gross rates of mineral N production and mineral N levels did not lead to increases in N<sub>2</sub>O fluxes in N-amended plots relative to the control (Table 1; Figure 1). This does not mean that denitrification was not occurring, though, but may instead be an indication that produced N<sub>2</sub>O was being further reduced to N<sub>2</sub>. In the <sup>15</sup>N tracing experiment, although denitrification was the main N<sub>2</sub>O source, emitted <sup>15</sup>N<sub>2</sub>O accounted for maximally 0.065% of soil <sup>15</sup>NO<sub>3</sub><sup>-</sup> (see Results: effects of nutrient addition on N<sub>2</sub>O). This low percentage suggests that there may have been further reduction of N<sub>2</sub>O to N<sub>2</sub>. Reduction to N<sub>2</sub> is possible in soils, given favorable anaerobic microsites, high soil carbon and low soil NO<sub>3</sub><sup>-</sup> levels (Weier et al., 1993). Such conditions were present in our sites, especially at the higher elevations with thick organic layers (see the discussion of water in organic layers above). Therefore, the combination of anaerobic microsites, with low NO<sub>3</sub><sup>-</sup> concentrations (Table 2) and presumably high labile carbon in organic layers, may have resulted in high N<sub>2</sub>/N<sub>2</sub>O ratios, where losses via denitrification were dominated by N<sub>2</sub> (Weier et al., 1993). Chronic N addition can cause increases in NO<sub>3</sub><sup>-</sup> levels and decreases in soil pH, which then inhibit N<sub>2</sub>O to N<sub>2</sub> reduction; this was observed in an Andosol soil from a montane forest in Panama (Koehler et al., 2009, 2012; Corre et al., 2014). The increases in NO<sub>3</sub><sup>-</sup> levels in our N-amended plots (Table 2), however, were much lower than those observed from the Panamanian montane forest soil, which received 4 years of 125 kg N ha<sup>-1</sup> yr<sup>-1</sup> (with resulting NO<sub>3</sub><sup>-</sup> levels as high as 50–60 mg N m<sup>-2</sup> in the organic layer and 112–183 mg N m<sup>-2</sup> in the mineral soil). Our moderate levels of nutrient addition were probably the reason why soil pH in our N-amended plots did not yet differ

significantly from the control plots even after 4 years (Baldos et al., 2015).

After the first 21 months of N addition to our sites, Martinson et al. (2013) reported that net nitrification at all elevations increased slightly and that these increases were accompanied by small increases in N<sub>2</sub>O fluxes during the second year of N addition. Although we again observed higher N cycling with N addition in Months 35–56 (Table 2), we did not measure higher N<sub>2</sub>O emissions with N addition (Table 1; Figure 1). Whether an increase in soil N availability (e.g., mineral N concentrations, net/gross rates of mineral N production) results in an increase in N<sub>2</sub>O fluxes can depend on inter-annual variations in climate. In our results, the positive correlation of N<sub>2</sub>O fluxes with WFPS at 1000 m, both in control plots (see Results: control plots) and across all treatments (Table 4), clearly indicates that there was a soil moisture control on N<sub>2</sub>O fluxes. Additionally, at 2000 m, the correlations across treatments showed that a combination of changes in soil mineral N concentrations and moisture contents was controlling N<sub>2</sub>O fluxes (Table 3). Corre et al. (2014) showed that the N<sub>2</sub>O response to chronic N addition in tropical forest soils will tend to be more pronounced in wet years as opposed to dry years. They also showed that even small changes in moisture can strongly affect N<sub>2</sub>O; a decrease of 7% in the seasonal average WFPS, corresponded to a 50% decrease in N<sub>2</sub>O emissions (Corre et al., 2014).

Martinson et al. (2013) reported N<sub>2</sub>O flux measurements from the initial 21 months of our study, but they had no information regarding the processes responsible for the observed emissions. Given the additional information from this study, we can now infer that the fluxes measured by Martinson et al. (2013) were predominantly denitrification-related and that gaseous N loss was likely dominated by N<sub>2</sub> rather than the small emissions of N<sub>2</sub>O that they observed. We also know that WFPS from Months 35 to 56 (43–71%; Table 1) was lower than that measured from Months 1 to 21 of nutrient manipulation (63–88% WFPS; Martinson et al., 2013). Thus, the difference in the response of N<sub>2</sub>O fluxes to N addition between the two study periods could be reflecting a general dampening of denitrification activity from the slightly wetter Months 1–21 to the slightly drier Months 35–56, which effectively removed the measurable response to N addition (i.e., the already low N<sub>2</sub>O fluxes), while N<sub>2</sub> emissions may have continued to be elevated.

### P-addition Effects on Soil N<sub>2</sub>O Fluxes

As we hypothesized, we began to see P-addition effects on N<sub>2</sub>O fluxes during our study period (Months 35–56), which were not present during the initial 2 years (Months 1–21) of nutrient manipulation (Martinson et al., 2013). At 1000 and 2000 m, soil N<sub>2</sub>O fluxes in P plots were significantly lower than fluxes from N-amended plots, with the same trend—although not significant—when compared to control (Table 1; Figures 1A,B). This may have been related to changes in aboveground net primary production (ANPP) with P addition. Across our elevation gradient, ANPP was limited by P and/or co-limited by N+P, as shown by the trend toward higher basal area increment, which was already evident after 1 year of P addition (at 1000 and 2000 m) and N+P addition (at all elevations) (Homeier et al.,

2012, 2013). If P addition increased ANPP, there may have been an increase in plant uptake of other soil nutrients, including soil mineral N. Since P addition did not change net (Table 3) or gross (Baldos et al., 2015) rates of mineral N production, an increase in uptake of soil mineral N by plants would lead to lower mineral N levels in P plots; although not always significant, we did measure lower mineral N concentrations (especially NO<sub>3</sub><sup>-</sup>) in soils of P plots at 1000 and 2000 m (Table 2). Since NO<sub>3</sub><sup>-</sup> was the main substrate for N<sub>2</sub>O production across our elevation gradient, decreased NO<sub>3</sub><sup>-</sup> concentrations in P plots may have led to reduced N<sub>2</sub>O fluxes. Similar results were observed in a 6-year old leguminous tree plantation in Indonesia, where P addition alleviated plant P limitation and increased root N uptake, resulting in decreased mineral N concentrations and N<sub>2</sub>O fluxes (Mori et al., 2013).

At 3000 m, we possibly observed the same mechanism (i.e., P addition catalyzing N uptake) with slightly different results. At this elevation, there were significant decreases in NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> concentrations (Table 2) with no significant change to N<sub>2</sub>O fluxes. We attribute this to the fact that initial fluxes of N<sub>2</sub>O were too low to detect a decrease (Table 1; Figure 1C). This idea is supported by the fact that N<sub>2</sub>O fluxes at 3000 m were not correlated with any of the measured soil factors, neither for control plots nor across all treatments (Tables 3, 4), which again suggests that the N<sub>2</sub>O fluxes were too low (mostly fluctuating around zero; Figure 1C) to generate any significant relationships with the soil factors known to control N<sub>2</sub>O fluxes.

As shown in the N-addition section above, the effects of N+P addition on net N cycling and soil mineral N concentrations followed the same trends as those for N addition alone (Table 2). However, it is notable that the increases as a result of N addition in the N+P plots were not as strong as the increases in the N plots, presumably because of the opposing effect of P addition. Although N-cycling responses to P addition appeared to be delayed as compared to responses to N addition (i.e., there were no significant effects of P addition during the initial 21 months reported by Martinson et al., 2013), the presence of P in atmospheric deposition could be an important long-term control on N<sub>2</sub>O fluxes in ecosystems where deposition of both nutrients is occurring. For example, although our study did not show any nutrient-addition effects on N<sub>2</sub>O fluxes, Baral et al. (2014) in a different study related to N<sub>2</sub>O fluxes also observed that added P addition increased plant N uptake; in their case this resulted in significantly less N<sub>2</sub>O emissions with N+P addition as compared to N addition alone.

## CONCLUSION

We have shown that soil N<sub>2</sub>O fluxes in our study sites were among the lowest measured in TMFs and that denitrification was the main source of N<sub>2</sub>O, which was possibly being produced in anaerobic microsites. We attribute the low N<sub>2</sub>O fluxes to the conservative soil N cycling along our elevation gradient (Baldos et al., 2015), and the combination of low NO<sub>3</sub><sup>-</sup> concentrations and presumably high available C in the organic layers (at 2000 and 3000 m) which could favor the already low gaseous N losses to be dominated by N<sub>2</sub>. In contrast to the first 21 months of this study

(Martinson et al., 2013) we did not detect significant increases in N<sub>2</sub>O fluxes in Months 35–56, despite an increase in soil N availability. This may be due to the generally low N<sub>2</sub>O fluxes during our measurement period, which we in turn attribute to the lower rainfall and soil moisture contents during our study period. However, we did detect a reduction in soil mineral N concentrations and N<sub>2</sub>O fluxes with P addition, in contrast to the first 21 months when no effects were observed (Martinson et al., 2013). The significant P effect during our study period was probably due to increased uptake of soil mineral N by vegetation after an extended period of P addition, since P is a limiting element for ANPP at our sites (Homeier et al., 2012, 2013). Nitrogen plus phosphorus addition showed similar trends in net rates of mineral N production, mineral N concentrations and N<sub>2</sub>O fluxes to those with N addition alone, although to a lesser degree because of the counteracting effects of P addition. This 5-year study (the work of Martinson et al., 2013 together with our results), strongly illustrated that effects of nutrient addition on soil N<sub>2</sub>O fluxes are not always linear with time of exposure. We observed large inter-annual variation in N<sub>2</sub>O responses, which we primarily attributed to changes of soil moisture conditions, combined with soil characteristics such as texture and organic C content. Without this multiple-year study we would not have been able to detect these changes in nutrient response over time.

## AUTHOR CONTRIBUTIONS

Substantial contributions were achieved by all listed authors and were as follows:

Conception and design of the work: AKM, MC, EV

Acquisition of data: AKM, ALM

Analysis and Interpretation of data: AKM, ALM, MC, EV

Drafting and revising the work: AKM, ALM, MC, EV.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/feart.2015.00066>

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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