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NITROUS OXIDE NITRIFICATION AND DENITRIFICATION ^{15}N ENRICHMENT FACTORS FROM AMAZON FOREST SOILS

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Abstract. The isotopic signatures of ^{15}N and ^{18}O in N_2O emitted from tropical soils vary both spatially and temporally, leading to large uncertainty in the overall tropical source signature and thereby limiting the utility of isotopes in constraining the global N_2O budget. Determining the reasons for spatial and temporal variations in isotope signatures requires that we know the isotope enrichment factors for nitrification and denitrification, the two processes that produce N_2O in soils. We have devised a method for measuring these enrichment factors using soil incubation experiments and report results from this method for three rain forest soils collected in the Brazilian Amazon: soil with differing sand and clay content from the Tapajos National Forest (TNF) near Santarém, Pará, and Nova Vida Farm, Rondônia. The ^{15}N enrichment factors for nitrification and denitrification differ with soil texture and site: −111‰ ± 12‰ and −31‰ ± 11‰ for a clay-rich Oxisol (TNF), −102‰ ± 5‰ and −45‰ ± 5‰ for a sandier Ultisol (TNF), and −10.4‰ ± 3.5‰ (enrichment factor for denitrification) for another Ultisol (Nova Vida) soil, respectively. We also show that the isotopomer site preference ($\delta^{15}N^{\alpha} - \delta^{15}N^{\beta}$, where α indicates the central nitrogen atom and β the terminal nitrogen atom in N_2O) may allow differentiation between processes of production and consumption of N_2O and can potentially be used to determine the contributions of nitrification and denitrification. The site preferences for nitrification and denitrification from the TNF-Ultisol incubated soils are: 4.2‰ ± 8.4‰ and 31.6‰ ± 8.1‰, respectively. Thus, nitrifying and denitrifying bacteria populations under the conditions of our study exhibit significantly different ^{15}N site preference fingerprints. Our data set strongly suggests that N_2O isotopomers can be used in concert with traditional N_2O stable isotope measurements as constraints to differentiate microbial N_2O processes in soil and will contribute to interpretations of the isotopic site preference N_2O values found in the free troposphere.

Key words: Amazon forest soils; denitrification; isotopic enrichment factors; isotopomers; nitrification; nitrous oxide; site preference.

Introduction

Nitrous oxide (N_2O) is a greenhouse gas that has increased in the troposphere at a rate of 0.25% ± 0.05% per year between 1980 and 1998 (Ehhalt et al. 2001). There are large uncertainties in the budget of N_2O, and especially in the factors that are causing the N_2O atmospheric increase (Cicerone 1989, Ehhalt et al. 2001). The major N_2O sources are bacterial production during nitrification and denitrification in tropical rain forest soils, agricultural fields and oceans. The major sink is destruction by photolysis and reaction with $O(^1D)$ in the stratosphere (Khalil and Rasmussen 1992). Microbial activity in soils is the largest single source of N_2O but is difficult to characterize because soil emissions have high such spatial and temporal variability. The combination of large variability and sparse measurements contributes substantially to the uncertainties in the global N_2O budget.

Comparison of the stable isotopes of N (including the intramolecular distribution of ^{15}N in N_2O) and O in atmospheric N_2O, its sources and in firn air (air preserved in deep snow by slow diffusion) provides

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another tool for constraining the N_2O budget (Kim and Craig 1993, Rahn and Wahlen 1997, 2000, Naqvi et al. 1998, Rahn et al. 1998, Pérez et al. 2000, 2001, Kaiser et al. 2002, Rockmann et al. 2003, Sowers et al. 2003, Park et al. 2004). Recent measurements of the isotopic composition of N_2O from firn air found that from preindustrial times until the present, the ^{15}N and ^{18}O isotopic composition of tropospheric N_2O have decreased by 1.9‰ and 2.9‰, respectively (Rockmann et al. 2003, Sowers et al. 2003). This isotopic shift supports the hypothesis that a significant (~30%) increase in agriculturally derived N_2O (which on average is relatively light in ^{15}N and ^{18}O with respect to atmospheric N_2O) during the last century is responsible for changes in the mixing ratio of N_2O in the atmosphere. Although these results demonstrate the promise of using isotopes to constrain the global N_2O budget, the model used by Sowers et al. (2003) has large uncertainties that stem from uncertainties in the isotopic signatures of various source components, especially the soil source. Thus, additional characterization of the isotopic signatures of N_2O produced in soils is required if stable isotopes are to be used to further constrain the global N_2O budget.

Two processes regulate N_2O emissions from soils: nitrification and denitrification. The "hole-in-the pipe" model proposed by Firestone and Davidson (1989) provides a useful framework for explaining the controls on N_2O and nitric oxide (NO) emissions. During nitrification, NH_4^+ is oxidized to NO_3^- and produces both NO and N_2O, dissolved gases that can escape to soil air and the overlying atmosphere (leak out of the pipe). Under anaerobic conditions, NO_3^- is reduced to N_2 by denitrifiers. NO and N_2O are intermediates in the reduction that again can leak into the soil pore space and escape to the overlying atmosphere. Conditions favoring N_2O production via nitrification and denitrification in soils differ according to the "hole-in-the-pipe" model with (1) availability of nitrogen as NH_4^+ and NO_3^- (amount of flow through the "pipe"; Keller et al. 1988), (2) efficiency of N_2O production during nitrification or denitrification (size of the leaks allowing gases to escape to the atmosphere, or size of the "hole in the pipe"), and (3) efficiency of gas transfer from soil to atmosphere (e.g., the ability of the N_2O produced to escape to the atmosphere before it gets reduced to N_2; Matson and Vitousek 1990, Matson et al. 1990, Davidson 1992, Keller and Reiners 1994, Davidson and Schimel 1995).

The microbial enzymatic pathways associated with the N_2O emissions from nitrification and denitrification and their combined effects on the isotopic composition of the emitted N_2O have been discussed in a recent review (Stein and Yung 2003). The ^{15}N enrichment factors (ε) values, defined as $\varepsilon = \delta^{15}N\text{-}N_2O_{emitted} - \delta^{15}N_{substrate}$, for nitrification and denitrification measured in pure culture bacteria studies showed more negative values for nitrifying bacteria ($\varepsilon = -45‰$ to $-66‰$) compared to those produced by denitrifying bacteria ($\varepsilon = -12‰$ to $-28‰$) (Wahlen and Yoshinari 1985, Yoshida 1988, Yoshinari and Koike 1994, Webster and Hopkins 1996, Barford 1997, Yoshinari et al. 1997, Barford et al. 1999). Nitrification leads to N_2O that is highly depleted in ^{15}N ("lighter" N_2O) whereas denitrification is less discriminating against ^{15}N ("heavier" N_2O). Given these distinct signatures, it is therefore possible, in principle, to use the isotopic signature of emitted N_2O, in combination with N_2O concentration, to partition the N_2O production between nitrification and denitrification in soils with similar ^{15}N in substrates.

The determination of ^{18}O in the N_2O emitted from soils should also yield information on the microbial process producing N_2O. The $\delta^{18}O$ value of nitrification-derived N_2O should reflect the oxygen isotope composition of hydroxylamine (NH_2OH), soil air molecular oxygen, and soil water whereas the $\delta^{18}O$ value of denitrification-derived N_2O should reflect the isotopic composition of the substrate (NO_3^-) and intermediate by-products, and the oxygen isotope effects associated with each denitrification step (Pérez 2005). Sutka et al. (2003, 2004) evaluated under aerobic conditions N_2O production from hydroxylamine by methanotrophic nitrification (*Methylococcus capsulatus*) and nitrification (*Nitrosomonas Europaea*). They found two distinctive ^{15}N and ^{18}O isotope fingerprints and suggested that the enzymatic pathway of N_2O production via nitrification can be differentiated in this manner. Enrichments in ^{18}O have been observed in the N_2O remaining after reduction to N_2 has occurred (Barford 1997, Yoshinari et al. 1997, Dore et al. 1998, Naqvi et al. 1998). Therefore, differences in the microbial pathways in soils should also be reflected in the ^{18}O isotopic composition of emitted N_2O.

In addition to the determination of the bulk ^{15}N and ^{18}O isotopic compositions of N_2O, measurement of the nitrogen isotopic compositions of the central vs. terminal nitrogen atom in N_2O may also serve as an additional tool for constraining the global N_2O isotope budget (Toyoda et al. 2001, Kaiser et al. 2002, Rockmann et al. 2003, Sutka et al. 2003, 2004, Park et al. 2004). As N_2O is a linear molecule with two nitrogen atoms with the structure NNO, different fractionations may occur at the two non-equivalent nitrogen positions. The ^{15}N position nearest the oxygen atom in the N_2O molecule is referred to as the α site (or position 2) and the terminal N atom referred to as the β site (or position 1), then $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ may have different values depending on the mechanisms of N_2O production (e.g., Yoshida and Toyoda 2000, Perez et al. 2001). If different N_2O sources (e.g., nitrifying and denitrifying bacterial sources) exhibit a different site preference (i.e., $\delta^{15}N$ alpha or $\delta^{15}N$ beta), then site preference may be used in a manner analogous to using the overall $\delta^{15}N\text{-}N_2O$ values to differentiate between processes of production and consumption of N_2O. The site preference differences found in the study done by Sutka et al. (2003, 2004), for example, allowed

them to propose two different nitrifying enzymatic pathways for N_2O production, demonstrating the usefulness of the site preference measurements.

Presently, the limitations associated with the use of the stable isotopes of N_2O to differentiate between the microbial pathways of N_2O emitted from soils include the following: (1) Variations in the isotopic composition of emitted N_2O depend on the factors mentioned above in the hole-in-the-pipe model description; thus, in order to interpret measured N_2O isotopic values, characterization of the isotopic composition of substrates is required. (2) We do not know the ^{15}N and ^{18}O isotopic enrichment factors associated with nitrification and denitrification for real soil conditions. Published values of ^{15}N enrichment factors are all based on studies of pure bacterial cultures, rather than the consortia of bacteria and fungi found in soils. (3) The reported values of the intramolecular distribution of ^{15}N in the N_2O molecule from different studies were mostly derived from pure culture studies and they are difficult to compare because of the lack of a unified isotope standard in the scientific community.

To date, there is no controlled study in the literature documenting the nitrogen and oxygen isotope effects associated with N_2O formation via nitrification and denitrification in soils. Perez et al. (2000 and 2001), found significant isotopic differences in the bulk ^{15}N and ^{18}O isotopic composition of N_2O emitted from soils, based on work done in tropical rain forest soils and an agricultural field. Natural tropical rain forest soils showed large differences associated with soil texture, with lighter N_2O isotopic composition in a sandy Ultisol soil than a clay Oxisol. These results suggested that the ^{15}N of N_2O emitted from soils becomes more enriched when the N_2O has a higher probability of being reduced to N_2 (as in clay soils). Nitrous oxide emissions from agricultural soils are significantly more depleted in ^{15}N than natural soils (Perez et al. 2001), possibly because of larger available supplies of substrate N for nitrifying and denitrifying microbial communities in fertilized soils. In these agricultural soils, the site preference of the N_2O emitted increased in the days after being irrigated, suggesting that a change in microbial enzymatic pathway might be responsible for the site preference shifts. Perez et al. (2001) also found that the $\delta^{18}O$ values of N_2O emitted from agricultural soils was a few per thousand higher than that of atmospheric O_2 ($\delta^{18}O$ of $O_2 = 23.5‰$ relative to SMOW, standard mean ocean water), suggesting that incorporation of oxygen from molecular O_2 during N_2O formation via nitrification was greater than that of oxygen from soil water ($\delta^{18}O$ $-26‰$ to $-20‰$).

Based on the previous work done by Perez et al. (2000 and 2001) and from other studies, it is clear that ^{15}N enrichment factors for each process producing N_2O might be different in different soil types and that their values may allow a partitioning between nitrification and denitrification in a particular soil to be studied (Perez 2005). Here, we report a method for measuring ^{15}N isotope enrichment factors for nitrification and denitrification for three tropical forest soils using simple incubation methods. The incubation study also yields information on how ^{18}O isotopic signatures and the ^{15}N site preference in N_2O differs with nitrification and denitrification in each soil. The usefulness of the obtained ^{15}N enrichment factors per soil is that by means of an isotope mass balance it will be possible to differentiate the relative contribution of nitrification and denitrification from these soils by simply measuring the ^{15}N isotopic composition of emitted N_2O and the emission size in the field. This method is non invasive, in contrast with the currently used methods for differentiating these microbial processes in the field (such as ^{15}N labeling or acetylene (C_2H_2) inhibitions).

Methods

Soil collection

We used three soil types for our incubation studies. Soils were sampled from areas of active measurement of soil nitrogen trace gas fluxes located in (1) the Tapajos National Forest (TNF), near Santarém, Para state, Brazil (2°64′ S, 54°59′ W) and (2) the Nova Vida Farm, in Rondonia state, Brazil (10°30′ S, 62°30′ W). At the TNF, we collected soils from two sites, clay-rich (Oxisol) and sandy loam (Ultisol) soils (described in Silver et al. 2000, Telles et al. 2003). Nova Vida Farm soil has been classified as red-yellow podzolic latosol in the Brazilian classification and as Kandiudult (Ultisol) in the U.S. classification (Moraes et al. 1995).

At all three sites, the soils were collected in primary forest areas. Both N_2O and NO emissions have been performed at each site (Table 1). For all three soil types, the NO emission values were higher in the dry season while N_2O emission values were higher in the wet season. The relative amounts of N_2O emissions in each site during the rainy season were as follows: TNF Oxisol > Nova Vida Ultisol > TNF Ultisol. The TNF Oxisol N_2O emissions were twice those in the Nova Vida Ultisols and 10 times higher than those in the TNF Ultisols. The NO emission amount during the dry season was: TNF Oxisol = TNF Ultisol > Nova Vida Ultisol. The NO emissions at Nova Vida Ultisol had values that were half those from the TNF soils.

Soils were sampled by coring (10 cm inner diameter) the 0–10 cm depth interval. TNF soils were sampled in March 2002, while Nova Vida soils were sampled in April 2002. Soil samples (2 kg) were stored in plastic resealable freezer bags and refrigerated (at 4°C) until the second week of April 2002 when the incubations were performed.

Soil incubations for the determination of the nitrification and denitrification ^{15}N enrichment factors

We determined the ^{15}N enrichment factors for nitrification (NH_4^+ to N_2O) and for the "first" step of denitrification (NO_3^- to N_2O) with our experimental

TABLE 1. NO and N_2O soil emissions from the study sites.

Site	NO emissions by season (ng NO-N·cm^{-2}·h^{-1})		N_2O emissions by season (ng N_2O-N·cm^{-2}·h^{-1})		References
	Dry	Rainy	Dry	Rainy	
Nova Vida Ultisol sandy					Garcia-Montiel et al. (2001)
1998	3.3	0.5	0.1	7.6	
1999	NA	1.0	NA	6.7	
TNF Oxisol clay					Keller et al. (2005)
2000	12.5 (3.1)	NA	2.7 (1.0)	13.0 (0.9)	
2001	10.4 (4.4)	7.7 (3.4)	1.2 (0.3)	12.8 (1.2)	
TNF Ultisol sandy					Keller et al. (2005)
2000	9.4 (1.1)	NA	1.0 (0.1)	2.3 (0.2)	
2001	15.3 (9.9)	2.3 (1.3)	1.2 (0.4)	2.0 (0.4)	

Note: Values in parentheses are the standard errors of 15 dates when samples were collected; NA indicates that data were not available.

setup. We made soil incubations using 10 kPa of C_2H_2 as an inhibitor to block N_2O emissions from nitrification and from N_2O to N_2 reduction via denitrification (Tiedje et al. 1989, Mosier and Klemedtsson 1994). The "control" incubations (without C_2H_2 addition) produced N_2O molecules derived from both nitrification and the "first" step of denitrification (NO_3^- to N_2O); thus, they exhibited an isotopic fingerprint characteristic of a combination of both processes. In the acetylated soils, the emitted N_2O had the isotopic signature that can be ascribed to the "first" step of denitrification alone. An isotope mass balance therefore allowed us to calculate the ^{15}N isotopic enrichment factor associated with nitrification from the difference between the acetylated (denitrification only) and the control (nitrification + denitrification) incubations.

For this experimental approach to work, we needed to ensure that negligible amounts of N_2O were reduced to N_2 in the control experiments, so that the N_2O isotopic fingerprint in the control experiments was not affected by an additional microbial pathway (i.e., reduction of N_2O to N_2). When soils are incubated at low soil water filled pore space (WFPS), i.e., under less extremely reducing conditions, the fraction of N_2O being reduced to N_2 is negligible (Davidson et al. 1986, Weier et al. 1993, Del Grosso et al. 2000). In addition, we can use the ratio of NO to N_2O emitted as a guide to the aerobic conditions of the soil as described in the hole-in-the-pipe model (Davidson 1993); and by experimental results of soil incubation studies (Davidson et al. 1986, Weier et al. 1993, Del Grosso et al. 2000). According to Firestone et al. (1979), NO:N_2O ratio values higher than 1 imply that nitrification is the dominant process in the soils. In order to minimize the possibility of N_2O to N_2 reduction in our control experiments, we did the following: (1) worked with WFPS values lower than 0.5, (2) used homogenized and sieved soils to reduce the heterogeneity of the soils which potentially could provide anaerobic microsites to induce N_2O to N_2 reduction (despite the fact that the WFPS values are small), (3) worked with the field soil water content to make sure the water distribution in the soil was homogeneous (to diminish the likelihood of N_2O to N_2 reduction in the places where water was unevenly distributed), (4) ascertained that the NO to N_2O ratio was significantly higher than 1, thus ensuring that the conditions were aerobic, and (5) kept the duration of the incubation experiment short enough (15 h) to avoid the production of an anaerobic atmosphere inside the jars due to the high decomposition rates in soils. See the Appendix for details that justify this assumption is correct for our experimental setup.

Soil samples were homogenized and sieved (4 mm) to remove large roots, and uniform amounts (100 or 200 g, depending on the N_2O emission associated with the soil sample) were weighed and placed in 2-L jars (18 jars in all). Nine jars were exposed to 10 kPa of C_2H_2 and nine remained under natural conditions. The jars with 10 kPa of C_2H_2 were closed and left for 4 h. The use of C_2H_2 has two potential problems for the incubation experiments: (1) the presence of acetone as an impurity and (2) C_2H_2 itself could be an additional carbon source for the microbial population. We avoided the acetone contamination by purifying the gas with an activated charcoal trap before exposing the soils. We also aired the soils for 1 h after C_2H_2 exposure to minimize the possibility of the unreacted acetylene acting as an additional carbon source and also to minimize its interference with the measurements of NO by the chemiluminescence method. We took triplicate gases and soil samples of the control and acetylated soils at 0, 5, 10, and 15 h of incubation. The parameters to characterize for each time of collection were soil water content, N_2O and NO mixing ratios, KCl-extractable NH_4^+ and NO_3^-, total nitrogen (TN) content; ^{15}N isotopic composition for TN, NH_4^+, and NO_3^- and ^{18}O isotopic composition of H_2O. The isotopic characterization of N_2O was done at 5, 10, and 15 h of incubation because at time 0 there was too little N_2O for isotopic analysis.

Sampling and analysis for NO and N_2O mixing ratios

Fluxes of NO were measured with a Unisearch Associates LMA-4 NO_2 analyzer (Unisearch Associates,

Concord, Ontario, Canada; see Garcia-Montiel et al. 2001 for a more detailed description). Headspace gas samples were collected with 10 mL glass syringes covered with aluminum foil and equipped with stopcocks and immediately injected in a NO_2/NO free air stream. The sampled air containing the NO that entered the analyzer was oxidized to NO_2 by reaction with CrO_3, and the air stream was passed across a fabric wick saturated with luminol solution, which is oxidized when in contact with NO_2 to produce chemiluminiscence. This chemiluminiscence is measured by a photomultiplier tube (Unisearch Associates) and is directly proportional to the mixing ratio of the NO_2. The ambient NO_2 and NO entering the air stream were scrubbed through a gas washer filled with activated charcoal and equipped with a diffuser stone. The output from the NO analyzer was collected at 0.5-s intervals with a CR10X Campbell data logger (Campbell Scientific, Logan, Utah, USA). Estimation of NO concentration at each injection used the peak height method, and NO fluxes were calculated from the rate of increase or decrease of NO concentrations in the atmosphere inside a glass jar. Calibration of the LMA-4 NO_2 was done using a 49.2 ppbv NO standard obtained by dilution of a 1.032 ppmv NO standard in O_2-free N_2 (Scott-Marrin, Riverside, California, USA) with NO/N_2O-free air. This same standard was sequentially diluted with NO-free air to develop calibration curves for estimation of NO concentrations. The data recorded by the data logger was stored in a computer.

N_2O mixing ratios were determined by collection of jar incubation headspace air with 20 mL nylon syringes and measurement by electron capture detector (ECD) gas chromatography. Calibration curves with a N_2O standard (985 ppb N_2O in air, Scott Specialty Gases, Plumsteadville, Pennsylvania, USA) were made each day prior to analysis of the samples collected. The relationship of NO to N_2O emission is a sensitive indicator of nitrification vs. denitrification (Firestone et al. 1979).

N_2O stable isotope collection

Once an aliquot of the sample had been collected for N_2O mixing ratio determinations, a N_2O collection system was attached to one of the valves of the incubation jar (Fig. 1). The collection system consisted of an evacuated stainless steel canister attached to a tee with a septum in one end and a drierite/ascarite trap (for removal of CO_2 and H_2O) at the other end. The other end of the drierite/ascarite trap was connected to the aforementioned sample jar via a valve and tubing. To collect the N_2O sample, a syringe with needle was inserted into the septum of the tee, then valve 1 was opened and a vacuum was pulled with the syringe between the collection system and the jar. This procedure was repeated three times to ensure that ambient air content in the collection system was expunged. The jar valve (2) was opened and closed,

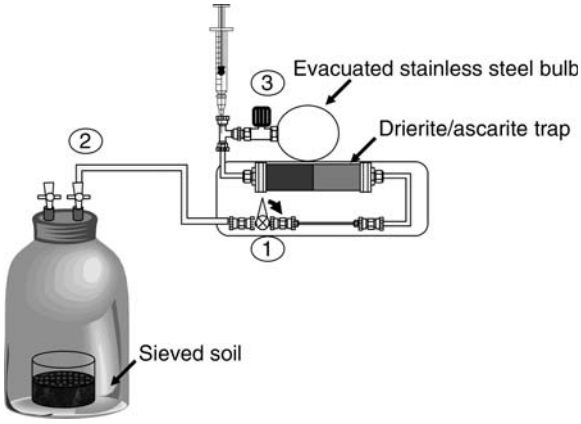

FIG. 1. Soil incubation N_2O collection system for stable isotope analysis.

and the same vacuum procedure with the syringe was repeated. Then the jar valve was opened and the stainless steel canister valve (3) was opened so that air from the jar headspace was transferred inside the canister after passing through the drierite/ascarite trap. After a 2-min equilibration period, the canister valve was closed and the sample stored until it was analyzed at the University of California-Irvine for bulk ^{15}N and ^{18}O analysis. The samples were then sent to the University of California–Berkeley for the determination of the ^{15}N intramolecular distribution of N_2O.

Soil water content and inorganic nitrogen concentrations

Immediately after the collection of N_2O in the stainless steel canisters, the jars were opened and the soils extracted to measure the concentrations of extractable NH_4^+, NO_3^-, and TN, as well as their isotopic compositions. An aliquot of 10 g of soil was added to 100 mL of 2 mol/L KCl and shaken for 1 h. The solution was filtered with a KCl-prewashed Whatman 42 filter. An aliquot of 15 mL of the extract was preserved with 100 μL of a solution of phenyl mercuric acetate (PMA; 0.06 mg/mL) and stored at 4°C prior to analysis for NH_4^+ and NO_3^- concentrations at the Laboratório de Biogeoquímica Ambiental at the Centro de Energia Nuclear na Agricultura (CENA) of the University of São Paulo, Brazil. Another aliquot of 85 mL of the KCl extract was preserved with H_2SO_4 (2 mL/L) and stored at 4°C for stable isotope analysis. The concentrations of NH_4^+ and NO_3^- were determined by modified salicylate–hypochlorite and modified Griess-Illosvay methods, respectively (Mulvaney 1996) using a lab-built continuous flow autoanalyzer, consisting of a peristaltic pump connected to a conductimeter and a spectrophotometer.

Approximately 30 g of each soil sample were weighed and oven dried at 105°C. After drying for 48 h, the samples were weighed again and gravimetric water content was determined by weight loss. Ten grams of

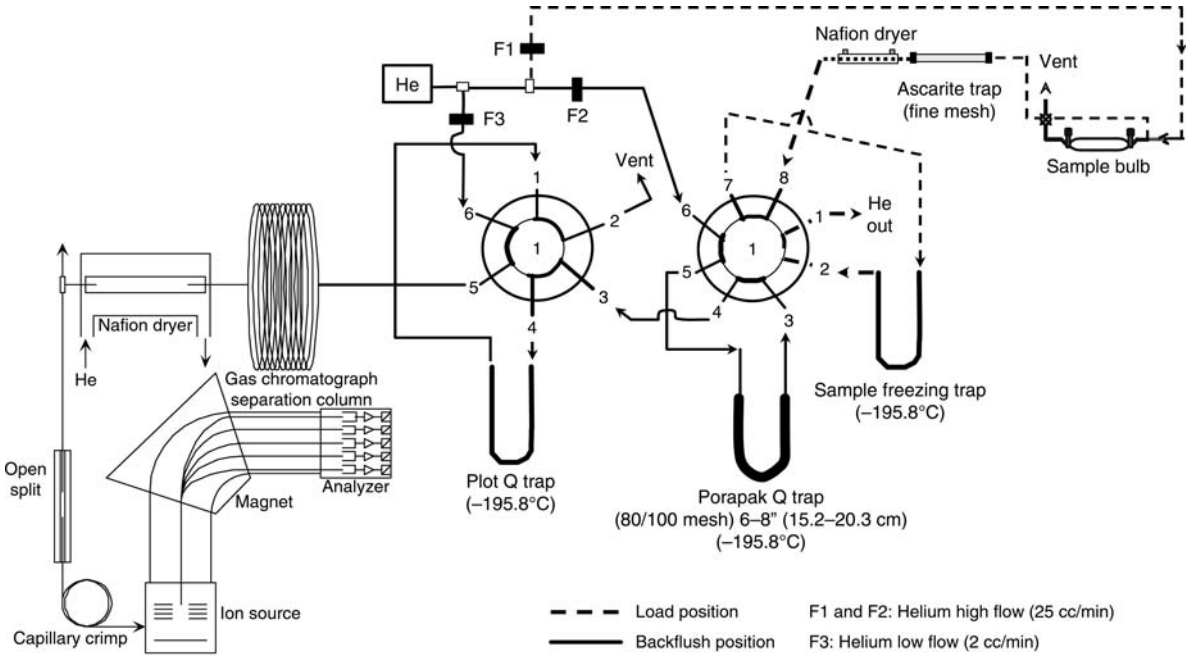

FIG. 2. N_2O pre-concentrator for mass spectrometry analysis of ^{15}N and ^{18}O in N_2O. In the figure, the six-port valve is in the load position while the N_2O is being trapped cryogenically in the Plot Q trap. After that procedure has happened, the valve is switched to the inject position, and the helium (He) carries the sample to the gas chromatograph.

the fraction of the unweighed portion of each soil sample was stored in a glass vial with an air-tight lid and frozen for ^{18}O analysis of H_2O. The remaining unweighed portion of soil was stored at 4°C for TN analysis in the lab.

N_2O purification and isotope measurement of $\delta^{15}N$ and $\delta^{18}O$ of N_2O

The samples stored in the stainless steel canisters were transferred into glass bulbs (either 100 mL or 250 mL in volume) with two valves. The smaller glass bulbs were chosen for the samples with the highest N_2O concentration. A sample placed in a bulb was connected to the inlet of a custom-built gas pre-concentrator for N_2O stable isotope analysis (Fig. 2). High-flow ultra-high purity helium (25 mL/min) carried the sample first to an ascarite and then to a $MgClO_4$ trap (to remove CO_2 and H_2O), then to a Nafion dryer (to further remove H_2O; Perma Pure, Toms River, New Jersey, USA), and finally to the next trap in line (LN1) where the N_2O was condensed cryogenically (195°C) and the other non-condensable gases were removed (N_2, O_2, CH_4, CO). Enough helium was used to flush the sample bulb volume three times and ensure that all the sample was extracted from the bulb. The N_2O on the LN1 trap was released by warming it to room temperature and transferring it cryogenically to a Porapak Q trap (Alltech, Deerfield, Illinois, USA) at room temperature to remove the hydrocarbons remaining in the sample. Finally, the sample was transferred into a cryofocusing trap (Poraplot Q, Alltech) before its injection into a gas chromatograph (GC). The sample was transferred to the GC by a stream of low-flow UHP helium (3 mL/min) and the N_2O was separated from remaining traces of CO_2 by a 25-m Poraplot Q capillary column (Alltech). Finally its $\delta^{18}O$ and average $\delta^{15}N$ isotopic compositions were measured at the University of California-Irvine by a Finnigan MAT model Delta XL+ isotope ratio mass spectrometer (ThermoElectron, Waltham, Massachusetts, USA) connected to the GC via an open split. The method uncertainty, determined by repeated measurements of an N_2O isotope standard, was $\pm 0.2‰$ and $\pm 0.3‰$ for ^{15}N and ^{18}O, respectively.

Measurement of the intermolecular nitrogen isotopic composition of N_2O

The $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ data presented here were measured at University of California-Berkeley using a Finnigan CH_4/N_2O gas pre-concentrator connected to a Finnigan MAT model 252 isotope ratio mass spectrometer. This mass spectrometer has a relatively high magnetic field and a large dispersion, conditions that allow detection of the NO^+ fragments needed to do the N_2O isotopomer measurements. Details of the method are explained elsewhere (Kaiser et al. 2004, Park et al. 2004). The external long-term measurement precision on air samples containing ~1.3 nmol of N_2O is $\pm 0.8‰$ for $\delta^{15}N^{\alpha}$. Samples are first measured relative to the University of California–Berkeley N_2O working standard, which has an average ^{15}N isotopic composition of $\delta^{15}N^{bulk} = 0.07‰ \pm 0.06‰$ relative to air-N_2 and an ^{18}O isotopic composition of $\delta^{18}O = 41.55‰ \pm 0.20‰$

relative to V-SMOW, and measured isotopic compositions were then converted to the relevant international isotope scales. For $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$, calibration of the University of California-Berkeley working standard on the international air-N_2 isotope scale was carried out by a mass spectrometric method based on addition of different amounts of doubly labeled $^{15}N_2O$ to pure N_2O gas (Kaiser et al. 2004), yielding $\delta^{15}N^{\alpha} = 12.2‰ \pm 0.6‰$ and $\delta^{15}N^{\beta} = -12.0‰ \pm 0.6‰$ relative to air-N_2. However, we note that a calibration method based on site-specific chemical conversion of HNO_3 and NH_4OH to N_2O (Toyoda and Yoshida 1999, Yoshida and Toyoda 2000) resulted in significantly different values for $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ of tropospheric N_2O relative to air-N_2 from those measured at the University of California-Berkeley: $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ of tropospheric N_2O from the Tokyo group were $16.4‰ \pm 1.6‰$ and $-2.4‰ \pm 1.6‰$ relative to air-N_2, respectively, vs. $27.0‰ \pm 0.9‰$ and $-14.5‰ \pm 1.0‰$ relative to air-N_2, respectively, for the Berkeley group, despite the fact that $\delta^{15}N^{bulk}$ and $\delta^{18}O$ isotopic compositions of tropospheric N_2O are in good agreement between the two groups. These discrepancies in the site-specific $\delta^{15}N$ isotopic compositions of tropospheric N_2O scaled to air-N_2 have yet to be completely resolved. Until these differences are resolved, we note that tropospheric N_2O can serve as a secondary standard for interconversion between data sets, if necessary, as suggested in Park et al. (2004).

^{15}N isotopic composition of extractable NH_4^+, NO_3^-, and TN and ^{18}O isotopic composition of soil water

Isotopic composition of NO_3^- and NH_4^+.—The stored KCl extracts were processed using the diffusion technique described by Sigman et al. 1997 and Holmes et al. 1998. The final $(NH_4)_2SO_4$ salt samples fixed in the acid traps were placed in tin cups and analyzed for ^{15}N content by continuous flow elemental analyzer isotope ratio mass spectrometer (CF-EA-IRMS), consisting of a Fisons 5200 elemental analyzer (Fisons, Valencia, California, USA) connected to a Finnigan Delta XL at the Laboratorio de Ecología Isotópica del Centro de Energía Nuclear na Agricultura (CENA), University of São Paulo, Piracicaba, Brazil.

Amount and isotopic composition of TN.—The fresh soils samples stored at 4°C were taken to the Laboratorio de Ecología Isotópica at CENA where they were dried at 60°C for 24 h. Samples were sieved (2 mm) and milled, and total carbon and nitrogen content and ^{15}N isotopic composition was determined by CF-EA-IRMS. The nitrogen content analyzed this way is the sum of organic and inorganic N and is reported as percentage of total soil mass.

^{18}O isotopic composition of soil H_2O.—We determined the $\delta^{18}O$ values of soil water by means of a CO_2 microequilibration method (Moreira et al. 1997). The $\delta^{18}O$ values are expressed relative to the Vienna-standard mean ocean water (V-SMOW).

Results

Calculation of the nitrification and denitrification ^{15}N enrichment factors

Because the incubation experiment was conceived as a closed system, we first assume that the reactions can be modeled as a Rayleigh distillation process (Rayleigh 1896, Mariotti et al. 1981). The isotope ratio of the initial bulk composition of the substrate (R_s) and the instantaneous isotope ratio of the product (R_p) are related to the fraction of the residual substrate (f) and the isotope fractionation factor ($\alpha_{p\text{-}s}$) associated with the process by the following equation: $R_p/R_s = f^{\alpha\text{-}1}$. When the substrate availability is considered an infinite reservoir (f is close to 1), then the enrichment factor from product to substrate ($\varepsilon_{p\text{-}s} = 1000(\alpha_{p\text{-}s} - 1)$) can be approximated as $\varepsilon_{p\text{-}s} = \delta_p - \delta_s$, where δ_p is the isotopic composition of the product (the accumulated and instantaneous product are equal) and δ_s the isotopic composition of the substrate, both expressed in the delta notation ($\delta = [(R_{sam}/R_{std}) - 1] \times 1000$, where "sam" stands for sample and "std" stands for standard; Mariotti et al. 1981).

The enrichment factors in our incubation experiments were determined by the following steps:

1) Calculation of an isotope mass balance to get the weighted isotope signature of N_2O (δ_p) for each process:

$$\delta^{15}\text{N-N}_2\text{O}_{tot} \times [\text{N}_2\text{O}]_{tot} = \delta^{15}\text{N-N}_2\text{O}_{nit} \times [\text{N}_2\text{O}]_{nit} + \delta^{15}\text{N-N}_2\text{O}_{den} \times [\text{N}_2\text{O}]_{den}$$

where $\delta^{15}N\text{-}N_2O_{tot}$, $\delta^{15}N\text{-}N_2O_{nit}$, and $\delta^{15}N\text{-}N_2O_{den}$ are the N_2O isotopic signature of the total, nitrification-derived, and denitrification-derived emitted N_2O, respectively, and $[N_2O]_{tot}$, $[N_2O]_{nit}$, and $[N_2O]_{den}$ are the sum of N_2O mixing ratio in the jar headspaces during the incubation time from total, nitrification-derived, and denitrification-derived N_2O, respectively. In this equation, $\delta^{15}N\text{-}N_2O_{tot}$, $\delta^{15}N\text{-}N_2O_{den}$, $\delta^{15}N\text{-}N_2O_{nit}$, and $[N_2O]_{nit}$ are unknowns, and we calculated $[N_2O]_{nit}$ by $[N_2O]_{nit} = [N_2O]_{tot} - [N_2O]_{den}$ since the amount of N_2O produced from total (control soils) and denitrification-derived (from the soils incubated under 10 kPa C_2H_2) were both known.

2) Calculation of $\delta^{15}N\text{-}N_2O_{tot}$ and $\delta^{15}N\text{-}N_2O_{den}$ was done by taking advantage of the strong correlation between N_2O isotope signature and N_2O mixing ratios. We got the source N_2O fingerprint by means of "Keeling plots" where the intercept represents the isotopic value of the measured processes (Fig. 3).

3) Determination of the ^{15}N isotope composition of substrates (δ_s) for nitrification ($\delta^{15}N\text{-}NH_4^+{}_{nit}$) and denitrification ($\delta^{15}N\text{-}NO_3^-{}_{den}$) to calculate ε_{p-s}. Both substrates can be considered infinite reservoirs in the case of our incubations (see Appendix for details). The $\delta^{15}N\text{-}NH_4^+{}_{nit}$ value used is the average of the ^{15}N isotopic values taken at 5, 10, and 15 h (Table 2). The

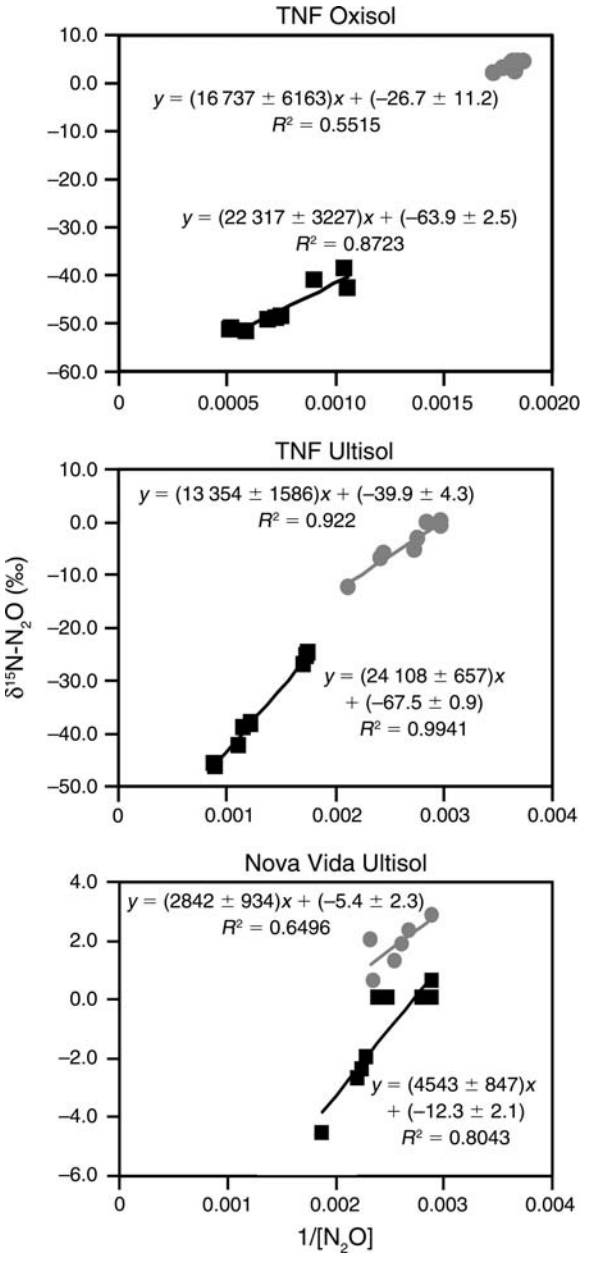

FIG. 3. ^{15}N "Keeling plots" from a textural gradient of forest soils in the Amazon basin. Control and acetylated incubated soils are represented by squares and circles, respectively. The *y*-intercepts in the linear regressions are the $\delta^{15}N\text{-}N_2O_{tot}$ and $\delta\ ^{15}N\text{-}N_2O_{den}$ for each soil.

$\delta^{15}N\text{-}NO_3^-$ was the average of the ^{15}N isotopic values taken at 0, 5, 10, and 15 h (Table 2).

4) Using the average value for δ_s ($\delta^{15}N\text{-}NH_4^+{}_{nit}$ and $\delta^{15}N\text{-}NO_3^-{}_{den}$) and δ_p ($\delta^{15}N\text{-}N_2O_{nit}$ and $\delta^{15}N\text{-}N_2O_{den}$), ε_{p-s} was calculated with the equation $\varepsilon_{p-s} = \delta_p - \delta_s$.

For all soil incubations, we found that the N_2O production rate in the control jars was significantly higher than in the acetylated soils (Table 2), suggesting that the inhibition of nitrification in the acetylated soils was successful.

The ^{15}N enrichment factors for nitrification were calculated for the TNF Oxisol and TNF Ultisol soils (Table 3) because the condition of an infinite supply of substrate was met in the control experiments (see Appendix). Due to the fact that in the Nova Vida Ultisol soils the NH_4^+ concentration in the control experiment declined by 66% during the incubation time, the substrate could not be considered an infinite reservoir. Therefore, we cannot calculate de ^{15}N enrichment factors for these soils. However, we determined the ^{15}N enrichment factors for the "first" step of denitrification for all the studied soils because the infinite substrate reservoir condition was met (see Appendix).

The nitrification-derived N_2O emitted from the TNF Oxisol and TNF Ultisol was 70% and 61% of the total N_2O emitted, respectively, whereas the Nova Vida Ultisol was only 13% (Table 3). The difference was mainly due to the low NH_4^+ concentrations in the NV soils which made the nitrification process be limited by the substrate availability. For all three soils, the "Keeling plots" for ^{15}N and the ^{15}N internal distribution of N_2O showed linear correlations with significant R^2 values (Figs. 3 and 4). Also, the calculated enrichment factors for nitrification were larger (i.e., more negative) than those for denitrification for all the studied soils, as previously shown by other studies (see Table 3 and references therein). The ^{15}N enrichment factors for nitrification for the TNF Oxisol and TNF Ultisol soils were about the same (Table 3) although the magnitude of the fractionation is larger than the range reported in the literature (i.e., more negative enrichment factors).

The ^{15}N enrichment factor for denitrification for the Nova Vida Ultisol soils is within the range reported in the literature (Table 3). In contrast, the ^{15}N enrichment factors for denitrification for the TNF Oxisol and TNF Ultisol showed a larger fractionation (more negative values) in comparison with the reported values for denitrification in the literature.

The $\delta^{18}O$ of the emitted N_2O

The $\delta^{18}O\text{-}N_2O$ values of the nitrification plus denitrification N_2O source in the control experiment were determined by the shift in $\delta^{18}O$ in N_2O during the incubation period. As with ^{15}N, we used "Keeling plots" to determine the $\delta^{18}O$ isotopic fingerprint for nitrification and denitrification of the emitted N_2O (Fig. 5). We found a significant linear correlation in the TNF soils control incubations (Fig. 5). The $\delta^{18}O\text{-}N_2O$ values of the control incubations determined by the intercept of the Keeling plots linear regressions were 14.7‰ ± 1.8‰ ($R^2 = 0.62$), 17.8‰ ± 1.0‰ ($R^2 = 0.95$), and 30.5‰ ± 4.7‰ ($R^2 = 0.32$) for TNF Oxisol, Ultisol, and NV Ultisol, respectively. In the acetylene incubations of TNF Oxisol and NV Ultisol (where denitrification is the only process happening), Keeling plots did not yield enough data to determine the ^{18}O isotopic fingerprint of denitrification

TABLE 2. Measured parameters (mean ± SD) in the soils during the incubation period.

Parameters	TNF Oxisol (clay)		TNF Ultisol (sandy loam)		Nova Vida Ultisol (sandy)	
	Control	Acetylated	Control	Acetylated	Control	Acetylated
N_2O (ng N/gds)†	19.4 ± 1.8	0.4 ± 0.3	11.8 ± 0.4	2.1 ± 1.1	2.1 ± 0.4	1.1 ± 0.3
NO (ng N/gds)†	673 ± 21	111 ± 8	870 ± 34	99 ± 21	67.5 ± 0.2	11.5 ± 4.4
NO:N_2O	34.7 ± 0.1	264.1 ± 0.6	74.0 ± 0.1	45.9 ± 0.5	32.1 ± 0.2	10.3 ± 0.5
cm^3 H_2O/cm^3 soil	0.28 ± 0.00	0.27 ± 0.06	0.10 ± 0.01	0.10 ± 0.0	0.23 ± 0.02	0.23 ± 0.02
Water-filled pore space‡	0.47 ± 0.07	0.46 ± 0.07	0.16 ± 0.02	0.16 ± 0.01	0.36 ± 0.08	0.38 ± 0.07
TN (%)‡	0.21 ± 0.01	0.21 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.09 ± 0.01
NH_4^+ (μg N/gds)‡	2.9 ± 0.6	5.8 ± 0.7	3.9 ± 0.5	5.2 ± 0.5	0.3 ± 0.2	2.4 ± 0.3
NO_3^- (μg N/gds)‡	11.4 ± 3.0	9.2 ± 1.9	4.2 ± 1.4	3.8 ± 1.2	4.1 ± 0.5	2.4 ± 0.8
C:N‡	12.0 ± 0.6	12.1 ± 0.5	13.6 ± 0.5	13.4 ± 0.7	12.2 ± 1.4	11.93 ± 0.79
δ^{15}N-TN (‰)‡	10.1 ± 0.2	10.0 ± 0.2	9.4 ± 0.6	9.6 ± 0.3	10.6 ± 0.6	10.6 ± 0.4
δ^{15}N-NH_4^+ (‰)‡	31.6 ± 2.5¶	16.1 ± 2.8	16.8 ± 2.0¶	10.8 ± 0.5	−2.1 ± 4.4	10.1 ± 2.5
δ^{15}N-NO_3^- (‰)§	4.2 ± 0.7	4.0 ± 0.9	3.8 ± 1.0	5.3 ± 1.6	5.3 ± 0.8	5.0 ± 1.6
δ^{18}O-H_2O (‰)§	−5.6 ± 0.5	−5.7 ± 0.7	−5.4 ± 0.6	−5.0 ± 0.5	−11.4 ± 0.5	−11.2 ± 0.3
δ^{18}O-N_2O (‰)	14.7 ± 1.8	22.0 ± 10.6	18 ± 1	27.2 ± 1.7	30.5 ± 4.7	45.3 ± 7.6

† N_2O flux during 15 hours of incubation; gds, grams of dry soil. Nine jars were incubated per flux calculated. NO fluxes were determined in additional incubations using 15 g of soils during a shorter period of time due to the high emissions found in the soils.

‡ For these parameters, $n = 12$; the $\delta^{15}N$ values are expressed relative to atmospheric N_2. TN, total nitrogen.

§ For these parameters, $n = 10$; the $\delta^{18}O$ values are expressed relative to Vienna-standard mean ocean water (V-SMOW).

¶ Due to the fact that NH_4^+ concentration in the control experiment reached an equilibrium before the first 5 hours of the experiment, the average of $\delta^{15}N$-NH_4^+ values was calculated for 5, 10, and 15 h (see Appendix for details).

(Fig. 5). Only in the TNF Ultisol soils did the acetylene incubation Keeling plot yield a linear correlation with an intercept value of 27.2‰ ± 1.6‰, which represents the $\delta^{18}O$ value of the denitrification-derived N_2O. For this soil we determined the $\delta^{18}O$-N_2O nitrification-derived signal by means of the following equation:

$$\delta^{18}\text{O-N}_2\text{O}_{\text{tot}} \times [\text{N}_2\text{O}]_{\text{tot}} = \delta^{18}\text{O-N}_2\text{O}_{\text{nit}} \times [\text{N}_2\text{O}]_{\text{nit}} + \delta^{18}\text{O-N}_2\text{O}_{\text{den}} \times [\text{N}_2\text{O}]_{\text{den}}$$

where the only unknown is $\delta^{18}O$-N_2O_{nit}. We found a value of 9.5‰ ± 1.9‰ for $\delta^{18}O$-N_2O_{nit}.

^{15}N internal distribution of N_2O for nitrification and denitrification

We measured the position-dependent ^{15}N isotopic composition of N_2O produced in the incubations and calculated the site preference for each process by means of Keeling plots (Fig. 4) and mass balance calculations similar to those for the bulk ^{15}N-N_2O data (Table 4). The Keeling plots for the $\delta^{15}N^{\alpha}$-N_2O and $\delta^{15}N^{\beta}$-N_2O had generally good linear correlations in the control and acetylated TNF Ultisol soils (R^2 values of 0.98, 0.84, 0.98, and 0.90 for the control and acetylated soils, respectively). The acetylated TNF Oxisol soils have the greatest uncertainty in the determination of the ^{15}N positional dependence of the emitted N_2O ($R^2 = 0.24$ and $R^2 = 0.12$ for alpha and beta ^{15}N-N_2O, respectively) and there were few data points for the Nova Vida Ultisol soils (Fig. 4). Therefore, we decided not to calculate for the TNF Oxisol and Nova Vida Ultisol soils the ^{15}N positional dependence values for nitrification and denitrification. However, we mention the ^{15}N positional preference for the control and acetylated experiments where we have good linear correlations (Table 4). To our knowledge these are the first ^{15}N intramolecular distribution in N_2O for nitrification and denitrification in incubated natural soils (Table 4).

DISCUSSION

^{15}N enrichment factors for nitrification

The fact that the ^{15}N enrichment factors for nitrification in the TNF Oxisol and TNF Ultisol were similar

TABLE 3. Calculated enrichment factors for nitrification and denitrification from Amazon forest soils and the relative contribution of each process to the total emitted N_2O in the jars.

Parameters	TNF Oxisol (clay)		TNF Ultisol (sandy loam)		Nova Vida Ultisol (sandy)	
	Nitrification	Denitrification	Nitrification	Denitrification	Nitrification	Denitrification
δ^{15}N-N_2O, δp (‰)	−80.1 ± 11.5	−26.7 ± 11.2	−85.4 ± 4.3	−39.9 ± 4.3	−59.3 ± 3.8	−5.4 ± 2.3
δ^{15}N-substrate, δs (‰)	31.6 ± 2.5	4.0 ± 0.9	16.8 ± 2.0	5.3 ± 1.6		5.0 ± 1.6
$\varepsilon_{p\text{-}s} = \delta p - \delta s$ (‰)	−112 ± 12	−31 ± 11	−102 ± 5	−45 ± 5		−10 ± 4
$\varepsilon_{p\text{-}s}$ literature range (‰)	−66 to −42	−12 to −35				
(Mean $\varepsilon_{p\text{-}s}$ literature)†	(−55)	(−24)				
Relative contribution (%)	69.7	30	60.8	39.2	12.8	87.2

Note: The ^{15}N enrichment factor ε is calculated as $\varepsilon_{p\text{-}s} = \delta_p - \delta_s$, where δ_p is the isotopic composition of the product (the accumulated and instantaneous product are equal) and δ_s is the isotopic composition of the substrate.

† Values from Yoshida (1988), Wada and Ueda (1996), Barford et al. (1999), Ueda et al. (1999).

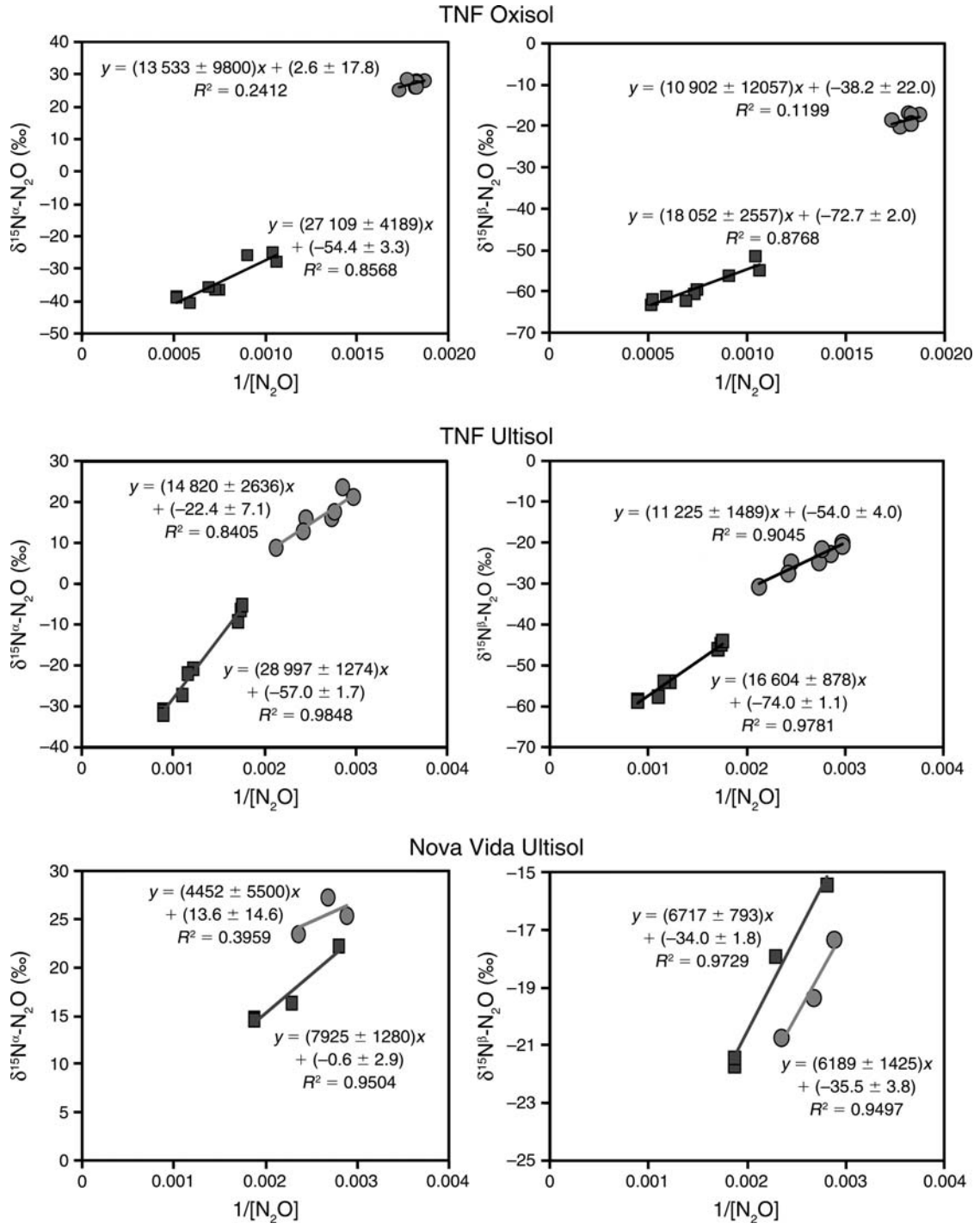

FIG. 4. "Keeling plots" of ^{15}N-N_2O positional dependence from a textural gradient of forest incubated soils in the Amazon basin. Control and acetylated incubated soils are represented by squares and circles, respectively.

cannot be attributed to similarities in the NH_4^+ concentrations for both soils because they are significantly different (Student's t test, $P < 0.0002$), with higher values in the TNF Ultisol soils. Also, the WFPS values, which would affect the ability of nitrifying bacteria to produce N_2O, were also significantly different (Student's t test, $P < 0.0001$) between these soils, with smaller WFPS values in the TNF Ultisol soil (Table 2). Therefore, we propose that the nitrifying bacteria in both soils use enzymatic pathways that fractionate similarly, leading to similar

^{15}N of produced N_2O, despite the fact that soil texture, water content, and nutrient availability might be different. Another possible factor that we cannot assess at this time is that a similar microbial community is responsible for nitrification in both soils. Future studies would benefit from including some characterization of the microbial communities in incubated soils.

The ^{15}N enrichment factors we calculated for nitrification are significantly larger (i.e., more negative) compared to reported values based on pure culture studies (Table 3). One possible explanation might be related to the fact that the NO emissions in the control experiment are six to nine times higher than in the acetylated experiment (Table 3). This can influence the N_2O isotopic composition of the denitrification-derived N_2O in the control experiment because NO is an intermediate in the denitrification pathway. A recent study by Daiber et al. (2002) found that the reduction of NO by a nitric oxide reductase enzyme ($P450_{NOR}$) extracted from *Fusarium oxysporum* (denitrifying fungus) is affected by the NO concentration. They suggest an intermediate is formed from NO to make N_2O. That intermediate is formed by the reduction of the primary enzyme–substrate complex $[Fe\text{-}NO]^{3+}$ by NADH in a rate limiting step as long as the NO concentrations are high. After this intermediate is formed another free NO molecule gets in the intermediate complex and generates the N_2O molecule. Therefore, if the NO concentration is not high enough that second step become rate limiting. These results imply that the NO to N_2O reduction by this fungus is limited by the NO concentration.

If the denitrifying bacteria in the TNF Oxisol and TNF Ultisol soils follow the same enzymatic pathway as the denitrifying fungus in Daiber et al. (2002) experiment, they could use the nitrification-derived NO to generate N_2O. If the nitrification-derived NO is very depleted in ^{15}N, the use of that NO as an intermediate in producing N_2O via denitrification will cause either (1) more depleted $\delta^{15}N\text{-}N_2O$ values in the resulting denitrification-derived N_2O in the control experiment compared to the acetylated experiment where the NO concentration was one-sixth to one-ninth of the control experiments; and/or (2) an overall increase in the amount of denitrification-derived N_2O in the control experiment compared to the acetylated experiment. This will influence the calculation of the $\delta^{15}N\text{-}N_2O$ value associated with nitrification-derived N_2O, and as a result the ^{15}N enrichment factors values we calculate for nitrification would be more negative (larger fractionation). At present, there is no way to assess this effect because there is no information in the literature on the ^{15}N isotopic fractionation accompanying the reduction of NO to N_2O during denitrification. Further, the analytical techniques to measure the ^{15}N isotopic composition of emitted NO from soils are not yet available, though clearly they would have been a useful addition to this study.

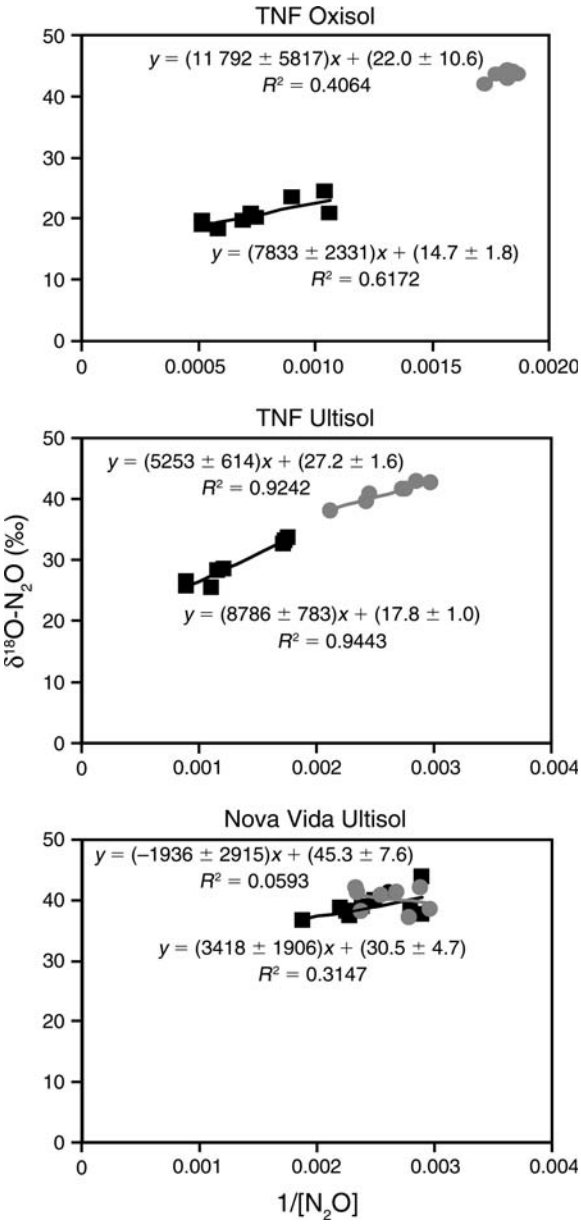

FIG. 5. "Keeling plots" of ^{18}O from a textural gradient of forest soils in the Amazon basin. Control and acetylated incubated soils are represented by squares and circles, respectively.

^{15}N enrichment factors for denitrification

The ^{15}N enrichment factors for denitrification were similar between the TNF Oxisol (–31‰ ± 11‰) and TNF Ultisol (–45.2‰ ± 4.5‰) soil incubations, but both were significantly different (ANOVA, $P < 0.001$) from the Nova Vida Ultisol (–10.4‰ ± 3.5‰) values. As in the case of the ^{15}N enrichment factor for nitrification, substrate availability, WFPS, and texture do not seem to play an important role in the values of ^{15}N enrichment factor of denitrification because they

TABLE 4. Measured ^{15}N internal distribution of N_2O in the control and acetylated soils and calculated site preference values (all values are ‰) for nitrification and denitrification (NO_3^- to N_2O).

Soil type	Control experiment			Acetylated experiment		
	$\delta^{15}N^{\alpha}$	$\delta^{15}N^{\beta}$	Control site preference	$\delta^{15}N^{\alpha}$	$\delta^{15}N^{\beta}$	Denitrification site preference
TNF Oxisol	−54.4 ± 3.3	−72.7 ± 2.0	18.3 ± 4.0			
TNF Ultisol	−57 ± 1.7	−74.0 ± 1.1	17.0 ± 2.0	−22.4 ± 7.1	−54.0 ± 4.0	31.6 ± 8.1
Nova Vida Ultisol	−0.6 ± 2.9	−34.0 ± 1.8	33.4 ± 3.4			

Note: Data are expressed relative to atmospheric N_2 for comparison with other work. Empty cells indicate that calculations were not done because the uncertainty was too large for that soil type.

differ significantly among the three soils. It is suggested that different enzymes that participate in denitrification (such as nitrite reductase and nitric oxide reductase) might produce N_2O with a different ^{15}N isotopic fingerprint given the experimental conditions and NO concentrations of each incubated soils. A study that has shown the ^{15}N isotopic fractionation factor for the denitrification step of NO_3^- to N_2O of a denitrifying bacteria culture (*Paracoccus denitrificans*) shows values similar than the one we obtained for the Nova Vida Ultisol soils. These results suggest that these soils might have the same enzymatic pathway (by nitrite reductase and nitric oxide reductase) of N_2O production via denitrification as the bacteria *Paracoccus denitrificans*. The fact that the TNF Oxisol and Ultisol have large ^{15}N discrimination for a denitrification process in comparison with the Nova Vida Ultisol soils might be related to the high NO concentrations found in the acetylated TNF soils (10 times higher than the Nova Vida Ultisol soils, Table 2), suggesting that in the TNF soils denitrification enzymatic pathway might be more sensitive to the NO concentration levels than the Nova Vida-Ultisol.

Based on the differences in the ^{15}N enrichment factor values for nitrification and denitrification found for the studied soils, we conclude that in order to differentiate the relative contribution of nitrification and denitrification for a particular soil using stable isotopes, we need to determine enrichment factors for each soil, since the values reported in the literature based on pure bacteria cultures may not be representative of the bacterial populations present in soils (Table 3).

$\delta^{18}O$ of emitted N_2O

In order to determine the ^{18}O enrichment factors for nitrification and denitrification, it is necessary to measure the ^{18}O isotopic composition of the oxygen sources for these microbial processes (Pérez 2005). We were not able to calculate ^{18}O enrichment factors with this experimental setup because of the lack of such measurements and due to the fact that the linear correlations of the Keeling plots yielded not significant correlation in most of the acetylated soils. However, we determined the ^{18}O composition of soil H_2O to see how they related to the $\delta^{18}O$ values of emitted N_2O. The TNF Oxisol and Ultisol soils have similar $\delta^{18}O$ values of emitted N_2O in the control experiment (Table 3) and were significantly lighter than the Nova Vida $\delta^{18}O$-N_2O values. The $\delta^{18}O$-H_2O values for the TNF soils in the control and acetylated soils were similar (~5‰) whereas that for the Nova Vida soils the value were significantly lighter (~−11‰). Based on these results, the $\delta^{18}O$-N_2O values of the TNF control experiment soils have a higher contribution of the ^{18}O form water than the Nova Vida soils. We conclude that the determination of oxygen ^{18}O enrichment factors by means of incubation techniques is more complicated than the ^{15}N enrichment factor determinations. Measurements of all the oxygen sources and the application of label ^{18}O sources (in water or molecular oxygen) will contribute to determine the ^{18}O enrichment factors associated with nitrification and denitrification using a similar procedure as the incubation technique applied here.

^{15}N isotopomeric site preference in N_2O for nitrification and denitrification

We were able to calculate the ^{15}N isotopomeric site preference of N_2O for nitrification and denitrification for TNF-Ultisol by using the same approach as described above for the ^{15}N isotopic source of nitrification and denitrification. The ^{15}N isotopomeric site preference for nitrification gives lower values than the site preference for denitrification (Table 4). We can infer from these results that the nitrifying enzymatic pathways for these soils might have step(s) to produce a relatively equal enrichment of ^{15}N at the terminal and central nitrogen.

Sutka et al. (2003, 2004), in a pure culture study where hydroxylamine was oxidated by *Nitrosomonas Eurapaea*, found an enrichment in the beta nitrogen of emitted N_2O. The authors propose a mechanism for dissimilatory nitrite reduction that leads to ^{15}N discrimination in the beta nitrogen. Our soils might exhibit a different trend than the one found by Sutka et al. (2003, 2004) due to the fact that we have a consortia of nitrifying bacteria in our soils that can produce as an overall an N_2O equally fractionated in both positions. Also, in our nitrification experiments, a fraction of the N_2O could be derived also from nitrifying denitrification that could shift the N_2O site preference of the observed values. The other alternative explanation for obtaining an equal

TABLE 4. Extended.

Nitrification calculated		
$\delta^{15}N^{\alpha}$	$\delta^{15}N^{\beta}$	Nitrification site preference
−87.3 ± 7.3	−91.5 ± 4.1	4.2 ± 8.4

enrichment in both nitrogen atoms in our soils is if the N_2O generating mechanism by nitrifiers in our soils is the simultaneous binding of NO to nitric oxide reductase (NOR). This will produce limited site preference because the NO molecules have equal positioning in the enzyme for loss of the oxygen atom and release of the N_2O molecule (Stein and Yung 2003).

The isotopomeric ^{15}N site preference for denitrification at TNF Ultisol has higher values than those that are nitrification derived. Because, in the acetylated soils, the N_2O emitted is denitrification derived, it is reasonable to infer that its production should be derived from an enzymatic pathway related to NOR. Stein and Young (2003) suggest that sequential binding of NO to NOR enzyme could explain larger values in the N_2O site preference. This mechanism will produce an accumulation of ^{14}N in the beta position and therefore a larger isotopomeric site preference.

Pérez et al. (2001) presented some limited data in support of isotopomer shifts associated with changes in the microbial processes in a urea fertilized agricultural field in Mexico. In this study, the N_2O ^{15}N isotopomeric site preference value of got larger as time progressed due to nitrification during the first four days of sampling (4.8‰ to 14.2‰, relative to a working standard). This enrichment suggests that the mechanism for sequential binding of NO to NOR pointed out by Stein and Young (2003) could also take place during nitrification. At present, there is no way of knowing the actual microbial enzymatic pathways for soils until a complete characterization of the isotopomeric shifts produced during different enzymatic pathways is available and studies of microbial communities that allow us to fill this gap are added.

It is difficult to compare the results found in this study with previous work due to the fact that there is no unified standard for isotopomers used across all the previous studies. If we compare the isotopomeric ^{15}N site preference for nitrification and denitrification (Table 4), we find substantial differences between them. Such large difference provides a new constraint to differentiate microbial enzymatic pathways of nitrification and denitrification. Future studies of the intramolecular distribution of ^{15}N from pure culture and field studies will provide new insights to differentiate mechanisms of N_2O production in soil.

CONCLUSIONS

From soil incubation experiments, we have shown that the ^{15}N fractionation factors and ^{15}N site preference for N_2O production are significantly different for nitrification vs. the first step of denitrification in tropical forest soils. These isotope effects could not be explained by soil texture, nutrient availability and water content. We hypothesize that differences between the microbial enzymatic pathways might be responsible for that.

Our measurements show the great potential for using N_2O isotopes to differentiate soil microbial processes and explain why N_2O fluxes may vary across sites. These new findings will help to develop a process level understanding as to why N_2O isotopic composition and ^{15}N positional dependence vary across a soil textural gradient and further suggest methods for using isotopic signatures to scale N_2O fluxes across larger regions. The advantage of knowing the isotope enrichment factors for these soils is that it allows us to determine the relative contribution of nitrification and denitrification to soil N_2O emissions by simply measuring the bulk ^{15}N isotopic composition of the emitted N_2O and applying an isotope mass balance. This method eliminates the need of using invasive methods in the field.

More N_2O bulk ^{15}N isotopic composition and ^{15}N intramolecular distribution studies of soil N_2O source will give a better understanding of the global N_2O budget. This in turn will allow us to produce better estimates of the relative contribution of the global soil source to the global N_2O budget.

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APPENDIX

Experimental details of the incubation experiment (*Ecological Archives* A016-069-A1).