

Stable isotope discrimination during soil denitrification: Production and consumption of nitrous oxide

Oleg V. Menyailo¹ and Bruce A. Hungate²

Received 5 April 2005; revised 19 May 2006; accepted 9 June 2006; published 27 September 2006.

[1] Measuring the stable isotope composition of nitrous oxide (N₂O) evolved from soil could improve our understanding of the relative contributions of the main microbial processes (nitrification and denitrification) responsible for N₂O formation in soil. However, interpretation of the isotopic data in N₂O is complicated by the lack of knowledge of fractionation parameters by different microbial processes responsible for N₂O production and consumption. Here we report isotopic enrichment for both nitrogen and oxygen isotopes in two stages of denitrification, N₂O production and N₂O reduction. We found that during both N₂O production and reduction, enrichments were higher for oxygen than nitrogen. For both elements, enrichments were larger for N₂O production stage than for N₂O reduction. During gross N₂O production, the ratio of $\delta^{18}\text{O}$ -to- $\delta^{15}\text{N}$ differed between soils, ranging from 1.6 to 2.7. By contrast, during N₂O reduction, we observed a constant ratio of $\delta^{18}\text{O}$ -to- $\delta^{15}\text{N}$ with a value near 2.5. If general, this ratio could be used to estimate the proportion of N₂O being reduced in the soil before escaping into the atmosphere. Because N₂O-reductase enriches N₂O in both isotopes, the global reduction of N₂O consumption by soil may contribute to the globally observed isotopic depletion of atmospheric N₂O.

Citation: Menyailo, O. V., and B. A. Hungate (2006), Stable isotope discrimination during soil denitrification: Production and consumption of nitrous oxide, *Global Biogeochem. Cycles*, 20, GB3025, doi:10.1029/2005GB002527.

1. Introduction

[2] The increase in the atmospheric concentration of nitrous oxide (N₂O) has attracted considerable scientific attention from different disciplines during the last 15–20 years, because N₂O is one of the major greenhouse gases [Yung *et al.*, 1976] and also participates in the destruction of the ozone layer [Crutzen, 1981]. Denitrification and nitrification are the main biological processes leading to N₂O formation and emission from the soil. Denitrification is known to be favored when soils are moist and anaerobic, whereas nitrification is favored under more mesic to xeric conditions [Davidson, 1991]. Understanding the relative contributions of each process to total N₂O emission is critical for modeling and predicting changes in N₂O fluxes under varying environmental conditions, including altered temperature and precipitation.

[3] The sources of N₂O can be identified using selective inhibitors, sterilization, or by adding substrates [Davidson and Schimel, 1995], but all of these methods are destructive and intrusive, and thus may not accurately reflect the

sources of N₂O [Stevens *et al.*, 1997]. Another way to identify the processes producing N₂O is to study the stable isotope composition of N₂O and how it changes in space and time [Yoshida, 1988; Yoshinari, 1990]. Because the stable isotope ratios of ¹⁵N/¹⁴N and ¹⁸O/¹⁶O of denitrifier-derived N₂O can differ from those of nitrifier-derived N₂O [Kim and Craig, 1990; Webster and Hopkins, 1996], the isotopic composition is thus a window through which we may understand biological processes underlying N₂O emission from the soil.

[4] Despite the potentially high value of the knowledge of isotopic composition, there are few published data describing the mechanisms and extent of isotopic fractionation of N₂O. Compared to denitrification, nitrification often produces N₂O that is ¹⁵N-depleted N₂O [Yoshida, 1988]. The reported values for isotopic enrichment for ¹⁵N (difference in the $\delta^{15}\text{N}$ between product and substrate) range from –13 to –27‰ for denitrification and up to –60‰ for nitrification [Yoshida, 1988]. Consequently, if $\delta^{15}\text{N}$ values of soil nitrate and ammonium are equal, $\delta^{15}\text{N}$ -N₂O derived from nitrifiers should be about 30‰ more negative than $\delta^{15}\text{N}$ of denitrifier-derived N₂O.

[5] Denitrifying bacteria also select isotopically light N₂O for reduction to N₂, enriching the remaining un-reacted N₂O in ¹⁵N [Barford *et al.*, 1999]. However, as for N₂O production, fractionation of oxygen isotopes during N₂O reduction has rarely been reported. Together, measuring both $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values in N₂O will likely provide more insight into the sources of N₂O than would either alone

¹Institute of Forest, Siberian Branch of Russian Academy of Sciences, Krasnoyarsk, Russia.

²Department of Biological Sciences and Merriam Powell Center for Environmental Research, Northern Arizona University, Flagstaff, Arizona, USA.

[Stein and Yung, 2003]. For example, we expect covariance between $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ in N_2O when denitrification is the dominant N_2O source: Because the N and O in N_2O originate primarily from the N and O in NO_3^- , according to the Rayleigh distillation model (see below) isotopes in both product and substrate become enriched during the reaction. By contrast, in nitrification, the N in N_2O originates from NH_4^+ , while O- N_2O has multiple sources, including H_2O , atmospheric O_2 and hydroxylamine [Pérez *et al.*, 2000; Sutka *et al.*, 2006]; therefore covariation of $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ in N_2O produced by nitrification is unlikely. N_2O reduction should also cause co-variation between $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ in the N_2O that remains, because both elements (N and O) are contained in the substrate (N_2O). Thus testing for covariance between $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ in soil-evolved N_2O could help distinguish the soil microbial processes responsible for N_2O efflux. The aim of this work was to describe enrichment in both isotopes for the two stages of denitrification, N_2O production and reduction, as well as to determine the ratios of $\delta^{18}\text{O}$ -to- $\delta^{15}\text{N}$ in N_2O evolved during the two stages of denitrification.

2. Methods

2.1. Research Site and Soil Samples

[6] We selected two tree species from the Siberian afforestation experiment, larch (*Larix sibirica*), and birch (*Betula pendula*). These species were selected because they differed strongly in their effects on the ratio of end products produced during denitrification, $\text{N}_2\text{O}/\text{N}_2$ [Menyailo *et al.*, 2002b], and thus were considered good candidates for testing the generality of isotope effects during N_2O formation and reduction. Soil samples were collected under larch and birch in the Siberian afforestation experiment, in which six common boreal forest tree species have been grown under common garden conditions for the past 30 years. Because this experiment began with initially uniform soils, and all plots are exposed to the same climatic conditions, differences in soil properties that arise over time can be fully attributed to the effects of plant species [Wedin and Tilman, 1990]. The research plots are located 50 km northwest from Krasnoyarsk and were established by the Laboratory of Soil Science of the Institute of Forest, Siberian Branch of the Russian Academy of Sciences [Menyailo *et al.*, 2002a]. The region is characterized by continental climatic conditions with average rainfall 500 mm yr^{-1} , average midday (12:00) summer temperature of 20°C, depth to permafrost of 70–170 cm, and soil temperature to 20 cm depth in winter -4° to -14°C , in summer 10° to 12°C . The soil is the gray forest type according to the Russian Soil Classification System and Greyzem according to *Food and Agriculture Organization* [1990]. In August 2001, each plot was subdivided into two subplots. From each subplot, two trees were randomly chosen and four soil samples were taken at 50 cm apart of the stem of each tree. Soil samples from each subplot were mixed. Overall, we had four composite soil samples, two from each of the two species.

2.2. N_2O -Reductase Activity

[7] The first incubation was performed to estimate parameters of kinetic fractionation (enrichment factors)

for nitrogen and oxygen isotopes in N_2O during N_2O reduction. Soils (20 g) were moistened and preincubated in 250-mL flasks at 25°C for 3 days to reduce the ambient NO_3^- concentration and to activate soil bacteria. Thereafter a glucose solution was added (2.5 mg C g^{-1}), and water content was adjusted to 60% of WHC. The flasks were closed with airtight rubber stoppers and fixed with clamps. Anaerobic conditions were induced by exchanging the gas phase with He for 15 min. 2.5 mL of pure N_2O were then added to the half of subsamples as a terminal electron acceptor. To the other half, the same amount of N_2O and 2.5 mL of C_2H_2 (10% v/v) was added. The last series of subsamples was necessary to estimate N_2O consumption by processes insensitive to C_2H_2 , presumably abiotic (e.g., dissolution in water).

[8] The incubation was conducted over 8 days. From each flask, 5 mL of headspace were removed with syringes after 0 hours and 12 hours and once per day thereafter for N_2O concentration measurements (GC-ECD, SRI 86100). The precision of the concentration measurements on the GC-ECD was better than 2% (maximum difference between five replicate values of the lab standard of N_2O at 100 ppm). At each sampling, 1 mL of headspace was also removed and injected into 20-mL flasks prefilled with He for subsequent isotopic analysis (see below).

[9] Because the isotope-ratio mass spectrometer operates in a 1000 times narrower range than the gas chromatograph, samples were stored until the N_2O concentrations had been determined and appropriate injection volumes could be calculated.

2.3. N_2O Production Through Denitrification

[10] The second incubation experiment was conducted when NO_3^- was the sole electron acceptor, and when N_2O reduction was blocked using acetylene. The conditions were the same as for the first incubation, except that C_2H_2 was added to all samples, and NO_3^- , rather than N_2O , was added as a terminal electron acceptor (500 mg N kg^{-1} soil added as KNO_3 in water). The incubation was conducted over 4 days and from each flask 5 mL of headspace were taken after 8, 16, 24, 32, 48, 60, 72 and 96 hours with syringes for N_2O concentration measurements and 1 mL for $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ measurements as described above.

2.4. Stable Isotope Analysis

[11] The ratios of the stable isotopes $^{15}\text{N}/^{14}\text{N}$ and $^{18}\text{O}/^{16}\text{O}$ in N_2O were determined using an online GC-IRMS system, consisting of a trace gas cryogenic preconcentration device (PreCon, ThermoQuest), gas chromatograph (ThermoQuest) with Plot Q capillary column (0.32 mm \times 30 m), and an isotope-ratio mass spectrometer (ThermoQuest Delta^{PLUS}).

[12] The ratios of masses 45:44 and 46:44 in N_2O samples were measured and used to estimate ratios of $^{15}\text{N}_2^{16}\text{O}/^{14}\text{N}_2^{16}\text{O}$ and $^{14}\text{N}_2^{18}\text{O}/^{14}\text{N}_2^{16}\text{O}$, respectively. We used N_2O as a reference gas (99.9990%, Linde). The $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ in N_2O were referenced to an N_2O standard (provided by T. Pérez, UC Irvine), which had been calibrated to an additional N_2O reference gas, prepared by Tadashi Yoshinari, New York State department of Health [Tanaka *et al.*, 1995]. Clearly, the lack of an international standard for isotopes in

N₂O complicates the use of natural abundance variations of the isotopic composition of N₂O. However, because isotopic separation and enrichment are expressed as differences (e.g., see equation (2), below), any absolute errors in calibration will have little influence on our estimates.

[13] Isotopic data are reported as δ values, where $\delta = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000$. $R = {}^{15}\text{N}/{}^{14}\text{N}$ or ${}^{18}\text{O}/{}^{16}\text{O}$. Delta values are reported as deviations from $\delta^{15}\text{N}$ of atmospheric N₂ and $\delta^{18}\text{O}$ of atmospheric O₂. The conversion for the $\delta^{18}\text{O}_{\text{atm}}$ standard to SMOW standard is $\delta^{18}\text{O}_{\text{atm}} = -23 + \delta^{18}\text{O}_{\text{SMOW}}/1.0235$ [Kim and Craig, 1990]. The precision of the isotopic composition measurements in N₂O was better than 0.5‰ for $\delta^{15}\text{N}$ and better than 1‰ for $\delta^{18}\text{O}$.

2.5. Definition of Enrichment Factor

[14] In unidirectional reaction ($S \rightarrow P$), the kinetic isotope fractionation factor is defined as

$$\alpha = R_p^i(t)/R_s(t), \quad (1)$$

where $R_p(t)$ and $R_s(t)$ are isotope ratios for P (product) and S (substrate) at time t , respectively, superscript i indicates isotope ratios for instantaneous products [Mariotti et al., 1981]. On the basis of the δ definition and approximation $\delta/1000 \ll 1$, equation (1) can be rewritten

$$\varepsilon = \delta_p^i(t) - \delta_s(t), \quad (2)$$

where ε is equal to $1000(\alpha - 1)$. This parameter (ε) is called the enrichment factor. During normal discrimination, where isotopes in the product are lighter (more negative) than in the substrate, values of ε are negative. This can be confusing when enrichments are described as large, but the values are negative; here, absolute values of enrichment should be understood. In the initial phase of the reaction, the concentration and isotopic composition of substrate is almost constant and, therefore, $\delta_p^i(t)$ is nearly equal to the isotope ratios of accumulated product, $\delta_p(t)$.

$$\varepsilon = \delta_p(t) - \delta_s(t=0). \quad (3)$$

[15] However, when the reaction has proceeded and substantial amount of substrate is consumed, equation (3) does not hold and isotopic composition is linked to concentrations of either substrate or product through the Rayleigh equation, which is, for the substrate

$$\delta_s(t) \sim \delta_s(t=0) + \varepsilon \ln f, \quad (4)$$

or for the product

$$\delta_p(t) \sim \delta_s(t=0) + \varepsilon f \ln f(1-f), \quad (5)$$

where f is the remaining fraction of substrate. According to Mariotti et al. [1981], equations (4) and (5) hold only if ε and α are constant.

2.6. Estimation of Enrichment Factor

[16] In our study, only the isotopic composition of N₂O was measured, which is both a product (for N₂O produc-

tion) and substrate (for N₂O reduction). For N₂O reduction, the enrichment factor was estimated according to equation (4); here $\delta_s(t)$ is the isotopic composition of residual N₂O and f is the proportion of N₂O remaining from the initial N₂O concentration. Because the direction of the isotope effect during N₂O reduction shifted during the experiment, we only calculated enrichment during the initial phases of N₂O reduction (first 72 hours for larch, first 144 hours for birch). Inaccurate determination of f is one possible source of error in estimating ε and can be in part responsible for the large variation in values of ε reported in the literature [Tong and Yankowich, 1957; Barford et al., 1999].

[17] The Rayleigh equation applies only for un-branched reactions [Peterson and Fry, 1987]. During nitrate reduction to N₂O or N₂, one atom of oxygen escapes from the reaction sequence at each step, so we have two factors contributing to oxygen isotope fractionation: biological selection of lighter molecules for reduction and possible preferential release of the lighter isotope ¹⁶O from the reaction [Toyoda et al., 2005]. Our use of the equations (2) and (5) for oxygen isotopes during N₂O production generates therefore an estimate of enrichment that reflects both fractionating processes. To make clear the distinction from enrichment factor (ε), we describe the observed enrichment in ¹⁸O-N₂O during N₂O production as the "reaction sequence enrichment."

[18] For N₂O production, we estimated the enrichment factor for $\delta^{15}\text{N}$ and reaction sequence enrichment for $\delta^{18}\text{O}$ according to equation (2) as the difference in the $\delta^{15}\text{N}$ or $\delta^{18}\text{O}$ values between the product (N₂O) and substrate (NO₃⁻) at the first measurement points, when N₂O was most depleted and the isotopic composition of NO₃⁻ was likely similar to that of the originally applied NO₃⁻. We used final values of $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ of N₂O produced during the incubations with nitrate to estimate the $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values in applied NO₃⁻, because at this late stage of incubation (after N₂O production had ceased) the isotopic values of the product should equal the initial isotopic values of the substrate (NO₃⁻). While less precise than direct determination of both $\delta^{15}\text{N}$ [Sigman et al., 2001] and $\delta^{18}\text{O}$ [Casciotti et al., 2002] in NO₃⁻, our estimate is also reasonable, as the values were near 0 ‰, consistent with measured values for most human-produced N fertilizers [Wrage et al., 2004; Mayer et al., 2001; Amberger and Schmidt, 1987; Voerkelius, 1990; Wassenaar, 1995].

[19] We estimated the enrichment factor using equation (5) for N₂O production. However, our data did not fit the Rayleigh distillation curve, possibly owing to a decline in the enrichment as the substrate became limiting; alternatively, the poor fit could be due to incorrect determination of f . Determining f is relatively simple for N₂O reduction but problematic for the N₂O production experiment, since the amount of applied NO₃⁻ may not be fully available for denitrification owing to immobilization. The amount of N₂O produced with C₂H₂, indicating the amount of NO₃⁻ denitrified, was always much lower than NO₃⁻ amended (Figures 1a and 1b). Because N₂O accumulation ceased during the later stages of incubation, we assume that all NO₃⁻ was denitrified or immobilized. Thus we used the maximum N₂O concentration in the C₂H₂ treatment to

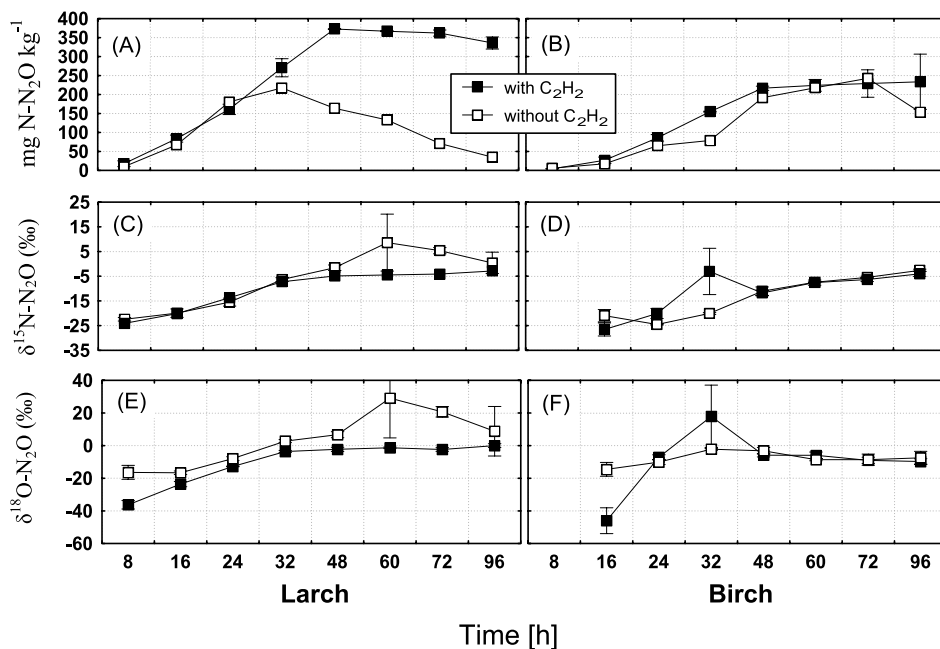


Figure 1. Production of (a, b) nitrous oxide, (c, d) cumulative isotopic signatures of N_2O : $\delta^{15}N$ and (e, f) $\delta^{18}O$ during the 96 h of incubation of soil samples beneath larch and birch.

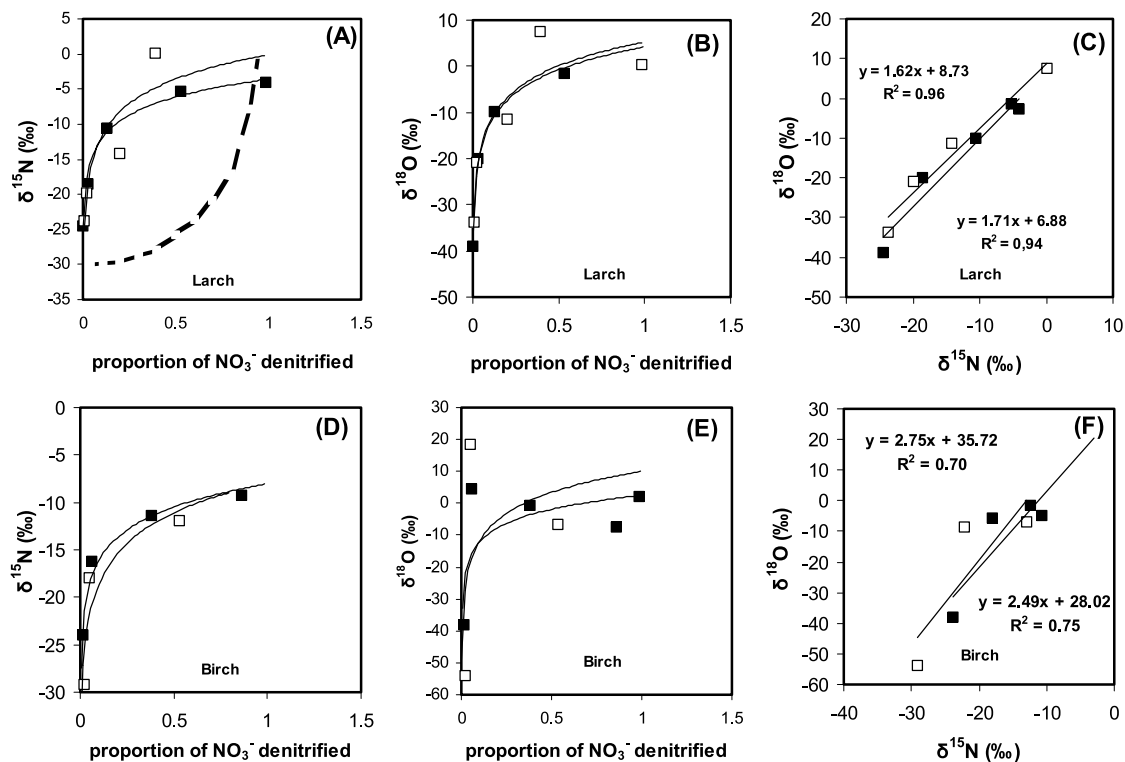


Figure 2. Isotopic signatures of N_2O versus the proportion of NO_3^- denitrified ($1 - f$) for C_2H_2 treatment. For larch (a) $\delta^{15}N$ and (b) $\delta^{18}O$. For birch (d) $\delta^{15}N$ and (e) $\delta^{18}O$. These data do not fit Rayleigh distillation model, approximate curve of which is shown in Figure 2a (dashed line). Scatterplots for $\delta^{18}O$ versus $\delta^{15}N$ are given for (c) larch and (f) birch. Open and solid squares are replicates.

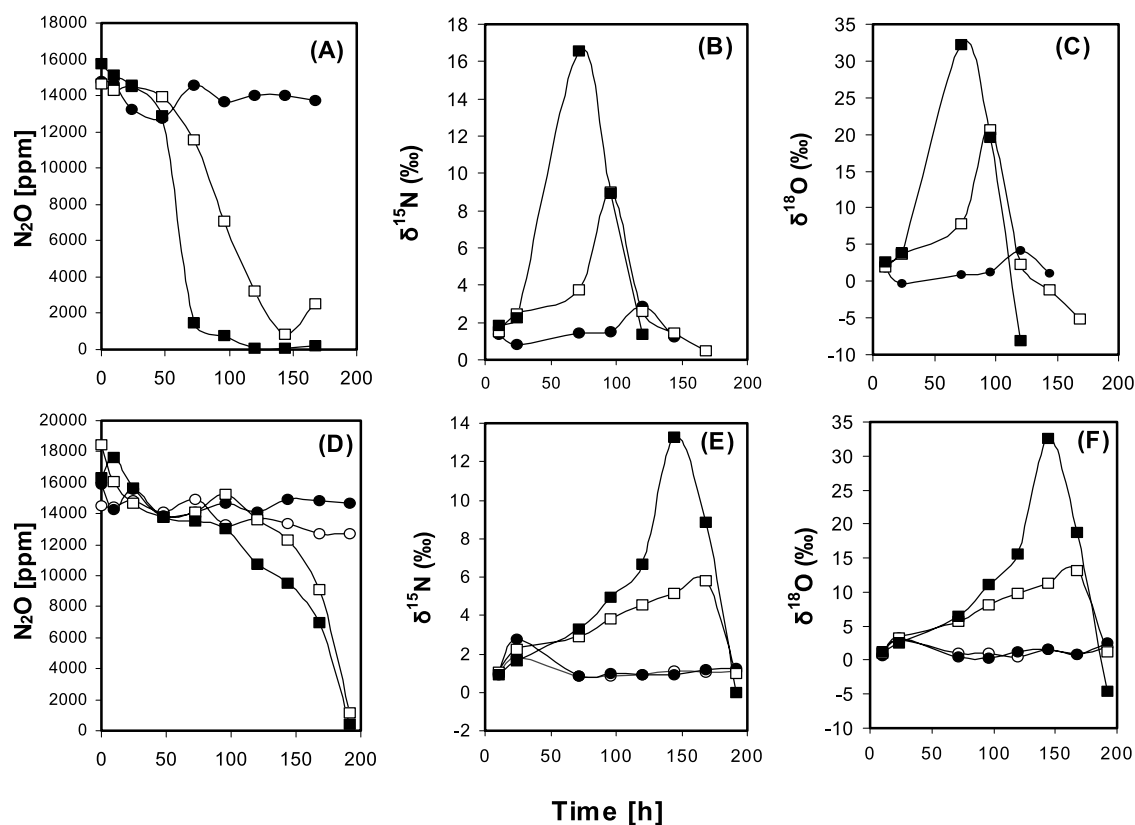


Figure 3. Changes in N_2O concentration and stable isotope values during the reduction experiment for soils from (a–c) larch and (d–f) birch. N_2O concentrations are shown in Figures 3a and 3d, $\delta^{15}\text{N}$ values in Figures 3b and 3e, and $\delta^{18}\text{O}$ values in Figures 3c and 3f. Circles indicate treatments with C_2H_2 , squares are those without C_2H_2 . Open and solid symbols are the replicates.

determine the total amount of NO_3^- available; all values of f were calculated accordingly.

3. Results

3.1. N_2O Production Experiment

[20] Acetylene (C_2H_2) inhibits activity of N_2O -reductase, the enzyme catalyzing the reduction of N_2O to N_2 , the last step of denitrification. Therefore N_2O production was measured with and without C_2H_2 because the difference in the amount of N_2O accumulated indicates the activity of N_2O -reductase, which is expected to contribute to enrichment of N_2O isotopes. A significant effect of C_2H_2 was observed only in soil samples under larch (Figure 1a), where after 32 hours of incubation, soils with C_2H_2 accumulated more N_2O due to increasing N_2O reduction in the flask without C_2H_2 . This supports our former conclusion that the activity of soil N_2O -reductase is depressed under deciduous species and is higher under coniferous species [Menyailo *et al.*, 2002b]. Thus the contribution of N_2O -reductase to isotopic fractionation can be expected only in soils under larch.

[21] Nitrogen isotopes in N_2O were depleted at the beginning of incubation in all soils and gradually became more enriched during the incubation (Figures 1c and 1d). Without C_2H_2 , active N_2O reduction in soils under larch

caused the $\delta^{15}\text{N}$ - N_2O to be up to 20‰ heavier (at 60h) compared to the incubations in which N_2O reduction was suppressed with C_2H_2 (Figure 1c). As the reaction proceeded, the difference in $\delta^{15}\text{N}$ - N_2O values between incubations with and without C_2H_2 decreased, and the values were almost equal by the end of incubation (96 hours), suggesting that N_2O -reductase enriches the isotopes in N_2O only at the beginning of the reaction (see below). The dynamics of isotopic values did not fit the Rayleigh distillation model (compare our data with the theoretical curve (dashed line) in Figure 2a). This might be due to declining enrichment as the reaction proceeds, as has been observed [Toyoda *et al.*, 2005], because the Rayleigh equations (equations (4) and (5)) hold only when enrichments (ϵ) are constant [Mariotti *et al.*, 1981]. The second possible explanation is that our estimates of f were incorrect (see section 2). Therefore we do not estimate enrichment factors for this experiment according to the Rayleigh equation (equation (4)), but rather use equation (2).

[22] The reaction sequence enrichments (absolute values) for oxygen were consistently larger (–34, –39‰ for larch and –39, –54‰ for birch) than the enrichment factors for nitrogen (–24, –25‰ for larch and –24, –29‰ for birch). Reflecting isotopic fractionation during N_2O reduction, the $\delta^{18}\text{O}$ values of the N_2O accumulated in incubations of soils from larch without C_2H_2 were consistently higher (more

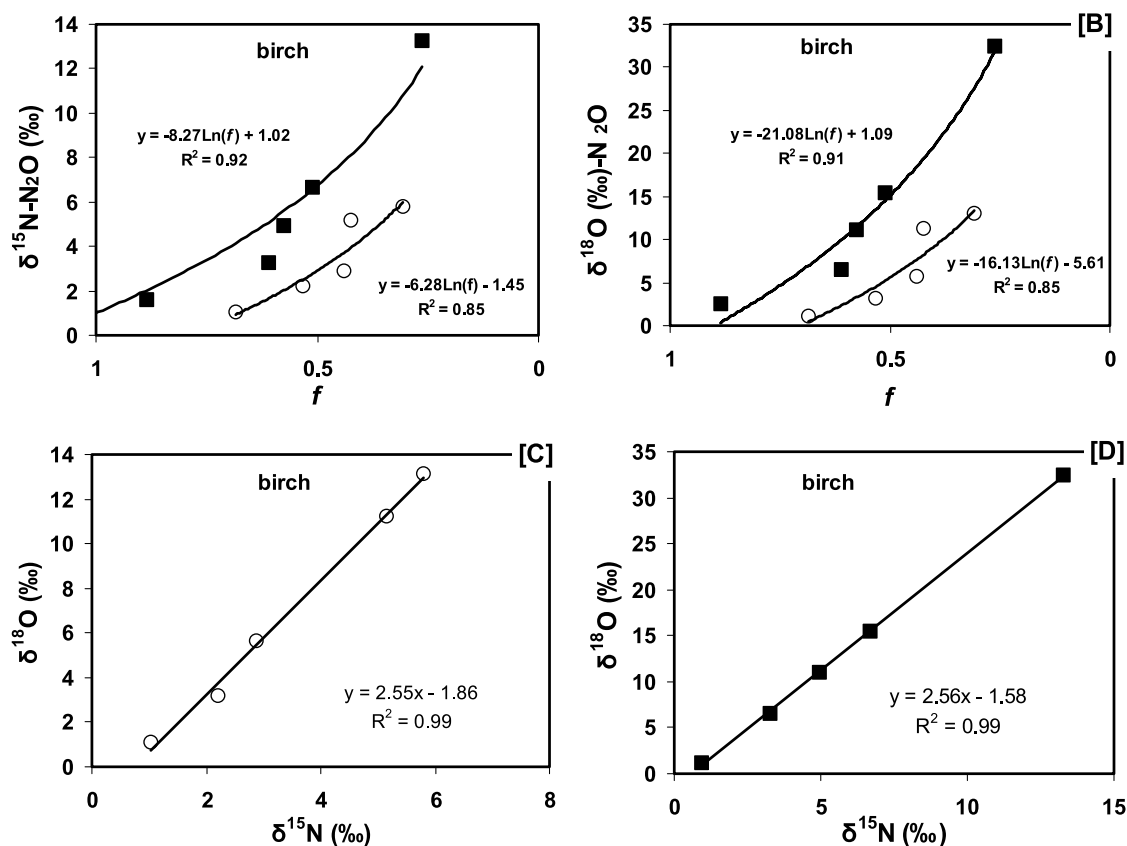


Figure 4. (a, b) Rayleigh fits to the isotopic composition of N₂O reduced by the two replicate samples taken under birch (solid squares and open circles show different replicates). (c, d) Relationship between $\delta^{18}\text{O}$ and $\delta^{15}\text{N}$ values of N₂O for both samples.

positive) than $\delta^{18}\text{O}$ values of N₂O accumulated in incubations with C₂H₂ (Figure 1e). This difference was not as apparent for $\delta^{15}\text{N-N}_2\text{O}$, indicating stronger isotopic fractionation for oxygen compared to nitrogen during N₂O reduction. Compared to $\delta^{15}\text{N}$, the $\delta^{18}\text{O}$ value of N₂O may provide a more powerful signature of in situ activity of N₂O-reductase.

[23] Despite differences in rates of potential denitrification and other aspects of N cycling between these tree species [Menyailo *et al.*, 2002b], they exhibited consistent enrichment values. While tree species did not differ significantly in their effects on enrichment factors for $\delta^{18}\text{O}$ and $\delta^{15}\text{N}$, a clear difference between soils under different species was found for the ratio of $\delta^{18}\text{O-N}_2\text{O}$: $\delta^{15}\text{N-N}_2\text{O}$ during N₂O production (Figures 2c and 2f). The ratio $\delta^{18}\text{O-N}_2\text{O}$ -to- $\delta^{15}\text{N-N}_2\text{O}$ was 1.6–1.7 (Figure 2c) for larch and 2.5–2.7 for birch (Figure 2f).

3.2. N₂O Reduction Experiment

[24] The consumption of N₂O has two clear stages, a lag-phase (enzyme synthesis) and an exponential growth phase (Figures 3a and 3b). During the lag-phase, a relatively small amount of N₂O was consumed by the N₂O-reductase already present in soils. But soils under different tree species significantly differed in the rate of initial N₂O consumption and in the time required for *de novo* synthesis

of N₂O-reductase. At this initial stage, soils under larch consumed N₂O two times faster than soils under birch. After 50 hours, soil under larch began the exponential phase of N₂O consumption, depleting the majority of N₂O from 72 to 144 hours, at which point the rate of consumption declined, likely owing to substrate (N₂O) limitation. For birch, the exponential phase did not begin until after 144 hours, indicating that tree species influenced the soil denitrifier community's capacity to synthesize the denitrifying enzyme, N₂O-reductase. During the lag-phase of N₂O consumption, $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of N₂O increased in soil from both species, following the expected pattern when N₂O-reductase reacts more rapidly with the lighter isotopes, enriching the substrate (N₂O) in the heavier isotopes.

[25] The enrichment factors for the initial phases of N₂O reduction experiment for $\delta^{15}\text{N-N}_2\text{O}$ were -6.3 , -9.8 ‰ and much higher for $\delta^{18}\text{O-N}_2\text{O}$ (-16 , -24 ‰) (Figures 4a and 4b and Figures 5a and 5b). The relationship between $\delta^{18}\text{O-N}_2\text{O}$ and $\delta^{15}\text{N-N}_2\text{O}$ values during initial N₂O reduction was strong and consistent, yielding a stable ratio of $\delta^{18}\text{O}$ -to- $\delta^{15}\text{N}$ with a value near 2.5. In other words, N₂O-reductase had a 2.5 greater effect on oxygen isotopes than on nitrogen isotopes (Figures 4c and 4d and Figure 5c). One sample gave lower ratio of 1.98 and was excluded because of the extremely high leverage of a single point (Figure 5d) and because the plot is built using only three points.

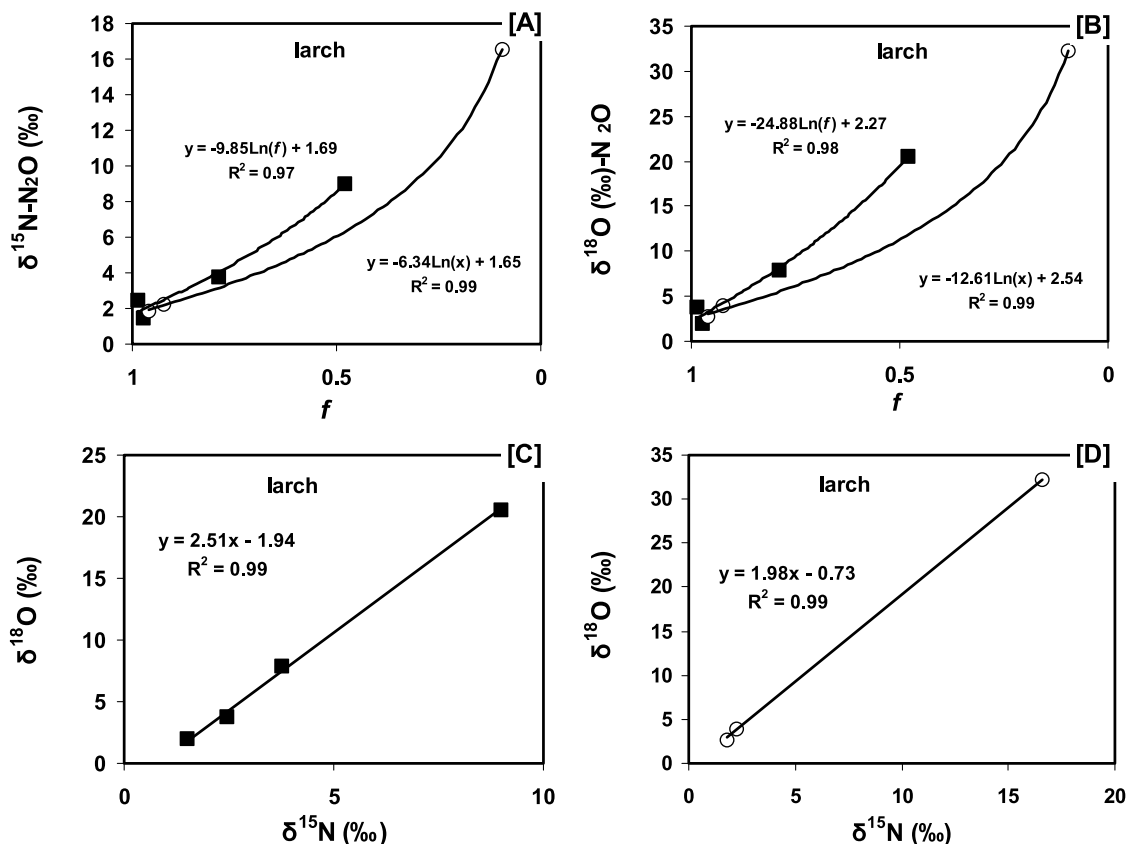


Figure 5. (a, b) Rayleigh fits to the isotopic composition of N_2O reduced by the two replicate samples taken under larch (dark squares and open circles show different replicates). (c, d) Relationship between $\delta^{18}O$ and $\delta^{15}N$ values of N_2O for both samples.

[26] After most of the N_2O had been consumed, $\delta^{15}N$ and $\delta^{18}O-N_2O$ values became lighter (at 96–120 hours for larch and at 168–192 hours for birch), indicating an inverse isotope effect late in the incubation. These effects were observed under both species and in all replicates, arguing against an artifact (Figure 3). During this period of inverse isotope fractionation, the fractionation of $\delta^{18}O$ was larger than for $\delta^{15}N$: enrichments for $\delta^{15}N$ were around 13–16.5‰ and around 2 times higher for $\delta^{18}O$ (33‰).

4. Discussion

[27] Stable isotopes in nitrous oxide have attracted the attention of both microbiologists and atmospheric chemists due to the potentially important information on N_2O origin and fate in the biosphere and atmosphere. This work has shown that (1) terrestrial sources produce lighter N_2O in both isotopes than tropospheric N_2O , (2) stratospheric loss of N_2O enriches N_2O in both ^{15}N and ^{18}O , and (3) there is a large “back flux” of heavy N_2O from stratosphere to the troposphere [Kim and Craig, 1993; Morgan et al., 2004]. All the above-mentioned processes result in a linear relationship between $\delta^{18}O-N_2O$ and $\delta^{15}N-N_2O$ of tropospheric and atmospheric N_2O , a line with a slope of 0.65–0.90 (Figure 6). By contrast, terrestrial sources of N_2O form a cloud if $\delta^{18}O-N_2O$ versus $\delta^{15}N-N_2O$ values are plotted

[Pérez et al., 2000]. Thus several terrestrial processes likely have distinct effects on the N and O isotopes in N_2O , making sources difficult to constrain until these effects are more fully characterized.

[28] We determined $\delta^{18}O-N_2O$ and $\delta^{15}N-N_2O$ evolved from soil under a rather limited suite of conditions: strictly anaerobic, with no variation in the starting $\delta^{15}N$ value of NO_3^- , and from only two field sites (birch or larch). However, our data span the range of documented variation in $\delta^{15}N$ and $\delta^{18}O$ from terrestrial N_2O sources (Figure 6). This is because isotopic signatures of N_2O , produced by the same soil and during only one process, have distinct signatures over the course of an incubation.

[29] Limited published data are available on N_2O fractionation during denitrification and nitrification. $\delta^{15}N-N_2O$ and $\delta^{15}N-NO_3^-$ have been reported to vary from 13 to 30‰ [Barford et al., 1999; Wada and Ueda, 1996]. Nitrification separates more strongly, causing differences between $\delta^{15}N-N_2O$ and $\delta^{15}N-NH_4^+$ that range from 45 to 66‰ [Ueda et al., 1999; Yoshida, 1988]. Barford et al. [1999] estimated enrichment values of –28.6‰ for *Paracoccus denitrificans* in pure culture, but no enrichments have been reported for soil-evolved N_2O . It is problematic to extrapolate fractionation parameters received for pure bacterial cultures to environmental samples such as soil, where a plethora of denitrifying or nitrifying genera is present and distinct

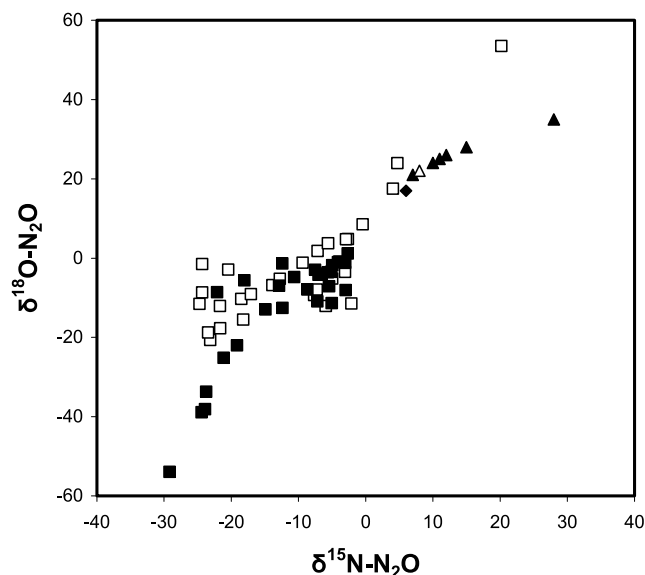


Figure 6. N₂O isotopic signatures for all time points produced during the N₂O production experiment by denitrification with C₂H₂ (solid squares) and without C₂H₂ (open squares). The isotopic signatures for N₂O emitted from the ocean surface (solid diamond) [Dore *et al.*, 1998], tropospheric N₂O (open triangle) and stratospheric N₂O (solid triangles) [Rahn and Wahlen, 1997] are shown for comparison.

genera might have different isotopic enrichments. Data on fractionation parameters for O isotopes during N₂O production is even more limited.

[30] Nitrous oxide reductase has been reported to enrich the unreacted N₂O in δ¹⁵N by 13 to 27‰ [Barford *et al.*, 1999; Wada and Ueda, 1996]. Yamazaki *et al.* [1987] reported the maximum ε of -39‰ for N₂O reduction by the N₂ fixer *Azotobacter vinelandii*, but this species possesses both N₂O reductase and nitrogenase, which also may reduce N₂O, making unclear which enzyme (nitrogenase or N₂O-reductase) was responsible for isotope enrichment in N₂O. For oxygen isotopes during N₂O reduction, Barford [1997] estimated an overall enrichment of -105‰, assuming no interaction of oxygen isotopes between N₂O and H₂O. Wahlen and Yoshinari [1985] determined that, during N₂O reduction by denitrifying organisms, δ¹⁸O increases by a range of 37 to 42‰. Thus reported enrichment values for oxygen for N₂O reduction are very different and may also reflect different organisms or incubation conditions.

[31] The enrichment factors for both oxygen and nitrogen isotopes during N₂O reduction by soil are reported in this paper. It was surprising that the direction of isotope fractionation during N₂O consumption depended on the growth stage of N₂O-reducing bacteria. Because we observed normal fractionation (preference for the lighter isotopes) at the beginning of the incubation, it is unlikely that the N₂O reductase active initially contributed to the apparent inverse isotope effect observed later. Rather, we suspect that the shift in the direction of the fractionation likely involves other processes. First, there may be an enzyme system capable of consuming N₂O that causes inverse fractionation, a phenomenon that has been observed for other enzyme systems [Alkema *et al.*, 2003; Jordan *et al.*, 1978]. As described above, the enzyme characteristics of nitrous oxide reductase have been characterized from only a few denitrifying bacteria. Possibly, an enzyme system specific to a particular group of denitrifiers that became active late in the incubation has an inverse isotope effect, particularly those adapted to higher concentrations of N₂O such as those used in our incubations. As described above, nitrogenase can also reduce N₂O to N₂ under anaerobic conditions. While traditional isotopic fractionation has been observed for *Azotobacter vinelandii* [Yamazaki *et al.*, 1987], whether this is general for nitrogenase is not known. Second, a process producing isotopically light N₂O could have begun late in the incubation, causing the appearance of an inverse isotope effect during net N₂O consumption. Nitrification is unlikely, because of the anaerobic conditions of the incubations. Denitrification is also unlikely, because we observed no N₂O production in the presence of C₂H₂ (Figures 3a and 3d). Nitrifier denitrification is another possibility [Wrage *et al.*, 2001, 2004]; nitrifier denitrification is known to produce N₂O but the isotopic kinetics of this process have not been characterized. The activity of nitrifier denitrification could also have been enhanced at the later stages of incubation due to progressive depletion of residual O₂ as mentioned above. These explanations are not mutually exclusive. The apparent inverse isotope effect requires further investigation.

[32] In the N₂O production experiment, we found that δ¹⁸O-N₂O and δ¹⁵N-N₂O values were most depleted at the beginning of the incubation, and then became more positive over time, likely owing to limitation of denitrification by nitrate. Overall, N₂O was most enriched in both isotopes in the incubations without C₂H₂, because of fractionation during N₂O reduction. Plotting δ¹⁸O-N₂O versus δ¹⁵N-N₂O values of N₂O evolved from denitrification (Figure 6) gives a clear picture of the contribution of N₂O-reductase to isotopic signatures of N₂O: when N₂O-reductase was suppressed, the isotopic composition of accumulated N₂O

Table 1. Summary of Fractionation Parameters Determined for the Two Stages of Denitrification: N₂O Production and N₂O Reduction

Isotopes Fractionation Characteristics	N ₂ O Production (NO ₃ ⁻ → N ₂ O)		N ₂ O Reduction (N ₂ O → N ₂)	
	Larch	Birch	Larch	Birch
ε for δ ¹⁵ N-N ₂ O	-24--25‰	-24--29‰	-6.3--9.8‰	-6.3--8.3‰
ε for δ ¹⁸ O-N ₂ O	-34--39‰	-39--54‰	-12.6--24.9‰	-16--21‰
Ratio δ ¹⁸ O-N ₂ O-to-δ ¹⁵ N-N ₂ O	1.6-1.7	2.5-2.7	2.51	2.55-2.56

was consistently lower compared to when N₂O-reductase was active (compare open versus solid squares in Figure 6). This confirms experimentally that N₂O-reductase enriches both isotopes in N₂O, making the N₂O evolved from the soil into the atmosphere heavier. Globally, atmospheric N₂O is becoming lighter [Rahn and Wahlen, 2000]. It is suggested that this is due to increased efflux of light N₂O from agricultural ecosystems [Pérez *et al.*, 2001]; because of high rates of inorganic N application, soil denitrifying and nitrifying bacteria are less limited by nitrogen, causing maximum isotopic separation. Alternatively, a general decline in N₂O reduction, also possibly driven by increased inorganic N fertilizer application, could contribute to the global isotopic depletion in N₂O. This idea warrants further investigation.

[33] We found higher enrichment for oxygen isotopes than for nitrogen in both stages of denitrification (Table 1). Although we only compared soils under two tree species, we found overlapping values for enrichment, suggesting that any differences in microbial denitrifying communities beneath birch and larch were likely not important for the fractionation associated with denitrification. However, the ratio of $\delta^{18}\text{O}$ -to- $\delta^{15}\text{N}$ of N₂O produced was different beneath larch (1.6–1.7) and birch (2.5–2.7); thus isotopic enrichment was stronger for oxygen than for nitrogen, and the difference varied among species. The ratios of $\delta^{18}\text{O}$ -to- $\delta^{15}\text{N}$ we found differ from those reported by others. For example, Bötscher *et al.* [1990] measured the isotopic composition of nitrate and found higher Rayleigh enrichment for nitrogen (15.9‰) than for oxygen (8.0‰) during NO₃⁻ reduction by denitrification in groundwater, contrary to our findings. Similarly, Pérez *et al.* [2001] found a value of 0.27 for the ratio of $\delta^{18}\text{O}:\delta^{15}\text{N}$ in N₂O evolved from agricultural soils, and Toyoda *et al.* [2005] even report negative values for this ratio due to an inverse isotope effect observed for oxygen. This large variation observed among studies (and in our case between tree species) could be explained by the fact that reduction of NO₃⁻ to N₂O is sequential (NO₃⁻ → NO₂⁻ → NO → N₂O), and steps in the sequence likely vary in isotopic enrichment and thus $\delta^{18}\text{O}$ -to- $\delta^{15}\text{N}$ ratios. The ratio of intermediate denitrification products, (NO₂ + NO)/N₂O, is highly variable [Tiltsner *et al.*, 2003] and this may account for differences in the ratios of $\delta^{18}\text{O}$ -to- $\delta^{15}\text{N}$ in N₂O. Thus, the ratios of $\delta^{18}\text{O}$ -to- $\delta^{15}\text{N}$ by N₂O production vary widely, possibly reflecting environmental conditions, or differences in denitrifying organisms. This variation suggests that it may be necessary to complement field measurements with incubation studies under conditions favorable for denitrification (as in our study) to determine for a particular site how denitrification affects the ratios of $\delta^{18}\text{O}$ -to- $\delta^{15}\text{N}$ in N₂O.

[34] N₂O-reductase caused a 2.5-fold greater enrichment in $\delta^{18}\text{O}$ compared to $\delta^{15}\text{N}$, and this ratio, in contrast to N₂O production, was consistent between species. The same ratio was reported for pure cultures of denitrifying bacteria and a soil by Ostrom *et al.* [2004]. This suggests that the determined ratio (2.5) is general and it could prove useful in estimating the proportion of N₂O being reduced in the soil before escaping into the atmosphere. As stated above, the most powerful method for characterizing the sources of

N₂O lies in its multiisotope signatures [Stein and Yung, 2003].

[35] In summary, we found both ¹⁵N and ¹⁸O isotopic enrichment in N₂O during N₂O reduction by denitrifiers. We also found that the two stages of N₂O transformation during denitrification, N₂O production and consumption, differ in isotope effects, potentially providing another means to assess their relative importance. Finally, the data received allowed us to estimate the ratio of $\delta^{18}\text{O}$ -N₂O-to- $\delta^{15}\text{N}$ -N₂O for both stages of denitrification, a ratio that in some cases may provide insight on the biological origin of N₂O. There are several excellent works utilized the positioning of ¹⁵N in the asymmetric molecule of N₂O (see review of Stein and Yung [2003]). Nitrifying and denitrifying bacteria show a different preference in enriching ¹⁵N in either central (¹⁴N¹⁵N¹⁶O) or terminal position (¹⁵N¹⁴N¹⁶O) in N₂O [Yoshida and Toyoda, 2000; Pérez *et al.*, 2001; Sutka *et al.*, 2006], and nitrification and nitrifier denitrification appear to differ in their effects on isotopomers of N₂O as well [Sutka *et al.*, 2006]. The combination of isotopomers, the $\delta^{18}\text{O}$ and $\delta^{15}\text{N}$ signatures, and the ratios of $\delta^{18}\text{O}$ -to- $\delta^{15}\text{N}$ of N₂O could improve our understanding in biological sources of N₂O and their relative importance for the global N₂O budget.

[36] **Acknowledgments.** We kindly thank Tibisay Pérez for providing N₂O stable isotopes standards for calibration of our reference gas and Rick Doucett for help with measurements of isotopic composition of N₂O. This work was supported by the U.S. Civilian Research and Development Foundation (CRDF) and the U.S. National Science Foundation (DEB-0092642).

References

- Alkema, W. B. L., E. de Vries, R. Floris, and D. B. Janssen (2003), Kinetics of enzyme acylation and deacylation in the penicillin acylase-catalyzed synthesis of beta-lactam antibiotics, *Eur. J. Biochem.*, 270(18), 3675–3683.
- Amberger, A., and H. L. Schmidt (1987), Natürliche Isotopengehalte von Nitrat als Indikatoren für dessen Herkunft, *Geochim. Cosmochim. Acta*, 51, 2699–2705.
- Barford, C. C. (1997), Stable isotope dynamics of denitrification, Ph. D. thesis, Harvard Univ., Cambridge, Mass.
- Barford, C. C., J. P. Montoya, M. A. Altabet, and R. Mitchell (1999), Steady-state nitrogen isotope effects of N₂ and N₂O production in *Paracoccus denitrificans*, *Appl. Environ. Microbiol.*, 65, 989–994.
- Bötscher, J., O. Stöbel, S. Voerkelius, and H.-L. Schmidt (1990), Using isotope fractionation of nitrate nitrogen and nitrate oxygen for evaluation of denitrification in a sandy aquifer, *J. Hydrol.*, 114, 413–424.
- Casciotti, K. L., D. M. Sigman, M. Galanter Hastings, J. K. Bohlke, and A. Hilker (2002), Measurement of the oxygen isotopic composition of nitrate in seawater and freshwater using the denitrifier method, *Anal. Chem.*, 74(19), 4905–4912.
- Crutzen, P. J. (1981), Atmospheric chemical processes of the oxides of nitrogen, including N₂O, in *Denitrification, Nitrification, and Atmospheric Nitrous Oxide*, edited by C. C. Delwiche, pp. 17–44, John Wiley, Hoboken, N. J.
- Davidson, E. A. (1991), Fluxes of nitrous oxide and nitric oxide from terrestrial ecosystems, in *Microbial Production and Consumption of Greenhouse Gases: Methane, Nitrogen Oxides and Halomethanes*, edited by J. E. Rogers and W. B. Whitman, pp. 219–235, Am. Soc. for Microbiol., Washington, D. C.
- Davidson, E. A., and J. P. Schimel (1995), Microbial processes of production and consumption of nitric oxide, nitrous oxide and methane, in *Biogenic Trace Gases: Measuring Emissions From Soil and Water*, edited by P. A. Matson and R. C. Harris, pp. 327–357, Blackwell Sci., Malden, Mass.
- Dore, J. E., B. N. Popp, D. M. Karl, and F. J. Sansone (1998), A large source of atmospheric nitrous oxide from subtropical North Pacific surface waters, *Nature*, 396, 63–66.
- Food and Agriculture Organization (1990), Soil map of the world, revised legend, map, Rome.

- Jordan, F., D. J. Kuo, and E. E. Monse (1978), Carbon kinetic isotope effects on pyruvate decarboxylation catalyzed by yeast pyruvate decarboxylase and models, *J. Am. Chem. Soc.*, *100*(9), 2872–2878.
- Kim, K.-R., and H. Craig (1990), Two-isotope characterization of N₂O in the Pacific Ocean and constraints on its origin in deep water, *Nature*, *347*, 58–61.
- Kim, K.-R., and H. Craig (1993), Nitrogen-15 and oxygen-18 characteristics of nitrous oxide: A global perspective, *Science*, *262*, 1855–1857.
- Mariotti, A., J. C. Germon, P. Hubert, P. Kaiser, R. Letolle, A. Tardieux, and P. Tardieux (1981), Experimental determination of nitrogen kinetic isotope fractionation: Some principles. Illustration for the denitrification and nitrification processes, *Plant Soil*, *62*, 413–430.
- Mayer, B., S. M. Bollwerk, T. Mansfeldt, B. Hutter, and J. Veizer (2001), The oxygen isotope composition of nitrate generated by nitrification in acid forest floors, *Geochim. Cosmochim. Acta*, *65*, 2743–2756.
- Menyailo, O. V., B. A. Hungate, and W. Zech (2002a), Tree species mediated soil chemical changes in a Siberian artificial afforestation experiment, *Plant Soil*, *242*, 171–182.
- Menyailo, O. V., B. A. Hungate, and W. Zech (2002b), The effect of single tree species on soil microbial activities related to C and N cycling in the Siberian artificial afforestation experiment, *Plant Soil*, *242*, 183–196.
- Morgan, C. G., M. Allen, M. C. Liang, R. L. Shia, G. A. Blake, and Y. L. Yung (2004), Isotopic fractionation of nitrous oxide in the stratosphere: Comparison between model and observations, *J. Geophys. Res.*, *109*, D04305, doi:10.1029/2003JD003402.
- Ostrom, P. H., A. J. Pitt, R. L. Sutka, N. E. Ostrom, L. Fang, and H. Gandhi (2004), Characterization of isotopomer fractionation during consumption of nitrous oxide in pure culture and soils, *Eos Trans. AGU*, *85*(47), Fall Meet. Suppl., Abstract B21A-0852.
- Pérez, T., S. E. Trumbore, S. C. Tyler, E. A. Davidson, M. Keller, and P. B. de Camargo (2000), Isotopic variability of N₂O emissions from tropical forest soils, *Global Biogeochem. Cycles*, *14*(2), 525–535.
- Pérez, T., S. E. Trumbore, S. C. Tyler, P. A. Matson, I. Ortiz-Monasterio, T. Rahn, and D. W. T. Griffith (2001), Identifying the agricultural imprint on the global N₂O budget using stable isotopes, *J. Geophys. Res.*, *106*(D9), 9869–9878.
- Peterson, B. J., and B. Fry (1987), Stable isotopes in ecosystem studies, *Annu. Rev. Ecol. Syst.*, *18*, 293–320.
- Rahn, T., and M. Wahlen (1997), Stable isotope enrichment in stratospheric nitrous oxide, *Science*, *278*, 1776–1778.
- Rahn, T., and M. Wahlen (2000), A reassessment of the global isotope budget of atmospheric nitrous oxide, *Global Biogeochem. Cycles*, *14*(2), 537–543.
- Sigman, D. M., K. L. Casciotti, M. Andreani, C. Barford, M. Galanter, and J. K. Bohlke (2001), A bacterial method for the nitrogen isotopic analysis of nitrate in seawater and freshwater, *Anal. Chem.*, *73*(17), 4145–4153.
- Stein, L. Y., and Y. L. Yung (2003), Production, isotopic composition, and atmospheric fate of biologically produced nitrous oxide, *Annu. Rev. Earth Planet. Sci.*, *31*, 329–356.
- Stevens, R. J., R. J. Laughlin, L. C. Burns, J. R. M. Arah, and R. C. Hood (1997), Measuring the contributions of nitrification and denitrification to the flux of nitrous oxide from soil, *Soil Biol. Biochem.*, *29*, 139–151.
- Sutka, R. L., N. E. Ostrom, P. H. Ostrom, J. A. Breznak, H. Gandhi, A. J. Pitt, and F. Li (2006), Distinguishing nitrous oxide production from nitrification and denitrification on the basis of isotopomer abundances, *Appl. Environ. Microbiol.*, *72*, 638–644.
- Tanaka, N., D. M. Rye, R. Rye, H. Avak, and T. Yoshinari (1995), High precision mass spectrometric analysis of isotopic abundance ratios in nitrous oxide by direct injection of N₂O, *Int. J. Mass Spectrom. Ion Processes*, *142*, 163–175.
- Tilsner, J., N. Wrage, J. Lauf, and G. Gebauer (2003), Emission of gaseous nitrogen oxides from an extensively managed grassland in NE Bavaria, Germany, *Biogeochemistry*, *63*, 249–267.
- Tong, J. Y., and P. E. Yankowich (1957), Calculations of experimental isotope effects for pseudo first-order reactions, *J. Phys. Chem.*, *61*, 540–543.
- Toyoda, S., H. Mutohe, H. Yamagishi, N. Yoshida, and Y. Tanji (2005), Fractionation of N₂O isotopomers during production by denitrifier, *Soil Biol.*, *37*, 1535–1545.
- Ueda, S., C.-S. Go, Y. Suwa, Y. Matsui, F. Yamaguchi, T. Shoji, K. Noto, T. Sumino, A. Tanaka, and Y. Matsufuji (1999), Stable isotope fingerprint of N₂O produced by ammonium oxidation under laboratory and field conditions, paper presented at International Workshop on the Atmospheric N₂O Budget: An Analysis of the State of Our Understanding of Sources and Sinks of Atmospheric N₂O, Jpn. Natl. Inst. of Agro-Environ. Sci., Tsukuba, Japan.
- Voerkelius, S. (1990), Isotopendiskriminierungen bei der Nitrifikation und Denitrifikation: Grundlagen und Anwendungen der Herkunfts-Zuordnung von Nitrat und Distickstoffmonoxid, Ph.D. dissertation, 119 pp., Tech. Univ. of Munich, Munich, Germany.
- Wada, E., and S. Ueda (1996), Carbon, nitrogen, and oxygen isotope ratios of CH₄ and N₂O in soil ecosystems, in *Mass Spectrometry of Soils*, edited by T. W. Boutton and S.-I. Yamasaki, pp. 177–204, CRC Press, Boca Raton, Fla.
- Wahlen, M., and T. Yoshinari (1985), Oxygen isotope ratios in N₂O from different environments, *Nature*, *313*, 780–782.
- Wassenaar, L. I. (1995), Evaluation of the origin and fate of nitrate in the Abbotsford Aquifer using the isotopes of ¹⁵N and ¹⁸O in NO₃⁻, *Appl. Geochem.*, *10*, 391–405.
- Webster, E. A., and D. W. Hopkins (1996), Nitrogen and oxygen isotope ratios of nitrous oxide emitted from soil and produced by nitrifying and denitrifying bacteria, *Biol. Fertil. Soils*, *22*, 326–330.
- Wedin, D. A., and D. Tilman (1990), Species effects on nitrogen cycling: A test with perennial grasses, *Oecologia*, *84*, 433–441.
- Wrage, N., G. L. Velthof, M. L. van Beusichem, and O. Oenema (2001), Role of nitrifier denitrification in the production of nitrous oxide, *Soil Biol. Biochem.*, *33*, 1723–1732.
- Wrage, N., J. Lauf, A. del Prado, M. Pinto, S. Pietrzak, S. Yamulki, O. Oenema, and G. Gebauer (2004), Distinguishing sources of N₂O in European grassland by stable isotope analysis, *Rapid Commun. Mass Spectrom.*, *18*, 1201–1207.
- Yamazaki, T., N. Yoshida, E. Wada, and S. Matsuo (1987), N₂O reduction by *Azotobacter vinelandii* with emphasis on kinetic nitrogen isotope effects, *Plant Cell Physiol.*, *28*, 263–271.
- Yoshida, N. (1988), ¹⁵N-depleted N₂O as a product of nitrification, *Nature*, *335*, 528–529.
- Yoshida, N., and S. Toyoda (2000), Constraining the atmospheric N₂O budget from intramolecular site preference in N₂O isotopomers, *Nature*, *405*, 330–334.
- Yoshinari, T. (1990), Emissions of N₂O from various environments—The use of stable isotope composition of N₂O as tracer for the studies of N₂O biogeochemical cycling, in *Denitrification in Soil and Sediment*, edited by N. P. Revsbech and J. Sorensen, pp. 129–150, Springer, New York.
- Yung, Y. L., W. C. Wang, and A. A. Lacis (1976), Greenhouse effect due to atmospheric nitrous oxide, *Geophys. Res. Lett.*, *3*(10), 619–621.

B. A. Hungate, Department of Biological Sciences and Merriam Powell Center for Environmental Research, Northern Arizona University, Box 5640, Flagstaff, AZ 86011, USA. (bruce.hungate@nau.edu)

O. V. Menyailo, Institute of Forest Siberian Branch of Russian Academy of Sciences (SB RAS), Krasnoyarsk 660036, Russia. (menyailo@hotmail.com)