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

Oleg V. Menyailo[1] and Bruce A. Hungate[2]

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[1] Measuring the stable isotope composition of nitrous oxide (N_2O) evolved from soil could improve our understanding of the relative contributions of the main microbial processes (nitrification and denitrification) responsible for N_2O formation in soil. However, interpretation of the isotopic data in N_2O is complicated by the lack of knowledge of fractionation parameters by different microbial processes responsible for N_2O production and consumption. Here we report isotopic enrichment for both nitrogen and oxygen isotopes in two stages of denitrification, N_2O production and N_2O reduction. We found that during both N_2O production and reduction, enrichments were higher for oxygen than nitrogen. For both elements, enrichments were larger for N_2O production stage than for N_2O reduction. During gross N_2O production, the ratio of $\delta^{18}O$-to-$\delta^{15}N$ differed between soils, ranging from 1.6 to 2.7. By contrast, during N_2O reduction, we observed a constant ratio of $\delta^{18}O$-to-$\delta^{15}N$ with a value near 2.5. If general, this ratio could be used to estimate the proportion of N_2O being reduced in the soil before escaping into the atmosphere. Because N_2O-reductase enriches N_2O in both isotopes, the global reduction of N_2O consumption by soil may contribute to the globally observed isotopic depletion of atmospheric N_2O.

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1. Introduction

[2] The increase in the atmospheric concentration of nitrous oxide (N_2O) has attracted considerable scientific attention from different disciplines during the last 15–20 years, because N_2O 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 N_2O 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 N_2O emission is critical for modeling and predicting changes in N_2O fluxes under varying environmental conditions, including altered temperature and precipitation.

[3] The sources of N_2O 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 N_2O [*Stevens et al.*, 1997]. Another way to identify the processes producing N_2O is to study the stable isotope composition of N_2O and how it changes in space and time [*Yoshida*, 1988; *Yoshinari*, 1990]. Because the stable isotope ratios of $^{15}N/^{14}N$ and $^{18}O/^{16}O$ of denitrifier-derived N_2O can differ from those of nitrifier-derived N_2O [*Kim and Craig*, 1990; *Webster and Hopkins*, 1996], the isotopic composition is thus a window through which we may understand biological processes underlying N_2O 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 N_2O. Compared to denitrification, nitrification often produces N_2O that is ^{15}N-depleted N_2O [*Yoshida*, 1988]. The reported values for isotopic enrichment for ^{15}N (difference in the $\delta^{15}N$ between product and substrate) range from −13 to −27‰ for denitrification and up to −60‰ for nitrification [*Yoshida*, 1988]. Consequently, if $\delta^{15}N$ values of soil nitrate and ammonium are equal, $\delta^{15}N$-N_2O derived from nitrifiers should be about 30‰ more negative than $\delta^{15}N$ of denitrifier-derived N_2O.

[5] Denitrifying bacteria also select isotopically light N_2O for reduction to N_2, enriching the remaining un-reacted N_2O in ^{15}N [*Barford et al.*, 1999]. However, as for N_2O production, fractionation of oxygen isotopes during N_2O reduction has rarely been reported. Together, measuring both $\delta^{15}N$ and $\delta^{18}O$ values in N_2O will likely provide more insight into the sources of N_2O than would either alone

[1]Institute of Forest, Siberian Branch of Russian Academy of Sciences, Krasnoyarsk, Russia.
[2]Department of Biological Sciences and Merriam Powell Center for Environmental Research, Northern Arizona University, Flagstaff, Arizona, USA.

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[*Stein and Yung*, 2003]. For example, we expect covariance between $\delta^{15}N$ and $\delta^{18}O$ 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 $\delta^{15}N$ and $\delta^{18}O$ in N_2O produced by nitrification is unlikely. N_2O reduction should also cause co-variation between $\delta^{15}N$ and $\delta^{18}O$ in the N_2O that remains, because both elements (N and O) are contained in the substrate (N_2O). Thus testing for covariance between $\delta^{15}N$ and $\delta^{18}O$ 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 $\delta^{18}O$-to-$\delta^{15}N$ 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, N_2O/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 $\delta^{15}N$ and $\delta^{18}O$ measurements as described above.

2.4. Stable Isotope Analysis

[11] The ratios of the stable isotopes $^{15}N/^{14}N$ and $^{18}O/^{16}O$ 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 × 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 $^{15}N_2^{16}O/^{14}N_2^{16}O$ and $^{14}N_2^{18}O/^{14}N_2^{16}O$, respectively. We used N_2O as a reference gas (99.9990%, Linde). The $\delta^{15}N$ and $\delta^{18}O$ 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

N_2O complicates the use of natural abundance variations of the isotopic composition of N_2O. 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 $\delta = [(R_{sample}/R_{standard}) - 1] \times 1000$. $R = {}^{15}N/{}^{14}N$ or ${}^{18}O/{}^{16}O$. Delta values are reported as deviations from $\delta^{15}N$ of atmospheric N_2 and $\delta^{18}O$ of atmospheric O_2. The conversion for the $\delta^{18}O_{atm}$ standard to SMOW standard is $\delta^{18}O_{atm} = -23 + \delta^{18}O_{SMOW}/1.0235$ [*Kim and Craig*, 1990]. The precision of the isotopic composition measurements in N_2O was better than 0.5‰ for $\delta^{15}N$ and better than 1‰ for $\delta^{18}O$.

2.5. Definition of Enrichment Factor

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

$$\alpha = R^i_p(t)/R_s(t), \tag{1}$$

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

$$\varepsilon = \delta^i_p(t) - \delta_s(t), \tag{2}$$

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, $\delta^i_p(t)$ is nearly equal to the isotope ratios of accumulated product, $\delta_p(t)$.

$$\varepsilon = \delta_p(t) - \delta_s(t = 0). \tag{3}$$

[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

$$\delta_s(t) \sim \delta_s(t = 0) + \varepsilon \ln f, \tag{4}$$

or for the product

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

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 N_2O was measured, which is both a product (for N_2O production) and substrate (for N_2O reduction). For N_2O reduction, the enrichment factor was estimated according to equation (4); here $\delta_s(t)$ is the isotopic composition of residual N_2O and f is the proportion of N_2O remaining from the initial N_2O concentration. Because the direction of the isotope effect during N_2O reduction shifted during the experiment, we only calculated enrichment during the initial phases of N_2O 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 N_2O or N_2, one atom of oxygen escapes from the reaction sequence at each step, so we have two factors contributing to oxygen isotopic fractionation: biological selection of lighter molecules for reduction and possible preferential release of the lighter isotope ^{16}O from the reaction [*Toyoda et al.*, 2005]. Our use of the equations (2) and (5) for oxygen isotopes during N_2O 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 ^{18}O-N_2O during N_2O production as the "reaction sequence enrichment."

[18] For N_2O production, we estimated the enrichment factor for $\delta^{15}N$ and reaction sequence enrichment for $\delta^{18}O$ according to equation (2) as the difference in the $\delta^{15}N$ or $\delta^{18}O$ values between the product (N_2O) and substrate (NO_3^-) at the first measurement points, when N_2O was most depleted and the isotopic composition of NO_3^- was likely similar to that of the originally applied NO_3^-. We used final values of $\delta^{15}N$ and $\delta^{18}O$ of N_2O produced during the incubations with nitrate to estimate the $\delta^{15}N$ and $\delta^{18}O$ values in applied NO_3^-, because at this late stage of incubation (after N_2O production had ceased) the isotopic values of the product should equal the initial isotopic values of the substrate (NO_3^-). While less precise than direct determination of both $\delta^{15}N$ [*Sigman et al.*, 2001] and $\delta^{18}O$ [*Casciotti et al.*, 2002] in NO_3^-, 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 N_2O 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 N_2O reduction but problematic for the N_2O production experiment, since the amount of applied NO_3^- may not be fully available for denitrification owing to immobilization. The amount of N_2O produced with C_2H_2, indicating the amount of NO_3^- denitrified, was always much lower than NO_3^- amended (Figures 1a and 1b). Because N_2O accumulation ceased during the later stages of incubation, we assume that all NO_3^- was denitrified or immobilized. Thus we used the maximum N_2O concentration in the C_2H_2 treatment to

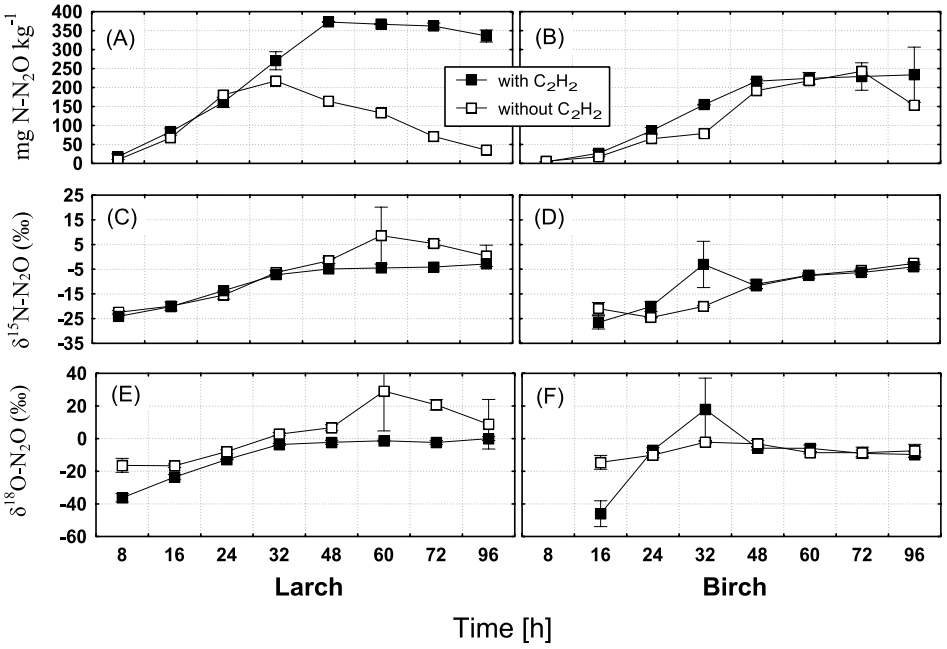

Figure 1. Production of (a, b) nitrous oxide, (c, d) cumulative isotopic signatures of N_2O: $\delta^{15}N$ and (e, f) $\delta^{18}O$ during the 96 h of incubation of soil samples beneath larch and birch.

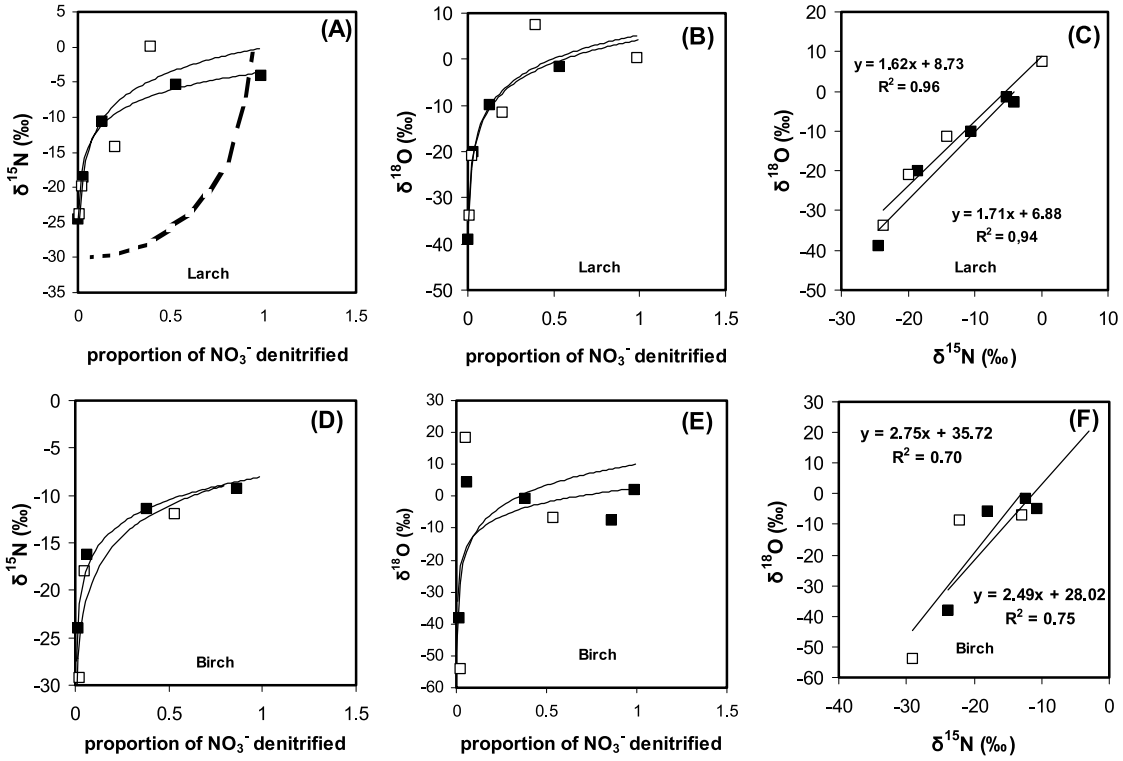

Figure 2. Isotopic signatures of N_2O versus the proportion of NO_3^- denitrified (1 − f) for C_2H_2 treatment. For larch (a) $\delta^{15}N$ and (b) $\delta^{18}O$. For birch (d) $\delta^{15}N$ and (e) $\delta^{18}O$. These data do not fit Rayleigh distillation model, approximate curve of which is shown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

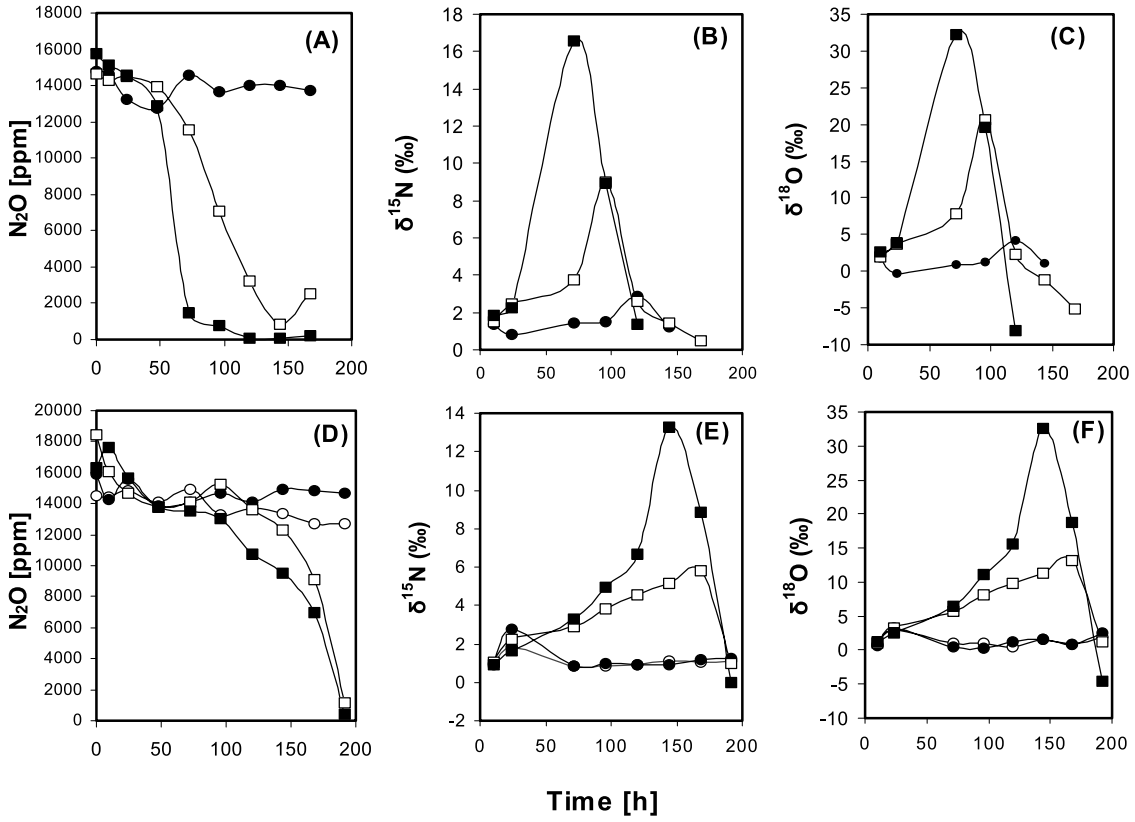

Figure 3. Changes in N_2O concentration and stable isotope values during the reduction experiment for soils from (a–c) larch and (d–f) birch. N_2O concentrations are shown in Figures 3a and 3d, $\delta^{15}N$ values in Figures 3b and 3e, and $\delta^{18}O$ values in Figures 3c and 3f. Circles indicate 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 $\delta^{15}N$-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 $\delta^{15}N$-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 $\delta^{18}O$ values of the N_2O accumulated in incubations of soils from larch without C_2H_2 were consistently higher (more

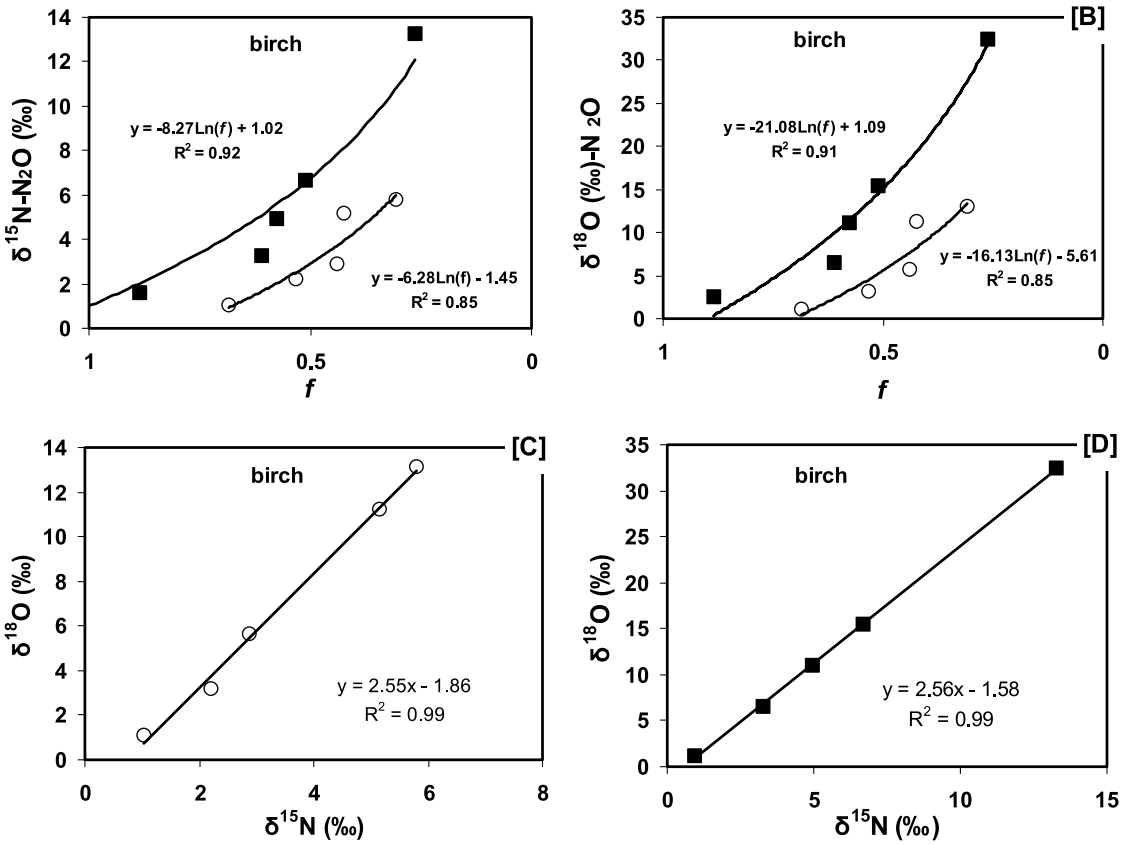

Figure 4. (a, b)Rayleigh fits to the isotopic composition of N_2O reduced by the two replicate samples taken under birch (solid squares and open circles show different replicates). (c, d) Relationship between $\delta^{18}O$ and $\delta^{15}N$ values of N_2O for both samples.

positive) than $\delta^{18}O$ values of N_2O accumulated in incubations with C_2H_2 (Figure 1e). This difference was not as apparent for $\delta^{15}N$-N_2O, indicating stronger isotopic fractionation for oxygen compared to nitrogen during N_2O reduction. Compared to $\delta^{15}N$, the $\delta^{18}O$ value of N_2O may provide a more powerful signature of in situ activity of N_2O-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 $\delta^{18}O$ and $\delta^{15}N$, a clear difference between soils under different species was found for the ratio of $\delta^{18}O$-N_2O: $\delta^{15}N$-N_2O during N_2O production (Figures 2c and 2f). The ratio $\delta^{18}O$-N_2O-to-$\delta^{15}N$-N_2O was 1.6–1.7 (Figure 2c) for larch and 2.5–2.7 for birch (Figure 2f).

3.2. N_2O Reduction Experiment

[24] The consumption of N_2O 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 N_2O was consumed by the N_2O-reductase already present in soils. But soils under different tree species significantly differed in the rate of initial N_2O consumption and in the time required for *de novo* synthesis of N_2O-reductase. At this initial stage, soils under larch consumed N_2O two times faster than soils under birch. After 50 hours, soil under larch began the exponential phase of N_2O consumption, depleting the majority of N_2O from 72 to 144 hours, at which point the rate of consumption declined, likely owing to substrate (N_2O) 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, N_2O-reductase. During the lag-phase of N_2O consumption, $\delta^{15}N$ and $\delta^{18}O$ values of N_2O increased in soil from both species, following the expected pattern when N_2O-reductase reacts more rapidly with the lighter isotopes, enriching the substrate (N_2O) in the heavier isotopes.

[25] The enrichment factors for the initial phases of N_2O reduction experiment for $\delta^{15}N$-N_2O were −6.3, −9.8‰ and much higher for $\delta^{18}O$-N_2O (−16, −24‰) (Figures 4a and 4b and Figures 5a and 5b). The relationship between $\delta^{18}O$-N_2O and $\delta^{15}N$-N_2O values during initial N_2O reduction was strong and consistent, yielding a stable ratio of $\delta^{18}O$-to-$\delta^{15}N$ with a value near 2.5. In other words, N_2O-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.

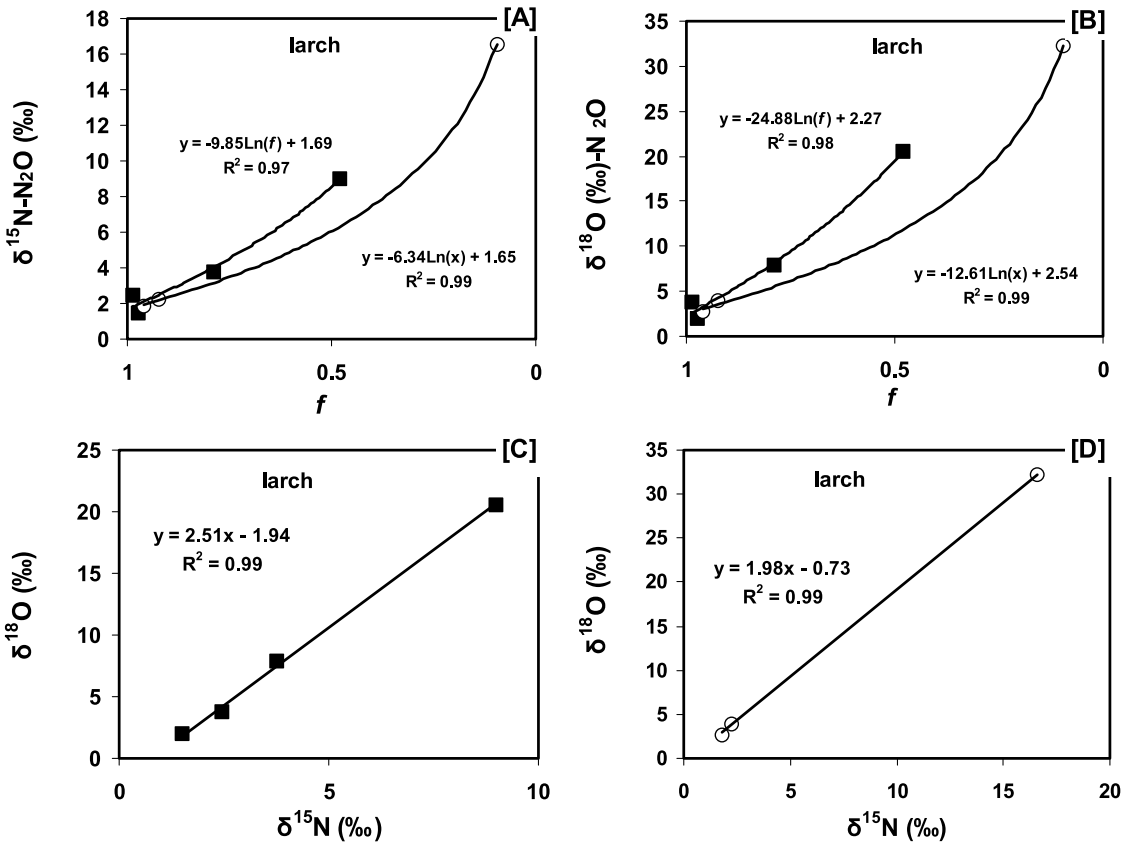

Figure 5. (a, b) Rayleigh fits to the isotopic composition of N_2O reduced by the two replicate samples taken under larch (dark squares and open circles show different replicates). (c, d) Relationship between $\delta^{18}O$ and $\delta^{15}N$ values of N_2O for both samples.

[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

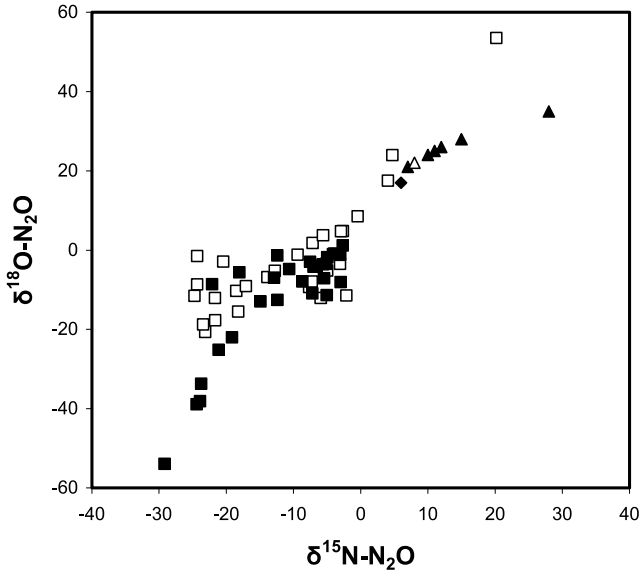

Figure 6. N_2O isotopic signatures for all time points produced during the N_2O production experiment by denitrification with C_2H_2 (solid squares) and without C_2H_2 (open squares). The isotopic signatures for N_2O emitted from the ocean surface (solid diamond) [*Dore et al.*, 1998], tropospheric N_2O (open triangle) and stratospheric N_2O (solid triangles) [*Rahn and Wahlen*, 1997] are shown for comparison.

genera might have different isotopic enrichments. Data on fractionation parameters for O isotopes during N_2O production is even more limited.

[30] Nitrous oxide reductase has been reported to enrich the unreacted N_2O in $\delta^{15}N$ by 13 to 27‰ [*Barford et al.*, 1999; *Wada and Ueda*, 1996]. *Yamazaki et al.* [1987] reported the maximum ε of −39‰ for N_2O reduction by the N_2 fixer *Azotobacter vinelandii*, but this species possesses both N_2O reductase and nitrogenase, which also may reduce N_2O, making unclear which enzyme (nitrogenase or N_2O-reductase) was responsible for isotope enrichment in N_2O. For oxygen isotopes during N_2O reduction, *Barford* [1997] estimated an overall enrichment of −105‰, assuming no interaction of oxygen isotopes between N_2O and H_2O. *Wahlen and Yoshinari* [1985] determined that, during N_2O reduction by denitrifying organisms, $\delta^{18}O$ increases by a range of 37 to 42‰. Thus reported enrichment values for oxygen for N_2O reduction are very different and may also reflect different organisms or incubation conditions.

[31] The enrichment factors for both oxygen and nitrogen isotopes during N_2O reduction by soil are reported in this paper. It was surprising that the direction of isotope fractionation during N_2O consumption depended on the growth stage of N_2O-reducing bacteria. Because we observed normal fractionation (preference for the lighter isotopes) at the beginning of the incubation, it is unlikely that the N_2O 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 N_2O 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 N_2O such as those used in our incubations. As described above, nitrogenase can also reduce N_2O to N_2 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 N_2O could have begun late in the incubation, causing the appearance of an inverse isotope effect during net N_2O consumption. Nitrification is unlikely, because of the anaerobic conditions of the incubations. Denitrification is also unlikely, because we observed no N_2O production in the presence of C_2H_2 (Figures 3a and 3d). Nitrifier denitrification is another possibility [*Wrage et al.*, 2001, 2004]; nitrifier denitrification is known to produce N_2O 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 O_2 as mentioned above. These explanations are not mutually exclusive. The apparent inverse isotope effect requires further investigation.

[32] In the N_2O production experiment, we found that $\delta^{18}O$-N_2O and $\delta^{15}N$-N_2O 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, N_2O was most enriched in both isotopes in the incubations without C_2H_2, because of fractionation during N_2O reduction. Plotting $\delta^{18}O$-N_2O versus $\delta^{15}N$-N_2O values of N_2O evolved from denitrification (Figure 6) gives a clear picture of the contribution of N_2O-reductase to isotopic signatures of N_2O: when N_2O-reductase was suppressed, the isotopic composition of accumulated N_2O

Table 1. Summary of Fractionation Parameters Determined for the Two Stages of Denitrification: N_2O Production and N_2O Reduction

	N_2O Production ($NO_3^- \rightarrow N_2O$)		N_2O Reduction ($N_2O \rightarrow N_2$)	
Isotopes Fractionation Characteristics	Larch	Birch	Larch	Birch
ε for $\delta^{15}N$-N_2O	−24 – −25‰	−24 – −29‰	−6.3 – −9.8‰	−6.3 – −8.3‰
ε for $\delta^{18}O$-N_2O	−34 – −39‰	−39 – −54‰	−12.6 – −24.9‰	−16 – −21‰
Ratio $\delta^{18}O$-N_2O-to-$\delta^{15}N$-N_2O	1.6–1.7	2.5–2.7	2.51	2.55–2.56

was consistently lower compared to when N_2O-reductase was active (compare open versus solid squares in Figure 6). This confirms experimentally that N_2O-reductase enriches both isotopes in N_2O, making the N_2O evolved from the soil into the atmosphere heavier. Globally, atmospheric N_2O is becoming lighter [*Rahn and Wahlen*, 2000]. It is suggested that this is due to increased efflux of light N_2O 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 N_2O reduction, also possibly driven by increased inorganic N fertilizer application, could contribute to the global isotopic depletion in N_2O. 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 δ^{18}O-to-δ^{15}N of N_2O 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 δ^{18}O-to-δ^{15}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 NO_3^- reduction by denitrification in groundwater, contrary to our findings. Similarly, *Pérez et al.* [2001] found a value of 0.27 for the ratio of δ^{18}O:δ^{15}N in N_2O 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 NO_3^- to N_2O is sequential ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O$), and steps in the sequence likely vary in isotopic enrichment and thus δ^{18}O-to-δ^{15}N ratios. The ratio of intermediate denitrification products, $(NO_2 + NO)/N_2O$, is highly variable [*Tilsner et al.*, 2003] and this may account for differences in the ratios of δ^{18}O-to-δ^{15}N in N_2O. Thus, the ratios of δ^{18}O-to-δ^{15}N by N_2O 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 δ^{18}O-to-δ^{15}N in N_2O.

[34] N_2O-reductase caused a 2.5-fold greater enrichment in δ^{18}O compared to δ^{15}N, and this ratio, in contrast to N_2O 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 N_2O being reduced in the soil before escaping into the atmosphere. As stated above, the most powerful method for characterizing the sources of N_2O lies in its multiisotope signatures [*Stein and Yung*, 2003].

[35] In summary, we found both ^{15}N and ^{18}O isotopic enrichment in N_2O during N_2O reduction by denitrifiers. We also found that the two stages of N_2O transformation during denitrification, N_2O 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 δ^{18}O-N_2O-to-δ^{15}N-N_2O for both stages of denitrification, a ratio that in some cases may provide insight on the biological origin of N_2O. There are several excellent works utilized the positioning of ^{15}N in the asymmetric molecule of N_2O (see review of *Stein and Yung* [2003]). Nitrifying and denitrifying bacteria show a different preference in enriching ^{15}N in either central ($^{14}N^{15}N^{16}O$) or terminal position ($^{15}N^{14}N^{16}O$) in N_2O [*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 N_2O as well [*Sutka et al.*, 2006]. The combination of isotopomers, the δ^{18}O and δ^{15}N signatures, and the ratios of δ^{18}O-to-δ^{15}N of N_2O could improve our understanding in biological sources of N_2O and their relative importance for the global N_2O budget.

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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)