

Tree species and moisture effects on soil sources of N₂O: Quantifying contributions from nitrification and denitrification with ¹⁸O isotopes

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Received 28 May 2005; revised 11 February 2006; accepted 17 March 2006; published 21 June 2006.

[1] Nitrous oxide (N₂O) is an important greenhouse gas and participates in the destruction of stratospheric ozone. Soil bacteria produce N₂O through denitrification and nitrification, but these processes differ radically in substrate requirements and responses to the environment. Understanding the controls over N₂O efflux from soils, and how N₂O emissions may change with climate warming and altered precipitation, require quantifying the relative contributions from these groups of soil bacteria to the total N₂O flux. Here we used ammonium nitrate (NH₄NO₃, including substrates for both processes) in which the nitrate has been enriched in the stable isotope of oxygen, ¹⁸O, to partition microbial sources of N₂O, arguing that a molecule of N₂O carrying the ¹⁸O labeled will have been produced by denitrification. We compared the influences of six common tree species on the relative contributions of nitrification and denitrification to N₂O flux from soils, using soils from the Siberian afforestation experiment. We also altered soil water content, to test whether denitrification becomes a dominant source of N₂O when soil water content increases. Tree species altered the proportion of nitrifier and denitrifier-derived N₂O. Wetter soils produced more N₂O from denitrification, though the magnitude of this effect varied among tree species. This indicates that the roles of denitrification and nitrification vary with tree species, and, that tree species influence soil responses to increased water content.

Citation: Menyailo, O. V., and B. A. Hungate (2006), Tree species and moisture effects on soil sources of N₂O: Quantifying contributions from nitrification and denitrification with ¹⁸O isotopes, *J. Geophys. Res.*, *111*, G02022, doi:10.1029/2005JG000058.

1. Introduction

[2] N₂O is a major greenhouse gas and also participates in the destruction of stratospheric ozone [Crutzen, 1981]. Denitrification and nitrification are the main biological processes leading to N₂O formation and emission from the soil [Davidson, 1991]. Because denitrification is favored when soils are moist and anaerobic, whereas nitrification is favored under more mesic to xeric conditions, 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 precipitation patterns and soil moisture regimes. In the past, soil sources of N₂O have been identified using selective inhibitors, sterilization, or by adding substrates [Davidson and Schimel, 1995; Stevens et al., 1997]. Another potential way to identify the processes producing N₂O is to measure the natural abundance stable isotope composition of N₂O [Yoshida, 1988; Yoshinari, 1990]. The isotopic composition (i.e., ratios of ¹⁵N/¹⁴N and ¹⁸O/¹⁶O) of denitrifier-derived N₂O often differs from that of nitrifier-

derived N₂O, especially under laboratory conditions [Kim and Craig, 1990; Webster and Hopkins, 1996]. However, using this difference to distinguish the biological processes underlying production of N₂O in the field is problematic, owing to uncertainties with the level of fractionation by denitrification and nitrification [Pérez et al., 2000] as well as numerous other sources of variation in the isotopic composition of N₂O [Menyailo et al., 2003; Schmidt et al., 2004]. Potentially, more information can be received if the positioning of ¹⁵N within the N₂O molecule is considered [Stein and Yung, 2003; Sutka et al., 2006], but also here there are many uncertainties.

[3] Isotope tracer approaches have the advantage that biological kinetic fractionations are much smaller compared to the isotopic signature of enriched substrates, and so fractionations become negligible sources of variation in tracer studies [Panek et al., 2000]. Several attempts have been undertaken to distinguish the sources of N₂O using ¹⁵N [Panek et al., 2000; Baggs et al., 2003]. However, after several hours of ¹⁵NH₄⁺ application, nitrification produces ¹⁵N-NO₃⁻, causing N₂O formed by denitrifiers to also be enriched and therefore mistakenly considered to be nitrifier-derived. Similarly, if ¹⁵NO₃⁻ is applied, dissimilative reduction of nitrate to ammonium (DRNA) can enrich NH₄⁺, and N₂O produced from nitrification will be enriched but incorrectly considered to be denitrifier-derived.

[4] Using oxygen isotopes in NO₃⁻ could address some of these issues. The oxygen atom in nitrous oxide produced

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through nitrification is derived from either atmospheric O₂ or from H₂O [Dua *et al.*, 1979; Hollocher *et al.*, 1981; Andersson and Hooper, 1983; Kumar *et al.*, 1983; Ostrom *et al.*, 2000]. Thus we can expect that oxygen in nitrifier-derived N₂O should reflect the isotopic composition of oxygen of both O₂ and H₂O. By contrast, in denitrification, oxygen in the N₂O product originates mostly from the oxygen in NO₃⁻, with minor contributions from soil H₂O [Tilsner *et al.*, 2003]. Using isotope-ratio mass spectrometry, it should be possible to measure the contribution of each process to total N₂O efflux, even if the impact of one of the processes is small. Wrage *et al.* [2005] reported the application of enriched ¹⁸O-H₂O in combination with ¹⁵N to distinguish the sources of N₂O, but to our knowledge no studies have reported the use of enriched ¹⁸O-NO₃⁻ for this purpose.

[5] To test the applicability of ¹⁸O for distinguishing N₂O sources, we used soils from a Siberian afforestation experiment, in which six common Siberia tree species were planted and allowed to grow for about 30 years on initially homogeneous soil. In this experiment, we have already documented tree species effects on denitrification and net nitrification potential [Menyailo *et al.*, 2002b]. Thus differences between tree species in the relative contributions of denitrification and nitrification to soil N₂O production can be expected. Furthermore, we incubated soil samples collected under different tree species at two moisture levels to additionally alter the proportion of denitrifier- versus nitrifier-derived N₂O and to test whether the isotopic signatures of oxygen in N₂O will be more enriched due to hypothesized increase of denitrifier-derived N₂O at higher soil moisture. The aims of this work were (1) to assess the use of ¹⁸O labeling in NO₃⁻ to distinguish the sources of N₂O, (2) to clarify if tree species alter proportion of denitrifier-versus nitrifier-derived N₂O and (3) to estimate the effect of altered soil moisture on the sources of N₂O and to check if these effects depend on tree species.

2. Methods

2.1. Sites and Soil Samples

[6] 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 upper 0–50 cm of soil of a 1.5-ha area were removed, mechanically homogenized to minimize vertical and spatial heterogeneity of chemical, physical and biological properties, and subsequently returned to the site prior to experimental planting. In 1971–1972, 2- to 3-year-old seedlings of spruce (*Picea abies*), birch (*Betula pendula*), Scots pine (*Pinus sylvestris*), aspen (*Populus tremula*), larch (*Larix sibirica*) and Arolla pine (*Pinus cembra*) were sown into individual pure species plots, each occupying 2400 m². An area of 9600 m² was left for grassland as a control, and the soil under grass was not mechanically homogenized. The region is characterized by continental climatic conditions with average rainfall 500 mm yr⁻¹, average daily summer temperature of 20°C (at 12:00), depth to permafrost 70–170 cm, and soil temperature to 20 cm depth in winter –4° to –14°, in summer 10° to 12°. The soil is the gray forest type according to the Russian Soil Classification System and Greyzem according to Food and Agriculture Organization

(FAO) [1990]. In August 2001, each plot was subdivided into three parts: A, B and C (as in work by Menyailo *et al.* [2002a]). From each subplot, two trees were randomly chosen and four soil samples (0–10 cm) were taken at a distance of 50 cm from the stem of each tree in cardinal directions. In the grassland plot, three subplots (each of 2 m²) were chosen along the forest plantation; at each subplot six soil samples were taken from the 0–10 cm depth. Soil samples from each subplot were mixed. The total number of soil samples was 21: six species plus grassland by three subplots. All soil samples were air-dried and sieved (2 mm).

2.2. Incubation Experiments

[7] The first experiment was carried out with one soil sample collected from grassland to determine the maximum instantaneous ¹⁸O enrichment of N₂O during denitrification, and thereby to provide the isotopic end-member for calculations of the relative contributions of nitrification and denitrification. Fifteen grams of each of four subsamples were placed in 250-mL glass flasks, moistened with distilled water and preincubated at 25°C for 3 days to initiate microbial activity and to reduce the concentrations of background NO₃⁻ and NH₄⁺. After that, NH₄NO₃ was added to each subsample in 1 mL of water, enough to bring soil water holding capacity (WHC) to 90%. Care was taken to moisten soils evenly. The rate of N addition was 500 mg N-NH₄NO₃ kg⁻¹ dw; the target enrichment for ¹⁸O-NO₃⁻ was 1.6 atom%. Half of the subsamples received 25 mL (10% v/v) of C₂H₂, to inhibit N₂O-reductase and nitrification.

[8] The samples were incubated for 2 days at 25°C. After 2, 4, 8, 16, 26, 36 and 48 hours, the headspace of each flask (5 mL) was sampled for the analysis of N₂O and CO₂ using a gas chromatograph (Agilent 6890), equipped with electron capture detector (ECD ⁶³Ni), flame ionization detector (FID) and in-line methanizer. Results were recorded as mg N-N₂O kg⁻¹ and mg C-CO₂ kg⁻¹. Additionally, 1 mL of the headspace was taken and injected in 20-mL glass vials filled with helium for later δ¹⁸O-N₂O measurements. The storage was necessary because the isotope-ratio mass spectrometer (IRMS) operates in 1000 times narrower concentration ranges than the gas chromatograph, and the concentration of N₂O injected into the IRMS should be known before analysis for dilution, if necessary.

[9] Because the flasks were not opened between sampling times, the measured isotopic values are cumulative, including probably some nitrifier-derived N₂O due to aerobic conditions (even in the presence of C₂H₂). We expected that at the end of incubation, when more of the O₂ has been reduced, the conditions will be more anaerobic and the denitrifier-derived N₂O will dominate. Therefore the instantaneous enrichment of δ¹⁸O-N₂O was estimated and a further assumption was made that δ¹⁸O values of N₂O derived from the soil at the latest stage of incubation with C₂H₂ corresponds to 100% denitrifier-derived N₂O. The maximum instantaneous enrichment of oxygen in N₂O evolved was estimated using the mass balance equation for all sampling points, beginning with the second (4 hours),

$$AP = (M_2 \times AP_2 - M_1 \times AP_1) / (M_2 - M_1), \quad (1)$$

where M₁ and M₂ are concentrations of N₂O in the flask at a given time and subsequent time, and AP₁ and AP₂

are the ¹⁸O enrichment in N₂O at a given time and subsequent time.

[10] The second incubation experiment was carried out using all 21 soil samples, using the protocol described above, with the difference that instead of the C₂H₂ treatment, all soils were incubated at two levels of soil moisture: 30% of WHC (low moisture) and 90% of WHC (high moisture). The headspace from the flasks was sampled after 10, 26, 50, 74 and 142 hours of incubation.

2.3. Isotopic Measurements in N₂O

[11] The ratios of the stable isotopes ¹⁸O/¹⁶O in N₂O emitted from soils were determined using an on-line 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 46:44 in N₂O samples were measured and used to estimate ratios of ¹⁴N₂¹⁸O/¹⁴N₂¹⁶O. We used N₂O as reference gas (99.9990%, Linde). The δ¹⁸O in N₂O were referenced to another N₂O standard provided by T. Pérez (UC Irvine). Using the natural abundance variations of the isotopic composition of N₂O is seriously complicated by the lack of an international standard for isotopes in N₂O. However, because our studies used artificially enriched work, an exact calibration to natural abundance values is not necessary.

2.4. Statistical Data Analysis

[13] The first incubation experiment was performed with two duplicates for one soil sample. Total number of flasks is four (one sample, with and without C₂H₂, two replicates). The second incubation experiment was done without duplicates for each soil sample, thus yielding three replicates (three soil samples for one tree species). The total number of flasks for the second experiment was 42: three samples for each of the six species and the grassland by two levels of soil moisture). For rates of CO₂ and N₂O production, the effects of tree species, soil moisture, and their interactions were determined with two-way ANOVA. The low moisture treatment resulted in much less N₂O production, not always yielding enough for isotopic analysis (thus some time points were missing). Because of an incomplete data set for ¹⁸O-N₂O for the low soil moisture treatment, and because our primary interest was the effects of species and moisture, we averaged ¹⁸O-N₂O values throughout the incubation and performed two-way ANOVA with species and moisture as main effects. The effects of species and moisture on ¹⁸O in N₂O were calculated with two-way ANOVA. Where the main effect was significant, LSD post hoc comparisons were used to determine significant differences between treatments. We considered the effect significant at $P < 0.05$. All statistics were carried out with the statistical package STATISTICA (5.0 for Windows) [StatSoft, 1997].

3. Results and Discussion

3.1. Determination of the Maximum ¹⁸O Enrichment During Denitrification in Grassland

[14] The maximum enrichment of ¹⁸O-N₂O during denitrification was 1.33 atom% ¹⁸O-N₂O. The production

of CO₂ was linear during 48 h of incubation, suggesting no limitation of soil heterotrophic microorganisms, including denitrifying bacteria, by C availability in grassland (Figure 1a). The application of C₂H₂ had no effect on CO₂ production, indicating that it is unlikely that soil microorganisms used C₂H₂ as additional C source. The N₂O production was not affected by C₂H₂ during the first 8 hours of incubation, but afterwards the flasks without C₂H₂ accumulated significantly less N₂O, indicating that N₂O-reductase activity increased through time (Figure 1b).

[15] The cumulative and calculated instantaneous ¹⁸O-N₂O values are shown in Figures 1c and 1d. In both treatments (with and without C₂H₂), N₂O became enriched in ¹⁸O throughout the incubation, indicating an increase in the relative proportion of denitrifier-derived N₂O. This is likely due to depletion of O₂ in the flasks, creating anaerobic conditions favorable for denitrification. This was even more pronounced for instantaneous values of ¹⁸O-N₂O (Figures 1c and 1d), which show the actual temporal shift in the relative contributions of denitrifiers and nitrifiers to N₂O production. The instantaneous ¹⁸O-N₂O values without C₂H₂ were slightly higher at the end of incubation than in the flasks with C₂H₂, likely as a result of N₂O-reductase activity. The calculation of instantaneous ¹⁸O-N₂O relies on N₂O concentrations (see equation (1)). Thus, by reducing N₂O concentrations in the flasks with little effect on the isotopic composition of N₂O (in enrichment studies such as this one, the fractionation has a negligible effect on atom% values), the increasing activity of N₂O reductase contributed to higher instantaneous ¹⁸O-N₂O values. Because natural abundance of ¹⁸O-N₂O is about 0.2 atom%, corresponding to 100% of nitrifier-derived N₂O (no ¹⁸O enriched substrate is incorporated into N₂O via nitrification), the contribution of each of the two processes to N₂O production can be calculated using the equation shown in Figure 2.

3.2. Species Effects on CO₂ and N₂O Fluxes

[16] In the second experiment, soil samples under different tree species and grassland were incubated at two levels of soil moisture. CO₂ production was measured to determine whether low moisture limited microbial activity and whether the water solution was uniformly distributed over the entire soil sample at low moisture treatment. CO₂ and N₂O production were more or less linear throughout the incubation period. Overall, species strongly affected CO₂ production (Figure 3), mostly owing to very high rate in grassland ($P < 0.001$). Soil moisture had no effect on CO₂ production rate indicating no limitation of soil microorganisms by water. Increasing soil water content enhanced N₂O production by a factor of 10–100 ($P < 0.001$, Figure 4). The effect of tree species on net N₂O production (main effect, $P = 0.010$) depended on soil moisture (species × moisture interaction, $P = 0.013$). At low soil moisture, species had no effect on net N₂O production. At high soil moisture, soil beneath aspen produced more N₂O than soils beneath Scots pine ($P = 0.028$) and larch ($P = 0.047$). This is important for predictions of future N₂O efflux from the Siberian forest ecosystems in response to changing tree species composition. Both spruce and larch forests cover large territories in Russian Siberia. If these coniferous species are replaced by hardwood aspen, as predicted in response to global climate change [Pastor and

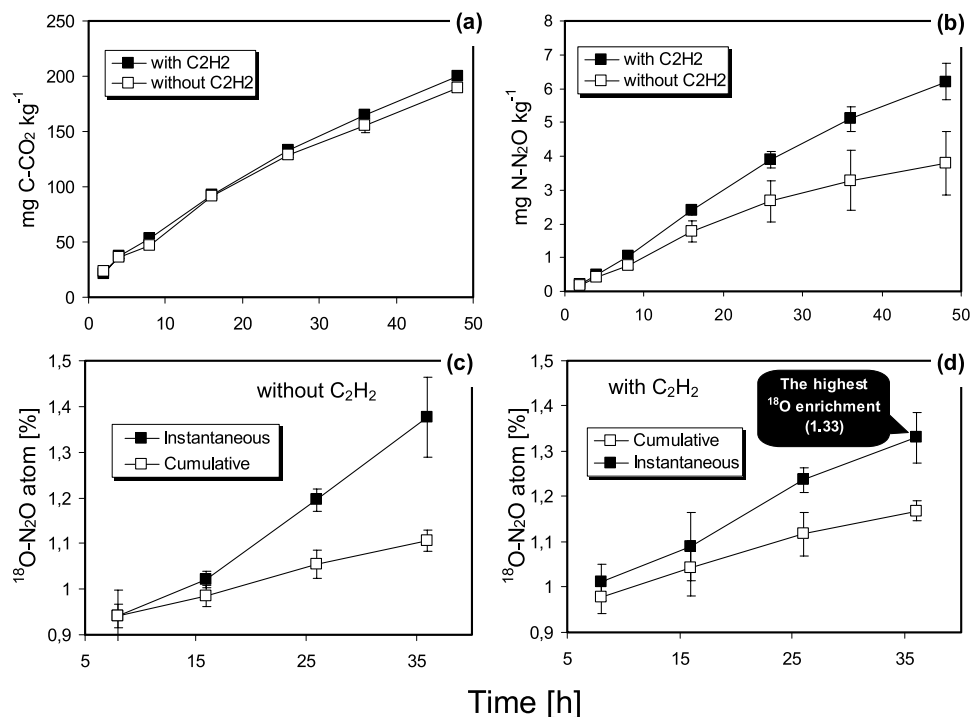


Figure 1. Accumulation of (a) CO₂, (b) N₂O, and the cumulative and instantaneous ¹⁸O enrichment in N₂O (c) without and (d) with C₂H₂ in the incubation experiment with soil samples from grassland (n = 2). Highlighted is the highest instantaneous enrichment (1.33) of ¹⁸O-N₂O observed during denitrification, which was used as the isotopic end-member for denitrifier-derived N₂O.

Post, 1998], the capacity of Siberian forest soils for N₂O production might increase.

3.3. Enrichment ¹⁸O-N₂O Values During Incubation

[17] At the high level of soil moisture, ¹⁸O-N₂O varied between 0.74 and 0.95 atom% at the beginning of incubation, becoming enriched to 0.96 and 1.25 atom% at later stages of incubation (Figure 5). As in the grassland (Figures 1c and 1d), in the forests the relative contribution of denitrifiers to total N₂O efflux increased and the contribution of nitrifiers decreased throughout the incubation. Considering the weighted average for all time points together, tree species affected ¹⁸O enrichment: the effect was significant ($P < 0.001$) mostly owing to spruce, in which the ¹⁸O composition of N₂O was 0.1–0.2 atom% lower compared to all other tree species ($P < 0.050$). Thus, at high soil moisture, soil under spruce had the lowest ¹⁸O-enrichment and thus probably the highest contribution of nitrification to total N₂O production (Figure 6). For comparison, the ¹⁸O-N₂O values for the low moisture treatment are also presented here (Figure 6). At low moisture, tree species also affected oxygen enrichment in N₂O. Aspen had higher ¹⁸O-N₂O values than spruce ($P = 0.011$), Arolla pine ($P = 0.008$) and birch ($P = 0.037$). Also, grassland had higher ¹⁸O-N₂O values than Arolla pine ($P = 0.045$).

[18] While tree species differed in ¹⁸O enrichment ($P < 0.001$), soil moisture also had a large effect on ¹⁸O-N₂O ($P < 0.001$) (Figure 6). Increased soil moisture caused an increase ¹⁸O-N₂O enrichment under all tree species by 120–165%, likely because wetter soils enhanced denitrification and decreased nitrification, increasing the contribution of denitrifiers to total N₂O efflux.

[19] The difference in ¹⁸O enrichment between low and high moisture levels was species-dependent (interaction species versus moisture $P < 0.001$), at least in part because differences among tree species were larger at low moisture. This is the first evidence that the relative importance of the processes responsible for N₂O efflux differs under different tree species, and that they respond in distinct ways to altered precipitation patterns and soil moisture regimes. Using the equation given in Figure 2, the proportions of denitrifier- and nitrifier-derived N₂O were calculated for low moisture

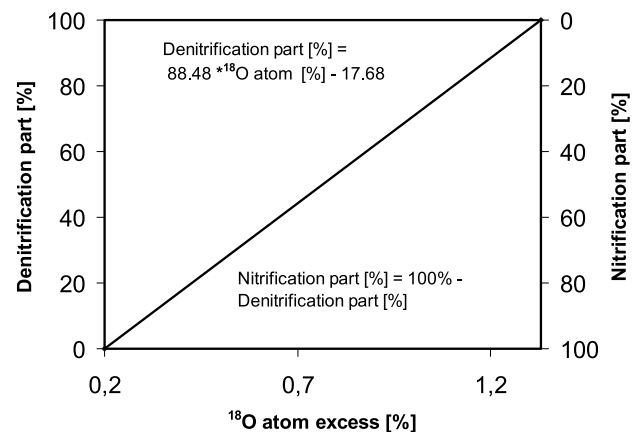


Figure 2. Conceptual scheme for estimating the contribution of nitrification and denitrification to total N₂O efflux. The values are expected to range between 0.2 atom%, when contribution of denitrification is 0%, and 1.33 atom% when contribution of denitrification is 100%.

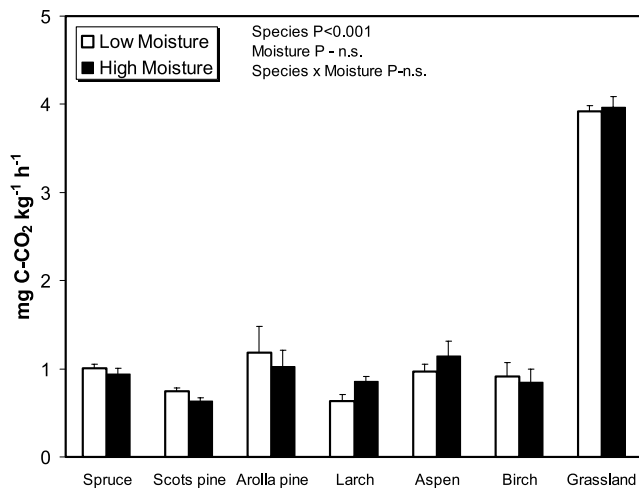


Figure 3. Rate of CO₂ production under 6 tree species and grassland during 144-hour incubation at two levels of soil moisture (low: 30% of WHC, and high: 90% of WHC). We found no effect of soil moisture but strong effect of species, mostly due to high rates of CO₂ production in grassland (n = 2).

and for high moisture at the beginning and at the end of the incubation (Figure 7). At low soil moisture, the contribution of denitrification varied between 7% and 38% depending on species. With increasing soil moisture, the contribution from denitrification increased to 52–63% at the beginning of the incubation, and, with the depletion of O₂ during the course of the incubation, increased further to 75–93%. With increasing soil moisture and net N₂O efflux, denitrification becomes the main source of N₂O. At the same time, only at high soil moisture did species significantly affect net N₂O production. Varying contributions of denitrifier-derived N₂O under different species does not appear to explain species effects on N₂O efflux, because species that differed in net N₂O production at high moisture (Figure 4) did not differ in the relative contributions of denitrification and nitrification to total N₂O production at high moisture (Figures 6 and 7). Alternatively, aspen soils may support higher rates of N₂O reductase activity than soils under Scot pine and larch [Menyailo *et al.*, 2002b], possibly explaining the lower net N₂O production observed under aspen than under conifers.

3.4. Possible Limitations of ¹⁸O Application

[20] We note four possible limitations of the method presented: (1) contributions of nitrifier denitrification; (2) limitation of denitrifying bacteria by available C, (3) dilution of the applied ¹⁸O-NO₃⁻ by nitrification and (4) exchange of oxygen between intermediates produced in denitrification and water.

[21] Our approach does not account for nitrifier denitrification, a process which can contribute to N₂O production as revealed in pure culture studies [Shaw *et al.*, 2006]. However, knowledge about the contributions of nitrifier denitrification to N₂O efflux from soils is limited [Wrage *et al.*, 2001]. Use of our technique in combination with parallel incubations using ¹⁵N-NO₃⁻ and ¹⁸O-H₂O [Wrage *et al.*, 2005] could provide insight on the contributions of all three processes to total N₂O efflux.

[22] In the experiments we conducted, our goal was to assess the proportions of nitrifier- and denitrifier-derived N₂O in the absence of substrate limitations. Addition high amounts of NO₃NH₄ should relieve NO₃⁻ limitation of denitrifiers and NH₄⁺ limitation of autotrophic nitrifiers. However, the shift in resource balance could induce C limitation of heterotrophic denitrifying bacteria, causing their contributions to total N₂O efflux to be underestimated. This limitation is not so easy to overcome since addition of C-source would most likely promote rapid growth of denitrifying bacteria making denitrification a dominant source of N₂O.

[23] The third possible limitation is easier to overcome by adding high amounts of NO₃⁻. Nitrification, by forming new unlabeled NO₃⁻ and diluting the applied ¹⁸O-NO₃⁻, was likely a minor source of error in the present study because we applied very high amounts of NO₃⁻ (250 mg kg⁻¹) compared to gross nitrification rates in these soils: 1–2 mg kg⁻¹ d⁻¹ [Menyailo and Hungate, 2006]. Avoiding dilution of applied ¹⁸O-NO₃⁻ was the major reason for high N application. However, future studies at lower N application

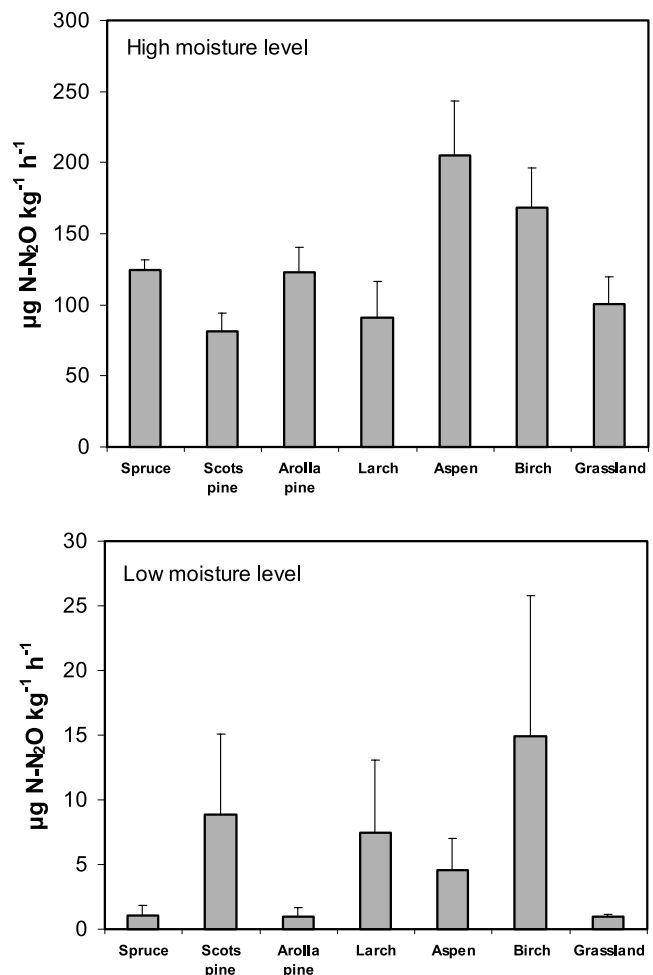


Figure 4. Rate of N₂O production at the same two moisture levels in soil samples under six tree species and grassland (n = 2). Soil moisture enhanced the efflux of N₂O by 10–100 times. When the flux is high, the largest amount of N₂O evolved from soils beneath hardwood tree species (aspen and birch).

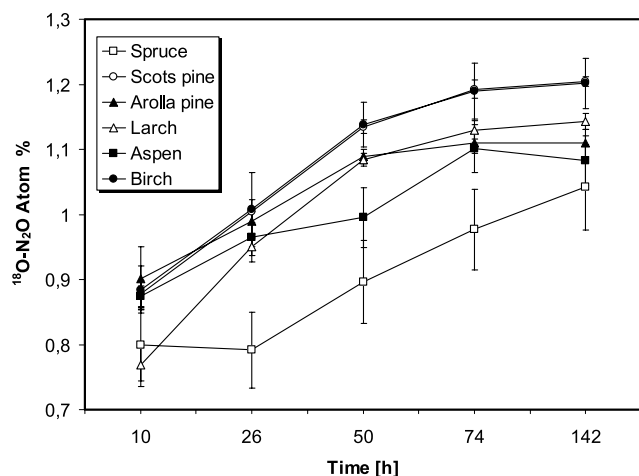


Figure 5. Oxygen isotopic enrichment (¹⁸O) of N₂O evolved from the soil samples under different tree species at high soil moisture during 142-hour incubation (n = 2). The significant increase in ¹⁸O-N₂O with incubation time indicates the increase in denitrifier-derived N₂O.

should also estimate the contribution of nitrification to ¹⁸O-NO₃⁻ dilution. This can be easily achieved by measuring gross nitrification rate with isotope pool dilution technique.

[24] The fourth limitation, exchange of oxygen between water and denitrification intermediates (NO₂⁻ and NO), also deserves consideration. The exchange of water oxygen with nitrite and nitric oxide has been demonstrated for some denitrifying bacteria [Garber and Hollocher, 1982; Ye et al., 1991; Shearer and Kohl, 1988], but the degree of exchange varies greatly among bacterial strains and may be related to biochemistry of nitrite reduction [Ye et al., 1991]. Bacteria

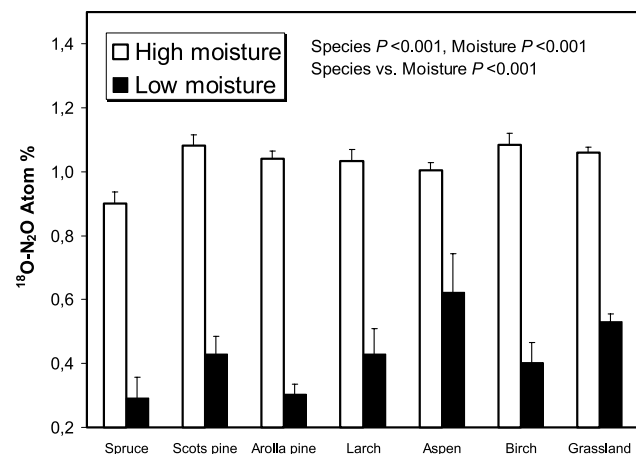


Figure 6. Oxygen isotopic enrichment (¹⁸O) of N₂O evolved from the soil samples under different tree species at high and low moisture levels. The mean and standard errors for all sampling points are presented. High soil moisture increases the ¹⁸O-N₂O values compared to low soil moisture, reflecting the higher contribution of denitrifying bacteria to N₂O efflux under the presumably more anaerobic conditions.

possessing the heme-type nitrite reductase, as *Pseudomonas chlororaphis* does [Ye et al., 1993], were shown to catalyze relatively large amounts of exchange (39–76%) [Ye et al., 1991], while *Pseudomonas aureofaciens*, known to possess the copper-type nitrite reductase [Glockner et al., 1993] was shown to cause relatively little incorporation of oxygen atoms from water into N₂O (around 6%) [Ye et al., 1991]. Casciotti et al. [2002] recently reported the low incorporation of oxygen isotopes of water into the N₂O by *Pseudomonas aureofaciens*: While in some cases up to 10% of oxygen in N₂O originated from water, in most cases incorporation was frequently less than 3%. It is thus possible that the relative abundance of denitrifying bacteria possessing either heme- or copper-type nitrite reductase will determine the applicability of ¹⁸O isotopes for separation nitrification and denitrification. However, the ecological significance and relative abundance of the two groups of nitrite reductases is poorly understood.

[25] We have two lines of evidence that the exchange of oxygen between nitrogen oxides and water is minor in the

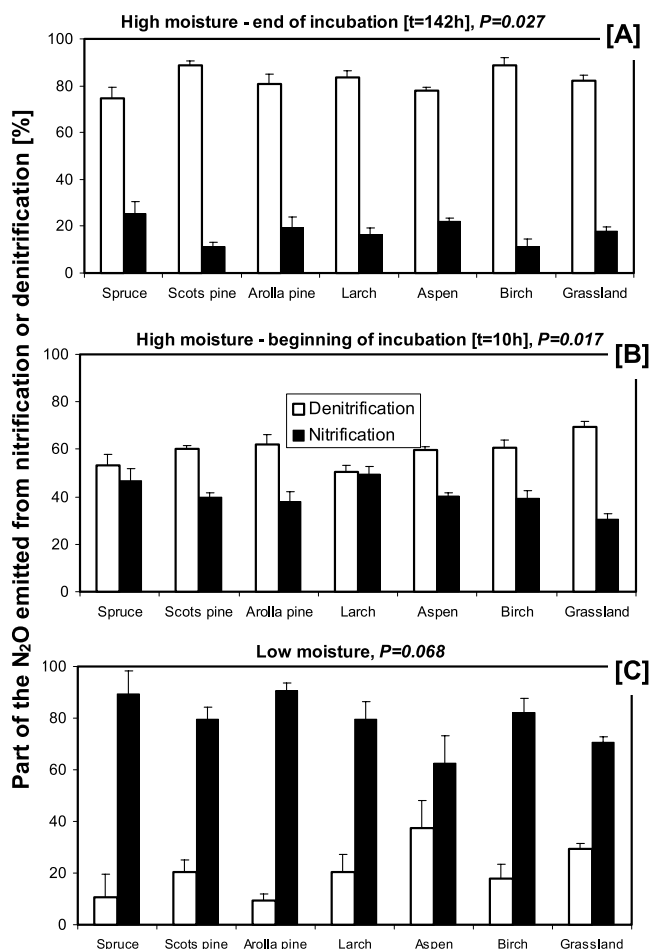


Figure 7. Estimated proportion of nitrifier- and denitrifier-derived N₂O for the high moisture treatment (a) at the beginning and (b) at the end of the incubation, and (c) for the low soil moisture treatment. The highest contribution of denitrification was at high soil moisture and at the final stage of the incubation. Nitrification contributes to N₂O efflux at low soil moisture, but when the rate of N₂O itself is very low (Figure 4).

studied soils. First, if large rates of exchange had occurred we would not have observed the progressive enrichment of ¹⁸O-N₂O as our incubation proceeded (Figure 5). Rather, the ¹⁸O-N₂O would have decreased or remained more or less constant. The enrichment in ¹⁸O in concert with presumed increased O₂ deficiency, promoting denitrification, suggests that the exchange of oxygen between denitrification intermediates and water is minor. The second argument for defending our method is that in our recent study of N₂O isotope discrimination at natural abundance [Menyailo and Hungate, 2006] we demonstrated parallel enrichment of nitrogen and oxygen isotopes in N₂O under strictly denitrifying conditions (absence of oxygen and C₂H₂ presence), in the same soil samples we used in the present study. Both oxygen and nitrogen isotopes followed the Rayleigh distillation and were most depleted at the beginning of the incubation; as denitrification proceeded and NO₃⁻ became limiting, both ¹⁵N-N₂O and ¹⁸O-N₂O gradually became more enriched.

[26] These two arguments provide evidence that oxygen exchange was likely not a problem in these soils, probably owing to dominance of denitrifying species not actively exchanging oxygen of water with gaseous intermediates or, again, owing to high amounts of NO₃⁻ applied since O-isotopes exchange appears to be more important when NO₂⁻ or NO are final electron acceptors [Ye *et al.*, 1991]. However, future studies should address the questions of distribution and abundance of denitrifying bacteria possessing heme- and copper-type nitrite reductases (with varied exchange rates of oxygen) in different soils and ecosystems.

4. Conclusion

[27] Use of ¹⁸O isotopes in NO₃⁻ provides additional insight in distinguishing the major biological sources of soil-derived N₂O, nitrification and denitrification. Our method was sensitive and allowed tracking a shift in denitrification and nitrification even within a relatively short incubation time (several days). Although our measurements were conducted under laboratory conditions, the principles ought to be applicable to studies targeting the separation of nitrifier- and denitrifier-derived N₂O in the field. Our results also suggest that tree species influence not only the rate of N₂O production but also the mechanisms of N₂O production. We demonstrated that the response to altered soil moisture of the processes responsible for N₂O production depends on the tree species. This has importance for predicting future N₂O fluxes from forest ecosystems with altered tree species composition and precipitation patterns.

[28] **Acknowledgments.** We kindly thank Tibusay Pérez for providing N₂O stable isotopes standards for calibration of our reference gas, and Rick Doucett for help with the isotope-ratio mass spectrometry. This work was supported by the U.S. Civilian Research and Development Foundation (CRDF grant RG1-2537-KY-03) and by the U.S. National Science Foundation (DEB-0092642).

References

Andersson, K. K., and A. B. Hooper (1983), O₂ and H₂O are each the source of one O in NO₂⁻ produced from NH₃ by Nitrosomonas: ¹⁵N evidence, *FEBS Lett.*, *164*, 236–240.

Baggs, E. M., M. Richter, G. Cadisch, and U. A. Hartwig (2003), Denitrification in grass swards is increased under elevated atmospheric CO₂, *Soil Biol. Biochem.*, *35*, 729–732.

Casciotti, K. L., D. M. Sigman, M. Galanter Hastings, J. K. Bohlke, and A. Hilkert (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 Emission From Soil and Water*, edited by P. A. Matson and R. C. Harris, pp. 327–357, Cambridge Univ. Press, New York.

Dua, R. D., B. Bhandari, and D. J. D. Nicholas (1979), Stable isotopes studies on the oxidation of ammonia to hydroxylamine by Nitrosomonas europaea, *FEBS Lett.*, *106*, 401–404.

Food and Agriculture Organization (1990), Soil map of the world, revised legend, map, Rome.

Garber, E. A. E., and T. C. Hollocher (1982), N-15, O-18 tracer studies on the activation of nitrite by denitrifying bacteria—Nitrite water-oxygen exchange and nitrosation reactions as indicators of electrophilic catalysis, *J. Biol. Chem.*, *257*(14), 8091–8097.

Glockner, A. B., A. Jungst, and W. G. Zumft (1993), Copper-containing nitrite reductase from *Pseudomonas aerofaciens* is functional in mutationally cytochrome-cd (1)-free background (nirs-) of *Pseudomonas stutzeri*, *Arch. Microbiol.*, *160*(1), 18–26.

Hollocher, T. C., M. E. Tate, and D. J. D. Nicholas (1981), Oxidation of ammonia by Nitrosomonas europaea: Definitive ¹⁸O-tracer evidence that hydroxylamine formation involves a monooxygenase, *J. Biol. Chem.*, *256*, 10,834–10,836.

Kim, K. Y., 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.

Kumar, S., D. J. D. Nicholas, and E. H. Williams (1983), Definitive ¹⁵N NMR evidence that water serves as a source of O during nitrite oxidation by *Nitrobacter agillis*, *FEBS Lett.*, *152*, 71–74.

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

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.

Menyailo, O. V., B. A. Hungate, J. Lehmann, G. Gebauer, and W. Zech (2003), Tree species of the Central Amazon and soil moisture alter stable isotope composition of nitrogen and oxygen in nitrous oxide evolved from soil, *Isot. Environ. Health. Stud.*, *39*, 41–52.

Ostrom, N. E., M. E. Russ, B. Popp, T. M. Rust, and D. M. Karl (2000), Mechanisms of nitrous oxide production in the subtropical North Pacific based on determinations of the isotopic abundances of nitrous oxide and di-oxygen, *Chemosphere*, *2*, 281–290.

Panek, J. A., P. A. Matson, I. Ortiz-Monasterio, and P. Brooks (2000), Distinguishing nitrification and denitrification sources of N₂O in a Mexican wheat system using ¹⁵N, *Ecol. Appl.*, *10*(2), 506–514.

Pastor, J., and W. M. Post (1998), Response of northern forests to CO₂-induced climate change, *Nature*, *334*, 55–58.

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*, 525–535.

Schmidt, H. L., R. A. Werner, N. Yoshida, and R. Well (2004), Is the isotopic composition of nitrous oxide an indicator of its origin from nitrification or denitrification? A theoretical approach from referred data and microbiological and enzyme kinetic aspects, *Rapid Commun. Mass Spectrom.*, *18*, 2036–2040.

Shaw, L. J., G. W. Nicol, Z. Smith, J. Fear, J. I. Prosser, and E. M. Baggs (2006), Nitrosospira spp. can produce nitrous oxide via a nitrifier denitrification pathway, *Environ. Microbiol.*, *8*(2), 214–222.

Shearer, G., and D. H. Kohl (1988), Nitrogen isotopic fractionation and O-18 exchange in relation to the mechanism of denitrification of nitrite by *Pseudomonas-stutzeri*, *J. Biol. Chem.*, *263*, 13,231–13,245.

- StatSoft, (1997), *STATISTICA for Windows (Computer Program Manual)*, Tulsa, Okla.
- 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.
- 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.
- 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.
- 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. W. van Groenigen, O. Oenema, and E. M. Baggs (2005), A novel dual-isotope labelling method for distinguishing between soil sources of N₂O, *Rapid Commun. Mass Spectrom.*, 19, 3298–3306.
- Ye, R. W., I. Toro-Suarez, J. M. Tiedje, and B. A. Averill (1991), (H₂O)-O-18 isotope exchange studies on the mechanism of reduction of nitric-oxide by denitrifying bacteria—Evidence for an electrophilic nitrosyl during reduction of nitric-oxide, *J. Biol. Chem.*, 266, 12,848–12,851.
- Ye, R. W., M. R. Fries, S. G. Bezborodnikov, B. A. Averill, and J. M. Tiedje (1993), Characterization of the structural gene encoding a copper-containing nitrite reductase and homology of this gene to DNA of other denitrifiers, *Appl. Environ. Microbiol.*, 59, 250–254.
- Yoshida, N. (1988), ¹⁵N-depleted N₂O as a product of nitrification, *Nature*, 335, 528–529.
- 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.

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