JOURNAL OF GEOPHYSICAL RESEARCH, VOL. 112, G02005, doi:10.1029/2006JG000287, 2007

Isotopologue effects during N_2O reduction in soils and in pure cultures of denitrifiers

Nathaniel E. Ostrom,[1] Adam Pitt,[1] Robin Sutka,[2] Peggy H. Ostrom,[1] A. Stuart Grandy,[3] Kristin M. Huizinga,[4] and G. Philip Robertson[3]

Received 9 August 2006; revised 3 December 2006; accepted 22 December 2006; published 13 April 2007.

[1] Site preference (SP), the difference in $\delta^{15}N$ between the central and outer nitrogen atoms in N_2O, is a powerful approach for apportioning fluxes of N_2O from soils to nitrification and denitrification (Sutka et al., 2006). A critical aspect of the use of SP data to apportion sources of N_2O to nitrification and denitrification is the need to evaluate data for isotope shifts that may have occurred during N_2O reduction in soils prior to its escape to the atmosphere. We present data on the isotopologue effects during reduction of N_2O during anaerobic incubation of soils and pure cultures of denitrifying bacteria. Isotopic enrichment factors for N_2O reduction in soil mesocosms experiments varied between −9.2 and −1.8‰ for nitrogen and between −25.1 and −5.1‰ for oxygen. In pure cultures of *Psuedomonas stutzeri* and *Psuedomonas denitrificans* we observed isotopic enrichment factors for SP of −5.0 and −6.8‰, respectively. We further find that N_2O consumption produces consistent relationships between $\delta^{18}O$ and $\delta^{15}N$ and $\delta^{18}O$ and the $\delta^{15}N$ of the central N atom in N_2O of 2.5 and 1.6, respectively, which are clearly diagnostic of this process. Our results indicate that SP may be altered during reduction of N_2O and thus bias evaluations of its origins. To understand the impacts of N_2O reduction in soil flux studies on source isotope signals we modeled the isotope effects of N_2O production occurring simultaneous with reduction and find increasingly curvilinear relationships between $\delta^{18}O$ and $\delta^{15}N$ and $\delta^{18}O$ and $\delta^{15}N^{\alpha}$ with increased reduction. Consequently, a deviation from the linear mixing relationship between soil-derived and atmospheric N_2O is an indication of extensive reduction. On the basis of our characterization of isotopic fractionation during N_2O reduction, we show that the rate of reduction would have to be substantially greater than 10% of that of production to impact SP estimates of N_2O from denitrification by more than a few percent. Nonetheless, reduction results in a small, but potentially important, increase in SP away from values proposed for bacterial denitrification (0‰) toward those associated with production from nitrification (33‰) (Sutka et al., 2006). On this basis, estimates of the proportion of N_2O derived from denitrification obtained from SP values are underestimates and therefore conservative.

Citation: Ostrom, N. E., A. Pitt, R. Sutka, P. H. Ostrom, A. S. Grandy, K. M. Huizinga, and G. P. Robertson (2007), Isotopologue effects during N_2O reduction in soils and in pure cultures of denitrifiers, *J. Geophys. Res.*, *112*, G02005, doi:10.1029/2006JG000287.

1. Introduction

[2] Nitrous oxide is an important greenhouse gas that is strongly influenced by microbial production and consumption. Agricultural soils annually add about 2.5 Tg of N_2O-N to the atmosphere [*Intergovernmental Panel on Climate Change*, 2001] and N_2O abatement in agriculture has been identified as one of several high-impact strategies for stabilizing atmospheric greenhouse gas concentrations [*Caldeira et al.*, 2004]. Effective mitigation, however, requires a process-level understanding of N_2O sources, and even though agricultural soils represent >50% of anthropogenic N_2O emissions worldwide [*Mosier et al.*, 1998a, 1998b; *Robertson*, 2004], the microbial source of N_2O in most soils remains ambiguous. Both nitrification and denitrification produce N_2O, and because these processes are under very different environmental controls [*Robertson and Groffman*, 2006] they are responsive to very different management strategies [*Matson et al.*, 1989]. Understanding the microbial source of N_2O in high-flux soils is thus an important research objective.

[1]Department of Zoology and Biogeochemistry Program, Michigan State University, East Lansing, Michigan, USA.
[2]GV Instruments, Wythenshawe, UK.
[3]W. K. Kellogg Biological Station and Department of Crop and Soil Sciences, Hickory Corners, Michigan, USA.
[4]Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, Michigan, USA.

0148-0227/07/2006JG000287$09.00

[3] Efforts to determine microbial pathways of N_2O production have primarily relied upon the application of nitrification inhibitors such as acetylene [*Berg et al.*, 1982; *Robertson and Tiedje*, 1987; *Davidson*, 1992], isotopically enriched substrates [*Well et al.*, 2006; *Menyailo and Huntgate*, 2006b], or observation of characteristic changes in the natural isotopic abundances of ^{15}N and ^{18}O in N_2O [*Webster and Hopkins*, 1996; *Ostrom et al.*, 2000; *Yamulki et al.*, 2000, 2001; *Perez et al.*, 2000, 2001; *Wrage et al.*, 2004a]. These approaches have significant limitations. Acetylene-inhibition is limited to short-term experiments and presents methodological challenges related to microsite heterogeneity, unequal diffusion of inhibitor into microsites, and unintended effects of inhibitors on microbial activity that collectively can bias results [*Tiedje et al.*, 1989; *Tilsner et al.*, 2003; *Wrage et al.*, 2004b, 2004c; *Bateman and Baggs*, 2005]. Use of isotopically enriched substrates can alter activity by changing substrate concentrations and can provide erroneous signals if substrates are being produced from natural abundance sources over the time course of measurement [*Madsen*, 1998]. Consequently, a number of studies have been directed at distinguishing N_2O derived from nitrification and denitrification based on the magnitude of fractionation during N_2O production in the absence of inhibitors [*Webster and Hopkins*, 1996; *Mandernack et al.*, 2000; *Yamulki et al.*, 2000, 2001; *Perez et al.*, 2000, 2001; *Bol et al.*, 2003, 2004; *Tilsner et al.*, 2003]. These efforts are based on determination of the difference in isotopic composition (fractionation) of the substrate (ammonium or nitrate) and N_2O during production by the soil microbial community. Quantitative estimates of the relative importance of nitrification and denitrification based on this approach are confounded because the $\delta^{15}N$ of the substrates can fluctuate spatially and temporally [e.g., *Ostrom et al.*, 1998] and the magnitude of isotopic fractionation for microbial N cycling pathways tend to be variable [e.g., *Ostrom et al.*, 2002].

[4] Recently, the intramolecular distribution of isotopes within N_2O has been used in addition to bulk isotope data to constrain origins and global budgets [*Toyoda and Yoshida*, 1999; *Brenninkenmeijer and Röckmann*, 2000; *Yoshida and Toyoda*, 2000; *Toyoda et al.*, 2001]. Nitrous oxide is an asymmetric molecule consisting of two N atoms with unique covalent bonds. Equilibrium and kinetic isotope effects in natural reactions result in distinct abundances of ^{15}N within the central (α) or outer (β) N atoms [*Miller and Yung*, 2000; *Yoshida and Toyoda*, 2000]. The difference in $\delta^{15}N$ between the α and β atoms in N_2O is termed site preference (SP). In pure microbial incubation studies, SP has been found to have unique values reflecting the microbial production pathway [*Sutka et al.*, 2003, 2006; *Toyoda et al.*, 2005]. Production of N_2O via nitrite or nitrate reduction, whether by nitrifying or denitrifying organisms, has been shown to result in particularly low SP values in most studies (−5‰ [*Toyoda et al.*, 2005]; average of 0‰ [*Sutka et al.*, 2006]) with one notable exception (−23‰) that may have been affected by inorganic reduction [*Toyoda et al.*, 2005]. Production of N_2O via hydroxylamine oxidation by nitrifying or methane oxidizing microorganisms, has been shown to have SP values that average 33‰ that is clearly distinct from the SP associated with production by nitrate or nitrite reduction [*Sutka et al.*, 2006]. Furthermore, SP is constant over the course of reactions and independent of substrate isotopic composition whereas $\delta^{15}N$ and $\delta^{18}O$ values change markedly during reactions and are dependent upon the isotope values of the substrates [*Sutka et al.*, 2003, 2006; *Toyoda et al.*, 2005]. Thus, while pure culture studies of N_2O production using microorganisms using the full suite of reduction enzymes is still yet to be done [*Schmidt et al.*, 2004], SP is emerging as a noninvasive tool for apportioning N_2O flux to specific microbial pathways [*Well et al.*, 2006; *Sutka et al.*, 2006].

[5] While the SP values of N_2O have the potential to reveal microbial production pathways, consumption (or reduction) of N_2O during denitrification can impart an isotope effect that could bias or obscure source isotope values [*Tilsner et al.*, 2003; *Wrage et al.*, 2004a; *Yamagishi et al.*, 2007]. Rates of N_2O reduction independent of gross denitrification are rarely determined in soils; hence it is difficult to know the importance of this process a priori [e.g., *Bandibas et al.*, 1994; *Cavigelli and Robertson*, 2001]. Denitrification results in cleavage of the N and O covalent bond in N_2O, which based on kinetic isotope theory, is expected to result in an increase in the ^{15}N content of the α position and, therefore, an increase in SP [*Popp et al.*, 2002; *Yamagishi et al.*, 2007]. The increase in SP in response to reduction would result in a shift away from values associated with denitrification (0‰) toward those associated with nitrification (33‰) [*Sutka et al.*, 2006] and result in an overestimate of the importance of nitrification.

[6] Characterization of fractionation during N_2O reduction may resolve the importance of this process and provide an ability to correct for resulting isotope shifts. Fractionation during N_2O reduction is, at minimum, a two step process involving diffusion into the cell and enzymatic reduction. Consequently, the degree of fractionation will reflect the relative importance of the isotope effect during diffusion, which is likely small, and that associated with enzymatic reduction which is expected to be large [e.g., *Ostrom et al.*, 2002]. The relative importance of diffusion and enzymatic reduction of N_2O is expected to vary with the degree of soil water saturation and other soil properties such that fractionation is small when soils are saturated (supply limited by diffusion) and large when soils are dry (diffusion nonlimiting). Thus an important goal of our research is to constrain fractionation during reduction of N_2O as a means to correct for the influence of isotope effects on source apportionment. In this study we use the term "isotopologue" to refer to both bulk nitrogen (average of $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$) and oxygen isotope values and the $\delta^{15}N$ of the individual nitrogen atoms within the N_2O molecule. Given that molecules of $^{15}N^{14}N^{16}O$ and $^{14}N^{15}N^{16}O$ differ in the position of ^{15}N enrichment but not mass, these molecules are appropriately referred to as "isotopomers." We present results on isotopologue effects during N_2O reduction in both pure culture and soil mesocosm experiments and an assessment of the importance of N_2O reduction on source apportionment studies.

2. Methods

2.1. Site Description

[7] Our field experiments were conducted at the Kellogg Biological Station Long Term Ecosystem Research

(KBS LTER) site located in southwest Michigan, USA (42°24′ latitude, 85°24′ longitude). Native vegetation in the area is beech-maple and oak-hickory forests interspersed with open oak savannas [*Burbank et al.*, 1992]. Most of the area was cleared for agriculture in the mid-1800s and modern agronomic yields are typical of those in the North Central region as a whole. Soils at the site are Kalamazoo (fine-loamy) and Oshtemo (coarse-loamy) mixed, mesic typic Hapludalfs developed from glacial outwash deposited at the end of the Wisconsinan glaciation (J. R. Crum and H. P. Collins, KBS soils, 1995, available at www.lter.kbs.msu.edu/soil/characterization). The main experimental design of the KBS LTER site consists of a series of replicated ecosystems arranged along a management intensity gradient and this design facilitates an understanding of management history on N_2O fluxes and production pathways (http://lter.kbs.msu.edu).

2.2. N_2O Reduction Experiments Within Soil Mesocosms

[8] Soils for N_2O reduction experiments were collected from the KBS LTER in June of 2002 from 3 replicates of 3 different treatments: (1) a conventionally managed corn-soybean-wheat row-crop ecosystem; (2) a historically cultivated early successional field abandoned after spring planting in 1989; and (3) a late successional deciduous forest. Approximately 4 kg of soil were collected from each of the nine experimental plots by taking 20 cores from the upper 25 cm of soil from each of three different treatment plots from the replicated series of cropped and unmanaged ecosystems. A 4 mm sieve was used to homogenize soil. Aliquots were covered and allowed to air dry for ca. three weeks. Dry soil (100 g) was added to mesocosms (1 L glass Mason jars bearing lids fitted with butyl rubber septa) and packed to a volume of ~80 mL for a target bulk density of 1.2 g dry soil cm^{-3}. Water was added to achieve ~85% water filled pore space [*Bergsma et al.*, 2002]. Heterogeneities in the soil, however, resulted in differences in water filled pore space and subsequent measurements revealed values between 91 and 100%. To prevent N_2O production over the course of the N_2O reduction experiments soils were incubated for 2 weeks in the absence of oxygen to exhaust alternative substrates (primarily nitrate) for denitrification after which production was not evident. To initiate N_2O reduction and maintain anoxic conditions, sealed jars were flushed with pure N_2 and then amended with 500 μL of pure N_2O that was delivered using a gas tight syringe (Hamilton). This resulted in a headspace concentration appreciably greater than that observed in typical soil environments but assured sufficient gas for isotopologue analysis. These conditions also assure that concentration is not a factor in contributing to the diffusional limitation of enzymatic reduction within cells (see section 3.1). Headspace samples of 500 μL were taken periodically and stored in vacutainers (BD Diagnostics) previously purged with pure N_2 and brought to atmospheric pressure. Samples were stored for no longer than 1 month prior to isotopic analysis.

2.3. N_2O Reduction Within Pure Culture Experiments

[9] *Pseudomonas stutzeri* (provided by J. M. Tiedje) and *Pseudomonas denitrificans* (ATCC 13867 purchased from ATCC, Manassas, Virginia) were cultured from a frozen stock and maintained in Citrate Minimal Medium (CMM) [*Anderson et al.*, 1993]. These suspensions were derived from 100 mL cultures in CMM incubated at 30°C in the case of *P. denitrificans* and 25°C for *P. stutzeri*. A single culture of each species was grown to late log phase of growth and concentrated (centrifugation: 10,000 g for 10 min at 5°C). The cell pellet was washed twice (20 mL, sterile CMM) and resuspended in 20 mL of sterile CMM. For each culture, a 12 mL exetainer vial (Labco, UK) was prepared with 2 mL of the concentrated cell suspension and 1 mL of a 0.01 M KNO_3 stock solution. The tubes were sealed with a N_2 headspace and incubated at 30°C and 22°C for *P. denitrificans* and *P. stutzeri*, respectively. Isotopically characterized N_2O (20 μL) was injected into the headspace and an initial sample was taken after the headspace gases were allowed to mix for 2 min. The headspace was sampled for N_2O approximately every 20 min using a 100 or 500 μL gas tight syringe. The gas sample was immediately analyzed for isotopologue abundances.

2.4. Analysis of Isotopologues

[10] Gas samples were analyzed on a multicollector GV Instruments IsoPrime Mass Spectrometer interfaced with a continuous flow Trace Gas Inlet System for purification and concentration of N_2O. Isotopic analysis of N_2O involves removal of CO_2 and water using chemical scrubbers (Carbosorb and magnesium perchlorate) and cyrogenic trapping followed by chromatographic separation on a Poraplot Q gas chromatographic column with He as the carrier gas within the Trace Gas system. The effluent from the Trace Gas system is subsequently allowed to enter the mass spectrometer for isotopic characterization. Determination of N_2O concentrations were based on the intensity of the ion current generated by the mass 44 detector in comparison to standards consisting of air spiked with known quantities of pure N_2O. Calibration was performed daily and precision of N_2O concentrations for replicate standards and samples were less than 5%. The multicollector mass spectrometer is able to simultaneously monitor 5 masses of interest for N_2O isotopologues; 30, 31, 44, 45 and 46. We follow the convention of *Toyoda and Yoshida* [1999] in defining the central and outer nitrogen atoms as α and β, respectively, elsewhere identified as atoms 2 and 1, respectively [*Breninkenmeijer and Röckmann*, 2000].

[11] Values for $\delta^{15}N$, $\delta^{18}O$ and $\delta^{15}N^{\alpha}$ are obtained from the ratio of the 45:44, 46:44 and 31:30 ion beam ratios, respectively. We applied corrections for the contribution of ^{17}O to masses 31 and 45 and for a small degree of rearrangement of ^{15}N between the α and β positions within the ion source [*Toyoda and Yoshida*, 1999; *Breninkenmeijer and Röckmann*, 2000; *Sutka et al.*, 2003, 2004a]. The value of $\delta^{15}N^{\beta}$ is calculated given that $\delta^{15}N$ is the average of $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ [*Toyoda and Yoshida*, 1999; *Breninkenmeijer and Röckmann*, 2000]. Final $\delta^{15}N$, $\delta^{18}O$, $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ values were calculated using the approach outlined by *Toyoda and Yoshida* [1999] and reported with respect to Air as the international standard with the exception of $\delta^{18}O$ values that are reported with respect to VSMOW.

[12] There is disagreement with respect to the $\delta^{15}N^{\alpha}$ and SP of tropospheric air [*Toyoda and Yoshida*, 1999; *Kaiser et al.*, 2004] that has recently been resolved [*Westley et al.*, 2007]. We report here our data correction method and internal laboratory standard values so that additional cor-

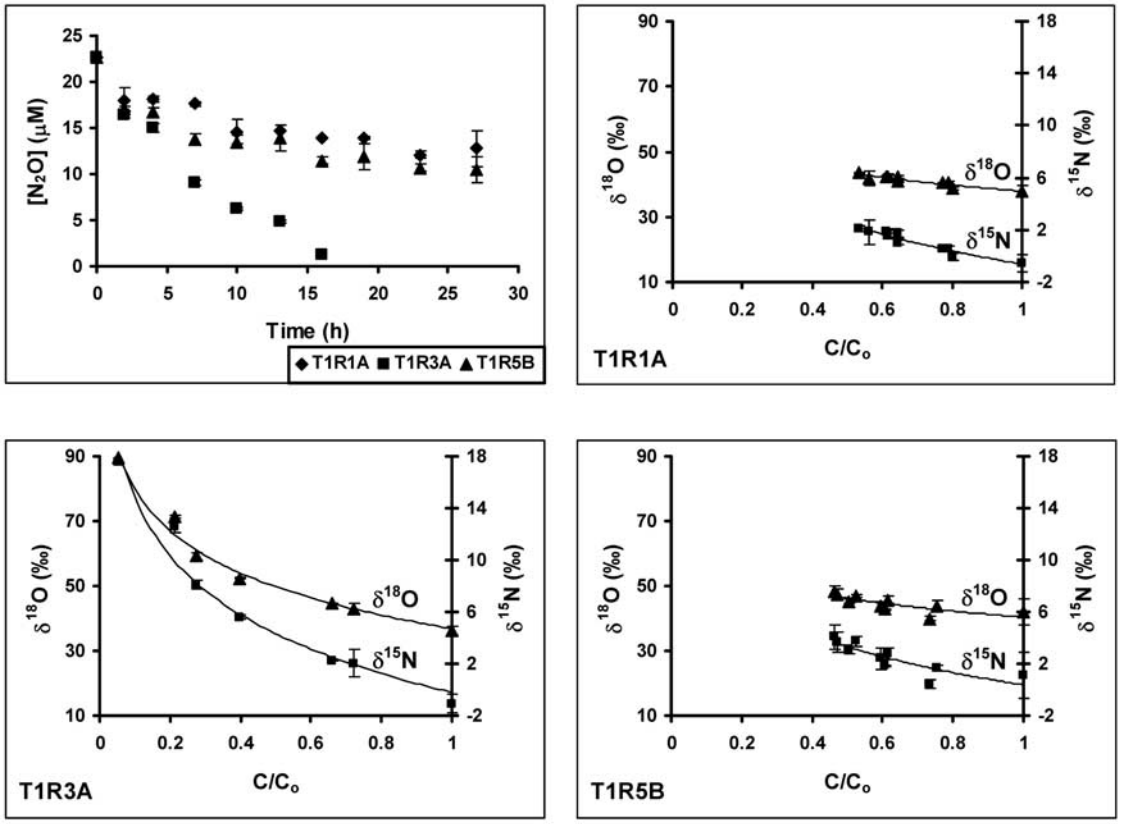

Figure 1. Concentration, $\delta^{15}N$, and $\delta^{18}O$ data for N_2O within soil mesocosm N_2O reduction experiments using soils under conventional agricultural treatment from three replicate plots within the KBS LTER main site. Isotope values are expressed in relation to the concentration (C) at any point in time divided by the initial concentration (C_o) following the Rayleigh model (equation (3)). The slope of a natural log fit to the data equates to the fractionation factor for each experiment and these values are listed in Table 1. Error bars represent 2 standard deviations around the mean of three replicates for each treatment. For most data points, the error bars are less than the size of the markers.

rections can be made, if needed. Our internal laboratory standard was calibrated in collaboration with S. Toyoda and N. Yoshida at the Tokyo Institute of Technology using the equations of *Toyoda and Yoshida* [1999], and we commonly obtain the values of $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ of 16.4 and 2.4‰, respectively, reported by these authors for tropospheric air. The $\delta^{15}N$, $\delta^{15}N^{\alpha}$ and $\delta^{18}O$ of our laboratory pure N_2O standard is 1.6, 14.9 and 41.7‰, respectively.

[13] The multicollector mass spectrometer that we use is the first to determine all masses needed for determination of N_2O isotopologues simultaneously. On other instruments samples are analyzed twice with adjustments to ion source parameters between analyses [e.g., *Breninkenmeijer and Röckmann*, 2000; *Röckmann et al.*, 2003]. While the multicollector approach offers significant time savings it challenges the analyst to demonstrate appropriate accuracy in several isotope ratios over a wide range of sample concentrations (defined as ''linearity''). We have found, in practice, that the optimal ion source parameters to assure precision and linearity for $\delta^{45/44}$ and $\delta^{46/44}$ are not optimal for $\delta^{31/30}$. Consequently, we have optimized analysis for $\delta^{31/30}$, since these are the masses of lowest intensity, which results in a small deterioration in the precision of $\delta^{45/44}$ and $\delta^{46/44}$ as well as some loss in sensitivity. Similar challenges in isotopologue linearity were reported by *Röckmann et al.* [2003]. With this adjustment we are routinely analyzing samples as low as 3.5 nmol N_2O with precisions for $\delta^{15}N$, $\delta^{18}O$, $\delta^{15}N^{\alpha}$, $\delta^{15}N^{\beta}$, and SP better than 0.3, 0.7, 0.9, 0.9, and 1.3‰, respectively. Prior to the initiation of our revised ion source tuning protocol the instrument was linear for $\delta^{45/44}$ and $\delta^{46/44}$ but not linear for $\delta^{31/30}$ below 6 nmol N_2O. Samples from the soil mesocosm experiments did not reach this threshold. Consequently, we report $\delta^{15}N$ and $\delta^{18}O$ values for these samples but do not report $\delta^{15}N^{\alpha}$, $\delta^{15}N^{\beta}$ or SP.

3. Results and Discussion

3.1. N_2O Reduction in Soil Mesocosms

[14] The concentration of N_2O in all soil mesocosm experiments exhibited declines over the course of the experiment consistent with microbial reduction though rapid initial declines in some treatments may reflect initial equilibration between the headspace and soil (Figures 1 and 2). Correlations of isotopologue values with the natural log of the change in concentration is a good indication that reduction is the predominant process occurring in the mesocosms [*Ostrom et al.*, 2002]. A rapid decline in N_2O was evident in

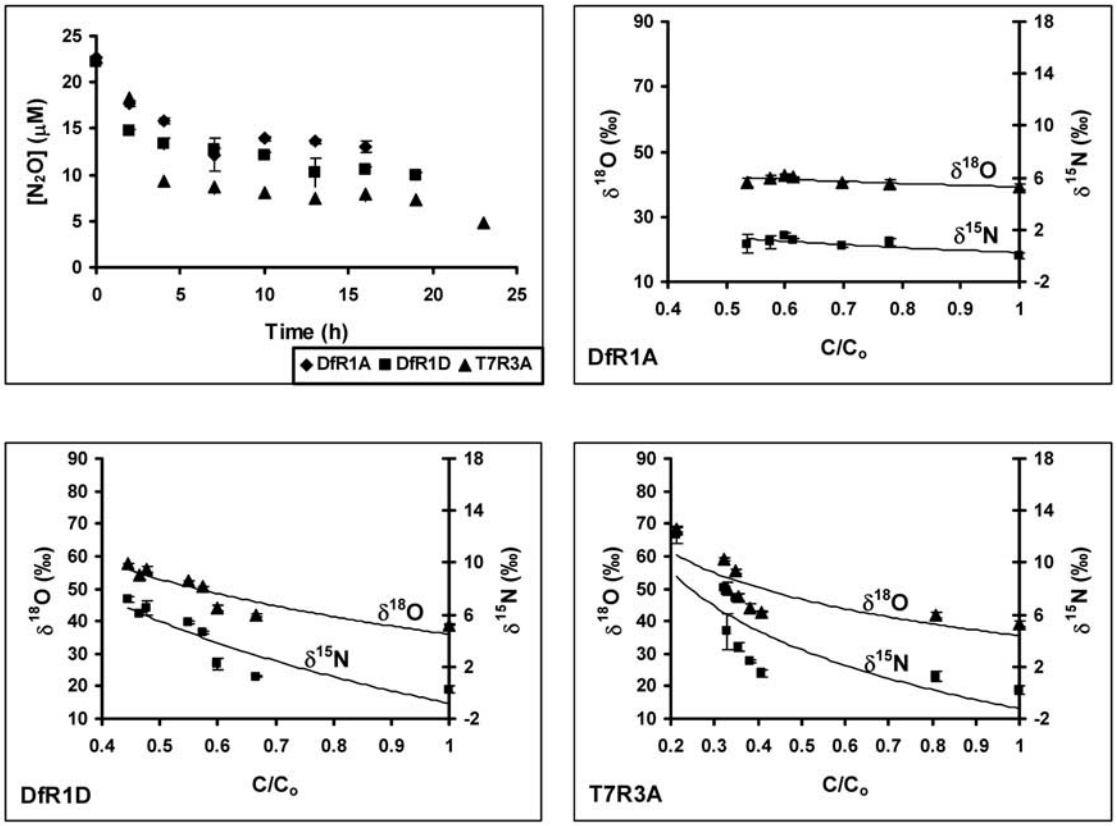

Figure 2. Concentration, $\delta^{15}N$, and $\delta^{18}O$ data for N_2O within soil mesocosm N_2O reduction experiments using soils from two replicate plots dominated by deciduous forest (DfR1A and DfR1D) and an early successional treatment (T7R3A) within the KBS LTER main site. Isotope values are expressed in relation to the concentration (C) at any point in time divided by the initial concentration (C_o) following the Rayleigh model (equation (3)). The slope of a natural log fit to the data equates to the fractionation factor for each experiment and are listed in Table 2. Error bars represent 2 standard deviations around the mean of three replicates for each treatment.

the soil mesocosm from the conventional agricultural field, T1R3A, which illustrates heterogeneity in N_2O reduction rates across plots with similar management histories. Rates of N_2O reduction ranged from 64 to 241 $\mu mol \cdot h^{-1} \cdot m^{-1}$ (Table 1), which are between 1 and 5 times the rates of denitrification previously measured by *Cavigelli and Robertson* [2000] in conventional agricultural fields at the KBS LTER. N_2O was the only oxidized N substrate available for denitrification in these mesocosms, so the rates of N_2O reduction observed also represent gross denitrification. Furthermore, the artificial conditions imposed by homogenization of soils, saturation, and maintenance of anaerobic conditions indicate that these rates should be considered potential rather than actual rates of N_2O reduction and, consequently, are likely in excess of what can be expected in the field. Despite their departure from field conditions, our experiments are appropriate for constraining the isotope effects associated with N_2O reduction by soil microbial communities.

[15] In this paper, we follow the convention of *Mariotti et al.* [1981] by defining the magnitude of isotopic fractionation during a specific reaction as the fractionation factor α,

$$\alpha = k_2/k_1, \tag{1}$$

where k_1 and k_2 are the reaction rates for the light and heavy isotopically substituted compounds, respectively (although some authors use the inverse of this ratio). We further define an isotopic enrichment factor, ε, which approximates for N_2O reduction the difference in isotopic composition between N_2 and N_2O,

$$\varepsilon = (\alpha - 1)*1000. \tag{2}$$

Isotopic fractionation during many microbial reactions, including denitrification, has been described using a Rayleigh distillation equation in which the isotopic composition of the residual substrate of a reaction (δ_s) is related to that of the initial substrate (δ_{so}) an isotopic enrichment factor (ε), and the ratio of the observed to initial substrate concentration (C/C_o) [*Mariotti et al.*, 1981],

$$\delta_s = \delta_{so} + \varepsilon \ln(C/C_o). \tag{3}$$

Equation (3) describes a relationship by which the isotopic composition of the substrate, N_2O, is linearly related to the natural log of its concentration. Within each of the mesocosm experiments both $\delta^{15}N$ and $\delta^{18}O$ were correlated with the natural log of C/C_o ($r^2 > 0.65$, $P < 0.05$) (Figures 1

Table 1. Rates of N_2O Reduction (Denitrification) and Isotopic Enrichment Factors for Mesocosm Experiments Using Soils From a Deciduous Forest (DfR1A and DfR1D), Early Successional (T7R3A), and Conventional Agricultural (T1R1A, T1R3A, and T1R5B) Treatment Histories[a]

Soil Mesocosm	Rate, μmol m^{-2} h^{-1}	$\varepsilon^{15}N$	$\varepsilon^{18}O$
DfR1A	87.1 ± 22.8	−1.8 ± 0.4	−5.1 ± 1.5
DfR1D	114.3 ± 2.9	−9.2 ± 0.3	−25.1 ± 0.5
T7R3A	137.0 ± 3.5	−6.8 ± 0.8	−15.3 ± 3.4
T1R1A	63.6 ± 9.1	−4.5 ± 0.4	−8.6 ± 0.7
T1R3A	240.8 ± 4.4	−8.3 ± 1.8	−19.8 ± 1.3
T1R5B	91.5 ± 11.9	−4. 7 ± 0.6	−11.1 ± 2.4

[a]All treatments were conducted in triplicate and the error shown represents 1 standard deviation.

and 2). Thus even while rates of reduction and isotope values differed markedly between mesocosm experiments, the significant correlations observed indicate that the fundamental constraints of the Rayleigh model, namely a unidirectional reaction and maintenance of a closed system, were followed.

[16] Under ideal conditions, an isotopic enrichment factor is a constant that should not vary with experimental conditions. Isotope effects controlled by biological processes, however, fundamentally violate the assumptions of the Rayleigh model in that transformations rarely consist of a single reaction. Denitrification, for example, consists of a series of steps involving diffusion of nitrate into the cell, reduction of nitrate to nitrite, nitrite to nitric oxide, nitric oxide to N_2O, and N_2O to N_2 [*Firestone and Davidson*, 1989]. Fractionation factors will vary depending on environmental conditions (e.g., degree of water saturation and availability of electron donors) and with which step in the overall denitrification process is rate limiting. The observed fractionation factor is most often that associated with the rate limiting step but may reflect variation in the relative importance of individual reaction steps [*Bryan et al.*, 1983; *Ostrom et al.*, 2002]. During N_2O reduction, the most important rate limiting steps are diffusion into the cell and enzymatic reduction. While fractionation during diffusion is generally small [e.g., *Brandes and Devol*, 1997] fractionation during enzymatic reduction is large [*Yoshida et al.*, 1984; *Yamazaki et al.*, 1987]. The low observed fractionation factors for nitrogen in our soil mesocosm experiments (−9.1 to −1.7‰) (Table 1) relative to those reported previously (−39 to −27‰) [*Yoshida et al.*, 1984; *Yamazaki et al.*, 1987] indicate that diffusion is important in limiting the expression of the enzymatic fractionation in our experiments. The variability in observed fractionation factors suggests that the balance between diffusion and enzymatic reduction in controlling fractionation is variable between mesocosm experiments. Such observed variability in the expression of fractionation is a confounding factor that often limits application of natural abundance stable isotope studies in source apportionment.

3.2. N_2O Reduction in Pure Culture Experiments

[17] Within pure cultures of *Pseudomonas stutzeri* and *Pseudomonas denitrificans* correlations between isotope values and the natural log of C/C_o during N_2O reduction substantiate the use of the Rayleigh model requirements of a closed system (Figure 3). The observed isotopic enrichment factors for $\delta^{15}N$ and $\delta^{18}O$ data (Table 2) were within the range of those obtained in soil mesocosms. Experimental conditions enabled the determination of isotopic enrichment factors for $\delta^{15}N^{\alpha}$, $\delta^{15}N^{\beta}$ and SP in addition to those for bulk isotopes. Isotopic enrichment for the β position were quite small (~2‰), whereas those associated with the α position were greater than those associated with the bulk $\delta^{15}N$. These results indicate that the majority of N isotope fractionation during reduction is associated with the α position in N_2O. This is consistent with cleavage of the N-O covalent bond during denitrification. The fractionation factor for SP during N_2O reduction was small (~6‰) but of sufficient magnitude that if reduction of N_2O is a prevalent process changes in SP can be expected. Based on the Rayleigh model using a fractionation factor for SP of 6‰, for example, 50% consumption of an existing N_2O pool would result in a 4‰ increase in SP. While small, this change is of sufficient magnitude to affect estimates of the relative importance of nitrification and denitrification to N_2O fluxes determined on the basis of SP.

3.3. Relationships Among Isotopic Enrichment Factors for N_2O Reduction

[18] A critical question for apportionment studies is whether changes resulting from N_2O reduction can be recognized and/or potentially corrected for. Our results indicate that reduction of N_2O prior to its evolution to the atmosphere has the potential to result in changes in $\delta^{15}N$, $\delta^{18}O$ and SP of sufficient magnitude to compromise calculation of sources based on these values. Correction of isotope values for fractionation during reduction is theoretically possible if the extent of the reaction and isotopic enrichment factors are known [*Ostrom et al.*, 2002]; however, this approach, based on bulk isotope data, is not possible in this case owing to the wide variation of ε values we observe for N_2O reduction. Furthermore, reduction results in ^{15}N enrichment, which serves to decrease the difference in $\delta^{15}N$ between soil nitrate or ammonium and N_2O, which is the basis of the apportionment approach commonly used in bulk natural abundance isotopologue studies [*Perez et al.*, 2000, 2001; *Bol et al.*, 2003]. Reduction also results in a small but appreciable increase in SP, which shifts values away from the SP value for denitrification (0‰) and toward that of nitrification (33‰) [*Sutka et al.*, 2006]. Consequently, we conclude that the isotope effects resulting from reduction cannot be ignored in isotope based source apportionment studies.

[19] We note, however, that despite a wide range of fractionation factors evident for N_2O reduction within the soil mesocosm and pure culture data there is a remarkable and significant relationship between $\varepsilon^{18}O$ and $\varepsilon^{15}N$ that is characterized by a slope of 2.5 (Figure 4). Similarly, we find a relationship of 2.6 between $\delta^{18}O$ and $\delta^{15}N$ for all of the data in our reduction experiments (Figure 4) which is expected given that fractionation factors are derived from individual isotope values (equation 3). The slight difference between the slopes of $\delta^{18}O$ versus $\delta^{15}N$ and $\delta^{18}O$ versus $\varepsilon^{15}N$ in Figure 4 is a consequence of unequal numbers of samples in experiments used to calculate fractionation factors and thus we believe the ratios of fractionation factors are a more accurate descriptor of the isotopologue relationships during N_2O reduction. The value of 2.5 we report is in

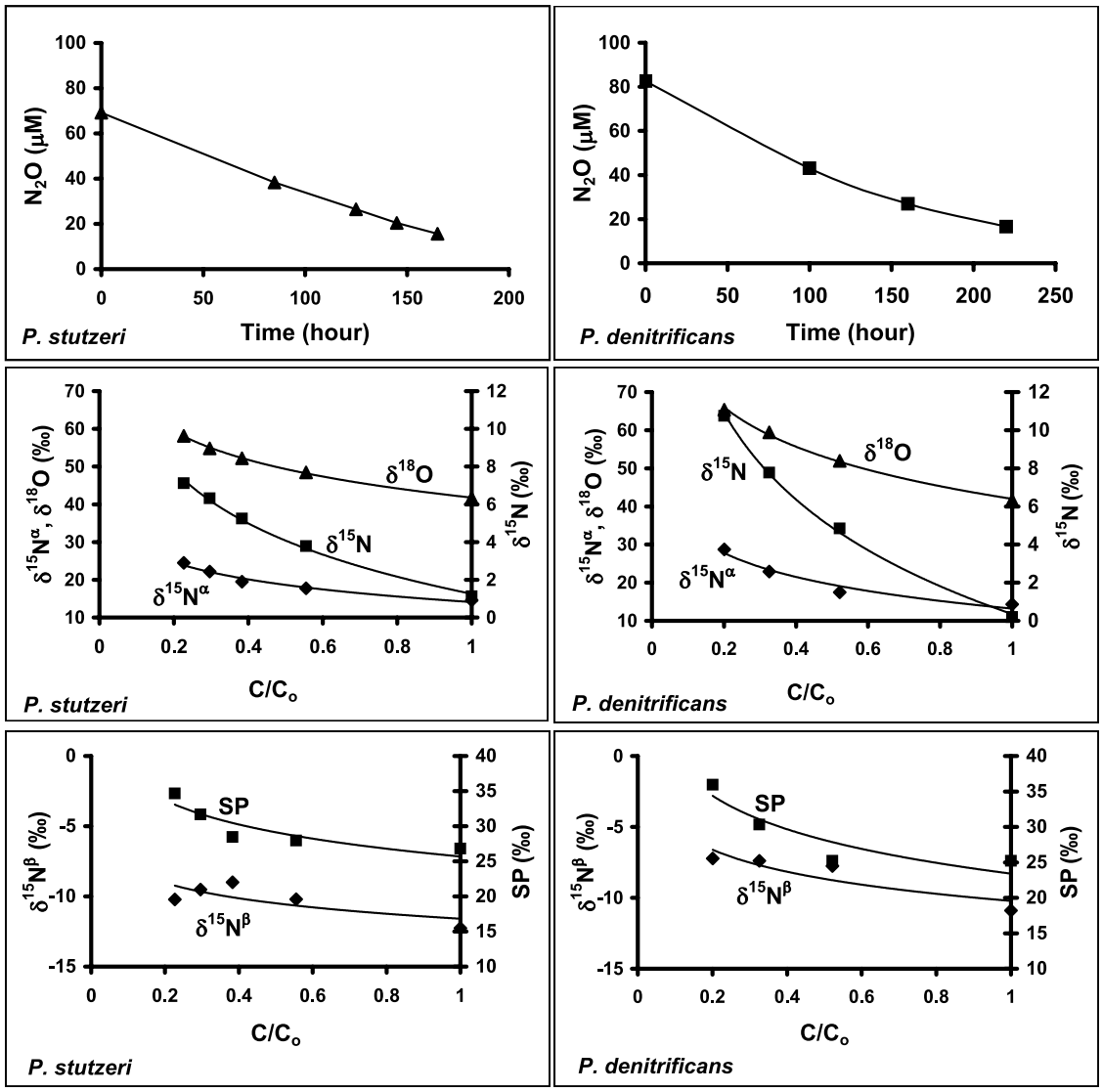

Figure 3. Concentration, $\delta^{15}N$, $\delta^{18}O$, $\delta^{15}N^{\alpha}$, $\delta^{15}N^{\beta}$, and SP values for N_2O reduction within pure culture experiments of *P. stutzeri* and *P. denitrificans*. Isotope values are expressed in relation to the concentration (C) at any point in time divided by the initial concentration (C_o) following the Rayleigh model (equation (3)). The slope of a natural log fit to the data equates to the fractionation factor for each experiment and isotopomer and are listed in Table 2.

remarkable agreement with the values of 2.5 reported for reduction in Siberian soils [*Menyailo and Huntgate*, 2006a], 2.0 reported for reduction in soils [*Webster and Hopkins*, 1996; *Mandernack et al.*, 2000] and pure culture [*Webster and Hopkins*, 1996], and 2.6 that is obtained by taking the ratio of $\varepsilon^{18}O$ and $\varepsilon^{15}N$ obtained during reduction in anoxic ocean waters [*Yamagishi et al.*, 2007]. Furthermore, ratios of $\varepsilon^{18}O$ to $\varepsilon^{15}N^{\alpha}$ and $\varepsilon^{18}O$ to SP (which are analogous to the slope) within the pure culture experiments, while only based on two cultures, were in remarkable agreement with values of 1.7 and 2.2, respectively (Table 2), that is similar to a value of 1.5 in ocean waters [*Yamagishi et al.*, 2007]. A similar constant relationship between oxygen and nitrogen isotope fractionation in nitrate for denitrification has been reported [*Böttcher et al.*, 1990; *Durka et al.*, 1994]. While fractionation factors for N_2O reduction experiments or in the field are susceptible to considerable variation, the relationships between $\varepsilon^{15}N$, $\varepsilon^{15}N^{\alpha}$ and $\varepsilon^{18}O$ covary in a predictable and diagnostic manner. Consequently, reduction of N_2O can be clearly recognized when relationships between $\delta^{18}O$ and $\delta^{15}N$, $\delta^{18}O$ and $\delta^{15}N^{\alpha}$, and $\delta^{18}O$ and SP approach values of 2.5, 1.7 and 2.2, respectively.

3.4. N_2O Reduction and Isotopologue-Based Source Apportionment

[20] Mass balance mixing models are commonly used in flux chamber studies to determine the isotopologue compo-

Table 2. Isotopic Enrichment Factors for N_2O Reduction (Denitrification) in Pure Culture Experiments

Denitrifier Species	$\varepsilon^{15}N$	$\varepsilon^{18}O$	$\varepsilon^{15}N^{\alpha}$	$\varepsilon^{15}N^{\beta}$	εSP
P. stutzeri	−4.1	−10.9	−6.6	−1.6	−5.0
P. denitrificans	−6.6	−15.0	−9.1	−2.2	−6.8

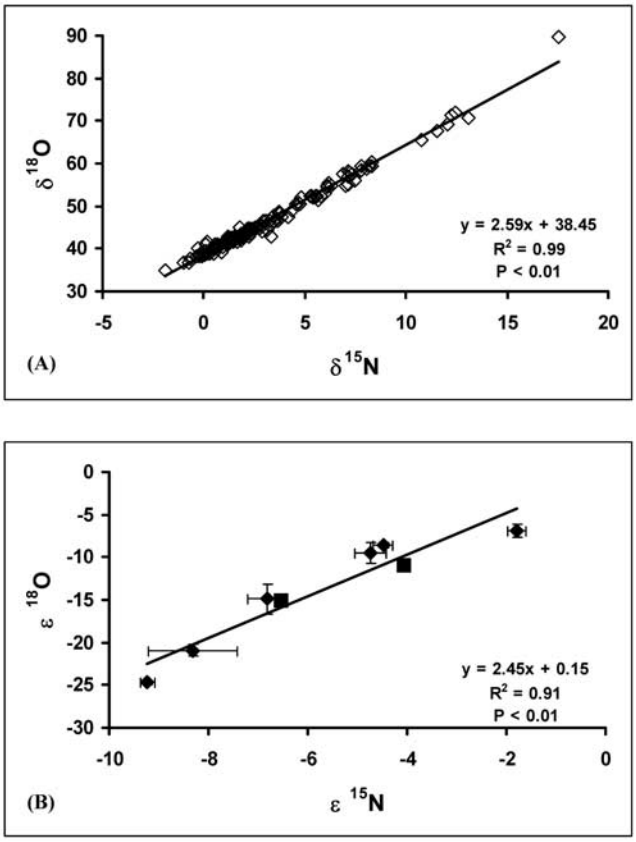

Figure 4. Relationships between (a) $\delta^{18}O$ and $\delta^{15}N$ values for all soil mesocosm (diamonds) and pure culture (triangles) N_2O reduction experiments and (b) between the fractionation factors of oxygen and nitrogen isotopes during N_2O reduction in the soil (diamonds) and pure culture (squares) experiments (ε values from Tables 1 and 2). The error bars in Figure 4b indicate 1 standard deviation based on triplicate experiments for each soil type in the mesocosms experiments. The pure culture experiments were not replicated.

sition of soil-derived N_2O [e.g., *Well et al.*, 2006]. If, however, reduction of N_2O is significant, the isotope affects associated with this process will result in enrichment in ^{15}N and ^{18}O and an inaccurate assessment of the isotopologue values for soil-derived N_2O. Consequently, it is desirable (1) to know when the isotope affects associated with reduction are important and (2) to correct for such shifts if possible. We can establish the affect of N_2O reduction in flux chamber studies on isotope values in a hypothetical manner based on the relationships between $\delta^{18}O$, $\delta^{15}N$ and $\delta^{15}N^{\alpha}$ established within our mesocosm and pure culture studies in comparison to flux chamber data collected previously at the KBS LTER [*Ostrom et al.*, 2004] (Figure 5). During a typical flux chamber deployment, the concentration of N_2O will rise steadily representing the addition of soil-derived N_2O to that initially present in the atmosphere (320 ppbv). With continued input of soil-derived N_2O the isotopologue composition of N_2O within the chamber will shift away from values characteristic of the atmosphere (labeled "troposphere" in Figure 5) toward values reflecting the isotopologue composition of soil-derived N_2O and data will lie along a line connecting the isotopologue values for the troposphere and soil-derived N_2O (labeled "mixing line"). If N_2O reduction is the only process affecting the isotopic composition of N_2O then the concentration of N_2O would decline steadily from tropospheric values (320 ppbv)

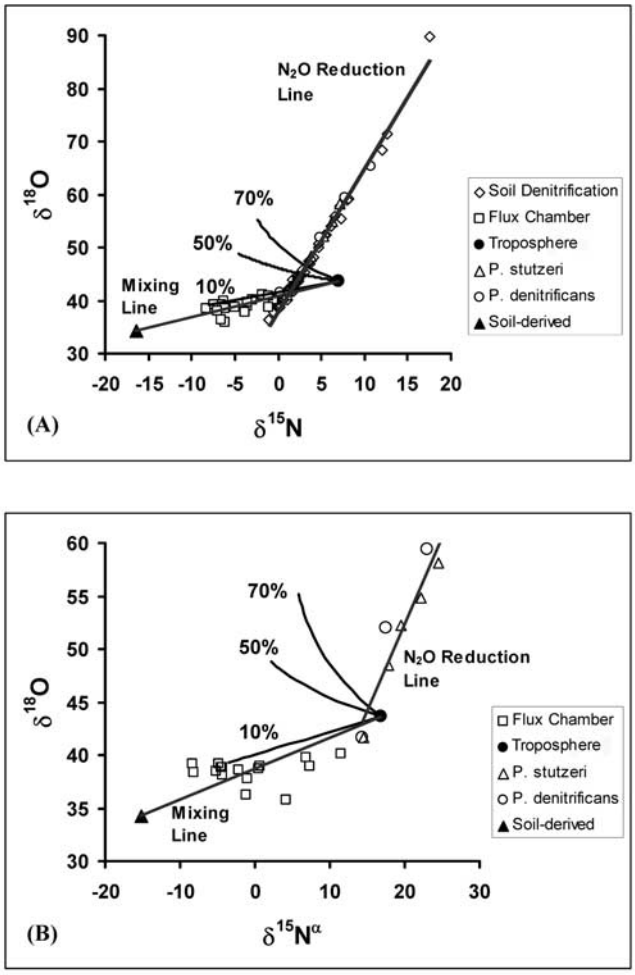

Figure 5. Relationships between the (a) $\delta^{18}O$ and $\delta^{15}N$ and (b) $\delta^{18}O$ and $\delta^{15}N^{\alpha}$ of N_2O for N_2O production and reduction in soils. Data from cultivated successional soil flux chambers at the KBS LTER [*Ostrom et al.*, 2004] are indicated in open squares and lie along a "mixing line" between the values for tropospheric N_2O (solid circle) [*Yoshida and Toyoda*, 2000] and calculated values for soil-derived N_2O (solid triangle). Isotopomer values from soil mesocosm (open diamond) and pure culture studies of *P. stutzeri* (open triangles) and *P. denitrificans* (open circle) combine to form the "N_2O reduction line." The slope of the N_2O reduction lines, 2.5 for $\delta^{18}O$–$\delta^{15}N$ and 1.7 for $\delta^{18}O$–$\delta^{15}N^{\alpha}$, would result when N_2O reduction occurs in the absence of production. The N_2O reduction line does not intersect the isotopomer values for the troposphere but originates with values of the pure N_2O standard that was used in the headspace of all reduction experiments. Solid lines represent the trends expected within flux chamber studies at the production rates and soil-derived isotopologue values from *Ostrom et al.* 2004 when simultaneous production and consumption occur, at 10, 50, and 70% the rate of production, based on a bulk nitrogen isotopic enrichment factor of 10‰.

and isotopologue values would rise steadily. These data would like on a line with a slope of 2.5 for the plot of $\delta^{18}O$ versus $\delta^{15}N$ and 1.7 for the plot of $\delta^{18}O$ versus $\delta^{15}N^{\alpha}$ (identified as "N_2O reduction line" in Figure 5). In that soil-derived N_2O is commonly depleted in ^{15}N and ^{18}O relative to tropospheric N_2O [*Mandernack et al.*, 2000; *Perez et al.*, 2000, 2001; *Tilsner et al.*, 2003; *Wrage et al.*, 2004a], samples representing a mixture of soil-derived and atmospheric sources lie on a mixing line with a slope less than 1 (0.41 in the flux chamber study shown in Figure 5). Linear relationships between $\delta^{18}O$ and $\delta^{15}N$ of less than 1 have been commonly associated with N_2O production within flux chamber studies [*Webster and Hopkins*, 1996; *Mandernack et al.*, 2000]. Similarly, the low $\delta^{15}N^{\alpha}$ values for soil-derived N_2O from the *Ostrom et al*. [2004] study also produce a linear $\delta^{18}O$ versus $\delta^{15}N^{\alpha}$ mixing relationship with atmospheric N_2O with a slope of 0.30 (Figure 5b). These values differ markedly from the slopes obtained in our reduction experiments that produce relationships between $\delta^{18}O$ and $\delta^{15}N$ and $\delta^{18}O$ and $\delta^{15}N^{\alpha}$ of 2.5 and 1.7, respectively (shown as "N_2O reduction lines" in Figure 5). Consequently, a qualitative indicator of N_2O reduction are slopes for the relationships between $\delta^{18}O$ and $\delta^{15}N$ and $\delta^{18}O$ and $\delta^{15}N^{\alpha}$ that approach values of 1 or greater.

[21] We can predict, in a theoretical manner, how the relationships between $\delta^{18}O$ and $\delta^{15}N$ and $\delta^{18}O$ and $\delta^{15}N^{\alpha}$ will respond if reduction of N_2O occurs simultaneously with production. In the absence of production, isotope data in flux chambers will follow the "mixing line" shown in Figure 5 owing to the addition of soil-derived N_2O to tropospheric N_2O. The precise changes in $\delta^{18}O$ and $\delta^{15}N$ are determined from a basic isotope mixing model [e.g., *Well et al.*, 2006],

$$\delta_{meas} = (\delta_{ATM}Q_{ATM} + \delta_{SD}Q_{SD})/Q_{meas}, \quad (4)$$

where, meas, ATM and SD refer to the isotope values (δ) of and quantity (Q) of N_2O measured, that is present initially in the atmospheric (ATM), and that added from soil-derived (SD) production, respectively. We use actual flux rate and soil-derived isotope values that were obtained from a series of flux chamber deployments at the KBS LTER [*Ostrom et al.*, 2004] with the assumption that this data is unaffected by N_2O reduction. On the basis of the average rate of production and isotopologue composition of soil-derived N_2O, we predict, from equation (4), how the isotopic composition of N_2O measured (δ_{meas}) changes as a function of time over a typical 1 hour closing of a flux chamber. The solution to δ_{meas} for each point in time becomes the initial isotopic composition of N_2O (δ_{so}) in equation (3) that evaluates the isotopologue shifts resulting from N_2O reduction. For the C/C_o term in equation (3) we use 0.1, 0.3 or 0.7 to represent a rate of reduction equivalent to 10, 30 or 70% of the rate of production. We use a bulk nitrogen fractionation factor ($\varepsilon^{15}N$) for N_2O reduction of $-10‰$ to predict the resulting $\delta^{15}N$ values of the chamber N_2O. Fractionation factors for $\varepsilon^{18}O$ and $\varepsilon^{15}N^{\alpha}$ are based on the constant relationships between $\delta^{18}O$ and $\delta^{15}N$ and $\delta^{18}O$ and $\delta^{15}N^{\alpha}$ of 2.5 and 1.7, respectively, which were found in the soil and pure culture N_2O reduction experiments. A $\varepsilon^{15}N$ of $-10‰$ was chosen as a reasonable worst-case-scenario; being slightly greater than the largest degree of fractionation observed in the soil N_2O reduction experiments (Table 1). The net result of simultaneous production and consumption of N_2O is a deviation from a linear mixing relationship between soil-derived and atmospheric N_2O to progressively more curvilinear relationships between $\delta^{18}O$ and $\delta^{15}N$ and $\delta^{15}N^{\alpha}$ with increasing importance of reduction. Consequently, an important line of evidence for an alteration of isotope values due to reduction is the deterioration of a linear relationship between $\delta^{18}O$ and $\delta^{15}N$ and $\delta^{18}O$ and $\delta^{15}N^{\alpha}$. The magnitude of deviation from linearity may thus serve as a tool for potentially correcting isotopologue data for isotope shifts due to reduction.

[22] A small degree of reduction of 10% results in only a slight deviation from the mixing lines while maintaining a linear relationship (Figure 5). Over the course of 1 hour at the production rates observed the net affect of 10% reduction and a $\varepsilon^{15}N$ of $-10‰$ is a shift in $\delta^{15}N$ of only 0.7‰. Given the proportional relationships between isotopologue enrichment factors we have established for reduction, this small shift in $\delta^{15}N$ corresponds to increases in $\delta^{18}O$ and SP of 1.9 and 0.9‰, respectively. *Sutka et al.* [2006] indicate that SP values of 0 and 33‰ can be used as conservative tracers of N_2O production from denitrification and nitrification, respectively. On this basis, the small shift in SP that would result from a reduction rate equivalent to 10% of the production rate would result in an underestimate of the proportion of N_2O from denitrification of only 2.7%. In this manner, the proportional relationships between isotopologues provides a basis to correct for shifts resulting from N_2O reduction, however, a precise correction requires knowledge of the soil-derived isotopologue values a priori which is unlikely. Nonetheless, our approach defines a means for establishing if the affect of N_2O reduction on isotopologue values in soil flux chamber studies is appreciable and provides a semi-qualitative approach for understanding the magnitude of such shifts.

[23] While our experiments are quite useful in quantifying isotope effects in response to N_2O reduction in natural soil microbial communities it is important to understand that our experimental design specifically addresses reduction of N_2O that enters microbial cells from the surrounding soil or headspace. Our design does not address N_2O reduction that occurs within the cells of nitrifying or denitrifying microbes following production of N_2O but prior to its escape from the cell. In other words, N_2O that is produced by soil bacteria can undergo 3 possible routes: (1) escape from the cell to the atmosphere, (2) reduction within the cell prior to its escape, or (3) reduction upon reentering the cell prior to escape to the atmosphere. Our study specifically simulates step 3 but does not address reduction within the cell prior to its escape (step 2). This distinction is subtle but important as studies commonly report the ratio of N_2O production to the sum of N_2O and N_2 production ($N_2O/(N_2O + N_2)$) [*Dendooven and Anderson*, 1995; *Dendooven et al.*, 1996; *Bergsma et al.*, 2002; *Well et al.*, 2006] which measures total N_2O reduction and, therefore, differs from our experimental design by including reduction via step 2 as well as step 3. Ratios of $N_2O/(N_2O + N_2)$ across ecosystems have been shown to be quite variable [*Dendooven and Anderson*, 1995; *Dendooven et al.*, 1996; *Well et al.*, 2006] and our previous studies using soils from the KBS LTER found values between 0.3 to 0.4 [*Bergsma et al.*, 2002]. Our study does not

specifically address the isotopologue effects associated with step 2 but rather targets the isotope effects during step 3. Given that steps 2 and 3 are both measures of N_2O reduction within microbial cells we expect that the isotopologue effects associated with steps 2 and 3 will be quite similar and that our results apply to N_2O reduction whether or not the N_2O reduced originates from within or external to the cell.

[24] While it is clear from measurements of the $N_2O/(N_2O + N_2)$ ratio that substantial rates of N_2O reduction relative to production can be expected in soils, we believe most isotopologue studies of N_2O soil flux are often biased by methodological limitations to periods of high flux when rates of N_2O reduction are minor and not of sufficient magnitude to alter isotopologue data. The low abundance of N_2O in the atmosphere creates an analytical challenge to mass spectrometrists such that isotopologue studies of soil flux occur most often when fluxes are high [*Tilsner et al.*, 2003]. Furthermore, error propagation in the calculation of soil-derived isotopologue values from mixing models (equation (3)) increases when the proportion of N_2O from soils versus the atmosphere is low. Reduction of N_2O clearly results in enrichment in ^{15}N and ^{18}O, however, our theoretical exercise indicates that minor rates of reduction relative to production have only a small affect on isotopologue data; most notably on SP. Thus we believe the tendency for soil-derived N_2O to be characterized by depletions in ^{15}N and ^{18}O relative to atmospheric N_2O [*Mandernack et al.*, 2000; *Perez et al.*, 2000, 2001; *Yamulki et al.*, 2001; *Bol et al.*, 2003; *Tilsner et al.*, 2003; *Wrage et al.*, 2004a] and slopes for the relationships between $\delta^{18}O$ and $\delta^{15}N$ and $\delta^{18}O$ and $\delta^{15}N^{\alpha}$ of less than one are key lines of evidence for the lack of substantial isotopologue shifts resulting from reduction. Such depletions contrast markedly with studies in anoxic marine waters that show substantial enrichments in ^{15}N and ^{18}O that are clearly the result of N_2O reduction (e.g., $\delta^{18}O > 100‰$ [*Yoshinari et al.*, 1997; *Naqvi et al.*, 1998]). Furthermore, our theoretical exercise demonstrates that if reduction occurs at rates of production of 30% or more there is a substantial deviation from the linear relationships between $\delta^{18}O$ and $\delta^{15}N$ and $\delta^{18}O$ and $\delta^{15}N^{\alpha}$. Thus the linear relationships between $\delta^{18}O$ and $\delta^{15}N$ [*Webster and Hopkins*, 1996; *Mandernack et al.*, 2000; *Menyailo and Huntgate*, 2006a] and $\delta^{18}O$ and $\delta^{15}N^{\alpha}$ [*Sutka et al.*, 2006] is strong evidence that reduction was a minor process in these studies. This conclusion is in contrast to studies of the $N_2O/(N_2O + N_2)$ ratio that indicate rates of reduction in excess of 60% that were reported previously at our study site [*Bergsma et al.*, 2002], however, N_2O reduction may be enhanced in incubation studies (by the addition of glucose, for example) and there is a paucity of data on variation in the $N_2O/(N_2O + N_2)$ ratio across broad temporal and spatial scales. Consequently, our expectation that N_2O reduction is a minor process during periods of high soil N_2O flux needs to be confirmed by rate measurements of both N_2O production and consumption across seasonal and spatial scales and, in particular, by isotopologue studies during periods of low N_2O flux.

[25] Resolving production rates and sources of N_2O production continues to be an ongoing effort of many researchers and we have established that SP is an important means for understanding microbial N_2O production and consumption. While a natural abundance level approach, such as the use of SP, is advantageous to tracer level studies in that the artificial conditions imposed by incubation are not needed, the microbial N cycle adds considerable complexity. For example, dissimilatory nitrate reduction to ammonium and fungal denitrification have been implicated as sources of N_2O [*Smith*, 1982; *Robertson and Tiedje*, 1987; *Laughlin and Stevens*, 2002]. While we expect that the conditions that favor these processes (such as high soil organic content and lack of tilling) are not likely operative in our study clearly more research is warranted. Preliminary research in our laboratory indicates that the SP associated with N_2O production during fungal denitrification is uniquely enriched in ^{15}N in the α position (>35‰) [*Sutka et al.*, 2004b]. Nonetheless, the low SP value for N_2O production from denitrification of approximately 0‰ is unique relative to all other production pathways measured thus far (nitrification via hydroxylamine production and fungal denitrification) [*Sutka et al.*, 2006]. Furthermore, reduction of N_2O results in an increase in the ^{15}N content of the α position and SP. Consequently, low values for SP approaching 0‰ can only be the result of production from denitrification during times when an influence from N_2O reduction is minor. Consequently, calculations of the proportion of N_2O from denitrification based on SP values remain conservative as all other production pathways and N_2O reduction cause increases in SP.

[26] **Acknowledgments.** We thank J.M. Tiedje for denitrifier cultures, and J. Breznak for inspiration and assistance with microbial cultures. We especially appreciate the comments and reviews provided by Kevin Mandernack and Reinhard Well. Financial support was provided by the National Science Foundation (DEB and LTER) and the Michigan Agricultural Experiment Station.

References

Anderson, I. C., M. Poth, J. Homstead, and D. Burdige (1993), A comparison of NO and N_2O production by the autotrophic nitrifier *Nitrosomonas europaea* and the heterotrophic nitrifier *Alcaligenes faecalis*, *Appl. Environ. Microbiol.*, *59*, 3525–3533.

Bandibas, J., A. Vermoesen, C. J. De Groot, and O. Van Cleemput (1994), The effect of different moisture regimes and soil characteristics on nitrous oxide emission and consumption by different soils, *Soil Sci.*, *158*, 106–114.

Bateman, E. J., and E. M. Baggs (2005), Contributions of nitrification and denitrification to N_2O emissions from soils at different water-filled pore space, *Biol. Fertil. Soils*, *41*, 379–388.

Berg, B., B. Wessen, and G. Ekbohm (1982), Nitrogen level and decomposition in Scots pine needle litter, *Oikos*, *38*, 291–296.

Bergsma, T. T., G. P. Robertson, and N. E. Ostrom (2002), Influence of soil moisture and land use history on denitrification end-products, *J. Environ. Qual.*, *31*, 711–717.

Bol, R., S. Toyoda, S. Yamulki, J. M. B. Hawkins, L. M. Cardenas, and N. Yoshida (2003), Dual isotope and isotopomer ratios of N_2O emitted from a temperate grassland soil after fertilization, *Rapid Commun. Mass Spectrom.*, *17*, 2550–2556.

Bol, R., T. Röckmann, M. Blackwell, and S. Yamulki (2004), Influence of flooding on $\delta^{15}N$, $\delta^{18}O$, $^{1}\delta^{15}N$ and $^{2}\delta^{15}N$ signatures of N_2O released from estuarine soils—A laboratory experiment using tidal flooding chambers, *Rapid Commun. Mass Spectrom.*, *18*, 1561–1568.

Böttcher, J., O. Strebel, S. Voerkelius, and H.-L. Schmidt (1990), Using isotope fractionation of nitrate-nitrogen and nitrate-oxygen for evaluation of microbial denitrification in a sandy aquifer, *J. Hydrol.*, *114*, 413–424.

Brandes, J. A., and A. H. Devol (1997), Isotopic fractionation of oxygen and nitrogen in coastal marine sediments, *Geochim. Cosmochim. Acta*, *61*, 1793–1801.

Breninkenmeijer, C. A. M., and T. Röckmann (2000), Mass spectrometry of the intramolecular nitrogen isotope distribution of environmental nitrous oxide using fragment-ion analysis, *Rapid Commun. Mass Spectrom.*, *13*, 2028–2033.

Bryan, B. A., G. Shearer, J. L. Skeeters, and D. H. Kohl (1983), Variable expression of the nitrogen isotope effect associated with denitrification of nitrite, *J. Biol. Chem.*, *258*, 8613–8617.

Burbank, D. H., K. S. Pregitzer, and K. L. Gross (1992), Vegetation of the W. K. Kellogg Biological Station, *Rep. 510*, Mich. State Univ. Agric. Exp. Stn. Res., Lansing.

Caldeira, K., M. G. Morgan, D. Baldocchi, P. G. Brewer, C. T. A. Chen, G.-J. Nabuurs, N. Nakicenovic, and G. P. Robertson (2004), A portfolio of carbon management options, in *The Global Carbon Cycle*, edited by B. Field and M. R. Raupach, pp. 103–130, Island, Washington, D. C.

Cavigelli, M. A., and G. P. Robertson (2000), The functional significance of denitrifier community composition in a terrestrial ecosystem, *Ecology*, *81*, 1402–1414.

Cavigelli, M. A., and G. P. Robertson (2001), Role of denitrifier diversity in rates of nitrous oxide consumption in a terrestrial ecosystem, *Soil Biol. Biochem.*, *33*, 297–310.

Davidson, E. A. (1992), Sources of nitric-oxide and nitrous-oxide following wetting of dry soil, *Soil Sci. Soc. Am. J.*, *56*, 95–102.

Dendooven, L., and J. M. Anderson (1995), Maintenance of denitrification potential in pasture soil following anaerobic events, *Soil Biol. Biochem.*, *27*, 1251–1260.

Dendooven, L., L. Duchateau, and J. M. Anderson (1996), Gaseous products of the denitrification process as affected by the antecedent water regime of the soil, *Soil Biol. Biochem.*, *28*, 239–245.

Durka, W., E.-D. Schultze, G. Gebauer, and S. Voerkelius (1994), Effects of forest decline on uptake and leaching of deposited nitrate determined from ^{15}N and ^{18}O measurements, *Nature*, *372*, 765–767.

Firestone, M. K., and E. A. Davidson (1989), Microbial basis of NO and N_2O production and consumption in the soil, in *Exchange of Trace Gases Between Terrestrial Ecosystems and the Atmosphere*, edited by M. O. Andreae and D. S. Schimel, pp. 7–21, John Wiley, Hoboken, N. J.

Intergovernmental Panel on Climate Change (2001), *Climate Change 2001: The Scientific Basis*, Cambridge Univ. Press, New York.

Kaiser, J., S. Park, K. A. Boering, C. A. M. Brenninkmeijer, A. Hilkert, and T. Röckmann (2004), Mass spectrometric method for the absolute calibration of the intramolecular nitrogen isotope distribution in nitrous oxide, *Anal. Bioanal. Chem.*, *378*, 256–269.

Laughlin, R. J., and R. J. Stevens (2002), Evidence for fungal dominance of denitrification and codenitrification in a grassland soil, *Soil Sci. Soc. Am. J.*, *66*, 1540–1548.

Madsen, E. L. (1998), Epistemology of environmental microbiology, *Environ. Sci. Technol.*, *32*, 429–439.

Mandernack, K. W., T. Rahn, C. Kinney, and M. Wahlen (2000), The biogeochemical controls on the $\delta^{15}N$ and $\delta^{18}O$ of N_2O produced in landfill cover soils, *J. Geophys. Res.*, *105*, 17,709–17,720.

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.

Matson, P. A., P. M. Vitousek, and D. S. Schimel (1989), Regional extrapolation of trace gas flux based on soils and ecosystems, in *Exchange of Trace Gases Between Terrestrial Ecosystems and the Atmosphere*, edited by M. D. Andreae and D. S. Schimel, pp. 97–108, John Wiley, Hoboken, N. J.

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

Menyailo, O. V., and B. A. Huntgate (2006b), Tree species and moisture effects on soil sources of N_2O: Quantifying contributions from nitrification and denitrification with ^{18}O isotopes, *J. Geophys. Res.*, *111*, G02022, doi:10.1029/2005JG000058.

Miller, C. E., and Y. L. Yung (2000), Photo-induced isotopic fractionation of stratospheric N_2O, *Chemosphere Global Change Sci.*, *2*, 255–266.

Mosier, A. R., J. M. Duxbury, J. R. Freney, O. Heinemeyer, and K. Minami (1998a), Assessing and mitigating N_2O emissions from agricultural soils, *Clim. Change*, *40*, 7–38.

Mosier, A., C. Kroeze, C. Nevison, O. Oenema, S. Seitzinger, and O. van Cleemput (1998b), Closing the global N2O budget: Nitrous oxide emissions through the agricultural nitrogen cycle: OECD/IPCC/IEA phase II development of IPCC guidelines for national greenhouse gas inventory methodology, *Nutr. Cycling Agroecosyst.*, *52*, 225–248.

Naqvi, S. W. A., T. Yoshinari, D. A. Jayakumar, M. A. Altabet, P. V. Narvekar, A. H. Devol, J. A. Brandes, and L. A. Codispotti (1998), Budgetary and biogeochemical implications of N_2O isotope signatures in the Arabian Sea, *Nature*, *394*, 462–464.

Ostrom, N. E., K. Knoke, L. O. Hedin, G. P. Robertson, and A. Smucker (1998), Temporal trends in nitrogen isotope values of nitrate leaching from an agricultural soils, *Chem. Geol. Isotope Geosci.*, *146*, 219–227.

Ostrom, N. E., M. E. Russ, B. Popp, T. M. Rust, and D. M. Karl (2000), Mechanisms of N_2O production in the subtropical North Pacific based on determinations of the isotopic abundances of N_2O and O_2, *Chemosphere Global Change Sci.*, *2*, 281–290.

Ostrom, N. E., L. O. Hedin, J. C. von Fisher, and G. P. Robertson (2002), Nitrogen transformations and nitrate removal at a soil-stream interface: A stable isotope approach, *Ecol. Appl.*, *12*, 1027–1043.

Ostrom, N. E., R. L. Sutka, A. S. Grandy, K. M. Huizinga, P. H. Ostrom, and G. P. Robertson (2004), Apportionment of nitrous oxide flux from a successional Midwest grassland to denitrification and nitrification based on isotopomers, *Eos Trans. AGU*, *85*(47), Fall Meet. Suppl., Abstract B21A-0855.

Perez, T., S. E. Trumbore, S. C. Tyler, E. A. Davidson, M. Keller, and P. B. de Camargo (2000), Isotopic variability of N_2O emissions from tropical forest soils, *Global Biogeochem. Cycles*, *14*, 525–535.

Perez, 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 N_2O budget using stable isotopes, *J. Geophys. Res.*, *106*, 9869–9878.

Popp, B. N., et al. (2002), Nitrogen and oxygen isotopometric constraints on the origins and sea-to-air flux of N_2O in the oligotrophic subtropical North Pacific gyre, *Global Biogeochem. Cycles*, *16*(4), 1064, doi:10.1029/2001GB001806.

Robertson, G. P. (2004), Abatement of nitrous oxide, methane, and other non-CO_2 greenhouse gases: The need for a systems approach, in *The Global Carbon Cycle*, edited by C. B. Field and M. R. Raupach, pp. 493–506, Island, Washington, D. C.

Robertson, G. P., and P. Groffman, (2006), Nitrogen transformations, in *Soil Microbiology, Biochemistry, and Ecology*, edited by E. A. Paul and F. E. Clark, pp. 341–388, Springer, New York.

Robertson, G. P., and J. M. Tiedje (1987), Nitrous oxide sources in aerobic soils: Nitrification, denitrification, and other biological processes, *Soil Biol. Biochem.*, *19*, 187–193.

Röckmann, T., J. Kaiser, C. A. M. Brenninkmeijer, and W. A. Brand (2003), Gas chromatography/isotope ratio mass spectrometry method for high-precision position-dependent ^{15}N and ^{18}O measurements of atmospheric nitrous oxide, *Rapid Commun. Mass Spectrom.*, *17*, 1897–1908.

Schmidt, H.-L., R. A. Werner, N. Yoshida, and R. Well (2004), Is the isotopic composition of nitrous oxide an indicator for 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.

Smith, M. S. (1982), Dissimilatory reduction of NO_2^- to NH_4^+ and N_2O by a soil Citrobacter sp., *Appl. Environ. Microbiol.*, *43*, 854–860.

Sutka, R. L., N. E. Ostrom, P. H. Ostrom, J. A. Breznak, and H. Gandhi (2003), Nitrogen isotopomer site preference of N_2O produced by Nitrosomonas europaea and Methylococcus capsulatus Bath, *Rapid Commun. Mass Spectrom.*, *17*, 738–745.

Sutka, R. L., N. E. Ostrom, P. H. Ostrom, J. A. Breznak, and H. Gandhi (2004a), Erratum: Nitrogen isotopomer site preference of N_2O produced by Nitrosomonas europaea and Methylococcus capsulatus Bath, *Rapid Commun. Mass Spectrom.*, *18*, 1–2.

Sutka, R. L., J. A. Breznak, N. E. Ostrom, P. H. Ostrom, and H. Gandhi (2004b), Using pure cultures to define the site preference of nitrous oxide produced by microbial nitrification and denitrification, *Eos Trans. AGU*, *85*(47), Fall Meet. Suppl., Abstract B21A-0861.

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. Exp. Microbiol.*, *72*, 638–644.

Tiedje, J. M., S. Simkins, and P. M. Groffman (1989), Perspectives on measurement of denitrification in the field including recommended protocols for acetylene based methods, *Plant Soil*, *115*, 261–284.

Tilsner, J., N. Wrage, J. Lauf, and G. Gebauer (2003), Emission of gaseous nitrogen oxides from an extensively managed grassland in NE Bavaria, Germany II. Stable isotope natural abundance of N_2O, *Biogeochemistry*, *67*, 249–267.

Toyoda, S., and N. Yoshida (1999), Determination of nitrogen isotopomers of nitrous oxide on a modified isotope ratio mass spectrometer, *Anal. Chem.*, *71*, 4711–4718.

Toyoda, S., N. Yoshida, T. Urabe, S. Aoki, T. Nakazawa, S. Sugawara, and H. Honda (2001), Fractionation of N_2O isotopomers in the stratosphere, *J. Geophys. Res.*, *106*, 7515–7522.

Toyoda, S., H. Mutobe, H. Yamagishi, N. Yoshida, and Y. Taniji (2005), Fractionation of N_2O isotopomers during production by denitrifier, *Soil Biol. Biochem.*, *37*, 1535–1545.

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.

Well, R., I. Kurganova, and H. Flessa (2006), Isotopomer signatures of soil-emitted N_2O under different moisture conditions—A microcosm study with arable loess soil, *Soil Biol. Biochem.*, *38*, 2923–2933.

Westley, M. B., B. N. Popp, and T. M. Rust (2007), The calibration of intramolecular nitrogen isotope distribution in nitrous oxide measured by isotope ratio mass spectrometry, *Rapid. Commun. Mass Spectrom.*, *21*, 391–405.

Wrage, N., J. Lauf, A. del Prado, M. Pinto, S. Pietrzak, S. Yamulki, O. Oenema, and G. Gebauer (2004a), Distinguishing sources of N_2O in European grasslands by stable isotope analysis, *Rapid Commun. Mass Spectrom.*, *18*, 1201–1207.

Wrage, N., G. L. Velthof, O. Oenema, and H. J. Laanbroek (2004b), Acetylene and oxygen as inhibitors of nitrous oxide production in Nitrosomonas europaea and Nitrosospira briensis: A cautionary tale, *FEMS Microbiol. Ecol.*, *47*, 13–18.

Wrage, N., G. L. Velthof, H. J. Laanbroek, and O. Oenema (2004c), Nitrous oxide production in grassland soils: Assessing the contribution of nitrifier denitrification, *Soil Biol. Biochem.*, *36*, 229–236.

Yamagishi, H., M. B. Westley, B. N. Popp, S. Toyoda, N. Yoshida, S. Watanabe, K. Koba, and Y. Yamanaka (2007), Role of nitrification and denitrification on the nitrous oxide cycle in the eastern tropical North Pacific and Gulf of California, *J. Geophys. Res.*, doi:10.1029/2006JG000227, in press.

Yamazaki, T., N. Yoshida, E. Wada, and S. Matsuo (1987), N_2O reduction by Azotobacter vinelandii with emphasis on kinetic nitrogen isotope effects, *Plant Cell Physiol.*, *28*, 263–271.

Yamulki, S., I. Wolf, R. Bol, B. Grant, R. Brumme, E. Veldkamp, and S. C. Jarvis (2000), Effects of dung and urine amendments on the isotopic composition of N_2O released from grasslands, *Rapid Commun. Mass Spectrom.*, *14*, 1356–1360.

Yamulki, S., S. Toyoda, N. Yoshida, E. Veldkamp, B. Grant, and R. Bol (2001), Diurnal fluxes and the isotopomer ratios of N_2O in a temperate grassland following urine amendment, *Rapid Commun. Mass Spectrom.*, *15*, 1263–1269.

Yoshida, N., and S. Toyoda (2000), Constraining the atmospheric N_2O budget from intramolecular site preference in N_2O isotopomers, *Nature*, *405*, 330–334.

Yoshida, N., A. Hattori, T. Saino, S. Matsuo, and E. Wada (1984), $^{15}N/^{14}N$ ratio of dissolved N_2O in the eastern tropical Pacific Ocean, *Nature*, *307*, 442–444.

Yoshinari, T., M. A. Altabet, S. W. A. Naqvi, L. Codispotti, A. Jayakumar, M. Kuhland, and A. Devol (1997), Nitrogen and oxygen isotopic composition of N_2O from suboxic waters of the eastern tropical North Pacific and the Arabian Sea—Measurement by continuous-flow isotope-ratio monitoring, *Mar. Chem.*, *56*, 253–264.

A. S. Grandy and G. P. Robertson, W. K. Kellogg Biological Station and Department of Crop and Soil Sciences, Hickory Corners, MI 49060, USA. (stuart.grandy@colorado.edu; robertson@kbs.msu.edu)

K. M. Huizinga, Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824, USA. (huizing9@msu.edu)

N. E. Ostrom, P. H. Ostrom, and A. Pitt, Department of Zoology and Biogeochemistry Program, Michigan State University, East Lansing, MI 48824, USA. (ostromn@pilot.msu.edu; ostrom@msu.edu; pittadam00@hotmail.com)

R. Sutka, GV Instruments, Crewe Road, Wythenshawe M23 9BE, UK. (robin.sutka@gvinstruments.co.uk)