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Isotopologue data reveal bacterial denitrification as the primary source of N_2O during a high flux event following cultivation of a native temperate grassland

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ABSTRACT

The source of N_2O in terrestrial ecosystems has long been debated. Both nitrification and denitrification produce N_2O but their relative importance remains uncertain. Here we apply site preference, SP (the difference in $\delta^{15}N$ between the central and outer N atom in N_2O), to estimate the relative importance of bacterial denitrification (including nitrifier denitrification) to total N_2O production from soil. We measured SP over a diurnal cycle following the third year of tillage of a previously uncultivated grassland soil at the Kellogg Biological Station (KBS) in southwestern Michigan. Fluxes of N_2O in our study ranged between 7.8 and 12.1 g N_2O–N ha^{-1} d^{-1} and were approximately 3 and 10 times greater than fluxes observed in managed agricultural and successional fields, respectively, at KBS. Consequently, our study captured a period of high flux resulting from the cultivation of a historically never-tilled soil. Concentration weighted SP values decreased from 12.9‰ in the morning to a minimum value of −0.1‰ in the afternoon.

Based on SP values reported for bacterial denitrification (−5 to 0‰; Toyoda et al., 2005; Sutka et al., 2006), hydroxylamine oxidation (nitrification) and fungal denitrification (33–37‰; Sutka et al., 2006) we found that production attributable to bacterial denitrification increased from between 52.9 and 60.9% in the morning to between 87.5 and 100% in the afternoon. Further, we observed diurnal variation in flux and SP that is consistent with increased production from bacterial denitrification associated with temperature-driven increases in respiration.

1. Introduction

Nitrous oxide is an important greenhouse gas and microbial activity promoted by agricultural practices is the primary cause of its increase during the industrial era (Forster et al., 2007). Effective mitigation requires a process-level understanding of N_2O sources; however, the microbial source of N_2O in most soils remains ambiguous (Mosier et al., 1998a,b, Robertson, 2004). Both nitrification and denitrification produce N_2O, and because these processes are under different environmental controls (Robertson and Groffman, 2006) they are likely responsive to different management strategies (Matson et al., 1989). Further, nitrifier denitrification and fungal denitrification are increasing being recognized as important production pathways in soils (Wrage et al., 2001; Crenshaw et al., 2008). Understanding the microbial source of N_2O in high flux soils is, thus, an important research objective.

Traditional approaches for apportioning N_2O production to nitrification and denitrification include acetylene inhibition and/or isotope labeling approaches that inherently alter microbial activity (Madsen, 1998; Groffman et al., 2006). The natural stable isotope abundance of N_2O also provides insight, however, $\delta^{15}N$ and $\delta^{18}O$ values vary with the isotopic composition of the substrates and in response to exchange with water (Kool et al., 2009a,b). Further, variation in the magnitude of fractionation during nitrification and denitrification can result in uncertain assignment of production to these processes (Ostrom et al., 2007; Well et al., 2008). Indeed, while tracer, inhibition, and natural abundance level isotope approaches have been widely used, the microbial origin of N_2O remains uncertain.

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Natural processes result in distinct abundances of ^{15}N within the central (α) and outer (β) N atoms that are commonly expressed as site preference (SP), the difference in $\delta^{15}N$ between the α and β atoms (Yoshida and Toyoda, 2000). A key advantage of SP in tracing origins of N_2O is that, in contrast to bulk isotope values, it is independent of the isotope values of the substrates of nitrification or denitrification (Toyoda et al., 2005; Sutka et al., 2006). Recently Sutka et al. (2006) found unique SP values for N_2O derived from hydroxylamine oxidation (nitrification) and N_2O produced via nitrite or nitrate reduction (denitrification and nitrifier denitrification) in bacterial cultures and suggested that SP could be use to apportion microbial sources of this gas. Production of N_2O via nitrite or nitrate reduction, whether by nitrifying or denitrifying bacteria, resulted in an average SP value of 0‰; compared to 33‰ for N_2O produced from hydroxylamine oxidation (Sutka et al., 2006). Toyoda et al. (2005) similarly report an SP value for N_2O produced from one species of denitrifying bacteria of −5.1‰. A second species produced N_2O with an SP of 23.3‰; however, these authors indicate that this culture occurred with a low growth rate and may have been influenced by inorganic production. Sutka et al. (2008) further demonstrated that fungal denitrification yields N_2O with an SP of 37‰, which is not distinct from that associated with nitrification but clearly distinct from that produced during bacterial denitrification. Collectively pure culture studies provide compelling evidence that an SP of 0‰ or less is indicative of N_2O produced by denitrifying bacteria whereas values of 33–37‰ indicates production from hydroxylamine oxidation or fungal denitrification.

A limitation of pure culture studies to evaluate N_2O production pathways is that the behavior of a few organisms under artificial conditions may not reflect the behavior of a diverse community in the natural soil environment. For this reason, several investigators have conducted soil incubations to evaluate the SP signature of N_2O produced by nitrification and denitrification using acetylene inhibition or ^{15}N tracer studies in both tropical and temperate soils (Perez et al., 2006; Well et al., 2006, 2008; Well and Flessa, 2009). These approaches, however, may also impose artificial conditions and results may not be directly comparable to those obtained in pure culture. The use of acetylene to define SP associated with specific production pathways can be problematic owing to the potential for (1) incomplete diffusion of acetylene into the soil and incomplete inhibition of N_2O reduction, (2) alteration of microbial activity, (3) production of N_2O via NO reduction in the presence of trace levels of O_2, and (4) production of N_2O from novel pathways such as fungal denitrification (Madsen, 1998; Groffman et al., 2006; Wrage et al., 2004, 2005; Crenshaw et al., 2008). As a result, there is a strong desire to avoid the use of acetylene and other inhibitors in N cycling studies (Wrage et al., 2004, 2005; Groffman et al., 2006). Acetylene at 10 Pa is commonly applied in soil incubation to arrest nitrification activity but as acetylene blocks ammonium monooxygenase activity both pathways of N_2O production from nitrification, hydroxylamine oxidation and nitrifier denitrification, are inhibited (Hynes and Knowles, 1982). In contrast, the use of SP to evaluate nitrification specifically measures production via the hydroxylamine pathway.

Soil incubations directed towards defining the SP values associated with nitrification and denitrification have found values distinct from those obtained in pure culture. Perez et al. (2006) conducted parallel incubations with acetylene to block N_2O production from nitrification and without acetylene in which production from both nitrification and denitrification occurred. The former incubations yielded SP values associated with denitrification only and, thus, provides a basis to calculate the SP associated with nitrification from the latter incubation. SP values associated with nitrification and denitrification, respectively, were found to be 4.2 ± 8.4 and 31.6 ± 8.1‰ that are reversed relative to those obtained in pure culture (Sutka et al., 2006). The value of 4.2‰ is consistent with the value of 0‰ for nitrifier denitrification obtained in pure culture. The value of 31.6‰ could readily be explained by production from fungal denitrification (Sutka et al., 2008). Further the values reported by Perez et al. (2006) need to be adjusted owing to recent calibration efforts (Westley et al., 2007). Well et al. (2008) recently reported a range of 18.3–36.3‰ for N_2O produced by nitrification in soils. The higher values are in agreement with the SP values obtained via hydroxylamine oxidation in pure culture (Sutka et al., 2006). The lower values are consistent with partial production from nitrifier denitrification which has been shown to have an SP in pure culture near 0‰ (Sutka et al., 2006). Thus, the variable SP signals attributed to nitrification are quite readily explained by a mixture of N_2O derived from both nitrification pathways; hydroxylamine oxidation and nitrifier denitrification. Two incubation studies using temperate soils yielded variable SP values for N_2O production from denitrification of 3.1–8.9‰ (Well and Flessa, 2009) and 2.3–16‰ (Well et al., 2006). The lower values are similar to those obtained in pure culture (−5 to 0‰; Toyoda et al., 2005; Sutka et al., 2006). The higher values could reflect production from fungal denitrification (Sutka et al., 2008) or, when acetylene was not used, an increase in SP due to reduction of N_2O (Ostrom et al., 2007; Jinuntuya-Nortman et al., 2008). These studies indicate that a significant challenge to soil incubation studies is to isolate specific production pathways in a complex soil community and the results obtained thus far are not inconsistent with SP endmember values obtained in pure culture.

The ultimate control on the SP of N_2O, however, may not be the bacterial species or pathways but the enzymes involved in its production, in particular, nitrite reductase (NIR) and nitric oxide reductase (NOR) (Stein and Yung, 2003; Schmidt et al., 2004). Sutka et al. (2006) cultured two species of *Pseudomonas* sp. Each species possessed one of the two known types of dissimilatory NIR. A similar SP was observed in N_2O produced by both species (0‰) even though the type of NIR enzyme involved differed. This result provides support for the suggestion that during bacterial denitrification NOR is more important in controlling SP than NIR (Stein and Yung, 2003). The three known classes of NOR enzymes are P-450, cNOR and qNOR. P-450 is found in denitrifying fungi, cNOR is associated with bacterial denitrification, and qNOR is associated with detoxification of NO in pathogenic bacteria (Hendriks et al., 2000). Highly contrasting SP values have been demonstrated for denitrifying fungi (37‰) and bacteria (0‰) consistent with a control of NOR on SP (Sutka et al., 2006, 2008). As the NIR and NOR enzymes in denitrifiers and nitrifiers are structurally and genetically related (Casciotti and Ward, 2001, 2005; Garbeva et al., 2007) it is not surprising that the SP for N_2O from bacterial denitrification and nitrifier denitrification are found to be similar. Bacteria using qNOR have not been cultured to evaluate SP. As this pathway is primarily used in detoxification of environmental NO it likely constitutes a small portion of N_2O production in soils (Hendriks et al., 2000). Thus the SP associated with bacterial denitrification (including nitrifier denitrification) via cNOR (−5 to 0‰) is distinct from other pathways (nitrification via hydroxylamine oxidation and fungal denitrification, 33–37‰) and has the potential, therefore, to act as a tracer of its production. In this study, we apply SP to identify the microbial origins of N_2O production during a period of unusually high flux resulting from the cultivation of a historically never-tilled temperate grassland.

2. Methods

2.1. Site description

Our field experiments were conducted at the W.K. Kellogg Biological Station Long Term Ecological 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 (Grandy and Robertson, 2006a; Grandy et al., 2006).

The main experimental design of the KBS LTER site consists of a series of replicated ecosystems arranged along a management intensity gradient (http://lter.kbs.msu.edu). Adjacent to the main site is a never-previously-cultivated field that was cleared of trees in 1956 and has since been maintained as a mid-successional grassland ecosystem by mowing once every fall. In June 2002, two tillage treatments (plowed and no-till control), each replicated four times, were assigned to 3 by 6 m plots in this field (Grandy and Robertson, 2006a,b). The four tilled plots in the cultivation experiment were moldboard plowed and disced once each spring between 2002 and 2004.

2.2. Flux chambers

We measured fluxes and isotopologue values of N_2O accumulating within a flux chamber placed in one of the cultivation experiment treatments, 15 days (DOY 187) after tillage in 2004. A stainless steel flux chamber (60d × 30w × 17h cm) was placed 5 cm deep and covered with an opaque Plexiglass lid (Ambus and Robertson, 1998). Following standard procedures, the chamber was vented to the atmosphere by a coiled piece of stainless steel tubing (0.5 m × 0.3 cm OD) to maintain atmospheric pressure while minimizing diffusive exchange (Livingston and Hutchinson, 1995). Samples were collected from the chamber through 0.1 cm ID × 20 cm Peek tubing to which a syringe needle was connected. A volume approximately equivalent to that of the sample tube was removed prior to sampling so as to replace the tube dead volume with chamber air. Samples were collected upon penetration of the butyl rubber septum (Geomicrobiology Technologies) of pre-evacuated 250 or 60 mL bottles. Concentration and isotopic integrity with this sampling procedure and storage for up to one month were demonstrated by multiple analyses of N_2O in air. Gas samples for flux and isotopologue measurements were taken during four chamber closing that occurred at ca. 4 h intervals beginning at approximately 8:30 AM and ending at 8:30 PM. During each chamber closing, samples were collected every 10 min for 1 h. Thus, N_2O flux and isotopologue compositions were obtained for a total of 4 time periods with 6 measurements within each period.

2.3. Analysis of isotope and isotopomer abundances

We used fragment-ion compound specific isotope analysis (FICISA) to determine the atom-specific, or isotopomer, composition of N in N_2O emitted from soils. This method takes advantage of the partial fragmentation of N_2O to NO within the ion source of the mass spectrometer (Röckmann et al., 2003; Sutka et al., 2003). Gas samples were analyzed on a multi-collector 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 cryogenic trapping followed by chromatographic separation on a Poraplot Q gas chromatographic column with helium 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 and precision was typically 3% (1SD). The multi-collector mass spectrometer is able to simultaneously monitor 5 masses of interest for N_2O isotopomers; 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 Röckman, 2000).

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 Röckman, 2000; Sutka et al., 2003). 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 Röckman, 2000). Final $\delta^{15}N$, $\delta^{18}O$, $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ values were calculated using the approach outlined in 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. The $\delta^{15}N$, $\delta^{15}N^{\alpha}$ and $\delta^{18}O$ of our laboratory N_2O standard is 1.6, 14.9 and 41.7‰, respectively.

We used a mixing model to determine the isotopic composition of N_2O emitted from the soil, based on measured concentrations and isotopic composition of N_2O in the flux chambers. The difference between the concentration measured in a chamber (C_{meas}) and that of the troposphere ($C_A = 319$ ppbv) (Forster et al., 2007) defines the concentration due to emission from the soil (C_S). Similarly, measured isotope values of N_2O (δ_{meas}) are a mixture of soil-derived (δ_S) and atmospheric (δ_A) sources, where the $\delta^{15}N$, $\delta^{18}O$ and SP values of N_2O in air are 7.0 ± 0.6, 43.7 ± 0.9 and 18.7 ± 2.2‰, respectively (Yoshida and Toyoda, 2000). The measured isotopic composition of N_2O in the chamber at any point in time can thus be described as:

$$\delta_{meas} = (\delta_A C_A + \delta_S C_S)/C_{meas} \quad (1)$$

Equation (1) can be rearranged and solved for the δ_S using concentration and isotope measures from each time point. However, because the abundance of soil-derived N_2O increases with time during a chamber closing, we achieve greater statistical confidence (see below) by calculating concentration weighted (CW) isotope values for each isotopomer over the course of the flux measurement as:

$$\delta_{CW} = \sum_{i=1}^{n}(C_i * \delta_i)/\sum_{i=1}^{n} C_i \quad (2)$$

where C_i and δ_i are the concentration and isotopologue values, respectively, measured at each time point within a chamber closing. The solution for δ_{CW} using Equation (2) is a single value based on all the data collected within a chamber closing and implicitly gives more weight to those values at the end of the flux measurement when the accumulation of soil-derived N_2O was greater.

2.4. Propagation of error in determining δ_{CW}

Analytical precision sets limits on the statistical confidence for how tightly our observations relate to the true isotopic composition

of soil-derived N_2O (i.e., δ_{CW}). To determine the magnitude of statistical confidence, we performed a series of Monte-Carlo simulations. Our simulations assumed a "true" N_2O flux rate of 586 ppbv/h (equivalent to the mean of our observed rates) and $\delta^{15}N$, $\delta^{18}O$, and SP values for each flux measurement from the results of Equation (2). We also incorporate within the Monte-Carlo simulation error reported for the concentration of tropospheric N_2O (3 ppbv; typical of sites within the NOAA Earth System Research Laboratory Flask Sampling Network: http://www.esrl.noaa.gov/gmd/ccgg/iadv/), and error surrounding the isotopologue values of tropospheric N_2O as reported by Yoshida and Toyoda (2000). Given these true flux rates and isotopic compositions, we generated a series of expected concentrations and isotope compositions for each of the six time points that we sample during each flux measurement using Equation (1). We then simulated chamber flux measures by adding analytical noise to the concentration and isotope values for each of the six time points. The amount of noise was determined by drawing a random value from a normal distribution of mean equal to zero and standard deviation equal to the analytical precision. The analytical precision (1SD) based on replication of field samples for N_2O concentration and $\delta^{15}N$, $\delta^{18}O$, and SP values is 16 ppbv, 0.5, 0.7, and 1.3‰, respectively. In this way, we generated 500 simulated chamber flux measures, and analyzed these simulated chamber measures for N_2O flux rates and for δ_{CW} following Equation (2). The simulated flux and δ_{CW} values fall into a normal distribution. We report our confidence as the standard deviation of this distribution and for $\delta^{15}N$, $\delta^{18}O$ and SP the uncertainty ranges between 0.7 and 0.8, 0.9 and 1.0, and 2.3 and 2.4‰, respectively. Variation in isotopologue values at field scales can be greater; for example, Opdyke et al. (2009) recently found variation in SP between replicate field plots to range between 1 and 7‰. The Monte-Carlo estimation of errors associated with the weighted average approach avoids bias introduced in a Keeling plot model by avoiding the assumptions (1) that the independent variable ($1/[N_2O]$) has no error associated with it and (2) that errors in the dependent variable are unrelated to those associated with the independent variable (Pataki et al., 2003).

3. Results and discussion

Marked increases in fluxes of N_2O have previously been observed upon cultivation of never-tilled grassland soils (e.g. Pinot et al., 2004; Grandy and Robertson, 2006a). Our study was conducted on one of the grassland plots studied by Grandy and Robertson (2006a) in the last year of their three year study in which they observed fluxes of N_2O that were 3–8 times greater than in control plots that remained uncultivated. From the increase in N_2O concentration with time in each chamber closing (Fig. 1) the flux of to the atmosphere is calculated and ranged between 2.33 and 3.60 μmol m^{-2} h^{-1} (7.8–12.1 g N_2O–N ha^{-1} d^{-1}) (Fig. 2). These rates are substantially greater than average emission rates from unmanaged successional (1.1 g N_2O–N ha^{-1} d^{-1}) and managed (3.2–3.7 g N_2O–N ha^{-1} d^{-1}) ecosystems at the KBS LTER (Robertson et al., 2000; Grandy et al., 2006) and occurred during a short period of exceptionally high flux (see Fig. 1 in Grandy and Robertson, 2006a). Consequently, our study captured a period of high flux resulting from the cultivation of a historically never-tilled soil.

Fluxes of N_2O were lowest in the morning, reached a maximum value in late afternoon and declined towards evening (Fig. 2). Changes in flux were not related to soil moisture as water filled pore space (WFPS) measurements collected in the surrounding soil at each closing remained constant at ca. 48% (20 g/g^{-1}) but the flux varied with soil temperatures (5 cm) that rose from 14 °C to 32 °C by the third measurement, and declined to 23 °C by the 4th. Similar observations of temperature-driven diurnal variation in N_2O fluxes have been observed previously (Christensen, 1983; Baggs et al., 2002; Flechard et al., 2005) and related to an increased respiratory demand for O_2 at higher temperatures that produces anoxic conditions favorable for denitrification (Baggs et al., 2002). Thus, our observation of diurnal variation in N_2O flux and temperature suggests that denitrification is the source of N_2O. Nitrification activity was nonetheless substantial in the cultivated plots as soil nitrate increased from <1 to as much as 12 μg g^{-1} and nitrifier enzyme activity was as much as 5-fold higher than in the control plots (Grandy and Robertson, 2006a). Thus, evidence suggests that

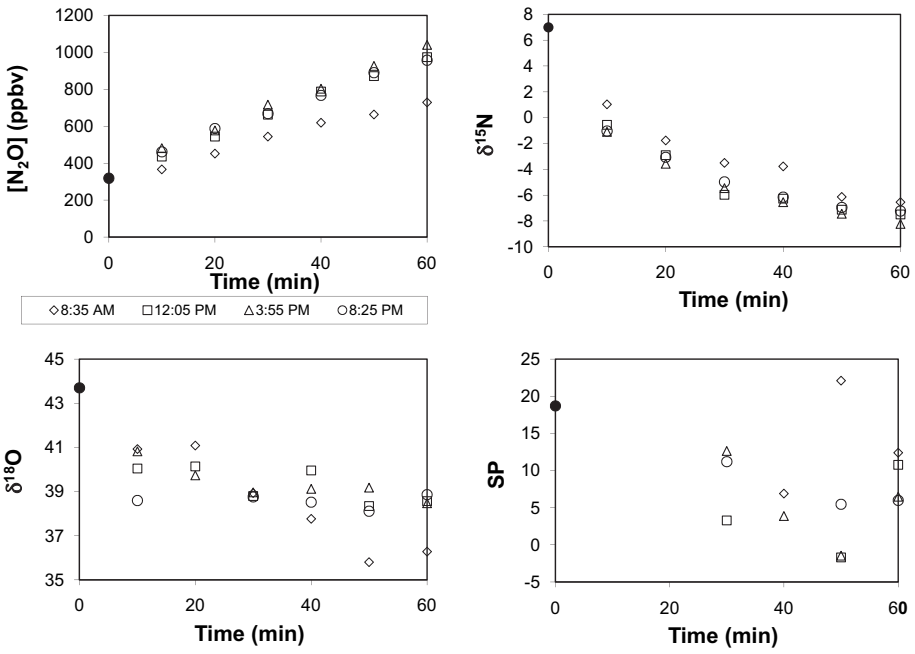

Fig. 1. Changes in the concentration, $\delta^{15}N$, $\delta^{18}O$ and SP of N_2O within the headspace of each flux chamber during each of four, 1 h chamber closings. Flux chambers were closed at 8:35 AM (T1), 12:05 PM (T2), 3:55 PM (T3) and 8:25 PM (T4) (eastern standard time) on DOY 187, 2004. Solid circles at time 0 indicate previously reported values for the concentration of atmospheric N_2O (Forster et al., 2007) and the $\delta^{15}N$, $\delta^{18}O$ and SP of N_2O in the atmosphere (Yoshida and Toyoda, 2000).

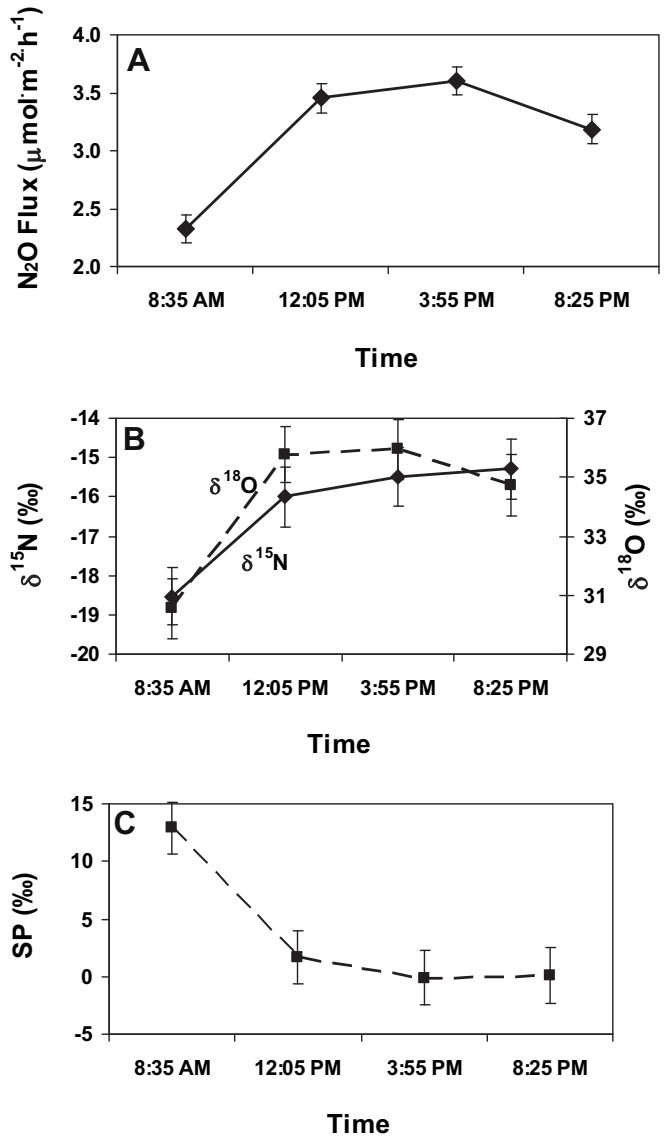

Fig. 2. Flux of N_2O and calculated concentration weighted isotopologue values for soil-derived N_2O from a recently tilled native soil at the KBS LTER on DOY 187, 2004. Soil-derived isotope values are determined by a mass balance mixing equation (Equation (1)) (A) Fluxes of N_2O. (B) Concentration weighted $\delta^{15}N$, $\delta^{18}O$ and (C) SP values for each chamber collection (Equation (2)). Error bars represent 2 SD based on a Monte-Carlo error propagation model.

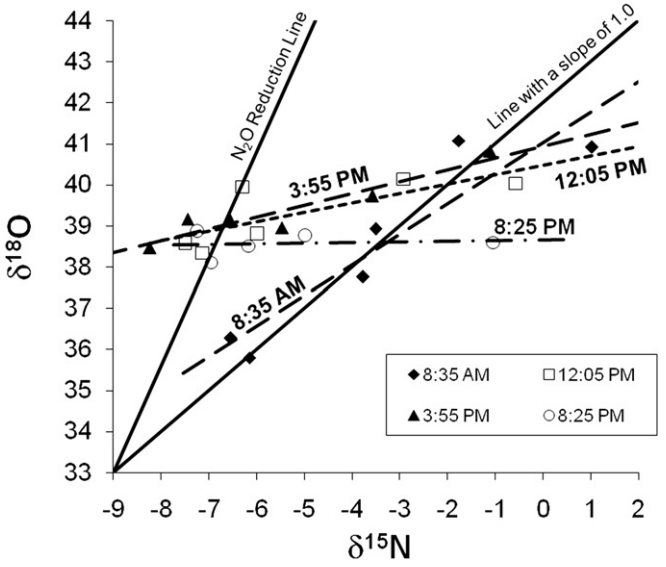

Fig. 3. Values for the $\delta^{18}O$ and $\delta^{15}N$ of N_2O within each of the 4 chamber closings that result from mixing of soil-derived and atmospheric N_2O. The regression lines are compared to lines with a slope of 2.6, expected if N_2O reduction was the only process affecting isotopologue values, and a line with a slope of 1 (the intercepts are chosen as the intersection of the *x*- and *y*-axes for illustration). A shift in SP due to reduction of N_2O is considered unlikely when the slope is less than 1.

both denitrification and nitrification are enhanced by cultivation but it remains uncertain which process is more important in N_2O production. To resolve this uncertainty we applied SP as a means to identify the origins of N_2O.

Within each chamber closing the isotopologue values shift from those of tropospheric N_2O (time zero) towards those of soil-derived N_2O as a function of time. The magnitude of this shift is a function of (1) strength of the N_2O flux, (2) duration of chamber closing, and (3) the difference in isotopologue composition of atmospheric and soil-derived N_2O. The mixing relationship described by Equation (1) dictates that the isotopologue values will change with concentration (and time) in a curvilinear manner such that isotope shifts will be greatest in the early time intervals of sampling. A curvilinear trend with time is most evident for $\delta^{15}N$ but not clearly evident for $\delta^{18}O$ (Fig. 1) which reflects that fact that the $\delta^{18}O$ of soil-derived and atmospheric N_2O in our study differed only slightly. A curvilinear relationship in SP is also not clearly evident in SP which (1) reflects the greater analytical uncertainty associated with SP relative to bulk isotope values and (2) the fact that SP in the early time intervals (10 and 20 min) within each closing was not measured due to insufficient gas for accurate analysis of $\delta^{15}N^{\alpha}$. It is important to note that the isotopologue values observed at each time point in Fig. 1 are not replicates and would not be unless the flux and soil-derived isotopologue values within each chamber closing were identical (the data shown in Fig. 2 demonstrates this is not the case). An anomalous value for SP (and also for $\delta^{15}N$) is evident at 50 min for the first chamber closing (8:35 AM); however, we have no *a priori* reason for eliminating this data point. The changes in isotopologue values as a function of concentration are used in Equation (2) to calculate the concentration weighted isotope values for each chamber closing and shown in Fig. 2. In this way, a single isotopologue value is obtained from each chamber closing and the error reported is derived from the Monte-Carlo model.

The low and unique SP value associated with production of N_2O from bacterial denitrification (−5 to 0‰; Toyoda et al., 2005; Sutka et al., 2006) clearly distinguishes this process and provides a basis to apply an isotope mixing model (e.g. Well et al., 2006) to determine the proportion of N_2O derived from this source:

$$F_{BD} = (SP_{meas} - SP_{OS}F_{OS})/SP_{BD} \quad (3)$$

Where F refers to the fraction of N_2O from the bacterial denitrification (BD) relative to other sources (OS) and the sum of F_{BD} and F_{OS} equals 1. The percentage of N_2O derived from bacterial denitrification is obtained by multiplying F_{BD} by 100. Here, we assume a endmember SP value of 0‰ to indicate production from bacterial denitrification and a value of 33‰ to the define the collective endmember value for production from nitrification and fungal denitrification. Because fungal denitrification produces N_2O with higher SP values, the choice of 33‰ for this endmember results in a small underestimate of production from bacterial denitrification in the mixing model. We find that N_2O derived from denitrification changed initially from 60.9% to approximately 100% for all subsequent measures. If a value of −5‰ is used instead of 0‰ in the mixing model for the SP of N_2O resulting from bacterial denitrification then the contribution of N_2O from this source ranges

between 52.9 and 87.1%. These results indicate the N_2O production in our study is primarily derived from bacterial denitrification. SP and the proportion of N_2O from bacterial denitrification, however, can be altered by diffusion and reduction of N_2O. Fractionation during diffusion through soil can affect SP; however, the maximum expected shift due to a diffusion effect, 1.6‰, is relatively minor (Well and Flessa, 2008). Reduction of N_2O has been shown to increase SP values and would act to shift the results of the mixing model in favor of the non-bacterial denitrification sources. The shift in SP due to N_2O reduction can be evaluated based on the relationship between $\delta^{18}O$ and $\delta^{15}N$ (Ostrom et al., 2007; Jinuntuya-Nortman et al., 2008) and, if need be, quantified (Opdyke et al., 2009). This approach is based on isotopologue shifts that result from the reduction of N_2O external to the cell (from the surrounding soil environment or introduced to the soil from the atmosphere) and, thus, is independent of the N_2O to N_2 production ratio (e.g. Bergsma et al., 2002).

A linear relationship between $\delta^{18}O$ and $\delta^{15}N$ characterized by a slope of 2.6 is indicative of N_2O reduction (Ostrom et al., 2007; Jinuntuya-Nortman et al., 2008). This relationship is quite distinct from that produced upon mixing of atmospheric and soil-derived N_2O which commonly yields a slope of less than 1.0 (Ostrom et al., 2007). On this basis, Ostrom et al. (2007) proposed that a slope greater than one for the line describing the relationship between $\delta^{18}O$ and $\delta^{15}N$ is a criterion to identify the influence of N_2O reduction on SP and that from this relationship an estimate of the magnitude of the shift in SP due to reduction can be obtained (Opdyke et al., 2009). Within each of our chamber closings the slope of the line relating $\delta^{18}O$ and $\delta^{15}N$ varied between 0.02 and 0.74 and, thus, did not indicate a shift in SP due to reduction of N_2O (Fig. 3). If reduction had occurred, SP would have increased and, in effect, reduced our estimates of N_2O derived from denitrification on the basis of Equation (3). Consequently, our values for the proportion of N_2O derived from denitrification are conservative.

If the isotopic composition of the substrates for N (nitrate or ammonium) or O (O_2 or water) varies over the time course of sampling then an assessment of the importance of N_2O reduction on SP values would be invalid. Variation in the isotopic composition of the substrates, however, is unlikely within the time frame of a chamber closing (1 h) and would cause a deviation from linearity in the regression data. Within 3 out of 4 closings, a linear relationship was observed ($R^2 = 0.086$ and $P = 0.008$, $R^2 = 0.76$ and $P = 0.08$, $R^2 = 0.88$ and $P = 0.005$ for T1, T2, and T3, respectively). In the remaining closing, the slope of the line was zero. Thus, there was no indication that the isotopic composition of the substrates varied within the sampling intervals. Further, there is no evidence indicating that SP was altered by N_2O reduction and, thus, there was no need to correct our estimates of the proportion of N_2O derived from bacterial denitrification due to this process.

Soil water content has been recognized as an important control on N_2O flux and maximum fluxes are generally associated with WFPS values of 60–80% (Dobbie et al., 1999; Bateman and Baggs, 2005). The predominantly anaerobic conditions that prevail within this range of water filled pore space also favor production of N_2O from denitrification (Davidson, 1991; Bateman and Baggs, 2005). Production from nitrification prevails when WFPS is between 35 and 60% and production from denitrification prevails at lower WFPS but is characterized by considerably lower flux (Bateman and Baggs, 2005). Consequently, our observation of SP values which are consistent with a predominant origin from bacterial denitrification at a WFPS of 48% is in contrast to previous studies. We believe that the soil conditions resulting from tillage of a native soil, such as high rates of mineralization, nutrient supply, and enzyme activity, create optimal conditions for N_2O flux at moderate WFPS values (Grandy and Robertson, 2006a,b). This is most likely the result of high rates of microbial respiration that produce anaerobic conditions at levels of WFPS that normally favor nitrification. Our observation of low SP values that indicate N_2O predominantly derived from bacterial denitrification are, therefore, consistent with enhanced anaerobic conditions in response to tillage of this previously never-tilled soil.

The endmember approach we provide (Equation (3)) provides insight into sources of N_2O production in previous studies. In a study of marsh soils Bol et al. (2004) found SP values for flooded and non-flooded soils of 30.8–41.8‰ and 14.8–16.3‰, respectively. The SP values in the non-flooded soils are consistent with approximately half of the total N_2O production being derived from bacterial denitrification. Production from nitrification would not be expected under the flooded and likely anoxic conditions; therefore, the high SP values could be indicative of production from fungal denitrification or could be the result of N_2O reduction. In a study of temperate grassland soils, Bol et al. (2003) reported SP values ranging between 1.3 and 13.8‰ that are indicative of a predominance of production from bacterial denitrification. Similarly in temperate grassland amended with urine SP values of approximately −3 to 9‰ are consistent with a bacterial denitrification source (Yamulki et al., 2001). Recently in agricultural and successional fields at the KBS LTER Opdyke et al. (2009) found average SP values between 2.6 and 14.6‰ that indicate 61 and 92% of production was from bacterial denitrification. Overall SP values in many previous studies identify bacterial denitrification as an important source of N_2O.

4. Summary

Our study captured a brief period of high N_2O flux resulting from the cultivation of a historically never-tilled soil. We observed diurnal variation in N_2O flux that has previously been attributed to production from denitrification resulting from an increase in respiration and concomitant reduction in soil O_2 concentrations (Baggs et al., 2002). On the basis of SP, we found that the range in N_2O derived from bacterial denitrification increased from between 52.9 and 60.9% to between 87.5 and 100% between the morning and afternoon. Production from bacterial denitrification occurred at a WFPS of 48% which would not normally be considered conducive to this process. The production of N_2O from bacterial denitrification at this WFPS, was likely due to lowered soil O_2 levels in response to enhanced mineralization and respiration that results from the tillage of a native soil (Grandy and Robertson, 2006a).

Quantitative N_2O source apportionment is challenged by the need to better constrain endmember SP values. Moreover, other and as yet untested pathways of N_2O production provide additional uncertainty. Nonetheless, at present, production of N_2O via the cNOR pathway of bacterial denitrification stands alone with a low and unique SP value. Despite increases in nitrifier enzyme activity noted by Grandy and Robertson (2006a), the low SP values in our study provide compelling evidence that bacterial denitrification (specifically via cNOR), was the primary source of N_2O upon cultivation of a historically never-tilled soil.

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