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THE RELATIONSHIP BETWEEN DENITRIFICATION AND NITROUS OXIDE FLUX FROM SOIL

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Timothy Todd Bergsma

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Major professor

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THE RELATIONSHIP BETWEEN DENITRIFICATION AND NITROUS OXIDE FLUX FROM SOIL

By

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ABSTRACT

THE RELATIONSHIP BETWEEN DENITRIFICATION AND NITROUS OXIDE FLUX FROM SOIL

By

Timothy Todd Bergsma

Denitrification in soil is the major source of atmospheric nitrous oxide (N_2O), a potent contributor to global warming and a regulator of stratospheric ozone. Flux of N_2O to the atmosphere is poorly understood. There is a serious imbalance in the global N_2O budget (missing sources), and N_2O flux at the field scale is difficult to predict, even when the rate of denitrification has been adequately characterized. In this dissertation, I review the literature pertaining to the relationship between denitrification and N_2O flux, emphasizing a major source of uncertainty: relative production of N_2O and N_2 (dinitrogen) during denitrification. I explore the ecological factors that influence the nitrous oxide mole fraction (N_2O / [N_2O + N_2]), an aspect of denitrification that expresses relative production of N_2O and N_2. Second, I develop theory that facilitates the evaluation of N_2O and N_2 flux using ^{15}N-labeled compounds and mass spectrometry. My heuristic model of labeled N-gas flux from soil simplifies the process of drawing inferences from isotope data. Third, I report new procedures for measuring fluxes of labeled N_2O and N_2 for the same incubation using mass spectrometry. My procedures could lead to improved estimates of

the nitrous oxide mole fraction. I illustrate these procedures with field data. Finally, I describe a laboratory experiment in which I combine traditional and isotope methods to test for effects of moisture history (antecedent soil moisture) and ecosystem management history on nitrous oxide mole fraction, while controlling soil type and moisture. I find that response of mole fraction to differences in short-term (48 h) soil moisture history is different for soils from ecosystems with different management histories. A cropped soil had a high (~0.9) mole fraction after rapid transition from air-dry to 85% water-filled pore space but a low (~0.3) mole fraction when 80% of added moisture was applied 48 h in advance of the incubation. However, soil from a successional system generated a nitrous oxide mole fraction of about 0.3 regardless of short term moisture history. Progress in understanding the relationship between denitrification and nitrous oxide flux from soil seems still to be methods-limited.

For Qiaobing. *Wo ai ni!*

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INTRODUCTION

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The structure of this dissertation reflects the objectives listed above. Chapter 1 reviews current knowledge regarding factors that influence N_2O mole fraction directly and indirectly. Chapter 2 is reprinted from a publication in which we present theory and equations that facilitate the interpretation of ^{15}N data for N-gas fluxes from soil. Chapter 3 explains our development of methods for measuring N-gas flux and the N_2O mole ratio. We combine chamber methods and mass spectrometry in a way that allows very sensitive measurement of N_2 and N_2O flux from ^{15}N labeled soil. The goals were to improve on the statistical uncertainty of traditional methods that require

separate chambers for N_2 and N_2O measurements and to mitigate the inherent bias of ^{15}N methods by using identical methods for N2 and N_2O when estimating ratios. Chapter 4 deploys both traditional and isotope methods to examine an empirical question: what are the effects of short term moisture history and ecosystem management history on N_2O mole fraction? Though many questions remain, I am convinced that we have made significant progress.

Chapter 1

FACTORS INFLUENCING THE RELATIONSHIP BETWEEN DENITRIFICATION AND NITROUS OXIDE FLUX FROM SOIL.

Background

Human domination of ecosystems and biogeochemical cycles has become widely recognized (Vitousek *et al.* 1997). Anthropogenic changes in ecosystems can generate changes in the Earth's atmosphere, which in turn can have widespread consequences. Nitrous oxide is one of several atmospheric trace gases that have attracted the attention of the scientific community. It is produced in soils by microbial denitrification (Robertson 1999), nitrification (Firestone and Davidson 1989) and other biological processes (Robertson and Tiedje 1987). The concentration of N_2O in the Earth's atmosphere is currently about 312 ppb_v (= $312 \cdot 10^{-9} L \cdot L^{-1}$) and is increasing at a rate of 0.5 ppb per year (IPCC 1996). Pre-industrial levels were < 290 ppb, suggesting that current levels reflect a significant anthropogenic influence. Nitrous oxide has a long (~120 y) half-life in the stratosphere. It indirectly catalyzes the destruction of stratospheric ozone, increasing the hazard of UV exposure at the Earth's surface (Hahn and Crutzen 1982). Nitrous oxide is also a radiatively active gas; about 5% of the observed radiative forcing of climate change over the last 100 years can be attributed to N_2O (Bouwman 1990).

International concern over rising levels of atmospheric nitrous oxide has stimulated research regarding its global sources and sinks. An estimated 14 Tg N_2O-N are consumed or stored in the atmosphere annually, while only about 7.9 Tg of sources have been identified at the Earth's surface (Davidson 1991). A large part of the imbalance in the nitrous oxide budget may be due to uncertainty in flux estimates, especially for tropical regions of the world. In particular, contributions from agriculture may be underestimated (Mosier *et al.* 1998, Kroeze *et al.* 1999).

Most of the increase in nitrous oxide flux to the atmosphere may be related to land use change, especially conversion to agriculture. Deforestation produces pulses of nitrous oxide, whether by clear-cutting (Robertson and Tiedje 1988) or burning (Davidson 1991). Nearly 100 Tg of anthropogenic fertilizer N is applied to crops globally each year (Eichner 1994). Most of the anthropogenic N applied to watersheds in the North Atlantic drainage basin is eventually denitrified (Howarth *et al.* 1996). If even a small fraction of fertilizer N results in N_2O flux, contribution to the global budget could be significant. Eichner (1994) estimates that as much as 2.1 Tg N_2O-N $\cdot$ y^{-1} may derive from fertilizer; similarly Matthews (1994) estimates the fertilizer source at 2.0 Tg N_2O-N $\cdot$ y^{-1}. Mosier *et al.* (1998) place emission of N_2O-N from agricultural soils for 1989 at 2.1 Tg.

Challenges for research

Although denitrification is well understood, the relationship between denitrification and N_2O flux from soil is not simple. First, other processes,

especially nitrification, produce N_2O in soil (Firestone and Davidson 1989). While nitrification dominates in well-aerated soils and denitrification dominates under anaerobic conditions, both can contribute simultaneously to N_2O flux in soils of intermediate aeration (e.g. Stevens *et al.* 1997, Panek *et al.* 2000), or even low aeration (Wolf and Russow 2000). The relationship between soil moisture and relative importance of nitrification and denitrification for N_2O production is uncertain (Davidson 1991). Over large temporal and spatial scales, flux of N_2O from nitrification is smaller and more predictable, while denitrification accounts for the bulk of N_2O flux but is highly variable temporally and spatially.

Second, denitrification has another major product: dinitrogen. Dinitrogen, unlike nitrous oxide, is formed only by denitrification, and represents closure of the nitrogen cycle. The relative proportions of N_2O and N_2 produced during denitrification vary widely. Field studies have shown the inadequacy of using a simple conversion factor to estimate N_2O flux from denitrification (Vinther 1984) or denitrification from N_2O flux (Weier *et al.* 1993). The N_2O mole fraction during denitrification (i.e., $N_2O/[N_2O + N_2]$) can vary from zero to one (e.g. Rolston *et al.* 1982), is influenced by a suite of physical and chemical factors (Firestone and Davidson 1989), and is unstable over time (Letey *et al.* 1980a).

Third, N_2O flux during denitrification in soil is partly uncoupled from N_2O production *per se* by factors which regulate the transfer of N gases from soil solution to the atmosphere. N_2O may be released from soil hours or days after

it is formed, while N_2 produced at the same time may diffuse away from the source more quickly due to lower solubility in soil water (Letey *et al.* 1980b). Other factors, such as freeze/thaw cycles and sudden changes in barometric pressure, may also modulate N_2O flux independently of N_2O production.

Fourth, measurement of denitrification is difficult. Chamber methods and gas chromatography suffice for estimates of N_2O flux. However, changes in headspace concentration of N_2 are nearly impossible to detect against the high atmospheric background. Two methods of N_2 measurement have been recommended: acetylene inhibition and ^{15}N dilution (Mosier and Klemedtsson 1994). Both have disadvantages, which vary in importance depending on the specifics of implementation.

This dissertation focuses on the second and fourth of the challenges listed above: factors influencing N_2O mole fraction and methods for measurement of denitrification. N_2O mole fraction is briefly reviewed below. The two chapters following explore theory and methods, respectively, for measuring denitrification products. A final chapter advances understanding of the relationship between denitrification and N_2O flux by testing for ecosystem differences and effects of soil moisture history on N_2O mole fraction.

Factors influencing N_2O mole fraction during denitrification

Theory

Denitrification is an anaerobic microbial process that converts nitrate to gases (Paul and Clark 1996) by means of the cellular enzymes nitrate

reductase (NR), nitrite reductase (NiR), nitric oxide reductase (NOR), and nitrous oxide reductase (NOS). It can be summarized as follows:

$2[NO_3^-] \xrightarrow{NR} 2[NO_2^-] \xrightarrow{NiR} 2NO \xrightarrow{NOR} N_2O \xrightarrow{NOS} N_2$

Nitrate serves as an electron acceptor, most commonly for the anaerobic respiration of organic matter. Almost all denitrifying organisms use O_2 preferentially, with the result that denitrification is restricted to anoxic sites (Paul and Clark 1996). Genera with denitrifiers are found among the organotrophs, chemolithotrophs, photolithotrophs, diazotrophs, thermophiles, and archaea, although organotrophs are the principal denitrifiers in soil.

In the denitrification sequence (above), NO and N_2O are free intermediates that may or may not be further reduced, depending on conditions. Proximally, N_2O production depends on the relative status of NOR and NOS. If NOS is fully active, denitrification may result in N_2 only; however, if NOS is inhibited relative to NOR activity, N_2O is produced. It is convenient to express the relationship between N_2O and N_2 production as N_2O mole fraction: $N_2O / [N_2O + N_2]$. Alternative formulations of mole fraction giving essentially the same information appear in the literature (e.g. N_2 / N_2O etc.).

A suite of proximal controls on N_2O mole fraction have been consistently identified, with few variations (Colbourn and Dowdell 1984, Sahrawat and Keeney 1986, Firestone and Davidson 1989, Arah and Smith 1990, Bouwman 1990, Aulakh *et al.* 1992, Hutchinson and Davidson 1993). Table 1.1 lists factors hypothesized or demonstrated to influence N_2O mole fraction. Not

surprisingly, most of the factors that influence N_2O mole fraction also influence the overall rate of denitrification (e.g., Paul and Clark 1996).

By what mechanisms do soil physical and chemical factors influence N_2O mole fraction? Change in N_2O mole fraction at the cellular level results from a change in the relative rates of production and consumption of N_2O. Environmental factors can change N_2O mole fraction by altering the effective availability of substrates, by altering the relative production and maintenance of enzymes, or by inhibiting the function of enzymes. Betlach and Tiedje (1981) demonstrated the importance of substrate availability. They manipulated soil characteristics and followed each pool in the reductive sequence using ^{13}N label. They found that their results could be predicted qualitatively using a simple Michaelis-Menten model for each reductive step (cf. Dendooven *et al.* 1994). Experimentation with the model system led to the generalization that any change that slows the overall rate of denitrification leads to an accumulation of N_2O. Additionally, they concluded that this effect alone was sufficient to account for their experimental results; differential inhibition of enzymes need not be invoked. The Betlach and Tiedje paradigm is consistent with most other experimental findings.

An important implication of the Betlach and Tiedje paradigm is that, for many controlling factors, influence on denitrification rate and influence on N_2O mole fraction are twin aspects of a single phenomenon: general modification of reaction rates. However, conceptual independence is warranted because there are important exceptions (e.g. the effect of nitrate concentration on N_2O

mole fraction). The hole-in-the-pipe model (Firestone and Davidson 1989, Davidson 1991) maintains a functional distinction between denitrification and N_2O mole fraction as controls on N_2O flux. According to the model, N_2O flux is subject to controls at 3 levels: factors influencing overall rates of nitrification and denitrification (flow through the pipes), factors influencing N_2O mole fraction (the size of the holes in the pipes) and factors influencing transport of gases out of the soil. Figure 1.1 shows explicit relationships among these three levels of influence and the proximal factors listed in Table 1.1. Notice that if a factor has opposite effects on rate and N_2O mole fraction, changes in N_2O flux tend to be buffered. Sahrawat and Keeney (1986) provide a rather comprehensive review of these factors. Below, their work is summarized, with supplemental material from other sources.

Temperature

Temperature generally has a positive effect on total denitrification and a negative effect on N_2O mole fraction. Some have questioned the importance of temperature for predicting N_2O mole fraction (Lensi and Chalamet 1982). Temperature has a general influence on a wide range of biochemical processes; its influence is likely to be complicated (Sahrawat and Keeney 1986).

Available carbon

Organic carbon added to soils can stimulate denitrification and decrease N_2O mole fraction (Sahrawat and Keeney 1986). Greater supply of electron donors may create a demand for electron acceptors, causing N_2O to

be reduced. This is consistent with early work by Nommik (1956). Weier et al (1993) reported a decrease in N_2O mole fraction with increasing carbon availability. However, Dendooven *et al.* (1996a) discovered an increase in N_2O mole fraction with added glucose.

pH

Soil pH is an important regulator of enzyme activity. Early work showed an increase in N_2O mole fraction at pH < 6 (Wijler and Delwiche 1954, Nommik 1956); this finding was supported by later research (see Sahrawat and Keeney 1986, and references therein). Focht (1974) modeled N_2O flux from soils using Nommik's data: he incorporated an explicit effect of pH on N_2O mole fraction. Blackmer and Bremner (1978) disputed Focht's model; they found that pH had no effect in the absence of nitrate. Whereas Wijler and Delwiche (1954) had suggested that low pH inhibits the reduction of N_2O to N_2, Blackmer and Bremner (1978) claimed rather that nitrate inhibits the activity of nitrous oxide reductase although it stimulates its production (cf. Blackmer and Bremner 1979) and that low pH interacts with nitrate to increase its inhibitory effect. Koskinen and Keeney (1982) attribute the pH effect on N_2O mole fraction to either the greater sensitivity of N_2O reductase to pH or to changes in species diversity at low pH. They note (*sensu* Blackmer and Bremner 1978) that there is no pH effect at low nitrate concentrations. They suggest that low pH may inhibit NOS indirectly by favoring high nitrite concentrations. Weier and Gilliam (1986) noted large fluxes of N_2O from low pH soils; fluxes were correlated with

accumulation of NO_2^-. In general, pH control of N_2O mole fraction has not been demonstrated in the field (Davidson 1991).

Nitrate and nitrite

It is universally reported that high nitrate concentrations increase N_2O mole fraction (Nommik 1956, Cooper and Smith 1963, Blackmer and Bremner 1978, Vinther 1984, Weier *et al.* 1993). Firestone *et al.* (1979) found, however, that nitrite was a more potent inhibitor of nitrous oxide reduction than nitrate (cs. Gaskell *et al.* 1981, cited in Coulbourn and Dowdell 1984), and suggested that small amounts of nitrite produced from large pools of nitrate may in fact be responsible for the inhibition usually observed.

Since nitrate, when limiting (Sahrahwat and Keeney 1986) is positively correlated with denitrification and yet increases N_2O mole fraction at high concentrations, it does not conform in a strict sense to the Betlach and Tiedje model. This suggests that its influence is not via simple substrate kinetics. Probably nitrate concentration is not high and limiting at the same time; nitrate may have a small but unreported kinetic effect on N_2O mole fraction at low concentrations but must exert influence by a different mechanism at high concentrations. Two proposed mechanisms are that nitrate competes with N_2O as an electron acceptor or that it directly or indirectly inhibits NOS. Firestone *et al.* (1979) favored the first mechanism for nitrate but the second for nitrite. This question has not been resolved, although the fact that pH interacts with nitrate concentration suggests the priority of enzyme inhibition.

Soil Water and Oxygen

Three mechanisms are responsible for the influence of soil water on N_2O mole fraction. First, at very low soil moistures, metabolic stress may limit denitrification (Sahrawat and Keeney 1986) and N_2O mole fraction may be influenced concomitantly. Second, at high soil moistures, O_2 availability in soil microsites is limited by diffusional transport of oxygen; denitrification and N_2O mole fraction are affected. Third, high soil water content limits the diffusion of N gases away from the sites of formation, increasing the probability of further reduction of N_2O-N. The first two mechanisms are consistent with the kinetic model of denitrification (Betlach and Tiedje 1981) and all three proceed in the same direction with respect to the effect of soil moisture on N_2O mole fraction.

Water-filled pore space, when expressed as a percentage of the total pore space, is the most informative way of describing soil moisture because it integrates absolute water content (e.g. gravimetric) with bulk density (Linn and Doran 1984). It is probably the single most useful predictor of N_2O mole fraction, largely because of its inverse relationship with soil aeration. Denitrification is anaerobic, and all denitrification enzymes are sensitive to (inhibited by) oxygen.

Lower soil water contents favor N_2O production over N_2 (Nommik 1956, Weier *et al.* 1993). However, the effect of water status is inseparable from its effect on soil aeration. Oxygen inhibits denitrification but increases N_2O mole fraction (Firestone *et al.* 1979). Focht (1974) included aeration as a

determinant of N_2O mole fraction in his model of denitrification. Implications for enzyme status are discussed below.

Enzyme status

The principal effect of oxygen is to inhibit denitrification enzymes. The most sensitive of these is the terminal enzyme NOS. As oxygen increases, reduction of N_2O to N_2 slows sooner than reduction of NO to N_2O, thereby increasing N_2O mole fraction (McKenney *et al.* 1994). Notwithstanding, the Betlach and Tiedje model predicts increasing N_2O mole fraction with increasing O_2, regardless of enzyme sensitivity. If a well-aerated soil is sufficiently wetted that it becomes anaerobic, denitrification enzymes are induced sequentially (see Letey *et al.* 1980a). Thus some N_2O accumulates before NOS is completely induced, and N_2O mole fraction should decrease with time. The ever-present potential for synthesis and inactivation of enzymes makes N_2O mole fraction inherently dynamic. Enzymes may be induced, repressed, de-repressed, re-repressed, or destroyed. Any difference in the time-dependent behavior of the last two reduction enzymes leads to a time dependent difference in accumulation of the last two reductive products -- N_2O and N_2. Just as water status is usually inseparable from O_2 status, enzyme status is inseparable from its temporal dependency.

Time

Changes in N_2O mole fraction may lag behind changes in factors that control it, creating complex temporal dependency. Rolston *et al.* (1978) found that N_2O mole fraction was highest at the initiation of denitrification, and

decreased thereafter. Jacinthe *et al.* (2000) manipulated water tables in soil columns (various drainage classes) to stimulate denitrification; N_2O mole fraction was 0.95 four days after raising a water table to 10 cm below the soil surface, but dropped to 0.35 one week later. Firestone and Tiedje (1979) reported that after the onset of anaerobiosis, N_2O mole fraction was initially low, then rose significantly, and then declined. They attributed this effect to the staggered synthesis of enzymes, and speculated that it reflected the sequence of events following O_2 depletion due to rainfall or irrigation. Letey *et al.* (1980a) reported high initial values for N_2O mole fraction during denitrification, with a subsequent decrease to zero. They attributed this effect to differential rates of induction of nitrate reductase and nitrous oxide reductase. Rolston *et al.* (1982) found that "nitrous oxide mole fractions tended to be smallest immediately after irrigation and increased as the soil water redistributed and became less anoxic". They also found a decrease in N_2O mole fraction with successive irrigation cycles. Hallmark and Terry (1985) reported that N_2O mole fraction following irrigation was initially high, then dropped over a period of six weeks. Weier *et al.* (1993) reported N_2/N_2O ratios rising (i.e., N_2O mole fraction dropping) over a 5-day period. Dendooven and Anderson (1994) found that *de novo* synthesis of nitrate reductase and nitrous oxide reductase began one and 16 hours after anaerobiosis was imposed, respectively. Dendooven and Anderson (1995) found that upon return of soil to aerobic conditions, N_2O mole fraction increased with time over a 70 day period (from 51% to 100%). Conversely, soil cores submerged for 96 h had smaller subsequent N_2O mole

fraction than soils submerged for 6 h (Dendooven *et al.* 1996b). In general, it is difficult to predict N_2O mole fraction without knowing both the antecedent water regime and the time since change in water regime.

Effects of moisture cycles on denitrification and N_2O mole fraction merit consideration. Arnold (1954) suggested that moisture fluctuation should stimulate gaseous N loss from soils. High denitrification rates are sometimes attributed to extreme drying-wetting cycles (Peterjohn and Schlesinger 1991). Smith and Patrick (1983) found that 7 and 14 day aerobic/anaerobic cycles produced far more N_2O than either condition alone. A simple explanation is that the cycling systems represent intermediate levels of aeration, such that denitrification is supported, but not complete reduction to N_2. However, moisture cycles may support more complex dynamics. Groffman and Tiedje (1988) showed that hysteresis (dependence of the response on the direction of change) is important for the overall rate of denitrification. Hutch *et al.* (1999) found N_2O emission under a fluctuating moisture treatment 4 to 9 times higher than under constant low or high moisture. The final chapter of this dissertation describes an investigation of the temporal dependence of N_2O mole fraction on soil moisture, i.e., the effect of moisture history.

Prospectus

The task of understanding the relationship between N_2O flux and denitrification will have been accomplished when N_2O from denitrification can be predicted reasonably well from a discrete set of measurable factors. Soil water status will likely continue to be a focus of investigation because it is

easily measurable and correlates well with oxygen status. Oxygen status, though more difficult to measure in spatially heterogeneous soil environments, is the most important proximal regulator of denitrification enzyme status. Dynamic, differential variations in enzyme status are the principal cause of uncertainty regarding the relationship between N_2O flux and denitrification because of the potential for production of the alternative product N_2. From an empirical perspective, useful approaches will be (1) to investigate the response of N_2O and N_2 flux to differences in soil moisture (2) to assess the temporal dependence of the relationship between flux and moisture, and (3) to test for the robustness of the perceived patterns across varying environments. In the work that follows, I seek to implement these approaches by developing techniques for observing N_2O and N_2 flux, and by testing explicitly the temporal dependence of flux on change in moisture, for soils from contrasting ecosystems.

Table 1.1. Summary of factors potentially influencing N_2O mole fraction. The generally-accepted direction of the correlation is indicated. WFPS = water-filled pore space.

factor	correlation
temperature (0 - 30 °C)	-
water (60-100% WFPS)	-
Time since water addition	-
nitrate, nitrite	+
available organic carbon	-
pH (4 - 7)	-
pO_2	+
enzyme status	variable
rhizosphere/ plants	?
depth of activity	-
soil structure	+

Figure 1.1. Conceptual diagram: factors influencing flux of N_2O from soils. WFPS = water-filled pore space. C = organic carbon availability. P = phosphorus availability.

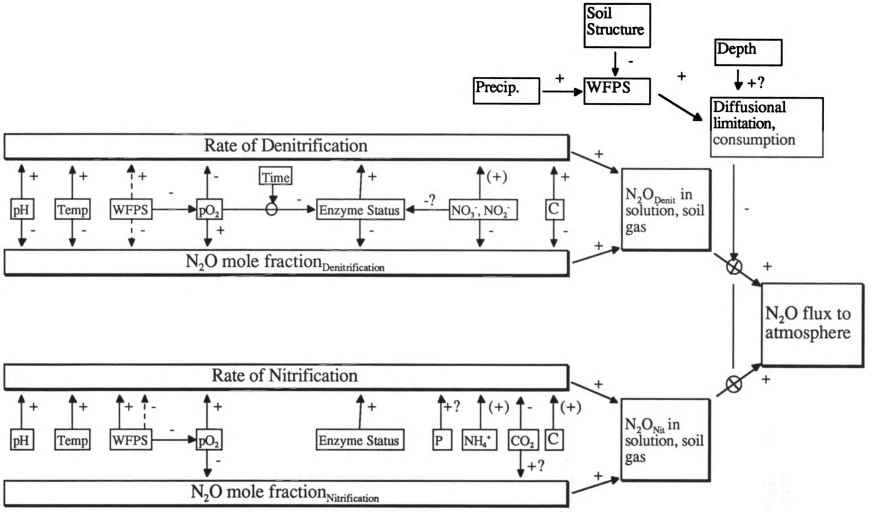
Soil Structure
Depth
Precip.
WFPS
Diffusional limitation, consumption
Rate of Denitrification
pH
Temp
WFPS
pO2
Time
Enzyme Status
NO3-, NO2-
C
N2O mole fraction Denitrification
N2O Denit in solution, soil gas
N2O flux to atmosphere
Rate of Nitrification
pH
Temp
WFPS
pO2
Enzyme Status
P
NH4+
CO2
C
N2O mole fraction Nitrification
N2O Nit in solution, soil gas

Figure 1.1.

Chapter 2

A HEURISTIC MODEL FOR THE CALCULATION OF DINITROGEN AND NITROUS OXIDE FLUX FROM NITROGEN-15-LABELED SOIL

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A Heuristic Model for the Calculation of Dinitogen and Nitrous Oxide Flux from Nitrogen-15-Labeled Soil

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ABSTRACT

Very sensitive measurements of N_2 and N_2O flux from soil are possible when gas evolved from ^{15}N-labeled soil is analyzed by isotope ratio mass spectrometry. This approach is useful for studying the fate of nitrogen fertilizer and for studying soil microbial processes contributing to the atmospheric increase of nitrous oxide, a radiatively active trace gas that can contribute to global warming and ozone depletion. Most systems of equations that relate isotopic analysis to gas flux are sufficiently complex that certain limitations and potentials of the ^{15}N approach may be overlooked. We describe a graphical representation of labeled N-gas flux that illustrates the equations and encourages critical thinking regarding the implementation of related experiments. This model is used to interpret underestimation that occurs if flux derives from multiple pools of differing enrichment. A statistical derivation is presented for a previously published simulation of underestimation due to multiple pools. The same equations are applied to field data to explore whether temporal variation in soil nitrate enrichment is likely to cause significant underestimation. Two sampling strategies are proposed that may eliminate the assumption of pool uniformity, thereby eliminating a potential source of underestimation.

DINITROGEN AND NITROUS OXIDE are alternative end products of microbial denitrification. Quantifying their flux from soil can help explain fertilizer losses from agricultural systems (Mosier et al., 1986; Eichner, 1990; Weier et al., 1993) as well as the atmospheric buildup of N_2O — an important greenhouse gas in the troposphere and ozone-destructive catalyst in the stratosphere (Bouwman, 1990; IPCC, 1996). However, field studies of denitrification have been hampered by the insensitivity of standard instrumentation to N_2 increases, e.g., under soil covers (Mosier and Klemedtsson, 1994).

Mass spectrometric analysis of gas from ^{15}N-labeled soil is a sensitive method for quantifying the flux of N_2, as well as N_2O, because of the low natural abundance of ^{15}N. Isotopic data for headspace gases can also estimate the enrichment of the source N pool, can help identify the source of N for N_2O, and can be used to check assumptions of the flux method (e.g., Stevens et al., 1997; Arah, 1997). Recent advances in spectrometer sensitivity and affordability have generated new interest in using ^{15}N for soil biogeochemical investigations. Given the complexity of the system of equations normally used for interpreting the isotope data, it is useful to have conceptual tools which make the principles of the system more intuitive.

We present here a heuristic model that illustrates the estimation of source pool enrichment and estimation of soil-derived headspace gas for N_2 or N_2O from ^{15}N-labeled soil. A convenient graphical representation of N isotope data is identified, from which a complete set of equations is derived by geometric inference. These equations are similar in form and identical in function to those of others (Siegel et al., 1982; Mulvaney, 1984; Arah, 1992). To illustrate utility, the model is applied to the problem of underestimation that occurs when flux derives from multiple pools of differing enrichment.

Measuring Flux

Flux of N_2 produced by denitrification in a ^{15}N-enriched soil can be measured by monitoring the increase in enrichment of headspace gas in a chamber placed over the soil. Proper analysis requires measurement of the abundance of all three molecular masses of N_2 (28, 29, 30). When atoms of ^{15}N are distributed randomly among a sample of N_2 molecules, measurement of any two masses suffices, because the abundance of the third mass can be predicted statistically. However, a mixture of N_2 from two differently labeled sources (e.g., enriched N_2 from the soil and unenriched atmospheric N_2 in an enclosure) is not in isotopic equilibrium (Hauck et al., 1958); that is, the isotopes of N are not randomly distributed among the three molecular fractions. This means that all three masses must be measured, which has the additional advantage of providing an indirect estimate of the average enrichment of the soil N pool (Hauck and Bouldin, 1961). The estimation of enrichment is convenient because it is non-destructive and is a time-weighted mean.

Equations for the determinations of flux and source enrichment, by isotope ratio mass spectrometry, are well established (Siegel et al., 1982; Mulvaney and Boast, 1986; Mulvaney, 1984; Arah, 1992). These equations are designed to measure total flux of N_2 and assume that the gas is derived from a single, uniformly labeled pool of soil N. (In the absence of pool uniformity, flux derived from added label—e.g., fertilizer—can be estimated, but not by these equations.) The same principles apply for N_2O; for simplicity, most of this discussion is limited to N_2.

Graphical Representation

We adopt, wherever possible, the notations and definitions of Arah (1992). Briefly, the ^{15}N *atom fraction* ^{15}a of any sample of N is the total number of ^{15}N atoms

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divided by the total number of N atoms (informally, the *enrichment*). The *molecular fractions* ${}^{28}x$, ${}^{29}x$, and ${}^{30}x$ are the fractions of the total number of N_2 molecules in a sample with masses 28, 29, and 30, respectively. A sample of N_2 is in *isotopic equilibrium* if the molecular fractions follow a binomial distribution:

$$ {}^{28}x = ({}^{14}a)^2 \quad [1a] $$

$$ {}^{29}x = 2({}^{14}a)\,({}^{15}a) \quad [1b] $$

$$ {}^{30}x = ({}^{15}a)^2 \quad [1c] $$

where

$$ {}^{14}a = 1 - {}^{15}a \quad [1d] $$

All N_2 (or N_2O) derived from uniform pools of molecules with single N atoms (e.g., nitrate) by microbial and chemical processes (e.g., denitrification) are expected to be in isotopic equilibrium, even if isotopic fractionation is significant (i.e., even if one isotope is inherently favored by the process). All mixtures of two or more equilibrium samples with different ${}^{15}N$ atom fractions are necessarily *nonequilibrium mixtures*. Atmospheric N_2 is assumed to be in isotopic equilibrium.

Additionally, we define the *isotopic character* of an N_2 sample as the relative proportions of the three molecular fractions. We represent the isotopic character of N_2 by plotting ${}^{29}x$ versus ${}^{15}a$. The fact that such a plot is equivalent to a ternary plot of ${}^{28}x$, ${}^{29}x$, and ${}^{30}x$ (Fig. 1; Note 1) is both a mathematical convenience and an informal proof that ${}^{29}x$ and ${}^{15}a$ completely characterize N_2 isotopically. The isotopic character of a single sample may be expressed as the coordinate pair $({}^{15}a, {}^{29}x)$.

Figure 2 represents graphically the calculation of source pool enrichment and flux from a plot of isotopic character. The downward-opening parabola is called the equilibrium curve, and has the form of Eq. [1b]; it represents the isotopic characters of all possible N_2 samples that are in isotopic equilibrium. Whenever N atoms are paired randomly from a source of given enrichment, ${}^{29}N_2$ will be a quadratic function of ${}^{15}N$, with no ${}^{29}N_2$ produced when all or none of the source atoms have mass 15.

The symbol A represents the isotopic character of atmospheric N_2 already present in a chamber headspace. P represents the isotopic character of N_2 derived from the soil pool; since the soil pool consists of uniformly enriched mineral N, Component P is initially in isotopic equilibrium. M represents the isotopic character of a mixture of atmospheric and soil-derived N_2 in a chamber headspace (this mixture is not in isotopic equilibrium). Plotted thus, the isotopic character of any mixture is a linear interpolation between its two constituents, and its distance from either is inversely proportional to the relative contribution from that constituent. Thus M falls on a line between A and P, and its position along that line indicates the mixing ratio of A and P.

In practice, a soil cover is deployed, and gas samples are taken at the beginning and end of an incubation. A and M are the isotopic compositions of these samples, respectively. P is unknown initially, but must fall somewhere on the ray drawn from A through M, and must also fall on the equilibrium curve. Thus, the intersection of the curve and the ray identifies the character of P. The atom fraction of the soil N pool, identical (barring fractionation) to that of P, is displayed on the horizontal axis. The relative contribution of P to the mix is given by the "travel" of M along the ray (the length of segment AM), divided by the total length of the segment AP. Since the ray has constant slope, the relative contribution can be determined simply from the relative enrichments (${}^{15}N$) of A, M, and P (i.e., the proportion collapses to a single axis).

Equations

From the concept illustrated in Fig. 2, equations for gas flux and enrichment of source pool can easily be derived by geometric inference. Formally, the isotopic character of a gas mixture and the assumed or measured character of one of its pre-mixing components (e.g., atmospheric N_2 already present in a chamber headspace) can be used to calculate both ${}^{15}a_p$, the atom fraction of the second component (e.g., soil-derived N_2), and d, the fractional contribution of the second component to the mixture. This is achieved by assuming that the second component is initially in isotopic equilibrium (as stated earlier). The atom fraction of the second component is found by simultaneous solution of an equation for the mixing line

$$ {}^{29}x = C + s\,{}^{15}a \quad [2a] $$

and an equation for the equilibrium curve [1b], which results in a quadratic expression:

$$ {}^{15}a_p = [-B \pm (B^2 - 4AC)^{1/2}] / 2A \quad [2b] $$

where

$$ A = 2 \quad [2c] $$

$$ B = s - 2 \quad [2d] $$

$$ C = {}^{29}x_m - s({}^{15}a_m) = {}^{29}x_a - s({}^{15}a_a) \quad [2e] $$

$$ s = ({}^{29}x_m - {}^{29}x_a)/({}^{15}a_m - {}^{15}a_a) \quad [2f] $$

and the Subscripts a, p, and m refer respectively to the initial component (atmospheric N), the second component (soil pool N), and the mixture. C is the intercept for the mixing line, and s is the slope of the mixing line. The relative contribution of N_2 from the second component is

$$ d = ({}^{15}a_m - {}^{15}a_a)/({}^{15}a_p - {}^{15}a_a) \quad [3] $$

Absolute flux can be calculated from relative flux (relative contribution) by associating some absolute measure with initial or final quantity of headspace gas: for example, if final N_2 concentration is 0.8, and chamber volume is 1 L, then a value of 0.01 for d implies evolution of (0.8)(1 L)(0.01) = 0.008 L of N_2. Note that a quadratic expression is used to find ${}^{15}a_p$, which in turn is used to find d. Arah (1992) used a quadratic to find d and used d to find ${}^{15}a_p$. The two systems give identical results. The relevant root in Eq. [2b] can be identified by inspection of Fig. 2 and is necessarily the greater; the other root is identical to a_a in this case (cf., Boast et al., 1988 Eq.

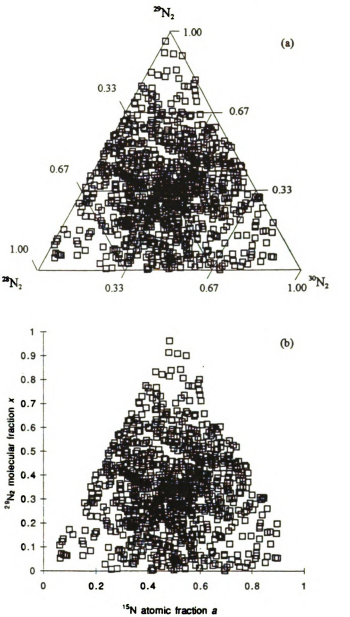

Fig. 1. Illustration of the equivalence of ternary and Cartesian pots. (a): One thousand simulated N_2 samples, with each molecular fraction independently randomized, plotted as a ternary graph. (b): These same one thousand samples plotted as ^{29}x vs. ^{30}a.

[26], [31] *ff.*). Note again that all mixtures of equilibrium gases necessarily lie under the equilibrium curve. This is true regardless of the number of equilibrium components in a mixture. As an informal proof, consider stepwise additions of many equilibrium components to an accumulating mixture. The first two components create a mixture below the curve; subsequent additions displace the mixture toward some point in the curve, but never past it. The formal proof has been provided by Boast et al. (1988; see Eq. [19]).

Expectation of Error Due to Multiple Pools

The utility of the heuristic model outlined above can be illustrated by revisiting the principle assumption of

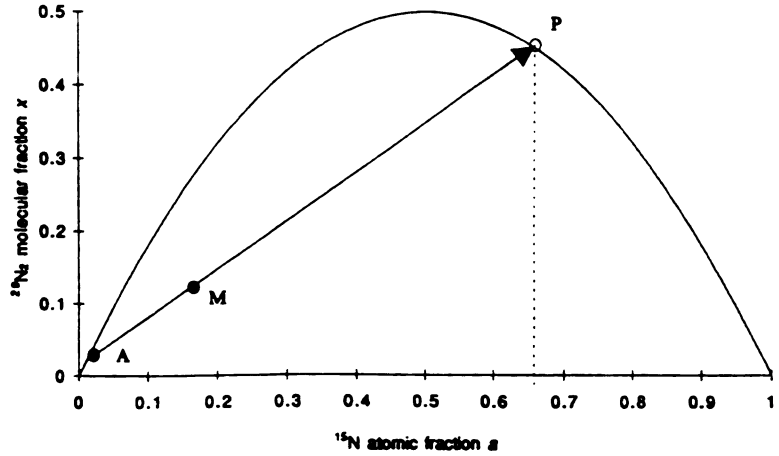

Fig. 2. Visualization of the heuristic model (Eq. [1], [2], and [3]). *A* represents the atmosphere and *M* represents the sample: positions of both are determined analytically. The ray drawn from *A* through *M* intersects the equilibrium curve, implicating a pool *P* of gas derived from the soil, having an equilibrium distribution of masses.

disequilibrium methods of N_2 or N_2O flux analysis (Hauck and Bouldin, 1961; Siegel et al., 1982; Mulvaney, 1984; Arah, 1992). Any method that does not measure enrichment of the soil mineral N substrate directly, but infers it from isotopic data, assumes that soil N_2 or N_2O derives from a single, uniformly labeled pool. The importance of this assumption has been debated (Focht, 1985; Mulvaney and Kurtz, 1985) and evaluated in the lab (Mulvaney, 1988) and field (Mulvaney and Vanden Heuvel, 1988) and tested by simulation (Vanden Heuvel et al., 1988; Arah, 1992). Addition of ^{15}N-labeled material to native soil pools creates the possibility of at least two differently labeled pools. Theory suggests that concurrent flow from multiple pools of different enrichment should usually lead to an overestimation of soil N enrichment, and should always lead to an underestimation of flux (Boast et al., 1988; Arah, 1992).

Arah (1992; see Fig. 2a) simulated the underestimation that occurs when the assumption of pool uniformity fails. Estimated mixing ratio d was compared with actual mixing ratio for 1000 runs in which number of pools was randomized on the Interval [1, 50] (cf., Vanden Heuvel et al., 1988, for simulations based on two pools) and pool enrichment was randomized on the Interval [γ, 1] (where γ is the natural abundance of ^{15}N in the atmosphere, or $^{15}a_a$). The plot of estimated vs. actual showed very little scatter about a line with a slope of 0.76; thus, for the conditions that were simulated, the isotopic method consistently underestimated N_2 flux by about 24%. This non-intuitive result begs for a more fundamental interpretation.

We have discovered that Arah's result can be calculated directly from statistical principles, without recourse to simulation. It is instructive to begin by visualizing how underestimation occurs (Fig. 3). If a gas sample P^{actual} is itself a mixture of N_2 that derives from two or more soil N pools, then the character of P will fall somewhere below the equilibrium curve (Fig. 3). The ray approaching P^{actual} from the direction of A will necessarily "overshoot" P, intersecting the curve beyond P. Thus, $^{15}a_p$ will always be overestimated if $^{15}a_p > {}^{15}a_a$ (as shown by Boast et al., 1988; and Arah, 1992). It is apparent from Fig. 3 (and from Eq. [3]) that d is always underestimated: the segment $AP^{calculated}$ is always longer than the segment AP^{actual} (as per Arah, 1992).

The underestimation can be quantified if the isotopic character of P^{actual} is specified. Regarding Arah's (1992) simulation, it is appropriate to associate P^{actual} with the statistical expectations of ^{15}a and ^{29}x for a mixture of equilibrium gases, whose values of ^{15}a are randomly and uniformly distributed on the interval [g, h]. With P^{actual} thus specified, the expectation of underestimation can be calculated. The answer derived here is independent of the number of contributing gases (pools) because statistical expectation $E(x)$ is independent of the number of samples. For generality, we take the case where each pool has equal weight. For a random variable uniformly distributed on the interval [g, h],

$$E(^{15}a) = (g + h)/2 \quad [4]$$

$$\mathrm{Var}(^{15}a) = (h - g)^2/12 \quad [5]$$

$$E(^{15}a^2) = E^2(^{15}a) + \mathrm{Var}(^{15}a) \quad [6]$$

Considering the dependence of ^{29}x on ^{15}a (Eq. [1b]), the expectation of ^{29}x is given by

$$E(^{29}x) = E(2\ ^{15}a - 2\ ^{15}a^2) \quad [7a]$$

$$= 2E(^{15}a) - 2E(^{15}a^2) \quad [7b]$$

Substituting [5] in [6] and [6] in [7b] gives

$$E(^{29}x) = 2E(^{15}a) - 2\,[E^2(^{15}a) + (h - g)^2/12] \quad [8]$$

Thus, given a range of enrichments, the expected isotopic character of a random mixture ($^{15}a^{expectation}$, $^{29}x^{expectation}$) can be determined from [4] and [8]. To calculate the resulting underestimation, an atom fraction $^{15}a_p^{calculated}$ is found by substituting $^{29}x^{expectation}$ and $^{15}a^{expectation}$ for $^{29}x_m$ and $^{15}a_m$ in Eq. [2]. A coefficient of estimation e can be expressed as

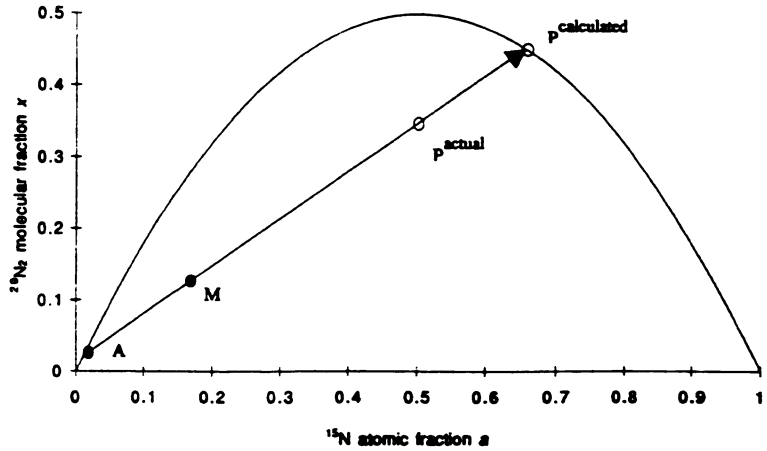

Fig. 3. Visualization of underestimation due to multiple soil N pools of differing enrichment. If P is in fact a mixture of equilibrium gases (resulting from multiple pools) then it falls below the equilibrium curve. Thus $P^{calculated}$ always overestimates P^{actual}, when $^{15}a_p > {}^{15}a_s$. Since the segment $AP^{calculated}$ is longer than AP^{actual}, flux is always underestimated (since flux $\propto$ AM / AP).

$$e = d^{calculated}/d^{expectation} \quad [9a]$$

$$= ({}^{15}a_p{}^{expectation} - {}^{15}a_s)/({}^{15}a_p{}^{calculated} - {}^{15}a_s) \quad [9b]$$

Eq. [9b] can be proven from Eq. [3]. For the case where the range of enrichments is [0,1], ${}^{15}a^{expectation} = (\frac{1}{2})$ by Eq. [4] and ${}^{29}x^{expectation} = (1/3)$ by Eq. [8] (Note 2). Interestingly, if the lower bound of the interval g is equal to γ (${}^{15}a_s$; cf. Arah, 1992), $e = 0.75$, regardless of the range of enrichment; this value depends neither on the value of γ nor h (Note 3).

In summary, we have provided a more fundamental interpretation of Arah's (1992) simulation (Fig. 2a). Our heuristic model for N-gas flux from ^{15}N-labeled soil shows why underestimation occurs. Our equations reduce the simulation to a relatively straight-forward calculation. We show that even under somewhat less restrictive conditions than Arah's (i.e., range not specified), the coefficient of underestimation e evaluates to 0.75. This value agrees well with the slope of 0.76 in Arah's Fig. 2a, which can also be interpreted as an index of underestimation. Simulation is a valuable tool for exploring systems of equations that defy direct solution; the discovery of a direct solution for such a system represents progress. While our solution may not represent any real set of field conditions, it does help predict how field conditions will influence the accuracy of isotopic methods for N-gas flux measurement.

The approach employed above can be used to explore other questions about labeled N_2 or N_2O fluxes. For instance, Hauck and Bouldin (1961) state that their system gives a value for ^{15}N that "represents the average isotope content of the material undergoing denitrification over a given period of time." But the concept of "average" implies that the enrichment is changing with time (e.g., by dilution from concurrent nitrification), and thus the assumption of pool uniformity is violated. In principle, it makes no difference whether the assumption is applied to space or time. Is change of source pool enrichment with time likely to be a significant source of error in field experiments?

We sampled N_2O over a 3.5-d period in April 1998 in a heavily labeled wheat plot (30 kg ha^{-1} as 99% $K^{15}NO_3$). Enrichment of the source pool, as inferred from N_2O isotope data, dropped gradually from 82 to 72% during this period. Even if this entire drop had occurred during a single incubation, the resulting underestimation would have been negligible. Let [g, h] be [0.7, 0.8] and let g = 0.003663. From Eq. [9b] (which invokes others), $e = 0.999$. For comparison, a drop from 80 to 60% during an incubation yields $e = 0.993$. We conclude that error from temporal changes in enrichment for our experiment must have been negligible, and is probably negligible in most cases.

DISCUSSION

The heuristic model presented above facilitates the design of N-gas flux experiments and the interpretation of isotopic data for N_2 and N_2O samples collected over ^{15}N-labeled soil. It is particularly useful for exploring the problem of underestimation that occurs when N_2 or N_2O analyzed by mass spectrometry derives in part from a soil pool that is not uniformly labeled: it illustrates how underestimation occurs. We reduced a published simulation of underestimation to a direct calculation based on statistical principles. We showed the general utility of our equations by evaluating a case where enrichment varied over time, rather than in space.

Isotopic methods for measuring N_2 and N_2O flux have general appeal because they are relatively non-disruptive of soil systems and because they represent the only practical direct method for measuring N_2. As isotope ratio mass spectrometry (IRMS) becomes more widely available, use of isotopic methods will continue to grow. Although flux equations and analytical methods have been available for decades, there still exists considerable uncertainty regarding the accuracy of the method when applied in the field. Most of the uncertainty pertains to the necessary assumption that empirical methods result in uniformly labeled soil mineral N pools. Underestima-

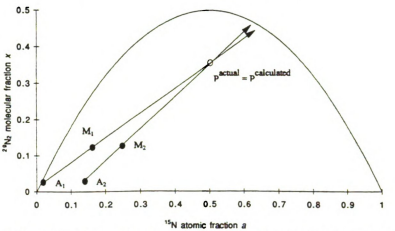

Fig. 4. Visualization of a method to circumvent the problem of pool non-uniformity. After an initial incubation in which A_1 and M_1 are sampled, the chamber is flushed, closed, and spiked with $^{30}N_2$. A_1 is displaced relative to A_2. Measurement of M_2 gives the trajectory of a second ray that converges on the first at p^{actual}. The principal assumption is constant isotopic character of the evolving gas. (Scale is illustrative only.)

tion is expected to result from the failure of the assumption, but the magnitude of the underestimation is difficult to predict.

Our work does not imply the existence of a theoretical method for correcting flux estimates from the field. "Statistical expectation", as used in our argument, has a precise mathematical definition that is not equivalent to expected error in field measurements, unless field conditions closely match the constraints of the mathematical model. The underestimation calculated by our equations for e will not likely be realized in the field unless (i) the number of pools is large, (ii) the enrichments of the pools are randomly distributed, and (iii) flux is distributed evenly among the pools. We doubt that any of these conditions is likely to be met in field settings, especially the third. Even if the conditions were met, it would seem impossible to know this a priori. Our experience with N_2O fluxes shows underestimation to vary within experiments and especially among experiments. When mass spectrometric flux estimates are compared to estimates made by gas chromatography, agreement ranged from 6 to 117% (MS/GC, unpublished data). In a systematic laboratory study of N_2O fluxes, Mulvaney (1988) found that differences between MS and GC usually were small (less than 10%) and probably resulted from analytical error. Mulvaney and Vanden Heuvel (1988) found no appreciable difference between MS and GC in the field, unless plots were relabeled. Can pool uniformity ever be assumed? We believe that when added N (labeled) far exceeds preexisting soil pool N, there is initially only one significant pool, which is practically uniform.

When uniformity of the soil N pool cannot be assumed, it should be demonstrated (e.g., Stevens et al., 1997). However, the conceptual model given here (e.g., Fig. 2 and 3) reminds us that it is not critical to know that the soil pool is uniform; rather it is critical to know the isotopic character of N gas derived from the soil pool. Uniformity merely makes this easy to calculate (by invoking the equilibrium curve as one constraint). The error that attends violation of the assumption of pool uniformity could be avoided if there were alternative means of assessing the isotopic character of the N gas derived from the soil.

It may be possible to assess independently the isotopic character of soil-derived gas. Consider two successive incubations of the same unit of soil. Suppose that, after sampling the headspace at the end of the first incubation, the enclosure is flushed, closed, and spiked with a small amount of $^{30}N_2$. The spike has the effect of displacing A_2, the base of the ray for the second incubation (Fig. 4). The rays for the two incubations necessarily converge at a point identifying the isotopic character of the total evolved gas, allowing accurate determinations of flux for both intervals (equations are outlined in Note 4). The principal assumption is that the isotopic character of the evolved gas is constant: probably a more robust assumption than pool uniformity. Another assumption is that change in headspace enrichment due to other processes (e.g., equilibration with soil pore space) is negligible. The amount of gas needed for the spike depends on the volume of the headspace and the sensitivity of the mass spectrometer. For N_2, such a spike will be relatively expensive until $^{30}N_2$ costs drop or sensitivity improves such that smaller changes in $^{30}N_2$ abundance become detectable. For N_2O, however, for which additions of labeled N such as $^{46}N_2O$ can readily be measured in a normal atmosphere, this approach is already an option.

Another approach for independently assessing the isotopic character of soil-derived gas is similar to that above. Two consecutive incubations are conducted, but before the second incubation, the headspace is purged of the gas of interest so that the final sample will contain only (mostly) soil-derived gas. This is, again, difficult for N_2 because of contamination problems, but is an interesting possibility for N_2O.

We believe the heuristic model described here is use-

ful for clarifying principles, for designing experiments, and for evaluating data related to N_2 or N_2O collected over labeled soil. ^{15}N approaches to measuring N_2 and N_2O fluxes are likely to become more common as associated materials and technology become more readily available. Application of conceptual tools to explore the limits and potential of isotopic methods is warranted.

Note 1

A Cartesian plot of ^{29}x vs. ^{15}a is graphically identical to a ternary plot of ^{28}x, ^{29}x, and ^{30}x if the ternary plot is bounded by an equilateral triangle, and if the abscissa is expanded by a factor of $\cos(30)^{-1}$ relative to the ordinate (about 15%). Equivalence, but not identity, is preserved even if both conditions are removed.

Consider an equilateral triangle of unit height and horizontal base. Specify three axes that bisect the three vertices and intersect their respective opposite sides at right angles. Scale these from 0 to 1, base to vertex. Beginning with the lower left vertex and proceeding clockwise, assign the axis bisecting the vertex to represent ^{28}x, ^{29}x, or ^{30}x, respectively. Additionally, specify a vertical axis and a horizontal axis (v and h) originating at the lower left vertex and scaled identically to the other three axes. Adopt the term "base" to represent a line normal to an axis, which passes through its origin.

For any given point, what is the relationship between its Cartesian coordinates (h, v) and its ternary coordinates (^{28}x, ^{29}x, ^{30}x)? It is clear by inspection that

$$v = {}^{29}x$$

because their bases are collinear. The distance of any point to the base of the h axis can be divided into two portions falling inside and outside the ternary plot, respectively. Trigonometric analysis shows that

$$h = (^{30}x)\cos(30)^{-1} + (^{29}x)\tan(30).$$

Multiplying both sides by cos(30),

$$(h)\cos(30) = (^{30}x) + (^{29}x)\sin(30).$$

Since all ^{15}N atoms occur either in the $29x$ or $30x$ fractions, and since only half of the atoms in the $29x$ fraction are ^{15}N, we can write

$$15a = (^{30}x) + (^{29}x)(0.5).$$

Noting that sin(30) evaluates to 0.5,

$$(h)\cos(30) = {}^{15}a \quad \text{and} \quad h = (^{15}a)\cos(30)^{-1}$$

Therefore, a plot of (^{29}x) vs. $(^{15}a)\cos(30)^{-1}$ is identical to a ternary plot of ^{28}x, ^{29}x, and ^{30}x. The factor $\cos(30)^{-1}$ merely scales the abscissa, and is largely irrelevant. The plot ^{29}x vs. ^{15}a also emulates a valid ternary plot, albeit with a compressed base.

Note 2

To illustrate this, we calculated the isotopic character of 1000 simulated gas mixtures by mass balance. Number of pools was randomized on the interval [2, 50]. Enrichment as well as relative weights of pools were randomized on the interval [0,1]. When plotted, the isotopic characters of the mixtures clustered around the coordinates (1/2, 1/3) for (^{15}a, ^{29}x). Mean coordinates for all values were (0.497, 0.334). Mean coordinates for 100 000 simulated mixtures were (0.5004, 0.3336).

Note 3

In other words, h can approach arbitrarily close to γ, and underestimation remains unchanged. When $h = \gamma$, however, e is undefined. The apparent suggestion is that an almost perfectly uniform pool still leads to significant underestimation, when h is very close to γ. The point is moot, since flux estimates could hardly be made from such a poorly labeled pool. Anyway, the reader is cautioned that this result only arises under the assumptions stated. The proof follows.

What is e for a mixture of equilibrium N_2 samples whose enrichments are uniformly distributed on the interval $[\gamma, h]$?

Definitions

$$\gamma = {}^{15}a_s$$

$$q = {}^{29}x_s = 2\gamma - 2\gamma^2$$

$$k = {}^{15}a_p$$

$$i = E(^{15}a) = (\gamma + h)/2$$

$$j = E(^{29}x) = 2i - 2[i^2 + (h - \gamma)^2/12]$$

$$k = \{-(m - 2) \pm [(m - 2)^2 - 8(q - m\gamma)]^{1/2}\}/4$$

$$m = (j - q)/(i - \gamma)$$

$$e = (i - \gamma)/(k - \gamma)$$

Substitutions

$$j = 2 \times [(\gamma + h)/2] - 2[(\gamma + h)^2/4 + (h - \gamma)^2/12] = (-4h^2 - 4\gamma^2 - 4h\gamma + 6\gamma + 6h)/6$$

$$j - q = (-4h^2 - 4\gamma^2 - 4h\gamma + 6\gamma + 6h)/6 - (2\gamma - 2\gamma^2) = (-4h^2 + 8\gamma^2 - 4h\gamma - 6\gamma + 6h)/6$$

$$m = (j - q)/[(\gamma + h)/2 - \gamma] = (-4h^2 + 8\gamma^2 - 4h\gamma - 6\gamma + 6h)/[3(h - \gamma)]$$

$$m - 2 = [(-4h^2 + 8\gamma^2 - 4h\gamma - 6\gamma + 6h) - 2(3h - 3\gamma)]/[3(h - \gamma)] = (-4h^2 + 8\gamma^2 - 4h\gamma)/[3(h - \gamma)]$$

$$q - m\gamma = 2\gamma - 2\gamma^2 - \{(-4h^2 + 8\gamma^2 - 4h\gamma - 6\gamma + 6h)/[3(h - \gamma)]\}\gamma = (-2\gamma^3 - 2\gamma^2 h + 4\gamma h^2)/[3(h - \gamma)]$$

$$(m - 2)^2 = \{(-4h^2 + 8\gamma^2 - 4h\gamma)/[3(h - \gamma)]\}^2 = (16h^4 + 64\gamma^4 - 48h^2\gamma^2 + 32h^3\gamma - 64h\gamma^3)/[9(h - \gamma)^2]$$

$$(m - 2)^2 - 8(q - m\gamma) = (16h^4 + 64\gamma^4 - 48h^2\gamma^2 + 32h^3\gamma - 64h\gamma^3)/[9(h - \gamma)^2] - 8(-2\gamma^3 - 2\gamma^2 h + 4\gamma h^2)/[3(h - \gamma)] = (16h^4 + 16\gamma^4 + 96h^2\gamma^2 - 64h^3\gamma - 64\gamma^3 h)/[9(h - \gamma)^2] = (4h^2 + 4\gamma^2 - 8h\gamma)^2/[9(h - \gamma)^2]$$

$$[(m - 2)^2 - 8(q - m\gamma)]^{1/2} = (4h^2 + 4\gamma^2 - 8h\gamma)/[3(h - \gamma)]$$

$$k = \{-(m - 2) \pm [(m - 2)^2 - 8(q - m\gamma)]1/2\}/4 = \{-\{(-4h^2 + 8\gamma^2 - 4h\gamma)/[3(h - \gamma)]\} \pm \{(4h^2 + 4\gamma^2 - 8h\gamma)/[3(h - \gamma)]\}\}/4$$

$k_1 = (4h^2 - 8\gamma^2 + 4h\gamma + 4h^2 + 4\gamma^2 - 8h\gamma)/[12(h - \gamma)]$
$= (2h^2 - \gamma^2 - h\gamma)/[3(h - \gamma)] = (\gamma + 2h)/3$

$k_2 = (4h^2 - 8\gamma^2 + 4h\gamma - 4h^2 - 4\gamma^2$
$+ 8h\gamma)/[12(h - \gamma)] = \gamma$

For k_2:

$$e = (i - \gamma)/(k - \gamma) \ (undefined)$$

For k_1:

$e = (i - \gamma)/(k - \gamma) = [(h + \gamma - 2\gamma)/2]/[[(\gamma + 2h)/3]$
$- \gamma] = [(h - \gamma)/2] \times [3/(2h - 2\gamma)] = [3(h - \gamma)]/$
$[4(h - \gamma)] = 3/4$

Note 4

Using the symbology in Fig. 4, fractional contribution of the soil pool to the final mix is calculated as

$$d_n = ({}^{15}a_{Mn} - {}^{15}a_{An})/({}^{15}a_P - {}^{15}a_{An})$$

where d is the fractional contribution, n is the incubation number, ${}^{15}a$ is the enrichment of the sample, A is the initial chamber headspace, M is the final mix, and P is the soil-derived component. The enrichment of P can be found by solving for the intersection of the two rays as follows:

$$(s_1)({}^{15}a) + C_1 = (s_2)({}^{15}a) + C_2$$
$$({}^{15}a)(s_1 - s_2) = C_2 - C_1$$
$${}^{15}a = (C_2 - C_1)/(s_1 - s_2)$$

where s is slope, C is intercept (by analogy to Eq. [2a]) and the subscripts reference the two incubations.

Slope is calculated as

$$s_n = ({}^{29}x_{Mn} - {}^{29}x_{An})/({}^{15}a_{Mn} - {}^{15}a_{An})$$

where ${}^{29}x$ is the mole fraction of singly-substituted molecules.

Intercept is calculated as

$$C = {}^{29}x_{Mn} - s({}^{15}a_{Mn}) = {}^{29}x_{An} - s({}^{15}a_{An}).$$

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Chapter 3

A NOVEL METHOD FOR DIRECT DETERMINATION OF NITROUS OXIDE AND DINITROGEN FLUX FROM ^{15}N-LABELED SOIL

Summary

Field measurements of denitrification gas products are useful for studying both the contribution of nitrous oxide to global climate change and the loss of fertilizer nitrogen as nitrous oxide or dinitrogen. Understanding the relationship between N_2 and N_2O fluxes may lead to process-based models that improve prediction of N gas fluxes. We report here an analytical method that uses a single conceptual approach for independent analysis of N_2 and N_2O for the same field incubation. Sequential samples are removed from a chamber cover placed over ^{15}N-labeled soil. A small amount of each sample is analyzed for the isotopic composition of N_2, and the remainder for N_2O. Each aliquot passes through an ascarite trap to remove water and a LiOH trap to remove CO_2; N_2O is cryogenically focused in a liquid nitrogen trap. Aliquots are then carried by He through chromatographic columns to further resolve interfering masses. Finally, a triple-collector mass spectrometer detects peaks for m/z 28, 29, and 30 (N_2) or 44, 45, and 46 (N_20). The peaks are integrated and expressed as ratios relative to a laboratory standard. Flux is determined by the change in isotopic character of chamber N_2 or N_2O with time. Unlike other published methods for N_2, O_2 is separated chromatographically rather than removed chemically. For N_2O, this is the first report of a method that infers flux

directly from the change in isotopic character of headspace N_2O. Since the method is equivalent for the two denitrification products, a subsequent estimate of N_2O mole fraction (N_2O / [N_2O + N_2]) may be free of a well-known systematic error that attends such methods when gases derive from non-uniformly enriched substrate.

Introduction

The observed rate of increase in the concentration of N_2O in the Earth's atmosphere of 0.25% per year has raised concern over its contribution to global warming (IPCC 1996) and to the destruction of stratospheric ozone (Hahn and Crutzen 1982). A significant proportion of the increase in N_2O may derive from terrestrial environments (Bouwman 1990), especially as a consequence of agricultural activity (Eichner 1990, Mosier *et al.* 1998). Denitrification and nitrification are responsible for most N_2O flux from soils (Firestone and Davidson 1989) although their relative importance is still unclear (e.g. Stevens *et al.* 1997).

Microbial denitrification in soils (Paul and Clark 1996, Robertson 1999) results in variable proportions of its end-products, N_2O and N_2. The relationship between denitrifier N_2O production and total denitrification is conveniently defined as the N_2O mole fraction: N_2O / [N_2O + N_2]. The N_2O mole fraction is highly variable spatially and temporally (Letey *et al.* 1980, Hutchinson and Davidson 1993, Weier *et al.* 1993). Single estimates of N_2O mole fraction cannot be used reliably to relate denitrification and N_2O flux (Vinther 1984, Aulakh *et al.* 1992, Weier *et al.* 1993) because of the variability in mole fraction.

Improved methods of measuring N_2 and N_2O emissions from soil can foster the understanding of global N_2O emissions, loss of fertilizer N from the plant-soil system during denitrification, and the relative contributions of nitrification and denitrification to soil N_2O flux.

Direct methods for measuring fluxes of both gases are few. Chamber techniques prevail for N_2O (Mosier 1989) because its concentration is readily determined by gas chromatography using an electron-capture detector. Dinitrogen can also be quantified by gas chromatography, but owing to its abundance in the atmosphere its concentration is usually not detectably altered during chamber incubations. The acetylene inhibition technique (AIT) is often employed to measure total denitrification gas flux (e.g. Mosier and Klemedtsson 1994) since acetylene blocks reduction of N_2O to N_2 (Yoshinari and Knowles 1976). Dinitrogen flux can be determined by difference, if a control chamber or soil core is used to measure N_2O alone; however, the potential variability between chambers introduces uncertainty, which must be accommodated by adequate replication. Also, AIT may be ineffective for heavy textured soils (Arah *et al.* 1993). Furthermore, acetylene blocks nitrification at levels lower than those that inhibit N_2O reductase, which can cause denitrification to be underestimated because of substrate depletion (Bollmann and Conrad 1997). Finally, some microorganisms metabolize acetylene (Topp and Germon 1986).

An alternative method for determining N_2 flux is to label soil with $^{15}NO_3^-$ or $^{15}NH_4^-$ and analyze a time series of chamber gas samples by isotope ratio

mass spectrometry (IRMS) (Hauck and Bouldin 1961, Siegel *et al.* 1982). This method, known as the ^{15}N isotope dilution technique, is becoming more common (Mulvaney and Kurtz 1984, Mosier *et al.* 1986, Stevens *et al.* 1993). One modification involves reducing N_2O to N_2 chemically before analysis to measure total denitrification (Mosier *et al.* 1990, Arah *et al.* 1993, Mosier and Klemedtsson 1994). If total denitrification is measured, the N_2O mole fraction can be determined if N_2O flux is independently assessed (e.g. Arah *et al.* 1993). Dinitrogen flux has also been determined by radioactive (^{13}N) isotopic labeling (Firestone *et al.* 1979, Speir *et al.* 1995) but the radioactive tracer is short-lived and limited in availability.

The procedure described here estimates fluxes of N_2O and N_2 from soil independently by detecting changes in isotopic composition of headspace N_2O and N_2. Data from a field experiment are provided as an illustration. This is the first demonstration of N_2O and N_2 flux concurrently measured by equivalent methods. Similarity of method and sampling of the same headspace may reduce error in the estimate of the N_2O mole ratio.

Materials and Methods

Overview

The method may be summarized as follows. $^{15}NO_3^-$ is added in aqueous solution to soil. A headspace is confined over the soil surface and N_2O and N_2 are allowed to accumulate (e.g. by denitrification). The headspace is sampled using evacuated glass vessels fitted with stopcocks. Samples are

purified in the laboratory, and then analyzed by isotope ratio mass spectrometry.

'Isotopic character' is defined as the relative abundances of all isotopic masses of a gas (Bergsma *et al.* 1999). The shift in isotopic character of a headspace gas (either N_2 or N_2O) indicates the relative contributions (to the final mixture) of soil-derived gas and atmospheric gas initially present. Changes in isotopic character also indicate the enrichment of the soil N pool undergoing denitrification. The rate of emission can be calculated from the relative contribution of the soil source as a function of elapsed time, chamber area, headspace volume, and initial or final concentration. The initial concentration of N_2O or N_2 may be assumed, but for N_2O it is more accurate (and not very difficult) to obtain a measurement.

Vessels and Sampling

Pyrex vessels are used to collect headspace samples. The 500-mL volume of the vessels provides sufficient N_2O for analysis at ambient concentrations in air. Analysis of N_2 is performed on a subsample from each vessel, prior to N_2O analysis. The vessels are oblong, with stopcocks at each end. The stopcocks, constructed of glass barrels and pistons, are sealed with VITON O-rings and terminate in 1/4" (6.4 mm) O.D. glass tubing. Vessels are pre-evacuated to a pressure of less than 1.3 Pa. In the field, the vessels are fitted to a chamber cover using latex tubing. Samples are collected by rapidly opening the chamber-side stopcock for ~10 s.

Analysis

For isotopic characterization of N_2 and N_2O, samples are processed using a preparation system interfaced to a mass spectrometer (Figure 3.1). Sample vessels are attached to the terminus of the system using 1/4" ID CAJON Ultratorr unions. The system is then evacuated. For analysis of N_2, the sample trap is isolated from vacuum; the vessel stopcock is opened for 10 s and sample diffuses into the sample trap (0.5 m x 1/16" large internal diameter nickel tubing, coiled). The remaining sample (the majority) is reserved in the vessel for later N_2O analysis. For both gases, the sample passes through a 10 cm column of ascarite (10 mm I.D.) for removal of water, then to a 20 cm column (4 mm I.D.) of anhydrous lithium hydroxide for removal of CO_2, which has the same mass spectrum as N_2O.

For N_2O analysis, the sample trap remains open to vacuum and the complete contents of the sample vessel pass through the chemical scrubbers at a regulated rate. In contrast to analysis of N_2, however, the sample trap is chilled with liquid nitrogen to retain N_2O. The evacuation of the vessel is regulated by an electronic mass flow controller (25 mL min^{-1}) to maintain the efficiency of the sample trap. After trapping of either N_2 or N_2O, the sample trap is isolated by pneumatically operated valves (and then thawed, in the case of N_2O). A stream of He (50 psi head pressure) then carries the contents of the trap onto the column of a gas chromatograph. For analysis of N_2O, a J.W. Scientific GS-Q column is used to assure separation from CO_2. A molecular sieve column (Alltech, 8 m by 1/8" O.D., 5Å) is used to separate N_2 from CO_2, Ar,

and O_2 (oven temperature, 50° C). The column terminates at the inlet of the isotope-ratio mass spectrometer. Prior to arrival of a sample peak, the mass spectrometer analyzes a pulse of reference gas (Note 1). The ratios 2/1 and 3/1 are calculated for both the sample and reference by routines which integrate the signals from beams 1,2, and 3 (masses 44, 45, and 46 for N_2O or 28,29, and 30 for N_2). For N_2 analysis, a pressure regulator adds make-up helium (30 psi) into the flow path just before the inlet of the mass spectrometer (Figure 3.1) to dilute the N_2 for optimal peak size.

Because of the low abundance of N_2O and high abundance of N_2 in air, typical fluxes from an isotopically enriched source (e.g. soil NO_3^-) to a chamber headspace cause a marked increase in the ^{15}N enrichment of N_2O, but only mildly perturb the level of enrichment in N_2. Therefore, the potential range of isotope ratios is greater for N_2O than for N_2. The Micromass Prism mass spectrometer used in this study has a second head amplifier that is readily interchanged with the first. Initially these were both configured such that the two minor ion beams were amplified 100 times more than the major beam (resistor values $5 \cdot 10^8\ \Omega$, $5 \cdot 10^{10}\ \Omega$, and $5 \cdot 10^{10}\ \Omega$). This configuration anticipates that the minor beams will be much smaller than the major, which is usually the case for natural abundance measurements and is still the case for our analysis of N_2 in our chamber experiments. We modified our second head amplifier with the result that all three resistors were of equal but intermediate sensitivity ($1 \cdot 10^9\ \Omega$). This configuration makes no assumptions about the relative strengths of the major and minor beams and is appropriate for

enriched gases (>5%). Normally all samples in a set are analyzed for N_2 before reconfiguring the mass spectrometer for N_2O.

Data Processing

The equations of Arah (1992) were modified by Bergsma *et al.* (1999) to calculate fluxes and estimate enrichments of the gas source. Application of these equations to N_2O is completely analogous to their use for N_2. However, because of naturally occurring isotopes of oxygen, the molecular fractions $^{45}N_2O$ and $^{46}N_2O$ do not strictly correspond to the molecular fractions [$^{14}N^{15}NO$+$^{15}N^{14}NO$] and $^{15}N^{15}NO$. We have derived equations that express $^{29}(N_2)O/^{28}(N_2)O$ and $^{30}(N_2)O/^{28}(N_2)O$ as functions of $^{45}N_2O/^{44}N_2O$, $^{46}N_2O/^{44}N_2O$, $^{17}O/^{16}O$ and $^{18}O/^{16}O$; thus the equations for N_2 can be used directly for N_2O (Note 2).

Since the equations estimate *d* (the fraction of mixed gas derived from the soil source), estimation of absolute flux requires some estimate of absolute abundance. If *a* represents gas from the atmosphere (pre-existing headspace gas) and *p* represents gas derived from the soil mineral pool, then

$$d = p / [a + p]. \qquad [1]$$

Therefore

$$p = d * [a + p]\ ; \quad \text{alternatively} \qquad [2]$$

$$p = da / [1-d]. \qquad [3]$$

Since concentration and therefore actual volume of headspace N_2 hardly changes during a typical incubation,

$$[a + p] \approx a \quad \text{and therefore} \qquad [5]$$

$p \approx da.$ [6]

For N_2O flux, however, Eq. [3] must be used rather than Eq. [6]; *a* is the average abundance of N_2O in the atmosphere (~$3.1 \cdot 10^{-9}$ $L \cdot L^{-1}$) multiplied by chamber volume. Alternatively, concentration can be measured by gas chromatography at the beginning and end of an incubation, so exact values of *a* (beginning; Eq. [3]) and [*a* + *p*] (end; Eq. [2]) are known. In the field experiment described later, similar results were obtained whether Eq. [3] was used with an estimated *a*, Eq. [3] was used with a measured *a*, or Eq. [2] was used with a measured [*a* + *p*].

Verification

Tests were conducted to assess the performance of the mass spectrometer and gas purification system. To determine whether isotopic analysis is influenced by the size of the sample, replicate samples of N_2O reference gas were analyzed using the traditional head amplifier at four different intensities for the major beam, covering the range of valid sample sizes for this system (Table 3.1).

Our mass spectrometer is designed for analysis of samples having isotope ratios near natural abundance. To test for a memory effect (i.e., whether analysis of highly enriched samples potentially biased the analysis of subsequent samples by contaminating some portion of the system with residual ^{15}N) we analyzed a sample of N_2O at natural abundance (45/44 only) immediately following analysis of a highly enriched sample. Enriched samples were prepared by mixing natural abundance N_2O with $^{45}N_2O$, as described

elsewhere in this study. Samples were injected by gas tight syringe. Measured enrichments are reported in δ (per mil) notation (Table 3.2) to facilitate interpretation:

$$\delta = [(R_{SAM} / R_{STD}) -1] 1000$$

where R is the ratio of m/z 45 to 44, R_{SAM} is the sample ratio, and R_{STD} is the ratio of the standard. Since the laboratory standard is identical to the reference gas, a measurement of 0 ‰ (0 per mil) was expected for the standard gas, and the subscripts refer to the sample and the standard.

We tested the response of the mass spectrometer for linearity over a moderate range of enrichments using the traditional head amplifier. Natural abundance N_2O and purified $^{45}N_2O$ (>98% $^{15}N^{14}NO$) were mixed on a vacuum line. Natural abundance N_2O was measured with a calibrated glass bulb (~20 mL) and enriched N_2O was measured with a gas-tight syringe (0-10 μL). Gases were frozen into a 1L flask submerged in liquid nitrogen. After thawing, aliquots of mixture could be removed from the flask and analyzed, or the mixture could be refrozen for further dilution or enrichment. Aliquots (~0.3 mL) were analyzed using the preparation system described above. Results are reported as a calibration line (Figure 3.2).

The response of the mass spectrometer was tested for linearity over a wide range of enrichments using the modified head amplifier. Natural abundance N_2O, purified $^{45}N_2O$, and/or $^{46}N_2O$ were mixed in a flask in ratios of small whole numbers. In order to prevent previously observed fractionation effects at low pressure, flask pressure was increased to ~$1 \cdot 10^5$ Pa (1 atm.) by

adding He (99.999%). Replicate aliquots were removed from the flask for analysis, with highly repeatable analytical results (Table 3.3).

The N_2O analysis system was tested for long-term stability, for an effect of the presence of a LiOH column on measured isotope ratios, and for an effect of water in the LiOH column. A mixture of natural abundance N_2O, $^{45}N_2O$, and $^{46}N_2O$ was prepared in a ratio of 1:1:2 and analyzed. Three weeks later, more aliquots were analyzed using each of the following: no LiOH column, a hydrous LiOH column, and a column of LiOH dried by heating on a vacuum line overnight (Table 3.4). The between-date comparison is also a test of the stability of laboratory mixtures.

Rapid movement of gas through narrow apertures can cause fractionation. We tested whether significant fractionation occurs during filling of the Pyrex sampling vessels described previously. Three vessels were left open in the laboratory for several days, and three were evacuated and filled rapidly at the time and location that the other three were closed. N_2O in the vessels was analyzed as described, using the modified head amplifier (Table 3.5).

Laboratory Denitrification

The equations used for data processing allow calculation of the mean isotopic enrichment of the soil mineral N pool from which evolved N_2 or N_2O is derived. We conducted a laboratory experiment to verify the accuracy of calculated enrichments. Fresh soil was collected in July 1998 from a wheat plot at the Kellogg Biological Station, MI, that had not been fertilized at planting.

Water-extractable NO_3^- in this soil was ~2.8 μg · g^{-1} dry soil. Ten g of fresh sieved soil (8% moisture) were added to each of fourteen 600 mL Erlenmeyer flasks fitted with septa. Each flask also received ~1 g steel wool activated with detergent solution (as a sink for trace levels of O_2 -- Parker, 1955; cited in Kaspar and Tiedje, 1994) and 10 mL of ~1.0 mM sodium succinate. Flasks were fitted with evacuated sampling vessels using one-holed stoppers, then flushed with high purity nitrogen (via the septa, using a source needle and vent needle) and monitored for N_2O production. Quantitative consumption of native soil NO_3^- was suggested by cessation of N_2O accumulation and confirmed by selective destructive sampling for soil NO_3^-. Stock solutions of 99.93 atom percent ^{15}N-KNO_3 and natural abundance KNO_3 were prepared and mixed to give secondary solutions of ~0, 10, 20, 40, or 100% ^{15}N target enrichment. Each flask was flushed with N_2, and then received 1 mL of a secondary solution, to deliver ~5 μg N as NO_3^-. When the N_2O concentration in the flask headspace reached 1-2 ppm, samples for isotopic analysis were collected by opening the vessel stopcocks. Samples were analyzed within two days, plotted as ^{29}x vs. ^{15}a (Figure 3.3) and tabulated (Table 3.6). The process was repeated for analysis of N_2 production, using 20 g fresh soil per flask (1.4 μg NO_3^--N · g dry $soil^{-1}$), 20 mL H_2O, 1 mL of 0.1 M sodium succinate, and 1 mL of 0.1 M secondary solution. Flasks were flushed thoroughly with high purity N_2. Four blank vessels were flushed with N_2 as a reference. Evolution and subsequent disappearance of N_2O in the headspace suggested active denitrification. Anaerobic conditions were confirmed by monitoring headspace O_2 by gas

chromatography. After several days, headspaces were sampled by opening vessel stopcocks. Destructive sampling of one vessel showed ~2 ug N as NO_3^- remaining (cs. ~1.4 mg added). N_2 in sample vessels was analyzed for isotopic abundances. Average enrichments were calculated by the equations of Bergsma *et al.* (1999), using the average isotopic character of the reference flasks to represent initial headspace (Table 3.6).

Field Demonstration

In April 1998 a field of winter wheat at Kellogg Biological Station, MI was fertilized with NH_4NO_3 at a rate of 30 kg N · ha^{-1}. Fertilizer was excluded from six microplots (0.25 m^2) nested within treatment plots (32 m^2), three of which were clipped. The microplots received 99% ^{15}N-KNO_3 at a rate of 30 kg N · ha^{-1}. Aluminum frames (0.0846 m^2) were installed in each microplot as bases for gas sampling chambers. A clipped microplot was selected for intensive study.

Rain fell sporadically over the two week period following fertilization. Nine one-hour incubations were distributed around these rain events. A lid (30 cm x 30 cm x 14 cm) was placed over the selected frame (sealed with a moat of water). At the beginning and end of each incubation, gas samples were collected for analysis by gas chromatography (GC), infrared gas analysis (IRGA), and mass spectrometry (IRMS). Samples for GC and IRGA were collected by syringe to 3 mL Vacutainers (4 mL overpressure). For MS analysis, pre-evacuated glass vessels were connected to the chamber lid using latex hose and opened at appropriate intervals.

To minimize the pressure artifact at the soil surface associated with opening the evacuated vessels, "barostatic" chamber lids were used. Each barostat consists of a resealable polyethylene bag (44.4 um thick, 0.94 L capacity) and a short length of threaded pipe (I.D. 7 mm) which attaches one wall of the bag to an internal wall of the lid (using a washer and 0-ring) and vents the bag to the exterior of the lid. The bag can be opened for assembly and resealed for deployment. The pipe can be stoppered, except during sample collection, to guard against leakage due to bag failure or diffusion. Normally two barostats are used per lid, and are pressure-tested prior to each incubation. For the experiment described above, only one unstoppered bag per lid was used.

Results

Verification

The data in Table 3.1 indicate that the measured isotope ratios for N_2O reference gas do not vary appreciably with sample size on our mass spectrometer although, as expected, larger samples provide better precision. When equipped with the traditional head amplifier, the mass spectrometer demonstrates linearity over a large range of moderate enrichments (Figure 3.2) with only a slight bias in favor of the heavy isotope (slope ~1.05). Apparently our system slightly overestimates the enrichment of a natural abundance sample immediately following analysis of a highly enriched sample (Table 3.2). In our judgment, the overestimation is negligible.

Agreement of measured and calculated values was usually within 1% for high levels of enrichment using the modified head amplifier (Table 3.3). We could not find evidence that the presence of LiOH or the presence of water in the LiOH significantly affects measured isotope values of N_2O (Table 3.4; P > F for the effect of LiOH condition on $^{45}N_2O$ and $^{46}N_2O$: 0.8306 and 0.8989, respectively). Furthermore, measurements are stable across periods as long as three weeks (P > F for the effect of date on $^{45}N_2O$ and $^{46}N_2O$: 0.4102 and 0.5362, respectively). We found no evidence that sample gas is significantly fractionated upon collection using evacuated vessels (Table 3.5). Repeatability was better when gas was collected quickly by opening pre-evacuated vessels (SE: $5.5 \cdot 10^{-6}$, $2.4 \cdot 10^{-6}$ for ion beam ratios 2/1 and 3/1, respectively) than upon collection over long time intervals by diffusion of ambient air (1.4×10^{-6} and 1.9×10^{-6} for 2/1 and 3/1, respectively).

Laboratory Denitrification

The measured and predicted enrichments for N_2O and N_2 produced from labeled soil in flasks agreed well (Table 3.6). The predictions are slightly different from the 'target' enrichments because of the assumption of 0.3663% ^{15}N in natural abundance KNO_3 (i.e., equal to the ^{15}N abundance in atmospheric N_2) and a measured 0.07% ^{14}N in stock ^{15}N-KNO_3. Despite evidence of statistically significant differences (Student's *t* test), absolute differences are small: in all but one case the means of measured enrichments are within 1% (0.01) of the predicted value. These results indicate the lower limit of the

accuracy of our method and eliminate systematic error as a possible explanation for unusual field results.

In the case of N_2O, the laboratory denitrification experiment provides a test of an important assumption: namely, that microbial denitrification of a uniform soil N pool results in an equilibrium mixture of masses (that is, N atoms with masses 14 and 15 are paired in a statistically random fashion, such that the abundances of singly-, doubly- or un-labeled molecules in the product pool can be predicted from the enrichment of the substrate; see Note 3). Since no headspace N_2O was initially present, sampled N_2O was entirely from a soil source with presumably uniform enrichment. Under these circumstances, the sampled gas should be in equilibrium. Figure 3.3 shows nine samples (three replicates at three enrichments) plotted with the equilibrium curve (see Bergsma *et al.* 1999). Since the data points lie very near the curve, the equilibrium assumption for microbial denitrification appears to be supported. Previous studies have tested the equilibrium character of N_2O by comparing ^{15}N content calculated from ^{45}R and ^{46}R (Stevens *et al.*, 1997; 1998a; 1998b). Here, information from ^{45}R vs. ^{46}R is combined to calculate isotopic character, a two-dimensional quantity, which can be compared to the equilibrium curve, a two-dimensional reference.

Field Demonstration

The labeled soil experiment (April 1998) is a field application of the analytical methods described above. Nitrous oxide and dinitrogen fluxes showed similar temporal trends; however, during the first two days of the

experiment N_2O and N_2 fluxes generally increased and decreased, respectively (Figure 3.4). The associated rise in N_2O mole fraction ($N_2O/[N_2O + N_2]$, Figure 4.5) is counter-intuitive: accumulation of soil water due to precipitation should decrease soil redox status and favor production of N_2. Enrichment of the soil pool undergoing denitrification was estimated non-destructively by analysis of the shift in isotopic character of headspace gases during each incubation (Hauck and Bouldin 1961, Bergsma *et al.* 1999). The apparent enrichment, based on N_2O isotope data, dropped from 0.82 to 0.72 during the 4 day period, suggesting that significant nitrification was occurring. Soil pool enrichment estimated from N_2 data was considerably more variable, perhaps reflecting lower sensitivity for N_2 (see precision and detection limits, below). Agreement of mass spectrometric and gas chromatographic flux estimates for N_2O was good (Figure 3.4), with 5 values of the MS/GC ratio between 94% and 107%, and three values between 66 and 77%. Differences could be due to analytical error, to non-uniform enrichment of the soil mineral N undergoing denitrification (cf. Mulvaney *et al.* 1988), or to other sources of N_2O production (e.g. Robertson and Tiedje 1987, Stevens *et al.*, 1998b).

Precision and Detection Limits

There is not as yet a universally recognized method for calculating detection limits when denitrification fluxes are quantified from shifts in isotopic character. However, means and standard deviations of ion ratios 2/1 and 3/1 (the primary output of isotope ratio mass spectrometers) are easily compared (Table 3.7). Precision for 2/1 (N_2) in this study is comparable to that in previous

reports, and for 3/1 is less. Precision for N_2O and N_2 in this study are similar. Mean 3/1 ratios for N_2 are substantially higher than those reported by others. This is probably an artifact of high mass 30 background due to the formation of NO in the spectrometer source (Stevens et al 1993). *Post hoc* correction of data represented in Figure 3.4 using a representative calibration curve lowered the mean N_2 flux by ~4%.

Detection limit has been defined as three times the standard deviation of the blank (Miller and Miller, 1988, cited in Stevens *et al.* 1993). Interpreting this to mean that ratios 2/1 and 3/1 in end-of-incubation samples must exceed initial ratios by three standard deviations (as tabulated), we calculated minimum detectable flux independently for 2/1 and for 3/1, and then selected the larger of the two. Detection limit is a function of analytical precision, chamber volume, chamber area, enrichment of the soil pool, and duration of the incubation. For the field demonstration above, headspace was ~14 L, area was 0.0846 m^2, enrichment averaged 0.77 ^{15}N, and duration was ~1 h; consequently our estimated detection limit for N_2 is 216 $g \cdot ha^{-1} \cdot d^{-1}$. This is larger than reported estimates of 5 $g \cdot ha^{-1} \cdot d^{-1}$ (Siegel *et al.* 1982) and 12 $g \cdot ha^{-1} \cdot d^{-1}$ (Stevens *et al.* 1993), but not directly comparable because sampling configurations were different. For N_2O, our detection limit was $2.72 \cdot 10^{-4}$ g N_2O-$N \cdot ha^{-1} \cdot d^{-1}$, which is equivalent to a minimum detectable change in headspace concentration of $6 \cdot 10^{-12}$ $L \cdot L^{-1}$. Stevens *et al.* (1993) report a minimum detectable change in headspace concentration of $2.1 \cdot 10^{-6}$ $L \cdot L^{-1}$ for N_2O. For N_2O analysis by gas chromatography, assuming a nominal CV of 1%

(perhaps optimistic), minimum detectable concentration change is ~ $1 \cdot 10^{-8}$ L · L^{-1}.

Discussion

Our results provide evidence that flux of N_2O and N_2 from ^{15}N-labeled soil can be reliably measured by isotopic analysis of chamber headspace gases using mass spectrometry to directly analyze N_2O and N_2 after chemical, cryogenic, and chromatographic purification. We verified that our analytical technique gives reasonably accurate, precise, and unbiased results. The field data show that our analytical technique can be an integral part of a complete experimental system. Although similar methods exist for N_2, flux of N_2O has never been analyzed in this way before. Usually N_2O is reduced to N_2 and analyzed in a mixture with sample N_2 (Mosier *et al.* 1990) or laboratory standard N_2 (Mulvaney and Kurtz 1982). Our method for N_2O gives lower detection limits and coordinates well with the ^{15}N dilution technique for N_2 because it involves the same assumptions regarding homogeneity of the soil mineral pool.

An isotopic method for measuring total N_2 flux from soil has been available for three decades (Hauck and Bouldin 1961) but it has only recently been exploited (e.g. Siegel *et al.* 1982, Mulvaney and Kurtz 1984, Stevens *et al.* 1993). Our technique for estimating N_2 flux differs in some details from those published. The conventional approach for purifying N_2 involves removing condensibles (e.g. CO_2) in a cold trap and removing oxygen with chemical traps or hot copper (e.g. Siegel *et al.* 1982, Mosier and Klemedtsson 1994). Boyd *et al.* (1994) claim superior convenience and efficiency for a mixture of

CaO granules and Cu for purifying nanomole quantities of N_2 (mainly removing CO_2 and O_2, respectively). We remove water and the majority of CO_2 using chemical and cold traps, and separate O_2, Ar, and trace CO_2 and CO from N_2 on a molecular sieve column during continuous flow mass spectrometry. Our purification system is efficient and inexpensive, and requires very little maintenance. The conceptual approach to the flux calculation is in principal the same as originally proposed (Hauck and Bouldin 1961) and uses the equations and notation of Arah (1992) as modified by Bergsma *et al.* (1999).

In early studies of N_2 flux, ^{15}N-labeled fertilizer was added to soil and evolved gas was evaluated for mass ratio 29/28 (e.g. Rolston *et al.* 1978, 1982). One limitation of this approach is that only the N_2 flux derived from fertilizer is determined, and not any flux derived from native soil mineral N (see Mosier and Klemedtsson 1994). Another way of describing this limitation is that the isotope ratio 29/28 does not completely characterize N_2 when the sample is a mixture of atmospheric N_2 and soil-derived N_2 of different enrichment; the ratio 30/28 must also be measured (Hauck *et al.* 1958) since such mixtures are not in isotopic equilibrium. Hauck and Bouldin (1961) showed that measurement of the ratio 30/28 allows calculation of the average enrichment of the N pool experiencing denitrification, which in turn allows a calculation of total N_2 flux (whether from native soil N, label, or both). The principle assumption of this approach is that flux derives from a single, uniformly labeled pool.

The N_2O molecule, like the N_2 molecule, can be singly or doubly substituted with respect to ^{15}N (giving masses 44, 45, and 46 instead of 28, 29, and 30). Therefore, it is possible, as we have shown, to estimate flux of N_2O by measuring shift in isotopic character (as for N_2). This has not been performed until now, probably because isotope ratio mass spectrometers, although more precise than gas chromatographs, may not have been sufficiently sensitive. In the past, as much as 0.1 mg N was needed for analysis, as compared to our 0.2 µg; see Mulvaney and Kurtz, 1982; 1985).

When measuring soil denitrification by chamber methods, it is usually of interest to determine both N_2 flux and N_2O flux. Five methods have been previously reported for determining N_2O flux from soil otherwise labeled for N_2 determination. Some researchers measure N_2 flux using the shift in isotopic character ("Hauck technique") and N_2O flux by gas chromatography (Mosier *et al.* 1986). Others measure N_2O by GC and [N_2O + N_2] by the Hauck technique, reducing N_2O over hot copper and thereby letting it mix with sample N_2 before analysis (Mosier *et al.* 1990, Arah *et al.* 1993). Third, some researchers calibrate their isotope ratio mass spectrometer so that the concentration of N_2O can be derived from the sum of ion currents above baseline for masses 44, 45, and 46 (Stevens *et al.* 1993). A fourth approach is to trap N_2O from the sample, mix it with a known quantity of standard N_2, and then reduce the N_2O to N_2 (Mulvaney and Kurtz 1982, Mulvaney and Kurtz 1984, Mulvaney and Vanden Heuvel 1988). Finally, a linear mixing equation can estimate N_2O flux if the

enrichments of the soil mineral N, the label, and the evolved N_2O have been independently determined (Brooks *et al.* 1993).

Our method for quantifying flux of N_2O has advantages relative to the five methods outlined above. The first three methods of flux estimation depend on a change in headspace concentration, a measure of net flux, whereas isotope methods generally measure gross fluxes (see discussion in Hart *et al.* 1994). Net positive flux of N_2O is only the same as total flux if significant quantities of N_2O are not consumed during incubation; consumption of N_2O has not been widely tested, and may be especially important under soil covers, within which N_2O concentration (and therefore likelihood of consumption) is increasing. The fourth and fifth methods are isotopic approaches, but assume negligible background N_2O in the chamber headspace. The atmospheric background is indeed small ($\sim 3.1 \cdot 10^{-9}$ L $\cdot$ L^{-1}) but negligible only if concentration due to flux is several orders of magnitude greater. Clearly a method that does not assume negligible background is potentially more sensitive. Our method requires larger samples (500 mL) and longer analysis times (40 min/sample) than those reported for automated methods (Brooks *et al.* 1993, Stevens *et al.* 1993) but sensitivity is greatly enhanced, no destructive sampling of soil is required (in contrast to the fifth method above), and gross flux rather than net flux is estimated.

In addition to the general advantages above, our method for determination of N_2 and N_2O carries specific advantages for determination of the N_2O mole ratio during denitrification. (1) Because both N_2 and N_2O are

measured by shifts in isotope ratios rather than changing concentrations, both flux estimates represent gross rather than net flow from the soil surface, resulting in an internally-consistent ratio. (2) When the soil mineral pool is not uniformly enriched, flux of N_2 is underestimated (see Boast *et al.* 1988, Vanden Heuvel *et al.* 1988, Arah 1992, Bergsma *et al.* 1999). To the extent that the same mineral N pool is the source for both N_2O and N_2, proportional underestimation of both will be similar when equivalent methods are used. Therefore the calculated ratio of the two fluxes will be relatively independent of this source of error. (3) Unlike the acetylene inhibition technique, the method described here and the others outlined above allow fluxes of both N_2 and N_2O (and therefore the N_2O mole ratio) to be determined from a single experimental unit, thus reducing statistical uncertainty due to natural variability among control and experimental units. The statistical need for replication is reduced.

While any given method for determining N_2O flux may have its advantages, important gains are often made by coordinating multiple methods. Mulvaney (1988) measured N_2O by GC and by MS to test the assumption that N_2O was derived from a uniformly labeled pool of soil N. Arah *et al.* (1993) used both GC and MS methods to test the suitability of acetylene inhibition for measuring denitrification in heavy-textured soils. Stevens *et al.* (1997) employed concentration and isotope distribution data from mass spectrometry to examine relative contributions of nitrification and denitrification to N_2O flux. As noted above, GC values that are lower than MS values may constitute evidence of concurrent production and consumption of N_2O by soil. As the

precision and convenience of these methods improve, so does the potential for characterizing fundamental controls on denitrification dynamics. Such improvements should lead to a more comprehensive perspective on regional and global N budgets, in addition to local insight regarding soil N cycling.

Notes

Note 1. For N_2O analysis, a well-characterized laboratory standard is used. For N_2 analysis, ~150 µL of $^{30}N_2$ are mixed with ~20 mL of laboratory standard to improve the stability of 30/28, since natural abundance N_2 has very little mass 30. The absolute abundances of m/z 28, 29, and 30 are measured manually: voltage shifts are used to sequentially place each mass in the same collector for three replicate cycles. After correction for background readings, the ratios of the means are calculated and used later to interpret the analytical data for the samples analyzed with this reference.

Note 2. Let x, y, and z represent the fractional abundances of $^{28}(N_2)O$, $^{29}(N_2)O$, and $^{30}(N_2)O$. Let r, s, and t represent the fractional abundances of $^{44}N_2O$, $^{45}N_2O$, and $^{46}N_2O$. Let c, d, and e represent the fractional abundances of ^{16}O, ^{17}O, and ^{18}O. That the mass spectrometer measures s/r and t/r, although y/x and z/x are of interest. Now,

$r = xc,$

$s = yc + xd$ and

$t = zc + yd + xe.$

Then

$s/r = [yc + xd] /[xc] = y/x + d/c$ and

$t/r = [zc + yd + xe]/[xc] = z/x + [yd]/[xc] + e/c.$

Thus,

$y/x = s/r - d/c$ and

$z/x = t/r - y/x * d/c - e/c.$

In terms of simple mass ratios,

$[^{29}(N_2)O/^{28}(N_2)O] = [^{45}N_2O/^{44}N_2O] - [^{17}O/^{16}O]$ and

$[^{30}(N_2)O/^{28}(N_2)O] = [^{46}N_2O/^{44}N_2O] - [^{29}(N_2)O/^{28}(N_2)O] * [^{17}O/^{16}O] - [^{18}O/^{16}O].$

In shorthand,

$^{29}R = {}^{45}R - {}^{17}R$ and

$^{30}R = {}^{46}R - {}^{29}R{}^{17}R - {}^{18}R.$

Literature values for $[^{17}O/^{16}O]$ and $[^{18}O/^{16}O]$ are used.

Note 3. Use of the term "equilibrium" in discussions of the ^{15}N isotope dilution technique is related to its classical use in discussions of chemical reactions. The reaction

$$^{28}N_2 + {}^{30}N_2 <--> 2\,[\,{}^{29}N_2\,]$$

proceeds spontaneously in both directions, but at a negligible rate because of high activation energies. Thus, the different molecular-mass fractions of an N_2 sample do not normally equilibrate, except perhaps at geologic time scales. The reaction rates increase, of course, if the sample is heated (> 1000 ° C) or in the presence of some other form of energy (e.g. microwave). At chemical equilibrium, the relative concentrations of products and reactants no longer change. The relative proportions of the three mass fractions is then approximately that which is predicted from the composite ^{15}N abundance of the

whole system (neglecting fractionation effects). Any sample meeting this criterion may be said to be in equilibrium, even if no "equilibration" has occurred.

Table 3.1. Test for stability of measured ratios with varying sample size. Variations were simulated by adjusting the strength of the major beam for analysis of N_2O laboratory standard gas. 2/1 and 3/1 refer to m/z ratios 45/44 and 46/44, respectively. SE is standard error, n is number of samples.

major beam (Amps)	2/1 mean	SE	3/1 mean	SE	n
$2.60 \cdot 10^{-9}$	$7.858 \cdot 10^{-3}$	$3.2 \cdot 10^{-6}$	$2.109 \cdot 10^{-3}$	$2.9 \cdot 10^{-6}$	3
$4.43 \cdot 10^{-9}$	$7.853 \cdot 10^{-3}$	$2.0 \cdot 10^{-6}$	$2.102 \cdot 10^{-3}$	$2.0 \cdot 10^{-6}$	4
$1.30 \cdot 10^{-8}$	$7.857 \cdot 10^{-3}$	$3.0 \cdot 10^{-7}$	$2.106 \cdot 10^{-3}$	$8.5 \cdot 10^{-7}$	3
$1.79 \cdot 10^{-8}$	$7.858 \cdot 10^{-3}$	$4.0 \cdot 10^{-7}$	$2.106 \cdot 10^{-3}$	$4.2 \cdot 10^{-7}$	3

Table 3.2. Test for a memory effect during analysis. Highly enriched N_2O was prepared by mixing various quantities of labeled and unlabeled N_2O. Analysis of laboratory standard N_2O immediately followed each analysis of enriched gas. Since the laboratory standard is the same as the reference gas, a value of 0 ‰ is expected if there is no memory effect.

date	enriched sample (‰)	standard sample (‰)
1	$223 \cdot 10^3$	16
1	$532 \cdot 10^3$	81
1	$577 \cdot 10^3$	59
2	$540 \cdot 10^3$	76
2	$517 \cdot 10^3$	43
3	$414 \cdot 10^3$	17
3	$279 \cdot 10^3$	37

Table 3.3. Summary of analyses of high-enrichment laboratory mixtures of N_2O. 44:45:46 represents the mixing ratio of natural abundance N_2O, $^{45}N_2O$, and $^{46}N_2O$. Predictions ^{15}a and $^{45}N_2O$ adjust for gas purity. ^{15}a is the atom fraction of ^{15}N in the sample, consistent with the notation of Arah *et al.* (1992). $^{45}N_2O$ is the molecular fraction of mass 45 in the sample. Measured ^{15}a and $^{45}N_2O$ are means for all samples where number of samples n is more than 1.

44:45:46	predicted ^{15}a	measured ^{15}a	predicted $^{45}N_2O$	measured $^{45}N_2O$	n
1:0:1	0.5022	0.5057	0.0040	0.0045	1
1:1:0	0.2499	0.2658	0.4937	0.5085	1
1:1:1	0.5013	0.5029	0.3283	0.3278	5
1:1:2	0.6265	0.6267	0.2460	0.2532	2

Table 3.4. Test for effects of the LiOH water trap, water in LiOH, and time on measured enrichment of a 1:1:2 mixture of natural abundance N_2O, $^{45}N_2O$, and $^{46}N_2O$. 'Hydrous' refers to hydrous LiOH used as a chemical trap, 'dried' refers to hydrous LiOH dried on a vacuum line overnight. $^{45}N_2O$ and $^{46}N_2O$ represent the mean calculated molecular fraction for N_2O of masses 45 and 46. SE is standard error.

Date	LiOH	$^{45}N_2O$	SE	$^{46}N_2O$	SE	n
1	dried	0.25318	0.00011	0.50011	0.00009	2
2	dried	0.25326	0.00026	0.49961	0.00048	3
2	hydrous	0.25320	0.00018	0.49980	0.00038	3
2	none	0.25310	0.00011	0.49998	0.00028	4

Table 3.5. Test for an effect of collecting sample gas quickly using pre-evacuated vessels. Air samples were collected by diffusion into open vessels or by rapid filling of pre-evacuated vessels when stopcocks were opened. Samples were analyzed for N_2O using the unmodified head amplifier. 2/1 and 3/1 are m/z ratios of 45/44 and 46/44, respectively. Means ± standard errors are reported; n is 3. P > |t| is the significance level for Student's *t* test of differences in means. Least significant number (LSN) is the smallest number of samples needed to demonstrate significant differences in means at a confidence level of $\alpha = 0.05$.

Ratio	Diffusion	Pre-evac	P >|t|	LSN
2/1	$7.835 \cdot 10^{-3} \pm 5.5 \cdot 10^{-6}$	$7.830 \cdot 10^{-3} \pm 1.4 \cdot 10^{-6}$	0.3965	28
3/1	$2.133 \cdot 10^{-3} \pm 2.4 \cdot 10^{-6}$	$2.129 \cdot 10^{-3} \pm 1.9 \cdot 10^{-6}$	0.2602	16

Table 3.6. ^{15}N enrichment of soil NO_3^- pools for laboratory denitrification experiments, calculated and measured . Calculations are based on the enrichments and mixing ratios of natural abundance KNO_3^- and highly enriched KNO_3^- (99.93 atom %). Measurements use the equations of Bergsma *et al.* (1999). 'n' is number of samples. Means ± standard errors are reported for separate experiments measuring N_2 and N_2O. P>|t| is the significance level for Student's *t* test.

calculated	N_2: measured	P > |t|	N_2O: measured	P > |t|	n
0.1032	0.1072 ± 0.0053	0.531	0.1026 ± 0.0003	0.210	3
0.2028	0.1969 ± 0.0031	0.199	0.2007 ± 0.0013	0.0336	3
0.4019	0.3827 ± 0.0007	0.001	0.3976 ± 0.0012	0.068	3
0.9993	0.9692 ± 0.0020	0.042	(ratio out of range)		2

Table 3.7. Comparison of spectrometer precision by study. Precision is reported for analysis N_2 or N_2O. 2/1 refers to m/z 29/28 or 45/44. 3/1 refers to 30/28 or 46/44, except as noted. STD is standard deviation and n is number of samples.

Citation	gas	ratio	mean	STD	CV	n
Siegel *et al.* 1982	N_2	2/1	$7.35 \cdot 10^{-3}$	$9.3 \cdot 10^{-7}$	$1.2 \cdot 10^{-4}$	15
Siegel *et al.* 1982	N_2	3/1	*$1.34 \cdot 10^{-5}$	$2.7 \cdot 10^{-7}$	$2.0 \cdot 10^{-2}$	15
Stevens *et al.* 1993	N_2	2/1	$3.50 \cdot 10^{-3}$	$5.3 \cdot 10^{-6}$	$1.5 \cdot 10^{-3}$	7
Stevens *et al.* 1993	N_2	3/1	$1.01 \cdot 10^{-5}$	$5.3 \cdot 10^{-7}$	$5.3 \cdot 10^{-4}$	7
this study	N_2	2/1	$7.22 \cdot 10^{-3}$	$4.8 \cdot 10^{-7}$	$6.7 \cdot 10^{-5}$	12
this study	N_2	3/1	$1.56 \cdot 10^{-4}$	$1.2 \cdot 10^{-6}$	$7.7 \cdot 10^{-3}$	12
this study	N_2O	2/1	$7.83 \cdot 10^{-3}$	$2.4 \cdot 10^{-6}$	$3.1 \cdot 10^{-4}$	3
this study	N_2O	3/1	$2.13 \cdot 10^{-3}$	$3.3 \cdot 10^{-6}$	$1.6 \cdot 10^{-3}$	3

*Siegel *et al.* measured 30/[29 + 28].

Figure 3.1. Gas purification system connected to mass spectrometer. Valves are configured such that during purification, He bypasses the cold trap and travels onto the GC column, while sample passes into the cold trap (N_2) or through the cold trap and then to vent via the mass flow controller (N_2O). During analysis, helium passes through the cold trap and onto the GC column, while the sample vessel remains open to vacuum via the mass flow controller. During N_2 analysis the sample trap is not chilled. Different columns for N_2 and N_2O are used. Interface helium is used only for N_2 analysis. The Penning valve passes about 10% of the gas stream to the spectrometer.

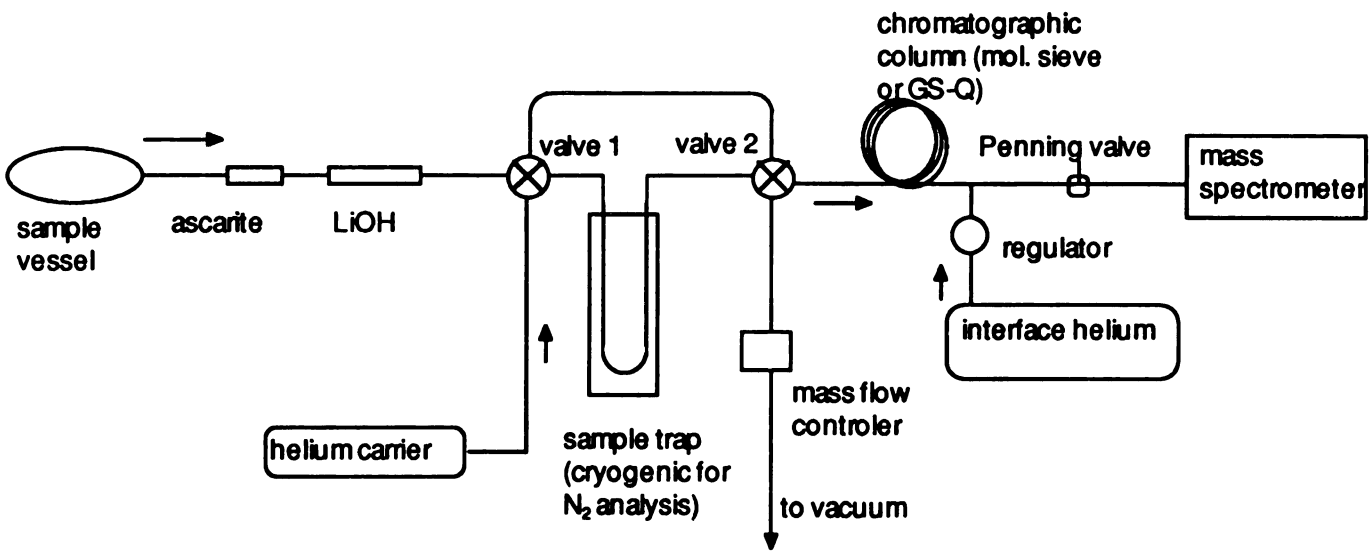
chromatographic
column (mol. sieve
or GS-Q)
valve 1
valve 2
Penning valve
mass
spectrometer
sample
vessel
ascarite
LiOH
regulator
interface helium
mass flow
controler
helium carrier
sample trap
(cryogenic for
N_2 analysis)
to vacuum

Figure 3.1.

Figure 3.2. Measured vs. calculated molecular fraction of $^{45}N_2O$ for moderate enrichments of N_2O, prepared volumetrically in the laboratory from enriched and unenriched standards; analyzed with the unmodified head amplifier.

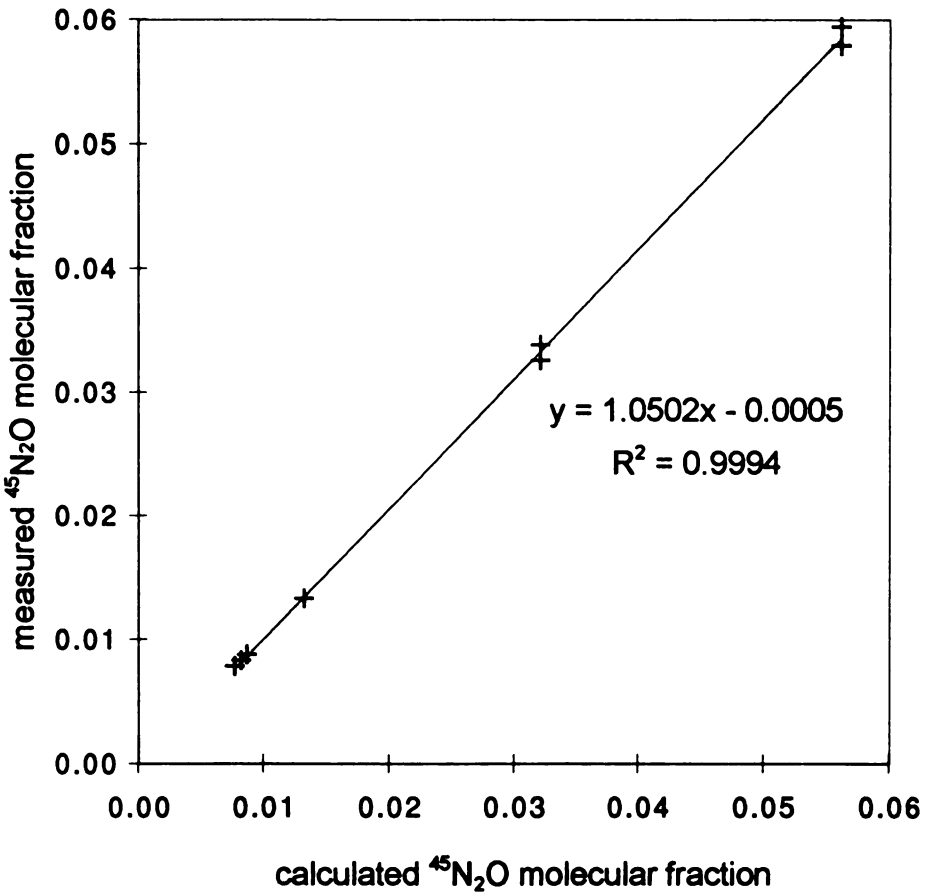
0.06
0.05
0.04
0.03
0.02
0.01
0.00
measured $^{45}N_2O$ molecular fraction
y = 1.0502x - 0.0005
R^2 = 0.9994
0.00
0.01
0.02
0.03
0.04
0.05
0.06
calculated $^{45}N_2O$ molecular fraction

Figure 3.2.

Figure 3.3. Isotopic character of nine N_2O samples from laboratory denitrification of prepared $^{15}NO_3^-$ label, plotted against the equilibrium curve. As expected, these samples are in equilibrium. Vertical bars intersect the curve at predicted enrichments, open circles represent measured values.

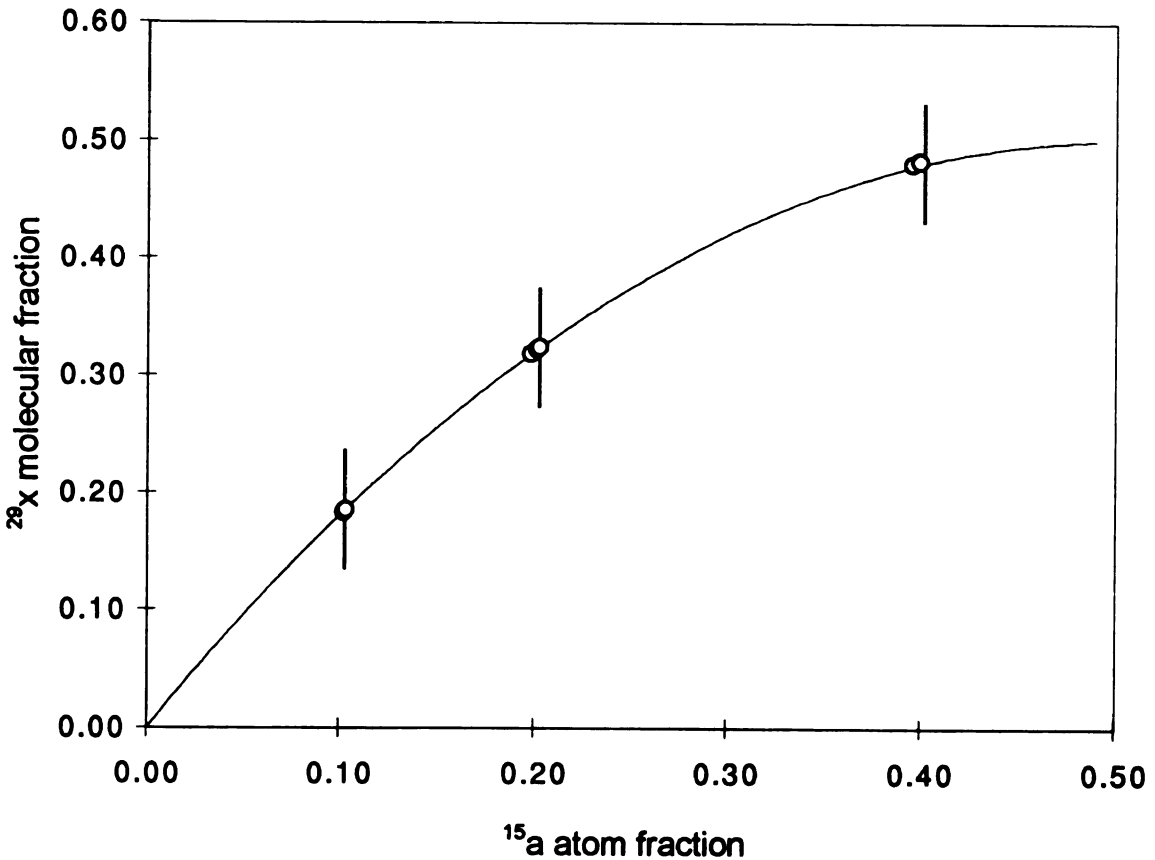
0.60
0.50
0.40
0.30
0.20
0.10
0.00
^{29}x molecular fraction
0.00
0.10
0.20
0.30
0.40
0.50
^{15}a atom fraction

Figure 3.3.

Figure 3.4. N_2 and N_2O fluxes with cumulative precipitation for nine field incubations over the same highly labeled plot.

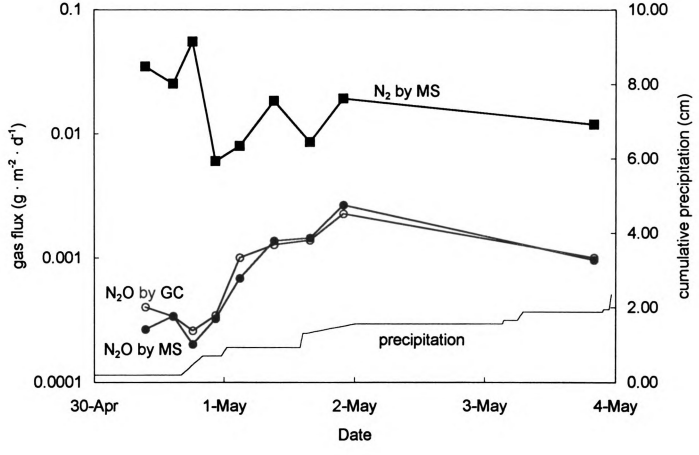
0.1
0.01
0.001
0.0001
gas flux (g · m-2 · d-1)
10.00
8.00
6.00
4.00
2.00
0.00
cumulative precipitation (cm)
N2 by MS
N2O by GC
N2O by MS
precipitation
30-Apr
1-May
2-May
3-May
4-May
Date

Figure 3.4.

Figure 3.5. Estimated soil enrichment, comparison of MS and GC, and N_2O mole fraction for nine incubations represented in Figure 3.4.

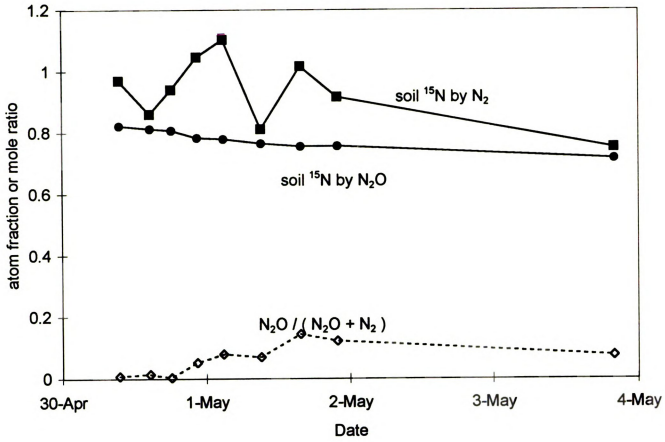
1.2
1
0.8
0.6
0.4
0.2
0
atom fraction or mole ratio
soil ^{15}N by N_2
soil ^{15}N by N_2O
N_2O / (N_2O + N_2)
30-Apr
1-May
2-May
3-May
4-May
Date

Figure 3.5.

Chapter 4

NITROUS OXIDE MOLE FRACTION DURING DENITRIFICATION IN SOIL: RESPONSE TO RECENT MOISTURE HISTORY VARIES AMONG ECOSYSTEMS

Summary

Very little is known concerning the effects of recent moisture history (antecedent moisture regime) and of ecosystem differences on the relative proportion of N_2O and N_2 produced during denitrification (N_2O mole fraction). We conducted laboratory incubations of sieved soil from cropped and successional ecosystems under two moisture histories. The soils were pedogenically identical but had been managed differently for the past decade. Fresh soils were air-dried, re-packed, and amended with nitrate, glucose, and sufficient water (about 85% water-filled pore space) to stimulate denitrification. One set of incubations received 80% of prescribed water 2 d before incubation and the remaining water at the start of the incubation; the other set of incubations received all water at the start of the incubation. Production of nitrous oxide and dinitrogen was estimated using acetylene inhibition (measuring resultant N_2O by gas chromatography) and also by ^{15}N isotope dilution (characterizing headspace samples by isotope ratio mass spectrometry). The response of N_2O mole fraction to recent moisture history varied by ecosystem. Mean N_2O mole fractions (N_2O / [N_2O + N_2]) measured using acetylene inhibition were 0.36 and 0.90 for cropped pre-wet and control soils, respectively, and were 0.34 and 0.33 for successional pre-wet and

control soils. Isotope data for N_2O showed that in most cases, the soil NO_3^- pool undergoing denitrification was nearly uniform in its N isotopic composition. ^{15}N isotope dilution consistently gave estimates of N_2 production that were about one-third of estimates by acetylene inhibition, suggesting that most of the N_2 came from an unlabeled source. Explicit recognition of ecosystem differences in response of N_2O mole fraction to recent moisture history may improve modeled estimates of global N_2O flux.

Introduction

The proportion of denitrification end product that is nitrous oxide (N_2O mole fraction) is an important aspect of the global budget of N_2O, a significant greenhouse gas (IPCC 1996) and regulator of stratospheric ozone (Hahn and Crutzen 1982). A major source of N_2O is microbial denitrification in soil, which produces dinitrogen and nitrous oxide in proportions that vary widely (Tiedje 1988, Robertson 1999). Many factors are recognized as influencing the N_2O mole fraction, including soil moisture, nitrate or nitrite concentration, pH, aeration, temperature, carbon availability, enzyme status, and moisture history (Colbourn and Dowdell 1984, Sahrawat and Keeney 1986, Firestone and Davidson 1989, Arah and Smith 1990, Bouwman 1990, Aulakh *et al.* 1992, Hutchinson and Davidson 1993). However, few experimental studies have considered the influence of moisture history on the nitrous oxide mole fraction (e.g. Dendooven and Anderson 1995, Dendooven *et al.* 1996) and we know of none that has looked for an interaction of moisture history and ecosystem management history. Since most N is probably lost from soils during brief

periods following irrigation or rainfall (Smith and Tiedje 1979, Sextone *et al.* 1985, Rolston *et al.* 1982, Mummey *et al.* 1994, see also Davidson 1991 and references therein) dependency of the N_2O mole fraction on short-term soil moisture history could have large consequences for the relationship between nitrous oxide production and total denitrification. The study of N_2O mole fraction is hampered primarily by the difficulty of analyzing N_2 flux from soil.

In this study, we estimated nitrous oxide mole fraction for incubations of soil from two ecosystems (row crop agriculture and early native succession field) and for two recent moisture histories in a factorial design. Our primary objectives were to determine the effect of recent moisture history on N_2O mole fraction and to determine whether the effect can vary among ecosystems. A secondary objective was to compare the use of the ^{15}N isotope dilution method with the acetylene inhibition method for estimating N_2O mole fraction.

Materials and Methods

Soil collection and processing

Soils (Kalamazoo/Oshtemo soil series; Austin 1979) were collected from the Long-term Ecological Research site at the W. K. Kellogg Biological Station, Hickory Corners, Michigan, 42° 24' N, 85° 24' W. The soils at this site are Typic Hapludalfs (fine-loamy, mixed, mesic) derived from glacial till that was deposited about 10,000 years ago. The ongoing LTER experiment at KBS is a randomized complete block design with 6 replicate blocks and 7 treatments on the main site, for a total of 42 1-ha plots. We sampled three replicates of two treatments: a high-input corn-wheat-soybean rotation and a

native succession treatment last plowed in 1988. The annual cropping system is tilled and receives conventional applications of fertilizer and pesticides. The successional treatment is managed only by occasional burning and/or removal of woody biomass.

Soil was collected in December 1999 from blocks 4-6 on the LTER site. For each of 6 plots (two treatments x 3 replicate blocks), 4 soil cores (2 cm diameter by 16 cm depth) were collected at each of 5 semi-permanent sampling stations. Soil was bulked by plot, sieved (4 mm mesh), air-dried for several weeks (to about 1% gravimetric moisture), and stored in bags at room temperature ("stock soil") until the start of the experiment. Due to analytical limitations, soil from the two ecosystems was tested on separate dates, four weeks apart. Stock soil was tested on both dates for nitrate and ammonium availability by KCl extraction (1M) followed by analysis using an Alpkem auto-analyzer.

Experiment and treatments

We incubated soil from each ecosystem for 24 hours in 1 L glass mason jars. Each jar received 150 g dry soil from one of three field-level replicates, packed to a volume of 125 mL (± ~5%) for a target bulk density of 1.2 g dry soil · cm^{-3}. Each jar within a replicate set was assigned to one of two moisture histories (pre-wet or control) and one of 4 sampling strategies (^{15}N-labeled soil, unlabeled soil, acetylene-amended soil, or soil for mineral N analysis). Two additional jars were established without soil to serve as blanks for gas analysis, for a total of 26 jars per ecosystem.

All soils received 9.75 mg of KNO_3 (about 9 μg NO_3^--N · g dry soil^{-1}), 20 mg glucose (about 53 μg C · g dry soil $^{-1}$), and 56.6 mL deionized water (for a target water-filled pore space of ~85%). "Pre-wet" soils received 80% of their prescribed water 48 hours before the start of the incubation, with remaining water reserved as a vector for nitrate and glucose. "Control" soils received all of their water and nutrients as a single solution at the start of the incubation. Blank jars received only 56.6 mL water (no soil). All soils received nitrate and glucose immediately prior to the start of the incubation. Solutions were delivered as a slow trickle down the edge of a tipped jar to minimize soil disturbance and air entrapment. The delivery method produced a wetting front that moved laterally across the soil within about 15 minutes.

The labeled soils received 9.84 mg $K^{15}NO_3$, the molar equivalent of the 9.75 mg $K^{14}NO_3$ received under the other three strategies. The acetylene jars received 80 mL C_2H_2 at the start of the incubation for a 10% headspace concentration, known to inhibit nitrous oxide reductase in these soils (Robertson and Tiedje 1987). All jars were fitted with air-tight lids; rubber septa and CAJON UltraTorr unions (custom o-ring seal) were added as necessary for syringe sampling and sampling to Pyrex vessels (0.5 L, pre-evacuated, with stopcocks) for ^{15}N analysis.

Sampling and analysis

The mineral-N soils were sampled destructively for analysis of nitrate and ammonium concentrations about 2 hours after the start of the incubation (10 g soil, dry weight equivalent, extracted in 100 mL 1M KCl). N_2O

concentrations in other jars were measured by gas chromatography at 0, 6, 12, and 24 hours after the start of the incubation.

At the close of the incubation (24 h) gas samples were collected for analysis by isotope ratio mass spectrometry (^{15}N-labeled and unlabeled treatments). The vessel stopcocks were opened for about 10 s and then sealed. Analysis was performed within two weeks, using methods described elsewhere (Bergsma *et al.* submitted). For N_2O, m/z ratios 46/44 and 45/44 were measured. For N_2, ratios 30/28 and 29/28 were measured. Equations for estimating the ^{15}N enrichment of the soil mineral N pool and the fraction of headspace gas derived from the soil mineral pool (*d*) require initial and final measurements of isotopic character (Arah 1992, Bergsma *et al.* 1999). Constraints of the experiment allowed only a final sampling. Therefore, each labeled sample was paired with its corresponding unlabeled sample to represent final and initial conditions, respectively. An advantage of this pairing is that it controls for (slight) biological and mechanical artifacts that could influence isotopic character under the experimental conditions described above. To guard against bias due to a label effect, N_2 flux was calculated as $[N_2O_{acetylene} - N_2O_{labeled}]$ for comparison to isotope data, but as $[N_2O_{acetylene} - N_2O_{unlabeled}]$ for all tests of treatment effects. Differences among treatment means were tested for statistical significance by ANOVA, using JMPIN software version 3.1.5 (Sall and Lehman 1996).

Results

Experimental design

We tested whether mineral N availability in stock successional soil changed during the interval between the date on which cropped soil was incubated and the date on which successional soil was incubated. Mean extractable nitrate in stock soil from the successional ecosystem dropped slightly between the two experimental dates, from 2.2 ± 0.4 μg N · g dry soil $^{-1}$ to 1.7 ± 0.4 μg N · g dry soil $^{-1}$ (statistically significant at the 0.05 level). Mean extractable ammonium was unchanged (15.2 ± 3.2 μg N · g dry soil $^{-1}$ and 15.2 ± 2.0 μg N · g dry soil $^{-1}$).

We tested whether pre-wet and control soils differed in available mineral N two hours after the start of incubation. Mean extractable nitrate in mineral-N jars was 2.3 μg N · g dry soil $^{-1}$ lower for pre-wet cropped soils than for controls (19.5 ± 2.5 and 21.8 ± 2.2 μg N · g dry soil $^{-1}$ respectively). For successional soils, pre-wet soils were only 0.8 μg N · g dry soil $^{-1}$ lower (9.0 ± 3.3 vs. 9.8 ± 0.1). For both ecosystems, mean extractable ammonium was sharply higher in pre-wet soils relative to controls: 3.1 μg N · g dry soil $^{-1}$ higher for cropped soils (4.8 ± 0.4 vs. 1.7 ± 0.3, respectively) and 12.7 μg N · g dry soil $^{-1}$ higher for successional soils (21.4 ± 2.3 and 8.7 ± 0.7).

We tested whether N_2O production in labeled soils differed significantly from N_2O production in unlabeled soils. Final N_2O concentration for each labeled jar was divided by final N_2O concentration in the corresponding unlabeled jar. On average, the labeled:unlabeled ratio was 0.98. However, the

average differs by ecosystem: 1.12 for cropped soil and 0.85 for successional soil. For this reason, production of N_2 by acetylene inhibition was calculated from concentration data for unlabeled jars for tests of treatment effects, but from labeled jars for comparison of acetylene inhibition with ^{15}N isotope dilution.

N_2O mole fraction

The time-course of change in concentration of N_2O in all jars (except blanks, where change was negligible) is summarized in Figure 4.1. N_2O accumulated more rapidly in the acetylene-amended jars than in the others. Since acetylene inhibits the reduction of N_2O to N_2, production of N_2 by the un-amended soils may be inferred by difference (Yoshinari and Knowles, 1976). Initially, total denitrification (represented by N_2O produced in acetylene jars) was less for control soil than for pre-wet soil, but was indistinguishable by the end of the incubation. For both ecosystems, pre-wet soil produced N_2O immediately and steadily throughout the incubation. Successional control soils did not respond differently than successional pre-wet soils. However, the response of the cropped control soils was almost identical to the response of the cropped acetylene-amended soils, diverging only slightly by the end of the incubation. The near-identity suggests that cropped control soils produced significantly less N_2 than soils for the other three combinations of ecosystem and recent moisture history.

Final N_2O concentrations were used to calculate total denitrification, N_2O production, and N_2 production (by difference) as $\mu g\ N \cdot g\ dry\ soil^{-1}$ (Figure 4.2).

Results were analyzed by ANOVA. When the block effect is included in the model, successional soils (grouped) had significantly higher total denitrification than cropped soils, at the 0.05 confidence level. Cropped controls had significantly higher N_2O production and lower N_2 production ($p = 0.02$ and 0.05, respectively). Although the difference in total N_2 production for successional pre-wet and successional control soils was not significantly different, review of time-series data for the entire incubation period shows that pre-wet soils consistently led their corresponding controls in N_2 production by a small margin (data not shown).

Nitrous oxide mole fraction was calculated as $N_2O / [N_2O + N_2]$ for each combination of ecosystem, recent moisture history, and block. Analysis of the results (ANOVA) is summarized in Table 4.1. Mean N_2O mole fractions were 0.36 and 0.90 for cropped pre-wet and control soils, respectively, and were 0.34 and 0.33 for successional pre-wet and control soils. In the analysis of variance, cropped soils showed a strong effect of recent moisture history, while successional soils did not. The difference accounts for the highly significant interaction term (ecosystem x history: $p = 0.012$) that results when the entire model is considered.

Isotopic data

For N_2 or N_2O, isotopic character has been defined as the relative proportions of the three (primary) molecular fractions ($^{28}N_2$, $^{29}N_2$, $^{30}N_2$ or $^{28}(N_2)O$, $^{29}(N_2)O$, $^{30}(N_2)O$; see Bergsma *et al.*, 1999). Isotopic character is conveniently represented by a plot of 29x vs. 15a: that is, the $^{29}(N_2)$ molecular fraction vs. the

composite ^{15}N atomic fraction. Figure 4.3 gives the isotopic character of N_2 at the end of the 24 hour incubation for unlabeled (lower left) and labeled (all other) jars. Each unlabeled/labeled pair (one line segment) represents one incubation unit. There is strong tendency toward colinearity for pairs of line segments representing paired pre-wet and control incubations, indicating similar soil nitrate enrichment (consistency within replicate field plots). Lengths of segments representing cropped controls are generally shorter than those for cropped pre-wet treatments, indicating smaller flux. Successional control and pre-wet segment lengths (and therefore fluxes) are similar for replicates 4 and 5, but not 6.

Figure 4.4 shows the final isotopic character of N_2O for unlabeled and labeled jars, paired to represent incubation units. Most of the labeled incubations resulted in equilibrium mixtures of N_2O masses. Differences between lengths of pre-wet and control segments were small.

If the soil mineral pool undergoing denitrification is not uniformly labeled, the enrichment of the soil pool is an overestimate (Boast *et al.* 1988, Arah 1992, Bergsma *et al.* 1999). Since uniformity was not assumed in this study, we adopt the convention of referring to the apparent enrichment of the soil mineral pool. Apparent enrichment for each incubation is shown in Table 4.2. For both cropped and successional soils, apparent enrichment calculated from N_2 isotope data agrees strongly with that calculated from N_2O isotope data. Enrichment of the soil mineral pool was predicted from the amount of label

added and the amount of nitrate initially present (Table 4.2). Predicted enrichments agree well with the calculated enrichments.

Production of N_2 estimated by isotope methods shows the same patterns as production of N_2 estimated by acetylene inhibition (Figure 4.5). However, the isotope method gives values consistently lower than the acetylene method. For each combination of ecosystem, moisture history, and replicate, the ratio MS/GC was calculated, where MS refers to N_2 flux by mass spectrometry (^{15}N isotope dilution method) and GC refers to N_2 flux by gas chromatography using acetylene inhibition. Analysis of variance showed no effect of block, ecosystem, or moisture history, and no interaction of ecosystem and moisture history ($P > 0.4$ for all effects). With one outlier removed (from 12 total values), mean and standard error for MS/GC is 0.34 ± 0.04.

Discussion

Experimental Design

Our experiment was designed to test for an effect of recent moisture history (antecedent moisture regime) and for an effect of ecosystem differences on N_2O mole fraction during denitrification in soil. Validation of the design requires that (1) tests performed on different soils were equivalent in other respects, (2) the moisture-history treatments were equivalent in other respects, and (3) the N_2O observed was the product of denitrification.

Successional soils were tested four weeks after the cropped soils. The very small (less than 0.5 $\mu g\ N \cdot g\ dry\ soil^{-1}$) drop in extractable NO_3^--N in stock soil during that interval, and the absence of change in NH_4^+-N, suggest that no

strong artifacts were introduced by the delay. The delay itself is small compared to the initial air-dry storage time for both soils (> 8 weeks).

To test whether the two patterns of moisture addition (pre-wet vs. control) had similar consequences for soil N status, we sampled destructively for extractable NO_3^- and NH_4^+ in the mineral-N jars as soon as possible (~2 h) after the start of the incubation. Mean NO_3^--N concentrations were 1-2 μg N · g dry soil $^{-1}$ lower for the pre-wet soils, a small difference relative to the amount of N added (9 μg N · g dry soil $^{-1}$) and the amount of NO_3^--N extracted (9-21 μg N · g dry soil $^{-1}$). Extractable ammonium was sharply higher in pre-wet soils than in control soils. However, the difference is due more to a reduction of control $[NH_4^+]$, relative to background, than to the enhancement of pre-wet $[NH_4^+]$ (data not shown). Apparently significant NH_4^+ was lost (possibly through volatilization or assimilation) when water first contacted the dry soil, but was replaced thereafter via mineralization. However, NH_4^+ is not the substrate for denitrification. Furthermore, since the pattern was similar across ecosystems, this artifact does not explain the interaction of ecosystem and moisture history in our results.

Was the observed N_2O a product of denitrification? Nitrous oxide can be produced by nitrification and dissimilatory nitrate reduction to ammonium as well as by denitrification (Tiedje 1988). However, nitrification is an aerobic process, for which optimum moisture ranges between 30% and 70% water-filled pore space (Davidson 1991). Many studies address the partitioning of N_2O production by source, including nitrification and denitrification (e.g.

Mulvaney and Kurtz 1984, Robertson and Tiedje 1987, Klemedtsson *et al.* 1988, Skiba *et al.* 1993, Mummey *et al.* 1994, Stevens *et al.* 1997, Stevens *et al.* 1998b, Hutsch *et al.* 1999, Panek *et al.* 2000, Wolf and Russow 2000) and also dissimilatory nitrate reduction to ammonium (e.g. Stevens *et al.* 1998a). With notable exceptions (e.g. Hutchinson *et al.* 1993) denitrification is usually the major source of nitrous oxide in saturated and nearly-saturated soils. Because WFPS was about 85% for this study, we assume that N_2O was a product of denitrification. Furthermore, for a majority of the incubations, N_2O derived from soil was in isotopic equilibrium, which implicates a single, uniformly labeled soil mineral pool (Figure 4.4; see Bergsma *et al.* 1999) and strongly supports denitrification as the only important source of N_2O. Also, there was good agreement between calculated and estimated enrichments for the soil NO_3^- pool contributing to flux (Table 4.2) suggesting that N_2O derived predominantly from NO_3^-.

N_2O mole fraction

For the successional soil, the N_2O mole fraction was about one-third. The pre-wet treatment brought mean soil moisture to approximately 68% WFPS, but apparently did not greatly enhance denitrification enzyme status relative to the controls. We conclude that denitrifying enzymes, especially nitrous oxide reductase, persisted well in the successional soil during several months of air-dry conditions (< 1% gravimetric moisture). Enzyme persistence in dry soils has been observed by others (e.g. Smith and Parsons, 1985).

For the cropped soils, however, the pre-wet treatment apparently enhanced the activity of nitrous oxide reductase relative to the controls. N_2O mole fraction was also about one-third for the pre-wet soils, but about 0.9 for the (previously dry) control soils. Although total denitrification was similar for the two moisture histories, a much greater fraction of N_2O was further reduced to N_2 in the pre-wet soils. We conclude that nitrous oxide reductase did not persist well in the cropped soil when air-dry, but that its activity was significantly enhanced by 48 hours of high soil moisture, achieving levels similar to those for successional soils. Since total denitrification was only slightly less for cropped soils than for successional soils, precursor enzymes (such as nitrite reductase and nitrate reductase) may have been less affected by drying than was nitrous oxide reductase. Our observation that -- under at least some conditions -- N_2O mole fraction may be higher for the cropped soils than for the successional soils may help explain field data showing threefold greater annual flux of N_2O from the cropped system (3.5 ± 0.21 g N_2O-N $\cdot$ $ha^{-1} \cdot d^{-1}$) than from the successional system (1.1 ± 0.05 g N_2O-N $\cdot$ $ha^{-1} \cdot d^{-1}$, Robertson *et al.* 2000).

To the best of our knowledge, no other published study of N_2O mole fraction has tested for a potential interaction between ecosystem effects and moisture history effects. However, there are some reports of effects of either ecosystem or moisture history on relative proportions of N_2 and N_2O. Merrill and Zak (1992) reported an N_2O mole fraction of 0.7 to 0.9 for well-drained sugar maple forests in northern lower Michigan; in contrast, the N_2O mole

fraction in a silver maple - red maple swamp was 0.25. Dendooven *et al.* (1996) found an effect of moisture history on relative production of nitrous oxide and dinitrogen (N_2O:N_2) for pasture soil, but the difference was small: 0.54 for soil cores previously submerged for 96 hours, and 0.4 for cores submerged for 6 hours. Conversion to nitrous oxide mole fraction yields values of 0.35 and 0.29: similar to the values presented here for successional soils and pre-wet cropped soil. Mulvaney and Kurtz (1984) studied N_2O and N_2 flux for three ^{15}N-amended soils subjected to wetting and drying cycles. We calculate from their Table 4.1 an average and standard error of 0.33 ± 0.02 (n = 12), similar to the result for our successional soils: 0.33 ± 0.04 (n = 6). In a study of three N-amended soils, Jacinthe *et al.* (2000) found that N_2O mole fraction was initially 0.68, increased to 0.95 with imposition of a water table at a depth of 10 cm, and decreased to 0.35 within one week.

Our results show that the dependency of nitrous oxide mole fraction on recent moisture history can vary among ecosystems, even when the ecosystems are pedogenically identical. The large difference in response between the successional soils and the cropped soils may be related to differences in soil physical properties, soil carbon patterns, and microbial community characteristics resulting from 10 years of contrasting soil management regimes. First, the cropped soil is plowed regularly and has poorer aggregation than the successional soil. Because the soil used here was sieved (4 mm), effect of aggregate structure would have been restricted to smaller size classes of aggregates. Physical differences may have influenced

N_2O mole fraction by altering the distribution of water and anaerobic microsites, where most denitrification may occur (McConnaughey and Bouldin 1985).

Second, the response difference between the two soil types may be related to differing soil carbon patterns. Although net primary productivity in the cropped ecosystem is about double that of the successional ecosystem (mean ± standard error = 9.24 ± 1.41 vs. 4.24 ± 0.37 $MT \cdot ha^{-1} \cdot y^{-1}$) the successional system is accumulating soil organic carbon while the cropped system is not (Robertson *et al.* 2000). After ten years, soil organic carbon content (0 to 7.5 cm depth) was unchanged for the cropped system (1.00 ± 0.05 %) but had risen significantly for the successional system (1.63 ± 0.06 %). In addition to greater absolute carbon content, the successional soil may have a greater variety of substrates for microbial heterotrophs because of greater plant species diversity. Differences in soil carbon can influence factors controlling N_2O mole fraction. Menyailo and Huwe (1999) found that 26 years of soil development under six species of trees caused changes not only in soil chemistry, but also in the persistence and dynamics of denitrifying enzymes. Carbon quality (C:N ratio) was the most important soil chemical factor in explaining differences in N_2O emission among soil types.

Finally, differences in the microbial communities between the cropped and successional ecosystems may account for the different responses to soil moisture history. It is possible, perhaps even likely, that differing soil properties caused functionally significant divergence in microbial community composition. For instance, nitrate availability is typically much lower for the

successional soil (0.63 ± 0.04 μg NO_3^--N · g^-1) than for the cropped soil (6.54 ± 0.53 μg NO_3^--N · g^{-1}; Robertson *et al.* 2000). In the successional soil, a hypothetical sub-group of denitrifiers with the ability to maintain enzyme status (especially NOS) during dry periods could have a competitive advantage in exploiting the flush of carbon that occurs on soil wet-up (e.g. Groffman and Tiedje 1988), since they could use N_2O as well as NO_3^- as a terminal electron acceptor if oxygen were limiting. In cropped soils, the incentive for NOS maintenance would be less, because of the abundance of the more energetically-favorable electron acceptor NO_3^-. Thus, if variation exists among denitrifier taxa in their ability to maintain NOS status during soil drying, then a putative mechanism of natural selection exists that could explain our results, in terms of differences in microbial community composition.

Variation may indeed exist among denitrifiers in their ability to maintain NOS status during soil drying. Cavigelli and Robertson (2000b) isolated 31 denitrifier taxa from two ecosystems: the cropped ecosystem studied here and a nearby never-tilled successional field. They showed that considerable variability exists among taxa for sensitivity of the NOS enzyme to varying levels of oxygen, a parameter clearly related to soil drying. Furthermore, Cavigelli and Robertson (2000a) found differences in denitrifying ability for whole soil microbial communities (slurry assay) for the cropped ecosystem and the never-tilled successional field. Denitrifying enzymes were more sensitive to oxygen levels in the agricultural soil, and nitrous oxide reductase was more active in the successional soil. Their results are consistent with our suggestion that the

microbial community in the successional soils may have experienced selection for denitrifiers with the ability to maintain the status of denitrification enzymes, especially NOS.

The story of microbial community for the KBS LTER treatments is, however, complicated. Cavigelli and Robertson (2000a, 2000b) compared the conventionally-tilled agricultural treatment (our "cropped" system) and a never-tilled successional treatment. Our study compared the conventionally-tilled system to a historically-tilled successional treatment. Buckley and Schmidt (2000) used biochemical techniques to characterize relative abundance of seven broad taxonomic groups in the microbial communities of KBS LTER treatments. They determined that the communities from the conventionally-tilled system and the historically-tilled system were much more similar to each other than to the community from the never-tilled system. Therefore, comparisons between our results and those of Cavigelli and Robertson should be made with appropriate reserve. Still, there is a strong possibility that at finer taxonomic levels, functionally significant differences exist between the microbial communities from the conventionally-tilled cropped ecosystem and the historically-tilled successional ecosystem (D. Buckley, personal communication). The existence of these differences and their importance for denitrification remain to be demonstrated.

Questions of mechanism notwithstanding, evidence of a role for moisture history in controlling N_2O mole fraction has an important place in the biogeochemistry of nitrogen. Many studies suggest that most N is lost from

soils during brief periods following irrigation or rainfall (Smith and Tiedje 1979, Sextone *et al.* 1985, Rolston *et al.* 1982, Mummey *et al.* 1994, Davidson, 1991). Dependency of the N_2O mole fraction on short-term soil moisture history could have large consequences for the relationship between nitrous oxide production and total denitrification. Since N_2O flux has been modeled for intervals as short as one day (e.g. Li *et al.* 1992a, 1992b), the time scale implied by "short-term" in our study (48 hours) is relevant for efforts to constrain the global N_2O budget (e.g. Bouwman 1990, Eichner 1990).

Isotope data

In principle, isotope data for N_2O allow an independent estimate of N_2O production. However, the estimate depends on a two-member mixing model (Bergsma *et al.* 1999) in which absolute contribution from both members is significant. Our concentration data (Figure 4.1) show that N_2O concentration changed by almost three orders of magnitude (0.3 μg N · g dry soil $^{-1}$ to ~200 μg N · g dry soil $^{-1}$) even in the least productive jars. We conclude that the final isotopic character of N_2O in the headspace of labeled jars essentially represents the isotopic character of soil-derived N_2O. Thus, the ^{15}N-N_2O data in this study are suitable for tests of equilibrium and estimates of soil enrichment, but not for independent estimates of N_2O production.

Estimates of N_2 production by mass spectrometry were typically only one-third of estimates by acetylene inhibition. One of the two methods may have been biased, or the methods may have reflected qualitatively different aspects of the experimental system. The acetylene method could have been

biased if denitrification was enhanced by the presence of acetylene. There is some evidence in the literature for the stimulation of denitrification by acetylene addition (e.g. Klemedtsson *et al.* 1988), but usually for longer incubations and to a lesser extent than would be the case here. Furthermore, the soils studied here were amended with glucose, which argues against one putative mechanism of denitrification enhancement by acetylene: namely, release from carbon limitation (see also Topp and Germon 1986). In oxic soils acetylene may cause denitrification to be underestimated due to scavenging of intermediate nitric oxide (Bollman and Conrad 1997). Our soils, however, were largely anaerobic, and the putative error (if any) is overestimation not underestimation.

Few studies have explicitly compared the acetylene inhibition technique with the ^{15}N isotope dilution technique for estimation of N_2 flux. In their seminal paper reviving interest in the ^{15}N isotope dilution method for N_2 flux, Siegel *et al.* (1982) confirm accuracy of enrichment estimates for their method, but not necessarily of flux estimates. Rolston *et al.* (1982), measuring only N-gas derived from fertilizer, found reasonable agreement between the acetylene method and a ^{15}N-accumulation method. Mosier *et al.* (1986) compared total denitrification under acetylene inhibition (N_2O only) with total denitrification by ^{15}N mass spectrometry ($N_2O + N_2$; N_2O catalytically reduced to N_2 prior to analysis). Acetylene-amended plots consistently gave higher fluxes (nominal MS/GC ~0.75): this was attributable to acetylene treatment itself, rather than analytical bias between the two methods, since no difference in methods was

found when both were applied to samples from the acetylene-amended plots (see Table 1, last column, in Mosier *et al.* 1986). Aulakh *et al.* (1991) reported very similar total denitrification for two acetylene methods and for the ^{15}N method of Mosier *et al.* (1986). Arah *et al.* (1993) found that acetylene inhibition consistently gave lower N_2 flux estimates than ^{15}N isotope dilution, and concluded that for heavy textured soils the acetylene block was incomplete. Mulvaney (1988) and Mulvaney and Vanden Heuvel (1988) compared fluxes of N_2O as measured by mass spectrometry (reducing N_2O catalytically to N_2) and by gas chromatography. Both studies found only modest differences, and no strong bias towards underestimation or overestimation of N_2O flux.

For our study, there is no strong justification for questioning the accuracy the N_2 measurements made by acetylene inhibition. The difference between the two methods is perhaps explained as bias in the ^{15}N-based measurements -- an explanation that is not entirely satisfying. Underestimation is expected from the ^{15}N-dilution technique when the soil mineral N pool undergoing denitrification is not uniform (Boast *et al.* 1988, Arah 1992, Bergsma *et al.* 1999). But how much underestimation? Simulation (Arah 1992) and direct calculation (Bergsma *et al.* 1999) show that if flux derives from a very large number of pools with enrichments randomly distributed from natural abundance levels to 100%, the central tendency of the underestimation is 0.75. Albeit theoretical, the value 0.75 serves as a convenient null hypothesis. It does not explain our nominal MS/GC ratio of 0.34. Also, Figure 4.4 shows that for most incubations, N_2O derived from soil

was probably in equilibrium, implying a well-mixed soil source. Since N_2O is the direct precursor of N_2 (Payne 1981), one would expect N_2 from soil also to be in equilibrium (Focht 1985) and therefore free of the underestimation ascribed to non-uniform pools. Furthermore, estimates of enrichment (of the soil mineral pool undergoing denitrification) based on N_2 in our study agreed well with estimates based on N_2O. Others have found similar results (Mulvaney and Kurtz 1984, Mosier *et al.* 1986).

Given the above considerations, the most satisfying explanation for the differences between the two methods of calculating N_2 production is that the methods gave qualitatively different information about the observed system: the acetylene method reported gross N_2 production while the ^{15}N dilution method reported only production from a highly enriched, uniformly labeled pool, i.e. the enriched N_2O or its substrate. A second, unenriched soil mineral N pool was also a source of N_2 (indeed the major source), but not a net source of N_2O. Under these circumstances, N_2 production would have been underestimated without affecting the estimate of enrichment for the labeled pool (see Focht 1985) which would explain the agreement of N_2 and N_2O data for estimates of pool enrichment (Table 4.2). Estimates of enrichment (of the soil mineral pool) based on isotopic data also agreed well with estimates predicted by mass balance (based on knowledge of extractable NO_3^- levels for stock soil and knowledge of the magnitude of the $K^{15}NO_3$ addition; Table 4.2). The agreement of the mass balance estimates and the ^{15}N estimates suggests further that the putative unlabeled source of N_2 is not a static, extractable NO_3^- pool. Perhaps

the unlabeled source is a dynamic result of mineralization and nitrification occurring as a consequence of rapid soil wet-up, tightly-coupled to denitrification. This scenario implies nitrification rates (a few μg N · g dry soil^{-1} · d^{-1}) that are about an order of magnitude greater than typical potential nitrification rates for these soils in the field (about 0.1 μg N · g dry soil^{-1} · d^{-1}; Robertson *et al.* 2000). More work is needed to account for the interesting differences between the acetylene inhibition method and the ^{15}N-dilution method for estimating N_2 flux as used in this study.

Conclusions

The design of the study as implemented was suitable to test for effects of recent moisture history (antecedent moisture regime) and ecosystem differences on N_2O mole fraction during denitrification. N_2O mole fraction in successional soils was not affected by moisture history, but in cropped soils it was sharply lower when soil moisture had been high for 48 hours prior to incubation. We suggest that persistence of nitrous oxide reductase in the successional soils was less sensitive to water stress during soil drying, perhaps because the lower level of native soil nitrate selects (in the successional microbial community) for denitrifier taxa with enhanced capacity for enzyme maintenance. Explicit recognition of ecosystem differences in response of N_2O mole fraction to recent moisture history may improve modeled estimates of global N_2O flux. Furthermore, understanding the impact of soil management regimes on mole fraction dynamics within ecosystems may lead to strategies that minimize flux of N_2O to the atmosphere.

The isotope data show that the mineral N pool undergoing denitrification was isotopically uniform in most cases. There is no conclusive explanation for the strong differences between estimates of N_2 production by acetylene inhibition and by ^{15}N isotope dilution. Perhaps, under the experimental conditions described, there existed an alternative substrate for production of N_2, but not for N_2O; the ^{15}N method may then have reported only production of labeled N_2, while acetylene inhibition would have reflected gross production of N_2. Factors likely contributing to the difference between methods are the complexity of the soil environment and the dynamic nature of N transformations during rapid re-wetting of soil.

Table 4.1. Nitrous oxide mole fraction (N_2O / [N_2O + N_2]) analyzed by ecosystem and recent soil moisture history. Estimates are mean ± standard error, in μg N · g dry soil $^{-1}$. Effects are P values (Prob. > F). "Ecosystem by history" is the interaction term.

Table 4.1.

ecosystem	pre-wet mean (μg N · g dry soil^{-1})	control mean (μg N · g dry soil^{-1})	combined mean (μg N · g dry soil^{-1})	block effect (P > F)	history effect (P > F)	ecosystem effect (P > F)	ecosystem by history (P > F)
cropped	0.36 ± 0.08	0.90 ± 0.19	-	0.1335	0.0377	-	-
successional	0.34 ± 0.10	0.33 ± 0.07	-	0.0656	0.7157	-	-
combined	-	-	0.48 ± 0.09	0.0449	0.0150	0.0085	0.0122

Table 4.2. Comparison, by ecosystem and replicate, of predicted soil NO_3^- enrichment (atom fraction ^{15}N) with apparent enrichment of the soil pool undergoing denitrification. "Predicted" is calculated by mass balance from the extractable NO_3^- levels in stock soil and the known addition of KNO_3. Apparent enrichments are from N_2 isotope data ("by N_2"), from N_2O isotope data ("by N_2O"), or "average" of N_2 and N_2O estimates.

Ecosystem	Rep.	predicted	by N_2	by N_2O	average
cropped	4	0.42	0.57	0.53	0.55
cropped	5	0.35	0.31	0.38	0.34
cropped	6	0.42	0.71	0.58	0.64
successional	4	0.90	0.87	0.87	0.87
successional	5	0.78	0.83	0.83	0.83
successional	6	0.84	0.84	0.86	0.85

Figure 4.1. Change in headspace concentrations (volume/volume) of nitrous oxide during incubation, with selected (for clarity) standard error bars. Dashed lines = successional soils, solid lines = cropped soils. Open symbols = control, filled symbols = pre-wet. Gray squares = + acetylene, black diamonds = - acetylene. Control and pre-wet curves diverge strongly for cropped soils (solid lines with open or filled diamonds) but not for successional soils (dashed lines with open or filled diamonds).

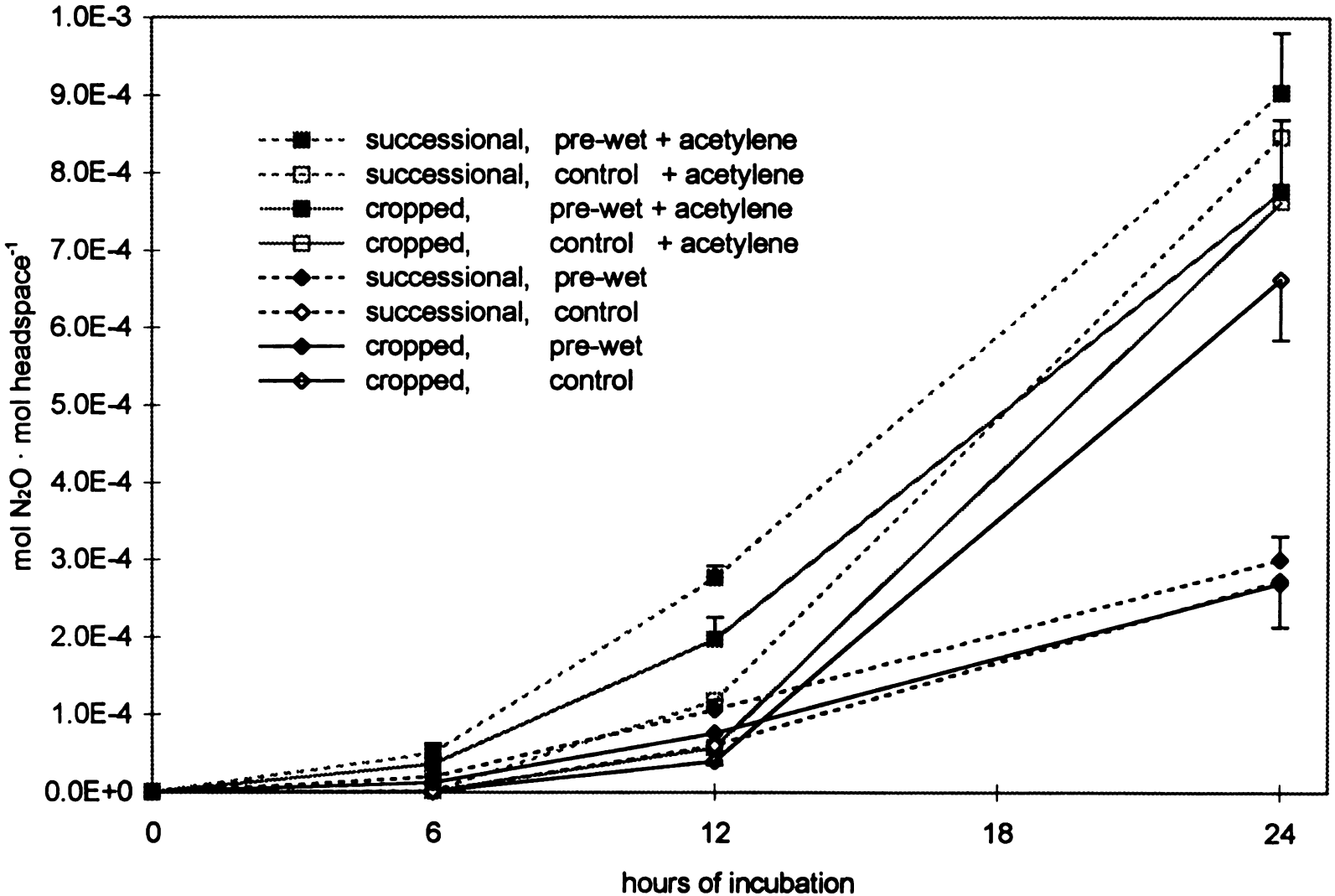
1.0E-3
9.0E-4
8.0E-4
7.0E-4
6.0E-4
5.0E-4
4.0E-4
3.0E-4
2.0E-4
1.0E-4
0.0E+0
mol N2O · mol headspace-1
successional, pre-wet + acetylene
successional, control + acetylene
cropped, pre-wet + acetylene
cropped, control + acetylene
successional, pre-wet
successional, control
cropped, pre-wet
cropped, control
0
6
12
18
24
hours of incubation

Figure 4.1.

Figure 4.2. Summary of production of N_2 and N_2O. "Total denitrification" is N_2O + N_2 by acetylene inhibition, "N_2O" is production of N_2O in the absence of acetylene, and "N_2" is the difference. Even though total denitrification did not differ significantly between moisture histories for cropped soils (bars with solid borders), the control incubations produced significantly more N_2O and correspondingly less N_2. Bars with dashed borders represent soils from successional plots.

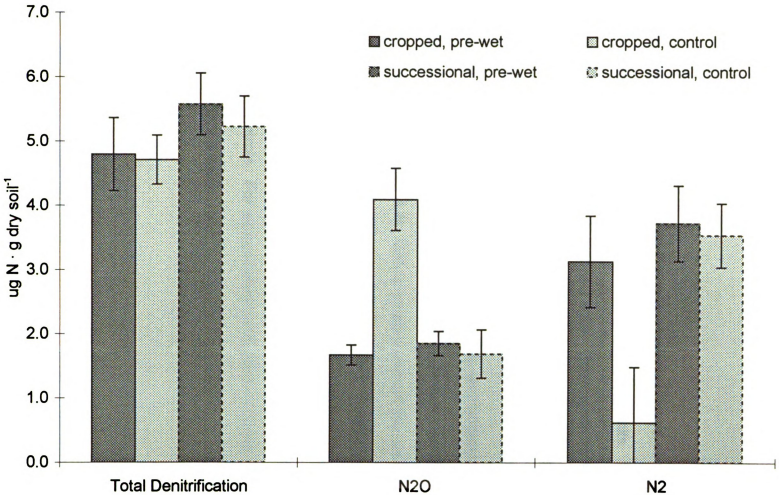
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
ug N · g dry soil-1
cropped, pre-wet
cropped, control
successional, pre-wet
successional, control
Total Denitrification
N2O
N2

Figure 4.2.

Figure 4.3. Isotopic character of N_2 for labeled and unlabeled jars at the end of the incubation, paired (by line segment) to represent incubation units. Dashed lines = successional soils, solid lines = cropped soils. Open symbols = controls, filled symbols = pre-wet. Circles = Replicate 4, squares = Replicate 5, triangles = Replicate 6. Due to the overwhelming abundance of unlabeled N_2 from the atmosphere relative to labeled soil-derived N_2, displacement of isotopic character during the incubation (i.e., length of the line segments) is much smaller than for N_2O (Figure 4.4). Only a small portion of the equilibrium curve (downward-opening parabola in Figure 4.4) is visible at this scale. For cropped soils, control segments (solid lines with open symbols) are much shorter than pre-wet segments (solid lines with closed symbols) indicating less N_2 production for controls. For successional soils (dashed lines) the opposite effect or no effect is seen.

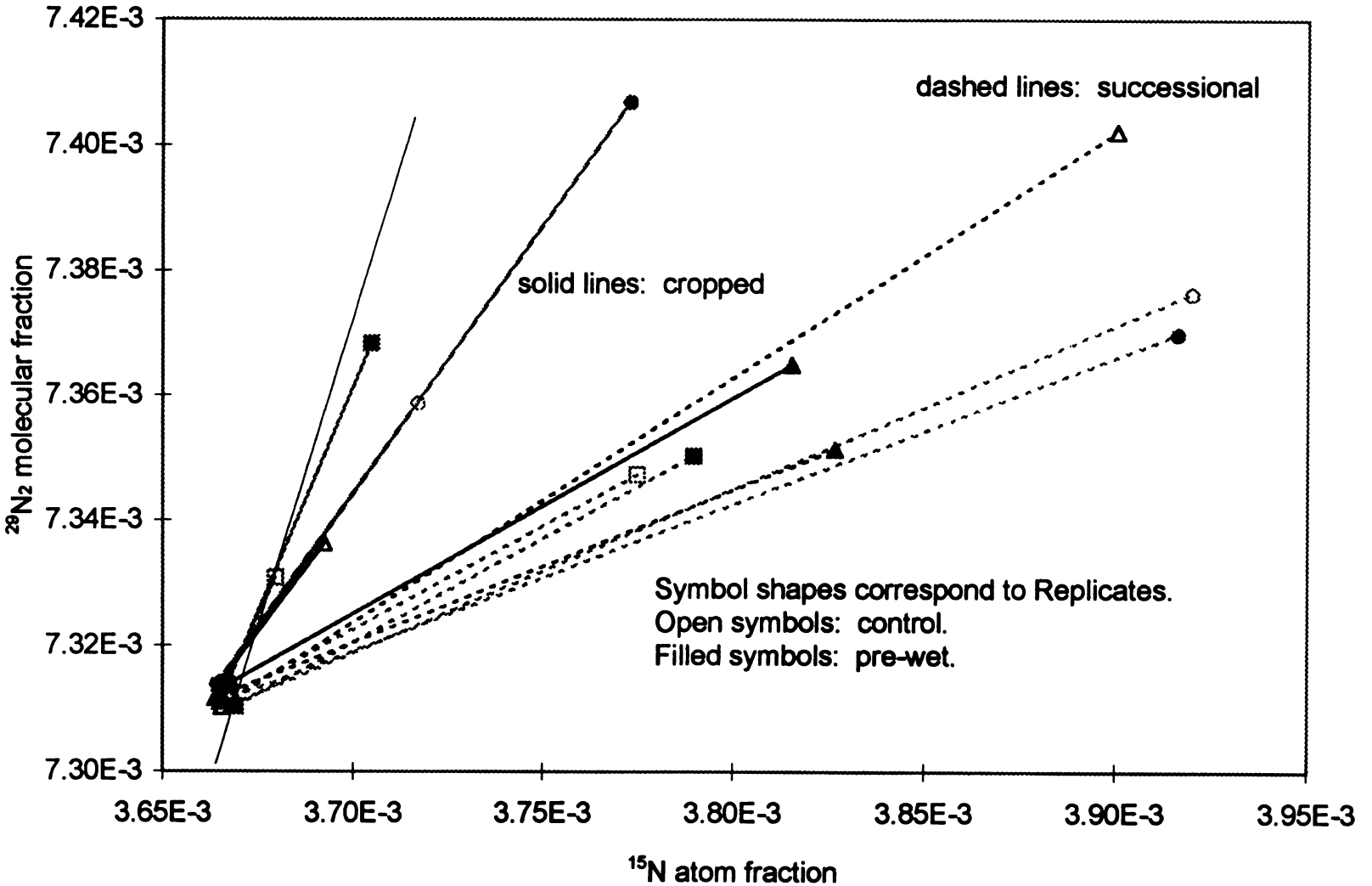
7.42E-3
7.40E-3
7.38E-3
7.36E-3
7.34E-3
7.32E-3
7.30E-3
$^{29}N_2$ molecular fraction
3.65E-3
3.70E-3
3.75E-3
3.80E-3
3.85E-3
3.90E-3
3.95E-3
^{15}N atom fraction
dashed lines: successional
solid lines: cropped
Symbol shapes correspond to Replicates.
Open symbols: control.
Filled symbols: pre-wet.

Figure 4.3.

Figure 4.4. Isotopic character of N_2O for labeled and unlabeled jars at the end of the incubation, paired (by line segment) to represent incubation units. Dashed lines = successional soils, solid lines = cropped soils. Open symbols = controls, filled symbols = pre-wet. Circles = Replicate 4, squares = Replicate 5, triangles = Replicate 6. Due to the overwhelming abundance of N_2O from soil relative to unlabeled atmospheric N_2O, enriched samples represent essentially the isotopic character of N_2O derived from soil. Samples falling on or near the equilibrium curve (downward-opening parabola) indicate a source in isotopic equilibrium, implying a single, uniformly labeled substrate pool (e.g. a homogenous mixture of native NO_3^- and $^{15}NO_3^-$ label).

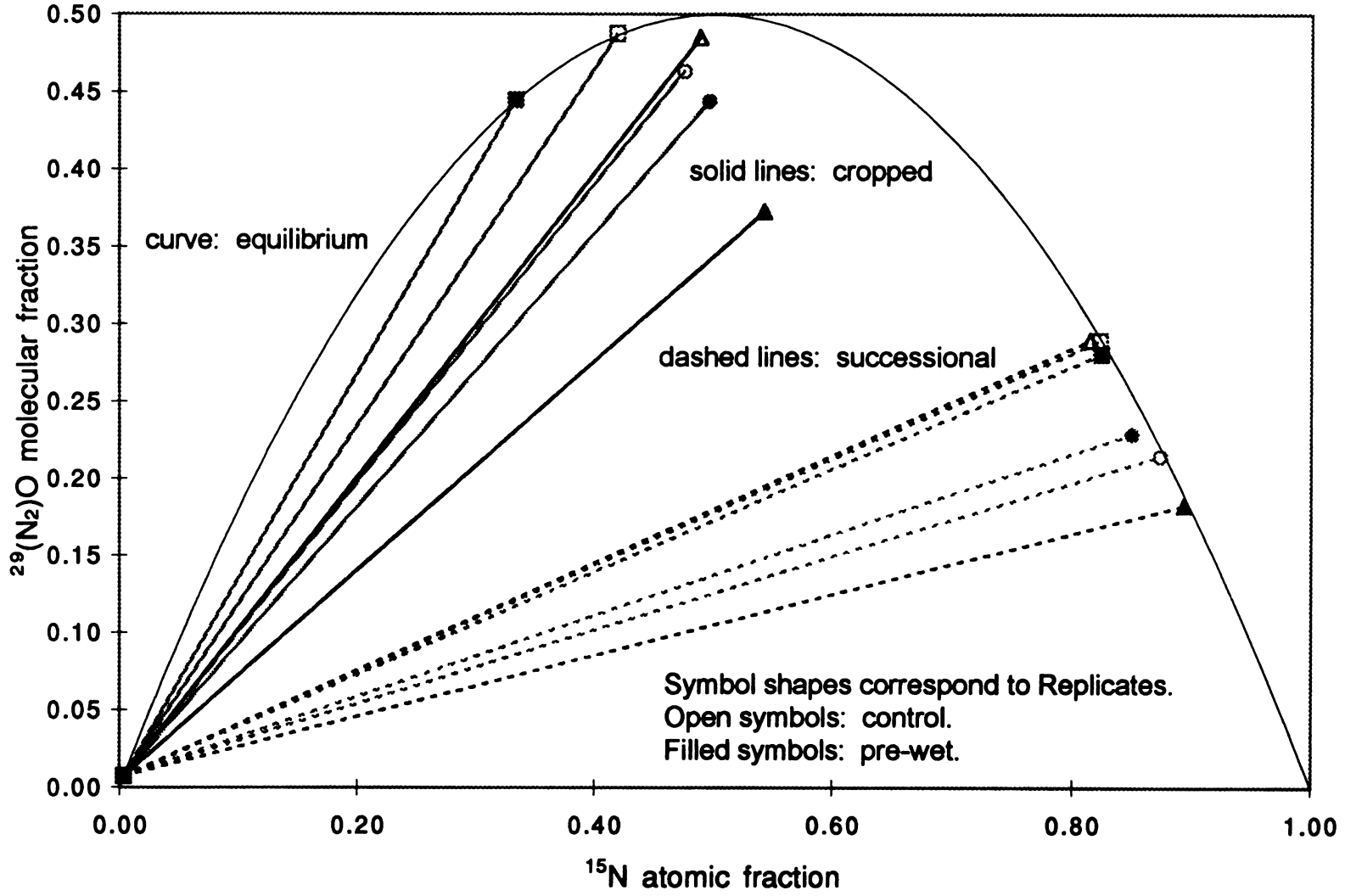
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
$^{29}(N_2)O$ molecular fraction
curve: equilibrium
solid lines: cropped
dashed lines: successional
Symbol shapes correspond to Replicates.
Open symbols: control.
Filled symbols: pre-wet.
0.00
0.20
0.40
0.60
0.80
1.00
^{15}N atomic fraction

Figure 4.4.

Figure 4.5. N_2 production calculated independently by the acetylene inhibition technique (AIT) and by mass spectrometry (MS), for both cropped and successional soils. In every case but one, MS gave a much smaller estimate of N_2 production than did AIT.

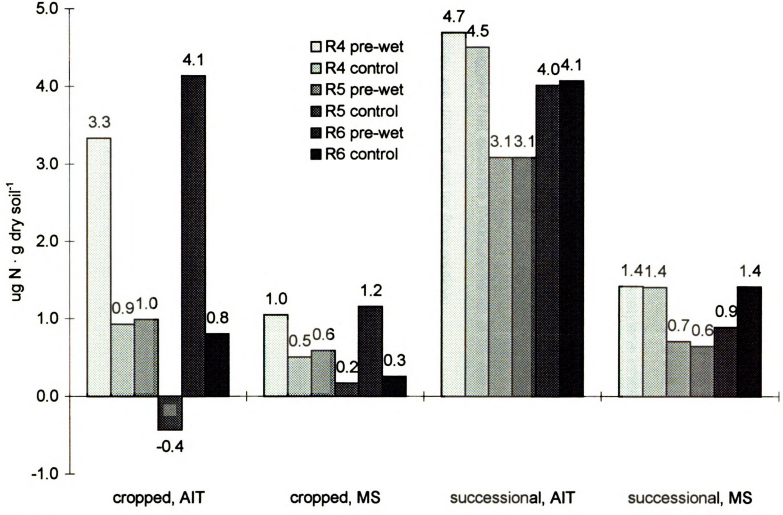
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
ug N · g dry soil-1
R4 pre-wet
R4 control
R5 pre-wet
R5 control
R6 pre-wet
R6 control
3.3
0.9
1.0
-0.4
4.1
0.8
1.0
0.5
0.6
0.2
1.2
0.3
4.7
4.5
3.1
3.1
4.0
4.1
1.4
1.4
0.7
0.6
0.9
1.4
cropped, AIT
cropped, MS
successional, AIT
successional, MS

Figure 4.5.

OVERALL CONCLUSION

A general conclusion that emerges from my research is that progress in understanding the relationship between denitrification and N_2O flux from soil is still methods-limited. The problem arises from the position that denitrification occupies in the nitrogen cycle: right on the brink of a sea of molecular N that constantly scours the beaches of inquiry. Acetylene inhibition and ^{15}N dilution are primarily devices to increase the sensitivity of measuring N_2 production, the missing piece of the puzzle. Unfortunately, both interact with the system they were designed to explore, thereby compromising the interpretation. I do not mean to suggest that accurate measurements of N_2 are rare or impossible, only that they are difficult and far from routine.

The good news is that when the tide goes out, the stories we find on the beach tend to be consistent. Variation in N_2 and N_2O production seems to abide proximally by principles of differential enzyme induction, and more distally by consistently-recognized ecological controls. The main question for the biogeochemistry community is, "How much needs to be known about ecological controls -- as distributed across ecosystems -- in order to adequately predict regional and global N_2O flux?" When the practical threshold for data gathering meets that need, a major breakthrough will be within reach. I hope that in some way my brief dashes among the waves will have made a contribution.

APPENDIX

APPENDIX

Table A1. Tests of significance (of differences among means) for Figure 4.2: probabilities of finding the observed differences in means by chance alone. Each row represents a single model of "Response", incorporating the effects for which there are column entries. In most cases, models returning $P > 0.05$ for an effect were re-run without that effect ("-"). Dropping the effect ("-") is a notational convenience only; statistically it is no different from grouping the responses across levels ("grouped"). Levels of block are [4, 5, 6]. Levels of ecosystem are [cropped, successional]. Levels of history are [pre-wet, control]. "NA" means the effect (column) is not meaningful for testing the response (row).

Table A1.

Response	block	ecosystem	history	block x history
total denitrification	0.0008	0.0085	0.2005	0.5702
total denitrification	0.0001	0.0030	0.1631	-
total denitrification	<0.0001	0.0035	-	-
N_2O production: cropped	0.2036	NA	0.0188	
N_2O production: cropped	0.0101	NA	-	
N_2O production: successional	0.1234	NA	0.4380	
N_2O production: successional	0.0705	NA	-	
N_2O production	0.0856	0.0856	0.0856	
N_2O production	-	0.0696	0.0696	
N_2O production	-	-	0.1038	
N_2O production	-	0.1038	-	
N_2O production: pre-wet	grouped	0.4676	NA	

Table A1 (continued).

N_2 production	0.0790	0.0297	grouped
N_2 production	-	0.0572	grouped
N_2 production: cropped	0.0190	NA	0.0074
N_2 production: successional	0.0068	NA	0.1994
N_2 production: successional	0.0025	NA	-
N_2 production: pre-wet	0.1170	0.3148	NA
N_2 production: pre-wet	0.0894	-	NA

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