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Abiotic immobilization of nitrate in forest soil: A double level approach

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ABIOTIC IMMOBILIZATION OF NITRATE IN FOREST SOIL: A DOUBLE LABEL APPROACH

BY

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THESIS

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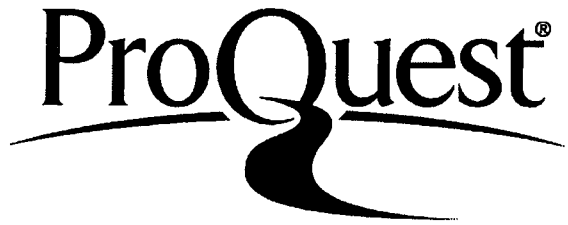

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Date

DEDICATION

This thesis is dedicated to my Grandmother for spending so much time teaching me how to write essays and arguments, to my parents for always supporting exploration of my interests, and to my wife for all of the love, support and insight she gave me in writing.

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ABSTRACT

ABIOTIC IMMOBILIZATION OF NITRATE IN FOREST SOIL: A DOUBLE LABEL APPROACH

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Abiotic immobilization of nitrogen may help explain nitrogen retention in soils under chronic nitrogen addition. Methodological limitations have made differentiating between abiotic and biotic immobilization in live soils difficult. This study attempted to make this differentiation with isotopically labeled nitrate, $^{15}N^{18}O_3^-$. My hypothesis was that during biological reduction and assimilation of ^{15}N, ^{18}O would be lost as labeled water, but some ^{18}O would be retained in abiotic reactions with soil chemicals. Lab incubations of soils from a *Pinus resinosa* stand were treated with 0.140 mg ^{15}N g^{-1} dry soil of $K^{15}N^{18}O_3$, for 0.25, 1 and 4 hours. Mean mass retained was 2.465 μg ^{15}N (±0.208 μg), and 7.875 μg ^{18}O (±0.677 μg). The ratio ^{18}O: ^{15}N was inconsistent with a hypothesized limit of 2:1 for abiotic immobilization of NO_3^-, suggesting either biotic assimilation of ^{18}O or unreacted $^{15}N^{18}O_3^-$. Further investigation of this method is required before drawing conclusions on abiotic immobilization.

INTRODUCTION

The combustion of fossil fuels and the use of fertilizers in industrial agriculture have resulted in the introduction of biologically available nitrogen (N) to the environment at more than twice the natural level (Vitousek, 1997; Galloway et al., 2003). Combustion creates airborne NO_x species, which can be transported far beyond their source. For example, coal burning power plants in the United States Midwest have increased N deposition in the Northeastern U.S. by up to five times pre-industrial levels (Ollinger et al., 1993; Galloway et al., 2003). While this increased deposition was anticipated to promote a corresponding increase in terrestrial sequestration of carbon (McNulty et al., 1996), chronic N inputs have demonstrated negative ecological consequences. Some of these negative effects include increased tree mortality in conifers, shifts in plant community composition, and soil and water acidification (Schulze, 1989; McNulty et al., 1996; Emmett et al., 1998; Ollinger et al. 2002; Fenn et al., 2003; Aber and Magill, 2004). Though varying by region, global rates of N deposition are expected to rise with increasing power demand and number of automobiles in use, pushing N deposition even further past already historic highs. Given these circumstances, it is important to understand how human caused N deposition will move through forest ecosystems and how these systems will react to chronic N deposition.

The chronic N addition experiment at the Harvard Forest in Massachusetts is one model for studying N deposition and forest response and has been ongoing for over 20 years (Aber, 1989). The experiment tests the N saturation hypothesis (Aber et al., 1989), which states that any terrestrial ecosystem has a maximum capacity for absorbing N, primarily through plant and microbial N demand, but also abiotic reactions between N and soil. Based on Smith (1972) and Bormann (1982), it was expected that the forest stands at the chronic N experiment would experience four stages of response. Initially, N deposition would fertilize growth as plants were relieved of N limitation. After this initial stage, subtle deleterious effects were expected to become evident. The third stage would be marked by obvious negative effects observable in the plant community. During the second and third stages, nitrate (NO_3^-) was expected to begin leaching into the surrounding streams and rivers as the system experienced saturation. However, the dissolved inorganic nitrogen (DIN) concentrations observed from lysimeters in the chronic N experiment remained low, and thus have not matched expectations (Magill, 2004).

Rather than observing the expected increase in NO_3^- leaching, Magill et al. estimated in 2004 that 70% of the added N at the chronic N pine plots was retained in the soil. The results suggest that the N saturation hypothesis does not account for all of the pools and fluxes of N in a system (Magill et al., 2004). Berntson and Aber (2000) proposed that the lag in saturation can be attributed to unaccounted denitrification or, possibly, abiotic immobilization. Independent observations of accumulating soil N under increasing deposition suggest an unexplained pool of N retention is in the soil (Magill et

al., 1997; Agren and Bosatta, 1998; Tietema et al., 1998). Short-term measurements of respiration during N immobilization suggest little increase in biological activity immediately after N addition (Micks et al., 2004), implying that soil retention of added N has an abiotic component.

Abiotic immobilization of N is the chemical reaction of mineral nitrogenous species with soil compounds outside of biochemical pathways. The nitrogenous species involved may be byproducts of biological processes, e.g. nitrite (NO_2^-) produced during nitrification, but the immobilizing reaction with soil chemicals happens without biological assistance. Abiotic immobilization has been suggested as a possible mechanism of N retention at the chronic N experiment based on observations of rapid ^{15}N retention during a tracer addition study (Berntson and Aber, 2000). That study, measured ^{15}N recovery in K_2SO_4 extracts from *in situ* soil, incubated with potassium nitrate ($K^{15}NO_3$) for 15 minutes. Over half of the N was immobilized in the soil and could not be extracted. They attributed this rapid retention to chemical reactions and not biological assimilation, but a subsequent slower N immobilization rate over the following 24 hours was attributed to microbial uptake. Similar rapid N immobilizations have been observed in multiple experiments (Smith and Chalk, 1980; Azhar, 1986a,c; Dail et al., 2001; Fitzhugh et al., 2003a,b)

Other research on ecosystem N retention has focused on accounting for all N inputs and outputs from systems ranging in size from forest stands to entire watersheds (Boyer et al., 2002; van Breemen et al., 2002; Goodale et al. 2003). For example, van Breemen et al. (2002) and Boyer et al. (2002) created N budgets for large watersheds in

the Northeast and found a discrepancy between the measurable inputs to the system and the sum of aquatic losses and changes in internal N stocks. The authors attributed this discrepancy to N lost to the atmosphere through denitrification. However, data to verify this assumption were absent and soil immobilization mechanisms could be invoked as a potential explanation.

Most soil biogeochemistry models do not include abiotic immobilization mechanisms and, therefore, may be overestimating the amount of N available to a given system (van Miegroet and Jandle, 2007). The current study examined the role of abiotic reactions of NO_2^- and soil humic compounds as a potentially important part of forest N cycles (Fig. 1). This mechanism has been well documented *in vitro* with soil suspensions, humic extracts, and synthetic humic compounds (Bremner, 1955, 1956; Bremner and Führ; 1966; Thorn and Mikita, 2000), but its importance in native soils is not well understood. Figure 1 shows a revised model of the N cycle illustrating the importance of NO_2^- as an intermediate in the commonly understood biotic transformations of N, but with abiotic immobilization pathways also included. This figure illustrates the importance of NO_2^- for multiple N cycling pathways, and how competition for NO_2^- can make it a limiting factor in microbial transformation of mineral N.

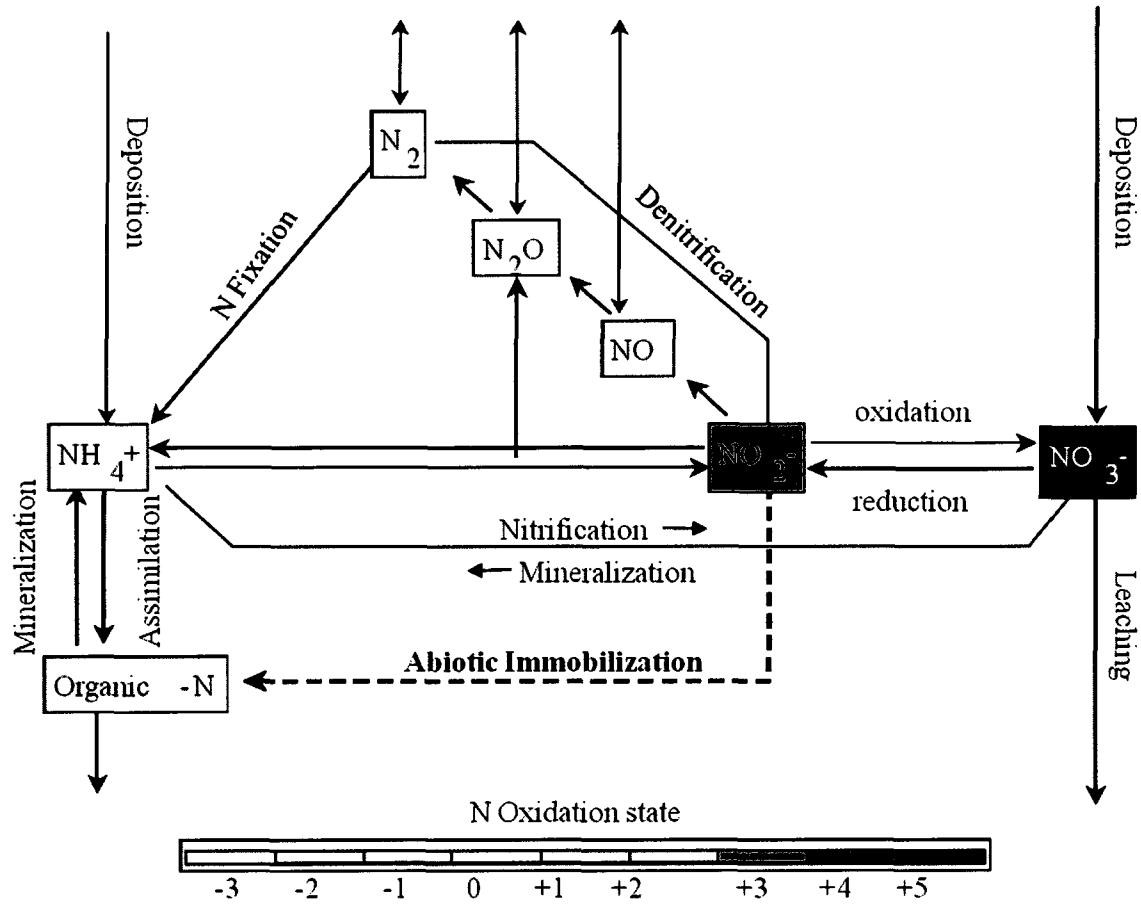

Figure 1. A model of nitrogen cycling in soils demonstrating the central role of NO_2^- as an intermediate between processes, including potential reduction of NO_3^- and abiotic immobilization of NO_2^- (dashed lines) (modified from Karl, 2002).

In theory, an abiotic sink of N would be limited by the amount of reactive soil organic matter present (Azhar et al., 1986b). The soil saturation point would be dependent on individual soil properties, including pH, humic acid content and organic soil mass (Nelson and Bremner, 1969; Azhar et al., 1986a,b,c). The presence of an additional fate for NO_3^- via abiotic immobilization would create an additional "buffer" against N deposition. Therefore, budgets including abiotic immobilization would have a saturation point different from that predicted by Aber et al. (1989), which was based on

plant and microbial demand only. Better understanding of the mechanisms that sequester N would allow for more accurate predictions of a system's response to N deposition. To date, attempts to examine the importance of abiotic mechanisms have been challenged by methodological limitations including extrapolating *in vitro* results to live soil, and chemical artifacts associated with soil sterilization.

History and Mechanism

Early observations suggesting abiotic immobilization of N by soil components were made by researchers using the van Slyke (also known as the van Slyke gasometric or manometric) method of free amino acids to analyze soil N (Bremner, 1952). The van Slyke method was designed to measure the amount of α-amino N that is present in a sample. In this method, nitrous acid is allowed to decompose in a vessel so that nitric oxide (NO) replaces the atmosphere in the reaction chamber. The sample solution (anything containing α-amino groups to be measured for N) is then added, and the reaction of the amino group with nitric acid evolves gaseous N_2 whose volume gives the value of α-amino N present in the sample (van Slyke, 1911). This method was originally developed for analysis of blood and urine in medical applications, and later adopted by biologists in other fields.

Bremner (1952) raised concerns over the van Slyke determination of humic N. At the time the lingo-protein theory, that humic N is derived from the chemical reaction of protein and lignin, was dominant in the literature (Bremner, 1954). Bremner's review (1954) questioned values reported for humic N content in the form of protein. Bremner

found that many studies used van Slyke's method to determine the total N in the humic fraction, and hypothesized that the high values returned were not a result of high protein content in the humic fraction, but in its chemical nature. Several researchers had already reported that phenolics, such as those found in tannins, would increase the N values obtained with the van Slyke method (Hulme, 1935; Stuart, 1935). This implied that some soil N was converted to a gas beyond the reaction with α-amino acids. Bremner (1956) applied NO_2^- to a sample of extracted wood lignin and the N content increased fourfold in only a few hours. He also found that the reacted N was very recalcitrant, with only 20-30% of it recovered after prolonged acid hydrolysis. Bremner concluded that there was a previously unknown abiotic reaction occurring in these samples.

Bremner (1955; 1956) performed further investigations to address the results of the van Slyke method on the humic fraction of soils using various soil types. Results from these studies demonstrated that N was fixed by the extracted humic acids, as indicated by the total N content, when determined by the micro-Kjeldahl method. At that point in time, the mechanism of the reaction was unknown. These papers marked the beginning of the investigation of NO_2^- reacting with the humic fraction of the soil abiotically. Bremner and Fuhr (1966) applied labeled potassium nitrite ($Na^{15}NO_2$) and $K^{15}NO_3$ to humic extracts from various agricultural and non-agricultural soils to confirm the reaction and try to determine a mechanism. The study concluded that NO_3^- was not reacting with the humic extract, but that NO_2^- was. While a specific mechanism was not determined in this work, Bremner and Fuhr were able to show that, as with the tannins in the plant extract experiment, the reactants were phenolics. They concluded that it was

most likely lignin derived chemicals that were reacting with the NO_2^-, and that the reaction led to the formation of nitroso functional groups.

Stevenson and Swaby (1964) found that reaction of NO_2^- and humic acids produced a mixture of molecular nitrogen (N_2), nitrous oxide (N_2O), carbon dioxide (CO_2), nitroso-methane (CH_3ONO), and NO (under anoxic conditions). Also during this period in the late 1950's early 1960's, the term chemodenitrification began to be used as a descriptor of the phenomenon of N_2 and N_2O generation in the absence of denitrifying microorganisms (Clark, 1962). Observations regarding the gas evolved allowed researchers to propose possible reaction pathways.

An exhaustive study of NO_2^- reactions with soil components conducted by Bremner (1968) eliminated most inorganic components as possible reactants, and supplied strong evidence that only organic matter and specifically phenolic hydroxyl groups were involved in fixation and chemodenitrification. Bremner (1968) proposed the first hypothetical mechanism for abiotic immobilization of N in soil (Fig. 2). According to the proposed reaction, NO_2^- in an acidic solution forms nitrous acid (HNO_2), and the nitronium ion of the acid is then able to attack the phenol (directed to the ortho or para position) through electrophilic substitution. Chemodenitrification can then occur through an additional electrophilic substitution of the newly created nitro group resulting in gaseous N_2 or N_2O (Bremner, 1968).

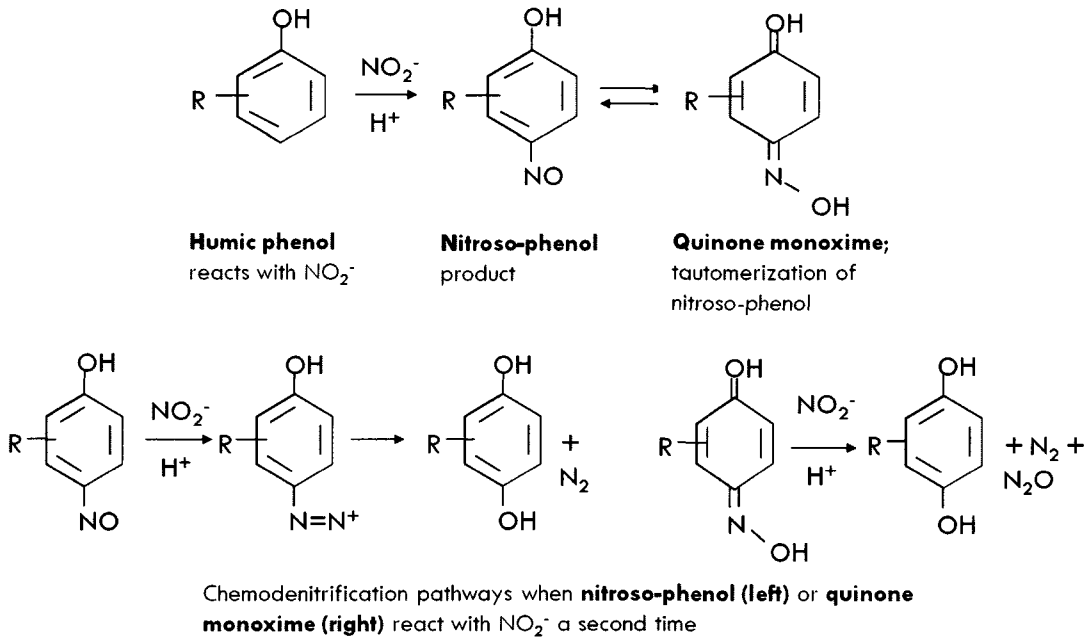

Figure 2. . Two proposed possible pathways involved in abiotic immobilization of nitrogen in soil. Pictured is the nitrosation of humic phenol by nitrite (NO_2^-) and chemodenitrification of nitrosated humic phenol by NO_2^-. (Stevenson and Swaby, 1964; Bremner, 1966).

At the time of Bremner's 1968 work, all of the relevant studies had been performed on extracted or model soil compounds. Subsequent studies began to use soil samples to attempt to further understand and quantify the reactions (Cawse and Cornfield, 1972; Smith and Chalk, 1980; Azhar et al., 1986a,b,c; Fitzhugh et al., 2003a,b). Cawse and Cornfield (1972) added NO_3^- to gamma irradiated soils, demonstrating chemodenitrification in an abiotic environment. Smith and Chalk (1980) applied $^{15}NO_2^-$ to soils of differing pH, confirming that fixation is inversely related to pH and directly related to organic matter content. Azhar et al. (1986a,b,c), in a series of experiments using labeled and unlabeled NH_4^+ and nitrapyrin, a nitrification inhibitor, inferred that N added to the humic fraction of their soil samples was derived from NO_2^- generated by the soil microbiota during nitrification. Fitzhugh et al. (2003a) realized that

the low pH and organic rich soils of northeastern forests was theoretically ideal for abiotic immobilization of NO_2^-. They added $^{15}NO_2^-$ to soil samples gathered from beneath different tree species in northeastern forests and found that the $^{15}NO_2^-$ was immobilized at time scales as short as a day, and that the dominant fate was incorporation into the soil organic matter (SOM), inferring abiotic immobilization. Another experiment (Fitzhugh et al., 2003b) added labeled NH_4^+, NO_3^-, and NO_2^-, to soils sterilized with mercuric chloride. This experiment demonstrated that in all three additions, some retention could be attributed to abiotic immobilization, but the magnitude of NO_2^- retained was much greater than NH_4^+ or NO_3^-.

Despite these efforts, at present, abiotic immobilization of NO_2^- is typically treated as a theoretical possibility in forest soils. It is rarely included in conceptual models of the N cycle (Aber et al., 1989; Boyer et al., 2002; van Breemen et al., 2002), likely due to the small pool size of NO_2^- commonly observed in forest soils (Venterea et al., 2003). Given the previous experiments on abiotic immobilization performed on soil samples, remaining questions limit our understanding of whether abiotic immobilization is a significant part of N cycling. Does the experiment significantly eliminate biotic factors? Is the availability of NO_2^- for reaction in the experiment realistic? If abiotic immobilization is occurring, what is the magnitude of abiotic N immobilization in nature?

To confirm of abiotic reactions in soil, several limitations arise in the use of sterilized soils. Every sterilization technique alters the soil chemistry to some degree, and several soil sterilization techniques do not completely suppress microbial respiration

(Wolf and Skipper, 1994). Most importantly, after sterilization, the only way to introduce NO_2^- into the soil is through direct application. NO_2^- is normally rare in forest soils and artificial addition creates an unrealistic pool size, and a drop in soil pH (Cawse and Cornfield, 1972; Fitzhugh et al., 2003b).

Pulse application of NO_2^- is meant to simulate continuous nitrification or denitrification input of NO_2^- to the soil, and do so on a shortened timescale. NO_2^- is normally only found in very small quantities in the soil, and in specific microsites where bacteria are present. Because of this, most researchers have assumed that microbial competition for NO_2^-, by oxidizers in the nitrification pathway and by denitrifiers, is so efficient that it does not allow NO_2^- to accumulate in soil and prevents the opportunity for abiotic reactions. Therefore, pulse applications of NO_2^- to soils limits inference of results in field conditions.

Special circumstances are required for NO_2^- accumulation in soils, usually related to grasslands with large herbivores, which can lead to decreases in the soil pH (Smith, 1980). In developing an experiment designed to investigate the limitations cited above, the ideal would be to introduce the NO_2^- at a more natural rate without eliminating the competing factors that occur in native soil. Azhar et al. (1986a,b,c) added $^{15}NH_4^+$ to stimulate NO_2^- production in nitrification, but this method does not eliminate the confounding effects of microbial immobilization and utilization of the labeled ^{15}N, i.e., some of the $^{15}NH_4^+$ would likely be assimilated rather than oxidized.

The goal of this study is to develop and apply a new method to test for abiotic immobilization of NO_3^- in soil while overcoming some of the limitations of previous studies. Past work using model soil compounds have a significant limitations, that their simplicity makes them unrealistic models of the soil environment. Stable isotope addition studies using ^{15}N can trace the total amount of $^{15}NO_3^-$ that is immobilized into the solid fraction of the soil, but do not differentiate between biological uptake and assimilation and abiotic immobilization. Limitations of soil sterilization techniques include ineffective sterilization of the microbial community, altering soil conditions, and the creation of chemical artifacts that could reduce or even stimulate N reactions (Wolf, 1994; Lotrario et al., 1995; Dail et al., 2001). Methyl chloroform sterilization results in a partial inhibition of microbial respiration, but not a full sterilization of microbiota in the soil, nor inhibition of extracellular enzymes. Irradiation is much more effective for total sterilization of a soil sample but also results in significant changes in the soil chemistry. Autoclaving soil samples, similar to irradiation, is effective at sterilization but results in significant chemical alterations, including an increase in soluble organic matter, soluble Fe(II) and other metals (Cawse and Cornfield, 1972; Wolf and Skipper, 1995; Dail et al., 2001).

The current study seeks to investigate abiotic immobilization using non-sterilized soils, while finding a way to differentiate between abiotic and biological immobilization. This study was based on the idea that double labeled nitrate, $^{15}N^{18}O_3^-$, allows differentiation between abiotic and biologic immobilization products using only standard

isotope measurement techniques, and without the need for isolating anything more than the solid fraction of the soil.

The Double Label Approach

The double label approach takes advantage of the biochemical pathways of biological NO_3^- use, assimilation and dissimilative denitrification (Beauchamp and Bergstrom, 1993; White, 2006). Both pathways involve the reduction of NO_3^-, whether it is for incorporation into microbial biomass, or for use in the electron transport chain. The initial step in both pathways is the reduction of NO_3^- to NO_2^-. This reduction is accomplished by the enzyme nitrate reductase, which cleaves one oxygen atom to produce water and NO_2^- (White, 2006). Further NO_2^- reduction then occurs, generating NO in the denitrification pathway, or ammonia (NH_4^+), in the assimilative pathway (Fig. 3). Either pathway involves a nitrite reductase enzyme, but the products of this step are dependent on the pathway involved, and in the case of denitrification, the soil conditions and microbial community (Firestone and Davidson, 1989). The reduction of NO_2^- is similar to the reduction of NO_3^-, the cleaving of oxygen to produce water and a reduced product. NO produced in denitrification is the product of reduced NO_3^- and NO_2^-, with the production of another water molecule. Any ^{15}N that assimilated by these pathways, is assimilated without associated ^{18}O, which has been lost as ^{18}O enriched water.

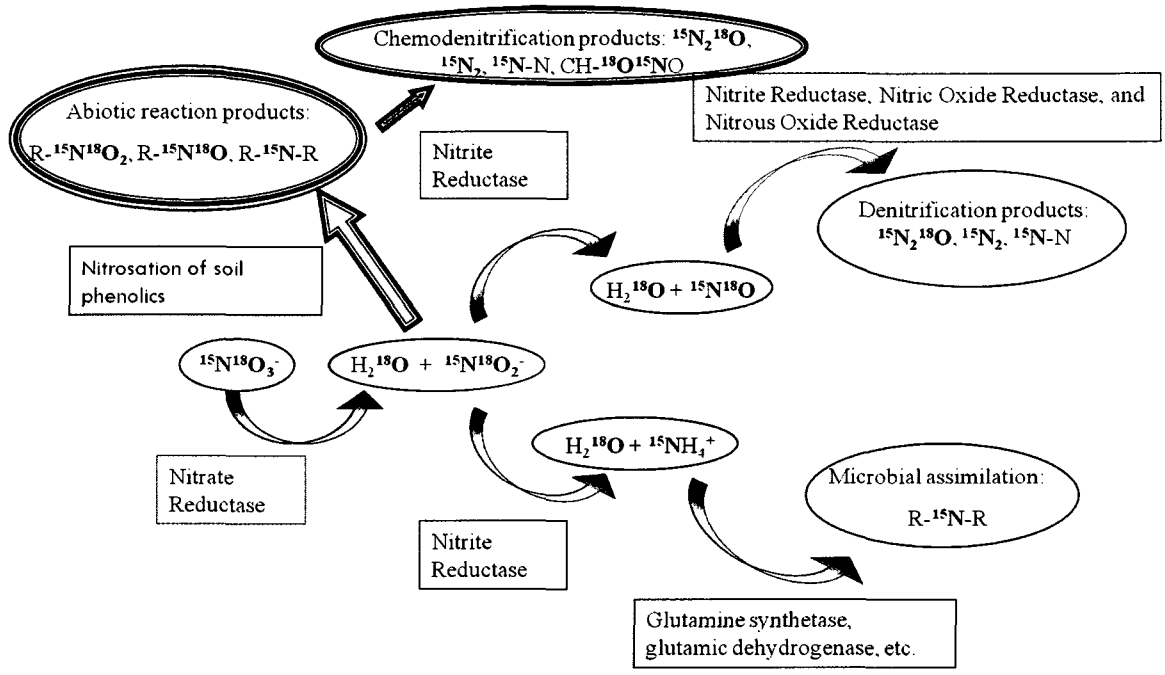

Figure 3. Possible fates of the labels in $^{15}N^{18}O_3^-$ when added to soil. Nitrate reduction occurs in both denitrification and nitrate assimilation. ^{18}O is cleaved to water during assimilation but not during abiotic immobilization, allowing differentiation between biotic (shaded arrows) and abiotic (double arrows) products in the soil organic matter (modified from Wray and Kinghorn, 1989).

It is possible that there will be microbial assimilation ^{18}O in water produced during microbial assimilation of $K^{15}N^{18}O_3$. Some microbial products contain water derived oxygen. Notably, during the citric acid cycle (TCA) condensation of a hydroxyl group is involved in the reactions forming citrate, succinate, and malate. TCA cycle intermediates can serve as precursors in the production of some lipids, proteins and nucleic acids. However, assuming a well mixed system, labeled water formed during the reduction of $K^{15}N^{18}O_3$ is added to a large pool of water, relative to ^{18}O added to the solid fraction. Biological processes will preferentially choose the lighter isotope, a process known as fractionation, further reducing the likelihood of biological incorporation of ^{18}O.

During abiotic immobilization, ^{18}O is retained in nitroso ($^{15}N^{18}O_2^-$) or nitro ($^{15}N^{18}O^-$) groups. The $K^{15}N^{18}O_3$ contains 98% ^{18}O enrichment, so abiotic O retention is functionally, an increase in ^{18}O and not ^{16}O. Even with subsequent isomerization, chemodenitrification, or decomposition of the initial abiotic products any small abiotic addition of ^{18}O to the solid fraction of the soil should be direct, indiscriminate, and easily detectable in the short term.

MATERIALS AND METHODS

Soils were collected at the Harvard Forest experimental forest, in Petersham, Massachusetts, USA. Soil was sampled at locations adjacent to the chronic N experiment, under a Red Pine (*Pinus resinosa*) dominant canopy in Typic Distrochrepts of the Canton or Montauk soil series. O horizon soil was sampled in late October 2006 from three sampling sites bordering the 150 kg $N \cdot h^{-1} \cdot yr^{-1}$ pine plot, not from within the plot. Soil was sampled close to the plot to attempt to increase the likelihood of observing similar rapid immobilization seen at the plots before (Berntson and Aber, 2000). Seven 10 x10 cm samples of the Oe and Oa horizon were taken and combined into one sample per location.

In the lab, soils were processed through a 2 mm sieve and stored at 4 °C. Before experimentation began, collected soil was tested for treatment effect from the fertilized plot by comparing total N and ^{15}N values to pine stand control plot values. There was no significant difference in N or ^{15}N between the collected and control values. Though not ideal, technical problems with the mass spectrometer delayed the experiment, so for four months of the storage period the soils were kept at 0 °C and then returned to storage at 4 °C for two months until experimental incubations began. This long storage was expected to introduce artifacts into the results, so to compensate an additional factor was included in statistical analysis to remove variance associated with this experimental error. On subsamples of the collected soils, soil water content and organic matter content were measured by oven drying at 65 °C for 24 hours and loss on ignition at 500 °C for 6 hours.

Humic acid content was measured by a 24 hour 0.5 *N* sodium hydroxide (NaOH) extraction followed by a 2 *N* hydrochloric acid (HCl) extraction (Schnitzer and Schuppli, 1989). After performing humic acid extracts the reproducibility of the results were to too poor to include these data in subsequent analysis.

A series of test incubations was performed to determine an appropriate labeling level; we sought to simultaneously, deliver a reasonable NO_3^- addition that would not be too unrealistic for soil, but also address the sensitivity of the instruments used to detect both labels. Additions of 0.005, 0.070, 0.14, and 0.28 mg of ^{15}N / g wet soil were incubated for 1 h and then washed for excess NO_3^-. The test incubations could not include the ^{18}O label due to temporary technical problems with the $^{18}O/^{16}O$ mass spectrometer. After analyzing the $\delta^{15}N$ of the test incubations, 0.070 mg ^{15}N / g field moist soil was determined to be the smallest addition that would result in a detectable ^{18}O signal. This addition level equates to roughly 0.14 mg ^{15}N g^{-1} dry soil, or 7 μg ^{15}N μg^{-1} soil NO_3^- N, a relatively large addition in a natural system, but thought necessary to ensure ^{18}O resolution.

The experiment utilized a 3x3x2 multi-factor design to test for abiotic immobilization in the three sampling sites, at three time intervals, and under oxic and anoxic conditions. A full factorial design called for 36 incubations, and with replication, 72 incubations total. Owing to the complexity and size of the experiment, the incubations occurred in two sets. The first incubation set was performed in June 2007. Technical difficulties with the mass spectrometer delayed the second set of incubations until August 2007. Twenty-four hours before incubation, soil samples were removed from the

refrigerator and 1.000 (±0.001) g of wet soil was placed in 30 ml serum vials and loosely covered. $K^{15}N^{18}O_3$ was dissolved in ultrapure deionized water (DI), and applied with a syringe. 0.66 ml of $K^{15}N^{18}O_3$ solution was added to each incubation to deliver 0.070 mg of ^{15}N or 0.437 mg of $K^{15}N^{18}O_3$. All serum vials were covered in aluminum foil for the length of the incubation. Before addition of the label to anoxic incubations, serum vials were capped with airtight rubber septa, evacuated under 500 mm Hg vacuum for 30 seconds and then flushed with N_2 for 60 seconds. At that point, with the N_2 still flushing the vial, an additional needle was inserted in the septa for 60 seconds and the headspace was allowed to reach atmospheric pressure. Before incubation, 0.66 ml of headspace was removed to prevent a positive pressure headspace during injection of the label solution.

Following the incubation period, 9.0 ml of 4 °C 1 M potassium chloride (KCl) was added to the serum vial, hand shaken for one minute and rinsed with an additional 1.0 ml of KCl into a centrifuge tube. The KCl extract was designed to remove unreacted $K^{15}N^{18}O_3$ and slow biological activity with a low temperature. The sample was spun at 9000 x g to sediment cells and all solid particles. The supernatant was then aspirated through a 2.7 μm pore filter and the filter scraped for retained particles, which were returned to the solid soil pellet. The supernatant was kept at 4 °C and then filtered through an additional 0.45 μm pore filter and later scraped for cells. 10 ml of Ultra pure DI was added to the centrifuge tube and the contents were resuspended with a vortex mixer. After this rinse, the tube was spun again at 9000 x g and aspirated. Once these rinse steps were completed, the tube and soil were frozen and then freeze dried. A ball

mill grinder was used to grind the samples for mass spectrometry. The full procedure for incubations can be found in Appendix A.

For isotope analysis, samples were ground in a ball mill grinder for 4 minutes, at which point the samples were pulverized to powder fineness. Ground samples were submitted to the University of New Hampshire Stable Isotope Lab for analysis. Total percent carbon (C) and N and N isotopes were analyzed on a Thermo-Finnigan elemental analyzer and continuous flow isotope ratio mass spectrometer (IRMS). Samples were introduced in tin cups and combusted at 900 °C. Soil samples were run with NIST 1515 apple leaf, NIST 1575a pine needles, and an internal soil standard. Total oxygen and oxygen isotopes were analyzed in a Thermo-Finnigan total combustion elemental analyzer. Samples were combusted in silver cups at 1400 °C. Standards were calibrated for O analysis with NIST 25039, the IAEA standard for solid ^{18}O analysis.

Initial results from the oxygen isotope analysis indicated a problem. Excess salt, remnant from the KCl extraction, and unreacted $K^{15}N^{18}O_3$ was interfering with the analysis. Another set of test incubations with unlabeled KNO_3 were performed for salt and NO_3^- analysis. Incubations were performed as above but after the DI rinse, a series of further DI rinses and centrifugations were performed. Each rinse supernatant was measured for NH_4^+ and NO_3^-/NO_2^- in a colorimetric autoanalyzer. A portion of the DI rinses were also submitted to the Water Quality Laboratory at UNH for chloride analysis. Once a DI rinse returned a NO_3^-/NO_2^- value below the detection level of the autoanalyzer, the sample was considered clean. Three additional DI rinses were required for complete removal of available NO_3^- from the sample. The three additional DI rinses also

drastically reduced the amount of chloride present in the sample. Based on these findings, all of the experimental incubations were given an additional three DI rinse and centrifuge cycles, for a total of five rinses and centrifuge cycles. The protocol for these additional DI rinses was the same as for the original KCl and DI rinse.

In December 2008, 0.050 to 0.060 mg of dried and pulverized soil from each incubation was weighed into silver weigh boats for ^{18}O analysis. Along with the samples of incubated soil, two standards were included at regular intervals: NIST 1515, and an internal standard of local white pine forest soil. In addition to the standards, two samples per run of 27 were duplicated and two samples of the total set of 72 were run with every run to check for value drift. Each run of samples was allowed to sit in the autosampler and purged with helium for 10 minutes to remove gaseous water from the atmosphere. The autosampler was then shut off to the atmosphere and allowed to dry for several hours before combustion to remove gaseous water from the air. All samples and standards were combusted at 1400 °C to ensure total combustion of the soil.

N isotope values were measured on a Thermo Finnigan EA continuous flow mass spectrometer. After being dried and pulverized, 5.00 to 5.40 mg of each experimental sample was weighed into tin weigh boats. Samples were duplicated similarly to samples measuring ^{18}O. In addition to the ^{15}N isotope data, the ratio of carbon to nitrogen (C:N) present in the sample was measured simultaneously.

To test retention response to the experimental factors and their interactions a multiple linear regression model was created with the following factors: whether the

incubation was oxic or anoxic (Atmosphere); which of the three sample sites the incubated soil originated (Site); and the length of the incubation, 0.25, 1, or 4 hours (Time). The model also included the full range of interactions between those factors, with incubation set, the factor describing when an incubation was performed, included as a blocking factor.

In addition, a second set of models was created with the unique soil C:N values substituted for sampling site. Sampling site is a general factor removing some variance from the model but provides little explanatory power. C:N is a biologically relevant factor that is often and easily measured in ecology. The unique C:N value was measured with ^{15}N analysis and provided the opportunity to build a model useful for studies not using Harvard Forest soil.

The data were tested for normality and heteroscedasticity before proceeding with the multiple linear regression model. Initially, the ratio of ^{18}O to ^{15}N retention was non-normal and not heteroscedastic. Two outliers were identified using the distribution of studentized residuals and removed from analysis, after which normality and heteroscedasity were demonstrated. Each model was tested for significance with a 0.05 chance of Type II error. If the model was statistically significant, each model parameter was tested for significance and the model paired down, starting at the highest interaction and working down to single factors, until only significant factors remained in the model.

RESULTS

Incubations with $K^{15}N^{18}O_3$ resulted in measureable enrichment of both isotope labels (Fig. 4). Retention for either label was less than 10 % (Fig. 5), with mass retained on the scale of micrograms. Mean mass retained of ^{15}N was 2.465 μg (±0.208 μg). The significant factors in the optimized model for ^{15}N retention are the sampling site, the interaction between Site and Atmosphere, and the experimental set factor (Set) (Table 1). Because Atmosphere has a significant higher order interaction, it is included as a single order factor. This optimized model is a significant predictor of the ^{15}N retention ($p<0.001$ $R^2=0.63$ for both labels). Set is a significant factor in the model response, with the earlier experimental set displaying higher retention rates than the latter. All three of the sampling sites have significantly different retention responses. Site C has the greatest retention, followed by A, and then B (Fig. 6). The interaction of Atmosphere and Site has two significantly different groupings of response interactions, which were a mix of sampling sites and atmospheres (Fig 7). Also note, time is not a significant factor, nor are any of its higher level interactions.

The model results for ^{18}O retention mirrored the results for ^{15}N retention (Table 2). Again, retention of the original addition is only small fraction; mean ^{18}O retained is 7.875 μg (±0.677 μg). The optimized model includes the same significant factors as that for the ^{15}N data, though the responses of the factors are different in this model. Site B has the lowest mean retention of ^{18}O, and retention at sites A and C are not significantly different (Fig. 8). The ^{18}O data for the interaction between the Atmosphere and the Site is similar to the ^{15}N data (Fig. 8).

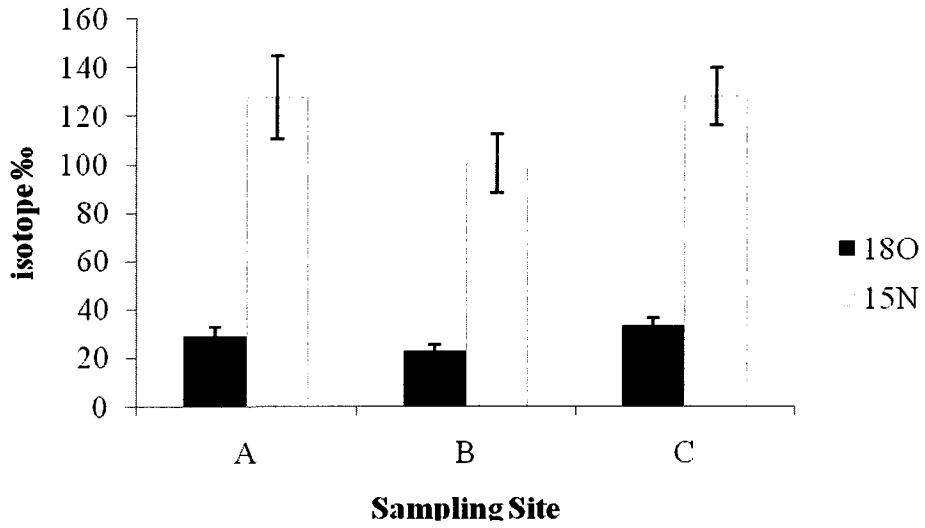

Figure 4. The mean per mil (‰) isotope value of $^{18/16}O$ and $^{15/14}N$ sorted by sampling site. Per mil values for oxygen are expressed in terms of VSMOW and for nitrogen compared to atmospheric air. Bars are one standard error of the mean.

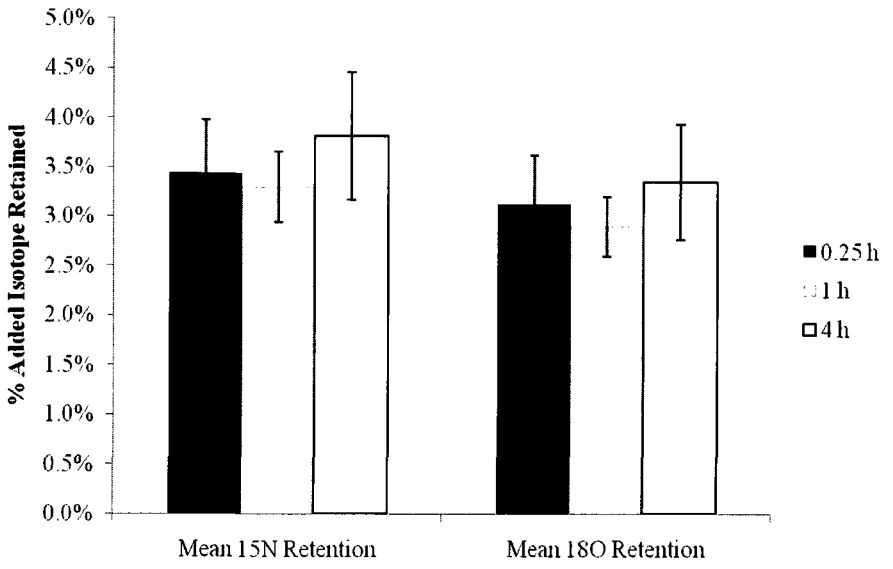

Figure 5. The percent retention of added ^{15}N and ^{18}O added as $K^{15}N^{18}O_3$ at a rate of 0.070 mg ^{15}N / 0.252 mg ^{18}O per g soil after 0.25, 1.0 or 4.0 hours incubation. There were no significant differences between incubation lengths.

Table 1. Summary statistics and model parameters for the original optimized prediction model of ^{15}N retention. RMSE is the root square mean error expressing variance of the data about the modeled response in percent added ^{15}N retained. Model effects denoted by an * are significant by t-test with a 0.05 chance of Type II error.

Statistic	**Value**
ANOVA P-value (whole model)	<0.0001
R squared	0.63
RMSE	0.0119
Model Effects	**P-value**
Atmosphere	0.0808
Site	0.0002*
Atmosphere Sampling Site Interaction	0.0058*
Experimental Set	0.0039*

Table 2. Summary statistics and model parameters for the original optimized prediction model of ^{18}O retention. RMSE is the root square mean error expressing variance of the data about the modeled response in percent added ^{18}O retained. Model effects denoted by an * are significant by t-test with a 0.05 chance of Type II error.

Statistic	**Value**
ANOVA P-value (whole model)	<0.0001
R squared	0.63
RMSE	0.0108
Model Effects	**P-value**
Atmosphere	0.0955
Site	0.0003*
Atmosphere Sampling Site Interaction	0.0029*
Experimental Set	0.0028*

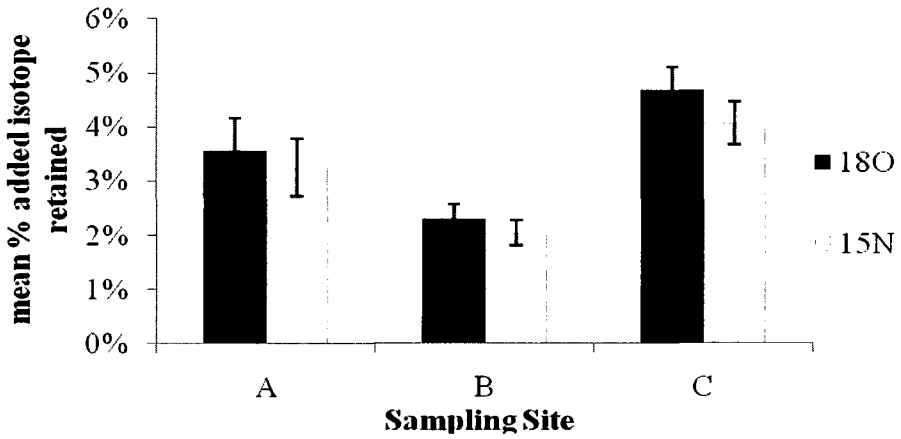

Figure 6. The mean percent retention of added ^{15}N, and ^{18}O averaged across atmosphere and incubation length organized by sampling site, error bars are one standard error of the mean. All means, comparing within isotope, are statistically different.

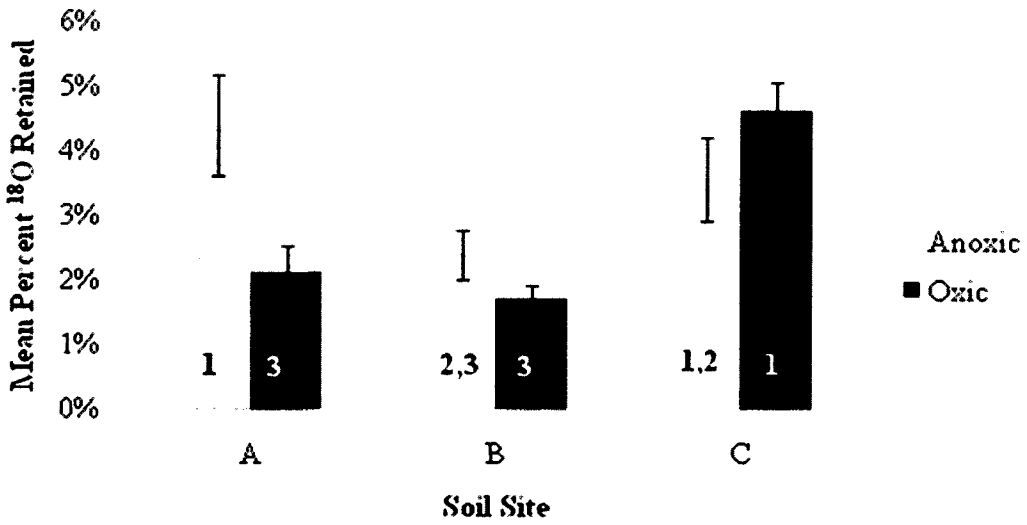

Figure 7. The mean percent retention of added ^{15}N split by bulk and atmosphere incubated to express the interaction between the two factors. Error bars are one standard error of the mean. Means not sharing a number are significantly different.

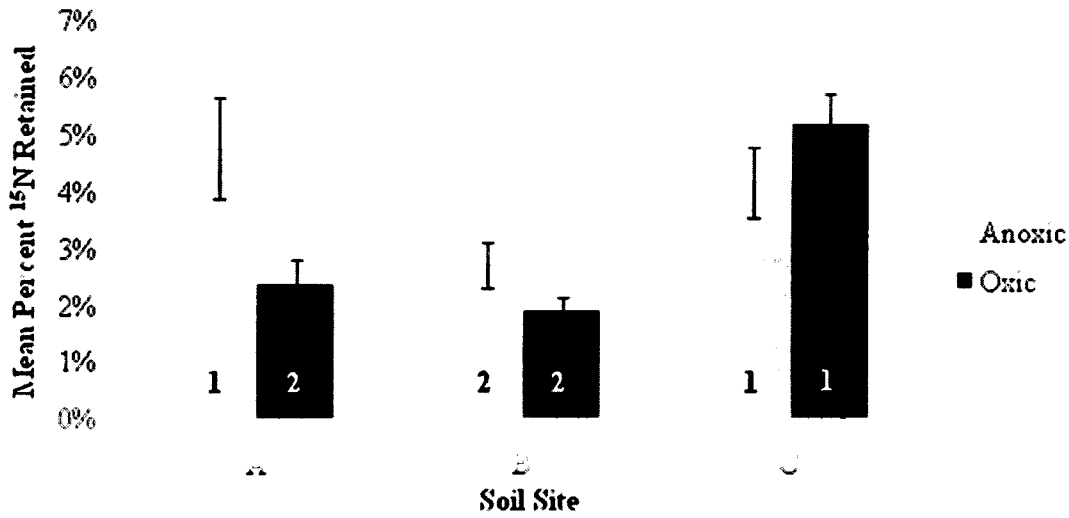

Figure 8. The mean percent retention of added ^{18}O split by bulk and atmosphere incubated to express the interaction between the two factors. Error bars are one standard error of the mean. Means not sharing a number are significantly different.

A second set oaf models created using the paired control carbon to nitrogen ratio (C:N) value substituted for the sampling site. C:N is a more descriptive factor than the arbitrary site label, and it was expected to be a predictive factor. When C:N was used as a factor in the model, the full model was included since the three-way interaction

between Atmosphere, Time, and C:N was significant for the ^{15}N retention (Table 3) and the ^{18}O retention (Table 4). While Time was not a significant factor in the previous model, in this model the interactions between Time and C:N and the three-way interaction were significant (Fig. 9, Fig. 10).

The molar ratio of retained ^{18}O to ^{15}N did not fit with the hypothesis of a nitrosation ^{18}O:^{15}N molar ratio maximum of two. The mean ratio is significantly greater than two, with only one sample having a ratio less than two (Fig. 11). As mentioned, two outliers were identified and removed from analysis. The low retention outlier is the only value less than the hypothesized maximum ^{18}O:^{15}N molar ratio of two. The high outlier is the only incubation with an ^{18}O:^{15}N molar ratio greater than the original molar ratio of $^{15}N^{18}O_3^-$, three. There were no significant differences between the treatments. The mean ^{18}O:^{15}N molar ratio (2.66) was significantly less than three.

As mentioned previously, nitrosation reactions result in the addition a nitroso group with two O and one N atom. This new nitroso group can then undergo chemo-denitrification and form a nitro group of one N and one O atom, or further to form an amine group. It follows that nitrosation products from doubly labeled N species should have an ^{18}O: ^{15}N ratio of two or less. Therefore, any ratio larger than two must be the product of an additional reaction of the labeled oxygen with the solid portion of the soil, or of ^{18}O water assimilated into microbial biomass not extracted from the solid fraction of soil, or the presence of uncreated $^{15}N^{18}O_3^-$.

Table 3. Summary statistics and model parameters for the C:N optimized prediction model of ^{15}N retention. RMSE is the root square mean error expressing variance of the data about the modeled response, in percent added ^{15}N retained. Model effects denoted by an * are significant by t-test with a 0.05 chance of Type II error.

Statistic	**Value**
ANOVA P-value (whole model)	0.0106*
R squared	0.49
RMSE	0.0145
Model Effects	**P-value**
Experimental Set	0.0013*
Atmosphere	0.4180
Time	0.1286
Atmosphere *Time	0.6782
C:N	0.3416
Atmosphere * C:N	0.0923
Time * C:N	0.0212*
Atmosphere * Time * C:N	0.0061*

Table 4. Summary statistics and model parameters for the C:N optimized prediction model of ^{18}O retention. RMSE is the root square mean error expressing variance of the data about the modeled response, in percent added ^{18}O retained. Model effects denoted by an * are significant by t-test with a 0.05 chance of Type II error.

Statistic	**Value**
ANOVA P-value (whole model)	0.0166*
R squared	0.47
RMSE	0.0134
Model Effects	**P-value**
Experimental Set	0.0012*
Atmosphere	0.4679
Time	0.1906
Atmosphere *Time	0.5301
C:N	0.3213
Atmosphere * C:N	0.1520
Time * C:N	0.0359*
Atmosphere * Time * C:N	0.0061*

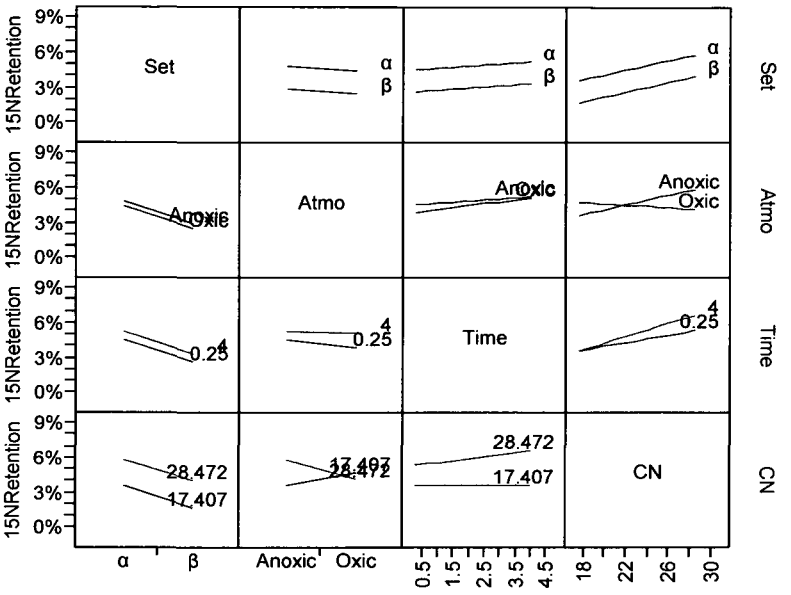

Figure 9. Interaction plots for the C:N optimized model for ^{15}N retention. Read these plots by matching a factor in a row with a factor in column. For the continuous factors of C:N and Time the lowest and highest value interaction is displayed.

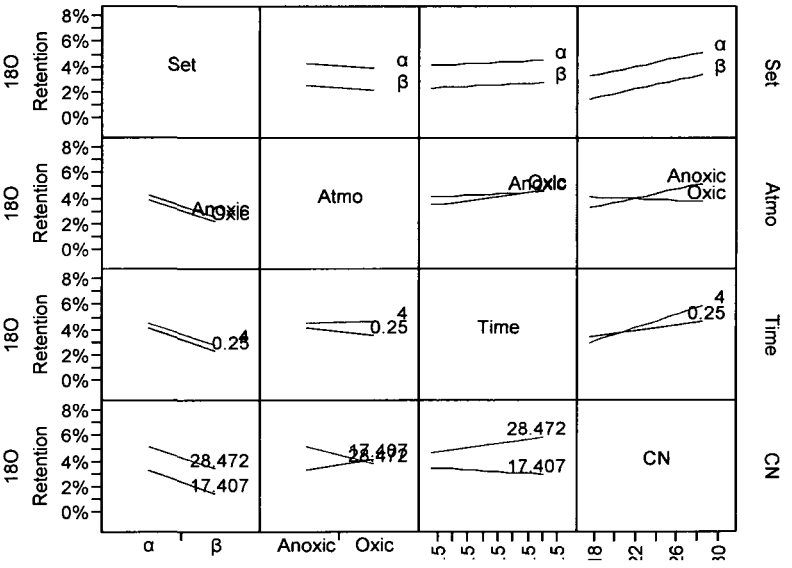

Figure 10 Interaction plots for the C:N optimized model for ^{18}O retention. Read these plots by matching a factor in a row with a factor in column. For the continuous factors of C:N and Time the lowest and highest value interaction is displayed.

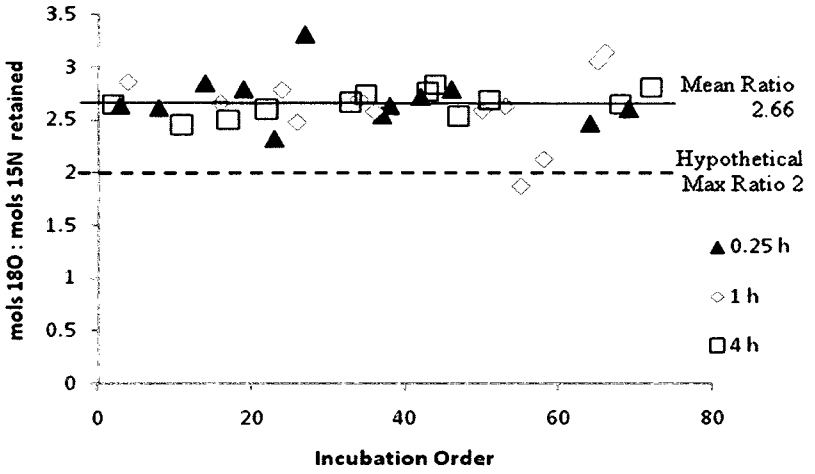

Figure 11. The distribution of the molar ratio of ^{18}O:^{15}N retained per incubation. Note that the theoretical max for this ratio if nitrosation is the only cause of ^{18}O retention is two.

DISCUSSION

The ^{18}O:^{15}N Molar Ratio

The assumption behind the use of ^{18}O to resolve abiotic from biotic immobilization was that, during the reduction of the $^{15}N^{18}O_3^-$ to glutamate during assimilation (or N_2 in denitrification), the ^{18}O label was lost to water and to the environment. The assumed reaction for abiotic immobilization of N is a nitrosation reaction of organic matter with NO_2^-. The nitrosation assumption establishes a hypothetical max ^{18}O:^{15}N molar ratio of two, if this is the only pathway for ^{18}O retention by the solid pool. The observation of a mean ^{18}O:^{15}N molar ratio of 2.66 exceeds the hypothetical maximum molar ratio and implies that something else must be occurring. Unfortunately, the source of this shift in the ^{18}O:^{15}N molar ratio cannot presently be determined, leaving the double label method for resolving abiotic immobilization in need of further examination.

There are several possible explanations for exceeding the theoretical nitrosation O:N molar ratio maximum. The presence of unreacted $K^{15}N^{18}O_3$ in the solid samples may be affecting the final molar ratio. After incubation, the initial isotope results suggested unreacted salt remaining in the samples. To determine the number of rinses required to remove the unreacted $K^{15}N^{18}O_3$, extracts from test incubations with KNO_3 were submitted for colorimetric NO_3^- analysis and chloride analysis. After an additional three rinses, the colorimetric analysis no longer detected NO_3^- and chloride in the extract

was negligible. A limitation of this method is that the NO_3^- detection limit of the colorimetric autoanalyzer is approximately 0.1 ppm compared to a reproducible difference of approximately 0.5 ppm from the isotope ratio mass spectrometer and a detection limit several orders of magnitude less. Unreacted $K^{15}N^{18}O_3$ may also be a result of microbial uptake of $K^{15}N^{18}O_3$ without assimilation. Then, during freeze drying, the $K^{15}N^{18}O_3$ is left behind by the sublimating cell water.

Assuming that isotopic labels are only present in the soil in the form of abiotic immobilization products or unreacted $K^{15}N^{18}O_3$, the relative contribution of each pool to the total O:N molar ratio can be calculated. Because the O:N molar ratio of the total system and unreacted $K^{15}N^{18}O_3$ was known, the relative contribution of abiotic immobilization to the total pool (x) could be calculated with the equation:

$$x = (2.66-b)/(a-b)$$

Where *a* is the O:N molar ratio of abiotic products and *b* the molar ratio of unreacted $K^{15}N^{18}O_3$ (or any pool). Given the assumption of no biologically retained ^{18}O, abiotic immobilization was responsible for 17% of the total retention of isotopic label in the soil and the remaining retention was $K^{15}N^{18}O_3$. Applying this to the mass ^{15}N in the sample, 0.42 μg ^{15}N was retained through abiotic immobilization.

Chemodenitrification may provide another explanation for the observed ^{18}O:^{15}N molar ratio. One step in the chemodenitrification mechanism may involve the removal of the ^{15}N in a nitro group (R-NO) but retain the ^{18}O (Stevenson and Swaby, 1964; Bremner, 1966). This retention of ^{18}O and loss of ^{15}N results in an increase of the final ^{18}O:^{15}N

molar ratio. *In vitro* reactions of NO_2^- with $^{18}O/^{15}N$ labeled quinone monoxime and nitroso-phenol would reveal if solution chemicals retain ^{18}O during chemodenitrification (Fig 2).

Biological assimilation of ^{18}O labeled water created during reduction of the $K^{15}N^{18}O_3$ would also increase the ^{18}O:^{15}N molar ratio. An example of one pathway for this biotic retention is the citric acid cycle (TCA). The TCA cycle involves hydration reactions forming citrate, D-isocitrate, and malate. While the processes of NO_3^-/NO_2^- reduction and the TCA cycle are occurring in different parts of eukaryotic cells, prokaryotic soil bacteria may have the processes occurring in close proximity. Close enough that the addition of labeled ^{18}O was not diluted in the soil water before interacting with pathways likely to retain ^{18}O in the solid fraction. The double label method assumes that, as NO_3^- is biologically reduced, the $H_2{}^{18}O$ produced joins a well-mixed pool before it can be involved in any hydration reactions and the ^{18}O assimilated into the microbial biomass. Because the added ^{18}O was added to a well-mixed pool, the relative availability of ^{18}O to be added biologically to the solid fraction should be quite small compared to the ^{18}O available to be added abiotically. However, with the data demonstrating an ^{18}O:^{15}N molar ratio that is significantly greater than two, the assumption of a well-mixed pool is questionable.

Between the possibilities of unreacted $K^{15}N^{18}O_3$ or biological uptake of ^{18}O, biological uptake might be the easier to test than the presence of unreacted $K^{15}N^{18}O_3$. Even after the 2 M KCl extraction and the subsequent four rounds of DI water rinses, there may be enough remaining unreacted $K^{15}N^{18}O_3$ to be a significant portion of the

measured enrichment. If that is the case, there may always be too much unreacted $K^{15}N^{18}O_3$ in the soil to see the theoretical abiotic ^{18}O:^{15}N molar ratio below two. Assuming that unreacted salts cannot be reliably removed from the system, testing the biological uptake of ^{18}O through culture becomes the next obvious step.

Although culturing of soil organisms is never an ideal system for the examination of soil biota, the approach could be useful in testing for microbial assimilation of ^{18}O. Culturing a variety of fungi and bacteria in separate media and then exposing the cultures with a small amount of $K^{15}N^{18}O_3$ should allow for a test of biological incorporation of ^{18}O. Microbial assimilation of the $K^{15}N^{18}O_3$ would come from tracking the ^{15}N, followed by measurement of ^{18}O assimilation. Detection of ^{18}O assimilation would immediately demonstrate that $K^{15}N^{18}O_3$ does not differentiate biotic and abiotic immobilization products. Unfortunately, an incubation that does not result in measurable assimilation of ^{18}O would not support $K^{15}N^{18}O_3$ for demonstration of abiotic immobilization. The majority of soil organism will not grow in lab incubations and so it would be difficult to extrapolate a negative result to live soil conditions.

Label Retention

While the $^{15}N^{18}O_3^-$ dose was large compared to the native pool of NO_3^- and the retention was low, the total mass retained was similar to previous studies. The capacity of soil to immobilize NO_3^-, by whatever mechanism, may be finite. Berntson and Aber (2000) conducted in situ incubations of Harvard Forest O and mineral horizon soil to track $^{15}NO_3^-$ recovery. They added $^{15}NO_3^-$ at approximately 16 μg ^{15}N g^{-1} soil and, based on label recovery from a 2M K_2SO_4 extraction, estimated 62% retention of added ^{15}N in

15 minutes, or roughly 10.2 μg ^{15}N g^{-1} soil. Dail et al. (2001) observed that approximately half of the much-smaller dose of $^{15}NO_3^-$ added was recovered as organic-N, and this occurred within 15 minutes of addition to Harvard Forest O horizon material. Unrecovered N amounted to about 2.5 μg ^{15}N g^{-1} soil. Assuming that this represents an upper limit for instantaneous abiotic NO_3^- retention, our observation of ~2-4% retention of 70 μg ^{15}N per g soil (our addition rate) is in close agreement (1.4-2.8 μg ^{15}N per g soil dm retained). Fitzhugh et al. (2003a), using soils from the Catskill Mountains in New York, saw retention of 7.5 μg ^{15}N g^{-1} soil in Mercury treated soils amended with $^{15}NO_2^-$ at 39.0 μg ^{15}N g^{-1} soil. The similar mass retention of our study and previous work suggests that the low percent retained has more to do with a high dose of ^{15}N g^{-1} soil than the freezing of the soil.

Model Factors and Interactions

The first set of models, which include the sampling location (Site) as a factor, were perhaps more interesting for what was missing than what was present. The incubation length (Time) was not a significant factor in the model (Fig. 5), nor were any of its interactions. A short period of N immobilization may indicate abiotic immobilization and saturation of the available reactive soil organic matter (SOM) pool. While the amount retained differs from the Harvard Forest *in situ* incubation, Berntson and Aber (2000) also observed rapid immobilization in the first fifteen minutes followed by little additional immobilization. One proposed mechanism for the abiotic reduction of NO_3^-, is the 'ferrous wheel' hypothesis (Davidson et al., 2003, 2008). This abiotic

method for introducing NO_2^- would allow for rapid abiotic immobilization of added NO_3^- without a corresponding rapid period of microbial reduction of NO_3^-.

A biological mechanism that could account for the lack of continuing NO_3^- immobilization (i.e. Time an insignificant factor) is denitrification. The reduction of NO_3^- to NO, N_2O or N_2 would not result in a measureable enrichment in the solid portion of the soil in either of the measured isotopes. Denitrification alone would not leave any label in the solid portion of the soil. ^{15}N would be lost as a gas ($^{15}N_2$, $^{15}N_2O$, ^{15}NO) and the ^{18}O would be lost either as gas ($N_2{}^{18}O$, $N^{18}O$) or in water. Future studies should include isotope measurements of incubation extracts and gaseous sampling of sealed incubations to test for ^{15}N and perhaps ^{18}O in N_2O generated in denitrification (Firestone and Davidson, 1996). Mass spectroscopy of the headspace of incubations would allow for the quantification of denitrification, and with the double label, the differentiation of nitrifier denitrification and denitrification might be possible (Wrage et al., 2005).

The most likely pathways of NO_2^- production, denitrification and the 'ferrous wheel' hypothesis, are anoxic processes relying on the chemically favorable reduction of NO_3^- (Davidson et al., 2003). Denitrification seems unlikely, however, because the presence of an oxic or anoxic atmosphere was not a significant factor. Only site A demonstrates increasing retention under anoxic conditions; sites B and C had no significant difference in retention by atmosphere. While both the 'ferrous wheel' hypothesis and denitrification can occur in anoxic microsites within oxic soils, inducing anoxic conditions experimentally was designed to maximize the potential for abiotic immobilization (Azhar et al., 1986a,b,c). Site A fit this expected pattern of greater

retention under anoxic conditions, and site B at least demonstrated a trend in that direction. Site C demonstrated no significant difference in retention under the different atmospheres, but suggests an opposite trend than site A. Site C had a large amount of decaying woody debris in it, and significantly greater %C, %N, and carbon loss on ignition than the other two sampling sites. The greater presence of complex phenolics derived from the woody debris may lead to a larger abiotic immobilization potential than the other two sampling sites (Bremner, 1968; Azhar et al. 1986a; Thorn and Mikita, 2000). While the data do not suggest that this is the case, site C did have the greatest retention, only the anoxic incubations of site A were not significantly lower. Site C also demonstrated a trend for greater retention in oxic conditions. This may be due to a greater role for biological uptake. In the highly organic soil, there may have been a larger amount of biomass available to fuel NO_3^- assimilation.

The C:N model presented a different set of significant factors than the sampling site model. While it might make sense to assume that the finer differentiation between incubations afforded by using C:N would reduce the variance in the model, both the R^2 was smaller and RMSE was larger than the RMSE of the sampling site (Site) model. Other soil factors, which would have been included in a blanket generic term like Site, must have contributed more explanatory power than the fine resolution of C:N alone. Other factors that should be considered for a fine scale analysis of each incubation include pH, humic acid content, particulate organic matter content, and aggregate content (Bremner, 1956, 1968; Azhar et al., 1986a).

Incubation length (Time) was not a significant factor, but its second and third order interactions with C:N were significant. The second order interaction demonstrates that the longer the incubation, the greater the rate of response to changes in soil C:N. This may indicate that while time was not a significant factor in the Site model, this could reflect the short incubation times. An incubation length greater than 4 hours may have revealed greater differences.

The interaction of C:N and atmosphere is notable for its opposite retention responses under anoxic and oxic environments. The C:N of the soil likely determines microbial nutrient investment, and microbial community activity, determining the suite of microbes most active to respond to the different atmosphere. Conversely, the different atmospheres dictate how microbes utilize available NO_3^- (Sterner and Elser, 2002). While this result might be expected, it should be noted that predictions of NO_3^- retention in oxic and anoxic condition should account for local C:N values.

A second attempt at investigation of abiotic immobilization of NO_3^- with the double label method would be worthwhile, starting with testing the assumptions of the method. Overcoming the difficulties of testing solid soil for ^{18}O was a large hurdle and should now allow for a more inclusive soil profile in incubations. An incubation such as that performed *in situ* by Berntson and Aber (2000) in combination with the double labeled NO_3^- may allow for the direct comparison of NO_3^- fate with more realistic soil conditions, including both humus and mineral soil content.

Conclusions

Despite retention of both isotopic labels, this study cannot conclude that abiotic immobilization was the mechanism responsible for the retention. The ^{18}O:^{15}N molar ratio exceeded the hypothetical limit for nitrosation products involved in abiotic immobilization. Exceeding that ratio violates the assumption that abiotic immobilization through nitrosation would result in a hypothetical max ^{18}O:^{15}N molar ratio of 2:1. Violation of this assumption eliminates certainty in using the ^{18}O to differentiate biotic and abiotic immobilization. These results suggest either the presence of unreacted $K^{15}N^{18}O_3$, or the biological uptake of ^{18}O.

The fact that the ^{18}O:^{15}N molar ratio was below three does imply that some of the ^{15}N and ^{18}O detected had reacted with soil. However, it cannot be determined whether the retained label was a result of biological uptake or abiotic immobilization. Further investigation of biological uptake and incorporation of ^{18}O will be required to determine if $^{15}N^{18}O_3^-$ could be effective in testing abiotic immobilization.

None of the incubations showed significant increases in retention past fifteen minutes. The rapid immobilization suggests the possibility of abiotic processes contributing to retention. Berntson and Aber (2000) saw similar rapid immobilization during their in situ ^{15}N addition study and suggested that abiotic immobilization was likely the source of ^{15}N retention given the short time span. The suggested possibility of abiotic immobilization being the source of some of the retained ^{18}O, leaves open the possibility that the $^{15}N^{18}O_3^-$ method may be useful in investigating abiotic immobilization.

LITERATURE CITED

Aber JD, Nadelhoffer KJ, Steudler P, Melillo JM. 1989. Nitrogen saturation in northern forest ecosystems. Bioscience 39(6): 378-386

Aber JD, Magill AH. 2004. Chronic nitrogen additions at the Harvard Forest (USA): the first 15 years of a nitrogen saturation experiment. Forest Ecology and Management 196(1): 1-5

Agren GI, Bosatta E. 1988. Nitrogen saturation of terrestrial ecosystems. Environmental Pollution 54: 185-197

Azhar E, Verhe R, Proot M, Sandra P. Verstraete W, 1986a. Binding of nitrite-N on polyphenols during nitrification. Plant and Soil 94(3): 369-382

Azhar E, Vandenabeele J, Verstrate W. 1986b. Nitrification and organic nitrogen formation in soils. Plant and Soil 94(3): 383-399

Azhar E, Vancleemput O, Verstraete W. 1986c. Nitrification mediated nitrogen immobilization in soils. Plant and Soil 94(3):401-409

Beauchamp EG, Bergstrom DW. 1993. Denitrification. In: Carter MR, editor. Soil Sampling and Methods of Analysis. Ann Arbor: Lewis Publishers. p 351-357

Berntson GM, Aber JD. 2000. Fast nitrate immobilization in N saturated temperate forest soils. Soil Biology and Biochemistry 32: 151-156

Borman FH, 1982. The effects of air pollution on the New England landscape. Ambio 11: 338-346

Boyer EW, Goodale CL, Jaworski NA, Howarth RW. 2002. Anthropogenic nitrogen sources and relationships to riverine nitrogen export in the northeaster U.S.A. Biogeochemistry 57/58: 137-169

Bremner JM. 1952. The nature of soil nitrogen complexes. Journal of the Science of Food and Agriculture 3: 497-500

Bremner JM. 1954. A review of recent work on soil organic matter II. Journal of Soil Science 5(2): 214

Bremner JM. 1955. Studies on soil humic acids: I. The chemical nature of humic nitrogen. Journal of Agricultural Science 46(2): 247-256

Bremner JM. 1956. Studies on soil humic acids: II. Observations on the estimation of free amino groups. Reactions of humic acid and lignin preparations with nitrous acid. Journal of Agricultural Science 48: 352-359

Bremner JM. 1968. The nitrogenous constituents of soil organic matter and their role in soil fertility. Pontificia Accademia Scientiarum Scripta Varia 32: 143-193

Bremner JM, Fuhr F. 1966. Tracer studies of the reaction of soil organic matter with nitrite. In: The Use of Isotopes in Soil Organic Matter Studies (Report of FAO-IAEA technical meeting, Brunswick-Völkenrode). New York: Pergamon Press. p. 347-348

Clark FE. 1962. Losses of nitrogen accompanying nitrification. International Soil Conference Transcripts, New Zealand. p 173-176

Cawse PA, Cornfield AH. 1972. Biological and chemical reduction of nitrate to nitrite in γ-irradiated soils, and factors leading to eventual loss of nitrite. Soil Biology and Biochemistry 4: 497-511

Dail DB, Davidson EA, Chorover J. 2001. Rapid abiotic transformation of nitrate in an acid forest soil. Biogeochemistry 54(2): 131-146

Davidson EA., Chorover J, Dail DB. 2003. A mechanism of abiotic immobilization of nitrate in forest ecosystems: the ferrous wheel hypothesis. Global Change Biology 9: 228-236

Davidson EA, Dail DB, Chorover J. 2008. Iron interference in the quantification of nitrate in soil extracts and its effect on hypothesized abiotic immobilization of nitrate. Biogeochemistry 90(1): 65-73

Emmet BA, Kjønaas OJ, Gundersen P, Koopmans C, Tietema A, Sleep D. 1998. Natural abundance of ^{15}N in forests across a nitrogen deposition gradient. Forest Ecology and Management 101(1-3): 9-18

Fitzhugh RD, Christenson LM, Lovett GM. 2003a. The fate of $^{15}NO_2^-$ tracer in soils under different tree species of the Catskill Mountains, New York. Soil Science Society of America Journal 67: 1257-1265

Fitzhugh RD, Lovett GM, Venterea RT. 2003b. Biotic and abiotic immobilization of ammonium, nitrite, and nitrate in soils developed under different tree species in the Catskill Mountains, New York, USA. Global Change Biology 9: 1591-1601

Fenn ME, Baron JS, Allen EB, Rueth HM, Nydick KR, Geiser L, Bowman WD, Sickman JO, Meixner T, Jonshon DW, Neitlich P. 2003. Ecological effects of nitrogen deposition in the western United States. Bioscience 53(4): 404-420

Frey, SD. 2010. Personal communication.

Galloway JN, Aber JD, Erisman JW, Seitzinger, SP, Howarth RW, Cowling EB, Cosby BJ. 2003. The Nitrogen Cascade. Bioscience 53(4): 341-356

Goodale CL, Aber JD, Vitousek PM. 2003. An unexpected nitrate decline in New Hampshire streams. Ecosystems 6(1):75-86

Hulme AC. 1935. Biochemical studies in the Nitrogen metabolism of the apple fruit: I. The estimation of amino-nitrogen by the Van Slyke method in the presence of tannin. Biochemical Journal 29: 263-271

Karl DM. 2002. Nutrient dynamics in the deep blue sea. Trends in Microbiology 10: 410-418

Lotrario JB, Stuart BJ, Lam T, Arands RR, Oconnor OA, Kosson DS. 1995. Effects of sterilization methods on the physical characteristics of soil – implications for sorption isotherm analyses. Bulletin of Environmental Contamination and Toxicology 54(5): 668-675

Magill AH, Aber JD, Currie WS, Nadelhoffer KJ, Martin ME, McDowell WH, Melillo JM, Steudler P. 2004. Ecosystem response to 15 years of chronic nitrogen additions at the Harvard Forest LTER, Massachusetts, USA. Forest Ecology and Management 196: 7-28

Magill AH, Aber JD, Hendricks JJ, Bowden RD, Melillo JM, Steudler PA. 2004. Biogeochemical response of forest ecosystems to simulated chronic nitrogen deposition. Ecological Applications 7(2): 402-415

McNulty SG, Aber, J.D., Newman, S.D. 1996. Nitrogen saturation in a high elevation New England spruce-fir stand. Forest Ecology and Management 84(1-3): 108-121

Micks P, Aber JD, Boone RD, Davidson EA. 2004. Short-term soil respiration and nitrogen immobilization response to nitrogen applications in control and nitrogen-enriched temperate forests. Forest Ecology and Management 196: 57-70

Nelson DW, Bremner JM. 1969. Factors affecting chemical transformations of nitrite in soils. Soil Biology and Biochemistry 1: 229-239

Ollinger SV, Aber JD, Lovett GM, Millham SE, Lathrop RG, Ellis JM. 1993. A spatial model of atmospheric deposition for the northeastern United-States. Ecological Applications 3(3): 459-472

Ollinger SV, Aber JD, Reich PB, Freuder RJ. 2002. Interactive effects of nitrogen deposition, troposhperic ozone, elevated CO_2 and land use history on the carbon dynamics of northern hardwood foreasts. Global Change Biology 8(6): 545-562

Schnitzer M, Schuppli P. 1989. Method for the sequential extraction of organic-matter from soils and soil fractions. Soil Science Society of America Journal 53(5): 1418-1424

Schulze ED.1989. Air pollution and forest decline in a spruce (*Picea abies*) forest. Science 244(4906): 776-783

Smith CJ, Chalk PM, 1980. Fixation and loss of nitrogen during transformations of nitrite in soils. Soil Science Society of America Journal 44(2): 288-1980

Sterner RW, Elser JJ. 2002. Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere. Princeton: Princeton University Press. 439 p.

Stevenson FJ, Swaby RJ. 1964. Nitrosation of soil organic matter: I. Nature of gases evolved during nitrous acid treatment of lignins and humic substances. Soil Science Society of America Journal 28(6): 773-778

Stuart NW. 1935. Determination of amino nitrogen in plant extracts. Plant Physiology 10: 135-148

Tietema A. 1998. Microbial carbon and nitrogen dynamics in coniferous forest floor material collected along a European nitrogen deposition gradient. Forest Ecology and Management 101: 29-36

Thorn KA, Mikita MA. 2000. Nitrite fixation by humic substances: Nitrogen-15 nuclear magnetic resonance evidence for potential intermediates in chemodenitrification. Soil Science Society of America Journal 64(2): 568-582

van Breemen N, Boyer EW, Goodale CL, Jaworski NA, Paustian K, Seitzinger SP, Lajtha K, Mayer B, van Dam D, Howarth RW, Nadelhoffer KJ, Eve M, Thorn KA, Mikita MA. 2000. Nitrite fixation by humic substances: Nitrogen-15 nuclear magnetic resonance evidence for potential intermediates in chemodenitrification. Soil Science Society of America Journal 64: 568-582

Miegroet, H, Jandle R. 2007. Are Nitrogen-fertilized forest soils sinks or sources of Carbon. Environmental Monitoring and Assessment 128(1-3): 121-131

van Slyke DD. 1911. A method for quantitative determination of aliphatic amino groups. Journal of Biological Chemistry 9:185-204

Venterea RT, Groffman PM, Verchot LV, Magill AH, Aber JD, Steudler PA. 2003. Nitrogen oxide gas emissions from emperate forest soils receiving long-term nitrogen inputs. Global Change Biology 9: 346-357

Vitousek PM, Aber JD, Howarth RW, Likens GE, Matson PA, Schindler DW, Schlesinger WH, Tilman DG. 1997. Technical report: Human alteration of the global nitrogen cycle: Sources and consequences. Ecological Applications 7(3): 737-750

White, D. 2006. Inorganic Metabolism. In: The Physiology and Biochemistry of Prokaryotes. 3rd ed. New York: Oxford University.

Wolf DC, Skipper HD. 1994. Soil Sterilization. in: Weaver, R.W, et al., (Eds.) Methods of Soil Analysis: Part 2. Soil Science Society of America book Series, no. 5, Madison, WI, pp 41-51

Wrage N, van Groenigen JW, Oenema O, Baggs EM. 2005. A novel dual-isotope labeling method for distinguishing between soil sources of N_2O. Rapid Communications in Mass Spectrometry 19(22): 3298-3306

Wray JL, Kinghorn JR, editors. 1989. Molecular and genetic aspects of nitrate assimilation. New York: Oxford Science Publications. 403 p.

APPENDICES

APPENDIX A

INUBATION PROTOCOLS

Purpose statement:

- To track the fate of $^{15}N^{18}O_3^-$ in forest soils to determine whether or not the nitrate is immobilized in the soil through abiotic immobilization.

Materials

- 50 ml Filter flasks (label for use with labeled compounds)
- Buchner funnels (label for use with labeled compounds)
- Polypropylene filters 47 mm diameter, 0.45 and 2.7 μm pore
- Aluminum foil
- Label tape
- Permanent marker
- 30 ml serum vials with butyl rubber stopper and aluminum sealing caps
- hand cap press and cap remover
- silicone sealant
- non coring syringes (label for use with labeled compounds)
- 2 ml glass vials with screw caps for ground soil (1 for each sample)
- 20 ml borosilicate glass scintillation vials for soil
- 20 ml HDPE scintillation vials with conical cap for extracts
- parafilm squares, 3 1/3rds for each incubation
- weigh paper
- Aspirator
    - 250 ml Erlenmeyer flask
    - 50 ml Erlenmeyer flask (1 for labeled and 1 for unlabeled samples)
    - Hand pump
    - Syringe
    - Tubing
- Micro-spatula
- Centrifuge tubes

Procedure

1. Label serum vials and cover in aluminum foil, label inside and out of foil.
2. Label glass scintillation vial for filtered soil, HDPE scintillation vial for extract, 2 ml sample vial for ground portion, label archive bags for filters, and centrifuge tubes.
3. Weigh glass scintillation vials, and centrifuge tubes.
4. Cut parafilm squares for each incubation to be run.

5. Cut weigh paper for temporary funnels.
6. Get ice.
7. Turn on centrifuge set temperature to 4 °C; 30 m required to reach temperature.
8. Ready solution of $K^{15}N^{18}O_3^-$, and untreated DI.
    a. Weigh ____ mg $K^{15}N^{18}O_3^-$ per incubation.
    b. Record actual amount weighed.
    c. Add to scintillation vial.
    d. Add Ultra Pure DI = (amount $^{15}N^{18}O_3^-$)*(1 ml DI / ____ mg $K^{15}N^{18}O_3^-$)
9. Weigh serum vial; tare scale with serum vial.
10. Weigh 1.00 ± 0.002 g soil into 30 ml serum vial and record weight of soil to 0.001 g, repeat steps 4-6 for each vial and sample.
11. **Cap and seal vial.**
12. **Evacuate vial headspace 30 s.**
13. **Flush/fill with N_2 120 s.**
14. Add nitrate or DI solution **and seal puncture with silicon sealant.**
15. Shake solution twice and let incubate for prescribed time (15 min, 1 hr, 4 hr).
16. Uncap and add 8 mL ice cold KCl.
17. Cover in parafilm.
18. Place in Styrofoam ice beaker on shaker table shake for 30 m at 180 rpm.
19. Transfer contents of serum vial to 50 ml plastic centrifuge tube.
20. Rinse serum vial with 2 ml ice cold KCl (for total 10:1 extractant to soil).
21. Centrifuge tube at 10,000 rpm for 15 m with acceleration set to 5.
22. Ready aspirator with appropriate label or unlabeled capture flask and ice bath.
23. Ready filter flasks with funnels and filters.
24. Aspirate most of the KCl from the centrifuge tube; keep supernatant on ice.
25. Add 10 mL ice cold DI to centrifuge tube.
26. Centrifuge tube at 10,000 rpm for 15 m with acceleration set at 5.
27. Aspirate as much DI as possible from the centrifuge tube.
28. Label tube and seal with parafilm.
29. Turn on vacuum.
30. Pour collected supernatant from aspirator into filter through 2.7 μm prefilter and 0.45 μm filter.
31. Pour filtered supernatant into HPDE scintillation vial and cap.
32. Place centrifuge tube and HPDE scintillation vials in freezer.
33. Place filters into corresponding archive bags and place in refrigerator.
34. Freeze soil for at least 18 h.

- BREAK

35. Freeze dry soil 8 h (longer if not dry by then), follow freeze drying protocol.

- BREAK

36. Transfer soil to borosilicate glass vials seal cap and move to dessicator.
37. Grind portion of soil and place in corresponding labeled glass sample vial, keep in desiccator.
38. Take ground soil to mass spec, follow mass spec protocol for tin cupping and analysis.

APPENDIX B

DATA

Table 1. Raw isotope data values for the ^{15}N measurement. All measurements were made on a Thermo Finnigan elemental analyzer and continuous flow mass spectrometer combusting at 900 °C. The column (#) is the order in which incubations were performed. Label column denotes addition of $K^{15}N^{18}O_3$ solution (1) or just water (0). Time is incubation length in hours. Site is which of three sampling sites the incubation came from. Set is which of two sets of incubations, separated by two months, the incubation was performed during. The column "samp" is the weight of the subsample submitted for combustion and isotope analysis, in mg. $\delta\ ^{15}N$ is the per mil delta value of ^{15}N in the sample compared with atmospheric air. N% and C% are the total Nitrogen and Carbon as percent of the total sample mass. Finally C:N is ratio of carbon to nitrogen present in the sample.

#	Label	Atmo	Time (h)	Site	Set	samp (mg)	$\delta\ ^{15}N$ (‰)	N%	C%	C:N
1	0	Anoxic	1.0	B	α	5.10	0.96	0.899	18.538	20.600
2	1	Oxic	4.0	C	α	5.28	183.22	1.308	37.240	28.472
3	1	Anoxic	0.25	A	α	5.31	241.61	0.832	17.970	21.604
4	1	Anoxic	1.0	A	α	5.01	136.79	0.859	18.291	21.286
5	0	Oxic	1.0	A	α	5.16	1.50	0.807	16.846	20.867
6	0	Anoxic	1.0	A	α	5.26	1.41	1.006	21.758	21.631
7	0	Oxic	0.25	B	α	5.19	0.92	0.804	14.453	17.974
8	1	Oxic	0.25	A	α	5.21	149.91	0.979	20.174	20.613
9	0	Oxic	4.0	A	α	5.43	1.56	0.911	18.845	20.675
10	0	Anoxic	1.0	C	α	5.21	-0.88	1.396	38.512	27.595
11	1	Anoxic	4.0	B	α	5.42	127.94	0.901	16.843	18.703
12	0	Anoxic	0.25	C	α	5.23	-0.89	1.425	39.784	27.912
13	0	Anoxic	0.25	A	α	5.37	1.43	0.958	19.770	20.635
14	1	Anoxic	0.25	B	α	5.33	166.58	0.730	12.704	17.407
15	0	Oxic	4.0	C	α	5.49	-1.60	2.260	82.568	36.542
16	1	Oxic	1.0	A	α	5.09	124.60	1.016	20.705	20.377
17	1	Anoxic	4.0	C	α	5.04	137.81	1.391	38.037	27.353
18	0	Oxic	4.0	B	α	5.47	0.94	0.874	16.926	19.356
19	1	Anoxic	0.25	C	α	5.17	161.19	1.386	37.639	27.164
20	0	Anoxic	4.0	A	α	5.47	1.50	0.942	19.264	20.442

#	Label	Atmo	Time (h)	Site	Set	samp (mg)	$\delta^{15}N$ (‰)	N%	C%	C:N
21	0	Anoxic	0.25	B	α	5.27	1.05	0.880	15.981	18.151
22	1	Oxic	4.0	B	α	5.30	81.38	0.876	17.199	19.631
23	1	Oxic	0.25	B	α	5.51	93.63	0.882	16.596	18.813
24	1	Anoxic	1.0	B	α	5.44	86.23	0.993	19.268	19.409
25	0	Oxic	1.0	C	α	5.49	-0.93	1.464	40.570	27.710
26	1	Anoxic	1.0	C	α	5.16	89.24	1.367	36.241	26.517
27	1	Oxic	0.25	C	α	5.10	176.23	1.479	39.687	26.834
28	0	Oxic	0.25	C	α	5.15	-0.84	1.428	38.653	27.060
29	0	Oxic	0.25	A	α	5.14	1.70	1.027	20.425	19.886
30	0	Anoxic	4.0	B	α	5.49	1.44	0.859	16.884	19.653
31	0	Oxic	1.0	B	α	5.44	1.11	2.695	59.834	22.204
32	0	Anoxic	4.0	C	α	5.03	-0.61	1.502	40.386	26.891
33	1	Oxic	4.0	A	α	5.21	103.40	0.980	22.378	22.845
34	1	Oxic	1.0	C	α	5.11	147.94	1.397	38.124	27.288
35	1	Anoxic	4.0	A	α	5.38	447.98	0.763	14.817	19.416
36	1	Oxic	1.0	B	α	5.27	118.64	0.971	21.089	21.724
37	1	Oxic	0.25	A	β	5.36	45.82	0.969	21.389	22.076
38	1	Anoxic	0.25	B	β	5.46	41.92	0.869	17.370	19.994
39	0	Anoxic	4.0	C	β	5.12	-0.79	1.442	39.285	27.244
40	0	Oxic	4.0	C	β	5.11	-0.88	1.418	39.240	27.668
41	0	Oxic	4.0	B	β	5.30	1.32	0.924	18.249	19.751
42	1	Anoxic	0.25	C	β	5.30	73.96	1.462	39.760	27.191
43	1	Anoxic	4.0	C	β	5.42	62.83	1.480	40.861	27.611
44	1	Oxic	4.0	B	β	5.20	61.79	0.927	18.783	20.263
45	0	Oxic	1.0	B	β	5.47	0.71	0.883	18.277	20.696
46	1	Anoxic	0.25	A	β	5.11	163.09	0.935	19.693	21.068
47	1	Oxic	4.0	C	β	5.06	141.65	1.427	38.756	27.150
48	0	Oxic	0.25	A	β	5.12	1.63	0.932	19.594	21.034
49	0	Anoxic	0.25	B	β	5.26	1.09	0.903	17.869	19.786
50	1	Anoxic	1.0	B	β	5.28	161.68	0.847	16.857	19.907
51	1	Anoxic	4.0	B	β	5.38	146.69	0.905	17.981	19.878
52	0	Oxic	0.25	B	β	5.48	0.85	0.915	18.802	20.559
53	1	Anoxic	1.0	C	β	5.33	157.23	1.377	37.757	27.429
54	0	Anoxic	0.25	C	β	5.14	-0.86	1.427	38.775	27.171
55	1	Anoxic	1.0	A	β	5.03	140.28	0.913	19.891	21.784
56	0	Anoxic	1.0	B	β	5.23	0.66	0.895	18.279	20.432

#	Label	Atmo	Time (h)	Site	Set	samp (mg)	$\delta^{15}N$ (‰)	N%	C%	C:N
57	0	Oxic	0.25	C	β	5.21	-0.64	1.320	36.238	27.460
58	1	Oxic	1.0	B	β	5.30	52.79	0.933	20.286	21.750
59	0	Anoxic	1.0	C	β	5.07	-0.54	1.439	39.433	27.412
60	0	Anoxic	4.0	B	β	5.31	1.66	0.841	18.438	21.915
61	0	Anoxic	0.25	A	β	5.48	1.66	1.014	23.594	23.278
62	0	Anoxic	1.0	A	β	5.38	1.95	0.957	22.109	23.109
63	0	Anoxic	4.0	A	β	5.40	1.67	0.953	22.060	23.150
64	1	Oxic	0.25	C	β	5.32	95.30	1.496	40.596	27.136
65	1	Oxic	1.0	C	β	5.19	103.35	1.338	37.106	27.736
66	1	Oxic	1.0	A	β	5.46	85.24	0.979	22.711	23.191
67	0	Oxic	1.0	C	β	5.44	-0.82	1.418	39.183	27.630
68	1	Anoxic	4.0	A	β	5.47	177.24	0.934	21.897	23.435
69	1	Oxic	0.25	B	β	5.38	80.78	0.867	18.335	21.147
70	0	Oxic	1.0	A	β	5.09	1.72	0.940	21.894	23.299
71	0	Oxic	4.0	A	β	5.21	1.60	0.955	21.593	22.599
72	1	Oxic	4.0	A	β	5.35	53.64	0.990	22.431	22.649

Table 2. The summary statistics of the isotopic standards including with the incubation samples during ^{15}N analysis. "Standard" denotes the type of material: an internal soil standard collected in College Woods on the University of New Hampshire campus in Durham, NH, IAEA NIST 1515 apple leaf, or IAEA NIST 1575a pine needle . The Statistic column denotes the summary statistic of that row. Amount is the mg of standard combusted for analysis. $\delta^{15}N$ (‰) is the per mil value of ^{15}N compared against atmospheric air. C:N is the ratio of carbon to nitrogen in the sample.

Standard	Statistic	Amount (mg)	$\delta^{15}N$ (‰)	C:N
Internal Soil	Mean	4.378	-2.94	28.978
	Count	11	11	11
	St Err	0.031	0.05	0.195
NIST 1515	Mean	4.488	0.66	22.071
	Count	9	9	9
	St Err	0.044	0.04	0.111
NIST 1575a	Mean	4.454	-3.94	46.333
	Count	18	18	18
	St Err	0.039	0.20	0.191

Table 3. Raw isotope data values for the ^{15}N measurement. All measurements were made on a Thermo Finnigan elemental analyzer and continuous flow mass spectrometer combusting at 1400 °C. The column (#) is the order in which incubations were performed. Label column denotes addition of $K^{15}N^{18}O_3$ solution (1) or just water (0). Time is incubation length in hours. Site is which of three sampling sites the incubation came from. Set is which of two sets of incubations, separated by two months, the incubation was performed during. The column "samp" is the weight of the subsample submitted for combustion and isotope analysis, in mg. $\delta\ ^{18}O$ is the per mil delta value of ^{18}O in the sample compared with ^{16}O in VSMOW.

#	Label	Atmo	Time (h)	Site	Set	samp (mg)	$\delta\ ^{18}O$ (‰)	%O
1	0	Anoxic	1	B	α	0.130	16.11	23.676
2	1	Oxic	4	C	α	0.088	66.20	29.811
3	1	Anoxic	0.25	A	α	0.142	67.21	22.539
4	1	Anoxic	1	A	α	0.130	46.77	25.068
5	0	Oxic	1	A	α	0.091	16.17	23.755
6	0	Anoxic	1	A	α	0.092	17.68	26.846
7	0	Oxic	0.25	B	α	0.093	17.69	18.232
8	1	Oxic	0.25	A	α	0.115	55.64	22.573
9	0	Oxic	4	A	α	0.113	16.67	23.453
10	0	Anoxic	1	C	α	0.089	18.26	39.955
11	1	Anoxic	4	B	α	0.121	42.85	21.832
12	0	Anoxic	0.25	C	α	0.097	18.70	34.624
13	0	Anoxic	0.25	A	α	0.132	16.22	25.749
14	1	Anoxic	0.25	B	α	0.099	55.70	22.207
15	0	Oxic	4	C	α	0.138	19.15	31.214
16	1	Oxic	1	A	α	0.098	44.79	24.454
17	1	Anoxic	4	C	α	0.121	53.65	32.021
18	0	Oxic	4	B	α	0.100	16.82	19.533
19	1	Anoxic	0.25	C	α	0.097	62.72	30.520
20	0	Anoxic	4	A	α	0.179	16.38	22.818
21	0	Anoxic	0.25	B	α	0.165	16.10	23.253
22	1	Oxic	4	B	α	0.156	34.07	25.056
23	1	Oxic	0.25	B	α	0.143	36.69	23.343
24	1	Anoxic	1	B	α	0.143	36.15	24.187
25	0	Oxic	1	C	α	0.151	18.67	31.587
26	1	Anoxic	1	C	α	0.120	44.39	29.648
27	1	Oxic	0.25	C	α	0.156	67.27	31.887
28	0	Oxic	0.25	C	α	0.122	19.28	33.460
29	0	Oxic	0.25	A	α	0.139	16.16	28.703

#	Label	Atmo	Time (h)	Site	Set	samp (mg)	$\delta^{18}O$ (‰)	%O
30	0	Anoxic	4	B	α	0.152	16.20	24.246
31	0	Oxic	1	B	α	0.165	15.99	22.093
32	0	Anoxic	4	C	α	0.128	18.82	32.111
33	1	Oxic	4	A	α	0.120	39.33	26.925
34	1	Oxic	1	C	α	0.186	57.58	29.098
35	1	Anoxic	4	A	α	0.184	116.73	20.081
36	1	Oxic	1	B	α	0.160	38.57	28.636
37	1	Oxic	0.25	A	β	0.156	25.90	24.958
38	1	Anoxic	0.25	B	β	0.144	24.52	22.688
39	0	Anoxic	4	C	β	0.142	18.83	32.915
40	0	Oxic	4	C	β	0.147	18.95	31.306
41	0	Oxic	4	B	β	0.178	15.62	22.963
42	1	Anoxic	0.25	C	β	0.144	32.41	33.511
43	1	Anoxic	4	C	β	0.139	32.17	32.931
44	1	Oxic	4	B	β	0.176	30.27	25.662
45	0	Oxic	1	B	β	0.147	15.92	23.957
46	1	Anoxic	0.25	A	β	0.129	53.48	28.207
47	1	Oxic	4	C	β	0.147	57.97	30.288
48	0	Oxic	0.25	A	β	0.117	16.14	22.896
49	0	Anoxic	0.25	B	β	0.130	15.49	21.721
50	1	Anoxic	1	B	β	0.142	53.87	19.240
51	1	Anoxic	4	B	β	0.161	50.73	20.830
52	0	Oxic	0.25	B	β	0.137	15.67	23.086
53	1	Anoxic	1	C	β	0.154	60.21	30.233
54	0	Anoxic	0.25	C	β	0.151	18.99	29.768
55	1	Anoxic	1	A	β	0.168	49.38	22.164
56	0	Anoxic	1	B	β	0.163	15.71	22.783
57	0	Oxic	0.25	C	β	0.138	19.02	30.080
58	1	Oxic	1	B	β	0.135	29.15	22.052
59	0	Anoxic	1	C	β	0.167	19.27	30.254
60	0	Anoxic	4	B	β	0.124	16.21	21.158
61	0	Anoxic	0.25	A	β	0.149	16.57	23.245
62	0	Anoxic	1	A	β	0.127	16.08	25.678
63	0	Anoxic	4	A	β	0.114	16.19	23.105
64	1	Oxic	0.25	C	β	0.144	45.29	33.148
65	1	Oxic	1	C	β	0.161	46.78	30.845
66	1	Oxic	1	A	β	0.146	35.50	24.268

#	Label	Atmo	Time (h)	Site	Set	samp (mg)	δ ^{18}O (‰)	%O
67	0	Oxic	1	C	β	0.124	18.69	31.464
68	1	Anoxic	4	A	β	0.133	53.75	25.702
69	1	Oxic	0.25	B	β	0.169	34.51	21.317
70	0	Oxic	1	A	β	0.164	16.76	25.384
71	0	Oxic	4	A	β	0.120	16.05	25.907
72	1	Oxic	4	A	β	0.126	28.79	24.863

Table 4. The summary statistics of the isotopic standards including with the incubation samples during ^{18}O analysis. "Standard" denotes the type of material, here an internal soil standard collected in College Woods on the University of New Hampshire campus in Durham, NH, or IAEA NIST 1515 apple leaf. Both standards were calibrated against NIST sucrose. The Statistic column denotes the summary statistic of that row. Amount is the mg of standard combusted for analysis. δ ^{18}O (‰) is the per mil value of ^{18}O compared against VSMOW.

Standard	Statistic	Amount (mg)	δ ^{18}O (‰)
Internal Soil	Mean	0.103167	20.88813
	Count	30	30
	St Err	0.004606	0.046923
NIST 1515	Mean	0.1001	22.87233
	Count	10	10
	St Err	0.009161	0.073857