RELATIVE STABILITY OF NITROGEN IN SOIL ORGANIC MATTER DEPENDS ON SOURCES OF INPUT

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Abstract. The nitrogen (N) cycle in temperate hardwood forest soils in the northeastern United States was closely examined to explore how different forms of N inputs affect the stability of N in soil organic matter (SOM). Despite years of receiving elevated levels of pollutant N from atmospheric deposition, recent inorganic N concentrations in forest stream runoff have been declining, possibly the result of poorly understood processes of N storage in SOM. To better understand N storage in forest soils, we conducted a ^{15}N tracer study to compare how the stability of N in recently formed SOM differs between N that is derived from decomposed plant organic material (representing the primary source of N inputs to soils in most undisturbed forests) versus N that is deposited in the inorganic form as nitrate (NO_3^-; representing the dominant N form in atmospheric deposition). We hypothesized that inorganic-N incorporated into SOM would be more stable and would be lost from the mineral-associated organic matter (MAOM) fraction at a slower rate than N derived from plant matter decomposition. Contrary to this hypothesis, we found that N derived from NO_3^- exhibited greater rates of loss from SOM while N derived from leaf litter appeared more stable over a two-week time period. These findings will contribute to a broader understanding of how atmospheric deposition of N is affecting the soil N cycle in forests.

INTRODUCTION

Of all essential nutrients, nitrogen (N) is required by plants in the largest quantity and is most frequently the limiting factor in plant productivity, yet it is easily lost from the soil system through hydrologic and gaseous loss pathways. Historically, N has been recognized as the nutrient most likely to limit forest growth in temperate and boreal ecosystems (Driscoll et al. 2003). Forests are known to have a high retention of added N (30-80%), in part dependent on the N saturation status of the system (Lovett and Goodale 2011; Weitzman and Kaye 2016). Nitrogen retention in the forest can occur through root uptake and storage in plant biomass and by microbial immobilization and abiotic soil processes, which result in storage in soil organic matter (SOM) pools (Driscoll et al. 2003). Although forests have been shown to generate greater amounts of stable N formation than other land uses (such as urban and agricultural systems) (Weitzman and Kaye 2016), within different forested systems soils with greater C content (such as old growth forests) rapidly integrate greater amounts of N into long-term storage than forest soils with lower C contents (Lewis et al. 2014).

Northeastern United States forests have received elevated inputs of reactive N for years as a result of atmospheric deposition, yet N concentrations in stream runoff from forests have been declining in many areas in recent decades (Driscoll et al. 2003). This surprising trend indicates poorly understood processes regulating N storage in forest soils over the long term. In most forest ecosystems the primary input of N to the soil is from internally cycled N from organic sources (leaf fall and root turnover), but in ecosystems downwind of industrial or automotive N emission sources, deposition from the atmosphere can also be a substantial N source to soils (Yanai et al. 2013). In soils, N can be in either particulate organic matter (POM) or mineral-associated organic matter (MAOM) pools (Hatton et al. 2012). Particulate organic matter (0.053-2 mm in size) includes materials such as dead roots, partially decomposed plants, and pollen (McClaran et al. 2008; Figueiredo et al. 2010), and can serve as an energy source for microbes. MAOM (<0.053 mm in size) is the fraction of SOM attached to clay and silt particles and tends to have

low nutrient availability (Cambardella and Elliot 1992; McClaran et al. 2008). This fraction of organic matter (OM) can remain highly persistent in soil and is the result of efficient microbial incorporation during the early stages of litter decomposition when litter loses mostly non-structural soluble compounds (Cotrufo et al. 2015; Hatton et al. 2015). MAOM can also be formed by microbial byproducts accreting to both mineral surfaces and already present MAOM (Vogel et al. 2014) and is considered to be resistant to mineralization (Castellano et al. 2012). Nitrogen retention and the rate of N immobilization decrease as the amount of MAOM accumulates (Castellano et al. 2012). A study by Hagedorn et al. (2005) concluded that 50–60% of the immobilization of ^{15}N occurred in the clay fraction smaller than 2 μm, which strongly suggests that N inputs were rapidly transferred to relatively stable SOM pools associated with soil minerals. While much of MAOM-N is assumed to have high stability, little is known about how variable the stability of newly formed MAOM-N is and whether stability depends on the source of N.

The goal of this study was an improved understanding of how N in mineral soils may be stabilized differently depending on whether the N comes from organic sources versus inorganic sources, which are characteristic of anthropogenic pollution. The objectives of this research were to 1) quantify the amount of N in newly formed MAOM using a ^{15}N isotopic tracer in a lab incubation, and to 2) evaluate the stability of that MAOM-N during a field incubation. We quantified how much of the newly formed MAOM-N remained in that fraction versus how much was lost or transferred to the POM pool during the field incubation.

Nitrogen derived from OM such as decomposing leaf litter can be incorporated into MAOM by leaching of dissolved organic N from plant material and subsequent adsorption onto MAOM, or by microbial uptake followed by adsorption of microbial byproducts (Bingham and Cotrufo 2016). Nitrate-N can be similarly incorporated into MAOM by microbial uptake and subsequent adsorption of microbial byproducts, but NO_3^-N can also be retained in MAOM by direct chemical reactions between NO_3^- or NO_2^- and carbon compounds in the MAOM (Davidson et al. 2003, Fitzhugh et al. 2003). We expected to see organic-derived ^{15}N on MAOM decline faster than inorganic-derived ^{15}N (meaning the OM-N is less stable) because it is the form of N that microbes should be best adapted to use. It is important to note that a different segment of the microbial community may be involved in the N cycling for the different inputs, because of how the N is accessed and immobilized.

METHODS

Site History and Land Management

Our study site was a mixed-species forest located on the grounds of the Cary Institute of Ecosystem Studies in Millbrook, NY (N 41.7865°, W 73. 7387°) immediately across Lovelace Drive from the Cary Institute weather station. The forest site has little understory and is mostly comprised of older, tall sugar maple (*Acer saccharum*) in the canopy. Agriculture abandonment in this area began in the 1890s, farming ceased entirely by 1939, and the forests have regrown from these pastures and woodlots without active management (Katz 2010). The growth of *Viburnum acerifolim* suggests this land was a former woodlot, as it is considered to be one of the best indicator species for former woodlots (Glitzenstein et al. (1990). Since 1939, the site has remained relatively undisturbed.

Sources of ^{15}N labeling for soil

The use of ^{15}N enrichment allowed us to quantify rates of N transformation in the soil and trace the fate of added N. The isotope tracer allows for precise detection of small changes against a large background, as ^{15}N is traceable at very low concentrations (Lewis et al. 2014). The two sources of ^{15}N for this experiment were ^{15}N- potassium nitrate (KNO_3) and ^{15}N-labeled leaves. Potted sugar maple saplings were initially labeled by applying ^{15}N labeled ammonium chloride ($^{15}NH_4Cl$, ≥98 atom % ^{15}N) to the pots in May of

2014, when the leaves were beginning to expand, with the leaf litter samples used in this study then collected the following year in 2015. The air-dried leaf litter was ground in June 2017 for purposes of this project.

Laboratory Incubation

Mineral horizon soil from 4-12 cm below the forest floor was collected from the study site in June 2017 and subsequently sieved (2 mm) to remove rocks and coarse roots. By utilizing ^{15}N-labeled leaf litter and ^{15}N-labeled nitrate, we sought to produce labeled MAOM from two sources. The incubations with 250 g of soil were split into five 50g aliquots to facilitate even mixing of the tracer materials into the soil. For the organic N labeling treatment, the 50g aliquots of field moist soil were mixed with 5g of dry crushed leaf litter. Our laboratory incubation method with labeled litter was based on that outlined by Vogel et al. (2014). The inorganic N soils of 50 g were enriched with 0.59 g $K^{15}NO_3^-$ (≥98 atom % ^{15}N) that was dissolved in 5 ml of water and homogeneously applied onto the soil surface of all samples and continuously mixed into soil for even distribution. The total amount of nitrogen applied to the soil in the KNO_3 was 112.4 mg and the total amount of ^{15}N was 29.4 mg. In the organic N treated soils, 5 g of leaf litter were added to each of the five 50 g soil aliquots and thoroughly homogenized. The leaf litter had an enrichment of $\delta^{15}N$ =1865‰. The total amount of nitrogen applied to the soil from the litter was 257.5 mg and the total amount of ^{15}N was 2.68 mg. The control soils remained untreated. Soil samples were incubated for a week at 18ºC and 75% relative humidity, and then the labeled samples were wet-sieved through a 53 μm sieve to separate the MAOM (<53 μm) from the POM (>53 μm) (Figueiredo et al. 2010). This allowed us to obtain ^{15}N labeled MAOM, which was then mixed back into unlabeled soil (1:10 ratio of MAOM to soil) to create whole soil with MAOM labeled by ^{15}N from an organic source (sugar maple leaf litter) or an inorganic source (KNO_3).

Field Incubation

Soils were returned to the field for an additional incubation in buried polyester-mesh bags (approximately 360 μm mesh size) in order to determine the *in situ* stability of the N in newly-formed MAOM in each soil mixture over time. A total of 24 soil incubation bags, each 5 x 10 cm in size, were buried for up to four weeks in the mineral horizon immediately beneath the forest floor. Each bag contained either control (unlabeled) soil, or soil in which the MAOM has been labeled with ^{15}N from NO_3^- or from leaf litter. Samples were divided into three categories: control (6 samples), inorganic treatment (from labeled (NO_3^-) - 9 samples) and organic treatment (from labeled leaf litter - 9 samples). In the field incubation, inorganic and organic treatments were paired closely together (at least 1 m apart in a matched environment), but separated far enough to avoid exchange of N. There were 3 time points for collection of the soil bags: initial, and after 2 and 4 weeks in the field. A total of 8 soil incubation bags (2 from control, 3 from inorganic, and 3 from organic treatments), was tested for initial concentrations of MAOM and POM. Recovered samples from the field had the MAOM separated from the bulk soil and POM by sieving. Samples were then centrifuged and dried overnight in a 60º C oven and ball-milled in preparation for the isotopic analysis.

Analytical Methods

For isotopic analysis, samples were sent to the Cornell University Stable Isotope Laboratory (Ithaca, NY) for ^{15}N and ^{13}C analysis on Thermo Delta V isotope ratio mass spectrometer (IRMS) interfaced to a NC2500 elemental analyzer.

Soil texture was determined be measuring the settling rates of the three sizes of primary soil particles as in an aqueous solution using a hydrometer (Schmidt 2008). Samples were pre-treated to remove soluble salts

and OM with subsequent dispersion using sodium hexametaphosphate. Corrections for temperature and for solution viscosity were made by taking a hydrometer reading of a blank solution.

The pH of the soils was measured electrometrically with a glass pH electrode in a slurry with a soil: water ratio of 1:5. After calibration of the electrode, the sample was thoroughly mixed/swirled, left undisturbed for 30 minutes, and then pH was measured (Westcott 1978).

Statistical Analysis

We made comparisons using 1-way ANOVA and the student's t test. For the 1-way ANOVA, we compared ^{15}N enrichment values ($\delta^{15}N$) for the MAOM and POM for each treatment over time. In addition, we compared ^{15}N enrichment values ($\delta^{15}N$) for the ^{15}N mass per g soil for POM + MAOM in the different treatments over time. For the student's t test, we compared ^{15}N enrichment values ($\delta^{15}N$) for the MAOM and POM for individual treatments over time. Results were considered significant at $\alpha = 0.05$.

RESULTS

Soil Characteristics

The soil pH was 4.53 and the soil texture was sandy loam with a composition of 66.2% sand, 28.9% silt, and 4.9% clay. The soil had 0.30% of total N and 4.27% total C (0.31% N and 4.57% C for POM fraction and 0.30% N and 3.97% C for MAOM fraction).

Production of labeled MAOM (lab incubation)

The results from the lab incubation demonstrate the difference in ^{15}N enrichment for soils 1 week after the treatments were applied. The isotopic enrichment of ^{15}N in MAOM in the inorganic treatment was markedly higher than in the organic treatment ($\delta^{15}N$ values of 2833.65‰ vs. 107.76‰), reflecting the likely higher enrichment of ^{15}N in the KNO_3 compared to the sugar maple leaves. MAOM in the control samples did not show any ^{15}N enrichment above the natural abundance background values ($\delta\ ^{15}N$ = 5.59‰).

Effects of treatments and time in field incubation

Here we refer to the soil with MAOM labeled by KNO_3 as "inorganic", and the soil with MAOM labeled by sugar maple leaves as "organic".

The ^{15}N tracer values for inorganic POM (Fig. 1) were highly variable and had no significant change ($p = 0.19$) with time. Organic POM showed a significant decrease in $\delta^{15}N$ between the initial sample and 2 weeks ($p = 0.04$), but not from 2 weeks to 4 weeks ($p = 0.09$).

The inorganic MAOM had a high initial $\delta\ ^{15}N$ label, but the $\delta\ ^{15}N$ declined significantly by week 2 and then remained essentially unchanged through week 4 (Fig. 2). Organic MAOM showed no change over time (Fig. 2).

Adding the mass fractions together allows for accounting of the total ^{15}N, which would not be affected by methodological limitation on the complete separation of SOM size fractions. In the organic treatment, there was a significant decline in the total ^{15}N through week 2, but this was followed by an increase so that there was no overall decline through week 4 (Fig. 3). For the inorganic treatment, there was a substantial decline between the initial samples and 2 weeks, but this change was not statistically significant (Fig. 3), perhaps because of the small sample size ($n = 3$).

DISCUSSION

Loss of N from MAOM and POM in the soil may occur if the N is leached out or taken up by microbes or roots. We expected to see the $\delta^{15}N$ of MAOM decline over time at a faster rate for the organic treatment than for the inorganic treatment, because decomposing plant material is the most abundant source of N in the forest soil and microbes and roots should be well adapted to use it. However, our results did not support this hypothesis, and the $\delta^{15}N$ on MAOM was higher initially and declined faster for the inorganic treatment than for the organic treatment.

Nitrate-N is immobilized directly by microbes and the N can transfer to MAOM through microbial exudates or compounds released during microbial turnover. Some soluble compounds can leach from decomposing leaves and either be stabilized directly on mineral particles or become accessible to microbes. Adsorption to mineral surfaces is the first step in long-term N storage and stabilizes the chemically labile microbial products (Bingham and Cotrufo 2016).

We expected that $\delta^{15}N$ values on POM would be stable over time, except if tracer N was transferred from MAOM to POM by microbial action, which would lead to an increase in $\delta^{15}N$ in POM over time. Instead, we observed a decrease in $\delta^{15}N$ in POM for the organic treatment. This may indicate that the sieving methods used in this experiment may have failed to fully or consistently separate the MAOM fraction from the POM fraction. This incomplete separation would result in tracer N in MAOM to incorrectly be accounted for in the POM fraction. It is possible that that some of the labeled MAOM became incorporated into aggregates that were too large to pass through the sieve. More aggressive methods for MAOM separation exist such as the use of sieves paired with sonication (Cambardella and Elliot 1993), mechanical dispersion of the soil by agitation in water with glass beads (Aoyama et al. 1999), and wet-sieving followed by density fractionation with sodium polytungstate (SPT) solution (McClaran et al. 2008). We used the less intensive sieving process to initially avoid getting any of the original leaf material (undecomposed) through the sieve, which would have been incorrectly accounted for as MAOM and artificially increased the ^{15}N value of the initial samples.

The inorganic treatment in the MAOM led to an initial high label and has a steep drop-off in the subsequent weeks (Fig. 2). This key finding from these results suggests that MAOM with the N derived from the NO_3^- tracer may be less stable over time, from initial sampling through 4 weeks. There is also the possibility that this result is an artifact of the methodology. If even a small amount of inorganic NO_3^- unused by microbes remained in the soil at the end of the laboratory incubation, the inorganic tracer would stay in the MAOM fraction absent sufficient leaching. A follow-up study would need to be performed with repeated washing of the labeled MAOM fraction to rule out or confirm this potential artifact.

The mass of ^{15}N in POM + MAOM did not change significantly over time for the organic treatment and the $\delta^{15}N$ values for are consistently lower for the organic than the inorganic treatment (Fig.3). This leads to a second key finding: the ^{15}N provided by the organic treatment has short-term stability for at least 4 weeks during the field incubation.

The mass of ^{15}N in POM + MAOM for the inorganic treatment (Fig. 3) mirrors the results from Fig. 2, however, it appears that the inclusion of POM masked the changes detected in MAOM alone (Fig. 1). Having an n value of only 3 allowed for high variation that limited our ability to detect differences.

SOM becomes more stabilized over time when it is protected in an aggregate form. The formation of new macroaggregates during the field incubation could explain why some of the MAOM was not included in the <53μm fraction where we expected it. If the MAOM was incorporated into the POM fraction by

aggregation, that suggests the need for other methodologies that further separate macroaggregates of labeled MAOM and POM more precisely.

The soils of undisturbed forest ecosystems are able to retain large volumes of N, absorb it as a pollutant and keep it from flowing into surface waters (Gundersen et al. 2006). The vast majority of N in forest soils is stored in organic forms requiring depolymerization by microbially-derived extracellular enzymes prior to root uptake (Phillips et al. 2011). Stabilization of N on MAOM increases the capacity of soils to retain N, and in soils that lack clay minerals that promote stabilization (e.g., sandy soils) the SOC and SON is less protected against microbial mineralization (Langley et al. 2009). The results of our experiment suggest that N retained from inorganic deposition may be less stable than N naturally cycling in an undisturbed forest. SON may cycle between microbial biomass and residues, adsorption and desorption from soil mineral particles, and dissolution and precipitation from the soil solution before it is held in the soil matrix (Bingham and Cotrufo 2016).

The results of this pilot experiment also demonstrate the potential to quickly produce highly labeled MAOM in soil that can be used to track stability in the field over time. To our knowledge, this has not been done before. It is a promising method, which can be used trace fates in ^{15}N in soils, including roots and microbial biomass. Although the results convey short-term stability, they can be used as an index for long-term stability studies. Taking this into account, more research should explore a longer timescale for nitrogen stabilization in MAOM within undisturbed and disturbed ecosystems and the effects of air pollution.

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APPENDIX

TABLE 1A. Inorganic-N, $\delta^{15}N$ pooled average ratio values (compared to the initial time point).

Time	POM	MAOM
Initial	1	1
2 weeks	0.43	0.32
4 weeks	0.62	0.46

TABLE 1B. Organic-N, $\delta^{15}N$ pooled average ratio values (compared to the initial time point).

Time	POM	MAOM
Initial	1	1
2 weeks	0.62	1.08
4 weeks	0.61	1.27

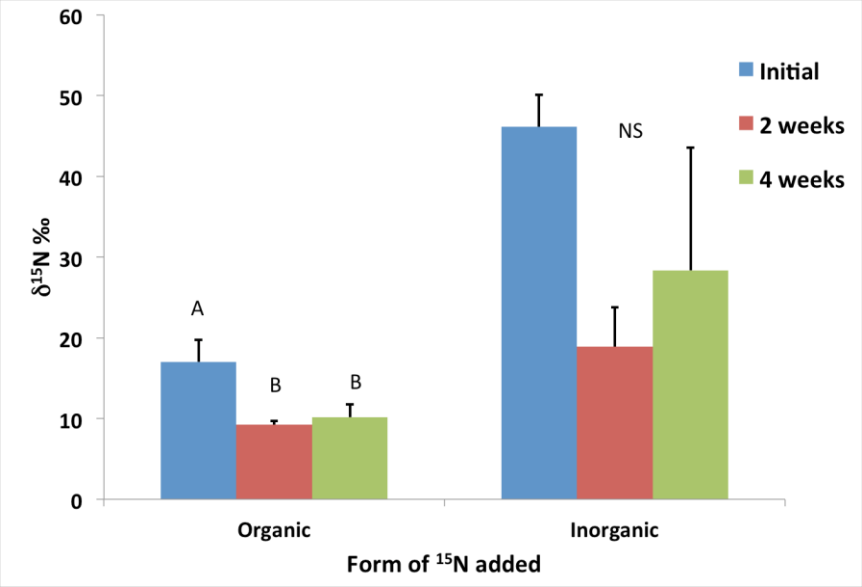

FIGURE 1. Mean $\delta^{15}N$ values for the POM fraction at each time point for both treatments. Vertical bars represent standard errors. Different letters above the bars denote significant differences between means ($p < 0.05$).

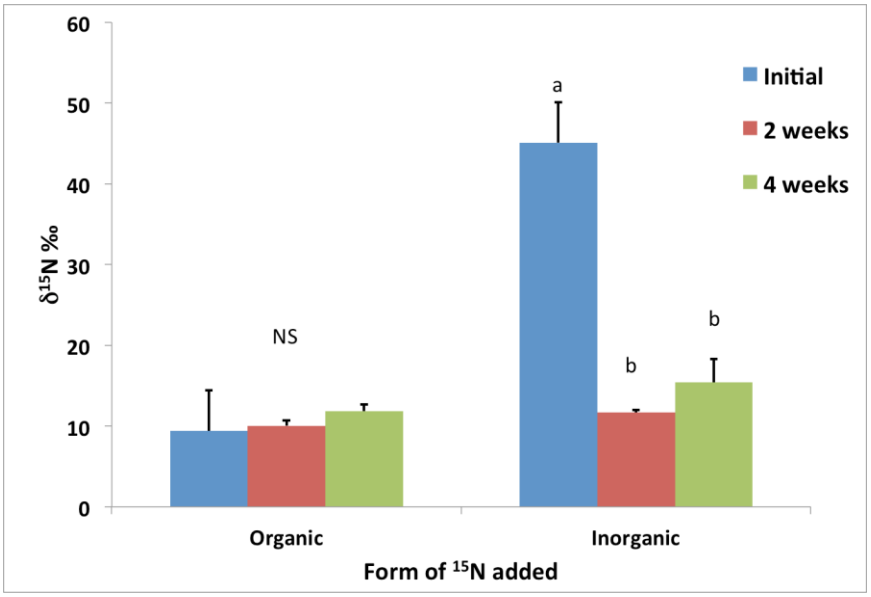

FIGURE 2. Mean $\delta^{15}N$ values for the MAOM fraction at each time point for both treatments. Vertical bars represent standard errors. Different letters above the bars denote significant differences between means ($p < 0.05$).

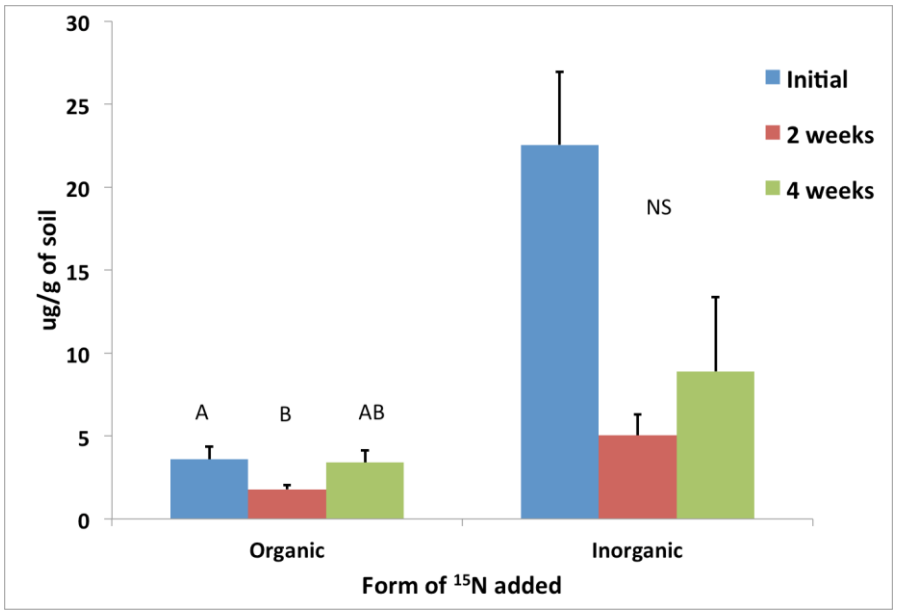

FIGURE 3. Mean mass values of ^{15}N for the POM + MAOM fractions at each time point for both treatments. Vertical bars represent standard errors. Different letters above the bars denote significant differences between means ($p < 0.05$).