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Atmospheric nitrate deposition and the microbial degradation of cellobiose and vanillin in a northern hardwood forest

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Abstract

Human activity has increased the amount of N entering terrestrial ecosystems from atmospheric NO_3^- deposition. High levels of inorganic N are known to suppress the expression of phenol oxidase, an important lignin-degrading enzyme produced by white-rot fungi. We hypothesized that chronic NO_3^- additions would decrease the flow of C through the heterotrophic soil food web by inhibiting phenol oxidase and the depolymerization of lignocellulose. This would likely reduce the availability of C from lignocellulose for metabolism by the microbial community. We tested this hypothesis in a mature northern hardwood forest in northern Michigan, which has received experimental atmospheric N deposition (30 kg NO_3^-–N ha^{-1} y^{-1}) for nine years. In a laboratory study, we amended soils with ^{13}C-labeled vanillin, a monophenolic product of lignin depolymerization, and ^{13}C-labeled cellobiose, a disaccharide product of cellulose degradation. We then traced the flow of ^{13}C through the microbial community and into soil organic carbon (SOC), dissolved organic carbon (DOC), and microbial respiration. We simultaneously measured the activity of enzymes responsible for lignin (phenol oxidase and peroxidase) and cellobiose (β-glucosidase) degradation. Nitrogen deposition reduced phenol oxidase activity by 83% and peroxidase activity by 74% when compared to control soils. In addition, soil C increased by 76%, whereas microbial biomass decreased by 68% in NO_3^- amended soils. ^{13}C cellobiose in bacterial or fungal PLFAs was unaffected by NO_3^- deposition; however, the incorporation of ^{13}C vanillin in fungal PLFAs extracted from NO_3^- amended soil was 82% higher than in the control treatment. The recovery of ^{13}C vanillin and ^{13}C cellobiose in SOC, DOC, microbial biomass, and respiration was not different between control and NO_3^- amended treatments. Chronic NO_3^- deposition has stemmed the flow of C through the heterotrophic soil food web by inhibiting the activity of ligninolytic enzymes, but it increased the assimilation of vanillin into fungal PLFAs.

Keywords: ^{13}C-Labeled substrates; ^{13}C-PLFA; Microbial enzymes

1. Introduction

Anthropogenic NO_3^- deposition is a global process that has increased N inputs to terrestrial ecosystems (Aber et al., 1989, 1998; Lovett, 1994; Fenn et al., 1998). Chronic deposition of NO_3^- will likely augment N availability in temperate forests, where N typically limits plant growth (Vitousek and Howarth, 1991). For example, northern hardwood forests in northeastern United States are receiving the greatest amounts of N deposition in North America (Fenn et al., 1998), ranging from 2 to 16 times background levels (Galloway et al., 1984). Increases in N availability in temperate forests is a primary concern, because it has the potential to alter ecosystem-scale C cycling by decreasing the decomposition of plant litter (Berg, 1986; Fog, 1988; Berg and Matzner, 1997).

Mounting evidence indicates that decreases in plant litter decomposition due to chronic N deposition can be partially explained by the inhibitory effect excess N has on extracellular enzymes that mediate microbial decomposition of plant litter (Berg, 1986; Carreiro et al., 2000; DeForest et al., 2004). Specifically, the rapid microbial assimilation of anthropogenic NO_3^- and its subsequent release as NH_4^+ (Zogg et al., 2000) has the potential to suppress the activity of white rot fungi, due to the inhibition of their ligninolytic enzymes by high levels of NH_4^+ (Berg, 1986; Fog, 1988). Because lignin protects plant tissue from

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decomposition, a reduction in lignin degradation could reduce the quantities C available for metabolism by other heterotrophic microorganisms. For example, cellulose is a major substrate for heterotrophic metabolism; however, the decomposition of plant litter containing cellulose associated with lignin is suppressed in soils with high N availability (Berg, 1986; Fog, 1988). Therefore, anthropogenic N deposition could potentially reduce the decomposition of lignin and other plant cell wall components.

The extent to which anthropogenic NO_3^- deposition has altered the flow of C through the microbial foodweb is poorly understood. We are unsure how the suppression of lignin-degrading fungi will alter soil microbial communities and, thus, the flow of C in soil. We have observed that chronic NO_3^- addition can reduce microbial biomass, indicating a reduction in substrate availability (DeForest et al., 2004). Furthermore, NO_3^- additions also can suppress enzymes responsible for cellobiose (β-glucosidase) and lignin (peroxidase and phenol oxidase) degradation, which suggests the microbial community has a reduced capacity to degrade recalcitrant plant litter (DeForest et al., 2004).

Based upon this knowledge, we investigated the hypothesis that NO_3^- additions, by reducing the depolymerization of lignocellulose, will also reduce the ability of soil microorganisms to metabolize and assimilate cellobiose and vanillin. Cellobiose is a disaccharide produced during the enzymatic hydrolysis of cellulose, which can be further metabolized to glucose by β-glucosidase (Eriksson and Wood, 1985). We reason that the observed reduction in β-glucosidase activity in N amended soils would limit the amount of energy obtained by the microbial community through cellulose degradation. Vanillin is a monophenolic compound that is a common product of lignin depolymerization, a process mediated by phenol oxidase and peroxidase (Pearl, 1967). The reduction in these ligninolytic enzymes would reduce the amount of monophenolic compounds available in the soil. Our objective was to determine if NO_3^- additions are reducing the flow of C from cellulose and lignin through the soil food web. To determine changes in the flow of C due to NO_3^- deposition, we used ^{13}C-cellobiose and ^{13}C-vanillin and traced the flow of ^{13}C through the microbial community and into common pools of C in the soil.

2. Materials and methods

2.1. Study site and soil sampling

Our study site was located (N 45°33′ by W 84°51′) in a mature northern hardwood forest in northern, Lower Michigan, USA. This stand is dominated by sugar maple (*Acer saccharum* Marsh.) and is representative of northern hardwood forest in the upper Great Lake States. Total basal area is 33 m^2 ha^{-1} and sugar maple represents 86% of the biomass. The mean annual temperature is 6.1 °C and the site receives 828 mm of precipitation annually. The soil is classified as a sandy, mixed, frigid Typic Haplorthod (MacDonald et al., 1995). Within this study site, we established six 30 × 30 m^2 experimental plots. Three plots serve as controls, whereas the remaining three plots receive ambient N deposition plus 30 kg NO_3^-–N ha^{-1} yr^{-1}. Since 1994, $NaNO_3$ pellets have been applied six times during the growing season. Over the 2002 growing season, extractable total inorganic N in control soils is 10.1 ± 1.3 μg N g^{-1} (Mean ± SE), whereas NO_3^- amended soil contain 17.3 ± 1.2 μg N g^{-1} (DeForest et al., in prep). Ammonium represents 76% of extractable inorganic N in control soils and 61% in NO_3^- amended soils. In late June, 2002, we collected twelve mineral soil cores (2-cm diameter and 10-cm deep; A and E horizons) in all six plots. In each plot, soil samples were composited and placed in plastic bags, kept at 4 °C, and processed within 24 h of field collection.

2.2. Soil enzyme analysis

We measured the activities of β-glucosidase, an enzyme responsible for the degradation of cellobiose, and the lignin degrading enzymes peroxidase and phenol oxidase. We used three analytical replicates for each enzyme for each plot. We prepared enzyme assays by mixing 2 g of soil in 150 ml of acetate buffer (50 mM, pH 5). β-glucosidase activity was fluorometrically measured in 96-well plates using 4-methylumbelliferone-β-D-glucoside as a substrate (Saiya-Cork et al., 2002). At the termination of the assay, we added 25 μl of NaOH (0.2 M) to each well to enhance fluorescence, which was measured using a F-max fluorometer (Molecular Devices Corp., Sunnyvale, CA); excitation energy was 355 nm and emission was measured at 460 nm. We measured peroxidase and phenol oxidase activity using L-dihydroxyphenylalanine (Saiya-Cork et al., 2002). After incubating the samples at 25 °C for 18 h in 96-well plates, the optical density (460 nm) of the oxidized reaction product was measured on a spectrophotometer (Bio-Tek Instruments, Winooski, Vt).

2.2.1. Labeling and incubation

We labeled field-fresh soil (12 g) from each control and N-amended plot with ^{13}C cellobiose (70 nmol ^{13}C g^{-1}) or ^{13}C vanillin (580 nmol ^{13}C g^{-1}). The cellobiose (atom % 27.5) was labeled with one ^{13}C with 99% purity (Omicron Biochemicals, Inc., South Bend, IN), whereas the vanillin (atom % 42.6) was ^{13}C ring-labeled with a 99% purity (Cambridge Isotope, Andover, MA). The ^{13}C substrate was delivered in 1 ml of deionized water, which did not cause saturation. We also amended soil subsamples from each plot with 1 ml of deionized water without any ^{13}C substrate to quantify the initial ^{13}C abundance in each soil pool. For each control and N-amended plot, two analytical replicates of each of the three treatments (^{13}C cellobiose, ^{13}C vanillin,

deionized water) were used to measure microbial respiration and subsequently were analyzed for phospholipid fatty acids. In addition, for each control and N amended plot, another two analytical replicates of each plot were used for sequential extractions to follow ^{13}C into dissolved organic carbon, microbial biomass, and soil organic C pools. The purpose for using two different sets of soils was to ensure an even distribution of labeled substrate while providing enough soil for all the analysis. In all, the 72 ^{13}C labeled and 36 non-labeled soil samples were incubated for 48 h at 25 °C.

2.2.2. Microbial respiration

On one half of the replicates, we determined microbial respiration by placing the samples (12 g field-fresh) in 50 ml vials within 1 qt mason jars. These jars had air-tight lids containing rubber septa for gas sampling. Using an air-tight syringe, we withdrew a 30 ml aliquot of headspace (940 ml) from each sample. Headspace CO_2 concentrations and $\delta^{13}C$ were determined by introducing headspace air into a Finnigan Delta Plus isotope ratio mass spectrometer (IRMS) with a Conflo II interface (Thermofinnigan, Bremen, Germany). We determine the initial headspace concentration of CO_2 and $\delta^{13}C$ from a sealed jar that contained no soil. The headspace concentration of CO_2 and $\delta^{13}C$ was measured once after the 48 h incubation period. The rate of respiration was calculated by using the time-linear change in headspace CO_2 (μmol CO_2 h^{-1}) during the 48 h incubation. We determined the amount of substrate ^{13}C respired by multiplying the moles of C respired by the atom percent excess (APE) ^{13}C. APE was calculated by subtracting natural abundance atom % ^{13}C from atom % ^{13}C. The natural abundance of ^{13}C respired was determined from the samples amended with deionized water.

2.2.3. Phospholipid fatty acid analysis

After determining microbial respiration, we freeze dried this soil (~10 g) for PLFA analysis To determine analytical recovery, we added 500 µl of an 11:0 and 21:0 fatty acid standards (50 pmol ml^{-1}; 99% purity) to each soil sample (Sigma Co., St Louis, MO). We used a solution containing 10 ml of CH_3OH, 5 ml of CH_3Cl, and 4 ml of PO_4^{3-} buffer to extract total lipids from 5 g of freeze-dried soil (White et al., 1979). The polar and non-polar lipids were separated by silicic acid chromatography. The separated polar lipids were subjected to an alkaline CH_3Cl–CH_3OH solution to form fatty acid methyl esters (FAMEs; Guckert et al., 1985). FAMEs were separated using gas chromatography and quantified using a Finnigan Delta plus mass spectrometer with a GC/C III interface (Thermofinnigan, Bremen, Germany). The GC column was an Agilent HP-1 column (50 m × 0.200 mm) and samples passed through the column with constant pressure. The initial temperature was 60 °C held for 2 min, which increased 10 °C min^{-1} until 150 °C, then increased 3 °C min^{-1} to 312 °C. The output of this process was a chromatogram where PLFA identity was establish from peak retention time; the area below the peak was used to determine C mass. Our analysis allowed us to quantify the $\delta^{13}C$ of each peak. The mass and $\delta^{13}C$ of each of the FAMEs was determined by a regression equation based on a standard solution that contained five common FAMEs (10Me16:0, 12:0, cy19:0a, i15:0, 15:0) of known mass of C and $\delta^{13}C$. This FAME mix was analyzed after every fifth sample. The excess ^{13}C recovered in each PLFA was determined as the product of the mass of PLFA (nmol C g^{-1}), the mass of soil (g), and APE.

We organized specific PLFAs into three microbial groups: bacterial PLFAs, fungal PLFAs, and PLFAs occurring in both bacteria and fungi. Bacteria specific PLFAs were i15:0, a15:0, i16:0, 10Me16:0, a17:0, i17:0, cy17:0, cy19:0a, 16:1ω9c, 18:1ω7t, and 18:1ω5c (Asselineau, 1966; Gitaitis and Beaver, 1990; Annous et al., 1999; Grayston et al., 2001). Fungal specific PLFAs were 18:1ω9c and 18:2ω6 (Federle et al., 1986; Feofilova et al., 1998). PLFAs that occur in bacteria and fungi include 14:0, 16:0, 18:0, 16:1ω7c, 18:1ω7c, and 16:1ω5c (Stahl and Klug, 1996; Funtikova et al., 1998; Feofilova et al., 1998; Schie and Young, 1998; Annous et al., 1999; Olsson, 1999). We used the sum of all these PLFAs to calculate total PLFA, a measure of living microbial biomass.

2.2.4. Sequential extractions

On the remaining group of replicates, we extracted DOC with 40 ml of K_2SO_4 (0.5 M) and passed the extract through a 1.2 µm glass fiber filter from ~10 g oven dried soil The remaining soil and filter were fumigated with CH_3Cl for 5 days, after which microbial C was extracted with 40 ml of K_2SO_4 (0.5 M). A 1 ml subsample of DOC and microbial C extracts were then evaporated, and the C content and $\delta^{13}C$ were determined by mass-spectrometry using a CE Elantech NC2500 interfaced to a Finnigan Delta Plus IRMS. The remaining soil was freeze-dried and pulverized with a ball mill prior to determining soil organic C content and $\delta^{13}C$. Carbon content and $\delta^{13}C$ was determined by mass-spectrometry as described above. The excess of ^{13}C recovered from DOC, microbial C, and SOM was determined by multiplying the product of C (μmol C g^{-1}) by the oven-dry mass of soil (g) and its APE.

2.3. Statistical analyses

We used an analysis of variance to determine the effect of chronic NO_3^- additions on enzyme activity, PLFA, and % recovery of ^{13}C substrates into C pools. Because moisture content was a major influence on enzyme activity, we used moisture content as a covariate in our analysis of enzyme activity. We used Tukey's post hoc test to determine significant differences among means. Significance for all statistical analyses was accepted at $\alpha = 0.05$.

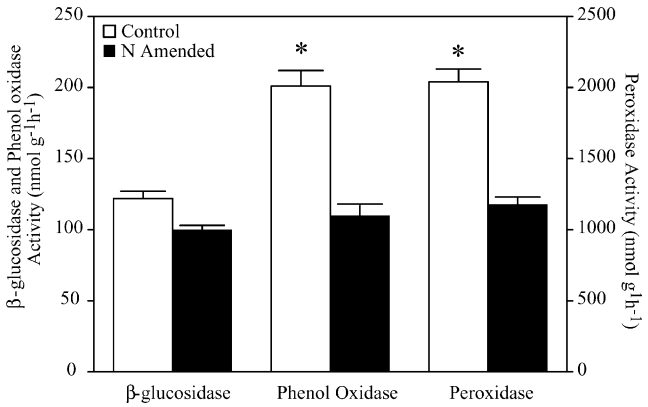

Fig. 1. The influence of N additions on enzyme activity in mineral soil. Means with an *are significantly different ($\alpha = 0.05$). Error bars are one standard error of the mean ($n = 3$).

3. Results

3.1. Soil enzyme analysis and organic C content

Nitrate additions consistently suppressed the activity of enzymes responsible for degrading cellobiose and lignin (Fig. 1). We found that β-glucosidase activity was suppressed by 21% in NO_3^- amended soils, but not to a significant degree. NO_3^- additions significantly suppressed peroxidase activity by 74% and phenol oxidase activity by 83% (Fig. 1). Moisture content was a significant covariate for β-glucosidase, peroxidase, and phenol oxidase (Table 1). NO_3^- amended soil total C was significantly ($P < 0.001$) higher then control soils. Control soils contained 2.2 ± 0.1 μmol C g^{-1} (mean ± SE) and NO_3^- amended soil has 3.9 ± 0.4 μmol C g^{-1}.

3.2. Microbial biomass and community composition

Microbial biomass, as indicated by total PLFA, was significantly ($P = 0.012$) lower in N amended soils (4.6 ± 0.6 nmol PLFA μg soil C^{-1}) than control soils (7.8 ± 1.0 nmol PLFA μg soil C^{-1}). Mean bacteria PLFAs were significantly reduced by 75%, whereas fungal PLFAs were significantly reduced by 48%. Nitrate addition did not significantly change the bacteria:fungal PLFA ratio. Directly extracted microbial C in control soils was 832 ± 58 (mmol C μg soil C^{-1}), compared to 521 ± 13 (mmol C μg soil C^{-1}) in N amended soils. The 68% reduction of total PLFA coincides with a 37% reduction in microbial C in N amended soils. NO_3^- addition did not have any observed effect on the microbial community composition as determined by the mole fractions of specific bacterial and fungal PLFAs (Fig. 2).

3.2.1. Substrate assimilation and processing

We found no treatment effect on the mean percent recovery of ^{13}C vanillin or ^{13}C cellobiose in SOC, microbial biomass, respiration, or DOC (Table 2) The majority of ^{13}C vanillin (~60%) was recovered in SOC, whereas the majority of recovered ^{13}C cellobiose was distributed evenly between microbial biomass (~22%) and SOC (~18%; Table 2). Almost three times more ^{13}C from cellobiose was recovered in microbial biomass than from vanillin, but ^{13}C recovered in microbial respiration was similar between cellobiose and vanillin (Table 2). The mean total recovery of ^{13}C vanillin was ~73%, whereas ^{13}C cellobiose was ~53%.

N additions significantly increased the incorporation of ^{13}C vanillin into 10:Me16:0, cy17:0, and 18:2ω6. We recovered significantly more ^{13}C vanillin in fungal PLFA in NO_3^- amended soils, as compared to soil from the control treatment (Fig. 3). Specific microbial respiration for ^{13}C vanillin decreased from 1.01 ± 0.52 (nmol $^{13}CO_2$ nmol total $PLFA^{-1}$) in N control soils compared to 0.37 ± 0.14 (nmol $^{13}CO_2$ nmol $PLFA^{-1}$) in N amended soils. We found no difference in the incorporation of ^{13}C cellobiose into any specific PLFA. In addition, there was no treatment effect in ^{13}C excess of any PLFA microbial group amended with ^{13}C cellobiose (Fig. 3). There was no observed difference in ^{13}C cellobiose specific respiration. Bacteria and fungal PLFAs and total PLFA also contained more ^{13}C vanillin, when compared to ^{13}C cellobiose.

4. Discussion

Our study provides evidence that NO_3^- additions can inhibit the ability of soil microorganisms to metabolize cellobiose or vanillin by suppressed the activity of lignin-degrading enzymes. However, this decrease does not appear to significantly decrease the ability of the microbial community to assimilate cellobiose or vanillin. The assimilation of ^{13}C cellobiose into fungal PLFAs was unaffected by NO_3^- deposition, whereas, more ^{13}C vanillin was incorporated into fungal PLFAs in NO_3^- amended soil. Although fungal PLFA was more enriched in ^{13}C from

Table 1
Analysis of covariance for the response β-glucosidase, peroxidase, and phenol oxidase activities to chronic NO_3^- additions and soil moisture content (covariate)

Source of Variation	df	β-glucosidase	Peroxidase	Phenol Oxidase
NO_3^- addition	1	0.802	0.082	<0.001
Soil moisture	1	<0.001	<0.001	<0.001
NO_3^- addition × soil moisture	1	0.567	<0.001	<0.001

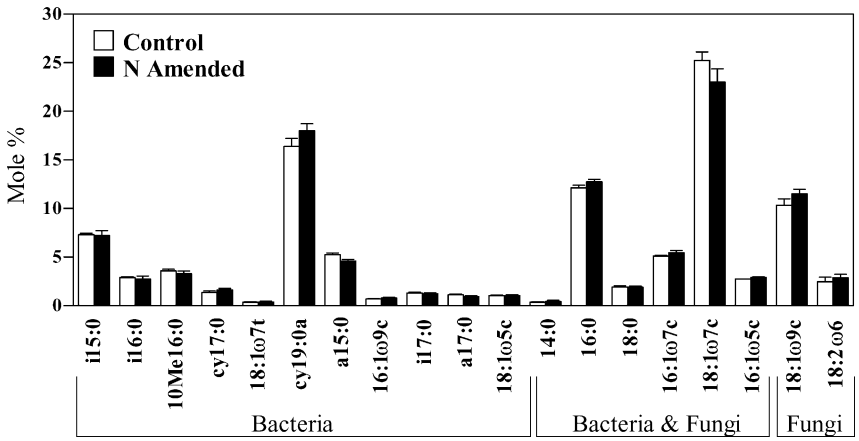

Fig. 2. The response of bacterial and fungal PLFAs to chronic NO_3^- additions in surface soils. Error bars are one standard error of the mean ($n = 3$). No significant difference in PLFA mole fraction existed between control and NO_3^--amended soils.

vanillin, we did not observe an increase in ^{13}C vanillin recovered in microbial C, estimated by direct extraction. A possible explanation is that the lignin-degrading organisms represents only a small fraction of total microbial biomass. The increase in ^{13}C vanillin incorporated into fungal PLFAs in NO_3^- amended soils cannot be explained by enzyme activities. Peroxidase and phenol oxidase activity can reflect the depolymerization of lignin and the production of vanillin, but not assimilation of vanillin (Kirk and Shimada, 1985). Therefore, it appears that NO_3^- additions can simultaneous inhibit lignin depolymerization and stimulate the assimilation of vanillin evident by the accumulation of ^{13}C in PLFAs.

Our results indirectly suggest that NO_3^- additions have altered the biochemistry of soil organic C. Previous research demonstrates that NO_3^- additions increased the decomposition of labile plant litter, whereas it decreased the decomposition of lignocellulose (Pinck et al., 1950; Berg, 1986; Fog, 1988; Carreiro et al., 2000). The suppression of lignolytic enzymes is likely to reduce the degradation of lignin, along with plant tissue protected by lignin. The 68% decrease in microbial biomass is consistent with the idea that less substrate is available for microbial growth in NO_3^- amended soils. It stands to reason that chronic NO_3^- deposition, by suppressing ligninolytic activity, has decreased the decomposition of lignin, thus increasing SOC. We have found that SOC is 76% higher in NO_3^- amended soils, then in control soils. NO_3^- amended soils from the same study site as this experiment exhibit a significant decrease in soluble phenolics (DeForest et al., in prep). The decreases in ligninolytic activity coupled with reductions in soluble phenolic monomers indicates that more C from lignin is remaining as polyphenolic compounds in NO_3^- amended soils rather than undergoing humification (Orlvo, 1986). However, we have no direct evidence of increases in humification of SOC. Determining if NO_3^- deposition has changed the biochemistry of SOC warrants future study.

Increases in soil organic carbon, decreases in microbial biomass, and potential increases in humification indicate that chronic NO_3^- deposition has stemmed the flow of C through the heterotrophic soil food web. The increase in SOC cannot be explained by greater leaf or root litter biomass, which has not changed in NO_3^- amended plots (Zak et al., 2003). Increases in SOC may be explained by decreases in peroxidase and phenol oxidase activity, which are responsible for degrading lignin in plant litter. The suppression of ligninolytic enzymes is a response to NO_3^- additions and this is a likely mechanism explaining decreases in decomposition of recalcitrant plant litter (Carreiro et al., 2000; Sinsabaugh et al., 2002). Other experiments found that the response of these ligninolytic

Table 2
The influence of N additions on the mean recovery of ^{13}C vanillin and ^{13}C cellobiose in soil organic matter, microbial biomass and respiration, and dissolved organic matter Means among various C forms with different letters indicate significant difference by Tukey's test $\alpha = 0.05$. Values in parentheses are standard error of the mean ($n = 3$)

	^{13}C Vanillin		^{13}C Cellobiose	
	Control	N Amended	Control	N Amended
Soil organic matter	64.4a (9.9)	53.6a (5.0)	20.3a (6.8)	14.9a (2.0)
Microbial biomass	6.6a (1.9)	6.9a (0.7)	21.8a (1.3)	22.0a (2.5)
Microbial respiration	6.8a (2.1)	7.4a (2.1)	7.8a (0.7)	8.5a (0.4)
Dissolved organic matter	0.7a (0.4)	0.3a (0.2)	3.7a (2.7)	5.3a (3.6)
Total recovery	78.4a (7.1)	67.8a (7.9)	55.2a (7.0)	50.5a (5.1)

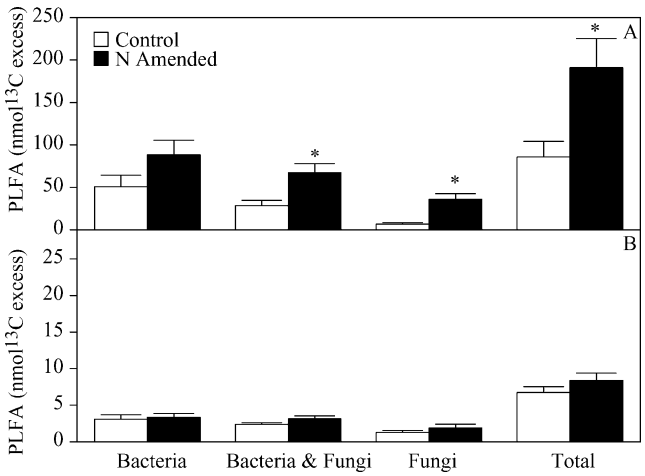

Fig. 3. The effect of chronic NO_3^- additions on the assimilation of (A) ^{13}C-labeled vanillin and (B) ^{13}C-labeled cellobiose into PLFA microbial groups. Means with an asterisks are significantly different ($\alpha = 0.05$). Error bars are one standard error of the mean ($n = 3$).

enzymes to chronic NO_3^- addition show similar decreases in enzyme activity (Carreiro et al., 2000; Saiya-Cork et al., 2002; Sinsabaugh et al., 2002; DeForest et al., 2004). It stands to reason that if lignolytic enzymes are inhibited, then SOC would accumulate. Therefore, it appears that NO_3^- deposition may have the capacity to increase soil C sequestration in northern hardwood forest.

In conclusion, the depolymerization of lignin by lignolytic enzymes has been reduced by NO_3^- additions. This, in turn, has probably altered SOC biochemistry by increasing the humification of SOC. This is consistent with the 76% increase in SOC in NO_3^- amended soils. The increases in SOC is not consistent with reductions in microbial biomass, which suggests a reduction in the availability of C from lignocellulose. Nevertheless, we have no evidence that NO_3^- additions have reduced cellobiose or vanillin metabolism or assimilation. Increases in incorporation of vanillin by the fungal community in NO_3^- amended soils indicated that the assimilation of lignin monomers are not directly coupled to lignin decomposition. Nevertheless, it is clear that NO_3^- additions has reduced the flow of C through the microbial community by increasing the amount of soil organic carbon.

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