

# Chronic nitrogen additions suppress decomposition and sequester soil carbon in temperate forests

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**Abstract** The terrestrial biosphere sequesters up to a third of annual anthropogenic carbon dioxide emissions, offsetting a substantial portion of greenhouse gas forcing of the climate system. Although a number of factors are responsible for this terrestrial carbon sink, atmospheric nitrogen deposition contributes by enhancing tree productivity and promoting carbon storage in tree biomass. Forest soils also represent an important, but understudied carbon sink. Here, we examine the contribution of trees versus soil to total ecosystem carbon storage in a temperate forest and

investigate the mechanisms by which soils accumulate carbon in response to two decades of elevated nitrogen inputs. We find that nitrogen-induced soil carbon accumulation is of equal or greater magnitude to carbon stored in trees, with the degree of response being dependent on stand type (hardwood versus pine) and level of N addition. Nitrogen enrichment resulted in a shift in organic matter chemistry and the microbial community such that unfertilized soils had a higher relative abundance of fungi and lipid, phenolic, and N-bearing compounds; whereas, N-amended plots were associated with reduced fungal biomass and activity and higher rates of lignin accumulation. We conclude that soil carbon accumulation in response to N enrichment was largely due to a suppression of organic matter decomposition rather than enhanced carbon inputs to soil via litter fall and root production.

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## Introduction

Of the estimated 8.7 Gt carbon (C) released to the atmosphere annually by human activities (Boden et al. 2012), only about half accumulates, with the remainder being absorbed by the oceans or taken up by the terrestrial biosphere (Denman et al. 2007). The terrestrial C sink represents as much as a third of annual CO<sub>2</sub> emissions (Myneni et al. 2001; Deng and Chen 2011; Pan et al. 2011), with a disproportionate sink in temperate and boreal regions due to forest re-growth and fire suppression in conjunction with CO<sub>2</sub> fertilization, climate warming, lengthening growing seasons, and atmospheric nitrogen (N) deposition (Schimel et al. 2001). Nitrogen deposition in northern forests results in the inadvertent fertilization of otherwise N-limited systems and has been established as a key driver of net forest C storage (Sutton 2008; de Vries et al. 2009). Anthropogenic emissions of reactive N to the atmosphere, primarily due to fossil fuel combustion and agriculture (Pinder et al. 2012), and the subsequent deposition to Earth's surface have increased nearly 200 % since the beginning of the industrial revolution and are projected to double yet again by 2050 in many parts of the world (Galloway et al. 2008). Nitrogen deposition in the absence of human influence is typically <0.5 kg N ha<sup>-1</sup> year<sup>-1</sup>; however, in heavily populated and industrialized regions, N deposition rates range from 1 to 100 kg N ha<sup>-1</sup> year<sup>-1</sup> (Hyvönen et al. 2007; de Vries et al. 2009).

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The C:N ratios of temperate forest woody biomass are high, such that large amounts of C can be fixed per unit N taken up by plants. Some tree species exhibit increased mortality with exposure to high levels of N fertilization (Thomas et al. 2010); however, growth enhancement appears to be the general response of forests to N deposition (Pinder et al. 2012). Estimates of N-induced tree C sequestration vary widely, but several recent data syntheses suggest the most ecologically plausible upper limit is about 60 kg C kg<sup>-1</sup> N (Hyvönen et al. 2007; de Vries et al. 2009; Thomas et al. 2010), with the C uptake rate for a given site being highly dependent on tree species (Thomas et al. 2010). Forest soils, which retain a larger and more persistent pool of C than that contained in vegetation, represent an additional and potentially important reservoir for enhanced terrestrial ecosystem C storage in response to N deposition (Pregitzer et al. 2008; Zak et al. 2008; Nave et al. 2009; Janssens et al. 2010; Lovett et al. 2013). A review of the literature shows a growing consensus that forest soils represent an important sink for added N, resulting in enhanced C storage beyond what is stored in tree biomass (summarized in Table 1). It might be expected that enhanced tree growth would facilitate soil organic matter accumulation through increased plant litter and root inputs. However, previous research suggests that a suppression of organic matter decay may be the primary mechanism by which soils accumulate C in response to long-term N additions (Pregitzer et al. 2008; Zak et al. 2008; Janssens et al. 2010; Zak et al. 2011). Despite a long history of research on soil responses to enhanced N availability, there have been relatively few efforts to comprehensively examine the relative contribution of trees versus soils to net ecosystem C storage in conjunction with the concomitant changes in soil biogeochemistry that could provide a clear explanation for why soils accumulate C in response to added N (but see Pregitzer et al. 2008; Zak et al. 2008; 2011). Soil C pools are still the least certain term of forest C budgets (Goodale et al. 2002), and a better understanding of N-induced soil C storage is critical to accurately predict the net response of terrestrial ecosystems to N deposition.

In this study, we used long-term tree inventory and litterfall data coupled with measurements of an extensive suite of soil properties to assess the relative importance of tree versus soil C accumulation for total ecosystem C storage following 20 years of

**Table 1** Carbon sequestration in temperate forests per unit nitrogen added

Study location	Study duration (year)	Nitrogen inputs (kg ha <sup>-1</sup> year <sup>-1</sup> )	Carbon response (kg C kg <sup>-1</sup> N)			Reference
			Trees	Soils <sup>a</sup>	Total	
N. America & Europe (9 sites)	1–3	4–58	25 <sup>b</sup>	21	46	Nadelhoffer et al. (1999)
Europe (121 forest plots)	40	2.8	33 <sup>c</sup>	15	48	de Vries et al. (2006)
Sweden (boreal forest)	28	34	25	3	28	Hogberg et al. (2006) <sup>d</sup>
Finland, Sweden (15 sites)	14–30	30–200	25	11	36	Hyvönen et al. (2008)
Northern US (4 hardwood sites)	10	30	17	23	40	Pregitzer et al. (2008)
Europe (400 forest plots)	7	1.2–61.5	15–38 <sup>e</sup>	–	–	Solberg et al. (2009); Laubhann et al. (2009)
North-central US (24 tree species)	~20	3–11	61	–	–	Thomas et al. (2010)
Global analysis (20 experiments)	–	28–300	–	19 <sup>f</sup>	–	Janssens et al. (2010)
Northeastern US (5 tree species)	6	50	0	14 <sup>g</sup>	14	Lovett et al. (2013)
Northeast US (hardwood stand)	20	50	10	10	20	This study
Northeast US (hardwood stand)	20	150	5	25	30	This study
Northeast US (pine stand)	20	50	–10	16	6	This study
Northeast US (pine stand)	20	150	–7	5	–2	This study

<sup>a</sup> These estimates of soil C storage were based on the organic horizon and some amount of mineral soil, though the depth of mineral soil sampling varied widely across studies. The following studies reported mineral soil sampling as being to a depth of 12 cm (Lovett et al. 2013), 20 cm (Hyvönen et al. 2008), 40 cm (our study; entire soil profile sampled), and 70 cm (Pregitzer et al. 2008). Hogberg et al. (2006) report sampling the organic horizon and 5–15 and 45–55 depth of mineral soil. The depth of mineral soil sampling was typically not specified in those studies where estimates of soil C storage were based on a synthesis or meta-analysis a large number of sites or experiments (Nadelhoffer et al. 1999; de Vries et al. 2006; Janssens et al. 2010)

<sup>b</sup> Estimates based on the short-term fate of <sup>15</sup>N tracer recovered after 1–3 years following N addition

<sup>c</sup> Derived from forest growth over 40 years (1960–2000) under ambient N deposition conditions (average of 2.8 kg N ha<sup>-1</sup> year<sup>-1</sup> additional N deposition since 1960)

<sup>d</sup> As estimated by de Vries et al. (2009)

<sup>e</sup> Derived from forest growth over 7 years (1993–2000) under ambient N deposition conditions

<sup>f</sup> Value estimated from the reduction in heterotrophic soil respiration in response to N fertilization

<sup>g</sup> Average from all experimental plots representing five different tree species

experimental N additions to two temperate forest stands (mixed hardwood and red pine) in Central Massachusetts, USA. We also examined factors underlying the observed soil response by assessing the relative importance of enhanced C inputs to soil via litterfall and roots versus reduced C losses due to a suppression of soil organic matter decomposition.

## Materials and methods

### Experimental site

Our research was conducted at the Chronic Nitrogen Amendment Experiment at the Harvard Forest Long-Term Ecological Research (LTER) site in Petersham,

MA, USA. This site was established in 1988 in two adjacent forest stands, one dominated by mature (~60 year-old), even-aged red pine (*Pinus resinosa*), the other by black (*Quercus velutina*) and red (*Q. rubra*) oak mixed with black birch (*Betula lenta*), red maple (*Acer rubrum*) and American beech (*Fagus grandifolia*) (Magill and Aber 1998; Magill et al. 2004). The soils are Typic Dystrudepts of the Gloucester series (Peterjohn et al. 1994) with no significant variation among N treatments in pH or concentrations of the base cations Ca and Mg (Turlapati et al. 2013). Mean monthly temperatures range from 19 °C in July to –5 °C in January and average annual precipitation is 112 cm, distributed relatively evenly throughout the year. The current rate of ambient N deposition at the site is ~8 kg N ha<sup>-1</sup> per year<sup>-1</sup> (Ollinger et al. 1993).

Three 30 × 30 m plots in each forest stand receive one of three N treatments: no N addition (N0; control); 50 kg N ha<sup>-1</sup> year<sup>-1</sup> (N50), or 150 kg N ha<sup>-1</sup> year<sup>-1</sup> (N150). The N fertilized plots are treated with an aqueous solution of ammonium nitrate applied monthly during the growing season. Each treatment plot is divided into thirty-six 5 × 5 m subplots with the outer subplots excluded from sampling to reduce edge effects. The N50 level is consistent with predictions for N deposition rates in large parts of the world by 2050 (Galloway et al. 2008). Even currently, some areas in Western Europe see rates of N deposition (total wet and dry) as high as 25–50 kg N ha<sup>-1</sup> year<sup>-1</sup> (Holland et al. 2004), and this is likely the case in parts of Asia as well. Thus the N50 treatment is a relevant simulation of current and future N deposition levels and is consistent with other long-term N addition experiments in the Northeastern U.S. (Lovett and Goodale 2011; Lovett et al. 2013) and elsewhere (Cusack et al. 2011). The high addition rate (N150) was not intended to be predictive, but to push the system toward saturation and to be a space-for-time substitution to evaluate underlying mechanisms/drivers in a high N world (J. Aber, personal communication).

#### Sample collection and analysis

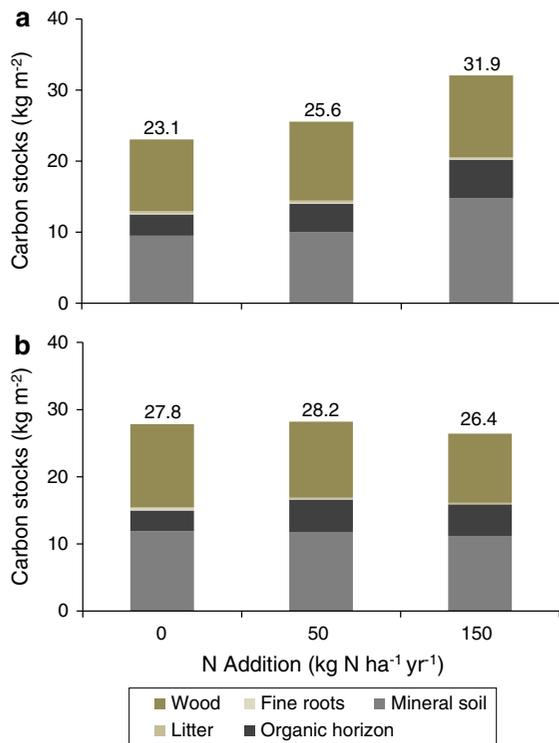
Long-term data from the site were obtained from the Harvard Forest data archive (<http://harvardforest.fas.harvard.edu/data-archive>) and included a tree inventory, foliar chemistry, and litterfall biomass. Tree biomass was determined from diameter at breast height (DBH) data collected every 3 years since 1988 on all trees greater than 5 cm diameter (Magill et al. 2004). Foliar litterfall was collected three times annually since 1988 according to Magill et al. (2004). Fine roots (<2 mm-diameter) were hand-picked from organic horizon and mineral soil samples collected in 2008, 2010, and 2011, dried at 60 °C, and weighed.

Soil samples were collected in 2008 from each of six 5 × 5 m subplots within each treatment plot to complement the long-term aboveground data and, specifically, to assess the effects of 20 years of N additions on soil C stocks and associated biological and chemical properties. Organic horizon samples were collected in each subplot by removing a 20 × 20 cm square of the Oe and Oa layers to the depth of the mineral soil. Mineral soil samples were then collected in the same location in 10 cm

increments to a depth of 40 cm (bottom of soil profile) using a gas-powered soil auger with a 9 cm diameter core. Samples were sieved (<2 mm) and roots, rocks and other debris >2 mm removed. Total soil organic C and N were determined by dry combustion on a Costech 4010 CHNS-O Analytical Combustion System. Bulk density, determined for each sample, was used to calculate volumetric soil C stocks (g C m<sup>-2</sup>). In situ soil respiration and temperature were measured nine times over the growing season (Apr 19, Jun 5 and 26, Jul 13 and 27, Aug 26, Sep 16, Oct 12 and 30) using a portable infrared gas analyzer.

Microbial biomass and community composition were determined on organic horizon samples (Oe and Oa layers) using phospholipid fatty acid (PLFA) analysis (Guckert et al. 1985). Microbial activity was assessed for the organic horizon and top 10 cm of mineral soil by measuring potential extracellular enzyme activity. Proteolytic enzyme activity was determined as the difference in amino acid concentrations in subsamples incubated for 4 h and initial subsamples that were terminated immediately (Brzostek et al. 2011). Both incubated and initial subsamples were amended with sodium acetate buffer (pH 5.0), toluene to inhibit microbial uptake, and a trichloroacetic acid solution to terminate the assay. Organic matter chemistry was assessed for the organic horizon (hardwood stand only) by determining the relative percentages and ratios of chemical classes determined using pyrolysis-GC/MS (Wickings et al. 2011). Samples were pyrolyzed at 600 °C and products transferred to a GC where compounds were separated on a 60 m capillary column with a starting temperature of 40 °C followed by a temperature ramp of 5 °C min<sup>-1</sup> to 270 °C followed by a final ramp (30 °C min<sup>-1</sup>) to 300 °C. Compounds were transferred to an ion trap mass spectrometer where they were ionized, detected via electron multiplier, and identified using a compound library from the National Institute of Standards and Technology (NIST).

The data were analyzed using rank analysis of variance (PROC RANK, PROC GLM, SAS Institute) with N addition treatment (control, N50, N150) as the main factor. A non-parametric procedure was selected because the data violated the assumptions of normality and homogeneity of variance for standard analysis of variance. The Ryan–Einot–Gabriel–Welsch multiple range test was used to determine significant differences among means at  $P < 0.05$ . Separate analyses



**Fig. 1** Total C ( $\text{kg m}^{-2}$ ) stored in tree biomass, leaf litter, fine roots ( $<2$  mm), organic horizon, and the mineral soil to a depth of 40 cm in control and N fertilized plots in the hardwood (a) and pine (b) stands at the Chronic Nitrogen Amendment Experiment at the Harvard Forest Long-term Ecological Research (LTER) site. Values above the bars indicate total ecosystem C storage, assuming a 50 % C content for plant components. Refer to Table 2 and Fig. 2 for within-treatment variability for plant and soil C stocks, respectively

were performed for each stand (hardwood versus pine) and soil horizon (organic versus mineral soil). In situ soil respiration, measured nine times over the growing season, was plotted as a function of soil temperature, and dummy variable regression analysis with slope and intercept interaction terms was used to determine significant differences among treatments ( $P < 0.05$ ). Nonmetric multidimensional scaling (NMDS) was used to visualize differences in microbial community composition and soil organic matter chemistry among N addition treatments.

## Results and discussion

Twenty years of N fertilization resulted in a significant increase (38 %) to a net loss (−5 %) of total

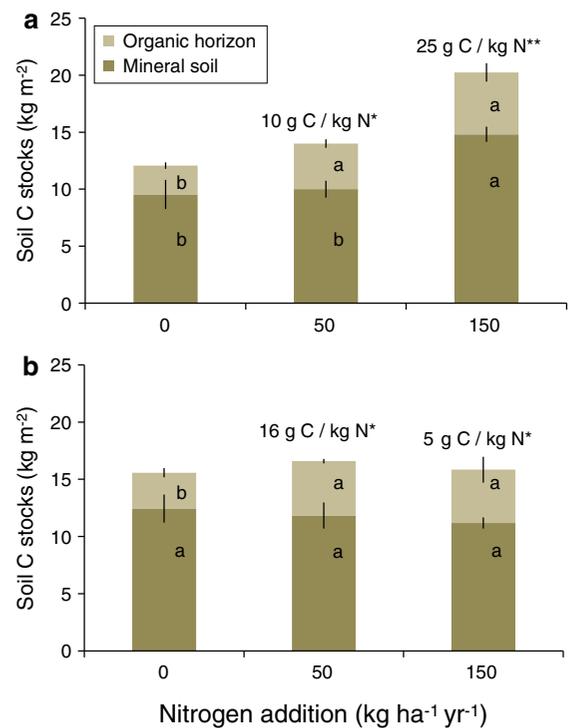
ecosystem C storage, with the response being dependent on stand type (hardwood versus pine) and N fertilization level (Fig. 1). The hardwood stand accumulated 20–30  $\text{kg C kg}^{-1}$  N added over the 20 year study (Table 1), with more than half (60–85 %) of this C storage due to an accumulation of soil C (Fig. 2). At the lower N addition rate ( $50 \text{ kg N ha}^{-1} \text{ year}^{-1}$ ), tree biomass in the hardwood stand increased by 10 % compared to a 33 % increase for soil C stored in the organic horizon (Fig. 2, Supplemental Table S1). At the highest N addition rate ( $150 \text{ kg N ha}^{-1} \text{ year}^{-1}$ ), hardwood trees showed little additional response, increasing their total biomass by only 3.7 % above the lower N treatment. Soil C levels responded to a much greater degree than did trees in the higher N treatment, with soil C in the organic horizon increasing by 80 % over the control treatment. Additionally, soil C stocks in the mineral soil (to 40 cm depth) significantly increased under the highest N addition rate, resulting in a total soil C accumulation (organic horizon plus mineral soil) for this treatment of  $25 \text{ kg C kg}^{-1}$  N added. The proportion of light fraction (free particulate) organic matter in the top mineral soil layer (0–10 cm) did not increase with N additions (Supplementary Table S2) and is consistent with the lack of response of fine root biomass to N addition (see below), suggesting that chemical association with mineral particles was the dominant and more persistent mechanism for the enhanced C storage at depth in the highest N treatment (as opposed to an increase in particulate organic matter).

Increases in soil C in the pine stand were largely offset by losses of tree biomass due to mortality. We observed an 8–16 % loss of total standing tree biomass and an increase of 50 % in soil C in the organic horizon, resulting in a slight net gain (1.4 %) to a small net loss (−5 %) at the lower versus higher N addition rate, respectively. The negative tree response observed for pine is consistent with recent measurements of forest inventory data showing that, of the 24 most common tree species in the Northeast US, *P. resinosa* (red pine) had the largest negative growth response to N deposition (Thomas et al. 2010). In contrast to the hardwood stand, soil C accumulation in the pine stand was only observed in the organic horizon with no significant change in mineral soil C levels.

These C stock results demonstrate that the soils at our site represent a C sink of equal or greater magnitude to that of woody tree biomass following

two decades of N enrichment. This finding is supported by a  $^{15}\text{N}$  tracer study at this site showing that tree biomass was a relatively small sink for added  $^{15}\text{N}$ , with most of the  $^{15}\text{N}$  being retained in the forest floor and to a lesser extent in the top 20 cm of mineral soil (Nadelhoffer et al. 1999, 2004; Templer et al. 2012). A review of published studies, where both the tree and soil response to ambient or simulated N deposition in temperate forests has been evaluated (Table 1), shows that N-induced soil C accumulation varies widely, ranging from 3 to 25 kg C kg $^{-1}$  N added and representing 10–100 % of the total C accumulation observed (trees + soils).

The amount of soil C sequestered in response to N deposition represents the balance between C inputs (via litterfall and root biomass turnover/exudation) and C losses from litter and soil organic matter decomposition. Carbon losses due to leaching from the mineral soil are small at our site (Currie et al. 1996; Evans et al. 2008) and erosional losses are likely minimal. To assess the relative contribution of soil C inputs to the observed soil C accumulation at Harvard Forest, we analyzed litterfall data collected annually over the 20 year study and examined root dynamics in response to the long-term N additions. Cumulative litterfall over the study duration in the hardwood stand was only 3 % higher in the N addition plots than in the control, and average annual litterfall was not significantly different among treatments (Table 2). Cumulative and annual litterfall in the pine stand increased significantly in response to N additions (18 %), primarily due to high tree mortality and reduced needle longevity (Bauer et al. 2004). Fine root biomass (to 20 cm depth) was not significantly different among treatments in the hardwood stand (Table 2), consistent with earlier estimates of root biomass at this site (Magill et al. 1997, 2004). Nitrogen additions in the pine stand resulted in a significant reduction in root biomass at all depths measured. Root activity, assessed by measuring root respiration, was not higher in the N-amended plots (hardwood or pine), and root tissue N concentration was generally a weak predictor of root respiration in N-enriched soils (Supplementary Table S3 and Fig. S1). We also did not observe any significant differences in root production and specific root length (Supplementary Table S4). Thus, our research does not provide strong evidence for enhancement of root productivity or root respiration in response to N additions, a finding similar to that



**Fig. 2** Total organic soil C (kg m $^{-2}$ ) in the organic horizon (Oe + Oa) and mineral soil to a depth of 40 cm in the hardwood (a) and pine (b) stands at the Chronic Nitrogen Amendment Experiment at the Harvard Forest LTER. Error bars represent  $\pm$  one standard error for each soil horizon. Different lower case letters within a soil horizon and across N treatments represent a significant difference at  $P \leq 0.05$ . Values above the bars represent the amount of C accumulated per unit of N added over the 20 year study. An \* indicates a significant accumulation of C with N addition in the organic horizon only, while \*\* indicates a significant accumulation in both the organic and mineral soil horizons ( $P \leq 0.05$ ). The primary data used for this analysis are provided in Supplementary Table S1

observed at a long-term N addition study in Michigan, USA (Pregitzer et al. 2008; Zak et al. 2008; Burton et al. 2012) and to results from a global meta-analysis demonstrating that long-term N additions did not increase fine root inputs to soil at boreal, temperate, and tropical forest sites (Liu and Greaver 2010). It has been suggested that the N-induced enhancement of aboveground tree biomass may be due to a shift in C allocation rather than an increase in C assimilation, a hypothesis that is supported by studies showing that N additions often do not enhance tree photosynthetic rates or N use efficiency (Talhelm et al. 2011; Smithwick et al. 2013). To summarize, our measurements show that N additions did not enhance C inputs

**Table 2** Total and live tree biomass, annual litterfall mass, fine root mass, and soil microbial biomass for control and nitrogen-amended plots at the Chronic Nitrogen Amendment Experiment at the Harvard Forest Long-Term Ecological (LTER) site

Ecosystem component	Hardwood			Pine		
	Nitrogen addition (kg N ha <sup>-1</sup> year <sup>-1</sup> )			Nitrogen addition (kg N ha <sup>-1</sup> year <sup>-1</sup> )		
	0	50	150	0	50	150
Vegetation (g m <sup>-2</sup> )						
Total tree biomass*	20,218	22,262	23,087	24,735	22,657	20,694
Live tree biomass*	17,529	18,961	18,105	20,721	18,230	5,577
Annual litterfall	305 (6)	317 (10)	321 (12)	316 (14) <sup>b</sup>	376 (19) <sup>a</sup>	374 (26) <sup>a</sup>
Fine roots						
O horizon	261 (43)	363 (40)	390 (84)	127 (31) <sup>a</sup>	82 (17) <sup>b</sup>	41 (18) <sup>b</sup>
0–10 cm mineral	227 (77)	230 (25)	295 (162)	386 (145) <sup>a</sup>	69 (15) <sup>b</sup>	ND
10–20 cm mineral	110 (17)	100 (13)	168 (63)	82 (17) <sup>a</sup>	65 (17) <sup>b</sup>	ND
Microbial biomass (nmol g <sup>-1</sup> )						
Bacteria						
Oe horizon	274 (31) <sup>a</sup>	306 (55) <sup>a</sup>	148 (23) <sup>b</sup>	329 (106) <sup>a</sup>	153 (19) <sup>b</sup>	109 (13) <sup>b</sup>
Oa horizon	85 (0)	75 (16)	42 (17)	106 (30) <sup>a</sup>	103 (29) <sup>a</sup>	15 (3) <sup>b</sup>
Fungi						
Oe horizon	220 (23) <sup>a</sup>	73 (8) <sup>b</sup>	31 (9) <sup>b</sup>	115 (28) <sup>a</sup>	44 (8) <sup>b</sup>	39 (5) <sup>b</sup>
Oa horizon	32 (2)	20 (3)	10 (5)	22 (6)	26 (8)	<1

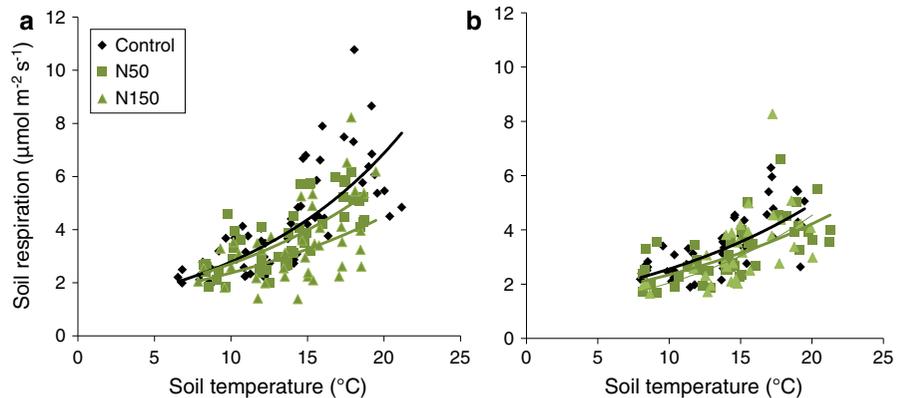
Values in parentheses are standard errors

Values followed by a different lowercase letter are significantly different at  $P \leq 0.05$

ND no data

\* Estimated for each 30 × 30 m plot based on diameter at breast height (DBH) data collected every 3 years since 1988 on all trees greater than 5 cm diameter as described by Magill et al. (2004)

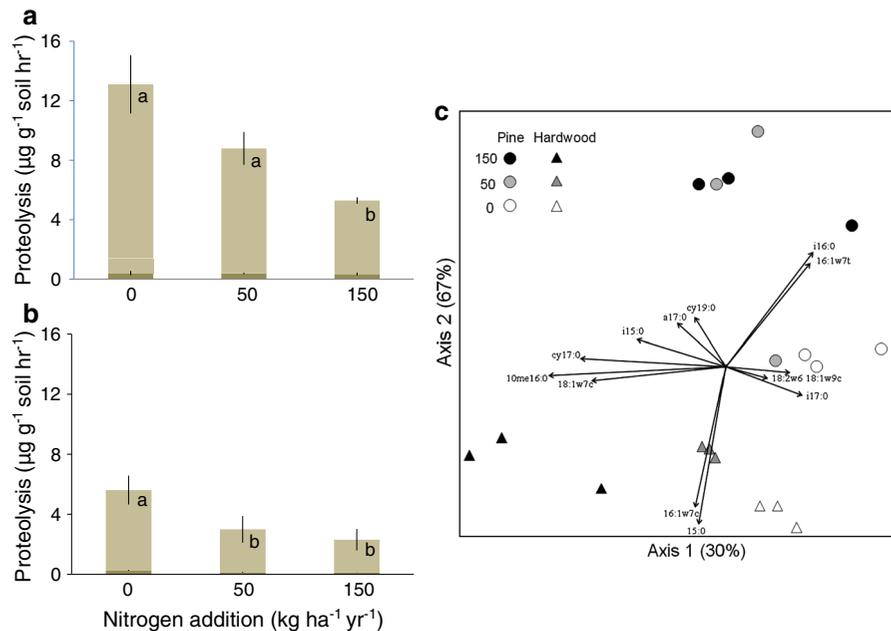
**Fig. 3** The temperature response of in situ soil respiration across N addition treatments in the hardwood (a) and pine (b) stands



(aboveground litterfall, root biomass, root production, and root respiration) to the hardwood stand. Nitrogen additions did induce significantly higher litterfall in the pine stand; however, this was not due to enhanced tree productivity, but rather to reduced tree vigor that caused needles to drop and which also resulted in reduced root biomass. We conclude, based on these

results, that an N-induced enhancement of plant C inputs is not the primary mechanism underlying the accumulation of soil C in our system.

In contrast, we have evidence that the observed C accumulation is due primarily to a suppression of organic matter decomposition. In situ soil respiration varied by stand and N treatment level, but was



**Fig. 4** Microbial activity as measured by proteolytic enzyme activity ( $\mu\text{g g}^{-1}$  soil  $\text{hr}^{-1}$ ) for the organic horizon (Oe and Oa layers) and the top 10 cm of mineral soil collected in the hardwood (a) and pine (b) stands. Bars represent means and standard errors for each soil horizon across N addition treatments. Different lower case letters within a soil horizon and across N treatments represent a significant difference at

consistently lower in the N addition plots (Fig. 3). Using the treatment specific relationship between soil temperature and respiration, we estimate that soil respiration in the N-amended plots was 7–22 % lower in the hardwood stand ( $P = 0.0096$ ) and 10–16 % lower in the pine stand ( $P = 0.0540$ ), results consistent with previous soil respiration measurements in these plots (Bowden et al. 2004). Although our field soil respiration measurements included both autotrophic and heterotrophic respiration components, the lack of difference in total root biomass, root production, and specific root respiration in the hardwood stand suggests that autotrophic respiration was largely unchanged by N additions in this stand and that the decline in total respiration in response to N fertilization was the result of reduced heterotrophic respiratory flux. Lab incubations of root-free soils collected from these plots showed that N addition greatly suppressed heterotrophic respiration (M. Weiss, unpublished data), further supporting this conclusion. It is more difficult to accurately assess the relative contribution of autotrophic versus heterotrophic respiration in the

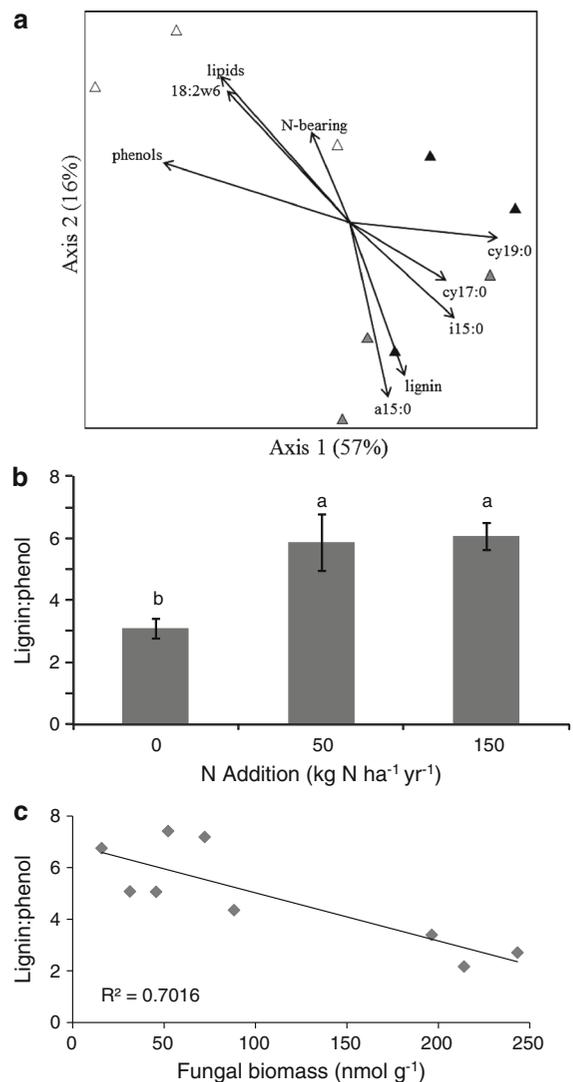
$P \leq 0.05$ . Microbial community composition of the organic horizon (c) was assessed by phospholipid fatty acid (PLFA) analysis and visualized using NMDS ordination using the individual PLFA biomarkers listed in Supplemental Tables S9 and S10. Arrows denote correlations between ordination scores and individual microbial markers

pine stand where increased tree mortality has resulted in an increase in needle inputs to the forest floor that have been offset by a reduction in root biomass and root respiration.

The suppression of in situ soil respiration with N additions coincided with significant changes in microbial biomass, enzyme activity, and community composition. Specifically, N enrichment significantly lowered bacterial and fungal biomass (46–86 %; Table 2) and proteolytic enzyme activity (47–60 %; Fig. 4a, b). The microbial communities in the hardwood and pine stands were distinct, with phospholipid fatty acid (PLFA) biomarkers from the hardwood plots ordinating primarily along axis one and the pine plots separating to a lesser degree along axis 2 of a non-metric multidimensional scaling (NMDS) ordination (Fig. 4c). Nitrogen addition in the hardwood stand was positively associated with the bacterial markers i15:0, cy17:0, 10me16:0, and 18:1w7c, and negatively associated with the bacterial marker i17:0 and the fungal marker 18:1w9c. Oxidative enzyme activity, measured in the hardwood stand only, was also

significantly reduced by N addition (42–87 %; Supplementary Table S5). Previous work at our site has demonstrated that N additions have resulted in reduced rates of leaf litter decay (Magill and Aber 1998; Knorr et al. 2005), microbial growth (Kamble et al. 2013), and the ability of the microbial community to utilize a suite of C substrates typically found in soil (Frey et al. 2004). Nitrogen additions have also resulted in a microbial community whose growth is substantially limited by the availability of readily assimilated C substrates (Kamble et al. 2013). Recent molecular analyses of the soil microbial community at our site have indicated significant N-induced shifts in the composition and diversity of both the bacterial (Turlapati et al. 2013) and fungal communities (E. Morrison and S.D. Frey, unpubl. data).

The largest N-induced responses in the microbial community were observed in the organic horizon of the hardwood stand where the greatest accumulation of C occurred and where marked changes in organic matter chemistry were also observed (Fig. 5a, b; Supplementary Tables S6–S8; Fig. S5). Nitrogen fertilization induced a significant reduction in the relative abundance of lipids (53–57 %) and N-bearing compounds (19–33 %), water-soluble dissolved organic C (40–70 %; Supplementary Table S7), and polyamine and amino acid concentrations (50 %; Supplementary Fig. S4). In contrast, the relative abundance of lignin and the lignin:phenol ratio were significantly higher under N addition (Fig. 5b). The lignin:phenol ratio represents an index of recalcitrant organic C decomposition, and a significant increase in this ratio indicates that relatively more C was present as unaltered lignin in the fertilized plots, with less lignin transformation resulting in a lower liberation of phenolic compounds, of which lignin decay is a major source. The biomass of soil fungi, the primary decomposers of lignin in temperate forests, was positively correlated with the relative abundance of lipids ( $r = 0.81$ ;  $P = 0.021$ ), phenols ( $r = 0.83$ ;  $P = 0.006$ ), and N-bearing compounds ( $r = 0.69$ ;  $P = 0.039$ ) and negatively correlated with the lignin:phenol ratio ( $r = -0.74$ ;  $P = 0.023$ ; Fig. 5c). To summarize, N enrichment has resulted in a shift in microbial community composition and organic matter chemistry such that the organic horizon of unfertilized soils has a greater relative abundance of fungi and



**Fig. 5** Soil chemistry and microbial community responses to N addition in the organic horizon of the hardwood stand. **a** Non-metric multidimensional scaling (NMDS) ordination of chemical classes determined using pyrolysis-GC/MS (final stress = 0.0485). Arrows denote significant correlations (Pearson) between ordination scores and organic matter chemistry classes or microbial biomarkers ( $P < 0.05$ ). *Open triangles* represent control plots and *closed triangles* the N50 (*grey symbols*) and N150 treatment (*black symbols*), respectively. **b** the lignin:phenol ratio as a function of N addition rate. *Bars* represent means and standard errors. *Different lower case letters* across N treatments represent a significant difference at  $P \leq 0.05$ . **c** the relationship between the soil fungal biomass and lignin:phenol ratio. Refer to the Supplemental Materials for the primary chemical class (Table S6) and microbial biomarker data (Table S9)

lipid, phenolic, and N-bearing compounds; whereas, the N-amended plots are associated with reduced fungal biomass and lignin accumulation. The shift to greater dominance by bacteria in the N amended plots may explain the larger amount of  $\text{NO}_3^-$  in soil solution attributed to nitrification, rather than N passing directly through from atmospheric deposition or fertilizer N inputs (Supplementary Fig. S4).

Our results, based on two decades of experimental N additions, document that long-term N fertilization of a temperate forest has resulted in an 11–38 % increase in ecosystem C storage in a hardwood stand, representing an accumulation of 20–30 kg C  $\text{kg}^{-1}$  N added. More than half of this C storage was attributable to an accumulation of soil organic matter, indicating that the soil has been more responsive to N additions than has tree growth. In the pine stand, a loss of tree biomass due to high tree mortality was offset by a significant increase in organic horizon C, resulting in no net change in ecosystem C balance. Soil C accumulation in both forest stands, primarily in the organic horizon, appears to be largely the result of suppressed microbially driven decomposition, rather than to increased litter or root inputs. A recent modeling exercise aimed at simulating the effects of N additions on soil C stocks at our site demonstrated that model outcomes most closely matched our observed C stock values when organic matter decomposition was aggressively suppressed (Tonitto et al. 2013), consistent with our experimental results. Soil C responses to long-term N additions have yet to be incorporated into global-scale C balance models (Whittinghill et al. 2012), but this is needed to accurately simulate future changes in terrestrial C storage in response to atmospheric N deposition (Thomas et al. 2013a, b).

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