

Long-term Nitrogen Addition Decreases Organic Matter Decomposition and Increases Forest Soil Carbon

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Eastern North American forests receive anthropogenically elevated nitrogen (N) deposition that alters soil processes and forest productivity. We examined N deposition effects on soil carbon (C) and N in temperate, N-rich forest plots fertilized annually ($100 \text{ kg N ha}^{-1} \text{ y}^{-1}$) since 1993. After nearly two decades, soil C in O, A, and upper 50 cm of B horizons of N-addition plots was 17% greater ($14.2 \pm 0.7 \text{ kg C m}^{-2}$) than control plots. Aboveground tree biomass growth and litterfall were not affected by fertilization. Fine root mass (0–1 mm) was 34% greater in N-addition plots, but did not explain soil C increases. Rather, reduced decomposition of litter and soil organic matter drove C increases in N-addition plots. Decomposition rates of black cherry, sugar maple, and mixed leaf litter were 43, 67, and 36%, greater, respectively, in control than N-addition plots. Light fraction organic matter was greater in N-addition plots than in control plots, due to either enhanced root production or decreased decomposition of soil organic matter. Soil respiration was reduced, and microbial biomass in O, A, and upper-B horizons was lower in N-addition plots than controls. The soil microbial community composition was also altered dramatically with N additions. Recalcitrant organic matter enzyme activity (peroxidase) was reduced in the O-horizon by N addition. Available Ca, Mg, and K were reduced in O and A horizons by N fertilization. These results suggest that chronic elevated atmospheric N inputs can increase forest soil C storage by decreasing decomposition, however the long-term stability of this additional C sequestration is unknown.

Core Ideas

- N additions alter soil microbial community composition and reduce forest soil microbial biomass and enzyme activity.
- Litter decomposition and soil organic matter degradation was slowed by N additions.
- Reduced decomposition increases soil C, but long-term effects on forest productivity are unknown.

Temperate forests continue to receive elevated rates of nitrogen (N) deposition (Zhang et al., 2012; Liu et al., 2013; Holland et al., 2005) that is derived from fossil fuel combustion and N fertilizer applications (Pinder et

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al., 2012), with global atmospheric N sources being about 60% anthropogenic in origin (Kanakidou et al., 2016). Even though N deposition rates have been declining in recent years in much of Europe and the United States due to air pollution legislation (Waldner et al., 2014; Li et al., 2016), large increases in N deposition rates are observed in other regions, particularly in Asia (Vet et al., 2014). Although nitrogen availability limits forest productivity on a global scale (LeBauer and Treseder, 2016), long-term deposition has rendered many regions as N-saturated, receiving N inputs at rates beyond the capacity of the system to store N within living biomass or abiotic ecosystem components (Pardo et al., 2011).

Carbon (C) and N dynamics are interrelated and complex, thus excessive N inputs can have a wide range of ecosystem effects (Compton et al., 2011), altering both forest N and C allocation (Nave et al., 2009). For example, increased N availability can increase overall plant productivity, leading to an increase in root biomass and consequently increased sequestration of soil C (Rasse et al., 2005). However, an abundance of N may reduce root to shoot ratios, allowing greater allocation of photosynthate to aboveground tissues (Ryan et al., 1996; Nadelhoffer, 2000; Jussy et al., 2004).

Within terrestrial ecosystems, soils are major global reservoirs of C, storing three-fold more C than is found in vegetation (Tian et al., 2015), and forest ecosystems in particular represent a dynamic and substantial C sink. Northern temperate forests, for example, capture 0.6 to 0.7 Pg C yr⁻¹ (Goodale et al., 2002), with approximately 75% of ecosystem C stored in soil pools. Small relative increases in soil C content, therefore, may result in large increases in the global C sink. Quantifying such sinks is important, especially given that on a global scale, recent work indicates that rates of soil C loss are increasing faster than rates of C uptake by plants (Bond-Lamberty et al., 2018).

Increases in net ecosystem C sequestration resulting from atmospheric N deposition have been documented (Nadelhoffer et al., 1999; Thomas et al., 2010; Yue et al., 2016), and recent work shows that N additions reduce organic matter decomposition (Frey et al., 2014; Zak et al., 2008), thus increasing storage of soil C. However, long-term effects of reduced decomposition are unknown. Slower decomposition may increase soil C storage, but at the same time may also depress C-storing biomass growth due to deficiencies of other nutrients that are locked up in soil organic matter that would otherwise cycle actively in decomposing organic matter pools. Reduced soil cation availability has been observed in a number of N addition experiments; a meta-analysis found that exchangeable cations decrease 24% following N fertilization (Lucas et al., 2011). Decreased cation availability may thus reduce important plant physiological functions, thereby reducing forest productivity (Schulze, 1989). Long-term atmospheric deposition, therefore, may lead to the enigma that excess additions of nitrogen, often a limiting nutrient in forests, can actually reduce forest productivity and increase tree mortality, a phenomenon that has been observed already (Schulze, 1989; Magill et al., 2004; McNulty et al., 2005; Wallace et al., 2007). Reduced productivity could then lead to declines in total ecosystem C.

Most studies of N-amendment decreases in decomposition have been conducted in forests with low to moderate levels of soil N, however soils are notoriously heterogeneous in N content and availability (García-Palacios et al., 2012). Hence, the influence of N additions on forests that are relatively high in soil N is not well known. Forests with high rates of N availability may be less sensitive to additional N inputs and may experience limited declines in decomposition in response to additional N inputs. However, additions of N, especially if as ammonium, may still have significant effects on soil pH and availability of other nutrient ions.

Early work that we conducted at the N-rich Bousson Experimental Forest in northwestern Pennsylvania, showed that after 10 years of annual experimental N additions at levels approximately 10-fold higher than current atmospheric deposition rates, soils had increased C and N concentrations in surface soils, and higher C to N ratios in the O and A horizons of N-addition soils compared to controls (Johnson, 2003). Increased soil N was not surprising, but the increased C did not seem to be due to increased tree productivity that could have provided more litterfall and root inputs to soil organic matter. To determine causes of increased soil C, we initiated a series of studies to determine if the increase in soil C was driven by increased forest productivity and subsequent inputs to soil organic matter, or by decreases in the decomposition of aboveground litter and soil organic matter (SOM). To evaluate the influence of increased forest productivity, we evaluated aboveground biomass growth and litterfall, and quantified root mass. To assess the influence of decreased decomposition on the increase in soil C, we examined litter decomposition, soil microbial biomass, soil microbial enzymes responsible for degrading soil organic matter, and light fractions of soil organic matter that are indicative of SOM degradation. We also sought to know if the soil C response was limited to the initially sampled surface soil or extended more deeply into the soil profile.

MATERIALS AND METHODS

Site Description

This experiment was conducted at the Allegheny College Bousson Experimental Forest (41°36' N, 80°2' W), which lies approximately 11 km southeast of Meadville, PA, at an elevation of 390 m in gently rolling topography with a westerly exposure. Soil parent material is Wisconsinian glacial till underlain by shale and sandstone, with a minor component of limestone (<10%); soils are Cambridge series silt-loam Alfisols (75% sand, 23% silt, 2% clay) (Bowden et al., 2014). Soils in this series contain O, A, B, and C horizons (Soil Survey Staff, 2017). At our site, the Oe and Oa horizons together are approximately 2 to 4 cm thick, the A horizon is approximately 12 to 15 cm thick, and the B horizon is 1 to 1.5 m thick. The Oi horizon (undecomposed litter) varies somewhat seasonally due to autumnal leaf senescence, but is 1 to 2 cm thick in mid-summer.

The region is characterized by temperate climate conditions with seasonally distributed precipitation (112.5 cm yr⁻¹) ranging from 6.6 cm in January to 11.5 cm in June, with mean

daily temperatures (1991–2011) of -4.1°C in January and 23.6°C in July, a 4-mo growing season, and approximately 4 mo of snow cover (Bowden et al., 2000, 2014). The mature, mid-successional forest is approximately 80 to 100 yr old, has a total aboveground tree biomass (control plots) of $690 \pm 188 \text{ Mg ha}^{-1}$ that is dominated by black cherry (*Prunus serotina* Ehrh.), sugar maple (*Acer saccharum* Marshall), red oak (*Quercus rubra* L.) and American beech (*Fagus grandifolia* Ehrh.), representing 42, 31, 24, and 3% of total biomass, respectively. Overstory canopy height is approximately 20 to 25 m. There were essentially no shrubs or tree seedlings or samplings in the understory. Annual litterfall (control plots) is $5.5 \pm 0.3 \text{ Mg ha}^{-1} \text{ yr}^{-1}$, with sugar maple, black cherry, American beech, and red oak representing 56, 24, 15, and 5%, respectively, of autumnal leaf litterfall. Nitrogen mineralization is approximately $120 \text{ kg N ha}^{-1} \text{ yr}^{-1}$, and 70% of ammonified nitrogen becomes nitrified (Bowden et al., 2000). The forest on this site likely regenerated on an abandoned pasture; there is no evidence of plowing in the upper soil horizon and the soil surface is characterized by pit and mound topography. The prevalence of shade-intolerant black cherry in the overstory indicates that the area was open at the time of forest regrowth.

Nitrogen Additions

The chronic N-addition study was established in summer of 1993 (Year 0 of the study). Three 15-m by 15-m treatment plots in the forest have been fertilized biweekly since establishment with NH_4NO_3 in June, July, and August, using backpack sprayers, providing a net input of $100 \text{ kg N ha}^{-1} \text{ yr}^{-1}$. This rate is approximately 10-fold greater than ambient N deposition, and was chosen to accelerate the process of atmospheric N additions. During several years when NH_4NO_3 was unobtainable, we used KNO_3 and NH_4Cl or commercial fertilizer. Three 15-m by 15-m control plots exist within 50 m of the N-addition plots; these are untreated except that in years when KNO_3 and NH_4Cl were applied to treatment plots, the control plots were sprayed with KCl at the same rate as K^+ was added to the N-addition plots.

Aboveground Biomass and Litterfall

Aboveground biomass and growth were estimated by using biomass regressions (Tritton and Hornbeck, 1982) based on tree diameter (at breast height [dbh], 1.35 m). Trees ($>2.5 \text{ cm dbh}$) were measured during winters in 1998, 2003, 2012, and 2015 (Years 5, 10, 19, 22, respectively). Annual litterfall mass was determined by collecting litter from 2014 through 2016 (Years 21–23) using two 1-m by 1-m litter traps on the forest floor within each plot, and were emptied monthly during snow-free months. Leaves, tree fruiting material, and woody litter $\leq 1 \text{ cm}$ diameter were included in litterfall. Litter was oven-dried for 48 h at 105°C .

Soil Carbon, Nitrogen, Roots, and pH

Three sampling locations were chosen randomly within each of the six study plots. At each location, soils were collected by horizon between June and November of 2010 (Year 17). For the O, A, and the upper 10 cm of the B horizons, 15-cm by

15-cm samples were excavated by hand. Horizon changes were determined in the field based on clearly discernible changes in soil color and texture. Deeper sections (10–20, 20–30, 30–50 cm) of the B horizon were sampled subsequently using a gas-powered auger (Earthquake, Model 9800B) equipped with a diamond-bit deep soil corer (9.8 cm diameter).

All fresh soil samples were passed through a 2-mm sieve. Roots that did not pass through the sieve were initially refrigerated (4°C) on collection. Total fresh soil sample mass (g m^{-2}) was measured for each soil depth; within each soil depth for each sample, fresh soil was mixed thoroughly, and then subsamples (approximately 10 g for surface samples and 100 g for deep samples) were removed, weighed, and dried at 105°C for at least 48 h to determine wet to dry ratios. Total mass for each soil depth in each sample was calculated using the wet to dry soil ratios.

Roots were rinsed, dried at 105°C for at least 48 h, sorted into two size classes ($\leq 1 \text{ mm}$, $>1 \text{ to } 2 \text{ mm}$), and weighed. To estimate the amount of small pieces of roots that passed through the 2-mm sieve in the O, A and top 10 cm of the B horizons, we removed a soil subsample ($\sim 5\%$ of total mass) and extracted and sorted small roots by hand. These roots were rinsed, dried at 105°C for at least 48 h, and weighed; the mass was then scaled to reflect the total mass of small roots in the sieved sample. This estimate was added to the mass of sieved roots. For deeper soils, extracted roots were rinsed, dried at 105°C for at least 48 h, sorted by size, and weighed. Very few roots existed in deeper soils, so we did not try to quantify roots that passed through the 2-mm sieve.

Concentrations of C and N were determined on dry soil ($\sim 0.5000 \text{ g}$ each) and leaf and root samples ($\sim 0.2000 \text{ g}$ each) using a LECO C-N-S analyzer. Soil and tissue standards were used for calibration. Because these soils contain low amounts of limestone, we assume that nearly all soil C is organically derived. Soil pH was determined in fall 2014 (Year 21) on fresh soil. O-horizon and A-horizon soil was collected from each quadrant in each plot, and homogenized by plot and depth. Soil slurries were made using a 1:2 ratio of soil mass to water volume (DI) for the O horizon, and a 1:1 slurry for A horizon. Slurries were mixed thoroughly, and allowed to sit for 30 min before pH was measured.

Litter Decomposition

We measured litter decomposition in 2002 (Year 9) and 2015 (Year 22). In Year 9, we measured 1-yr decomposition rates of sugar maple and black cherry, the dominant tree species at the site. Freshly fallen leaves were collected from the site in fall 2002 in four locations across the site. The collected leaves were composited and air-dried. Litterbags (15 cm by 15 cm) comprised of 1 mm fine mesh fiberglass were filled with the oven-dried equivalent of either 2 g of sugar maple or 2 g of black cherry leaves. On each of the control and N-addition plots, five randomly placed stakes received two each of the two different litterbags. The bags, deployed in early November when autumnal senescence had ended, were placed on top of the O horizon and covered with newly fallen leaf litter to resemble current forest conditions. Samples were retrieved 1 yr later; litter was oven-dried (105°C , 48 h) and weighed.

We again measured decomposition in Year 22, with a few modifications to the methods. First, to compare the treatments directly, we collected leaves from within each treatment, and returned those leaves to their original treatments to decompose for a year. Second, to separate potential effects of N fertilizer-induced leaf chemistry soil changes induced by N addition, we conducted a litter transfer experiment wherein leaves collected from the control plots were placed into litter bags and allowed to decompose on the control plots. We reciprocated the approach by collecting litter from the N-addition plots and allowing them to decompose on the N-addition plots. Leaves used for decomposition were collected during early autumn from screens placed on the forest floor in the middle of each N-addition plot, and screens located immediately outside each control plot. The leaves from the plots were combined and homogenized separately for control and N-addition plots.

Litter decomposition was measured separately for sugar maple and black cherry (the dominant litter inputs) as well as for the ambient mixed litterfall. Leaf pack composition for the mixed litter bags was determined through an autumnal collection in 2014 using two 1-m² screens within each plot. Leaves were sorted by species and oven-dried (105°C, 48 h) to determine the contribution of each species to total litterfall at the site.

Litterbags were constructed with the oven-dried equivalent of either 2 g of sugar maple or 2 g of black cherry leaves, and 2 g for mixed litter bags that contained 53.8% sugar maple, 29.5% black cherry, and 16.7% beech. Litter was placed into 15-cm by 15-cm, 0.18-mm mesh fine mesh bags. A smaller mesh size was used to ensure that litter was not lost from the bags, however this approach probably excluded some macroinvertebrate faunal decomposers. On each plot, one bag of each litter type and treatment origin was located within each quadrant of the plot, providing four bags per treatment per plot. The bags, deployed in mid-October, were placed on top of the O horizon and covered with newly fallen leaf litter. Samples were retrieved 1 yr later; litter was oven-dried (105°C, 48 h) and weighed.

Soil Respiration

Soil respiration was measured twice per month from May to November in 2002 and 2003 (Years 9 and 10) using the soda lime technique (Raich et al., 1990; Grogan, 1998). On each plot, a 27.5-cm diameter plastic ring was set 1 to 2 cm into the O horizon and left in place. During measurements, the ring was removed, and a 20 cm (height) by 27.5 cm (diameter) plastic chamber was set over an open soda lime (~60 g) container placed atop the soil for 24 h. The weight change in soda lime, corrected for water loss and CO₂ absorption during handling, was determined.

Enzymes

Soil samples for enzyme analysis were collected before and after leaf senescence in October and November, 2014 (Year 21) from each quadrant within each of the plots. O-horizon soil horizon was collected from 20-cm by 20-cm samples. A-horizon soil samples were obtained by using a 6 cm diameter soil corer

below the O-horizon samples. O and A-horizon soil samples were passed through a 4-mm sieve and 2-mm sieve, respectively. Soils were stored at 4°C on collection, and soil moisture content was determined (soils dried at 105°C, 48 h). Loss on ignition was performed using 5 g of dried soil in a 550°C muffle furnace for 4 h to estimate soil organic matter concentrations. Quadrant soil samples were homogenized, based on proportional mass per sample collected, to provide one sample per plot per horizon. Soil slurries were made fresh for each assay and kept at 27°C.

Phenoloxidase and Peroxidase

Phenoloxidase and peroxidase activities were measured based on modifications of the L-3,4-dihydroxyphenylalanine (DOPA) assays (Bach et al., 2013). Prior to the experiment, optimum reaction time and substrate concentration for the Bousson soils were determined. One hundred mL of 50 mM sodium acetate buffer, pH 5, were added to 1 g of each homogenized sample and emulsified for 30 s using a polytron probe. Two mL of the soil slurry were incubated at 27°C for 1 h with 2 mL of a 5-mM solution of L-DOPA (Sigma, CAS 59-92-7) in 50-mM sodium acetate buffer, pH = 5, for each assay. Additionally, the peroxidase samples had 0.2 mL of 0.3% H₂O₂ added during the incubation period. For each sample, there were three analytical replicates and one blank containing 2 mL of acetate buffer that replaced the L-DOPA substrate. After incubation, the samples were centrifuged (2500 rpm, 5 min), and the absorbance of the supernatant was read on a spectrometer at 460 nm (Beckman DU-530 UV-Vis spectrometer). Enzyme activity was calculated based on the known (μmol L-DOPA) oxidation calibration factor (Abs/1.66).

Beta-glucosidase

Beta-glucosidase activity was measured based on a modification of the Caldwell et al. (1999) p-nitrophenyl-ester based assay. In short, 30 mL of DI water were added to 5 g of soil and stirred for 5 min to create a soil slurry. One milliliter of soil slurry was incubated at 27°C with 1 mL of 20 mM-p-nitrophenyl-β-glucoside for 3 h, after which time 0.5 mL of 0.5 M CaCl₂ was added and the reaction was stopped using 2 mL of 0.1 M, pH = 12, tris(hydroxyl-methyl)aminomethane. For each sample, there were three analytical replicates and one blank, containing 1 mL of deionized water replacing the p-nitrophenyl substrate. The samples were centrifuged (2500 rpm, 5 min), and the supernatant absorbance was read on a spectrometer at 410 nm (Beckman DU-530 UV-Vis spectrometer). Enzyme activity was calculated based on a 4-nitrophenyl β-glucopyranoside standard curve (Sigma, CAS 2492-87-7).

Microbial Analyses

Soils from the O and A horizons, and the top 10 cm of the B horizon, were collected from two 20-cm by 20-cm soil blocks within each plot in 2016 (Year 23). Phospholipid fatty acids (PLFAs) were extracted using a modified Bligh-Dyer method followed by identification and quantification using gas chromatography-mass spectrometry (GC-MS) (Frostegård and

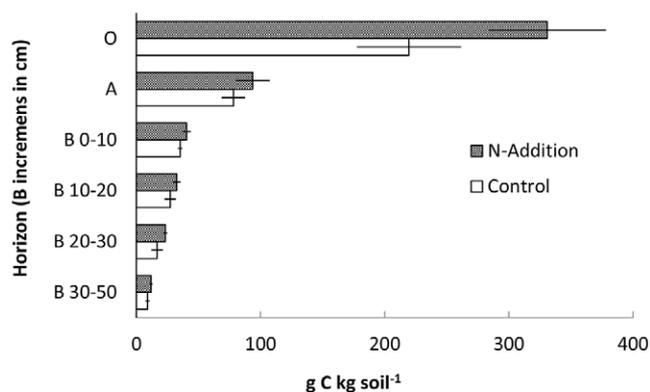


Fig. 1. Soil C concentration by sampling depth in control and N-addition plots at the Bousson Experimental Forest (mean \pm SE, $n = 3$ plots per treatment), Year 17. Nitrogen addition ($p < 0.004$) and depth ($p < 0.001$) differed significantly.

Bååth, 1996). Full details of the PLFA extraction and quantification by gas chromatography–mass spectrometry are given in Wang et al. (2019). Concentrations of all compounds were normalized to soil C content for comparisons between different soil horizons.

Light Fraction Soil Organic Carbon

Using subsamples of soil collected for microbial analysis, we followed the density fractionation method described by Sollins et al. (2006) to isolate the free light density SOC fraction (< 1.85 g cm⁻³) in Year 23. Moist bulk soil samples were suspended in a sodium polytungstate (SPT) solution (1:3 soil/SPT), shaken for 2 h on a shaker table, then centrifuged at a relative centrifugal force of 2560 g for 10 min. The density of the supernatant was checked and readjusted to the density target (± 0.01 g cm⁻³) if necessary. Floating material and supernatant were aspirated using a vacuum system. The free light fraction SOC was rinsed on Whatman GF/F filters and oven dried for 2 d at 55°C.

Soil Cations

Soils were collected monthly from October through December in 2016 (Year 23). O-horizon soils were collected from a 5-cm by 5-cm area, and A horizon soils were collected via a 3 cm diameter Oakfield corer. Four samples from each of the three treatment and the three control plots were collected. The O horizon was passed through a 4-mm sieve and the A horizon through a 2-mm sieve. The sieved samples were stored at 4°C for a maximum of 2 wk before being processed.

Cation availability methods were adapted from Robertson et al., (1999). To measure available Ca, Mg, and K, soil slurries were made adding 10 g of soil to 100 mL of 1 M NH₄Ac at pH 7.0. The pH of the NH₄Ac was adjusted to pH 7.0 using acetic acid or aqueous ammonium. Slurries were shaken on a shaker table for 1 h. Samples were then filtered through a 1.6 μ m Gelman A/E glass fiber filter. Non-extractable cations were measured using methods adapted from Friedland et al. (1984) and Yanai et al. (1999). Three grams of oven-dried soil from each subplot and soil horizon were combusted in a muffle furnace at 550°C for 5 h. The remaining metals were dissolved in 10 mL of 6 M HNO₃, and boiled on a hot plate for 20

Table 1. Mean (\pm SE) soil C and N content (kg m⁻²) in control and N-addition plots at the Bousson Experimental Forest ($n = 3$ plots per treatment), Year 17. Carbon differed significantly by depth ($p < 0.001$) and treatment ($p < 0.05$). Nitrogen differed significantly by depth ($p < 0.001$) but not treatment.

Horizon and depth	Carbon		Nitrogen	
	Control	Fertilized	Control	Fertilized
O	0.66 (0.10)	1.24 (0.16)	0.046 (0.007)	0.074 (0.011)
A	2.47 (0.43)	2.24 (0.27)	0.190 (0.047)	0.152 (0.017)
B, 0–10 cm	2.63 (0.13)	2.91 (0.27)	0.203 (0.027)	0.199 (0.021)
B, 10–20 cm	2.26 (0.19)	2.70 (0.14)	0.154 (0.017)	0.159 (0.008)
B, 20–30 cm	1.88 (0.33)	2.51 (0.18)	0.098 (0.020)	0.133 (0.009)
B, 30–50 cm	2.17 (0.22)	2.59 (0.19)	0.066 (0.020)	0.118 (0.018)
Total	12.09 (0.72)	14.20 (0.73)	0.757 (0.076)	0.836 (0.050)

min. The mixture was then diluted with 50 mL of deionized water and passed through a 0.45 μ m membrane filter. Extracts for available and non-extractable cations were stored at 4°C until analysis. Ca, Mg, and K concentrations of both extractions were measured using an inductively coupled plasma atomic emissions spectrometer.

Statistical Analyses

Treatment and depth comparisons of variables were analyzed using t tests, two- and three-way ANOVA, and RMANOVA; differences were elucidated using Tukey's or Holm-Sidak pairwise multiple comparison tests (SigmaPlot ver. 12.5, Systat Software). Data that were not normally distributed or did not have equal variances were ln-transformed for analysis.

RESULTS

Soil Carbon

Concentrations of soil C (Fig. 1) and N (not shown) both decreased significantly with depth ($p < 0.001$), and C and N concentrations were both greater in the N-addition plots (C, $p < 0.01$; N, $p < 0.05$). The O-horizon C concentration (330.8 ± 47.4 mg g soil⁻¹), was 50% greater in the N-addition plots than the controls; similarly, N concentrations in the O-horizon (19.8 ± 2.8 mg g soil⁻¹) were 29% greater in the N-addition plots than the control plots.

Total soil C (Table 1) was greater in the N-addition plots than in the control plots ($p < 0.001$) and declined by horizon. Total C in the N-addition plots at Year 17, 14.2 ± 0.7 kg m⁻², was 17% more than the control plots, resulting in an increase of 1.17 Mg ha⁻¹ yr⁻¹. The C content differed significantly only in the B horizon ($p < 0.01$). Soil N concentrations differed significantly between N addition and control plots, and total soil N declined significantly with depth (Table 1), however, there was no statistical difference in total N between the control and N-addition plots. Similarly, there was no significant difference in total soil mass between the treatments. Soil pH was significantly lower in the N-addition plots (O, 3.67; A, 3.53) than the controls (O, 4.11; A, 3.90), in both the O ($p < 0.001$) and A horizons ($p < 0.001$), and was lower in the A than the O horizon ($p < 0.05$).

Free light fraction SOC was three-fold greater in the A horizon of the N-addition plots (43.52 ± 3.34 g C kg soil⁻¹) than the controls (13.00 ± 4.98 g C kg soil⁻¹), but concentrations were

not different in the uppermost B horizon (control, 6.05 ± 1.97 g C kg soil⁻¹; N-addition, 6.80 ± 1.16 g C kg soil⁻¹). Concentrations were greater in the A horizon than the B horizon ($p < 0.01$).

Litter Decomposition

Year 9 Decomposition

During Year 9, when leaves originating from control plots were placed in both treatments, both black cherry and sugar maple leaves decomposed more slowly in N-addition plots than in control plots ($p < 0.001$; Fig. 2). Nitrogen-addition plots had 12.2% more black cherry litter mass remaining, and 14.7% more sugar maple litter mass remaining, than control plots. Black cherry leaves decomposed more quickly than sugar maple leaves in both control and N-addition plots ($p < 0.001$).

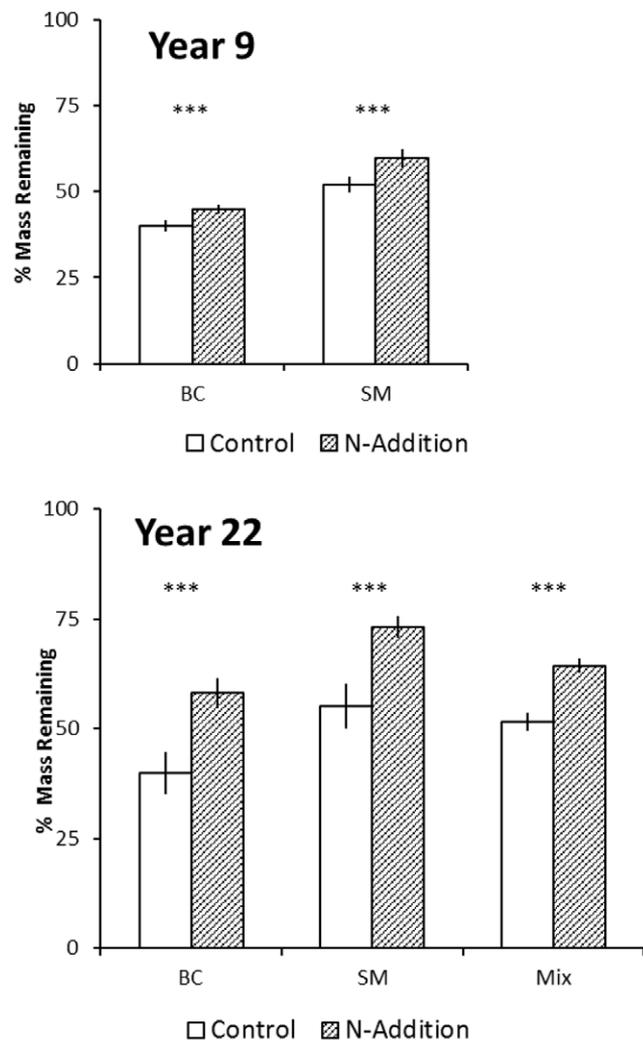


Fig. 2. One-year litter decomposition (mean \pm SE) of black cherry (BC), sugar maple (SM) and mixed litter (Mix) in control and N-addition plots at the Bousson Experimental Forest, Years 9 and 22. In Year 9, leaves obtained from the site (unfertilized areas) were allowed to decompose in each treatment. In Year 22, leaves collected from each treatment were decomposed in their “home” treatments. Decomposition differed significantly by treatment ($p < 0.001$) and leaf (Year 9: $p < 0.001$, Year 22: $p < 0.01$) in both years.

Year 22 Decomposition: Resident Analysis

All three litter types—sugar maple, black cherry, mixed—that originated from the N-addition plots decomposed more slowly in the N-addition plots than did control litter derived from the control plots (Fig. 2). Sugar maple, black cherry, and mixed litter had 32.6, 45.5, and 24.9% more mass remaining in the N-addition plots than the control plots. Across both treatments, black cherry decomposition rates were higher than rates for both sugar maple ($p < 0.01$) and mixed litter ($p < 0.05$).

Year 9 vs. Year 22

Decomposition rates of control leaves decomposing in control plots were the same in Year 9 as Year 22, however control leaves decomposed more slowly in N-addition plots in Year 22 than Year 9 ($p < 0.05$). In Year 9, after 1 yr of decomposition in the litterbags, there was 12.2% more black cherry litter in N-addition plots than in control plots; by Year 22, black cherry had 45.7% more mass remaining in the N-amended plots than in controls. Similarly for sugar maple, after a year of decomposition in the litterbags, the difference between the controls and N-addition plots in the amount of litter mass remaining increased from 14.7% in Year 9 to 32.8% in Year 22.

Year 22 Decomposition: Transfer Analysis

There were no differences in decomposition rates between transferred leaves (leaves grown in one plot type and transferred to the other plot type) and resident leaves for any litter species or for either plot type (Supplemental Fig. S1).

Microbial Biomass

The total concentration of PLFAs was significantly reduced in both the O and A horizons with N-addition (Fig. 3). In both the O and A horizons, total PLFA concentrations were markedly lower and reflect significant reductions in microbial biomass with chronic N-addition. PLFA concentrations were also lower in the B horizon but not statistically significant. The composition of the microbial community was also altered with long-term N-addition, especially in the O and A horizons (Fig. 3) where the amount of microbial biomass declined for gram-negative bacteria, gram-positive bacteria, fungi and actinomycetes. The microbial community composition in the B horizon was altered to a lesser extent.

Soil Respiration

Soil respiration was significantly lower in the N-addition plots than it was in the controls (Fig. 4). Annually, the CO₂ effluxes from the control plots were 1097.1 ± 99.2 and 1366.7 ± 82.8 g m⁻² y⁻¹ in Years 9 and 10, respectively, compared to effluxes from the N-addition plots, which were 1042.9 ± 47.8 and 1287.5 ± 19.8 g m⁻² y⁻¹ in Years 9 and 10, respectively. Over the 2 yr, respiration rates in the control plots were 5.7% greater than rates in the N-addition plots.

Enzymes

Peroxidase activity per gram of soil organic matter was significantly less in the N-addition O- horizon soils compared to the control soils ($p < 0.05$) (Fig. 5). Beta-glucosidase activities per gram of SOM were significantly higher in the N-addition soils compared to the control soils within the A horizon ($p < 0.05$).

Biomass, Litterfall, and Roots

Tree stem density (Table 2) changed somewhat over the course of the study. Tree mortality, ($7.6 \pm 0.7\%$; 1.0 tree plot $^{-1}$) in controls was significantly lower ($p = 0.02$) than in the N-addition plots ($16.5 \pm 2.3\%$; 2.3 trees plot $^{-1}$), however neither the diameters (control, 37.3 ± 17.9 cm; N-addition, 10.2 ± 1.6 cm) nor the mass (control, 81.8 ± 69.7 Mg ha $^{-1}$; N-addition, 5.34 ± 1.1 Mg ha $^{-1}$) of trees that died trees differed between the treatments. On one control plot, the single tree that fell represented 90% of the mass of all dead trees on the three control plots. There was no recruitment of new trees into any of the plots. Standing tree biomass varied among the plots and was two-fold higher in the N-addition plots than the control plots (Table 2), however the relative growth rate (growth [Mg] / standing biomass [Mg]) did not differ between the treatments. Over the period when growth was measured (Years 5–22), growth rates in the control plots (0.070 ± 0.025 Mg Mg $^{-1}$) and N-addition plots (0.072 ± 0.018 Mg Mg $^{-1}$) were nearly identical. During the last measurement interval (2012–2015), however, the relative rate of tree growth in N-addition plots was significantly lower in the N-addition plots than in the controls ($p < 0.05$).

Litterfall rates between the treatments were not different. Averaged over the three measurement years (2014–2017), litter inputs in the control plots were 478 ± 35 g m $^{-2}$, and in the N-addition plots, they were 492 ± 42 g m $^{-2}$.

Total 0- to 1-mm root mass (Table 3; Supplemental Table S1) was 33% greater in the N-addition plots than the control plots ($P < 0.05$). The 1- to 2-mm root mass did not differ by treatment. Root N concentrations differed significantly by both root size and treatment, with higher N concentrations in the N amended plots, and higher concentrations in the 0- to 1-mm roots than the 1- to 2-mm roots.

Cations

Concentrations of available Ca, Mg, and K were significantly higher in the controls than in the N-addition plots, in both the O and A horizons (Fig. 6). For

example, in the O horizon, available Ca was 40% greater in the controls than the N-addition plots; K was nearly 90% greater. Available Ca was greater in the O horizon than the A horizon in all plots. Non-extractable Ca concentrations decreased approximately 50% with N additions in the O and A horizons, but non-extractable Mg and K concentrations did not differ. There was no difference with depth in non-extractable cations.

DISCUSSION

The increase in soil C in response to N inputs that we observed is consistent with responses observed in N addition studies in other temperate forests. In the Catskills of New York, 6 yr of N additions caused substantial accumulations of C in the O horizon (Lovett et al., 2013), and following two decades of N additions, soil carbon in temperate coniferous and hardwood forested plots at the Harvard Forest increased up to 80%, depending on the level of addition and forest type (Frey et al., 2004). A latitudinal gradient of sugar maple stands in Michigan,

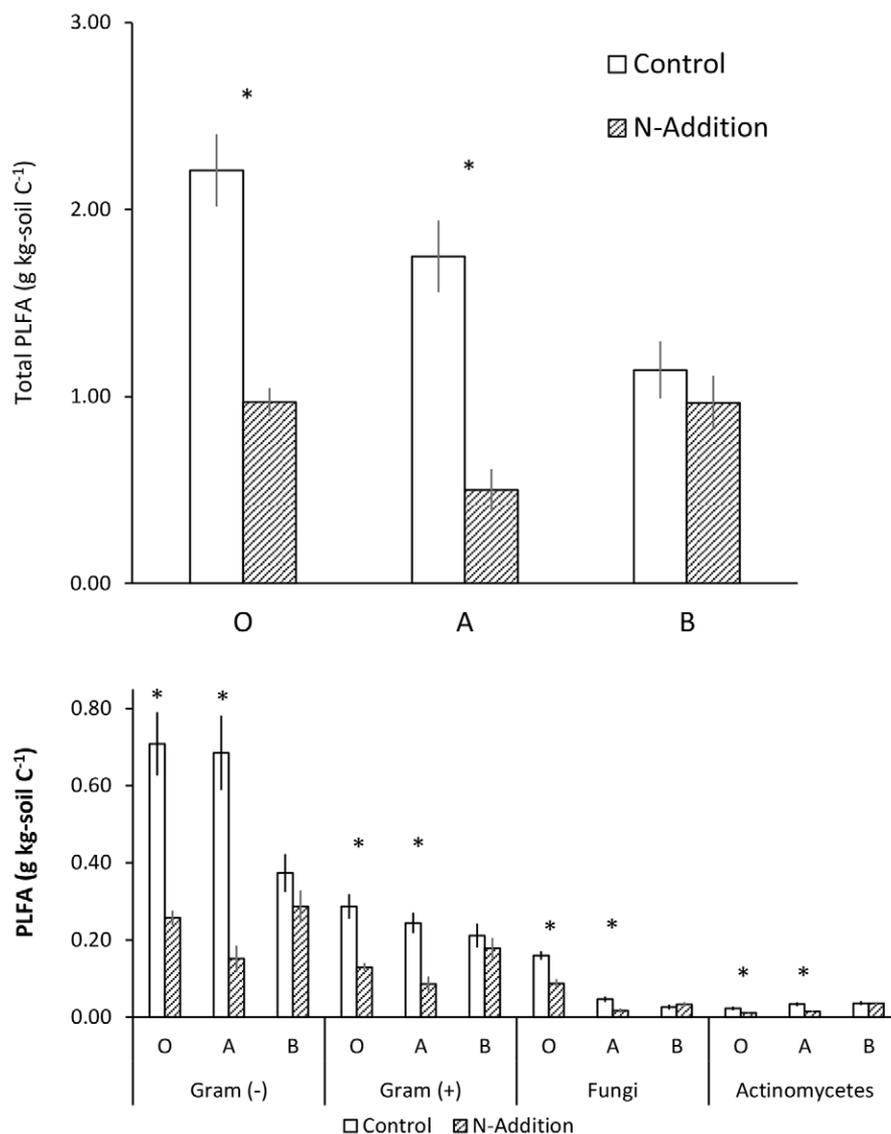


Fig. 3. Total PLFA and PLFA of microbial constituents in control and N-addition plots at the Bousson Experimental Forest. * Significant difference at $p < 0.05$.

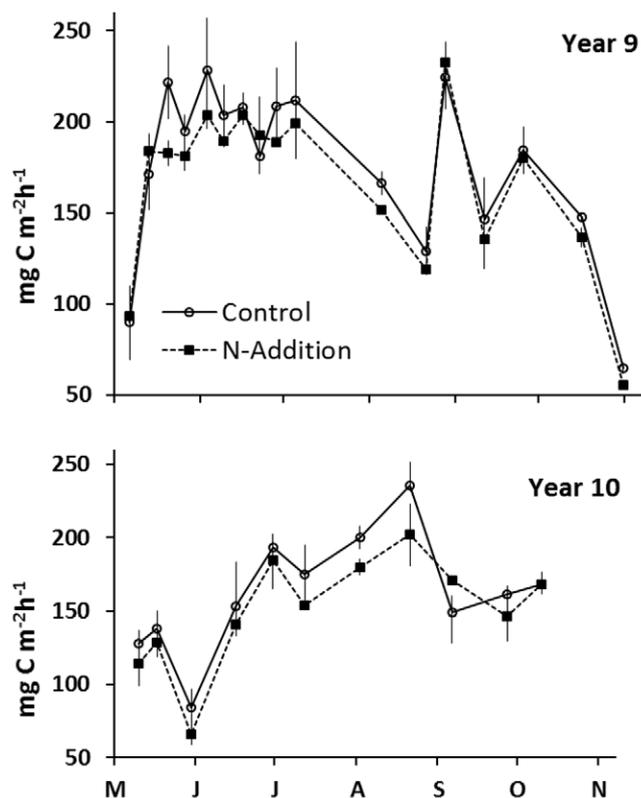


Fig. 4. Soil respiration in control and N-addition plots at the Bousson Experimental Forest. (Mean \pm SE, $n = 3$ plots per treatment), Years 9 and 10. Rates were significantly lower in N-addition plots than in control plots ($p < 0.001$).

USA showed similar results, with increased soil C in response to N addition (Waldrop et al., 2004b; Zak et al., 2008), where much of the increase occurred in the O horizon and upper portion of the mineral soil.

We found that the largest fraction of soil C storage occurred in the upper 10 cm of the B horizon, in agreement with results from the Michigan sugar maple gradient study (Pregitzer et al., 2008), where mean C content increased in the upper mineral soil, and at the Harvard Forest (MA), which found C increases in upper B-horizon soil, but not surficial mineral soils, in response to fertilization (Frey et al., 2014). Modeling efforts at the Harvard Forest also support the empirical observations of C accumulation in the B horizon (Tonitto et al., 2014). Furthermore, (Tonitto et al., 2014) suggested that long-term ambient atmospheric N deposition at the Harvard Forest has led to an increase in C in the O, A, and B horizons in both hardwood and pine stands.

There are many factors that could have caused increases in soil C, including increased aboveground litterfall deposition, increased root production and turnover, and decreased decomposition of litter and existing SOM. We found no difference in litterfall rates between the treatments, and even though there is evidence of a decline in biomass growth in those plots during the last measurement period (2012–2015), the tree biomass accumulation rates over the course of the study did not differ. There are also no obvious differences in coarse woody debris (CWD) inputs that might contribute inputs to

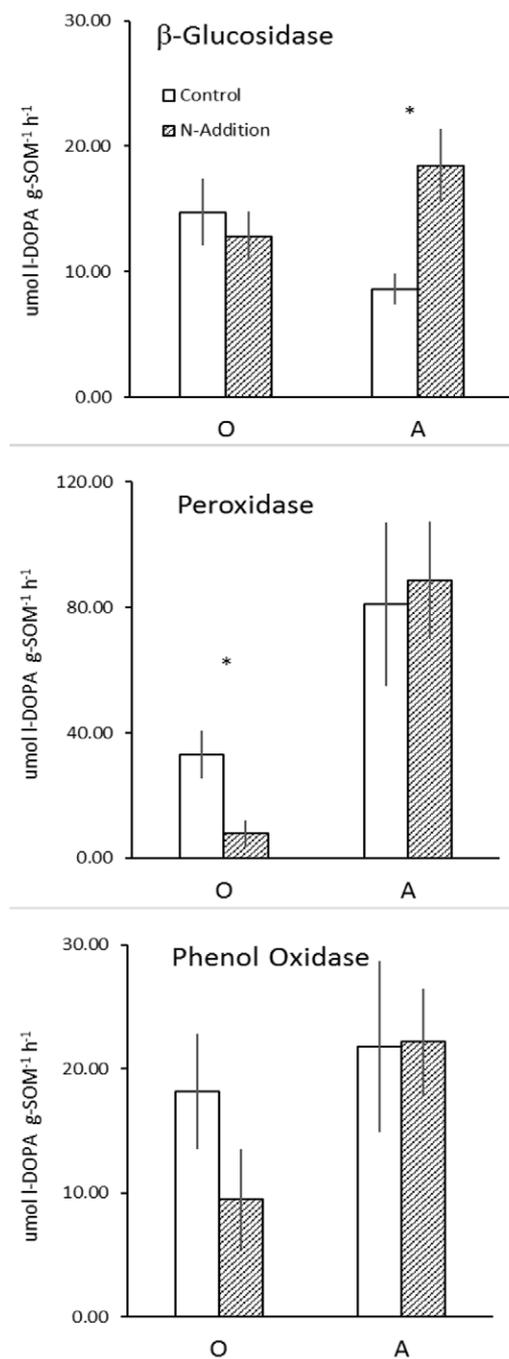


Fig. 5. Soil enzyme activity during autumn, in control and N-addition plots in the Bousson Experimental Forest. (Mean \pm SE, $n = 3$ plots per treatment), Year 21. * Significant difference at $p < 0.05$.

soil C. We did not measure CWD inputs directly, however, even though there is more tree mortality on the N-addition plots than the controls, there was no statistical difference between the treatments in the mass of trees that died over the course of the experiment. Hence, it is not likely that changes in litterfall, CWD, or inputs of large roots would have contributed to observed soil C increases.

Overall roots can provide up to 75% of soil C inputs (Gale and Cambardella, 2000; Puget and Drinkwater, 2001; Rasse et al., 2005; Kong and Six, 2010; Mendez-Millan et al., 2010), with contributions from dead roots, root exudates, and rhizospheric

inputs. We found significantly more roots (0–1 mm) in the N-addition plots than the controls, thus we sought to estimate whether or not fine roots might contribute importantly to the observed increases in soil C. If they did, we would expect that roots and soil C content are correlated, but that is not what we found. Rather, within the mineral soil, linear regressions between the mass of 0- to 1-mm roots and soil C by depth were not significant for either the control ($p = 0.08$) or the fertilized treatments ($p = 0.41$). This lack of a correlation may not be that surprising; if root turnover, microbial activity, and C stabilization rates vary by depth, then root content may not correlate directly to soil C.

Nonetheless, using our estimates of root mass and soil C, in conjunction with literature estimates of root turnover and root-derived soil C stabilization, we can provide an estimate of the importance of 0- to 1-mm roots (assumed to be the most active roots) in the measured increase in soil C. Total C of these roots (upper 10 cm of the B horizon) was $104.1 \pm 12.1 \text{ g m}^{-2}$ in the N-addition plots, and $55.0 \pm 3.0 \text{ g m}^{-2}$ in the controls, a net increase of 49.1 g C m^{-2} . Using an annual root turnover estimate of 30% measured in a northern hardwood forest (Tierney and Fahey, 2002), total root C inputs over the 18 years of this study would be 265 g C m^{-2} . For roots alone to explain the 275 g C m^{-2} measured increase in soil C, all of this increase in root inputs would need to be converted to stabilized SOC, which is clearly unrealistic.

Most estimates suggest that at best, only a small fraction of total organic matter inputs to soil become long-term, stabilized C. For example, Austin et al. (2017) estimated that after 17 mo in a cropping system, nearly all the belowground C inputs had been mineralized. If we assume that 5% the increased root C inputs (265 g C m^{-2}) at Bousson were stabilized over the 18 yr, then at best, 13.3 of the 275 g C m^{-2} soil C increase, or 4.8%, would be caused by root inputs. Even though there still exists considerable controversy and uncertainty in estimating root turnover and stabilization of root-derived C (Gaudinski et al., 2010; Brunner et al., 2013; Austin et al., 2017), organic matter contributions from root exudates and rhizospheric organic matter, or the increase in roots that we measured, do not seem sufficient to explain our observed increase in soil C.

We argue that most of the observed changes in soil C are probably driven by decreased organic matter decomposition at the surface and within mineral soil. A companion study (Wang et al., 2019) that analyzed the molecular composition of soil C from this experiment showed a suppression of plant litter processing with N addition. Furthermore, decomposition of fresh surface litter was significantly lower in the N-addition plots as measured during Years 9 and 22. Two overall conditions could drive the

Table 2. Aboveground biomass, stem density (trees, $\geq 2.5 \text{ cm dbh}$), and relative biomass growth in control and N-addition plots at the Bousson Experimental Forest. Values are means \pm SE ($n = 3$ plots per treatment). Relative growth did not differ by treatment.

Property	Year	5	10	19	22
Stem density (trees ha^{-1})	Control	593 (53)	593 (53)	563 (39)	548 (53)
	N-Addition	593 (155)	593 (155)	548 (146)	489 (118)
Biomass (Mg ha^{-1})	Control	502.2 (154.5)	559.7 (164.0)	572.2 (136.9)	690.1 (188.1)
	N-Addition	241.2 (117.7)	280.6 (130.8)	338.7 (158.8)	371.8 (175.9)
Relative growth ($\text{Mg Mg}^{-1} \text{ yr}^{-1}$)	Control	0.083 (0.019)	0.045 (0.014)	0.070 (0.025)	0.070 (0.013)
	N-Addition	0.109 (0.035)	0.055 (0.014)	0.016 (0.005)	0.072 (0.018)

Table 3. Mean (\pm SE) root mass, C mass, N mass and N concentration in control and N-addition plots at the Bousson Experimental Forest ($n = 3$ plots per treatment; 3 samples per plot), Year 17. In the 0- to 1-mm diameter class, mass ($p = 0.01$), C ($p = 0.047$), and N ($p = 0.007$) were significantly higher in the N-addition plots than the controls. In the 1- to 2-mm diameter class, root mass, C, or N did not differ by treatment. Root N concentrations are significantly greater ($p < 0.01$) in N-addition than control plots; 0- to 1-mm roots have higher N concentrations ($p < 0.001$) than 1- to 2-mm roots.

Root diameter	Treatment	Root mass g m^{-2}	C mass g C m^{-2}	N mass g N m^{-2}	N concentration g kg^{-1}
0–1 mm	Control	442.5 (45.5)	195.7 (17.2)	5.6 (0.24)	13.27 (0.74)
	N-addition	633.3 (54.6)	269.0 (22.1)	10.1 (1.40)	15.61 (0.87)
1–2 mm	Control	177.9 (17.5)	83.2 (8.2)	1.51 (0.08)	8.50 (0.36)
	N-addition	201.0 (28.7)	95.5 (13.5)	1.92 (0.25)	9.53 (0.27)

difference in decomposition rates: changes in foliar chemistry or changes in soil conditions. In our study, foliar N concentrations in Year 10 were $1.76 \pm 0.02\%$ for control plots compared to $1.93 \pm 0.09\%$ for the N-addition plots (Baldauf, 2004), and roots also had higher N concentrations (Table 3). Effects on decomposition of elevated N concentrations are complex. Recent reviews show that many studies have documented a strong, inverse linear relationship between initial N concentration in leaves and decomposition rates under ambient conditions, but following experimental N addition to soils, litter decomposition rates decrease with increasing duration and application rate of N (Zhang et al., 2012; Averill and Waring, 2017). Van Diepen et al. (2015) noted that decomposition of litter with high lignin concentrations was generally negatively affected by chronic N additions, while decomposition of litter with low lignin concentrations was enhanced by increased N availability. They also noted that simulated N deposition caused decreased leaf Al, B, Ca, Mg, Mn, P, and Zn concentrations which could also affect decomposition rates. Multiple studies have shown that elevated rates of N deposition repressed lignolytic enzymes and thus rates of microbial decomposition (Berg and Ekbohm, 1991; Lucas and Casper, 2008; Zak et al., 2008; Entwistle et al., 2018).

We think that it is also telling that the reduction in litter decomposition was observed in Year 9, and that the reduction was greater by Year 22, indicating that the magnitude of the effect changed during the course of the experiment. Suppression may have been lower prior to Year 9, but clearly the suppression was not constant over the course of the experiment, and hence the rate of C sequestration may not have been constant, either.

Because litter obtained from control and N-addition plots lost mass at the same rates regardless of whether it was placed into control or N-addition plots (Year 22), we conclude that

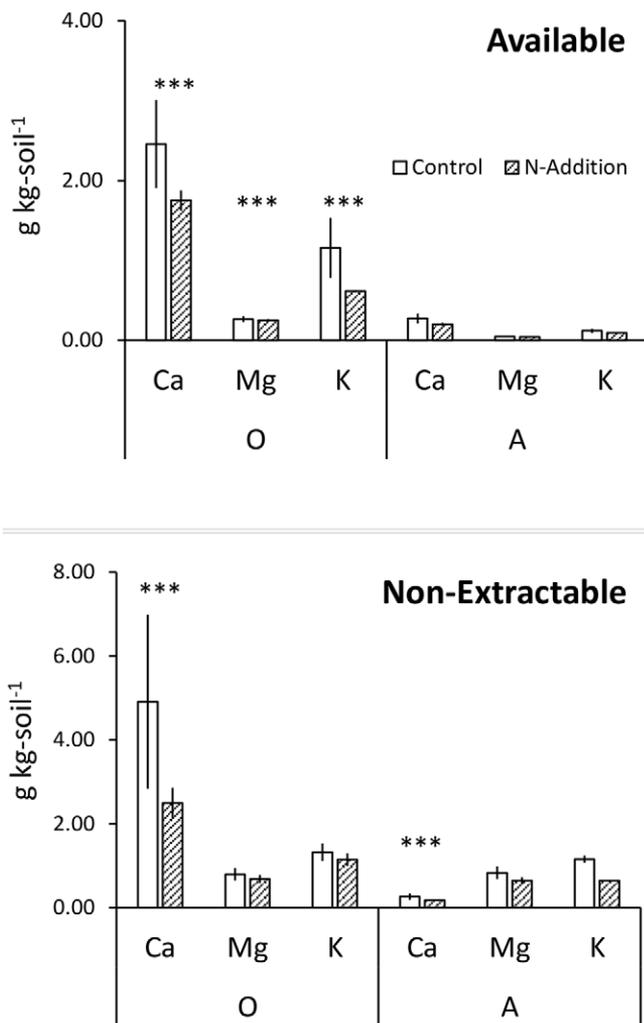


Fig. 6. Available and non-extractable cation concentrations (mean \pm SE) in control and N-addition plots during autumn, 2016 (Year 23), at the Bousson Experimental Forest. * Significant difference between control and N-addition plots ($p < 0.001$). Available Ca differed by horizon ($p < 0.05$).**

any difference in leaf chemistry at the onset of the year-long decomposition period is unlikely to be the major determinant of reduced decomposition. Rather, the decreased litter decomposition that we observed is likely due to changes in organic matter processes in soil (Wang et al., 2019), particularly reductions in microbial biomass and enzyme activity. Our evidence of reduced soil microbial biomass comes from the strong decline in soil PLFA concentrations and the accumulation of soil C. PLFAs are excellent indicators of living and active microbes because after cell death, PLFAs degrade in soil within days (Kindler et al., 2009; Dippold and Kuzyakov, 2016). With long-term N-enrichment, we observed a 50% decline in soil microbial biomass in both the O and A horizons. Our companion study (Wang et al., 2019) and other studies have shown that N-enrichment reduces soil microbial biomass and the processing of soil C inputs (Frey et al., 2004, 2014; Feng et al., 2010; Ramirez et al., 2012; Pisani et al., 2015; Morrison et al., 2016). Reduced soil pH may also reduce soil microbial biomass (Treseder, 2008; Boot et al., 2016); we did observe reductions in soil pH, likely due to deprotonation of

added ammonium and possibly to increased nitrification, which we did not measure, but which likely increased as a consequence of N additions (Nave et al., 2009).

The marked decline in soil microbial biomass is also supported by our measurement of suppressed soil respiration with N addition, a response observed in other N-addition studies (Bowden et al., 2004; Frey et al., 2014). Our finding of reduced soil respiration, occurring even though root mass increased by 37%, indicates that microbial processing of existing SOC is strongly depressed. Root respiration at our site contributes approximately 15% of total soil respiration (Lajtha et al., 2018), and an increase in roots would lead to greater root respiration. However, because total soil respiration decreased, there must have been a very large reduction in the heterotrophic component of respiration that offset any increases in autotrophic respiration. This result is in agreement with work at the Harvard Forest, where a laboratory analysis of soils from which roots had been removed showed reductions in respiration comparable to reductions in field-based rates at N-amended plots, thus demonstrating that the reduced respiration was driven primarily by reduced heterotrophic respiration (Bowden et al., 2004).

We also note, as others have found, evidence of a shift in the microbial community. Based on our PLFA data, gram-negative to gram-positive ratios were reduced in the N-addition plots by 25, 59, and 12% in the control plot O, A, and B horizons, respectively, and the fungal/bacterial ratio increased 75% in the A horizon (Wang et al., 2019). Our microbial results are consistent with efforts that show that microbial groups do not respond uniformly to N additions (Allison et al., 2008; Bhatnagar et al., 2018), and that carbon use efficiency (CUE) varies among microbial species and organic matter substrates (Frey et al., 2004; Billings and Ziegler, 2008; Allison et al., 2009; Liu et al., 2018).

Enzyme activity is a major determinant of microbial processing of organic matter, and can be influenced strongly by soil N. The increase in β -glucosidase activity we observed in the fertilized soils has been observed in other N addition studies (Carreiro et al., 2000; Saiya-Cork et al., 2002; Frey et al., 2004; Gallo et al., 2005; Zeglin et al., 2007). A forest in northern Michigan, for example, showed that β -glucosidase activity was 56% greater in N-addition soils than in control soils (Saiya-Cork et al., 2002); our soils showed a 114% increase in response to N additions. Changes in β -glucosidase activity in response to N addition are not unidirectional, however, with other studies showing no response or even reduced activity following N-deposition (DeForest et al., 2004; Waldrop et al., 2004a; Knorr et al., 2005; Wang et al., 2015; Boot et al., 2016). Comparable to our results, an N addition study in a boreal forest (Allison et al., 2008) found increased β -glucosidase activity, but also as we found, phenol oxidase activity remained the same or declined. Bhatnagar et al. (2018) suggest that mineral N (NH_4 , NO_3) enhances early stages of decomposition by favoring microbes that break down cellulose, which is consistent with our finding of increased β -glucosidase activity, but that lignin decay is suppressed later in the decomposition process, consistent with our finding of reduced peroxidase activity. Typically, phenol

oxidase and peroxidase are produced under N-limiting conditions (Waldrop et al., 2004b), and N additions decrease carbon enzyme activity within soils (Saiya-Cork et al., 2002; DeForest et al., 2004; Pregitzer et al., 2008; Zak et al., 2008; Hobbie et al., 2012; Frey et al., 2014; Weedon et al., 2014; Sun et al., 2016). When peroxidase is suppressed due to N fertilization, structurally complex SOC degradation will be reduced; the lack of a response in phenol oxidase activity to N fertilization that we observed may be attributed to a decrease of polysaccharides and phenolics produced during peroxidase-driven SOC decomposition (Knorr et al., 2005; Sinsabaugh, 2010). Recent work at the Harvard Forest Long-Term Ecological Research site reveals an important link between cation loss and microbial activity. Long-term N additions reduced levels of litter and soil Mn, thus increasing the relative abundance of weak fungal decomposers, resulting in reduced activity of lignin-decay enzyme activities (Whelan et al., 2018). This finding is consistent with results from our site, where we found that N additions altered the microbial community, decreased peroxidase activity, and increased lignin-derived compounds (Wang et al. (2019).)

At our site, evidence of reduced decomposition can be seen in the chemical signature of SOC. For example, the large N-driven increase in free light fraction organic matter in the A horizon indicates strongly that microbial processing is decreased not only in surface litter, as we have shown, but also in mineral SOC. Furthermore, N additions at our site resulted in an increase of plant-derived soil carbon compounds including steroids, lignin-derived, cutin-derived, and suberin-derived compounds that have anti-microbial properties or are less preferred microbial substrates (Wang et al., 2019). This overall shift in SOC composition is likely driven by reduced microbial biomass, lower enzyme activity, and by the shift in microbial community composition.

Long-term N additions may reduce cation availability due to decreased decomposition of fresh litter and stored SOM and to leaching (Mitchell and Smethurst, 2004; Lucas et al., 2011); long-term N addition studies have documented reductions in exchangeable soil cations in some locations (Hamburg et al., 2003; Bailey et al., 2005; Huntington, 2005; Hogberg et al., 2006; Vadeboncoeur, 2010). The relative impact of reduced cations on forest productivity will depend on total cation stocks and the rate at which cations are released via organic matter decomposition and weathering of soil minerals. We do not have estimates of relative cation abundance at our site, however, so we cannot assess long-term cation deficiencies and consequences to forest productivity.

As a result of long-term N additions at this site, we conclude that changes in soil microbial processes have fundamentally altered biogeochemical cycling and storage of SOC. Chronic N addition suppressed microbial biomass (Fig. 7) and altered the microbial community structure, thus reducing enzyme activity that slowed decomposition of both aboveground litter and soil organic matter. Reduced microbial activity altered soil carbon degradation rates and patterns, and increased soil C storage. Although N additions ultimately enhance C sequestration, the long-term stability of this increased organic matter is unknown. Soil organic C is protected by numerous biotic and abiotic processes and interactions that

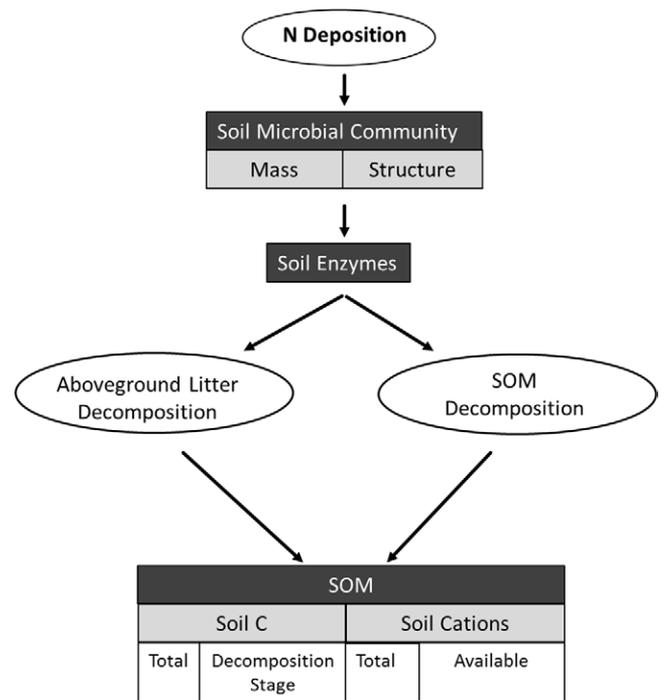


Fig. 7. Conceptual model of N addition effects on forest organic matter decomposition and soil C storage.

influence rates of stabilization (Rasse et al., 2005; Sollins et al., 2009; Poirier et al., 2018). Continued decreases in rates of atmospheric N deposition or incorporation of N deposition into unavailable biotic or abiotic N pools may at some point reduce the influence of N deposition on microbial processing of SOC, but experimental studies examining forest soil microbial and C dynamics in response to reduced N deposition are essentially nonexistent (Gilliam et al., 2019). If recently stored C does not become protected from microbial action, then recovery of the microbial pool could mineralize stored OM, releasing this stored C back to the atmosphere.

SUPPLEMENTAL MATERIAL

Supplemental material is available with the online version of this article. The supplement contains Fig. S1, One-year litter decomposition of black cherry, sugar maple, and mixed litter leaves decomposing in control and N-addition plots at the Bousson Experimental Forest, Year 22; and Table S1, Root mass, C, and N by depth in control and N-addition plots at the Bousson Experimental Forest, Year 17.

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