

The path less taken: Long-term N additions slow leaf litter decomposition and favor the physical transfer pathway of soil organic matter formation

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ABSTRACT

Understanding soil organic matter (SOM) formation as a balance between soil microbial access to organic plant inputs and protection by chemical recalcitrance and mineral associations can greatly improve our projections of this important terrestrial carbon pool. However, gaps remain in our understanding of the processes controlling the formation and destabilization of SOM and how these processes are affected by persistent global changes, such as nitrogen (N) deposition. To assess how elevated N deposition influences decomposition dynamics and the fate of plant inputs in a temperate deciduous forest, we coupled a reciprocal transplant leaf litter decomposition study with an analysis of the distribution of SOM in mineral associated and particulate organic matter fractions at a long-term, whole-watershed, N fertilization experiment. Nearly 30 years of N additions slowed leaf litter decomposition rates by about 11% in the fertilized watershed, regardless of the watershed from which the initial litter was collected. An apparent consequence of the altered rates of decomposition was that the proportion of SOM in light particulate organic matter in soil from the fertilized watershed was about 40% greater than that of the reference watershed, and was positively correlated with the bulk soil carbon to nitrogen ratio. Collectively, our results suggest that N saturation in a temperate forest alters SOM formation by slowing decomposition and favoring the accumulation of particulate organic matter as opposed to microbially processed mineral associated organic matter.

1. Introduction

Forest soils represent one of the largest terrestrial pools of carbon (C) (Pan et al., 2011; Ciais et al., 2013), and one whose rates of formation and loss may be significantly altered by prolonged changes in the global environment (e.g., soil warming; Melillo et al., 2017; Nottingham et al., 2020; Ofiti et al., 2021). While many recent studies have focused on the processes of soil C formation, we still lack a robust understanding of how the complex and interacting mechanisms responsible for soil C stabilization and destabilization will impact overall soil C stocks under future environmental and land management conditions (Friedlingstein et al., 2014; Bradford et al., 2016; Griscom et al., 2017; Bailey et al., 2019). An important factor controlling soil organic matter (SOM) dynamics is the nitrogen (N) status of an ecosystem. Numerous N addition studies in forest ecosystems suggest that elevated N inputs can slow the decomposition of plant inputs (especially lignin), reduce rates of soil CO₂ efflux, and may allow the accumulation of soil C with potentially greater C:N ratios (Pregitzer et al., 2008; Nave et al., 2009; Janssens et al., 2010;

Frey et al., 2014). However, plant residue decomposition and SOM properties are typically studied separately, hindering our understanding of how reductions in decomposition with N additions may translate to changes in overall C stocks and shifts in the nature of SOM.

The effects of N additions on SOM formation can be expressed through their influence on both the quality of organic inputs and the composition and function of the soil microbial community. Plant materials with lower C:N ratios and less molecular complexity (less lignin) are decomposed by microbes more efficiently, promoting mineral-associated organic matter (MAOM) through the sorption of microbial necromass and byproducts to soil mineral surfaces (Melillo et al., 1989; Kölbl and Kögel-Knabner, 2004; Talbot et al., 2012; Bradford et al., 2016; Winsome et al., 2017; Córdova et al., 2018). In contrast, plant inputs with greater recalcitrance to decomposition may form particulate organic matter (POM) simply through a lower tendency of microbes to decompose these components and their physical transfer through the soil profile (Von Lützow et al., 2008; Cotrufo et al., 2013, 2015).

In general, the POM fractions are more plant-like in chemistry, more

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vulnerable to disturbance, and are thought to have a faster turnover time than MAOM (Gregorich et al., 2006). Also within this view of SOM formation/destabilization, microbial activity and physical access to SOM regulates persistence and/or vulnerability of SOM pools. Consistent with these ideas, results from N addition experiments have shown decreased oxidative enzyme activity, as well as reductions in the relative abundance of fungal decomposers in the soil, which slow the degradation of lignin-containing plant inputs and can shift the pathway of SOM formation to favor POM accumulation (Frey et al., 2014; Averill et al., 2018; Carrara et al., 2018; Zak et al., 2019). Because different SOM pools may form through different processes and have different sensitivities to environmental controls, it is important to study how they individually respond to environmental changes such as N additions (Lavallee et al., 2020).

Long-term N addition experiments to forest ecosystems provide a unique opportunity to assess the linkages between litter quality, soil microbial processes, and SOM formation. For example, a recent synthesis of a 30-year, whole-watershed, N-addition study in a temperate forest found reduced belowground C allocation by plants, an accumulation of surface mineral soil C, and an increase in the C:N of SOM of surface mineral soil (Eastman et al., 2021a). Collectively, the pattern of observed changes from this synthesis suggests that the shift in C allocation with N fertilization influenced the soil microbial community and activity in ways that allowed an accumulation of high C:N SOM. This interpretation is supported by previous studies at this site and elsewhere that found reduced leaf litter decomposition (Adams and Angradi, 1996; Pregitzer et al., 2008; Frey et al., 2014; Argiroff et al., 2019; Wang et al., 2019), and reduced mycorrhizal colonization and ligninolytic enzyme activity with experimental N additions (Treseder, 2004; Carrara et al., 2018). These responses can reduce mid-to late-stage decomposition rates and favor POM formation through the physical transfer and accumulation of plant litter inputs that bypass microbial decomposition. Past studies suggest N addition alter POM accumulation in temperate forests (Von Lützow et al., 2008) but long term studies on how chronic N additions influence litter decomposition and the distribution of soil organic matter are rare. This is particularly important because soil C stabilization responses to environmental change may take decades to be fully expressed.

To examine how shifts in leaf litter quality and soil microbial activity due to experimental N additions influences rates of leaf litter decomposition and the distribution of SOM among distinct fractions, we paired a leaf litter decomposition study with a soil density fractionation analysis of the SOM at the Fernow Experimental Forest long-term N fertilization experiment (West Virginia, USA). Considering existing evidence for both a shift in leaf litter quality (lower C:N ratio) and soil microbial biochemistry (lower mycorrhizal colonization rates and reduced ligninolytic enzyme activity) in response to chronic N additions (Carrara et al., 2018; Eastman et al., 2021a), this site serves as a model system for understanding SOM formation and destabilization under conditions of elevated N inputs. We focused on testing three specific hypotheses: 1) Decomposition will be slower for leaf litter transplanted into N amended soil, especially for litter with high lignin and/or low N content; 2) There will be a greater proportion of POM in the surface mineral soils of the N addition watershed due to greater plant particulate litter that bypasses microbial decomposition; and 3) There will be a greater proportion of MAOM in the surface mineral soils of the N addition watershed due to greater microbial CUE with N amendments.

2. Material and methods

2.1. Site description

Both the litter decomposition and soil density fractionation studies were conducted at the Fernow Experimental Forest, WV, USA (39°1'48"N, 79°40'12"W), in two temperate deciduous forested watersheds that compose a long-term, whole-watershed fertilization

experiment (Adams et al., 2012). One watershed, +N WS3 (34 ha), received 35 kg N ha⁻¹ yr⁻¹ from 1989 to 2019 in the form of ammonium sulfate ((NH₄)₂SO₄). These N additions were about double the rate of ambient N in throughfall at the start of the experiment in 1989 (Helvey and Kunkle, 1986) and about quadruple the ambient rate towards the end of the experiment in 2019 (NADP <https://nadp.slh.wisc.edu/>; CASTNET <https://www.epa.gov/castnet>). An adjacent, similarly aged watershed, Ref WS7 (24 ha), serves as a reference to +N WS3. Land-use history for these watersheds has been previously described (see Kochenderfer and Wendel, 1983; Kochenderfer, 2006). A major difference between these watersheds was that Ref WS7 was cut in two phases and subsequently treated with herbicide for 3 or 6 years before recovery began in 1969, whereas +N WS3 was clear cut without herbicide treatment in 1970.

The study site is located in the Allegheny Mountain region of the Central Appalachian Mountains, with elevations ranging from 530 to 1115 m, and slopes from 20 to 50% (Adams et al., 2012). Mean annual precipitation is relatively evenly distributed throughout the year, averaging 146 cm annually, and mean annual temperature is 9.3 °C with the growing season lasting from May through October (Adams et al., 2012; Young et al., 2019). Soils are a shallow (typically <1 m), well-drained, silty loam, Typic Dystricrepts, derived from sandstone and shale parent material (Adams et al., 2012).

We conducted both studies at 10 circular, 0.04-ha plots per watershed, which were previously established to encompass the full range of elevation and slope aspect (Gilliam et al., 1994). Dominant tree species were similar in the selected plots in both watersheds and included sugar maple (*Acer saccharum* L.), tulip poplar (*Liriodendron tulipifera* L.), black cherry (*Prunus serotina* Ehrh.), and sweet birch (*Betula lenta* L.). However, the relative abundance of these dominant species differed between watersheds, as the +N WS3 had a greater abundance of black cherry and less tulip poplar by basal area than the Ref WS7 (see Eastman et al., 2021a).

2.2. Reciprocal litter decomposition experiment

We collected freshly fallen leaf litter of the four dominant species in October of 2017 from a single site in each watershed prior to any rain event. Leaf litter from each watershed was thoroughly mixed, sorted by species, then dried at 65 °C for >48 h. For two species (black cherry and sweet birch) sourced from Ref WS7, insufficient litter mass was collected, so we used dried and archived leaf litter collected in 2015 (<8% of total leaf litter used in this study) to supplement the 2017 freshly fallen leaf litter.

We measured rates of leaf litter decomposition using 1-mm mesh fiberglass litterbags (~20 cm × 10 cm) filled with 2 g (±0.25 g) of dried leaf litter of a single species and from a single source watershed. In March 2018, five replicate litterbags for each combination of tree species and source watershed were randomly assigned to each plot and placed flat on the surface of the mineral soil horizon after removing the litter layer. All litterbags in a plot were arranged in a 1-m x 1-m square, covered with coarse plastic mesh to prevent disturbance, and the litter layer was replaced. Litterbags were collected four times between deployment (March 2018) and the end of the study (March 2020). One litterbag of each species and watershed of origin was collected from each plot after 3, 6, and 12 months (10 replicates). After 24 months, two litterbags of each species and watershed of origin were collected from each plot for the final collection (20 replicates). Following collection, litter in each bag was gently brushed to remove soil, and roots and invertebrates were removed as best as possible without losing leaf litter material. Litter was then dried at 65 °C for >48 h and weighed.

Overall, the experimental design consisted of 2 watersheds of origin x 2 watersheds of transplant x 10 plots per watershed x 4 species x 5 time points for a total of 40 litterbags per plot and 800 litterbags in total. The reciprocal design of this experiment allowed us to assess whether any detectable differences in decomposition rates between the watersheds

were due to differences in litter chemistry between source watersheds or differences in the soil environment into which litter bags were transplanted.

2.2.1. Litter quality

To determine initial litter quality, three subsamples of freshly fallen litter collected for each species and watershed of origin were dried, ground, and analyzed for C and N content using Dumas combustion in an elemental analyzer (NA 1500 Series 2, Carlo Erba Instruments, Milan, Italy). Dried, partially decomposed leaf litter from all 800 litterbags collected during the two-year experiment were similarly ground and analyzed for C and N content.

Initial lignin and cellulose content of leaf litter from each species and watershed of origin were determined using an acid detergent digest method (Van Soest, 1963; as described by Holtzappple, 2003). In summary, 4–5 subsamples of dried, ground leaf litter were digested in an acid-detergent fiber digest solution to isolate cellulose, lignin, and ash. This residue was dried at 65 °C for >48 h and weighed. To remove and estimate cellulose in the residue, the samples were then soaked in 75% sulfuric acid, rinsed with deionized (DI) water, dried at 65 °C for >48 h, then weighed. Final residue was then heated in a muffle furnace at 525 °C for 2 h to determine ash-free dry weight. For the purposes of this study, we consider the ash-free mass remaining after the acid detergent and strong acid digests to be “lignin.” We similarly assessed lignin and cellulose content of final decomposed litter (after 24 months) by randomly selecting a subset of three litter samples for each species, source watershed, and watershed of transplant category (48 total).

We estimated the lignocellulose index (LCI) as lignin content/(lignin content + cellulose content) (Melillo et al., 1989). We also calculated the lignin:N ratio of initial and final leaf litter. Final leaf litter lignin:N was calculated for the subset of samples that were analyzed for lignin and % N (48 samples total). Because our initial litter chemistry analysis used a different number of subsample replicates for determination of % N ($n = 3$) and % lignin ($n = 4$ or 5), we paired every % N measurement with every % lignin measurement for a given species and source watershed category to determine the range and statistics of initial leaf litter lignin:N values.

2.2.2. Soil chemistry

The total C and N content of the 0–5 cm of mineral soil in each of the litter decomposition plots were measured on three 2.5-cm diameter soil cores collected in October 2018. The three soil cores per plot were combined, sieved (to pass a 2-mm mesh), dried at 65 °C for >48 h, and ground prior to analysis of C and N content by Dumas combustion.

2.2.3. Calculations

Percent mass remaining was calculated for each litterbag. Despite our efforts to clean the decomposed litter of soil, some soil could not be removed without potentially losing leaf tissue. To correct for soil contamination, we assumed that the C concentration of the leaf litter remains constant over decomposition. Thus, any decomposed leaf litter with a C concentration lower than the initial value was considered to be contaminated with mineral soil (Blair and Crossley, 1988; Janzen et al., 2002; Midgley et al., 2015). The following mixing model was used to determine the fraction of final mass that was litter:

$$f_{\text{Litter}} = (C_d - C_s) / (C_i - C_s)$$

where f_{Litter} = the fraction of the total litterbag sample mass that is actually litter; C_d = the decomposed litter C concentration; C_s = the mineral soil (0–5 cm) C concentration, previously obtained (see 2.2.2); C_i = the initial leaf litter C concentration. The mass of the decomposed litter sample was then multiplied by f_{Litter} to correct for soil contamination.

We calculated the decomposition rates of leaf litter using a single-pool negative exponential model,

$$M_t = \exp^{-kt}$$

where M_t is the proportion of initial mass remaining at a given time-point, k is the decomposition rate (year^{-1}) and t is the decomposition time (years) (Jenny et al., 1949; Olsen, 1963). To estimate the decomposition rate (k), an exponential model was fit to the proportion of mass remaining over time (years) for each combination of species, source watershed, and watershed of transplant. In this analysis, plots were the replicates ($n = 10$), and 160 models were fit to 160 sets of litterbags (4 species $\times$ 2 watersheds of origin $\times$ 2 watersheds of transplant $\times$ 10 plots). We also used a model structure with the intercept set to zero to avoid bias in single-pool decomposition models (Adair et al., 2010). R^2 values were >0.80 for >80% of model fits, and given the relatively short duration of this study, the single-pool exponential model is thought to best capture early-stage decomposition dynamics (Harmon et al., 2009).

2.3. Soil density fractionation study

2.3.1. Soil sampling

To assess how elevated N inputs may influence the fate of plant inputs, we separated SOM into three fractions: light POM, heavy POM, and MAOM. Soil was collected from four 5-cm diameter soil cores of the 0–15 cm of mineral soil in each plot in October 2018, for a total of 80 soil samples (2 watersheds $\times$ 10 plots $\times$ 4 soil cores). Soils were stored less than 6 weeks at 4 °C before being sieved (2 mm) to remove plant and rock material, homogenized, and dried at 65 °C for >48 h.

2.3.2. Fractionation procedure

We evaluated the nature of organic matter in the mineral soil in each plot using a three-pool soil density fractionation framework described by Lavalley et al. (2020). Briefly, SOM was separated into three pools based on their densities and sizes, which is thought to represent the degree of organic matter stabilization (Gregorich et al., 2006). Two steps were used to isolate the light POM fraction, which we define as plant-like residue with a density $<1.85 \text{ g cm}^{-3}$ because it is minimally bound to soil minerals. First, 5.5–6.0 g of dry soil subsamples were shaken for 15 min in DI water at ~ 100 oscillations per minute, centrifuged at 1874 g for 15 min, and then the supernatant was filtered through a 20 μm nylon filter to catch the light POM. Second, we isolated the rest of the light POM by shaking soils in a liquid of density 1.85 g cm^{-3} (sodium polytungstate, SPT) for 18 h to disperse soil macroaggregates. Samples were centrifuged at 1874 g for 30 min, the light POM that floated out of the dense liquid was aspirated onto a 20 μm nylon filter and rinsed thoroughly.

The heavy POM is defined as plant-like, chemically, but has some mineral association or microbial byproducts that increases its density and may protect the SOM in soil aggregates. Thus, the centrifuged soil pellets containing the heavy fractions ($>1.85 \text{ g cm}^{-3}$ density) were thoroughly rinsed and centrifuged with DI water at least three times to remove excess SPT. The heavy POM and MAOM that remained in the soil pellet were separated by size, suspending the pellet in DI water and sieving through a 53 μm sieve. The material remaining on the sieve was considered the heavy POM and sand ($>1.85 \text{ g cm}^{-3}$ density and $>53 \mu\text{m}$ in size), while the matter that passed through the sieve was considered the MAOM, silt and clay fraction ($<53 \mu\text{m}$).

All soil fractions were dried at 65 °C, and ground for C and N analysis. If 100% ($\pm 5\%$) of initial soil sample mass was not recovered in all fractions, then the fractionation procedure was repeated for that sample; this occurred for 7 of the 80 samples that were fractionated. Additionally, subsamples of the dried soil prior to fractionation, hereafter referred to as bulk soil, was analyzed for C and N.

2.4. Statistical analysis

Raw data and metadata associated with this study are publicly

available through Environmental Data Initiative (Eastman et al., 2021b). For the leaf litter decomposition study, we tested for differences in initial litter chemistry with a two-way ANOVA with species and source watershed as fixed effects and litter chemical properties as dependent variables (%C, %N, C:N ratio, %cellulose, %lignin, LCI, lignin:N ratio). To test for differences in final litter chemistry and decomposition rate, we conducted a 3-way ANOVA with litter species, source watershed, and watershed of transplant as fixed effects; and final litter chemical properties and decomposition rate as dependent variables (%N, C:N ratio, %cellulose, %lignin, LCI, lignin:N ratio, k). Robust two- and three-way ANOVAs (using the R package “rfit”; Hocking, 1985; as described in Kloeke and McKean, 2014) were performed to compare initial % lignin, lignin:N ratio and LCI, and final % N and C:N ratio, respectively, as the assumption of a normal distribution of residuals were not met by these dependent variables.

For the soil density fractionation study, we tested for differences in the chemistry of bulk soil between the watersheds with a one-way, nested ANOVA with watershed as a fixed effect, plot as a random nested effect (within WS), and bulk soil chemical properties as dependent variables (%C, %N, and C:N ratio). To test for differences in fraction of bulk soil in each density fraction and chemistry of individual fractions between watersheds, we conducted a one-way, nested ANOVA with watershed as the fixed effect, plot as the random nested effect (within WS), and fraction of total mass, total C, and total N, and chemical properties (%C, %N, C:N ratio) of each fraction as dependent variables. To test our hypothesis that a greater proportion of light POM may contribute to a greater C:N ratio in the bulk soil, we regressed the bulk soil C:N ratio against the fraction of total mass in the light POM.

For all parametric ANOVAs, comparisons among means were analyzed with Tukey-Kramer HSD *post hoc* tests, the normal distribution of residuals was tested using the Shapiro-Wilks test, and homogeneity of variance was tested using Levene's test. Variables that did not meet these assumptions were transformed using the natural logarithm prior to statistical analysis.

Replication of whole-watershed experiments is often logistically and financially challenging or impossible, and experimental treatments are commonly pseudoreplicated, as they are in this study (Hurlbert, 1984). Results should be interpreted with this in mind, but—given the duration and extent of the fertilization treatment—we consider the differences observed in leaf litter decomposition and soil density fractionation results to be primarily the result of the fertilization treatment. Furthermore, extensive differences have been previously observed between biogeochemical processes in these watershed, many of which were also observed in a nearby, fully-replicated field experiment (Adams et al., 2004; Fowler et al., 2015; Burnham et al., 2017; Carrara et al., 2018; Eastman et al., 2021a).

3. Results

3.1. Reciprocal litter decomposition experiment

3.1.1. Initial litter chemistry

The four species and two source watersheds of litter used in this experiment provided a sufficiently diverse array of tissue characteristics to examine the potential interaction between N additions and litter chemistry on decomposition rates. Initial litter chemistry varied among species for all chemical properties, and differences between source watersheds typically depended on the species (Table S1). Most notably, the % N of the initial litter ranged from 0.69% to 1.29%, the C:N ratio ranged from 37.7 to 70.9, and the lignin:N ratio ranged from 13.6 to 26.6 (Table 1). Specifically, red maple and tulip poplar leaf litter sourced from +N WS3 had greater % N and a lower C:N ratio, whereas black cherry litter from +N WS3 had lower % N and greater C:N than litter sourced from Ref WS7 (Table 1). All leaf litter sourced from +N WS3 had a lower lignin:N ratio than litter sourced from Ref WS7 (Table 1). In general, red maple and sweet birch had lower quality litter, as red maple

Table 1
Chemical composition of original freshly fallen leaf litter from four dominant tree species and two watersheds of origin. Mean (SE) values are reported; bold values indicate difference between watershed means; values with the same lowercase letters (a-d) are not different according to Tukey HSD test on WS * Species interaction; values with the same uppercase letters (A-D) are not different according to Tukey HSD test on Species differences.

Species	Watershed of origin	% N	% C	C:N	n	% cellulose	% lignin	LCI	lignin:N ^a
Red Maple	Ref WS7	0.69	48.6	70.9	5	19.9	18.3	0.47	26.6
	+N WS3	0.85	47.5	56.3	5	18.3	19.8	0.52	23.4
Sweet Birch	Ref WS7	1.30	49.1	37.7	4	19.3	30.4	0.61	23.3
	+N WS3	1.29	48.4	37.8	5	20.2	25.3	0.55	19.8
Tulip Poplar	Ref WS7	0.92	46.6	51.0	5	25.8	18.8	0.42	20.6
	+N WS3	1.14	48.2	42.6	4	24.4	16.8	0.40	14.9
Black Cherry	Ref WS7	1.26	49.0	38.8	5	18.6	19.8	0.51	15.7
	+N WS3	1.10	48.9	44.5	5	18.7	14.9	0.44	13.6

^a Lignin:N ratio calculated by combining all possible %lignin values with all possible %N values for each given species x watershed category.

litter had the lowest % N and greatest C:N and lignin:N ratios, while sweet birch litter had the greatest % lignin (Table 1; Tukey-Kramer HSD). In contrast, black cherry and tulip poplar had relatively high-quality litter, both with greater % N and lower % lignin than the other two species (Table 1; Tukey-Kramer HSD). The LCI only differed between tulip poplar and sweet birch litter, as tulip poplar had the lowest, sweet birch had the greatest, and red maple and black cherry had intermediate LCI (Table 1).

3.1.2. Final litter chemistry

After two years of decomposition in the field, we observed differences in final litter chemistry between watersheds of transplant (Table S2). Specifically, final leaf litter transplanted into +N WS3 had greater % N, % cellulose, and % lignin, and a lower C:N ratio than leaves transplanted into Ref WS7 (Table 2). These patterns were consistent for all species regardless of the watershed from which they originated (Table S2).

Additionally, we observed some differences in final litter % N, C:N ratios, and LCI between litter sourced from +N WS3 and Ref WS7, regardless of the watershed into which they were transplanted (Table S2). Specifically, final litter material that was sourced from +N WS3 had greater % N and a lower C:N ratio for all species (Table S3). Also, final litter material sourced from +N WS3 had a greater LCI after decomposition, meaning more lignin relative to cellulose remained at the end of the decomposition experiment for these litter bags (Table S3). The initial difference in the lignin:N ratio between source watersheds did not persist in the decomposed leaves, despite the greater final % N of litter sourced from +N WS3.

Comparing final litter chemistry among species, all litter chemical properties differed among species regardless of source watershed or watershed of transplant (Table S2). Similar to initial chemistry, final red maple litter had the lowest % N and LCI and the greatest C:N ratio of all species (Table 3). Interestingly, final black cherry litter had the highest % N yet also the greatest % lignin, lignin:N ratio, and LCI (Table 3). Final sweet birch litter also had high % N and LCI, but a low C:N ratio (Table 3). Final tulip poplar litter generally had an intermediate chemical composition in comparison with the other species (Table 3). Relative to the initial litter chemistry, final litter chemistry of all species had much greater % N (more than 2x), a much lower C:N ratio (about half) that was less variable among species, and generally greater % lignin and LCI (Table 3). The % lignin and LCI for sweet birch litter—the species with the greatest initial % lignin and LCI—did not change as much between initial and final litter chemistry compared to the other three species (Table 1; Table 3).

3.1.3. Soil chemistry

Mineral soil that was sampled at each litter decomposition plot ($n = 10$) had similar % C in both watersheds (~7%), while the % N was greater in the Ref WS7 (Table 4). The C:N ratio of the top 5 cm of mineral soil was significantly greater in +N WS3, 18.8, compared to a C:N ratio of 14.9 in Ref WS7 (Table 4). Similar results were found for the 0–15 cm soil sampled in the soil density fractionations sampling (Table 4). Likely due to the difference in soil sampling depth between the soil density fractionation samples and the litter decomposition soil samples (0–15 cm and 0–5 cm, respectively), we detected greater % C and % N in the litter decomposition soil samples, but similar C:N ratios from both samplings (Table 4; Fig. S1).

Table 2

Chemical composition and decay rates (k) of final leaf litter (decomposed for two years in field) summarized by watershed into which leaves were transplanted. Mean (SE) values are reported and bold values indicate difference between watershed of transplant means ($P < 0.05$).

Watershed of transplant	% N		C:N ratio		% cellulose		% lignin		lignocellulose index		lignin:N ratio		k (year ⁻¹)	
	$n = 393\text{--}399$		$n = 393\text{--}399$		$n = 24$		$n = 24$		$n = 24$		$n = 24$		$n_{\text{WS7}} = 73$	$n_{\text{WS3}} = 80$
Ref WS7	2.19	(0.1)	23.8	(0.02)	15.5	(0.13)	33.0	(0.27)	68.0	(0.002)	13.4	(0.13)	0.67	(0.02)
+N WS3	2.32	(0.1)	21.7	(0.01)	16.9	(0.12)	36.4	(0.23)	68.2	(0.002)	13.7	(0.08)	0.53	(0.02)

3.1.4. Decomposition rates

Decomposition rates did not differ between source watershed despite differences in initial litter chemistry between the source watersheds (Fig. S2, Table 1, S4). However, differences in decomposition rates were detected between watersheds of transplant and among species (Fig. 1; Tables 2,3; Table S4). As we expected, the annual rate of decomposition was ~20% lower for leaf litter transplanted to +N WS3 (Table 2). Decomposition rates also varied among species regardless of the watershed into which they were transplanted, and were faster for higher quality litters (black cherry and tulip poplar) and slower for lower quality litters (red maple and sweet birch; Fig. 1, Table 3). The greatest difference in average decomposition rates was between black cherry and sweet birch, with black cherry mass loss per year about twice that of sweet birch litter (Table 3).

3.2. Soil density fractionation

When comparing across soil density fractions, the light fractions in both watersheds had similar % C, % N, and C:N ratios (Fig. 2A-C). The % C and % N were lower in heavy POM from +N WS3, while the C:N ratio was greater compared to Ref WS7 (Fig. 2A-C). MAOM from +N WS3 also had a greater C:N ratio than MAOM from Ref WS7 (Fig. 2A-C).

We did not detect any watershed differences in the fraction of total mass attributed to the three soil fractions (Fig. 2D), but we did detect watershed differences in the fraction of total soil C and N in the light and heavy POM fractions. Specifically, the light fraction contributed a greater fraction of the total soil C and N in the +N WS3 compared to Ref WS7, consistent with our Hypothesis 2 (Fig. 2E,F). Heavy POM contributed less to the total soil C and N stocks in +N WS3 (Fig. 2E,F). In contrast to POM pools, there was no detectable difference in the contribution of MAOM to total soil C and N stocks between watersheds (Fig. 2E,F).

When considered the role of SOM distribution among fractions in the total bulk soil chemistry. We found a strong positive relationship between the C:N ratio of bulk soil and the proportion of total soil C in the light POM fraction for both watersheds (Fig. 3; $P < 0.001$, $R^2 = 0.55$), but no relationship for heavy POM nor MAOM fractions. We also found a weak positive relationship between the % N of bulk soil and the proportion of total soil C in the heavy POM fraction, but it explained little variance in bulk soil % N ($P < 0.001$, $R^2 = 0.38$).

4. Discussion

We paired a two-year leaf litter decomposition study with a density fractionation of the SOM in the top 15 cm of mineral soil to evaluate how long-term N additions impact pathways of SOM formation. From these studies, we found support for two of our three hypotheses, as chronic N additions led to reduced leaf litter decomposition rates (~11%) over a two-year period (Fig. 1) and a greater contribution of light POM to total SOM (~40%; Fig. 2). Together, these results suggest that the commonly observed reduction in decomposition with N fertilization can lead to differences in the composition and distribution of SOM fractions. Specifically, the physical transfer pathway of SOM formation (undecomposed plant inputs remaining in the soil) was favored over the microbial decomposition pathway with subsequent stabilization of microbial byproducts (Cotrufo et al., 2019). Additionally, the greater proportion of lignin remaining in the litter bags transplanted to +N WS3 (Table 2) was

Table 3

Chemical composition and decomposition rates (k) of final leaf litter (decomposed for two years in field) from four dominant tree species. Mean (SE) are reported and values with the same letter are not different among species (Tukey-Kramer HSD, $P < 0.05$).

Species	% N		C:N ratio		% cellulose		% lignin		lignocellulose index		lignin:N ratio		k (year ⁻¹)	
	$n = 197-200$		$n = 197-200$		$n = 12$		$n = 12$		$n = 12$		$n = 12$		$n = 40$	
Red Maple	2.05	(0.24)a	25.1	(0.04)c	18.4	(0.2)b	34.5	(0.3)a	0.65	(0.003)a	14.6	(0.2)bc	0.53	(0.03)b
Sweet Birch	2.41	(0.20)b	20.9	(0.02)a	14.1	(0.2)a	31.2	(0.4)a	0.69	(0.002)bc	11.4	(0.2)a	0.39	(0.02)a
Tulip Poplar	2.12	(0.21)a	23.3	(0.03)b	15.9	(0.3)ab	31.2	(0.4)a	0.66	(0.004)ab	12.8	(0.2)ab	0.67	(0.02)c
Black Cherry	2.44	(0.18)b	20.5	(0.02)a	16.4	(0.1)a	41.9	(0.3)b	0.71	(0.019)c	15.5	(0.1)c	0.81	(0.03)d

Table 4

Soil chemistry of litter decomposition plot samples and bulk soil density fractionation samples by watershed. Mean (SE) are reported and bold values indicate significant differences between watersheds.

Watershed	Soil sample depth (cm)	n	%C	%N	C:N
<i>Litter decomposition plot soil samples</i>					
Ref WS7	0–5	10	7.07 (0.5)	0.484	14.9
				(0.04)	(0.6)
+N WS3	0–5	10	6.82 (0.6)	0.363	18.8
				(0.03)	(0.8)
<i>Soil density fractionation bulk soil</i>					
Ref WS7	0–15	40	4.47	0.312	15.3
			(0.04)	(0.003)	(0.1)
+N WS3	0–15	40	4.00	0.224	17.9
			(0.03)	(0.001)	(0.1)

consistent with previous findings at this site of reduced ligninolytic enzyme activity with N additions (Carrara et al., 2018), a direct effect of N additions often observed elsewhere (Berg, 1986; Carreiro et al., 2000; DeForest et al., 2004). With N additions, lignin from aboveground litter may be a primary source of POM via physical transfer through the soil profile. Indeed, a meta-analysis by Chen et al. (2018) found a negative correlation between lignin-modifying enzymes and both the soil C stocks and the proportion of organic matter in the POM fraction across 40 N

addition experiments.

4.1. Decomposition rates and leaf litter lignin accumulation

When N is scarce, microbes produce lignin degrading enzymes to access N-containing molecules shielded by lignin; however, under N additions microbial C limitation may occur and, thus, enzyme production and activity may shift to favor cellulose degradation (Hobbie et al., 2012). Alternatively, as lignin accumulates during mid-to late-stage decomposition, non-ligninolytic enzyme activity can also slow because of the higher activation energy associated with accessing compounds shielded by lignin (Talbot and Treseder, 2012; Tan et al., 2020). These soil biogeochemical responses to elevated N could help explain the reduced decomposition rates of leaf litter transplanted to +N WS3 (Fig. 1) and are consistent with the reduced rates of soil respiration observed at this site (Eastman et al., 2021a). This reasoning also follows soil biogeochemical theory that increased N availability enhances microbial biomass growth relative to substrate mineralization (Schimel and Weintraub, 2003).

Furthermore, the reduced decomposition rate in +N WS3 was observed despite similar soil temperatures, greater % soil moisture (Eastman et al., 2021a), and lower C:N ratios of two dominant litter types (Table 1). Our results (data not shown) provide little indication that surface soil properties (i.e., % C, % N, C:N) contribute to the

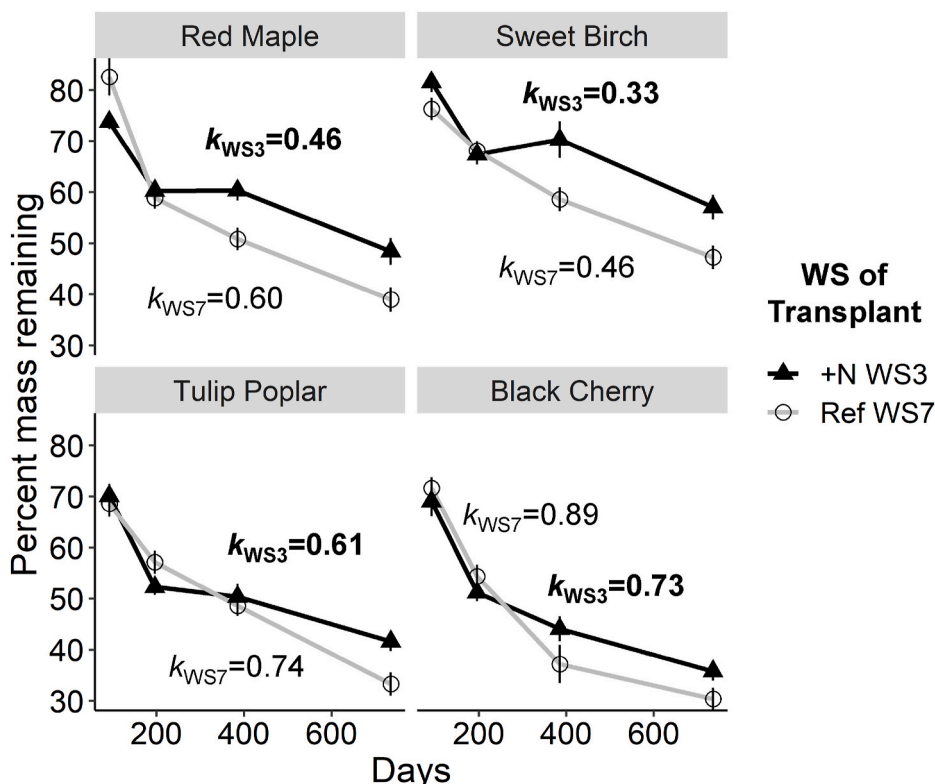


Fig. 1. Percent of initial leaf litter mass remaining over two-year litter decomposition in the field for four dominant tree species. Mean $\pm$ se of percent mass remaining for litter transplanted into the fertilized watershed (+N WS3; black triangles) and reference watershed (Ref WS7; open circles). Decomposition rates (k) displayed for each watershed and species combination. Decomposition rates (k) differed between watershed of transplant for all species, and there was no effect of watershed of litter origin on decomposition rates (but see Fig. S2).

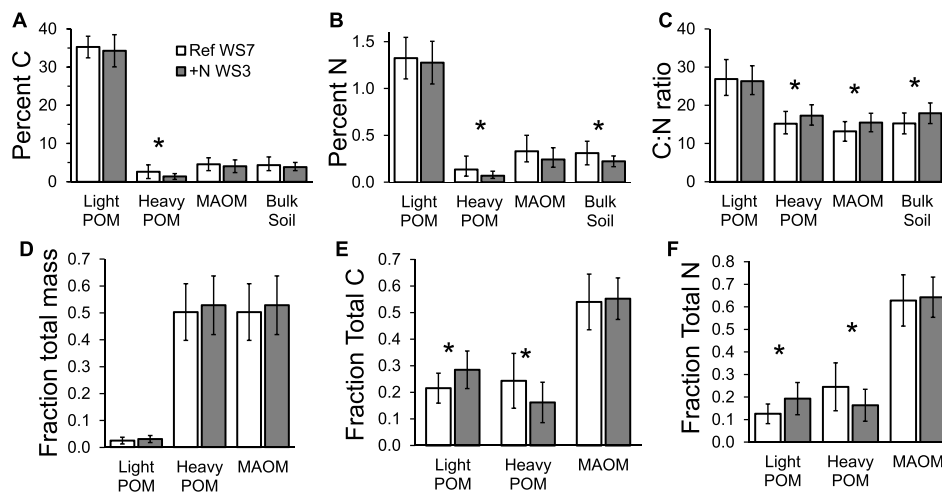


Fig. 2. Soil density fractionation results for the reference watershed (Ref WS7, white bars) and fertilized watershed (+N WS3, gray bars). Mean ($\pm$ se) of the percent C (A), percent N (B), and C:N ratio (C) of light particulate organic matter (POM), heavy POM, mineral-associated organic matter (MAOM) and bulk soil. The mean ($\pm$ se) fraction of total bulk soil mass (D), carbon (E), and nitrogen (F) for the three soil fractions. Asterisks denote significant difference between watersheds (ANOVA, $P < 0.05$).

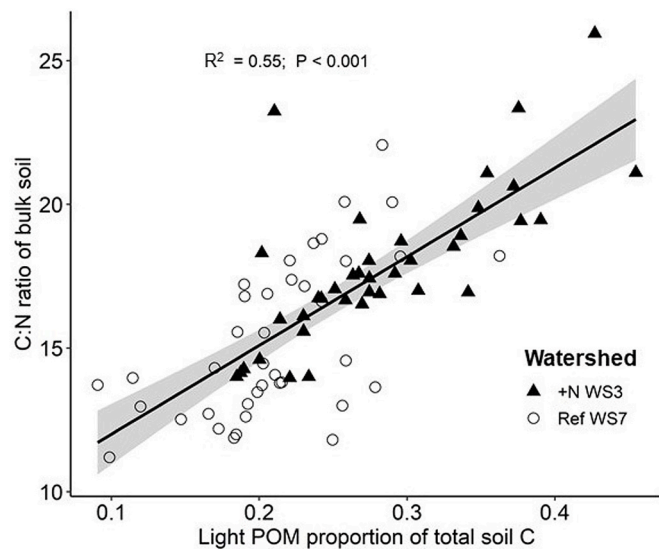


Fig. 3. Significant and positive relationship between the proportion of total soil C in the light particulate organic matter (POM) fraction and the C:N ratio of bulk soil from the reference watershed (Ref WS7, open circles) and fertilized watershed (+N WS3, black triangles). Black line represents the linear regression with standard error (gray shading).

variability in decomposition rates at our sites. This suggests that the microbial response to N additions, rather than the environment of the soils or the quality of litter, was responsible for the differences between watersheds. Indeed, N additions are known to alter the composition of soil microbial communities, decrease microbial biomass, increase the bacteria:fungi ratio, and reduce the abundance of soil microbes that typically degrade more chemically recalcitrant organic matter (DeForest et al., 2004; Ramirez et al., 2012; Moore et al., 2021). Apparently, any potential influence of litter quality differences between the watershed of origin on decomposition dynamics was overwhelmed by these shifts in soil microbial ecology. Although slight changes in litter quality with N fertilization did not affect decomposition rates (Fig. S2), the species of leaf litter had a strong influence on decomposition rates (range from k of 0.39–0.81). This highlights the importance of tree species composition—and any changes in species composition that may occur with chronic N additions—for decomposition dynamics, as opposed to the slight intraspecific changes in leaf litter chemistry (%N and C:N) that may result from N additions.

4.2. Fertilization effects on soil density fractions

Our observations of decreased decomposition rates of leaf litter in response to long-term N fertilization likely influenced the distribution of organic matter among surface soil density fractions. Specifically, N additions increased the light POM fraction, reduced the heavy POM fraction, and had little or no effect on the MAOM fraction. The greater fraction of organic matter in the light POM in the +N WS3 aligns with the greater C:N ratio of bulk soil in the +N WS3 (Figs. 2, 3). This greater C:N ratio persists despite higher N concentrations of some leaf litter (Table 1), greater inputs of inorganic N to the soil through experimental fertilization, and lower C:N ratio of final leaf litter in after two years of decomposition (Table 2). Eastman et al., 2021a proposed that reduced total belowground carbon flux by vegetation in the +N WS3 may have deprived mycorrhizae and soil microbes of labile carbon needed to decompose SOM and indirectly caused the observed increases in soil C stocks, increases in the C:N ratio of surface mineral soil, and reductions in soil CO₂ efflux. Similar patterns were also observed at other sites and at the global scale (Gill and Finzi, 2016; Phillips et al., 2012; Sulman et al., 2017).

The light POM in +N WS3 was likely protected from decomposition due to the direct effects of N additions on soil microbial communities and oxidative enzyme activity (Frey et al., 2014; Morrison et al., 2016; Carrara et al., 2018; Zak et al., 2019). However, as forests recover from chronic N deposition—and as demand for N increases with increasing atmospheric CO₂—nutrient acquisition strategies for plants may shift to promote the decomposition of the light POM fraction, possibly contributing to a loss in total soil C storage (Craine et al., 2018; Phillips et al., 2012; Terrer et al., 2017; Groffman et al., 2018). Thus, despite the current emphasis on more stable MAOM fractions as globally important C stocks, the sensitivity of light POM to environmental change can significantly impact the land-atmosphere exchange of C in the short term.

Less belowground C flux and, subsequently, lower mycorrhizal colonization rates in +N WS3 (Carrara et al., 2018; Eastman et al., 2021a) could also drive the 33% smaller proportion of SOM in the heavy POM fraction (Fig. 2). Root-derived and fungal byproducts can increase aggregation in soils (Six et al., 2004; Wilson et al., 2009), and thus the oft-observed reduction in fungal biomass and productivity under elevated N additions can cause less macro aggregation and greater heavy POM formation (Wallenstein et al., 2006; Morrison et al., 2016; Kemner et al., 2021). Thus, our results indicate a tradeoff may exist between heavy and light POM formation where more POM ends up in the light fraction relative to the heavy with N additions, which we observed as a negative correlation between heavy and light POM in this study ($r =$

–0.47; Fig. S3).

On the other hand, the MAOM and heavy POM fractions in +N WS3 also had greater C:N ratios than those from Ref WS7, likely contributing to the greater bulk soil C:N (Fig. 3). Though MAOM is often considered a relatively stable form of SOM derived from microbial byproducts (Cotrufo et al., 2013; Blankinship et al., 2018), recent studies suggest that MAOM may be equally or even more preferentially formed from plant-derived compounds that bypass microbial assimilation, especially in forest ecosystems (Mikutta et al., 2019; Angst et al., 2021). Alternatively, a greater C:N ratio of the MAOM and heavy POM fractions could indicate a shift in the microbial community (i.e., greater bacteria:fungi ratio; Fanin et al., 2013; Mooshammer et al., 2014; Midgley and Phillips, 2016) or a less active fungal community with a greater biomass C:N ratio biomass than their active counterparts (Camenzind et al., 2021). A closer look at the chemical composition of SOM (e.g., biomarkers) in each of the soil density fractions would help clarify the mechanisms and microbial controls of SOM stabilization in these watersheds (Angst et al., 2021).

An unexpected result from the soil density fractionation study was the similar proportion of MAOM to total SOM in both watersheds (Fig. 2), which contributed over 50% of C and over 60% of N in the top 15 cm of mineral soil from this study (Fig. 2). We hypothesized that MAOM pools would be greater in the +N WS3, based on theoretical predictions of greater microbial carbon use efficiency with greater N availability (Manzoni et al., 2012; Mooshammer et al., 2014). Nevertheless, there are some circumstances where we might expect the contribution of MAOM to be similar in these watersheds. First, levels of MAOM may saturate because of the limited surface area and binding sites of soil minerals (Castellano et al., 2015; Lavalley et al., 2020), further emphasizing the importance of POM that can theoretically grow infinitely in forest soils (Cotrufo et al., 2019). Additionally, because of the relatively shallow sampling depth (0–15 cm) in this study, any potential differences in MAOM fractions may become more evident if sampled to a greater depth with potentially more weatherable minerals. Alternatively, reduced root-derived C in +N WS3 (Eastman et al., 2021a) may limit MAOM formation in that watershed, consistent with the view that MAOM is most likely formed from belowground inputs that are closer in proximity to soil minerals and microbes (Sokol and Bradford, 2019; Sokol et al., 2019; Villarino et al., 2021). Finally, depleted concentrations of extractable Ca^{2+} and other soil cations in the +N WS3 (Gilliam et al., 2001; Adams et al., 2006) may decrease adsorption of organic matter to mineral surfaces (Chen et al., 2020).

5. Conclusions

This long-term N addition experiment at the Fernow Experimental Forest enhances our understanding of the processes driving SOM formation and destabilization by serving as a model system to consider the impacts of N deposition on plant input decomposition dynamics and different SOM formation pathways. Our results highlight significant effects of N fertilization through reduced rates of leaf litter decomposition and differences in the fate of plant inputs in different SOM fractions. Specifically, the greater light POM fraction present in +N WS3 suggests that N additions may increase the turnover time and stock of a C pool that can potentially accumulate indefinitely (Gregorich et al., 2006; Cotrufo et al., 2019), and emphasizes the need for a more process-oriented conceptualization of soil C cycling (Waring et al., 2020). The response of SOM stocks and associated soil biogeochemical processes to N additions are essential to predicting how global soil C stocks may respond to a changing environment. For example, if the pattern of increased light POM is widespread in regions of historically high N deposition, then the oft observed increases in forest soil C stocks with N addition may not persist under future conditions. If soil bacteria and fungi recover in ways that increases decomposition of light POM in order to access soil N, this soil C sink could become a C source. Thus, the complex response of plant-microbe interactions that link decomposition

and the stabilization of SOM to N deposition and availability is likely a key component of predicting the future terrestrial C stocks and making forest management decisions for C sequestration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.soilbio.2022.108567>.

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