The Effects of Chronic Nitrogen Deposition on Tree Leaf and Fine Root Decomposition

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Mengxue Xia

Major Professor: Kurt S. Pregitzer, Ph.D.

Committee Members: Zonglie Hong, Ph.D.; Kathleen L. Kavanagh, Ph.D.;

John D. Marshall, Ph.D.; Andrzej J. Paszczynski, Ph.D.

Department Administrator: Randall Brooks, Ph.D.

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Authorization to Submit Dissertation

This dissertation of Mengxue Xia, submitted for the degree of Doctor of Philosophy with a major in Natural Resources and titled "The Effects of Chronic Nitrogen Deposition on Tree Leaf and Fine Root Decomposition," has been reviewed in the final form. Permission, as indicated by the signatures and dates given below, is now granted to submit final copies to the College of Graduate Studies for approval.

Major Professor: _____ Date: _____
Kurt S. Pregitzer, Ph.D.

Committee Members: _____ Date: _____
Zonglie Hong, Ph.D.

_____ Date: _____
Kathleen L. Kavanagh, Ph.D.

_____ Date: _____
John D. Marshall, Ph.D.

_____ Date: _____
Andrzej J. Paszczynski, Ph.D.

Department Administrator: _____ Date: _____
Randall Brooks, Ph.D.

Abstract

Elevated atmospheric nitrogen deposition caused by human activity induces a forest carbon sink across broad parts of the Northern hemisphere. In addition to more rapid tree growth, this increase in carbon sequestration could be due to soil carbon accumulation caused by slower organic matter decomposition. The objective of this dissertation was to understand and compare how elevated nitrogen deposition affects decomposition of two major tree litter sources: leaf litter and fine roots. A long-term (>15 years) nitrogen deposition experiment enabled a three-year decomposition study across the span of the Northern Hardwood Biome in Michigan. Fine root and leaf litter biochemical composition and the contribution of leaves and roots to ecosystem biochemical fluxes was quantified. Fine roots were more chemically recalcitrant than leaf litter. At the ecosystem scale, fine roots dominated litter fluxes of acid-insoluble fraction (AIF, also known as Klason lignin) and condensed tannins to soil. Decomposition was estimated using a double-exponential model to describe litter mass loss. Annual litter production was combined with decomposition patterns to estimate how plant litters contribute to soil organic matter. Nitrogen additions increased the initial decomposition of leaf litter, but inhibited the later stages of fine root decomposition. Slower fine root decomposition caused a 23.8 % additional retention of root mass (g m^{-2}) after six years of decomposition. Wet chemistry and Fourier-transform infrared spectroscopy (FTIR) were used to quantify chemical changes of both litter types. Both gravimetrically-defined AIF and lignin/carbohydrate characteristic IR peak ratios indicated that lignin was selectively preserved under simulated nitrogen deposition. The slower degradation of AIF contributed 73.9 ± 5.2 % of additional root mass retention under simulated nitrogen deposition. Although nitrogen deposition studies have focused on leaf litter, these results highlight the dominant role of fine roots in plant-soil carbon fluxes and suggest that slower fine root decomposition is a major driver of soil organic mass accumulation under elevated nitrogen deposition.

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Chapter 1 Fine roots are the dominant source of recalcitrant plant litter in sugar maple dominated northern hardwood forests

This chapter was published as an article in *New Phytologist* (*See* Appendix A for the permission from the publisher to include the article in this dissertation). The original citation is as follows: Xia M, Talhelm AF, Pregitzer KS. 2015. Fine roots are the dominant source of recalcitrant plant litter in sugar maple-dominated northern hardwood forests. *New Phytologist* 208(3):715-726.

1.1 Abstract

- Most studies of forest litter dynamics examine the biochemical characteristics and decomposition of leaf litter, but fine roots are also a large source of litter in forests.
- We quantified concentrations of eight biochemical fractions and nitrogen in leaf litter and fine roots at four sugar maple (*Acer saccharum*) dominated hardwood forests in the north-central United States. We combined these results with litter production data to estimate ecosystem biochemical fluxes to soil. We also compared how leaf litter and fine root biochemistry responded to long-term simulated nitrogen deposition.
- Compared with leaf litter, fine roots contained 2.9-fold higher acid-insoluble fraction (AIF) and 2.3-fold more condensed tannins, both relatively difficult to decompose. Comparatively, Leaf litter had greater quantities of more labile components: non-structural carbohydrates, cellulose, and soluble phenolics. At an ecosystem scale, fine roots contributed over two-thirds of the fluxes of AIF and condensed tannins to soil. Fine root biochemistry was also less responsive than leaf litter to long-term simulated N deposition.
- Fine roots were the dominant source of difficult to decompose plant carbon fractions entering the soil at our four study sites. Based on our synthesis of the literature, this pattern appears to be widespread in boreal and temperate forests.

1.2 Introduction

Plant litter decomposition drives major flows of carbon in soil systems: carbon mineralization and carbon storage (Chapin *et al.*, 2011). As litter decomposes, carbon is returned to the atmosphere via respiration (Raich & Schlesinger 1992); the remaining carbon provides a source of heterogeneous soil organic carbon that accounts for approximately two-thirds of terrestrial carbon (Batjes, 1996). Knowledge of plant litter decomposition and its controlling factors are foundational elements of terrestrial biogeochemical models used to understand the effects of global change on carbon and nutrient cycling (McGuire *et al.*, 2001).

Plant litter is derived from various plant organs, such as leaves (Aber & Melillo 1982), roots (Gill & Jackson 2000), and woody stems (Dearden *et al.*, 2006). Of these organs, decomposition research has primarily focused on leaf litter, likely because leaf litter is a large and visible input to the soil that can be easily sampled. Consequently, leaf litter decomposition processes, including the rate, chemistry, and biology of decomposition are often assumed to be broadly representative of plant litter decomposition (Rasse *et al.*, 2005; Freschet *et al.*, 2013). This assumption has been used extensively in models of ecosystem carbon cycling (Schmidt *et al.*, 2011).

However, growing evidence suggests that our knowledge of leaf decomposition may be inadequate for the purpose of broadly understanding plant litter dynamics. First, it is now apparent that fine roots contribute a substantial portion of plant litter production. In a recent meta-analysis of litter inputs in tropical, temperate, and boreal/alpine forests, root litter accounted for 48% of annual plant litter inputs, greater than either leaf litter (41%) or fine stems (11%; Freschet *et al.*, 2013). In some forests, root litter was estimated to contribute over two-thirds of the litter production (Grier *et al.*, 1981). Second, evidence from litterbag and isotopic tracer studies has demonstrated that fine roots generally breakdown more slowly than leaves. Litter bag studies have shown that root litter decays slower than leaf litter across a range of terrestrial ecosystems, from boreal forests to sub-humid savannas (Taylor *et al.*, 1991; Lehmann *et al.*, 1995; Gholz *et al.*, 2000; Abiven *et al.*, 2005; Kelleher *et al.*, 2006, Vivanco & Austin 2006; Freschet *et al.*, 2013, van Huysen *et al.*, 2013*;* but see Ostertag

& Hobbie 1999). At the end of a decade-long litterbag experiment that used nine litter types and 27 sites across North and Central America, approximately one-third more root litter than leaf litter remained (estimated from Harmon *et al.*, 2009). In isotopic tracer studies, fine root inputs resulted in approximately one-third more soil carbon retention than leaf litter in a temperate forest (Bird *et al.*, 2006; 2008) and an acidic tundra soil (Loya *et al.*, 2004). In temperate deciduous forest soils, isotopic measurements estimated that root-derived carbon represented > 60% of microbial biomass after three years of litter addition (Kramer *et al.*, 2010). Rasse and colleagues (2005) found that roots contributed an average of two-fold more to soil organic carbon than leaf litter in a summary of agricultural studies using isotope tracer techniques. More recent research using pyrolysis and compound-specific isotope analysis showed that fine root-derived carbon was more abundant than leaf-derived carbon in the humin fraction, a recalcitrant part of soil organic matter (Mambelli *et al.*, 2011). Taken together, these lines of evidence demonstrate a need for biogeochemical models to incorporate more experimental data on fine root chemistry and decomposition dynamics.

It is not clear why fine roots are generally more resistant to decomposition than leaf litter. Decomposition is controlled by both exogenous factors, such as environmental conditions (Zhang *et al.*, 2008; Solly *et al.*, 2013), decomposer community composition (Wickings *et al.*, 2012), interactions with soil particles (Six *et al.*, 2002), and endogenous factors, such as tissue chemistry (Melillo *et al.*, 1982; Adair *et al.*, 2008). Because fine roots and leaves are initially added to the soil at different locations, exogenous factors likely account for some of the differences in decomposition (Rasse *et al.*, 2005). However, a number of experiments have observed that fine roots degrade slower than leaf litter when exogenous factors were held constant (*i.e.* both types were put in the same soil depth at the same locations, Taylor *et al.*, 1991, Abiven *et al.*, 2005; Bird *et al.*, 2008), and such a pattern persists even when fine roots were milled to fine particles before incubation in soil (Waid 1974). These results suggest that fine roots are more chemically-resistant to decomposition, which could be one of the mechanisms that contribute to the greater retention of root-derived carbon within soil. Consequently, we hypothesized that fine roots are more biochemically-resistant to

decomposition than leaf litter and that fine roots are the dominant source of recalcitrant plant materials returned to soil.

We tested this hypothesis by investigating the biochemistry of leaf litter and fine roots at four sugar maple dominated hardwood forests across a 500-km climate and air pollution gradient and by modeling inputs of specific classes of compounds to soil. We quantified the concentrations of nitrogen and eight major plant biochemical fractions/classes for both tissue types. Here, we refer to a plant tissue as 'recalcitrant' if it contains high concentrations of major biochemical fractions/classes resistant to microbial degradation and reported to retard litter decomposition, such as the acid-insoluble fraction (AIF, conventionally referred to as lignin, Melillo *et al.*, 1982; Taylor *et al.*, 1989; Sariyildiz & Anderson 2003; Cornwell *et al.*, 2008; Amin *et al.*, 2014) and condensed tannins (Wardle *et al.*, 2002; Hättenschwiler *et al.*, 2011). Likewise, recalcitrant tissues also have relatively low concentrations of easily degraded substrates, such as non-structural carbohydrates (Waldrop & Firestone 2004) and simple phenolics (Hättenschwiler *et al.*, 2011). We also calculated litter quality indices including the ratios of carbon and nitrogen (C:N), AIF and N (AIF:N), and AIF and the sum of AIF and holocellulose (lignocellulose index). These indices have been reported to be negatively correlated with decomposition rates across a large number of ecosystems (Taylor *et al.*, 1989; Preston *et al.*, 2000; Adair *et al.*, 2008; Zhang *et al.*, 2008). Our assessment of chemical recalcitrance was primarily based on C quality and largely neglected the role of mineral nutrient availability on litter decomposition (Paul, 2006). Mineral nutrient availability is less likely to influence decomposition on relatively fertile sites (Melillo *et al.*, 1982), such as those used in this study (Pregitzer *et al.*, 2008). Because forest productivity, root biomass, and root turnover at these sites are well-documented (Burton *et al.*, 2000; Pregitzer *et al.*, 2008), we were also able to estimate the relative contribution of leaves and fine roots to the total flux of recalcitrant and labile compounds returned to soil.

Because fine roots and leaves have different physiological functions, our second aim was to investigate if leaf litter and fine roots have unique biochemical responses to environmental change. For example, experimental soil warming decreased the amount of added ^{15}N that was allocated to old

leaves, but increased the ^{15}N recovery in fine roots (Hobbie & Chapin 1998). Thus, the response of leaf litter to environmental change may not represent the overall shift in litter chemistry. To compare how fine root and leaf litter biochemistry respond to environmental change, we took advantage of our long-term simulated N deposition experiment. We have already documented a number of responses to simulated N deposition at these sites, including increased soil organic C (Pregitzer *et al.*, 2008), an effect explained by slower litter decomposition (Zak *et al.*, 2008). Also, simulated N deposition significantly increased canopy leaf N concentration, but did not affect fine root N concentration (Zak *et al.*, 2008), suggesting that leaf litter and fine root chemistry have responded differently to simulated N deposition. Accordingly, we hypothesized that long-term simulated N deposition alters litter biochemistry to favor slower decomposition and that the biochemistry of leaf litter and fine roots responds differently to long-term simulated N deposition at these study sites.

1.3 Materials and Methods

1.3.1 Site description

The four study sites encompass the north-south distribution of the northern hardwood forest biome in the Great Lakes region of North America and occur along a 500-km temperature and N deposition gradient (Table 1.1; Fig. 1.1). These sites are heavily dominated by sugar maple (*Acer saccharum* M.) and similar in stand composition, age, and soil properties (Table 1.1). The $O_{e/a}$ horizon at these sites is permeated by a dense mat of sugar maple fine roots and contains a large amount of C (Zak *et al.*, 2008; Table 1.1). Soils are sandy (Kalkaska series, Typic haplorthod) and pH values range from 4.4 to 4.7 in the top 10 cm of mineral soil. Six 30-m × 30-m plots were established at each site, each plot surrounded on all sides by a 10-m buffer treated the same way as the main plot area. Since 1994, three plots at each site have received experimental additions of N at a rate (3 g N m^{-2} yr^{-1} as $NaNO_3$ in six equal increments across the growing season) similar to rates of N deposition occurring in some areas of Europe (Holland *et al.*, 2005).

1.3.2 Leaf litter and fine root sampling

Sugar maple leaf litter was collected in litter traps randomly located in each plot in the autumn of 2010 following the protocol of Pregitzer *et al.* (2008). Root mortality is relatively evenly-distributed over the growing season at these sites (Burton *et al.*, 2000), so we collected fine roots at two points in the growing season, October 2010 (autumn) and May 2011 (spring) to better describe the chemical characteristics of fine roots throughout the growing season. We used the mean of spring and autumn fine roots to represent fine roots unless otherwise stated. At six to eight random points within the buffer of each plot, we removed the O_i and excavated fine roots from the top 10 cm of soil, including the O_{e+a} horizon, A horizon, and a portion of the E horizon. Roots were sorted by hand and identified to the genus *Acer* by morphological characteristics. Maples other than the sugar maple contributed only an average of 7.5% to stand basal area in 2011. Young roots, visually identified as white and turgid, were removed to minimize the difference between the root tissue we sampled and root necromass. Because we are also conducting a decomposition study, the number of samples varied in order to collect approximately 300 g of fine roots per plot. We assumed that the chemical qualities of the leaf litter and fine roots we sampled represented the forest as a whole, because sugar maple represents 77% of the annual leaf litter flux at these sites and the genus *Acer*, whose fine roots we sampled, contributes 90% of overstory basal area and 83% of woody groundcover stems (Talhelm *et al.*, 2013).

Following initial processing to quickly remove mineral soil, organic matter, and roots of other species, the root samples of each plot were rinsed, homogenized, and flash frozen in liquid N_2, then packed with solid CO_2 for shipping to the University of Idaho, where we isolated first to third order roots for analysis (with the most distal root-tips defined as first order, Pregitzer *et al*., 2002, also *see* Appendix B). We used the first to third order roots because these roots represent the most short-lived and metabolically-active portion of the root system (Guo *et al.* 2008; Valenzuela-Estrada *et al.* 2008; Xia *et al.* 2010; McCormack *et al.*, 2015), better serving as the belowground counterpart to foliage (Li *et al.*, 2010a). In comparison, when the fine roots of trees are defined as those < 2 mm in

diameter, this pool includes a large number of roots that are longer-lived and tend to undergo secondary thickening (Xia *et al.,* 2010; McCormack *et al.,* 2015). Further, the first three order roots we sampled have mean diameters around 0.30 mm (Appendix C), similar to the mean diameter observed in minirhizotrons at our sites (~ 0.31 mm, Burton *et al.,* 2000). About 2 g DW of roots for each plot were used for the chemical analyses presented here.

1.3.3 Substrate biochemistry

Plant tissues were pulverized in a Wig-L-Bug grinder/mixer (Dentsply-Rinn, Elgin, IL) before being analyzed for total C and N, non-structural carbohydrates (NSCs), soluble phenolics, condensed tannins (CTs), soluble proteins, total lipids, AIF, and hemicellulose. Total C and N were analyzed with an elemental analyzer (ECS 4010, Costech Analytical, Valencia, US). For NSCs (sugars + starch), samples were extracted with 80% hot ethanol and analyzed for sugars using phenol-sulfuric acid (Chow & Landhäusser 2004; Quentin *et al.,* 2015). The residues were digested with a mixture of α-amylase/amyloglucosidase for starch determination. Starch-digesting enzymes exhibit more complete starch digestion than acid hydrolysis and lack the capability to inadvertently degrade structural polysaccharides (Chow & Landhäusser 2004). After digestion, the glucose hydrolyzates were measured colorimetrically with a peroxidase-glucose oxidase/o-dianisidine reagent (Chow & Landhäusser 2004). We noted that absolute estimates of NSCs may not be comparable among studies. Quentin *et al.* (2015) reported that absolute estimates of NSCs were highly variable among laboratories; however, their study supported that relative differences among treatments within a laboratory were meaningful. Soluble phenolics were extracted with 70% acetone and determined with Folin-Ciocalteu (FC) reagent as catechin equivalents (Booker *et al.,* 1996). This protocol quantifies phenolics as the overall capacity to reduce heteropolyphosphotungstates-molybdates to blue complexes (Singleton *et al.,* 1999); other reducing reagents that also react with FC reagent such as ascorbic acids and aromatic amino acids may also contribute to this reducing capacity. However, the occurrence of these compounds in mature leaves and fine roots are minor (mostly <0.05 %, Cyr *et al.,*

1990; Tschaplinski *et al.*, 1995; Chávez *et al.*, 2000; Chen & Gallie 2005), and further decreased in senesced litter (Buchanan-Wollaston 1997; Gergoff *et al.*, 2010). The extractable CTs were extracted with repeated sonication in 70% acetone (Yu & Dahlgren 2000), and determined by acid-butanol assay (Booker *et al.*, 1996). We prepared CT standards from apple fruits following the protocols of Li *et al.*, (2010b). Because there is no generally-accepted CT standard and different CT structures can react differently to the assay, the CT quantification in this study should be interpreted as a relative assessment of CT concentrations rather than an absolute quantification. However, Coq *et al.* (2010) observed a strong correlation between the acid butanol assay and HPLC quantification of CTs in 15 species of leaf litter (r = 0.934). The insoluble residues were freeze-dried, re-suspended in methanol, incubated at 95°C and determined for bound CTs (Booker *et al.*, 1996). Total CTs were the sum of extractable and bound fractions. Soluble proteins were extracted with 0.1 M NaOH and determined by Coomassie Protein Bradford Reagent (Thermo Fisher Scientific Inc., Rockford, US) with the addition of diluted polyvinylpyrollidone (Fisher BioReagent, Pittsburgh, US) to minimize the interference by brown quinones (Jones *et al.*, 1989). Bovine serum albumin (Thermo Fisher Scientific Inc., Rockford, US) was used to construct the standard curve. For total lipids, samples were homogenized using methanol/chloroform/water (Bligh & Dyer 1959). Water was added to the supernatants, which then separated into two layers. Lipid contents in the chloroform layer were determined gravimetrically after evaporating chloroform to dryness (Smedes & Thomasen 1996).

Extractive-free fraction, referred to as cell-wall fraction in this study, was prepared by processing samples with sequential extractions (*see* Appendix D for details). These washes removed both polar and non-polar extractives that are considered readily decomposable, leaving highly cross-linked cell wall components in the residues (Aber *et al.*, 1990; Hendricks *et al.*, 2000). The total of polar and non-polar extractives in this study is referred as "extractive fraction". The remaining residues were subsequently dried and weighed to determine the cell wall fraction. The extractive fraction is the difference between initial weight and the weight of the cell-wall fraction. The cell-wall fraction then was divided into acid-soluble and acid-insoluble fraction using a two-phase H_2SO_4

hydrolysis adapted from Booker *et al.*, (1996). The acid-soluble fraction, consisting of dominantly cell-wall polysaccharides, along with other compounds, *e.g.* phenolics and lipids, linked to cell wall via ester bonds (Iiyama *et al.*, 1994; Preston *et al.*, 2000), was hydrolyzed by H_2SO_4 incubation. The remaining residues were dried and weighed to determine the acid-insoluble fraction (AIF). For hemicellulose, the pellets remaining after tannins extraction were incubated with 10% KOH for 24 hours at 30°C (Dickson 1979; Chapman *et al.*, 2005). The extracts were mixed with 4 M acetic acid in ice-cold ethanol for 24 hours. The precipitate was dried to determine hemicellulose concentration. Cellulose was calculated as the cell-wall fraction concentration minus the concentration of AIF and hemicellulose. Lignocellulose index was the ratio of AIF to cell-wall fraction. Ash contents were determined after 4 h combustion in a muffle furnace at 500 °C. Leaf litter had an ash content of 7.6 ± 1.3%, and fine roots of 3.6 ± 1.3%. All concentrations were expressed on an ash-free dry mass basis.

1.3.4 Annual litter flux

The annual fluxes of biochemical classes to soil through leaf litter or fine roots were calculated as:

$$I_a = P_l \times C_a$$

I_a is the annual input of an individual biochemical class, P_l is the annual litter production of leaf litter or fine roots, and C_a represents the concentration of this biochemical class in leaf litter or fine roots.

The annual leaf litter production was estimated from the leaf litter trap collections in each plot (Pregitzer *et al.*, 2008) and was averaged for each plot from annual measurements of 1988-2011 for ambient plots, and 1994-2011 for N-amended plots (data available at Michigan Nitrogen Deposition Gradient Study database, http://webpages.uidaho.edu/nitrogen-gradient). Leaf litter production and fine root biomass at these sites have changed little through time (Talhelm *et al.*, 2012) or as a result of simulated N deposition (Burton *et al.*, 2004, Pregitzer *et al.* 2008). Similarly, simulated N deposition has not affected fine root turnover (Burton *et al.*, 2004). In the year we sampled the leaf litter for biochemistry (2010), leaf litter production was within ± 10% of the long-term average.

The annual litter production of fine roots included roots in the top 70 cm of the soil and was estimated as the standing biomass of fine roots within a specific soil depth increment multiplied by the corresponding fine root turnover rate for that soil increment for each plot. The fine root turnover rate at each soil depth in each plot was derived from minirhizotron observations at these sites (Burton *et al.*, 2000). Fine root biomass data for 0-10 cm, 10-30 cm, 30-50 cm, and 50-70 cm in soil depth were obtained by soil cores at each plot in 2004 and 2009 (data available at Michigan Nitrogen Deposition Gradient Study database), which classified roots by diameter, rather than branch order. We used the data from the smallest diameter class (<0.5 mm) in these surveys. We believe that the roots we sampled for biochemical analyses (the first three root orders) are analogous to those included in the estimates of root litter production because nearly all roots (~ 97%) among the small root branches of sugar maple are found within the first three root orders (Pregitzer *et al.*, 2002). Further, there is good correspondence between the mean diameter of the roots observed via minirhizotron (0.31 mm; Burton *et al.*, 2000) and the diameter of the three root orders we sampled (~ 0.30 mm, Appendix C). However, we are aware that the chemical traits of fine roots collected from the top 10 cm of soil may differ somewhat from those deeper in the soil. The influence of these differences on biochemical fluxes should be limited. Fine root production and turnover decrease with depth in temperate hardwood forests (Joslin *et al*., 2006); fine roots within the top 10 cm of soil represent 52% of the fine root biomass within the top 70 cm of soil and 72% of the root turnover in the top 50 cm of soil at our sites (Burton *et al.*, 2000). Further, this study focused on major C fractions rather than element concentrations, such as phosphorus, sodium, and potassium that vary strongly by soil depth.

1.3.5 Statistical analysis

We tested whether the biochemical traits and fluxes differ among tissue types (leaf litter vs. fine roots, or spring vs. autumn roots, $df = 1$), simulated N deposition ($df = 1$), and study sites ($df = 3$) using a split-plot design analyzed with mixed linear models (Proc Mixed, Little 2006), followed by Tukey's HSD tests for pairwise comparisons. In this model, sites, N treatments, and their interactions

were sources of whole-plot variation, while tissue type was the within-plot factor. The interaction terms of tissue type and treatment tested the hypothesis whether different tissue types responded differently to simulated N deposition. We also used a two-way ANOVA to determine whether simulated N deposition (df = 1), site (df = 3) or their interactions (df = 3) had effects on the leaf litter production, fine root mass turnover, total litter production, and the sum of leaf litter and fine root fluxes of each biochemical class. Data were log-transformed prior to being analyzed in SAS 9.3 (SAS institute, Inc., Cary, USA) to reduce the effects of variations increasing with means.

1.4 Results

1.4.1 Biochemical composition and nitrogen

We investigated the abundance of eight major biochemical fractions (representing ~ 90% of substrate dry mass, Table 1.2), N concentration, and three litter quality indices for leaf litter and fine roots collected from four sugar maple dominated hardwood forests in the north-central United States. Tissue type (leaf litter *versus* fine roots) resulted in a considerably greater variance than both site and simulated N deposition for all biochemical traits (Tables 1.2, Appendix E, F). Leaf litter had substantially higher concentrations of non-structural carbohydrates (NSCs) and lipids ($P < 0.001$, Table 1.2). Leaf litter also exhibited higher concentrations of cellulose and soluble phenolics than fine roots in general (Table 1.2), but the magnitude of difference varied among sites (tissue × site, $P < 0.05$, Appendix E, F). The amount of unidentified material was generally higher in leaf litter than in fine roots, a trend that was strongest at site C (tissue × site: $P = 0.013$, Tables 1.2, Appendix E, F).

In contrast, fine roots contained more acid-insoluble fraction (AIF), condensed tannins (CTs), and N than leaf litter ($P < 0.001$, Table 1.2). Fine roots averaged about ×2.9 higher AIF concentrations and about ×2.3 greater concentrations of CTs than those in leaf litter across four sites (Table 1.2). AIF was the most abundant of all eight biochemical fractions in fine root tissue, while cell wall polysaccharides (cellulose + hemicellulose) were the dominant constituent of leaf litter (Table 1.2). Fine roots had consistently higher N concentrations than leaf litter. This trend was

apparent at all sites, but was strongest at site C, leading to significant interactions of site × tissue on N concentration and N-related litter indices ($P < 0.05$, Appendix E, F). The AIF:N ratio and lignocellulose index (LCI) were higher in fine roots than leaf litter ($P < 0.001$, Table 1.2).

Relative to fine roots collected in spring, autumn fine roots had lower concentrations of AIF and hemicellulose, but higher concentrations of lipids, soluble proteins and N ($P < 0.05$, Appendix G). The most striking difference between spring and autumn roots was in the concentration of NSCs (Appendix G): sugar increased from 10.1 ± 0.7 mg g^{-1} in spring roots to 15.7 ± 0.8 mg g^{-1} in autumn roots, while starch was about twice as abundant in autumn roots (data not shown).

1.4.2 Biochemical fluxes

Leaf litter production across the four sites ranged from 324.0 to 447.1 g m^{-2} yr^{-1}, while fine root litter production ranged from 175.1 to 420.1 g m^{-2} yr^{-1} (Table 1.3). These two types of litter production together made up 89 ± 4% of total litter production at these sites (Table 1.3). Simulated N deposition did not affect fine root, leaf, or total litter production ($P > 0.1$, Appendix H).

Because the litter production of leaves and fine roots both varied among sites (Table 1.3), the magnitude of differences in each biochemical flux between leaf litter and fine roots also varied among sites (site × tissue; $P < 0.01$, Appendix I). However, there were considerable differences between the two tissues in all biochemical fluxes except hemicellulose (Tables 1.4, Appendix I). AIF and cell-wall polysaccharides were the two largest biochemical fluxes to the soil; each of the other biochemical classes accounted for < 10% of the total litter flux. Fine roots dominated the fluxes of AIF (~ 71% of the total flux) and CTs (~ 68%) across the four sites (Table 1.4). Assuming that there is no meaningful N resorption during root senescence, fine roots contributed more soluble protein and N than leaves to the soil across all sites ($P < 0.001$, Table 1.4). In contrast, leaf litter contributed considerably more cellulose, NSCs, soluble phenolics, and lipids to the soil ($P < 0.001$, Table 1.4).

1.4.3 Effects of simulated nitrogen deposition

Simulated N deposition generally decreased CT and increased N concentrations, as shown by significant main effects of N treatments on CT ($P = 0.030$) and N ($P < 0.001$, Tables 1.2, Appendix E). There were also significant overall effects of simulated N deposition on cell-wall fraction, AIF, soluble proteins, AIF:N, and C:N ($P < 0.05$, Table 1.2), but these effects were not consistent across either tissues, sites, or their interactions (Appendix E,F). The most consistent of these effects was that the decreases in C:N and AIF:N were more prominent in leaf litter than in fine roots (tissue × N: $P < 0.05$). In leaf litter at sites A, B, and C, simulated N deposition decreased the average cell-wall fraction from 69.3% to 58.1% and caused a corresponding increase in the extractive fraction (tissue × N × site: $P < 0.02$ for each; Appendix E,F). Within the cell-wall fraction at these sites, simulated N deposition decreased average AIF from 15.3% to 13.8% (tissue × N × site: $P = 0.008$), but decreased cellulose from 39.9% to 30.9% (tissue × N × site: $P = 0.091$). Within the extractive fraction at these three sites, the unidentified portion increased from an average of 5.8% to 14.3% (tissue × N × site: $P = 0.005$). These changes did not occur in leaf litter at site D or in fine roots (Table 1.2, Appendix F).

Other effects of simulated N deposition were more idiosyncratic. Soluble protein concentrations were generally lower under simulated N deposition ($P = 0.018$, Table 1.2), but this decrease did not occur at site C for leaf litter and site A for fine roots (tissue × N × site: $P = 0.031$, Appendix E,F). Soluble phenolics of leaf litter and fine roots showed both positive and negative responses to N deposition that depended on site (tissue × N × site: $P = 0.042$, Appendix E,F).

When combined with litter production, simulated N deposition increased N flux through leaf litter by an average of 29% ($P < 0.05$), but did not affect fine root N flux ($P > 0.05$, tissue × N: $P = 0.018$, Tables 1.4, Appendix I). Simulated N deposition generally decreased the fluxes of CTs ($P = 0.036$, Tables 1.4, Appendix I), but this effect was not observed for fine roots at site A and for leaf litter at site C (site × tissue × N: $P = 0.035$, Appendix I). Simulated N deposition also marginally decreased the flux of soluble protein ($P = 0.054$), an effect that was strongest for fine roots at site C (site× tissue × N: $P = 0.027$, Appendix I). When fluxes through leaf litter and fine roots were

combined, simulated N deposition marginally decreased the total fluxes of CTs, soluble proteins, and increased NSCs, and N ($P < 0.1$, Table 1.4), yet this increase of N was not apparent at site C (site × N: $P = 0.041$, Appendix J). Simulated N deposition increased the cellulose flux at site D, but decreased this flux elsewhere (site × N: $P = 0.011$, Appendix J).

1.5 Discussion

1.5.1 Differences in litter biochemical composition and flux

Consistent with our hypothesis, fine roots had greater concentrations of biochemical fractions associated with chemical recalcitrance than leaf litter, and this pattern persisted across sites and N treatments. Because leaves and fine roots contributed comparable litter fluxes to the soil in these forests, the large biochemical differences meant that the flux of an individual biochemical class was often dominated by a single litter type: *e.g.* more than two-thirds of the fluxes of AIF and condensed tannins (CTs) were attributed to fine root turnover (Table 4). In short, leaf litter and fine roots represented fluxes of very different substrates for decomposition within these forests. While exogenous factors also influence retention of detrital carbon within the soil, our observation that greater quantities of recalcitrant compounds are returned to soil through fine roots than leaf litter is consistent with previous work identifying fine roots as the major source of soil organic carbon (SOC).

The most striking difference between leaf litter and fine roots was that fine roots had considerably higher concentrations of AIF than leaf litter (Table 1.2). Acid-insoluble fraction (AIF) has often been referred to as lignin, an aromatic heteropolymer difficult to degrade because its complex structure limits degradation to only non-specific oxidative enzymes (Kirk & Farrell 1987). Lignin has been reported to persist longer in soil than plant-derived polysaccharides and proteins after one to several years of incubation of synthesized lignin (Martin *et al.*, 1980; Stott *et al.*, 1983) and plant materials (Kelleher *et al.*, 2006). However, AIF isolated in this and many studies is not purely lignin, but a mixture of lignin and other complex substrates such as cutins, suberins, and CT-protein complexes (Preston *et al.*, 1997). AIF may still be a good indicator of chemical recalcitrance because

these compounds are generally preserved in decomposing litter (Preston *et al.,* 2000). The recalcitrance of AIF has been empirically demonstrated by reports that a higher initial AIF concentration or AIF:N ratio results in slower litter decomposition (Taylor *et al.,* 1989; Berg, 2000; Sariyildiz & Anderson 2003; Amin *et al.,* 2014). In an empirical model derived from a 10-year decomposition study, AIF defined the recalcitrant litter pool, while the decomposition of the intermediate pool (acid-soluble fraction) decreased when LCI increased, an effect proposed to result from the protection of cellulose by lignin (Adair *et al.,* 2008). These metrics of recalcitrance (AIF concentration, AIF:N, and LCI) were higher in fine roots than leaf litter, supporting the idea that fine roots are more chemically resistant to decomposition than leaf litter.

To understand if the high concentration of AIF in fine roots was a widespread phenomenon, we compiled studies that quantified proportions of acid-insoluble, acid-soluble, and extractive fraction across a number of boreal and temperate forests (Fig. 1.2), encompassing more than 30 species on three continents. These proximate fractions are frequently reported because they have been associated with increasing rates of mass loss during decomposition (acid-insoluble < acid-soluble < extractive; Hendricks *et al.*, 2000; Adair *et al.,* 2008). Fine roots had AIF concentrations that were an average of 2.3-fold higher than in leaf litter, while leaf litter often had more extractive materials (Fig. 1.2). We conclude that chemically recalcitrant AIF is consistently more abundant in fine roots than leaf litter across a global sample of forests.

The greater CT concentrations in fine roots than leaf litter (Table 1.2) may add to the chemical recalcitrance of roots. Condensed tannins are less accessible to biodegradation than other plant phenolics (Bhat *et al.*, 1998) and higher CT concentrations has been associated with slower decomposition (Wardle *et al.*, 2002; Coq *et al.*, 2010; Hättenschwiler & Jørgensen 2010). This suppression of decay is probably because CTs can bind to proteins or cell wall components to form less-degradable complexes (Cai *et al.*, 1989; Northup *et al.*, 1995; Mutabaruka *et al.,* 2007) and because CTs can inhibit soil enzyme activity (Ushio *et al.*, 2013). When the CT and AIF

concentrations are combined with litter production data, it is clear that fine roots dominated the litter input of these chemically recalcitrant materials at our sites (Table 1.4).

Although leaf litter contained less CTs than fine roots, leaf litter contained higher concentrations of soluble phenolics (Table 1.2). Condensed tannins are a subclass of phenolics, but it is impossible to estimate the proportion of CTs in phenolics in this study because the assay we used to assess CTs provides a relative, not absolute, quantification of these compounds (see *Materials and Methods*). Aside from CTs, total phenolics also include hydrolyzable tannins (HTs) and low-molecular-weight phenolics, which we did not quantify separately in this study. Soil microorganisms often utilize these non-CT phenolics as labile C sources (Schimel *et al.*, 1998; Nierop *et al.*, 2006) and Hättenschwiler & Jørgensen (2010) suggested that these compounds were responsible for an observed positive correlation between total phenolics and leaf litter mass loss. In contrast, Triebwasser *et al.* (2012) reported that HTs contribute significantly to soil enzyme inhibition. Leaf litter also had higher concentrations of cell-wall polysaccharides and readily-decomposed NSCs. The decrease of carbohydrate-related signals in NMR spectra represented the most pronounced C loss during litter decay (Kelleher *et al.*, 2006; Preston *et al.*, 2009). Higher concentrations of C sources such as NSCs and polysaccharides in leaf litter than in fine roots suggests that leaf litter could be a more efficient substrate in priming decomposition.

A limitation of this study is that we used live fine roots because it was extremely difficult to identify large quantities of recently-senesced fine roots. Leaf senescence is a well-understood process that includes the breakdown of proteins, membrane lipids, and nucleic acids (Lim *et al.*, 2007) and which removes > 50% of foliar N and phosphorus pools (Aerts 1996). In comparison, little is known about the senescence of fine roots because it is difficult to isolate death from decay (Comas *et al.*, 2000). Resorption of nutrients during root senescence may occur, but nutrient transfers appear to be considerably smaller than those in leaves (Kunkle *et al.*, 2009) and there are observations that suggest that no nutrient resorption occurs (Nambiar 1987; Aerts 1990; Gordon & Jackson 2002). Notably, distal root segments have been observed to live after preceding root segments have died (Comas *et*

al., 2000), making an intracellular disassembly process similar to that in leaves seem unlikely. Further, fine roots are dominated by biochemical classes that are bound in cell walls and therefore are less likely to be retranslocated during senescence.

1.5.2 Implications for soil organic carbon transformation

Throughout this paper, we have used the concept of chemical recalcitrance to refer to forms of litter and biochemical fraction/classes that are resistant to mass loss in studies of plant litter decomposition. The decomposition studies that developed this concept typically track the fate of litter over months to years, or occasionally a decade (Adair *et al.* 2008). Generally, the decomposing litter in these studies has limited physical interactions with mineral soil. Within these contexts, the concept of chemical recalcitrance as a mechanism that leads to the accumulation of decomposing litter is supported both empirically (*e.g.*, Adair *et al.*, 2008; Grandy & Neff 2008) and mechanistically (Kirk & Farrell 1987). Our results show that in our sites and other temperate/boreal forests, fine roots are the dominant source of the recalcitrant plant biochemicals (Table 1.4; Fig. 1.1) that tend to have slow initial decay and accumulate as partially-decomposed litter.

However, biochemical characteristics that provide the basis for the chemical recalcitrance of decomposing litter cannot *directly* explain the long-term stabilization of carbon in pools associated with soil aggregates or mineral particles (Marschner *et al.*, 2008). At the time-scale of decades to millennia, compounds that are considered chemically recalcitrant are not preferentially preserved in soil over those considered as labile (Schmidt *et al.*, 2011). Nonetheless, there is evidence for *indirect* effects of substrate biochemistry on long-term carbon retention through other microbial mechanisms. Microbial products have been recognized as a major precursor to stable SOC (Mambelli *et al.*, 2011; Cotrufo *et al.*, 2013). More recalcitrant C fractions are generally less efficient than labile compounds in generating microbial biomass (Bahri *et al*., 2008; Blagodatskaya *et al*., 2011; Dijkstra *et al*., 2011), suggesting labile compounds are more important for the formation of stable SOC (Cotrufo *et al.*, 2013). However, fungi dominate lignin degradation (de Boer *et al.*, 2005) and fungal products are

thought to reside longer in soil than bacterial products (Bardgett *et al.*, 2014). Understanding the manner in which substrate biochemistry affects microbial products will reveal how different biochemicals in plant debris eventually affect long-term SOC stabilization.

1.5.3 Responses to simulated nitrogen deposition

Ecosystem responses to N deposition have drawn considerable interest because human activity increased atmospheric N deposition by an order of magnitude during the last century (Galloway *et al.*, 2004). At our sites, the first decade of simulated N deposition caused a 26% increase in the surface soil carbon pool (Pregitzer *et al.*, 2008). In part, this result motivated our research in the plant biochemistry associated with initial litter decay because the partially-decomposed litter in the forest floor horizon ($O_{e/a}$) represents a distinct portion of total soil organic matter in our forests and the slower turnover of this horizon is a major driver of soil organic matter accumulation under simulated N deposition (Zak *et al.*, 2008). Other long-term N addition experiments have also reported slower decomposition (Franklin *et al.*, 2003).

Slower decomposition with increased N supply is either due to decreased initial litter quality, the inhibition of microbial activity, or both. Contrary to our second hypothesis, simulated N deposition resulted in a somewhat 'better' litter quality: CT concentrations generally decreased and N concentrations increased, whereas AIF concentrations and AIF:N ratios decreased in leaf litter (Table 1.2). At an ecosystem scale, simulated N deposition marginally decreased the total fluxes of CTs, and increased fluxes of N and NSCs entering soil ($P < 0.1$, Table 1.4). Thus, there is no evidence that simulated N deposition slows litter decomposition by decreasing initial litter quality. Instead, previous research at our sites observed that simulated N deposition suppressed laccase gene expression and the activity of lignin-degrading enzymes (Deforest *et al.*, 2004; Edwards *et al.*, 2011).

The biochemistry of leaf litter and fine roots responded differently to simulated N deposition, supporting our third hypothesis. Simulated N deposition generally decreased the concentration of AIF and the overall cell wall fraction in leaf litter, but had little influence on any cell wall components in

fine roots (Table 1.2). Also, simulated N deposition dramatically decreased AIF:N ratios of leaf litter, yet did not affect these ratios in fine roots. Although simulated N deposition increased leaf litter N concentration, we did not observe an increase in soluble protein (Table 1.2), which is dominated by Rubisco in leaves (Evans, 1989). Consistent with this, the additional foliar N induced by simulated N deposition did not increase photosynthesis at our sites (Talhelm *et al.*, 2011). Additional foliar N induced by N deposition could be stored as free amino acids (Bauer *et al.*, 2004), which we have not quantified. Likewise, although simulated N deposition has dramatically decreased arbuscular mycorrhizal (AM) fungal biomass and the colonization of roots by AM fungi in our sites (van Diepen *et al.*, 2010), these changes were not manifest through changes in fine root biochemistry. The different response of leaf litter and fine roots to simulated N deposition indicates that the impacts of environmental change on litter biochemistry, and therefore decomposition, cannot be accurately predicted at the ecosystem-scale by solely analyzing leaf litter.

1.6 References

Aber JD, Melillo JM. 1982. Nitrogen immobilization in decaying hardwood leaf litter as a function of initial nitrogen and lignin content. *Canadian Journal of Botany* 60: 2263-2269.

Aber JD, Melillo JM, McClaugherty CA. 1990. Predicting long-term patterns of mass loss, nitrogen dynamics, and soil organic matter formation from initial fine litter chemistry in temperate forest ecosystems. *Canadian Journal of Botany* 68: 2201-2208.

Abiven S, Recous S, Reyes V, and Oliver R. 2005. Mineralisation of C and N from root, stem and leaf residues in soil and role of their biochemical quality. *Biology and Fertility of Soils* 42: 119-128.

Adair EC, Parton WJ, Del Grosso SJ, Silver WL, Harmon ME, Hall SA, Burke IC, Hart SC. 2008. Simple three-pool model accurately describes patterns of long-term litter decomposition in diverse climates. *Global Chang Biology* 14: 2636-2660.

Aerts R. 1990. Nutrient use efficiency in evergreen and deciduous species from heathlands. *Oecologia* 84: 391-397.

Aerts R. 1996. Nutrient resorption from senescing leaves of perennials: are there general patterns? *Journal of Ecology* 84: 597-608.

Amin BA, Chabbert ZB, Moorhead D, Bertrand I. 2014. Impact of fine litter chemistry on lignocellulolytic enzyme efficiency during decomposition of maize leaf and root in soil. *Biogeochemistry* 117: 169-183.

Bahri H, Rasse DP, Rumpel C, Dignac, M-F, Bardoux G, Mariotti, A. 2008. Lignin degradation during a laboratory incubation followed by ^{13}C isotope analysis. *Soil Biology & Biochemistry* 40: 1916-1922.

Bardgett RD, Mommer L, De Vries FT. 2014. Going underground: root traits as drivers of ecosystem processes. *Trends in ecology & evolution* 29: 692-699.

Batjes NH. 1996. Total carbon and nitrogen in the soils of the world. *European journal of soil science* 47: 151-163.

Bauer GA, Bazzaz FA, Minocha R, Long S, Magill A, Aber J, Berntson GM. 2004. Effects of chronic N additions on tissue chemistry, photosynthetic capacity, and carbon sequestration potential of a red pine (*Pinus resinosa*) stand in the NE United States. *Forest Ecology and Management* 196: 173-186.

Berg B. 2000. Litter decomposition and organic matter turnover in northern forest soils. *Forest ecology and Management* 133: 13-22.

Bhat TK, Singh B, Sharma OP. 1998. Microbial degradation of tannins-a current perspective. *Biodegradation* 9: 343-357.

Bird JA, Torn MS. 2006. Fine roots vs. needles: a comparison of ^{13}C and ^{15}N dynamics in a ponderosa pine forest soil. *Biogeochemistry* 79: 361-382.

Bird JA, Kleber M, Torn MS. 2008. ^{13}C and ^{15}N stabilization dynamics in soil organic matter fractions during needle and fine root decomposition. *Organic Geochemistry* 39: 465-477.

Blagodatskaya E, Yuyukina, T, Blagodatsky S, Kuzyakov Y. 2011. Turnover of soil organic matter and of microbial biomass under C_3–C_4 vegetation change: Consideration of ^{13}C fractionation and preferential substrate utilization. *Soil Biology & Biochemistry* 43: 159-166.

Bligh EG, Dyer WJ. 1959. A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology* 37: 911-917.

Booker FL, Anttonen S, Heagle AS. 1996. Catechin, proanthocyanidin, and lignin contents of loblolly pine (*Pinus taeda*) needles after chronic exposure to ozone. *New Phytologist* 132: 483-492.

Buchanan-Wollaston V. 1997. The molecular biology of leaf senescence. *Journal of Experimental Botany* 48:181-199.

Burton AJ, Pregitzer KS, Hendrick RL. 2000. Relationships between fine root dynamics and nitrogen availability in Michigan northern hardwood forests. *Oecologia* 125: 389-399.

Burton, AJ, Pregitzer KS, Crawford JN, Zogg GP, Zak DR. 2004. Simulated chronic NO_3^- deposition reduces soil respiration in northern hardwood forests. *Global change biology* 10: 1080-1091.

Cai Y, Gaffney SH, Lilley TH, Haslam E. 1989. Carbohydrate—polyphenol complexation. In: Hemingway RW, Karchesy JJ, eds. *Chemistry and significance of condensed tannins.* New York, US: Plenum Press, 307-322.

Chapin III FS, Chapin MC, Matson PA, Vitousek P. 2011. *Principles of terrestrial ecosystem ecology.* New York, USA: Springer.

Chapman JA, King JS, Pregitzer KS, Zak DR. 2005. Effects of elevated concentrations of atmospheric CO_2 and tropospheric O_3 on decomposition of fine roots. *Tree physiology* 25: 1501-1510.

Chávez AL, Bedoya J, Sánchez T, Iglesias C, Ceballos H, Roca W. 2000. Iron, carotene, and ascorbic acid in cassava roots and leaves. *Food & Nutrition Bulletin* 21: 410-413.

Chen Z, Gallie DR. 2005. Increasing tolerance to ozone by elevating foliar ascorbic acid confers greater protection against ozone than increasing avoidance. *Plant Physiology* 138: 1673-1689.

Chow PS, Landhäusser SM. 2004. A method for routine measurements of total sugar and starch content in woody plant tissues. *Tree physiology* 24: 1129-1136.

Comas LH, Eissenstat DM, Lakso AN. 2000. Assessing root death and root system dynamics in a study of grape canopy pruning. *New Phytologist* 147: 171-178.

Coq S, Souquet JM, Meudec E, Cheynier V, Hättenschwiler, S. 2010. Interspecific variation in leaf litter tannins drives decomposition in a tropical rain forest of French Guiana. *Ecology* 91: 2080-2091.

Cornwell WK, Cornelissen JH, Amatangelo K, Dorrepaal E, Eviner VT, Godoy O, Hobbie SE, Hoorens B, Kurokawa H, Parez-Harguindeguy N *et al*. 2008. Plant species traits are the predominant control on litter decomposition rates within biomes worldwide. *Ecology Letter* 11: 1065-1071.

Cotrufo MF, Wallenstein MD, Boot CM, Denef K, Paul E. 2013. The Microbial Efficiency-Matrix Stabilization (MEMS) framework integrates plant litter decomposition with soil organic matter stabilization: do labile plant inputs form stable soil organic matter? *Global change biology* 19: 988-995.

Cyr D, Buxton G, Webb D, Dumbroff E. 1990. Accumulation of free amino acids in the shoots and roots of three northern conifers during drought. *Tree physiology* 6: 293-303.

Dearden FM, Dehlin H, Wardle DA, Nilsson MC. 2006. Changes in the ratio of twig to foliage in litterfall with species composition, and consequences for decomposition across a long term chronosequence. *Oikos* 115: 453-462.

de Boer W, Folman LB, Summerbell RC, Boddy L. 2005. Living in a fungal world: impact of fungi on soil bacterial niche development. *FEMS microbiology reviews* 29: 795-811.

DeForest JL, Zak DR, Pregitzer KS, Burton AJ. 2004. Nitrate deposition and the microbial degradation of cellulose and lignin in a northern hardwood forest. *Soil Biology & Biochemistry* 36: 965-971.

Dickson RE. 1979. Analytical Procedures for the Sequential Extraction of ^{14}C-Labeled Constituents from Leaves, Bark and Wood of Cottonwood Plants. *Physiologia Plantarum* 45: 480-488.

Dijkstra P, Thomas SC, Heinrich PL, Koch GW, Schwartz E, Hungate BA. 2011. Effect of temperature on metabolic activity of intact microbial communities: evidence for altered metabolic pathway activity but not for increased maintenance respiration and reduced carbon use efficiency. *Soil Biology & Biochemistry* 43: 2023-2031.

Edwards IP, Zak DR, Kellner H, Eisenlord SD, Pregitzer KS. 2011. Simulated atmospheric N deposition alters fungal community composition and suppresses lignocellulolytic gene expression in forest floor of a northern hardwood forest. *PLoS One* 6: e20421.

Evans JR. 1989. Photosynthesis and nitrogen relationships in leaves of C_3 plants. *Oecologia* 78: 9-19.

Franklin O, Högberg P, Ekblad A, Ågren GI. 2003. Pine forest floor carbon accumulation in response to N and PK additions: bomb ^{14}C modelling and respiration studies. *Ecosystems* 6: 644-658.

Freschet GT, Cornwell WK, Wardle DA, Elumeeva TG, Liu W, Jackson BG, Onipchenko VG, Soudzilovskaia NA, Tao J, Cornelissen JHC *et al*. 2013. Linking litter decomposition of above- and below-ground organs to plant-soil feedbacks worldwide. *Journal of Ecology* 101: 943-952.

Galloway JN, Dentener FJ, Capone DG, Boyer EW, Howarth RW, Seitzinger SP, Asner GP, Cleveland CC, Green PA, Holland EA *et al*. 2004. Nitrogen cycles: past, present and future. *Biogeochemistry* 70: 153-226.

Gergoff G, Chaves A, Bartoli CG. 2010. Ethylene regulates ascorbic acid content during dark-induced leaf senescence. *Plant science* 178: 207-212.

Gholz HL, Wedin DA, Smitherman SM, Harmon ME, Parton WJ. 2000. Long-term dynamics of pine and hardwood litter in contrasting environments: toward a global model of decomposition. *Global Chang Biology* 6: 751-765.

Gill RA, Jackson RB. 2000. Global patterns of root turnover for terrestrial ecosystems. *New Phytologist* 147: 13-31.

Gordon WS, Jackson RB. 2000. Nutrient concentrations in fine roots. *Ecology* 81: 275-280.

Grandy AS, Neff JC. 2008. Molecular C dynamics downstream: the biochemical decomposition sequence and its impact on soil organic matter structure and function. *Science of the Total Environment* 404: 297-307.

Grier CC, Vogt KA, Keyes MR, Edmonds RL. 1981. Biomass distribution and above-and below-ground production in young and mature *Abies amabilis* zone ecosystems of the Washington Cascades. *Canadian Journal of Forest Research* 11: 155-167.

Guo DL, Mitchell RJ, Hendricks JJ. 2004. Fine root branch orders respond differentially to carbon source-sink manipulations in a longleaf pine forest. *Oecologia* 140: 450-457.

Guo DL, Xia MX, Wei X, Chang WJ, Liu Y, Wang ZQ. 2008. Anatomical traits associated with absorption and mycorrhizal colonization are linked to root branch order in twenty-three Chinese temperate tree species. *New Phytologist* 180: 673-683.

Harmon ME, Silver WL, Fasth B, Chen H, Burke IC, Parton WJ, Hart SC, Currie WS. 2009. Long-term patterns of mass loss during the decomposition of leaf and fine root litter: an intersite comparison. *Global Chang Biology* 15: 1320-1338.

Hättenschwiler S, Jørgensen HB. 2010. Carbon quality rather than stoichiometry controls litter decomposition in a tropical rain forest. *Journal of Ecology* 98: 754-763.

Hättenschwiler S, Coq S, Barantal S, Handa IT. 2011. Leaf traits and decomposition in tropical rainforests: revisiting some commonly held views and towards a new hypothesis. *New Phytologist* 189: 950-965.

Hendricks JJ, Aber JD, Nadelhoffer KJ, Hallett RD. 2000. Nitrogen controls on fine root substrate quality in temperate forest ecosystems. *Ecosystems* 3: 57-69.

Hobbie SE, Chapin III FS. 1998. The response of tundra plant biomass, aboveground production, nitrogen, and CO_2 flux to experimental warming. *Ecology* 79: 1526-1544.

Hobbie SE. 2005. Contrasting effects of substrate and fertilizer nitrogen on the early stages of litter decomposition. *Ecosystems* 8: 644-656.

Hobbie SE, Oleksyn J, Eissenstat DM, Reich PB. 2010. Fine root decomposition rates do not mirror those of leaf litter among temperate tree species. *Oecologia* 162: 505-513.

Holland EA, Braswell BH, Sulzman J, Lamarque JF. 2005. Nitrogen deposition onto the United States and Western Europe: synthesis of observations and models. *Ecol. Appl.* 15: 38-57.

Iiyama K, Lam TBT, Stone BA. 1994. Covalent cross-links in the cell wall. *Plant Physiology* 104: 315-320.

Jones CG, Hare JD, Compton SJ. 1989. Measuring plant protein with the Bradford assay. *Journal of chemical ecology* 15: 979-992.

Joslin J, Gaudinski JB, Torn MS, Riley W, Hanson PJ. 2006. Fine-root turnover patterns and their relationship to root diameter and soil depth in a ^{14}C-labeled hardwood forest. *New Phytologist* 172: 523-535.

Kelleher BP, Simpson MJ, Simpson AJ. 2006. Assessing the fate and transformation of plant residues in the terrestrial environment using HR-MAS NMR spectroscopy. *Geochimica et Cosmochimica Acta* 70: 4080-4094.

Kirk TK, Farrell RL. 1987. Enzymatic "combustion": the microbial degradation of lignin. *Annual Reviews in Microbiology* 41: 465-501.

Kramer C, Trumbore S, Fröberg M, Cisneros Dozal LM, Zhang D, Xu X, Santos GM, Hanson PJ. 2010. Recent (<4 year old) leaf litter is not a major source of microbial carbon in a temperate forest mineral soil. *Soil Biology & Biochemistry* 42: 1028-1037.

Kunkle JM, Walters MB, and Kobe RK. 2009. Senescence-related changes in nitrogen in fine roots: mass loss affects estimation. *Tree Physiology* 29: 715-723.

Lehmann J, Schroth G, Zech W. 1995. Decomposition and nutrient release from leaves, twigs and roots of three alley-cropped tree legumes in central Togo. *Agroforestry Systems* 29: 21-36.

Li A, Guo D, Wang Z, Liu H. 2010a. Nitrogen and phosphorus allocation in leaves, twigs, and fine roots across 49 temperate, subtropical and tropical tree species: a hierarchical pattern. *Functional Ecology* 24: 224-232.

Li C, Trombley JD, Schmidt MA, Hagerman AE. 2010b. Preparation of an acid butanol standard from fresh apples. *Journal of chemical ecology* 36: 453-460.

Lim PO, Kim HJ, Gil Nam H. 2007. Leaf senescence. *Annu. Rev. Plant Biol* 58: 115-136.

Little RC, Milliken GA, Stroup WW, Wolfinger RD, Schabenberger O. 2006. *SAS for mixed models.* Cary, US: SAS Institute Inc.

Loya WM, Johnson LC, Nadelhoffer KJ. 2004. Seasonal dynamics of leaf-and root-derived C in arctic tundra mesocosms. *Soil Biology & Biochemistry* 36: 655-666.

Mambelli S, Bird JA, Gleixner G, Dawson TE, Torn MS. 2011. Relative contribution of foliar and fine root pine litter to the molecular composition of soil organic matter after in situ degradation. *Organic Geochemistry* 42: 1099-1108.

Marschner B, Brodowski S, Dreves A, Gleixner G, Gude A, Grootes PM, Hamer U, Heim A, Jandl J, Ji R, *et al*. 2008. How relevant is recalcitrance for the stabilization of organic matter in soils? *Journal of Plant Nutrition and Soil Science* 171: 91-110.

Martin JP, Haider K, Kassim G. 1980. Biodegradation and stabilization after 2 years of specific crop, lignin, and polysaccharide carbons in soils. *Soil Science Society of America Journal* 44: 1250-1255.

McClaugherty CA, Pastor J, Aber JD, Melillo JM. 1985. Forest litter decomposition in relation to soil nitrogen dynamics and litter quality. *Ecology* 66: 266-275.

McCormack ML, Dicke IA, Fahey TJ, Guo D, Helmisaari H, Hobbie EA, Iversen CM, Jackson RB, Leppälammi-Kujansuu J, Norby RJ *et al.* 2015. Redefining fine roots improves understanding of belowground contributions to terrestrial biosphere processes. *New Phytologist* 207(3):505-18.

McGuire A, Sitch S, Clein J, Dargaville R, Esser G, Foley J, Heimann M, Joos F, Kaplan J, Kicklighter DW, *et al.* 2001. Carbon balance of the terrestrial biosphere in the twentieth century: Analyses of CO_2, climate and land use effects with four process-based ecosystem models. *Global Biogeochemical Cycles* 15: 183-206.

Melillo JM, Aber JD, Muratore JF. 1982. Nitrogen and lignin control of hardwood leaf litter decomposition dynamics. *Ecology* 63: 621-626.

Mutabaruka R, Hairiah K, Cadisch G. 2007. Microbial degradation of hydrolysable and condensed tannin polyphenol–protein complexes in soils from different land-use histories. *Soil Biology and Biochemistry* 39: 1479-1492.

Nambiar EKS. 1987. Do nutrients translocate from fine roots? *Canadian Journal of Forest Research* 17: 913-918.

Nierop KG, Verstraten JM, Tietema A, Westerveld JW, Wartenbergh PE. 2006. Short-and long-term tannin induced carbon, nitrogen and phosphorus dynamics in Corsican pine litter. *Biogeochemistry* 79: 275-296.

Northup RR, Yu Z, Dahlgren RA, Vogt KA. 1995. Polyphenol control of nitrogen release from pine litter. *Nature* 377: 227-229.

Ostertag R, Hobbie SE. 1999. Early stages of root and leaf decomposition in Hawaiian forests: effects of nutrient availability. *Oecologia* 121: 564-573.

Paul EA. 2006. *Soil microbiology, ecology and biochemistry.* San Diego, USA: Academic press.

Pregitzer KS, DeForest JL, Burton AJ, Allen MF, Ruess RW, Hendrick RL. 2002. Fine root architecture of nine North American trees. *Ecological Monographs* 72: 293-309.

Pregitzer KS, Burton AJ, Zak DR, Talhelm AF. 2008. Simulated chronic nitrogen deposition increases carbon storage in Northern Temperate forests. *Global Chang Biology* 14: 142-153.

Preston CM, Trofymow J, Niu J, Sayer BG. 1997. ^{13}C nuclear magnetic resonance spectroscopy with cross-polarization and magic-angle spinning investigation of the proximate-analysis fractions used to assess litter quality in decomposition studies. *Canadian Journal of Botany* 75: 1601-1613.

Preston CM, Trofymow J, the Canadian Intersite Decomposition Experiment Working Group. 2000. Variability in litter quality and its relationship to litter decay in Canadian forests. *Canadian Journal of Botany* 78: 1269-1287.

Preston CM, Nault JR, Trofymow J. 2009. Chemical changes during 6 years of decomposition of 11 litters in some Canadian forest sites. Part 2. ^{13}C abundance, solid-state ^{13}C NMR spectroscopy and the meaning of "lignin". *Ecosystems* 12: 1078-1102.

Raich J, Schlesinger WH. 1992. The global carbon dioxide flux in soil respiration and its relationship to vegetation and climate. *Tellus B* 44: 81-99.

Rasse DP, Rumpel C, Dignac M-F. 2005. Is soil carbon mostly root carbon? Mechanisms for a specific stabilisation. *Plant and soil* 269: 341-356.

Sariyildiz T, Anderson JM. 2003. Interactions between litter quality, decomposition and soil fertility: a laboratory study. *Soil Biology & Biochemistry* 35: 391-399.

Schimel JP, Cates RG, Ruess R. 1998. The role of balsam poplar secondary chemicals in controlling soil nutrient dynamics through succession in the Alaskan taiga. *Biogeochemistry* 42: 221-234.

Schmidt MW, Torn MS, Abiven S, Dittmar T, Guggenberger G, Janssens IA, Kleber M, Kogel-Knabner I, Lehmann J, Manning DA *et al*. 2011. Persistence of soil organic matter as an ecosystem property. *Nature* 478: 49-56.

Singleton VL, Orthofer R, Lamuela-Raventos RM. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods in enzymology* 299:152-178.

Six J, Conant RT, Paul EA, Paustian K. 2002. Stabilization mechanisms of soil organic matter: implications for C-saturation of soils. *Plant and soil* 241: 155-176.

Smedes F, Thomasen TK. 1996. Evaluation of the Bligh & Dyer lipid determination method. *Marine Pollution Bulletin* 32: 681-688.

Solly EF, Schöning I, Boch S, Kandeler E, Marhan S, Michalzik B, Schrumpf M. 2014. Factors controlling decomposition rates of fine root litter in temperate forests and grasslands. *Plant and Soil* 382: 203-218.

Stott DE, Kassim G, Jarrell W, Martin J, Haider K. 1983. Stabilization and incorporation into biomass of specific plant carbons during biodegradation in soil. *Plant and soil* 70: 15-26.

Sun T, Mao Z, Han Y. 2013. Slow decomposition of very fine roots and some factors controlling the process: a 4-year experiment in four temperate tree species. *Plant and soil* 372: 445-458.

Talhelm AF, Pregitzer KS, Burton AJ. 2011. No evidence that chronic nitrogen additions increase photosynthesis in mature sugar maple forests. *Ecol. Appl.* 21: 2413-2424.

Talhelm AF, Pregitzer KS, Burton AJ, Zak DR. 2012. Air pollution and the changing biogeochemistry of northern forests. *Frontiers in Ecology and the Environment* 10: 181–185.

Talhelm AF, Burton AJ, Pregitzer KS, Campione MA. 2013. Chronic nitrogen deposition reduces the abundance of dominant forest understory and groundcover species. *Forest Ecology and Management* 293: 39-48.

Taylor BR, Parkinson D, Parsons WF. 1989. Nitrogen and lignin content as predictors of litter decay rates: a microcosm test. *Ecology* 70: 97-104.

Taylor BR, Prescott CE, Parsons W, Parkinson D. 1991. Substrate control of litter decomposition in four Rocky Mountain coniferous forests. *Canadian Journal of Botany* 69: 2242-2250.

Triebwasser DJ, Tharayil N, Preston CM, Gerard PD. 2012. The susceptibility of soil enzymes to inhibition by leaf litter tannins is dependent on the tannin chemistry, enzyme class and vegetation history. *New Phytologist* 196: 1122-1132.

Tschaplinski T, D Stewart, Norby R. 1995. Interactions between drought and elevated CO_2 on osmotic adjustment and solute concentrations of tree seedlings. *New Phytologist* 131: 169-177.

Ushio M, Balser TC, Kitayama K. 2013. Effects of condensed tannins in conifer leaves on the composition and activity of the soil microbial community in a tropical montane forest. *Plant and soil* 365: 157-170.

Valenzuela-Estrada LR, Vera-Caraballo V, Ruth LE, Eissenstat DM. 2008. Root anatomy, morphology, and longevity among root orders in *Vaccinium corymbosum* (Ericaceae*). American Journal of Botany* 95: 1506-1514.

van Diepen LT, Lilleskov EA, Pregitzer KS, Miller RM. 2010. Simulated nitrogen deposition causes a decline of intra-and extraradical abundance of arbuscular mycorrhizal fungi and changes in microbial community structure in northern hardwood forests. *Ecosystems* 13: 683-695.

van Huysen TL, Harmon ME, Perakis SS, Chen H. 2013. Decomposition and nitrogen dynamics of ^{15}N-labeled leaf, root, and twig litter in temperate coniferous forests. *Oecologia* 173: 1563-1573.

Vivanco L, Austin AT. 2006. Intrinsic effects of species on leaf litter and root decomposition: a comparison of temperate grasses from North and South America. *Oecologia* 150: 97-107.

Waid JS. 1974. Decomposition of roots. In: Dickinson CH and Pugh GJF, eds. *Biology of Plant Litter Decomposition.* Volume 1. pp. 175–211. Academic Press, London UK.

Waldrop MP, Firestone MK. 2004. Microbial community utilization of recalcitrant and simple carbon compounds: impact of oak-woodland plant communities. *Oecologia* 138: 275-284.

Wardle DA, Bonner KI, Barker GM. 2002. Linkages between plant litter decomposition, litter quality, and vegetation responses to herbivores. *Functional Ecology* 16: 585-595.

Wickings K, Grandy AS, Reed SC, Cleveland CC. 2012. The origin of litter chemical complexity during decomposition. *Ecology letters* 15: 1180-1188.

Xia MX, Guo DL, Pregitzer KS. 2010. Ephemeral root modules in *Fraxinus mandshurica*. *New Phytologist* 188: 1065-1074.

Yu Z, Dahlgren RA. 2000. Evaluation of methods for measuring polyphenols in conifer foliage. *Journal of chemical ecology* 26: 2119-2140.

Zak DR, Holmes WE, Burton AJ, Pregitzer KS, Talhelm AF. 2008. Simulated atmospheric NO_3^- deposition increases soil organic matter by slowing decomposition. *Ecol. Appl.* 18: 2016-2027.

Zhang D, Hui D, Luo Y, Zhou G. 2008. Rates of litter decomposition in terrestrial ecosystems: global patterns and controlling factors. *Journal of Plant Ecology* 1: 85-93.

Table 1.1 Location, climate, and edaphic characteristics of the four northern hardwood forest study sites

Site characteristic	Site A	Site B	Site C	Site D
Latitude (N)	46°52’	45°33’	44°23’	43°40’
Longitude (W)	88°53’	84°51’	85°50’	86°09’
Mean annual precipitation (mm)[a]	873	871	888	812
Mean annual temperature (°C)[a]	4.7	6.0	6.9	7.6
Ambient wet + dry N deposition (g N m^{-2} yr^{-1}) [b]	0.68	0.91	1.17	1.18
Growing season length (days)[a]	134	150	154	157
Total basal area (m^2 ha^{-1})[c]	34	31	32	33
Sugar maple basal area (%)[c]	86	86	83	75
Overstory age (2016)	109	103	104	108
Ambient soil carbon content (0-70 cm, g m^{-2})[a]	8341	9259	7841	7470
O_{e+a}	350	625	720	640
0-10 cm soil depth	2427	3126	2560	2113
Soil texture, 0-10 cm depth (%sand-%silt-%clay)[a]	75-22-3	89-9-2	89-9-2	87-10-3
Soil texture, 10-70 cm depth (%sand-%silt-%clay)[a]	84-11-5	88-7-5	91-6-3	92-5-3

[a] Pregitzer *et al.* (2008); [b] MacDonald *et al.* (1992). [c] Burton *et al.* (2000)

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Table 1.2 Major biochemical components and litter quality indices of leaf litter and fine roots averaged across the four hardwood forest study sites receiving simulated nitrogen (N) deposition

Chemical characteristics	Leaf litter		Fine roots		Main effects
	Ambient	N deposition	Ambient	N deposition	
Cell-wall fraction (%)	68.2^{b} (7.6)	62.0^{a} (8.3)	83.6^{c} (1.2)	84.4^{c} (2.2)	Type, N
AIF	15.2^{b} (1.0)	14.0^{a} (1.0)	45.1^{c} (2.0)	45.8^{c} (1.2)	Type, Site, N
Hemicellulose	14.1^{ab} (1.8)	13.8^{a} (1.3)	15.8^{c} (1.3)	15.7^{bc} (1.1)	Type
Cellulose	38.9^{b} (5.9)	34.2^{b} (6.8)	22.7^{a} (2.8)	22.9^{a} (1.9)	Type
Extractable fraction (%)	31.8^{b} (7.6)	38.0^{c} (8.3)	16.4^{a} (1.2)	15.6^{a} (2.2)	Type
Soluble phenolics	12.1^{b} (2.2)	12.5^{b} (2.1)	3.9^{a} (0.6)	3.7^{a} (0.9)	Type
Condensed tannins†	5.7^{a} (2.5)	4.2^{a} (1.7)	13.6^{b} (1.9)	12.4^{b} (2.8)	Type, N
NSCs	4.40^{b} (0.55)	4.94^{b} (1.11)	1.87^{a} (0.22)	1.84^{a} (0.34)	Type, Site
Lipids	7.94^{b} (1.24)	7.85^{b} (0.62)	3.60^{a} (0.45)	3.41^{a} (0.32)	Type, Site
Soluble proteins	1.11^{a} (0.20)	1.00^{a} (0.20)	3.28^{b} (0.40)	2.95^{b} (0.38)	Type, N
Unidentified‡	6.25^{a} (4.55)	11.75^{b} (5.79)	3.71^{a} (0.84)	3.68^{a} (1.02)	Type
N (%)	0.65^{a} (0.05)	0.81^{b} (0.18)	1.55^{c} (0.18)	1.64^{c} (0.13)	Type, Site, N
Litter quality indices (ratio)					
AIF/N	23.6^{b} (2.2)	18.1^{a} (3.58)	29.5^{c} (3.5)	28.1^{c} (2.4)	Type, Site, N
C/N	75.9^{c} (6.6)	63.3^{b} (13.3)	33.4^{a} (4.0)	31.7^{a} (3.0)	Type, Site, N
Lignocellulose index	0.22^{a} (0.02)	0.23^{a} (0.02)	0.54^{b} (0.02)	0.54^{b} (0.01)	Type

Values are means (SD) of three replicated plots for each treatment at each of four sites (n = 12). Different letters in the same row indicate significant differences ($P < 0.05$). Significant main effects are shown ($P < 0.05$), with full statistical results in Appendix E. AIF: acid-insoluble fraction; NSCs: non-structural carbohydrates. †Condensed tannins (CTs) are a subset of plant phenolics. There is no generally-accepted CT standard for the acid-butanol assays used to determine CTs. Thus, the CT concentrations reported here should be interpreted more as relative comparisons between fine roots and leaf litter than absolute quantification. Extractive and bound CTs were separately reported in Appendix F. Bound tannins could be double-counted in AIF in this table, however, bound CTs only represented 11.8 % and 20.9 % of total CTs by average in fine roots and leaf litter respectively. ‡Unidentified portion is the difference between extractable fraction and the sum of soluble phenolics, non-structural carbohydrates, lipids, and soluble proteins.

Table 1.3 Litter production across the four hardwood forest study sites

Litter production ($g\ m^{-2}\ yr^{-1}$)		Site A	Site B	Site C	Site D
Leaf litter	Ambient	324.0 (10.4)	361.9 (12.7)	399.7 (15.0)	415.2 (39.0)
	N deposition	325.7 (15.7)	376.3 (3.7)	397.9 (25.4)	447.1 (30.2)
Fine roots	Ambient	372.2 (22.6)	285.2 (30.6)	233.7 (54.9)	404.6 (124.3)
	N deposition	420.1 (10.6)	291.0 (62.7)	175.1 (22.7)	368.6 (60.1)
Total litter	Ambient	794.1 (17.0)	687.4 (43.7)	737.6 (37.1)	931.4 (114.5)
	N deposition	839.5 (27.6)	707.4 (53.1)	667.9 (50.3)	920.1 (92.8)

Values are means (SD) of three ambient plots and three N treatment plots for each site (n = 3). Total litter production was the average of total aboveground litter (leaf litter, reproductive litter, and woody debris) from 1988 to 2011 for ambient plots and 1994 to 2011 for N treatment plots, plus the corresponding fine root litter production. Source data are available in the Michigan Nitrogen Deposition Gradient Study database, http://webpages.uidaho.edu/nitrogen-gradient. Simulated N deposition had no effects on estimates of leaf litter, fine root, or total litter production ($P > 0.05$), while leaf litter, fine root, and total litter production varied among sites ($P < 0.001$, Appendix H).

Table 1.4 Mean flux (g m^{-2} yr^{-1}) of each biochemical class to soil via leaf litter, fine roots, and their sum, followed by the proportion (%) of the combined flux of leaf litter and fine root flux contributed by fine roots

Biochemical class	Leaf litter flux		Fine root flux		Sum		Fine root (%)	
	Ambient	N	Ambient	N	Ambient	N	Ambient	N
AIF	56.7[a] (5.6)	54.5[a] (9.7)	146.0[b] (44.5)	143.2[b] (46.7)	202.8 (43.9)	197.7 (46.4)	71.0 (6.0)	70.9 (8.5)
Hemicellulose	52.7[a] (7.9)	53.7[a] (9.7)	51.5[a] (17.7)	49.3[a] (16.3)	104.2 (17.8)	102.9 (20.2)	48.5 (8.9)	46.8 (9.5)
Cellulose	145.6[b] (23.2)	134.3[b] (41.5)	72.5[a] (18.1)	70.9[a] (21.6)	218.1 (25.0)	205.2 (52.6)	33.3 (8.1)	34.8 (8.9)
Soluble phenolics	45.3[b] (7.9)	47.7[b] (5.0)	13.1[a] (5.6)	12.1[a] (5.6)	58.4 (9.9)	59.9 (4.7)	22.2 (7.0)	20.1 (8.6)
Condensed tannins¶	20.9[a] (7.8)	16.4[a] (6.6)	45.0[b] (17.0)	40.9[b] (20.5)	65.9 (18.4)	57.3[(*)] (16.8)	67.3 (12.0)	67.6 (18.8)
Non-structural carbohydrates	16.5[b] (2.6)	18.7[b] (2.7)	6.1[a] (2.0)	5.9[a] (2.4)	22.6 (3.4)	24.6[(*)] (3.0)	26.9 (6.9)	23.7 (8.2)
Lipids	29.6[b] (4.5)	30.3[b] (4.3)	11.9[a] (4.4)	10.8[a] (4.0)	41.5 (6.9)	41.1 (6.1)	28.3 (7.4)	25.8 (8.0)
Soluble proteins	4.1[a] (0.6)	3.9[a] (0.9)	10.5[b] (2.5)	9.4[b] (3.8)	14.6 (2.7)	13.3[(*)] (3.3)	71.1 (6.6)	68.3 (13.3)
Nitrogen	2.4[a] (0.3)	3.1[b] (0.5)	4.9[c] (1.0)	5.0[c] (1.4)	7.3 (1.0)	8.1[(*)] (1.7)	66.4 (6.1)	61.2 (7.2)

Biochemical fluxes and proportions are shown as means (SD) of three ambient plots or three simulated N deposition plots from four sites (n = 12). Different letters in the same row indicate significant differences ($P < 0.05$). Marginally significant effects of N deposition on the combined flux of leaf litter and fine root flux at $P < 0.1$ (Appendix J) are denoted with **(*)**. AIF: acid-insoluble fraction. ¶ Although *post hoc* tests did not show any significant differences of CT flux induced by simulated N deposition for either tissue type, N deposition was a significant main effect on the flux of CTs in the overall *F*-statistics test ($P = 0.036$, Appendix I).

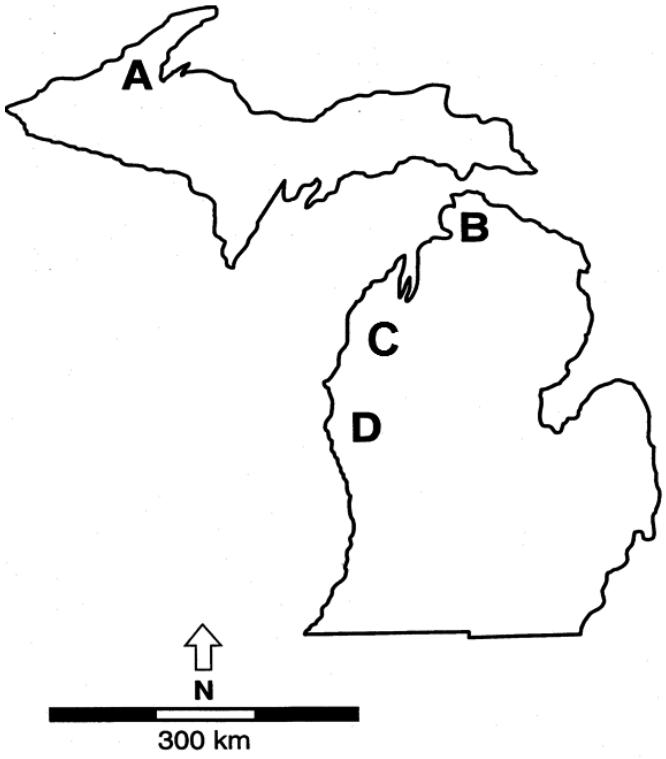

Fig. 1.1 Site locations of the four northern hardwood forest study sites.

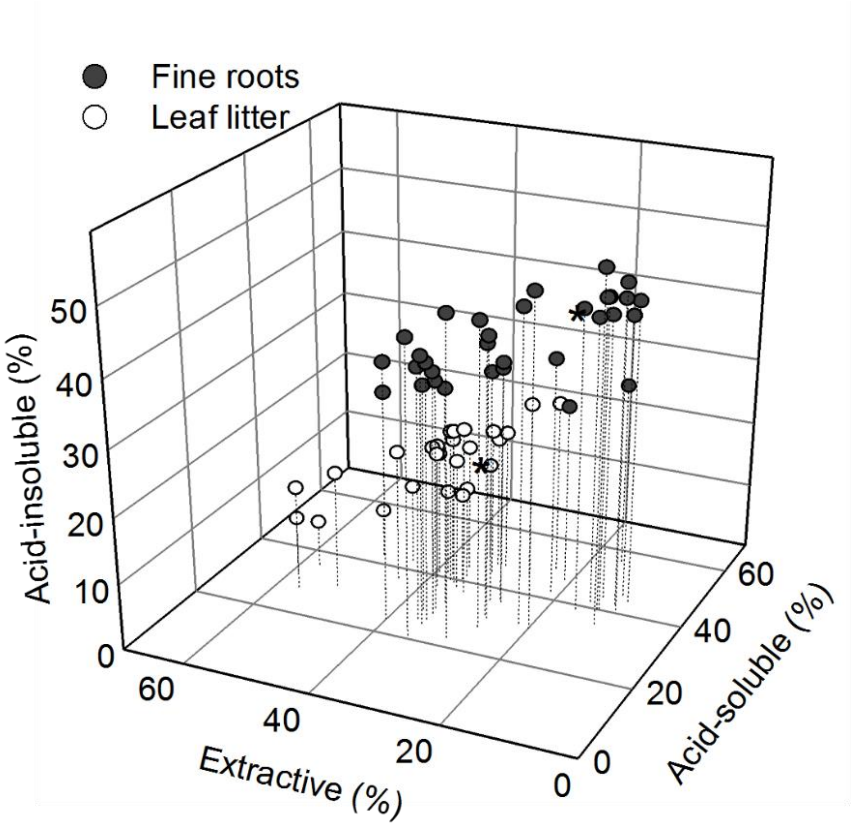

Fig. 1.2 Proximate fractions of leaf litter and fine roots taken from published data across a number of boreal and temperate tree species. Extractive fraction includes relatively labile compounds consisting of both polar and non-polar constituents. The acid-soluble fraction approximates structural polysaccharides and the acid-insoluble fraction includes lignin and other highly complex substrates such as cutin, suberin, and complexes formed between condensed tannins and proteins. Each dot denotes the proximate fractions of a specific species/genus or functional group (*e.g.* a hardwood stand) in an individual study. This synthesis includes more than 30 species from 14 genera. The data-points from our study are emphasized with '*'. The means (SD) for three fractions in fine roots are: extractive: 24.4 (9.3), acid-soluble: 33.6 (7.8), acid-insoluble: 41.9 (6.9), with n = 34. The means (SD) for three fractions in leaf litter are: extractive: 39.3 (9.0), acid-soluble: 40.6 (5.5), acid-insoluble: 18.4 (5.6), with n = 26. Data references: McClaugherty *et al.* (1985); Taylor *et al.* (1989); Hendricks *et al.* (2000); Guo *et al.* (2004); Hobbie (2005); Bird & Torn (2006); Harmon *et al.* (2009); Hobbie *et al.* (2010); Solly *et al.* (2014); Sun *et al.* (2013)

Chapter 2 Differential Effects of Simulated Nitrogen Deposition on Leaf and Fine Root Decomposition Rates and Consequences for Soil Carbon Accumulation

2.1 Abstract

- Atmospheric nitrogen deposition increases forest carbon sequestration across broad parts of the Northern Hemisphere. In addition to more rapid tree growth, this increase in carbon sequestration could also be caused by slower organic matter decomposition and increased soil carbon accumulation.
- We studied the effects of long-term (>15 years) simulated nitrogen deposition on litter decomposition at four hardwood forests in the north-central USA. At these sites, we previously observed that nitrogen additions increased soil organic carbon and altered litter chemistry. To understand the extent to which nitrogen additions altered decomposition and disentangled the effects of altered substrate chemistry and increased exogenous nitrogen availability, we deployed litterbags containing sugar maple (*Acer saccharum* Marsh.) leaf litter and fine roots for up to three years. We used double-exponential decay models to describe litter mass loss. Annual litter input of leaves and fine roots were combined with the observed and model-projected decomposition patterns to estimate how fine root and leaf litter contribute to soil organic matter.
- We found that nitrogen additions stimulated early-stage decomposition of leaf litter, an effect associated with previously documented changes in litter chemistry toward more labile constituents. In contrast, nitrogen additions did not affect the early decomposition of fine roots, but inhibited the later stages of decomposition. This late-stage effect is consistent with observed decreases in lignin-degrading enzyme activities with chronic nitrogen additions at these sites. We estimate that chronic nitrogen additions cause a 23.8 % additional retention in the amount (g m^{-2}) of fine root mass remaining after the six years of decomposition.
- *Synthesis*. Our results demonstrated that simulated nitrogen deposition altered two different parts of the decomposition process, creating contrasting effects on mass loss in leaf litter and fine roots.

Although previous nitrogen deposition studies have focused on leaf litter, our work suggests that slower fine root decomposition is a major driver of soil organic mass accumulation under elevated nitrogen deposition.

2.2 Introduction

Human activities currently convert more atmospheric nitrogen (N) gas to biologically-active forms of N than all natural processes combined (Gruber & Galloway 2008), and the potential of this anthropogenic N to alter biogeochemical cycles has raised world-wide research interest (e.g., Erisman *et al.* 2013; Fowler *et al.* 2013). Investigations across boreal and temperate forests in Western Europe and North America have shown that forest carbon (C) sequestration is strongly influenced by N deposition (Nadelhoffer *et al.*, 1999; Sutton *et al.,* 2008). Because N availability limits plant productivity in most terrestrial ecosystems (Vitousek *et al* 1997; LeBauer & Treseder 2008), greater tree growth due to higher N availability appears to contribute to the N deposition-induced C sink. However, forest soil also represents a large C pool in boreal and temperate biomes (Pregitzer & Euskirchen 2004) that could be sensitive to anthropogenic N deposition (*e.g.* Yue *et al.* 2016). Indeed, several chronic N deposition studies in northern temperate forests in Europe and North America have observed increased soil organic C storage (Franklin *et al.*, 2004; Hyvonen 2008; Pregitzer *et al.*, 2008; Zak *et al.*, 2008; Frey *et al.*, 2014, but Magill *et al.*, 1998). Given these observations, knowledge of how and why soil C pools respond to added N is crucial for understanding the extent to which terrestrial C cycling is altered by N deposition.

At four northern temperate forests in the north-central USA, simulated atmospheric NO_3^- deposition has been applied to replicated plots as part of the Michigan Gradient Study (MGS) since 1994. One of the major observations from this experiment is that long-term simulated NO_3^- deposition increased the C pool in the soil organic horizons and surface mineral soil by ~ 26% (Pregitzer *et al.*, 2008; Zak *et al.*, 2008). This increase in soil C content occurred without an increase in litter input, providing strong evidence for slower turnover of organic C under simulated N deposition (Zak *et al.*,

2008). This field observation is accompanied by evidence indicating that N additions can suppress heterotrophic respiration and decomposition in forest soils (e.g., Knorr *et al*., 2005; Janssens *et al.*, 2010).

Elevated N deposition could affect litter decomposition by altering initial litter chemistry, increasing soil N availability, or, via both mechanisms. Our previous work at MGS sites observed that simulated N deposition decreased concentrations of acid-insoluble fraction (AIF), AIF/N ratios, and condensed tannins in leaf litter (Xia *et al.*, 2015, also see Chapter 1). These litter quality metrics have been associated with slower initial rates of litter decomposition (Taylor *et al.*, 1989; Berg, 2000; Amin *et al.*, 2014; Coq *et al.*, 2010). In contrast, these chemical properties had little or no response to simulated N deposition in fine roots (Xia *et al.*, 2015, also see Chapter 1). Thus, it is possible that simulated N deposition has the altered chemical composition of leaf litter so that it decomposes more quickly than litter produced under ambient conditions.

The effects of substrate quality are complicated by external N availability, which could either enhance or counteract the effects of substrate quality on decomposition. Knorr *et al.* (2005) conducted a meta-analysis on the effects of N additions on leaf litter decomposition using 24 studies. The results indicated that added N inhibited decomposition when ambient N deposition was relatively high (5 – 10 kg N ha^{-1} yr^{-1}), when litter was lignin-rich, and, when experiments lasted more than two years, while added N stimulated decomposition when ambient N deposition was low, among lignin-poor litter substrates, and in studies lasting less than two years (Knorr *et al.*, 2005). Individual decomposition studies lasting more than five years have also found that added N stimulated initial decomposition, but inhibited the later stages of decomposition that were dominated by lignin degradation (Hobbie *et al.*, 2012; Sun *et al.*, 2016), with these inhibitory effects occurring regardless of the initial substrate lignin concentration (Hobbie 2008). Two major mechanisms have been proposed for the inhibition effect of exogenous N on litter decomposition (Fog 1988): N may react with lignin-degradation by-products (specifically polyphenolics) to form recalcitrant complexes; alternately, elevated soil N availability may repress the activity of extra-cellular enzymes involved in

lignin degradation. At MSG forests, simulated N deposition has decreased total microbial biomass, altered microbial community composition, and decreased the activity of two lignin-degrading extra-cellular enzymes: phenol oxidase and peroxidase activity (DeForest *et al* 2004; Edwards *et al.*, 2011). These changes in microbial community function suggest that suppression of lignin-degrading metabolism by increased external N availability could be slowing late stage decomposition at MGS sites.

The widely observed suppression of litter decomposition among lignin-rich substrates by added N (Berg & Matzner 1997; Carreiro *et al*. 2000; Sinsabaugh *et al.*, 2002; Knorr *et al.*, 2005; Janssens *et al.*, 2010; but Hobbie 2000) implies that N deposition may have different effects on leaf litter and fine root decomposition. Although decomposing fine roots and leaves each are sites of high metabolic activity and represent large litter fluxes, they have strong differences in chemistry that are consistent across temperate and boreal forests (Xia *et al.*2015, also see Chapter 1), including at the MGS sites (Xia *et al.* 2015, also see Chapter 1). Compared to leaf litter, fine roots are often lignin-rich materials (Rasse *et al.*, 2005). At MGS sites, fine roots contain 2.9-fold more abundant acid-insoluble fraction (AIF) than leaf litter. This AIF material is slow to decompose and has frequently been referred to as lignin, but also contains other recalcitrant substrates such as suberin and condensed tannin-protein complexes (Preston *et al.*, 1997). Therefore, N additions are likely to have stronger inhibitory effects on decomposition of fine roots than leaf litter. Because the annual fluxes of fine root and leaf litter are comparable in size at MGS sites and the fine roots contain substantially more AIF (Xia *et al.*, 2015, also see Chapter 1), it is possible that fine roots are the major driver of slower organic matter turnover under simulated N deposition.

The objective of this study was to compare the effects of simulated N deposition on leaf litter and fine root decomposition. We hypothesized that both altered substrate biochemistry and elevated soil N availability shape the overall effects of simulated N deposition on litter decomposition, but, their roles are different when comparing leaf litter and fine roots. Specifically, we hypothesized that N additions stimulate the decomposition of leaf litter because long-term N additions have caused

changes in litter chemistry that make leaves easier to degrade (Xia *et al.* 2015, also see Chapter 1) and because added N has been shown to stimulate the decomposition of "high quality" litter (*e.g.* Knorr *et al.* 2005). In contrast, we hypothesize that simulated N deposition will strongly inhibit the decomposition of fine roots, which are comparatively high in lignin. We tested the effects of simulated N deposition on leaf litter and fine root decomposition by conducting a three-year *in situ* decomposition study across the four MGS sites. We investigated the relationship between initial substrate chemical composition and decomposition rates for leaf litter and fine roots. Further, fine roots collected from ambient and NO_3^- amended plots were decomposed both *in situ* and in the plots of the opposite N treatment in order to disentangle the effects of substrate chemistry and soil N availability on litter decomposition. By combining annual litter input rates with estimates of litter mass loss, we were able to make ecosystem-scale estimates of how simulated N deposition affected the mass retention per unit area of fine root and leaf litter in the soil.

2.3 Materials and Methods

See 1.3 Materials and Methods in Chapter 1 for site information (1.3.1), leaf litter and fine root sampling (1.3.2), and chemical analysis (1.3.3).

2.3.1 Decomposition study

About ~1 g leaf litter or fine roots were weighed and sealed into 20 cm x 20 cm polyester litterbags. The mesh sizes of litter bags were 20 μm on the bottom and 300 μm on the top. The bottom mesh allowed the fungal hyphae to penetrate into the bags while minimizing the physical loss of decomposing plant debris (Hobbie 2005); the top mesh size permitted entry of most soil micro-fauna, but excluded entry of most of meso- and macro- fauna (Bradford *et al.*, 2002). Thus, this study mostly focused on the decomposition process driven by microorganisms, and caution remains as to the potential effects of soil animals on decomposition in this study.

Litter bags were returned to each of four MGS sites for a three-year decomposition study starting in July 2011. Leaf litter was deployed *in situ* to represent native leaf litter fall. Specifically, the leaf litter collected from three plots of a treatment at each site were homogenized and deployed to those three plots. Leaf litter bags were placed flat on the top of the forest floor. Additional litterbags with leaf litter were also placed at the interface between mineral soil and organic soil horizons, *i.e.* O/A interface, to rule out environmental effects in the differences in leaf litter and fine root decomposition. Fine roots collected from ambient and simulated N deposition plots were deployed at O/A interface both *in situ* and in the plots of the alternate treatment. Thus, two types of fine root litter bags were deployed at each plot: one set of bags filled with fine roots collected from that plot and one set of bags filled with roots collected from another plot at that same site receiving the opposite N treatment. Because root mortality occurs relatively evenly throughout growing season at these sites (Burton *et al.,* 2000), both autumn and spring roots were decomposed to better characterize the root decomposition pattern within a plot. Autumn and spring roots of a treatment type were placed in separate litterbags in a plot; the final mass remaining of that plot was calculated as the mean mass loss (%) of these two litterbags. Taken together, we had six types of litterbags (two leaf litters and four fine roots) in each plot at each site, with each type having 18 replicate bags in one plot.

Three bag replicates of each litter bag type in each plot were harvested after periods of one month to three years (August 2011, November 2011 August 2012, August 2013, and August 2014). Harvested bags were flash-frozen in liquid N_2 and transported to the University of Idaho on dry ice. We removed sample material from the litter bags, and cleaned them of soil, new root growth, and animal necromass using forceps. Decomposing samples were then freeze-dried and weighed. Mass loss for a litter bag was calculated as the difference between the oven-dry mass of initial substrate and decomposed substrate after harvest on the ash-free basis (500 °C for four hours). Plots are the replicates in the data analysis, so we averaged the mass remaining (%) of three bag replicates from one plot.

2.3.2 Data analysis

We evaluated the decomposition of leaf litter and fine roots using three commonly-used decomposition models (*e.g.* Weider & Lang 1982; Harmon *et al.*, 2009): a single exponential decay model developed by Olson (1963), $M_t = e^{-k_s t}$; a double exponential decay model from Hunt (1977), $M_t = Ae^{-k_1 t} + (1 - A)e^{-k_2 t}$; and an asymptotic model proposed by Howard and Howard (1974), $M_t = (1 - S)e^{-k_a t} + S$. In these models, M_t is the percentage of mass remaining at time *t* (year) and *k* is the decomposition rate of a certain substrate fraction. In single exponent models, the whole substrate decomposes at the rate k_s. In double exponent models, *A* is the kinetically-defined active fraction of the substrate that decomposes at a high rate k_1, while (1-*A*) is the fraction with a slower decomposition rate k_2. In asymptotic models, *S* represents a completely stable fraction with a decomposition rate of zero, while (*1*-*S*) has a decomposition rate k_a. These models make different biological assumptions about the decomposition process (Weider & Lang 1982). Single exponent models assume that substrates decompose at a constant decomposition rate. Both double exponent models and asymptotic models assume that substrates decompose as two pools, but asymptotic models assume that the slow pool is completely stable. The goodness of fit was estimated with adjusted coefficient of determination (R^2, n = 6) for each replicate of a litter type in the site × external N availability × substrate source combination. In addition, we used a site-specific AIC_C (n = 72) for each litter type to evaluate model performance with a penalty for the number of model parameters (Hurvich & Tsai 1989). When the overall "best-fit" model was determined, we fitted each experiment replicate to this model to estimate individual decomposition parameters. The active pool (*A*) in double exponential models is considered an initial litter trait (*sensu* Wider & Lang 1982), thus models of decomposition patterns of a litter type collected from the same plot were constrained to have the same *A* value (Table 2.1).

The effects of simulated N deposition on leaf litter and fine root decomposition were evaluated using model-fitted decomposition rates (y^{-1}) and final proportions of mass remaining after

three years. Because the effects of N additions may only be manifest in the later stage of decomposition, and be more pronounced beyond three years, we also extrapolated the mass remaining beyond the study period using the overall "best-fit" model at the plot level. Aber *et al.* (1990) stated that extrapolation of exponential decomposition models was valid until litter decomposition shifted to a more stable phase (phase II as in Aber *et al.,* 1989) when 13.8 to 26.1 % of initial leaf litter mass remained and ~20 % of fine root mass remained (Aber *et al.,* 1989). Previous long-term decomposition studies in similar temperate forests showed that decomposing leaf litter mass reached ~ 20 % and seemed to be stable or even increase after five to six years (Adair *et al.,* 2008; Harmon *et al.,* 2009). Further decomposition of leaf litter may be limited by physio-chemical interactions between organic matter and soil minerals (*sensu* Schmidt *et al.,* 2011). Therefore, we extrapolated decomposition to six years. We understand that extrapolation to six years should be interpreted with caution. In addition, annual litter inputs are multiplied with proportions of mass remaining to estimate the quantities of mass remaining per area unit (g m^{-2}) of an annual litter cohort in each plot over time. Litter input data for both leaf litter and fine roots were estimated at the plot level (see Table 1.3 in Chapter 1).

A two-way ANOVA was used to test if these decomposition metrics were different among sites (df = 3) and N treatment (df = 1), separately for leaf litter and fine roots. Our previous work has shown that simulated N deposition affected initial litter biochemistry (Xia *et al.*, 2015, also see Chapter 1). Here, the initial chemical traits of each experimental unit were used as continuous variables to relate NO_3^- additions induced chemical changes to the variation in decomposition rates. We conducted an ANCOVA analysis on decomposition metrics with site as the main effect and each of the initial chemical traits as a covariate tested in a separate ANCOVA analysis. A common slope model of all sites was used because initial ANCOVA failed to reject the hypothesis that slopes were equal across all sites (Littell *et al.,* 2006). To further disentangle the effects of elevated external N availability and altered substrate biochemistry from overall simulated N deposition, we analyzed decomposition metrics of fine roots decomposed both *in situ* and in the opposite N treatment, *i.e.* with

factorial arrangements of substrate and external N availability. We tested if substrate source (litter collected from NO_3^- amended vs. ambient, *df*=1), external N availability (litterbags deployed in NO_3^- amended vs. ambient, *df*=1), and study sites (*df*=3) affect fine root decomposition metrics with a mixed linear model (Proc mixed, Littell *et al.*, 2006) in a split-plot design. Sites and external N treatment and their interactions (*df* = 3) were tested on whole-plot experimental units (plots in the combination of site and external N treatment), while substrate source is the within-plot factor.

2.4 Results

The goals of our study were to understand decomposition patterns in leaf litter and fine roots at four northern hardwood forest sites and how these patterns were altered by long-term simulated N deposition. Averaged across all treatments and sites, fine roots had the highest percentage of ash-free mass remaining after three years (51.29 to 56.40 %), followed by leaf litter decomposed on the forest floor (24.60 to 25.41 %), and leaf litter decomposed at the O/A interface (14.96 to 15.06 %; Fig. 2.1, Table 2.2). All three exponential models exhibited strong fits (R^2 > 80% in most cases), but double exponential models generally exhibited the "best" fit (Fig. 2.1), and the double exponential models showed smaller AICc (smaller the better) than the other two models at all sites for all litter types. Therefore, we described the decomposition patterns of leaf litter and fine roots using double exponential model parameters (A, k_1, k_2). Double exponential models had a mean R^2 fit of 0.984 for fine roots, 0.988 for leaf litter decomposed on the forest floor, and 0.984 for leaf litter decomposed in the soil. The mean kinetically-defined active pools (A) are 15.5 ± 3.3 % in fine roots, and 44.8 ± 6.3 % in leaf litter (Table 2.1). Leaf litter had significantly different A values among sites (F = 107.42, P = 0.002, tested by a two-way ANOVA, Table 2.1), A values in fine roots differed marginally among sites (F = 2.55, P = 0.092).

We used metrics of *in situ* leaf litter and fine roots decomposition to test the effects of simulated N deposition and to estimate the amount of mass remaining per area (g m^{-2}) of an annual litter cohort. The effects of simulated N deposition on leaf litter decomposition were generally minor

(Fig. 2.1, Tables 2.2, 2.3). Simulated N deposition did not affect *A* values for leaf litter and fine roots (F = 0.26, P = 0.614; F = 2.6, P = 0.208; respectively), indicating no change in the kinetically-defined active pool size in both litter types. Simulated N deposition marginally stimulated the decomposition rates of the active pool (k_1) of leaf litter decomposed on the forest floor (5.17 to 5.74 y^{-1}, F = 3.46, P = 0.081, Table 2.2, 2.3), but there was no effect on the slow pool decomposition rate for leaf litter (k_2, F = 0.03, P = 0.870, Fig. 2.1, Table 2.2, 2.3) and no effect on the decomposition rate of either pool for leaf litter decomposed at the O/A interface (P > 0.376, Fig. 2.1, Table 2.2, 2.3). After three years of decomposition, simulated N deposition did not significantly alter the final proportion of the remaining leaf litter mass, either on the forest floor or at the O/A interface (P > 0.693, Fig. 2.1, Table 2.2, 2.3). When projected at the plot level to six years of decomposition using double exponential models, the proportions of leaf litter mass remaining, either decomposed on the forest floor or at the O/A interface, were not significantly affected by simulated N deposition (P > 0.444, Fig. 2.1, Table 2.2, 2.3). In contrast, simulated N deposition significantly decreased the decomposition rates of slow pools in fine roots (k_2, 0.175 to 0.144 y^{-1}, F = 12.3, P = 0.003, Fig. 2.1, Table 2.2, 2.3) and increased the final proportion of mass remaining from 51.29 to 56.40 % averaged across four sites (F = 21.2, P < 0.001, Table 2.2, 2.3). However, the increase of final mass remaining was less pronounced at site C than other sites (site × NO_3^-: F = 2.72, P = 0.079). When projected to six years of decomposition, simulated N deposition increased the proportion of fine root mass remaining from 29.7 to 36.0 % (F = 15.8, P = 0.001, Table 2.2, 2.3). To further test if N additions selectively preserved fine roots rather than leaf litter, we also conducted a two-way ANOVA (Site × NO_3^-) on the differences in the proportion of mass remaining between leaf litter (forest floor) and fine roots (% $MR_{R\text{-}L}$, Table 5). Here, simulated N deposition significantly enlarged the difference between mass remaining of leaf litter and fine roots (F = 5.3, P = 0.035, Table 2.4).

The initial chemical composition of both leaf litter and fine roots varied among sites and as a result of the NO_3^- amendments (Table 2.1; full results provided in Chapter 1). We used the initial chemical traits of each experimental unit as continuous variables to describe variation in

decomposition metrics. For leaf litter decomposed in the forest floor, we found that soluble phenolics concentrations were negatively correlated with k_1 values (ANCOVA, $P = 0.012$, Table 2.2), while leaf litter N concentrations were positively correlated with k_1 values ($P = 0.036$, Table 2.2). Notably, the soluble phenolics and substrate N were not significantly correlated ($F = 1.03$, $P = 0.348$, data not shown). In contrast, no initial chemical traits had significant relationships with fine root decomposition metrics (ANCOVA, $P > 0.380$, Table 2.2).

To further disentangle the effects of elevated external N availability and altered substrate biochemistry on fine root decomposition, fine roots were decomposed both *in situ* and in the opposite N treatment. Elevated external N availability significantly decreased the later stage decomposition rate (k_2, $F = 8.21$, $P = 0.011$, Table 2.5), while substrate source (collected from ambient vs. NO_3^- amended plots) had no effects on k_2 values ($F = 2.92$, $P = 0.107$, Table 2.5). Moreover, elevated external N availability significantly increased the final proportions of mass remaining after three years for fine roots ($F = 15.74$, $P = 0.001$, Table 2.5). Substrate source also showed effects on the proportions of mass remaining, but to a lesser degree ($F = 5.42$, $P = 0.033$, Table 2.5). In addition, fine roots collected from NO_3^- amended plots had a higher proportion of mass remaining at all sites except site C (Site × Substrate source, $F = 2.67$, $P = 0.083$, Table 2.5). To investigate which biochemical traits were related to the substrate effects on fine roots mass remaining, we conducted an alternative ANCOVA, with site and external N availability as main effects and each of the chemical traits as covariates on the proportions of the fine root mass remaining. However, none of the chemical traits listed in Table 2.1 had significant effects on the mass remaining (ANCOVA, $P > 0.235$, data not shown).

At the ecosystem scale, the mass (g m^{-2}) of an annual leaf litter cohort remaining after three years and six years was not affected by simulated N deposition ($P > 0.385$, Table 2.2, 2.3). In contrast, N additions marginally increased the estimated quantity of fine root mass remaining after three years from 158.99 to 176.51 g m^{-2} (average across sites; $F = 3.48$, $P = 0.081$, Table 2.2, 2.3). Simulated N deposition significantly increased the estimated fine root mass remaining after six years

of decomposition by 23.76 % (from 91.34 to 113.05 g m^{-2}, F =10.60, P = 0.005, Table 2.2, 2.3). The increase of fine root mass remaining after three and six years due to N additions were consistently pronounced at site A, B, and D, with the increase after three years ranging from 27.32 g m^{-2} at site B to 41.10 gm^{-2} at site A, and after six years ranging from 29.02 g m^{-2} at site B to 39.82 g m^{-2} at site D. However, such increases did not occur at site C ($3yr_{mass}$: site × NO_3^-, F = 2.92, P = 0.066; $6yr_{mass}$: site × NO_3^-, F = 3.66, P = 0.035). At site C, the averaged root mass remaining per unit area was generally lower under simulated N deposition, but not significantly, ($3yr_{mass}$: P = 0.750; $6yr_{mass}$: P = 0.921 in *post hoc* Tukey's HSD tests).

2.5 Discussion

We observed that simulated N deposition decreased the decomposition rate of fine roots, but had relatively minor effects on leaf litter decomposition (Fig. 2.1, Table 2.2). Excess N has been widely observed to alter litter decomposition (*e.g.* Knorr *et al*. 2005), particularly slowing the breakdown of complex biochemicals such as lignin (Fog 1988; Knorr *et al.* 2005). Previous studies devoted to understanding these responses have focused on changes in soil extracellular enzyme activity (*e.g.,* Carreiro *et al.* 2000; Saiya-Cork *et al.* 2002; Waldrop *et al.* 2004; Keeler *et al.* 2009) or changes in soil C pools (e.g., Neff *et al.* 2002; Mack *et al.* 2004; Liu *et al.* 2011), including past research at the MGS sites (DeForest *et al.* 2004; Pregitzer *et al.* 2008; Zak *et al.* 2008). Studies on the effects of exogenous N on decomposition have primarily focused on leaf litter (*e*.g. Aerts *et al.* 2006; Hobbie 2008; Hobbie *et al.* 2012). However, in forests, the annual flux of fine root litter to the soil is similar in magnitude or greater than the production of leaf litter (Xia *et al.,* 2015, also see Chapter 1) and these two dominant litter types represent markedly different decomposition substrates (Xia *et al.* 2015, also see Chapter 1). Given that simulated N deposition has had clear effects on later stages of fine root decomposition, but did not affect leaf litter decomposition (Fig. 2.1, Table 2.2) and has consistently suppressed the activity of the peroxidase and phenol oxidase soil enzymes that degrade lignin at these sites (DeForest *et al.,* 2004; Edwards *et al.,* 2011), it is apparent that biochemical

differences between these major litter sources play a large role in the accumulation of soil carbon at these study sites.

2.5.1 Mass loss and model projection

The double exponential model accurately described mass loss over time in both leaf litter and fine roots. Multiple pool models usually perform better than single-phase models in fitting litter decomposition, with double exponential models (*e.g.* Harmon *et al.,* 2009; Alexander & Arthur 2014) or asymptotic models (Hobbie *et al.,* 2012; Sun *et al.,* 2015) often best describing decomposition data. These multi-pool models are consistent with the idea that compounds such as sugars, starch, simple phenolics, and free amino acids form a labile pool that leaches or decomposes quickly, with subsequent decomposition limited by more recalcitrant polymers such as lignin, condensed tannins, cutin, and suberin (Wider & Lang 1982; Berg 2000; Preston *et al.,* 2009). The frequently used single decay model assumes a constant decomposition rate for litter material and tends to underestimate the mass remaining towards the later stages of decomposition (Manzoni *et al.*, 2012), meaning that the actual proportion of leaf litter remaining on the forest floor at the end of three years was substantially higher than the proportion predicted by the single exponential model (25.6 % to 11.8%, data not shown).

We compared the measured and model-projected mass remaining proportions of leaf litter decomposed on the forest floor to a long-term multi-site decomposition experiment (LIDET, Harmon *et al*, 2013) where leaf litter was also deployed on the ground surface. The proportion of leaf litter mass remaining at the end of our three-year study (25.0 %) and model-projected proportion for the sixth year at our study (12.1 %) were comparable to mass loss measured within LIDET at three other cool temperate broadleaf forests in the eastern USA (Coweeta Hydrologic Laboratory, Hubbard Brook Experimental Forest, and Harvard Forest). The mass remaining of sugar maple leaf litter at those three forests ranged from 19.5 to 41.1 % with a mean of 28.5 % at the third year of decomposition, and from 7.1 to 27.9 % with a mean of 15.4 % at the sixth year. In contrast, the leaf

litter mass loss at our sites and those three broadleaf forests within LIDET were much higher than at a temperate mixed conifer-hardwood forest (North Temperate Lakes site within LIDET) in the north-central USA where half of the initial leaf litter mass still remained in the tenth year (Harmon *et al.*, 2009).

For fine roots, the projected mass remaining after six years in our study seems higher than LIDET data: 25.6 % of *Drypetes glauca* fine root mass remained across those three temperate broadleaf forests after five years (Gholz *et al.*, 2000), but these roots had a much lower AIF fraction (9.2%) than the sugar maple roots in our study (Xia *et al.* 2015, also see Chapter 1). Also, the C fraction-specific decomposition models developed by Aber *et al.* (1989) predicted that only 20% of sugar maple fine root mass would remain after five to six years. Notably, these studies used fine roots with a diameter <2 mm, while we used the most distal three order roots, which are mostly < 0.5 mm in diameter (Xia *et al.*, 2015, also see Chapter 1). Multiple lines of evidence indicate that the distal small-diameter roots generally decompose slower than larger roots, probably due to lower C/N ratios, higher contents of AIF, and the presence of mycorrhizal colonization (Guo *et al.*, 2004; Fan & Guo 2010; Goebel *et al.*, 2011). Using roots < 0.5 mm, Sun *et al.* (2016) reported ~ 40 % of root mass remaining after five years averaged across five temperate broadleaf litters, which is similar to the model projection in this study (38.3 % after five years, data not shown).

2.5.2 The effects of simulated nitrogen deposition on leaf litter decomposition

Simulated N deposition had minor effects on long-term leaf litter decomposition. Nitrogen additions marginally stimulated initial leaf litter decomposition rates (k_1), but had no significant effect on subsequent leaf litter decomposition rates (k_2), with no effect of simulated N deposition on the proportion of mass remaining after three and six years (Fig. 2.1, Table 2.2, 2.3, 2.5). This agrees with other studies showing that N additions often have neutral or positive effects on initial decomposition of sugar maple leaf litter and other low-lignin litters (Carreiro *et al.*, 2000; Knorr *et al.*, 2005; Hobbie *et al.*, 2008). Combining leaf litter flux data with measured and model-fitted mass remaining,

simulated N deposition does not increase the pool of decomposing leaf litter mass relative to ambient conditions (Table 2.3).

The stimulation of initial leaf litter decomposition by simulated N deposition was associated with increased substrate N concentrations at all sites and decreased concentrations of soluble phenolics at three out of four sites (Table 2.1, 2.2). This supports our hypothesis that the effect of simulated N deposition on leaf litter decomposition can be attributed, at least partially, to the way it alters the initial leaf litter chemical composition. Although elevated external N availability has been observed to increase initial decomposition by enhancing cellulose-degrading enzyme activity in some ecosystems (Carreiro *et al.*, 2000; Sun *et al.*, 2015), this has not been observed at our study sites. Rather, N deposition suppressed *β*-glucosidase activity and had no effects on other major cellulolytic enzyme activities or the expression of cellulolytic gene *cbhl* (Deforest *et al.*, 2004; Edwards *et al.*, 2011). This suggests that external N availability is less important than initial substrate chemistry for stimulating early-stage leaf litter decomposition under simulated NO_3^- deposition at our sites.

High substrate N concentrations have been reported to stimulate early-stage decomposition (Melillo *et al.*, 1982), yet suppress later degradation (Berg 2000). The increase in leaf litter N concentrations caused by simulated N deposition stimulated early-stage decomposition, but did not slow later-stage decomposition in this and a similar study (Table 2.2, 2.3, also see Hobbie *et al.*, 2012); a larger range of N concentrations may be needed to reveal the differential effects of N on late-stage decomposition (*e.g.* 0.4 to 3.0 % in Berg 2000). The negative effect of soluble phenolics (Table 2.2) on decomposition is more difficult to interpret because phenolics comprise a large mixture of different compounds, among which are easily degradable low-molecular-weight phenolics and more recalcitrant CTs that can be toxic to microorganisms (Bhat *et al.*, 1998; Coq *et al.*, 2010).

2.5.3 The effects of simulated nitrogen deposition on fine root decomposition

As we expected, the decomposition rates of later stage (k_2) in fine roots were significantly lower under simulated NO_3^- deposition ($P = 0.019$, Table 2.2, 2.3), leading to an increase of mass remaining

at the end of this study from 51.29 % to 56.40 % (Table 2.2). The effect of simulated NO_3^- deposition on later stage decomposition was attributed to the elevated external N availability rather than altered initial substrate chemistry (Table 2.5). The decomposition of leaf litter deployed at the same layer as fine roots, i.e. O/A interface, was not inhibited by simulated N deposition (Table 2.2, 2.3), indicating that the response of leaf litter and fine roots to simulated N deposition was due to their biochemical differences rather than the contrasting physical environments in the soil.

High concentrations of exogenous N have been widely observed to suppress lignin degradation, which often dominates later stages (> 1 yr) of litter decomposition (Berg 2000). Under laboratory conditions, higher N availability repressed lignin-degrading activity of certain white-rot fungal species (Fenn *et al.* 1981; Boominathan *et al.* 1990; Van der Woude *et al.* 1993), while N limitation stimulated lignin-degrading activity (Reid *et al*. 1983). Field studies in temperate and boreal forests have found that N fertilization increased lignin accumulation (Magill & Aber 1998; Berg 2000). Previous work at the MGS sites observed that simulated N deposition suppressed the activity of the lignin-degrading phenol oxidase and peroxidase extracellular enzymes (DeForest *et al.*, 2004), decreased fungal to bacterial biomass ratios (van Diepen *et al.*, 2010), and down-regulated expression of the *lcc* gene that is responsible for the synthesis of laccase (Edward *et al.*, 2011). These results support the idea that simulated N deposition slows later stage decomposition of fine roots as a result of depressed lignin-degradation metabolism.

Although previous work showed that simulated N deposition did not alter annual litter input of fine roots at our sites (Burton *et al.*, 2002), simulated N deposition increased the retention of decomposing fine root mass. From our observations on decomposition and fine root turnover, we predict that at three out of four study forests, simulated N deposition increases the mass retention of decomposing fine roots by 27.3 to 41.1 g m^{-2} after three years of decomposition. No additional retention of decomposing root mass was observed at site C, which is partly due to lower estimated fine root turnover under simulated N deposition at this site (Xia *et al.*, 2015, also see Chapter 1). Consistently, in previous studies, site C had the least pronounced increase of organic horizon mass (g

m^{-2}) due to simulated N deposition among all four sites (Pregitzer *et al.*, 2008; Zak *et al.* 2008). Because fine roots are generally more lignin-rich than leaf litter in temperate and boreal forests (Reich *et al.*, 2000; Xia *et al.*, 2015), our results suggest that fine root litter could represent a growing C sink in temperate and boreal forests as high rates of anthropogenic N deposition continue to persist in North America and Europe, as well as become more widespread in Asia and other developing regions.

2.6 References

Aber JD, Melillo JM, McClaugherty CA.1990. Predicting long-term patterns of mass loss, nitrogen dynamics, and soil organic matter formation from initial fine litter chemistry in temperate forest ecosystems. *Canadian Journal of Botany* 68: 2201-2208.

Adair EC, Parton WJ, Del Grosso SJ, Silver WL, Harmon ME, Hall SA, Burke IC, Hart SC. 2008. Simple three-pool model accurately describes patterns of long-term litter decomposition in diverse climates. *Global Chang Biology* 14: 2636-2660.

Alexander H, Arthur M. 2014. Increasing Red Maple Leaf Litter Alters Decomposition Rates and Nitrogen Cycling in Historically Oak-Dominated Forests of the Eastern US. *Ecosystems* 17: 1371-1383.

Amin BA, Chabbert ZB, Moorhead D, Bertrand I. 2014. Impact of fine litter chemistry on lignocellulolytic enzyme efficiency during decomposition of maize leaf and root in soil. *Biogeochemistry* 117: 169-183.

Berg B, Matzner E. 1997. Effect of N deposition on decomposition of plant litter and soil organic matter in forest systems. *Environmental Reviews* 5: 1-25.

Berg B. 2000. Litter decomposition and organic matter turnover in northern forest soils. *Forest ecology and Management* 133: 13-22.

Boominathan K, Dass SB, Randall TA, Reddy CA. 1990. Nitrogen-deregulated-mutants of *Phanerochaete crysosprorium*–a lignin-degrading basidiomycete. *Archives Microbiology* 153: 521-527.

Bradford MA, Tordoff GM, Eggers T, Jones TH, Newington JE. 2002. Microbiota, fauna, and mesh size interactions in litter decomposition. *Oikos* 99: 317-323.

Burton AJ, Pregitzer KS, Hendrick RL. 2000. Relationships between fine root dynamics and nitrogen availability in Michigan northern hardwood forests. *Oecologia* 125: 389-399.

Carreiro MM, Sinsabaugh RL, Repert DA, Parkhurst DF. 2000. Microbial enzyme shifts explain litter decay responses to simulated nitrogen deposition. *Ecology* 81: 2359-2365.

Coq S, Souquet JM, Meudec E, Cheynier V, Hättenschwiler, S. 2010. Interspecific variation in leaf litter tannins drives decomposition in a tropical rain forest of French Guiana. *Ecology* 91: 2080-2091.

DeForest JL, Zak DR, Pregitzer KS, Burton AJ. 2004. Nitrate deposition and the microbial degradation of cellulose and lignin in a northern hardwood forest. *Soil Biology & Biochemistry* 36: 965-971.

Edwards IP, Zak DR, Kellner H, Eisenlord SD, Pregitzer KS. 2011. Simulated atmospheric N deposition alters fungal community composition and suppresses lignocellulolytic gene expression in forest floor of a northern hardwood forest. *PLoS One* 6: e20421.

Erisman JW, Galloway JN, Seitzinger S, Bleeker A, Dise NB, Petrescu AR, Leach AM, de Vries. W 2013. Consequences of human modification of the global nitrogen cycle. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* 368: 20130116.

Fan P, Guo DL. 2010. Slow decomposition of lower order roots: a key mechanism of root carbon and nutrient retention in the soil. *Oecologia* 163: 509-515.

Fenn P, Kirk TK. 1981. Relationship of nitrogen to the onset and suppression of ligninolytic activity and secondary metabolism in *Phanerochaete chrysosporium. Archives of Microbiology* 130: 59-65.

Fog, K. 1988. The effect of added nitrogen on the rate of decomposition of organic matter. *Biological Reviews* 63: 433-462.

Fowler D, Coyle M, Skiba U, Sutton MA, Cape JN, Reis S, Sheppard LJ, Jenkins A, Grizzetti B, Galloway JN, Vitousek P. 2013. The global nitrogen cycle in the twenty-first century. *Phil. Trans. R. Soc. B* 368: 20130164.

Franklin O, Högberg P, Ekblad A, Ågren GI. 2003. Pine forest floor carbon accumulation in response to N and PK additions: bomb ^{14}C modelling and respiration studies. *Ecosystems* 6: 644-658.

Frey SD, Ollinger S, Nadelhoffer K, Bowden R, Brzostek E, Burton A, Caldwell BA, Crow S, Goodale CL, Grandy AS, Finzi A. 2014. Chronic nitrogen additions suppress decomposition and sequester soil carbon in temperate forests. *Biogeochemistry* 121: 305-316.

Galloway JN, Dentener FJ, Capone DG, Boyer EW, Howarth RW, Seitzinger SP, Asner GP, Cleveland CC, Green PA, Holland EA, Karl DM. 2004. Nitrogen cycles: past, present, and future. *Biogeochemistry* 70: 153-226.

Gholz HL, Wedin DA, Smitherman SM, Harmon ME, Parton WJ. 2000. Long-term dynamics of pine and hardwood litter in contrasting environments: toward a global model of decomposition. *Global Chang Biology* 6: 751-765.

Goebel M, Hobbie SE, Bulaj B, Zadworny M, Archibald DD, Oleksyn J, Reich PB, Eissenstat DM. 2011. Decomposition of the finest root branching orders: linking belowground dynamics to fine-root function and structure. *Ecological Monographs* 81: 89-102.

Gruber N, Galloway JN. 2008. An Earth-system perspective of the global nitrogen cycle. *Nature* 451: 293-296.

Guo DL, Mitchell RJ, Hendricks JJ. 2004. Fine root branch orders respond differentially to carbon source-sink manipulations in a longleaf pine forest. *Oecologia* 140: 450-457.

Harmon ME, Silver WL, Fasth B, Chen HU, Burke IC, Parton WJ, Hart SC, Currie WS. 2009. Long-term patterns of mass loss during the decomposition of leaf and fine root litter: an intersite comparison. *Global Change Biology* 15: 1320-1338.

Harmon M. 2013. LTER Intersite Fine Litter Decomposition Experiment (LIDET), 1990 to 2002. Long-Term Ecological Research. Forest Science Data Bank, Corvallis, OR. [Database]. Available: http://andrewsforest.oregonstate.edu/data/abstract.cfm?dbcode=TD023 (17 February 2016).

Hobbie SE. 2000. Interactions between Litter Lignin and Soil Nitrogen Availability during Leaf Litter Decomposition in a Hawaiian Montane Forest. *Ecosystems* 3: 484-494.

Hobbie SE. 2005. Contrasting effects of substrate and fertilizer nitrogen on the early stages of litter decomposition. *Ecosystems* 8: 644-656.

Hobbie SE. 2008. Nitrogen effects on decomposition: A five-year experiment in eight temperate sites. *Ecology* 89: 2633-2644.

Hobbie SE, Eddy WC, Buyarski CR, Adair EC, Ogdahl ML, Weisenhorn P. 2012. Response of decomposing litter and its microbial community to multiple forms of nitrogen enrichment. *Ecological Monographs* 82:389-405.

Howard P, Howard D. 1974. Microbial decomposition of tree and shrub leaf litter. 1. Weight loss and chemical composition of decomposing litter. *Oikos*: 341-352.

Hunt HW. 1977. A simulation model for decomposition in grasslands. *Ecology*: 469-484.

Hurvich CM, Tsai CL. 1989. Regression and time series model selection in small samples. *Biometrika* 76:297-307.

Hyvönen R, Persson T, Andersson S, Olsson B, Ågren GI, Linder S.2008. Impact of long-term nitrogen addition on carbon stocks in trees and soils in northern Europe. *Biogeochemistry* 89: 121-137.

Janssens IA, Dieleman W, Luyssaert S, Subke JA, Reichstein M, Ceulemans R, Ciais P, Dolman AJ, Grace J, Matteucci G, Papale D. 2010. Reduction of forest soil respiration in response to nitrogen deposition. *Nature Geoscience* 3: 315-322.

Keeler BL, Hobbie SE, Kellogg LE. 2009. Effects of long-term nitrogen addition on microbial enzyme activity in eight forested and grassland sites: implications for litter and soil organic matter decomposition. *Ecosystems* 12: 1-15.

Knorr M, Frey S, Curtis P. 2005. Nitrogen additions and litter decomposition: a meta-analysis. *Ecology* 86: 3252-3257.

LeBauer DS, Treseder KK. 2008. Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. *Ecology* 89: 371-379

Little RC, Milliken GA, Stroup WW, Wolfinger RD, Schabenberger O. 2006. *SAS for mixed models.* Cary, US: SAS Institute Inc.

Liu X, Zhang Y, Han W, Tang A, Shen J, Cui Z, Vitousek P, Erisman JW, Goulding K, Christie P, Fangmeier A. 2013. Enhanced nitrogen deposition over China. *Nature* 494: 459-462.

Mack MC, Schuur EA, Bret-Harte MS, Shaver GR, Chapin FS. 2004. Ecosystem carbon storage in arctic tundra reduced by long-term nutrient fertilization. *Nature* 431: 440-443.

Magill AH, Aber JD. 1998. Long-term effects of experimental nitrogen additions on foliar litter decay and humus formation in forest ecosystems. *Plant and Soil* 203: 301-311.

Magill AH, Aber JD. 2000. Dissolved organic carbon and nitrogen relationships in forest litter as affected by nitrogen deposition. *Soil Biology & Biochemistry* 32: 603-613.

Magnani F, Mencuccini M, Borghetti M, Berbigier P, Berninger F, Delzon S, Grelle A, Hari P, Jarvis PG, Kolari P, Kowalski AS. 2007. The human footprint in the carbon cycle of temperate and boreal forests. *Nature* 447: 849-851.

McCormack ML, Dicke IA, Fahey TJ, Guo D, Helmisaari H, Hobbie EA, Iversen CM, Jackson RB, Leppälammi-Kujansuu J, Norby RJ *et al.* 2015. Redefining fine roots improves understanding of belowground contributions to terrestrial biosphere processes. *New Phytologist* 207(3):505-18.

Melillo JM, Aber JD, Muratore JF. 1982. Nitrogen and lignin control of hardwood leaf litter decomposition dynamics. *Ecology* 63: 621-626.

Mutabaruka R, Hairiah K, Cadisch G. 2007. Microbial degradation of hydrolysable and condensed tannin polyphenol–protein complexes in soils from different land-use histories. *Soil Biology & Biochemistry* 39: 1479-1492.

Nadelhoffer KJ, Emmett BA, Gundersen P, Kjønaas OJ, Koopmans CJ, Schleppi P, Tietema A, Wright RF. 1999. Nitrogen deposition makes a minor contribution to carbon sequestration in temperate forests. *Nature* 398: 145-148.

Neff JC, Townsend AR, Gleixner G, Lehman SJ, Turnbull J, Bowman WD. 2002. Variable effects of nitrogen additions on the stability and turnover of soil carbon. *Nature* 419: 915-917.

Northup RR, Yu Z, Dahlgren RA, Vogt KA. 1995. Polyphenol control of nitrogen release from pine litter. *Nature* 377: 227-229.

Olson JS. 1963. Energy storage and the balance of producers and decomposers in ecological systems. *Ecology* 44: 322-331.

Pregitzer KS, Euskirchen ES. 2004. Carbon cycling and storage in world forests: biome patterns related to forest age. *Global Change Biology* 10: 2052-2077.

Pregitzer KS, Burton AJ, Zak DR, Talhelm AF. 2008. Simulated chronic N deposition increases carbon storage in northern temperate forests. *Global Change Biology* 14: 142–153.

Preston CM, Trofymow J, Niu J, Sayer BG. 1997. ^{13}C nuclear magnetic resonance spectroscopy with cross-polarization and magic-angle spinning investigation of the proximate-analysis fractions used to assess litter quality in decomposition studies. *Canadian Journal of Botany* 75: 1601-1613.

Preston CM, Nault JR, Trofymow JA. 2009. Chemical changes during 6 years of decomposition of 11 litters in some Canadian forest sites. Part 2. ^{13}C abundance, solid-state ^{13}C NMR spectroscopy and the meaning of "lignin". *Ecosystems* 12: 1078-102.

Rassel DP, Rumpel C, Dianac M. 2005. Is soil carbon mostly root carbon? Mechanisms for a specific stabilization. *Plant and soil* 269: 341–356.

Reid ID. 1983. Effects of Nitrogen Supplements on Degradation of Aspen Wood Lignin and Carbohydrate Components by *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology* 45: 830-837.

Saiya-Cork K, Sinsabaugh R, Zak DR. 2002. The effects of long term nitrogen deposition on extracellular enzyme activity in an *Acer saccharum* forest soil. *Soil Biology & Biochemistry* 34: 1309-1315.

Schmidt MW, Torn MS, Abiven S, Dittmar T, Guggenberger G, Janssens IA, Kleber M, Kögel-Knabner I, Lehmann J, Manning DA, Nannipieri P. Persistence of soil organic matter as an ecosystem property. 2011. *Nature* 478: 49-56.

Sinsabaugh RL, Carreiro MM, Repert DA. 2002. Allocation of extracellular enzymatic activity in relation to litter composition, N deposition, and mass loss. *Biogeochemistry* 60: 1-24.

Sun T, Dong L, Wang Z, Lü X, Mao Z. 2016. Effects of long-term nitrogen deposition on fine root decomposition and its extracellular enzyme activities in temperate forests. *Soil Biology & Biochemistry* 93: 50-59.

Sutton MA, Simpson D, Levy PE, Smith RI, Reis S, Van Oijen M, De Vries WI. 2008. Uncertainties in the relationship between atmospheric nitrogen deposition and forest carbon sequestration. *Global Change Biology* 14: 2057-2063.

Talhelm AF, Burton AJ, Pregitzer KS, Campione MA. 2013. Chronic nitrogen deposition reduces the abundance of dominant forest understory and groundcover species. *Forest Ecology and Management* 293: 39-48.

Taylor BR, Parkinson D, Parsons WF. 1989. Nitrogen and lignin content as predictors of litter decay rates: a microcosm test. *Ecology* 70: 97-104.

van Diepen LT, Lilleskov EA, Pregitzer KS, Miller RM. 2010. Simulated nitrogen deposition causes a decline of intra-and extraradical abundance of arbuscular mycorrhizal fungi and changes in microbial community structure in northern hardwood forests. *Ecosystems* 13: 683-695.

Van der Woude MW, Boominathan K, Reddy CA. 1993. Nitrogen regulation of lignin peroxidase and manganese-dependent perioxidase production is independent of carbon and manganese regulation in Phanerochaete chrysosoporium. *Archives Microbiology* 160: 1-4

Vitousek PM, Aber JD, Howarth RW, Likens GE, Matson PA, Schindler DW, Schlesinger WH, Tilman DG. 1997. Human alteration of the global nitrogen cycle: sources and consequences. *Ecol. Appl.* 7: 737–750.

Waldrop MP, Zak DR, Sinsabaugh RL, Gallo M, Lauber C. 2004. Nitrogen deposition modifies soil carbon storage through changes in microbial enzymatic activity. *Ecol. Appl.* 14:1172-1177.

Wider RK, Lang GE. 1982. A critique of the analytical methods used in examining decomposition data obtained from litter bags. *Ecology*: 1636-1642.

Xia M, Guo DL, Pregitzer, KS. 2010. Ephemeral root modules in Fraxinus mandshurica. 2010. *New Phytologist* 188: 1065-1074.

Xia M, Talhelm AF, Pregitzer KS. 2015. Fine roots are the dominant source of recalcitrant plant litter in sugar maple-dominated northern hardwood forests. *New Phytologist* 208: 715-726.

Yue K, Peng Y, Peng C, Yang W, Peng X, Wu F. 2016. Stimulation of terrestrial ecosystem carbon storage by nitrogen addition: a meta-analysis. *Scientific Reports* 6: 19895.

Zak DR, Holmes WE, Burton AJ, Pregitzer KS, Talhelm AF. 2008. Atmospheric NO_3^- deposition increases soil organic matter by slowing decomposition in a northern hardwood ecosystem. *Ecol. Appl.* 18: 2016-2027.

Table 2.1 Chemical characteristics (%) and kinetically-determined active pool (A, %) of initial litter collected from ambient and NO_3^- amended plots.

Site	Litter type	Litter source	EXT	AIF	ASF	PHE	CTs	Lipids	NSCs	PRO	N	C/N	AIF/N	LCI	*A*
A															
	Leaf litter	Ambient	39.30	16.18	44.52	13.61	9.08	8.50	5.26	2.02	0.79	63.33	20.38	0.27	51.7
		NO_3^-	43.45	14.66	41.89	15.09	6.92	8.28	7.14	1.48	0.83	61.32	17.74	0.26	50.7
	Fine roots	Ambient	17.51 (1.18)	42.71 (1.09)	39.79 (1.07)	3.88 (0.21)	14.83 (0.77)	3.72 (0.29)	2.09 (0.08)	3.20 (0.07)	1.35 (0.02)	37.38 (0.59)	31.73 (0.68)	0.52 (0.01)	15.9 (2.0)
		NO_3^-	18.09 (0.51)	45.49 (1.76)	36.43 (1.64)	4.03 (0.11)	15.82 (1.13)	3.54 (0.33)	2.10 (0.32)	3.30 (0.26)	1.55 (0.06)	33.66 (1.43)	29.42 (1.44)	0.56 (0.02)	15.3 (1.7)
B															
	Leaf litter	Ambient	37.00	15.05	47.95	13.53	5.22	7.67	4.98	0.78	0.66	71.22	22.65	0.24	49.4
		NO_3^-	39.33	14.08	46.59	11.60	3.54	6.98	6.10	0.73	0.97	50.27	14.46	0.23	47.4
	Fine roots	Ambient	15.68 (0.50)	45.34 (0.49)	38.98 (3.14)	3.24 (0.24)	11.70 (1.25)	3.72 (0.21)	1.73 (0.24)	3.24 (0.25)	1.74 (0.11)	29.05 (1.80)	26.30 (1.92)	0.54 (0.01)	19.0 (2.8)
		NO_3^-	13.96 (0.22)	45.72 (0.81)	40.32 (2.75)	2.99 (0.34)	10.49 (0.50)	3.18 (0.43)	1.71 (0.10)	2.61 (0.28)	1.72 (0.06)	29.32 (1.02)	26.63 (1.31)	0.53 (0.01)	16.4 (0.8)
C															
	Leaf litter	Ambient	38.84	14.44	46.73	13.29	6.11	7.64	5.88	1.20	0.64	77.39	22.60	0.24	45.2
		NO_3^-	39.07	14.22	46.71	10.60	4.19	6.88	4.91	1.25	0.83	58.33	17.08	0.23	43.2
	Fine roots	Ambient	15.90 (1.71)	45.90 (1.37)	38.20 (1.62)	4.11 (0.73)	13.02 (2.54)	3.07 (0.56)	1.74 (0.07)	3.58 (0.72)	1.66 (0.05)	31.33 (1.01)	27.84 (1.61)	0.55 (0.01)	15.9 (5.1)
		NO_3^-	13.76 (1.11)	46.72 (0.72)	39.62 (4.32)	3.16 (0.28)	9.90 (1.50)	3.31 (0.32)	1.66 (0.29)	2.80 (0.28)	1.78 (0.04)	29.06 (0.63)	26.36 (0.27)	0.54 (0.01)	16.3 (5.5)
D															
	Leaf litter	Ambient	39.51	14.74	45.75	11.82	4.48	8.17	4.76	0.91	0.73	68.68	20.29	0.24	35.2
		NO_3^-	36.72	14.67	48.60	10.69	5.08	8.91	5.53	1.34	0.85	58.11	17.22	0.23	36.0
	Fine roots	Ambient	16.58 (0.90)	46.42 (2.36)	37.00 (4.96)	4.57 (0.47)	14.82 (1.02)	3.38 (0.27)	1.94 (0.23)	3.12 (0.34)	1.46 (0.15)	35.75 (3.99)	32.32 (4.78)	0.56 (0.03)	12.8 (1.0)
		NO_3^-	16.68 (2.35)	45.16 (1.27)	38.17 (1.92)	4.79 (0.96)	13.36 (2.42)	3.60 (0.07)	1.92 (0.52)	3.08 (0.39)	1.51 (0.12)	34.59 (2.98)	30.19 (3.20)	0.54 (0.01)	13.1 (1.3)

Values of leaf litter are homogenized leaf litter combined from three ambient or NO_3^- amended plots at each site. Values of fine roots are means (SD) for three ambient or NO_3^- amended plots (n=3), which have been shown in Chapter 1 (also *see* Xia *et al.* 2015). EXT: extractive fraction; AIF: Acid-insoluble fraction; ASF: acid-soluble fraction; PHE, soluble phenolics; CTs, condensed tannins; NSCs, non-structural carbohydrates; PRO, soluble proteins; N, nitrogen; LCI, lignocellulose index, AIF/(AIF+ASF). The details of chemical protocols were shown in Chapter 1.

Table 2.2. Decomposition rates (k_1, k_2), proportions of mass remaining ($3yr_{percentage}$, %) and quantities of mass remaining of an annual litter cohort ($3yr_{mass}$, g m^{-2}) for leaf litter and fine roots decomposed in situ after three years across four northern hardwood forests, and the model-fitted proportions ($6yr_{percentage}$, %) and quantities ($6yr_{mass}$, g m^{-2}) of mass remaining after six years.

Decomposition metrics	Leaf litter (O/A interface)		Leaf litter (O surface)		Fine roots	
	Ambient	NO_3^-	Ambient	NO_3^-	Ambient	NO_3^-
k_1 (y^{-1})	11.12 (2.46)	11.52 (2.50)	5.17 (0.69)	5.74$^{(*)}$ (1.22)	16.97 (10.70)	19.30 (7.55)
k_2 (y^{-1})	0.506 (0.105)	0.513 (0.087)	0.272 (0.082)	0.264 (0.096)	0.175 (0.024)	0.144 ** (0.018)
$3yr_{percentage}$ (%)	14.96 (3.97)	15.06 (3.02)	24.60 (3.46)	25.41 (4.97)	51.29 (2.39)	56.40***‡ (3.09)
$3yr_{mass}$ (g m^{-2})			91.97 (14.96)	97.47 (18.36)	158.99 (31.66)	176.51$^{(*)}$‡ (57.82)
$6yr_{percentage}$ (%)	2.95 (1.31)	2.82 (1.30)	11.64 (3.93)	12.65 (5.58)	29.68 (3.70)	35.94*** (3.95)
$6yr_{mass}$ (g m^{-2})			42.69 (11.69)	47.25 (16.97)	91.34 (17.75)	113.05**‡ (38.81)

Values are means (SD) of decomposition indices of leaf litter and fine roots decomposed *in situ* in each plot. (*), *, **, *** denote significant effects of simulated N deposition at $P < 0.10$, $P < 0.05$, $P < 0.01$. $P < 0.001$, respectively. The proportion of mass remaining after six years was predicted by double exponential decay models in each plot. The quantity of mass remaining of annual litter input as a cohort over time was estimated by multiplying litter input rates (see Chapter 1) with the proportion of mass remaining in each plot. Only leaf litter decomposed in the forest floor are used to construct the quantity of mass remaining. ‡Simulated N deposition significantly increased $3yr_{percentage}$, $3yr_{mass}$, and $6yr_{mass}$ for fine roots at all sites except site C, leading to a significant site × NO_3^- interaction ($P < 0.079$, Table 2.3, Fig. 2.1).

Table 2.3. Analysis of variance for the effects of site (*df* = 3), simulated N deposition (*df* = 1), and their interaction (*df* = 3) on k_1 values, k_2 values, proportions of mass remaining ($3yr_{percetage}$) and mass remaining per area unit ($3yr_{mass}$) after three years, model-projected proportions ($6yr_{percetage}$) and mass remaining per area unit after six years ($6yr_{mass}$) for leaf litter and fine roots decomposed *in situ* in four northern hardwood forests.

Source of variance	k_1		k_2		$3yr_{percetage}$		$3yr_{mass}$		$6yr_{percetage}$		$6yr_{mass}$	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Leaf litter (O/A)												
Site	19.3	**<.001**	16.3	**<.001**	2.11	0.140			7.78	**0.002**		
NO_3^-	0.83	0.376	0.21	0.653	0.01	0.932			0.11	0.749		
Site × NO_3^-	1.15	0.358	2.46	0.101	1.57	0.235			1.42	0.273		
Covariates¶	ns		ns		ns				ns			
leaf litter (forest floor)												
Site	10.8	**0.001**	17.9	**<.001**	1.76	0.194	0.14	0.935	12.6	**<.001**	5.01	**0.012**
NO_3^-	3.46	0.081	0.03	0.870	0.16	0.693	0.46	0.507	0.62	0.444	0.80	0.385
Site × NO_3^-	1.39	0.281	0.59	0.632	0.48	0.703	0.22	0.880	0.81	0.505	0.52	0.672
Covariates¶	$PHE^{(-)}$, $N^{(+)}$		ns		ns		ns		ns		ns	
Fine roots												
Site	2.96	0.063	0.24	0.869	0.22	0.882	24.6	**<.001**	0.12	0.944	18.3	**<.001**
NO_3^-	1.07	0.317	12.3	**0.003**	21.2	**<.001**	3.48	0.081	15.8	**0.001**	10.6	**0.005**
Site × NO_3^-	0.19	0.903	1.56	0.238	2.72	0.079	2.92	0.066	2.16	0.133	2.57	0.035
Covariates¶	ns		ns		ns		ns		ns		ns	

Annual leaf litter production data were combined with the decomposition rates of leaf litter decomposed in the forest floor, instead of those in top soil, to estimate the leaf litter mass remaining per unit area, so there are blanks in the $3yr_{mass}$ and $6yr_{mass}$ columns for leaf litter (O/A).
The significance of covariates¶ is tested in an alternative ANCOVA with site as the main effect to determine if the variations of decomposition rates at each site can be linked to the differential responses of initial chemical characteristics to simulated N deposition. Covariants with a significant level of 0.05 are listed in the table, followed by "(-)" or "(+)" to indicate a negative or positive correlation, with "ns" denoting no significant covariants. PHE: soluble phenolics; N: nitrogen.

Table 2.4 Analysis of variance on the differences between percentages of mass remaining of fine roots and leaf litter (% ML-R) among sites (*df* = 3) and simulated N deposition (*df* = 1).

Effects	n/m	*F*	*P*
Site	3/16	1.71	0.206
NO_3^-	1/16	5.30	**0.035**
Site × NO_3^-	3/16	2.32	0.115

The values of $\%M_L$ - $\%M_R$ are computed as the percentage mass remaining of fine roots after three years minus that of leaf litter decomposed on the forest floor in each plot. We used "$\%\ M_{L-R}$" to test if experimental NO_3^- amendment selectively reserve fine root mass over leaf litter. The degrees of freedom, numerator df / denominator df, are shown as "n/m".

Table 2.5 Mixed linear model analysis on a factorial split-plot design testing the differences of fine root decomposition rates (k_1, k_2) and percentages of mass remaining after three years (3yr %) among study sites, external N availability, and substrate source (fine roots collected from ambient vs. NO_3^- amended plots).

Source of variance		k_1		k_2		3yr %	
	n/m	*F*	*P*	*F*	*P*	*F*	*P*
Site	3/16	3.74	**0.033**	0.17	0.918	1.80	0.188
External N Availability	1/16	0.97	0.340	8.21	**0.011**	15.74	**0.001**
Site × N	3/16	0.26	0.852	0.80	0.512	1.14	0.362
Substrate Source	1/16	0.16	0.695	2.92	0.107	5.42	**0.033**
Site × Source	3/16	1.61	0.226	1.33	0.298	2.67	0.083
N × Source	1/16	1.01	0.329	0.03	0.860	2.07	0.169
Site × N × Source	3/16	0.31	0.815	1.01	0.413	1.86	0.178
Covariates¶		ns		ns		ns	

The degrees of freedom, numerator *df* / denominator *df*, are shown as "n/m". The significance of covariates¶ is tested in an alternative ANCOVA with site and external N availability as main effects and each of initial chemical traits as a covariate. "ns" means no significant covariates ($P > 0.05$) were found.

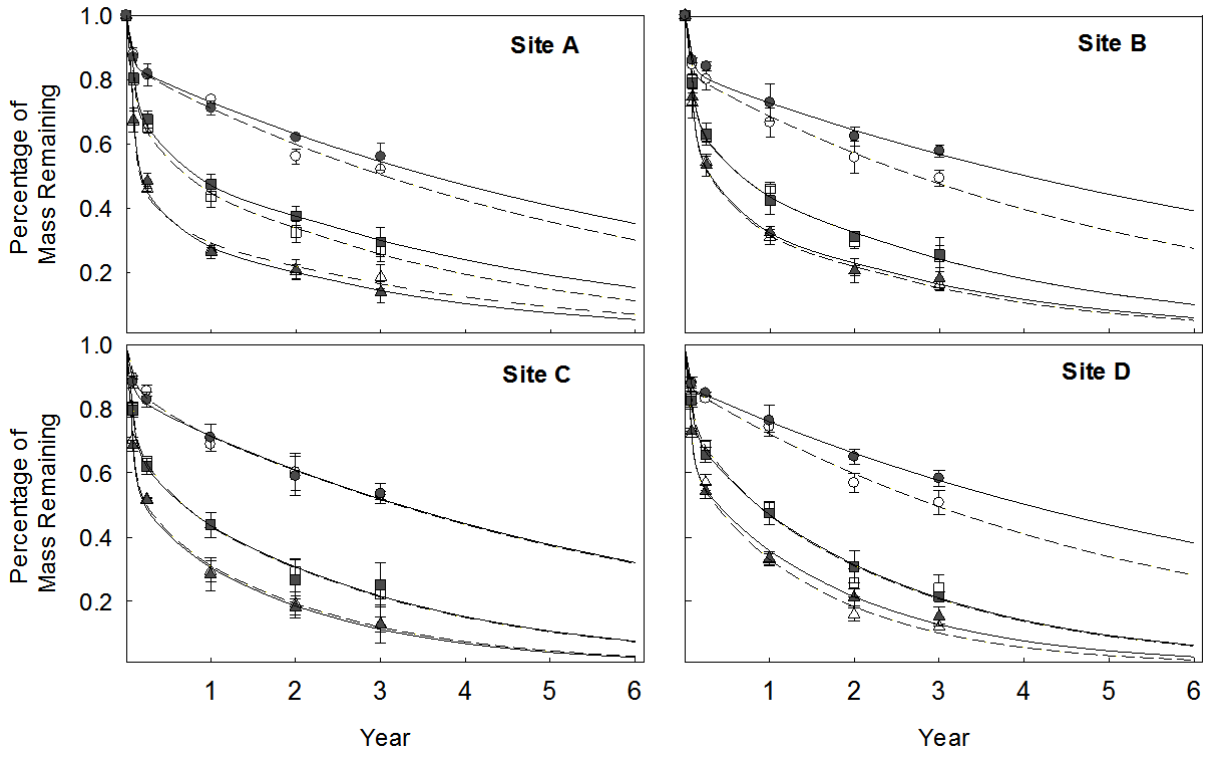

Fig. 2.1 Decomposition patterns of leaf litter and fine roots across four northern hardwood forests. Each data point is the mean with SD of three ambient or NO_3^- deposition plots at each site (n = 3). △, leaf litter (O/A interface) decomposed in ambient plots; ▲, leaf litter (O/A interface), NO_3^- amended plots; □, leaf litter (forest floor), ambient plots; ■, leaf litter (forest floor), NO_3^- amended plots; ○, fine roots, ambient plots; ●, fine roots, NO_3^- amended plots; solid lines are the double exponential model predicted decomposition patterns under simulated N deposition, while dash lines are the predicted patterns for ambient plots.

Chapter 3 The Effects of Simulated Nitrogen Deposition on Chemical Dynamics of Leaf Litter and Fine Roots During Decomposition

3.1 Abstract

- Atmospheric nitrogen (N) deposition induces a forest carbon sink across broad parts of the Northern Hemisphere. In part, this effect of N deposition may be due to soil carbon accumulation caused by slower litter decomposition.
- We used wet chemistry methods and Fourier transform infrared spectroscopy (FT-IR, on ground tissues) to study the effects of chronic simulated N deposition (>15 years) on chemical changes in leaf litter and fine roots during a three-year decomposition study at four hardwood forests in the north-central USA.
- Our results from gravimetrically-defined acid-insoluble fraction (AIF, also known as Klason lignin) and lignin/carbohydrate reference IR peak ratios both provide evidence that lignin is selectively preserved under simulated N deposition in fine roots. Lignin/carbohydrate peak ratios were highly correlated with AIF concentration and AIF/acid-soluble fraction ratios, suggesting that AIF is a good predictor of lignin. Because AIF was abundant in fine roots, the slower degradation of AIF in decomposing fine roots accounted for 73.9 % of the additional root mass remaining under simulated N deposition. Simulated N deposition also caused slower degradation of condensed tannins (CTs) and soluble proteins in fine roots. Similarly, N additions initially inhibited the decomposition of AIF, CTs, and proteins in leaf litter, but these effects disappeared at the late-stage of decomposition and did not increase the mass remaining at the end of the experiment.
- Our results suggest that chemical classes subject to oxidative degradation such as lignin and CTs are generally sensitive to N deposition, but the consequences of such responses on overall mass loss and organic matter accumulation can vary greatly among litter materials having very different initial concentrations of these chemical classes.

3.2 Introduction

The anthropogenic production of reactive nitrogen (Nr) has rapidly increased over the last century and exceeds global natural production of Nr (Ciais *et al.*,2014). A large portion of the Nr created by human activity is added to terrestrial ecosystems via atmospheric deposition, leading to a substantial increase in inputs of Nr across wide areas of Europe, North America, and Asia (Holland *et al*., 2005; Gruber & Galloway 2008; Liu *et al.,* 2014). Because carbon (C) and N are coupled in nearly all fundamental biological metabolic pathways and because N availability limits plant productivity in most terrestrial ecosystems (Chapin *et al.*, 2011), Nr deposition alters the biogeochemical cycling of C and other elements. Specifically, there are numerous reports of increased forest C sequestration in Western Europe and North America as a result of N deposition (*e.g.,* Hogberg 2007; Sutton *et al.,* 2008; Thomas *et al.* 2010). Chronic N deposition experiments have widely observed that N additions increase tree growth and soil organic C (Hyvonen e*t al.*, 2008; Pregitzer *et al.*, 2008; Zak *et al.*, 2008; Frey *et al.*, 2014), although all or part of these increases can be offset by higher tree mortality rates (Magill *et al.*, 2000; Frey *et al.*, 2014). The increase in soil organic C due to simulated N deposition has been associated with slower decomposition of plant litter and soil organic matter, rather than greater litter input (Zak *et al.,* 2008; Janssens *et al.*, 2010; also see Chapter 2).

Although N additions often slow litter decomposition, it is unclear how simulated N deposition affects the degradation of specific chemical fractions within plant litter. Here, we propose two hypotheses on the effects of elevated N deposition on chemical dynamics during litter decomposition. First, we hypothesize that as litter decomposition slows under elevated N deposition, the degradation of its chemical fractions are inhibited in similar ways. This hypothesis represents the idea that N additions suppress microbial activity in a non-specific way. Several mechanisms have been proposed for the relatively non-specific detrimental effects of N additions on microbial activity. For example, the introduction of additional ions could increase osmotic pressures in the soil solution that can be toxic to microorganisms (Broadbent 1965). Also, N deposition can lead to soil cation depletion and increase of aluminum toxicity by acidifying soil (Gundersen & Rasmussen 1990;

Vitousek *et al.* 1997; Hogberg *et al.,* 2006; Bowman *et al.,* 2008). A meta-analysis of 82 observations revealed that N additions decreased microbial biomass by an average of 15% (Treseder 2008), and a meta-analysis of 36 forest N manipulation studies found a significant suppression of heterotrophic soil respiration (Janssens *et al.,* 2010).

Alternatively, we hypothesize that N deposition selectively preserves certain compounds within decomposing litter. This pattern would be in line with the observations that N additions often selectively suppress the activity of oxidative extracellular enzymes that are involved in lignin degradation, such as phenol oxidase and peroxidase (Carreiro *et al.,* 2000; DeForest *et al.,* 2004; Sun *et al.,* 2016). Also, simulated N deposition has consistently down-regulated expression of the *lcc* gene that is responsible for synthesis of phenol oxidase (laccase, Edwards *et al.,* 2011) at four northern hardwood forests. Consistently, a meta-analysis on the effects of N additions on leaf litter decomposition found that N additions tend to inhibit decomposition of lignin-rich litter substrates, but had minor or positive effects on lignin-poor substrates (Knorr *et al.,* 2005). In contrast, N additions generally increased or caused little change in cellulolytic enzyme activities (Carreiro *et al.,* 2000; Deforest *et al.,* 2004; Sun *et al.,* 2016). Because of such contrasting effects of N additions on extracellular enzymes, we specifically hypothesize that simulated N deposition selectively preserved lignin relative to polysaccharides in decomposing litter. Although microbial responses to experimental N deposition have been well-studied, the evidence of slower degradation of specific chemical classes due to N additions is rare.

In 2011, we established a three-year decomposition study of leaf litter and fine roots at four northern hardwood forests in the north-central USA that since 1994 have received NO_3^- additions simulating enhanced atmospheric deposition. To understand changes in litter decay chemistry, we used a broad set of wet chemistry methods. In addition, we also used Fourier transform infrared (FT-IR) spectroscopy to investigate the lignin degradation relative to the polysaccharides. At these study sites, long-term simulated N deposition has increased organic carbon (C) in both soil organic horizons and surface mineral soils (Pregitzer *et al.,* 2008; Zak *et al.,* 2008), affected activity and community

composition of soil microbes (DeForest *et al.*, 2004; Edwards *et al.*, 2011), and decreased the decomposition rate of fine roots (see Chapter 2). Thus, this experiment provides a good opportunity to understand whether observed increases in soil C accumulation with chronic N deposition result from altered chemical dynamics during litter decomposition.

3.3 Materials and Methods

See 1.3 Methods and Materials in Chapter 1 for site information (1.3.1), leaf litter and fine root sampling (1.3.2), and chemical analysis (1.3.3). See 2.3 Methods and Materials in Chapter 2 for decomposition study (2.3.1).

3.3.1 Chemical analysis

Litter substrates were pulverized in a Wig-L-Bug grinder/mixer (Dentsply-Rinn, Elgin, IL) before chemical analysis. We determined concentrations of total C and N, acid-insoluble fraction (AIF), acid-soluble fraction (ASF), nonstructural carbohydrates (NSCs), soluble phenolics, condensed tannins (CTs), soluble proteins, and total lipids, for initial and harvested litters (harvested after 1 month, 3 months, 1 year, 2 years, and 3 years of decomposition). The initial chemical traits were documented in Table 2.1 in Chapter 2. The details of wet chemical methods have been shown in the subsection 1.3.3 of Chapter 1. Briefly, total C and N are analyzed with an elemental analyzer (ECS 4010, Costech Analytical, Valencia, CA). Extractive-free fraction was obtained with a sequential extraction procedure (Friend, 1992). Then, this fraction was subsequently divided into AIF and ASF using a two-phase H_2SO_4 hydrolysis adapted from Booker *et al.* (1996). For NSCs, we used phenol-sulfuric acid analysis to determine sugar concentrations (Chow *et al.*, 2004). Starch was determined colorimetrically with a peroxidase-glucose oxidase (PGO)/o-dianisidine reagent after *a*-amylase/amyloglucosidase digestion (Chow *et al*. 2004). Soluble phenolics were determined with Folin-Ciocalteu reagent based on catechin standards (Booker *et al*. 1996). Condensed tannins were determined with acid-butanol assay (Booker *et al*. 1996) with standards prepared from fresh apples

(Li *et al*. 2010). We used Coomassie protein Bradford Reagent (Thermo Fisher Scientific Inc., Rockford, IL) to measure concentrations of soluble proteins. Total lipids were extracted and determined according to Bligh and Dyer (1959).

The pulverized samples were also analyzed using attenuated total reflectance (SmartPerformer, ZnSe crystal) on a ThermoNicolet Avatar 370 FTIR spectrometer. We collected infrared spectra from pulverized material of both initial substrates and substrates from the final litter bag harvest (3 years). For each sample, we recorded 64 scans (4000 – 400 cm^{-1} wavenumber) at a resolution of 4 cm^{-1}. A blank spectrum of air was taken as background measured before each sample and automatically subtracted from each sample spectrum. The spectra were baseline corrected in a consistent manner for leaf litter and fine root samples, and normalized using OMNIC software v9.0 (Thermo Scientific). Peak heights were measured against the baseline to represent the intensity for the bands of interest. A peak around 1506 cm^{-1} that arises from aromatic skeletal vibration in lignin is frequently used as lignin reference (Faix 1991; Pandey & Pitman 2003). The relative intensity of this peak against the carbohydrates characteristics band have successfully predicted lignin concentrations in decayed wood (Rodrigues *et al.*,1998; Pandey & Pitman 2004). Polysaccharide characteristics peaks are often around 898 cm^{-1}, 1158 cm^{-1}, 1375 cm^{-1} (Pandey & Pitman 2003). Differences between decomposed substrates and initial substrates in the intensity of lignin/carbohydrate characteristic bands were used to quantify the relative preservation of lignin against carbohydrates during decomposition in this study. This method has been used to quantify the selective degradation of lignin by white-rot fungus in wood chemistry studies (*e.g.* Pandey & Pitman 2003; Fabiyi *et al.*, 2011).

3.3.2 Data analysis

We used the fraction of the original amount of a chemical class remaining to describe chemical changes during litter decomposition. The fraction (%) remaining (F_t) for a certain chemical class was calculated as follows:

$$F_t = \frac{C_t M_t}{C_0}$$

Where C_0, C_t are substrate concentrations for a certain chemical class in initial and decomposed litters respectively, and M_t is the percentage mass remaining. We tested the effects of site (*df* = 3), simulated N deposition (*df* = 1), and their interactions on mass remaining, fraction remaining of each chemical class at a harvest time point, and the differences of lignin/carbohydrates FTIR peak intensity ratios between decomposed and undecomposed litters ($\Delta I_{L/C}$) with a two-way ANOVA for leaf litter and fine roots. Because we decomposed fine roots both *in situ* and in the opposite N treatment in a split-plot design, we were also able to disentangle the effects of elevated external N availability and altered substrate quality from overall simulated N deposition effects on chemical dynamics for fine roots. We conducted a mixed linear model analysis (proc mixed, Littell *et al.*, 2006) to test if substrate source (litter collected from N-amended vs. ambient, *df* = 1), external N availability (decomposed in N-amended vs. ambient, *df* =1), and study sites (*df* = 1) affected the chemical fraction remaining at different time points for fine roots. Site and external N treatment and their interactions (*df* = 3) were tested on the whole-plot experimental units, while substrate source is the within-plot fraction.

Further, we applied a partial correlation analysis on fraction remaining of different chemical classes to reveal how chemical fluxes may couple with each other during decomposition. The partial correlation analysis controlled the effects of mass remaining to remove the monotonic correlations between mass remaining and the fraction remaining of nearly all chemical classes. Both Pearson's product-moment correlations and Spearman rank-order correlations were estimated in this study. We present Spearman rank-order correlations in this study when the assumptions for Pearson correlations did not hold for the data.

The $\Delta I_{L/C}$ values were calculated as follows (see Pandey & Pitman 2003; Fabiyi *et al.*, 2011):

$$\Delta I_{L/C} = \frac{I_{L.d}}{I_{C.d}} - \frac{I_{L.und}}{I_{C.und}}$$

Where $I_{L.d}$, $I_{C.d}$ $I_{L.und}$, $I_{C.und}$ represent the intensity of lignin reference peak, each of the carbohydrate reference peaks for decomposed litters (3 years) and initial substrates respectively. According to this

calculation, higher values of $\Delta I_{L/C}$ imply that more lignin is preserved relative to carbohydrates during litter decomposition (*sensu* Pandey & Pitman 2003). A correlation analysis was used to investigate the relationship among lignin/carbohydrates FTIR peak ratios, AIF concentrations, and AIF/ASF ratio. Data were analyzed in SAS 9.3 (SAS Institute Inc., Cary, NC, USA).

3.4 Results

Leaf litter, either decomposed in forest floor or at O/A interface, showed a greater mass loss than fine roots, with only 15.0 to 25.4 % mass remaining at the end of this study (Table 3.1).

During initial decomposition, leaf litter exhibited substantial loss of soluble phenolics, condensed tannins (CTs), and non-structural carbohydrates (NSCs). More than 70 % of the initial amount of these chemical classes were leached or degraded within three months (Table 3.1). At the end of this study, less than 5 % of the initial amount remained for these chemical classes (Table 3.1). In contrast, acid-insoluble fraction (AIF), soluble protein, and nitrogen (N) accumulated or remained near the initial amount for the first year of decomposition, and more than half of the initial amount still remained at the end of this study (Table 3.1). During early decomposition, fine roots did not show a rapid loss of soluble phenolics, CTs, and NSCs, or an increase of AIF, protein, and N. Fine roots maintained a considerably larger fraction of soluble phenolics, CTs, NSCs, lipids, and ASF than leaf litter throughout this study, with 24.1 to 52.3 % of the initial amount for these chemical classes still remaining at the end of this study (Table 3.2).

We used the fraction (%) of the original amount for a certain chemical class remaining at a time point to investigate the effects of N additions on litter decomposition. Although simulated N deposition did not affect leaf litter mass remaining during this study ($P > 0.750$, Table 3.1), the effects of N additions on leaf litter chemical changes were manifest in the early stage of decomposition. After three months, N additions significantly increased the fraction remaining of AIF *(*$F = 12.34$, $P = 0.003$), CTs ($F = 9.04$, $P = 0.008$), and soluble protein ($F = 9.04$, $P = 0.008$). In contrast, N additions decreased the fraction of NSCs remaining ($F = 14.05$, $P = 0.002$) for leaf litter

decomposed in the forest floor, indicating that N additions accelerated the degradation of NSCs. The effect of N additions on the fraction remaining of AIF, CTs, and protein disappeared at the first year, while the stimulation of NSC degradation caused by N additions disappeared at the third year. The accumulation of protein (*i.e.* fraction remaining > 100%) observed at the third month occurred at site A and B, but not at site C and D (Site × NO_3^-, $F = 9.61$, $P = 0.007$). The chemical dynamics of leaf litter decomposed at O/A interface showed a similar response to simulated N deposition with leaf litter decomposed in the forest floor (Table 3.1). Added NO_3^- decreased the decomposition of CTs ($F = 7.31$, $P = 0.015$), stimulated the degradation of NSCs ($F = 14.43$, $P = 0.002$ at three months; $F = 18.11$, $P < 0.001$ at one year), but such effects disappeared at the third-year harvest. Nitrogen additions decreased the fraction remaining of N at all sites but A, leading to a significant interaction of site and N treatment for both leaf litter in the forest floor and at the O/A interface within the first year of decomposition ($P < 0.009$, Table 3.1).

In contrast, N additions did not affect AIF degradation in fine roots early in decomposition, but N additions significantly increased the fraction of AIF remaining within fine root material at the third year harvest (69.3 vs. 60.1%, $F = 32.25$, $P < 0.001$, Table 3.2). Like in leaf litter, N additions increased the fraction remaining of CTs ($F = 7.09$, $P = 0.017$ at three months; $F = 9.72$, $P = 0.007$ at three years) and soluble proteins ($F = 12.53$, $P = 0.003$ at three months; $F = 7.48$, $P = 0.015$ at one year; $F = 10.06$, $P = 0.006$ at three years) in fine roots, and these trends remained throughout the study (Table 2.3). By the end of this study, N additions significantly increased the fine root mass remaining (from 51.3 to 56.4%, $F = 21.33$, $P < 0.001$, Table 3.2).

We estimated how the increase of AIF contributed to the additional mass remaining due to simulated N deposition at the end of study by combining the percentage of mass remaining, AIF concentrations, and litter input data in each plot (documented in Chapter 1, Table 1.3). Across three study sites that have accumulated more decomposing root mass (g m^{-2}) under simulated N deposition (site A, B, and D, see Chapter 2), the increase of AIF contributed 73.9 ± 5.2 % of additional mass retention due to N additions.

Because we decomposed fine roots both in the native plots and in the plots with the opposite N treatment, the effects of N additions on decomposition of AIF in fine roots were explored further by taking into account the effects of initial litter quality and external N availability. Elevated external N availability significantly increased the AIF fraction remaining in fine roots at the end of the study (F = 33.16, P < 0.001, Table 3.3), and this pattern persisted across four forests and fine roots collected in ambient or N-amended plots. Substrate source had minor effects on AIF, both positive and negative, that were site-specific (Site × substrate source: F = 5.01, P = 0.012). Both elevated external N availability and substrate source generally increased the fraction remaining of CTs and proteins (P < 0.052, analysis not shown).

The partial correlation analysis showed that a number of chemical fluxes exhibited significant correlations with each other, and their directions and magnitude can be different between leaf litter and fine roots (Table 3.4). The fraction remaining of AIF was generally negatively correlated with that of ASF, but the correlation coefficient was only significant in fine roots (R = - 0.395, P < 0.001, Table 3.4). In fine roots, the fraction of AIF remaining was also positively correlated with tissue N (R = 0.392, P < 0.001). The fraction of phenolics remaining was positively and significantly correlated with that for CTs for both leaf litter and fine roots (P < 0.001). Phenolics also significantly correlated with soluble proteins, but the direction of correlation was different between leaf litter and fine roots (Table 3.4); soluble phenolics were negatively correlated with soluble protein in leaf litter (P < 0.016,), but positively correlated in fine roots (P < 0.001, Table 3.4). The fraction of CTs remaining were positively correlated with that of soluble proteins for both leaf litter and fine roots, and this positive correlation was the strongest in fine roots (R = 0.400, P < 0.001). The fraction of soluble proteins remaining was negatively correlated with that of NSCs in leaf litter decomposed in the forest floor (R = - 0.242, P = 0.008), but these traits were positively correlated in fine roots (R = 0.330, P < 0.001, Table 3.4). The fraction of soluble protein remaining was also positively correlated with N fraction remaining (R > 0.337, P < 0.001) in leaf litter, but this correlation was not significant for fine roots (R = 0.084, P = 0.202, Table 3.4).

We presented the FTIR spectra for leaf litter and fine roots averaged across study sites in Fig. 3.1. The spectra of leaf litter and fine roots exhibited a typical broad and strong band for O-H stretch around 3300 cm^{-1}, a band for C_{SP3}-H stretch at 2800 cm^{-1}, and a number of distinct peaks in finger print region ranging from 600 to 1800 cm^{-1}. We identified lignin reference peak at 1511 cm^{-1} for aromatic skeleton vibration in lignin, while polysaccrides characteristics bands were identified at 898 cm^{-1} for C-H deformation in cellulose, 1158 cm^{-1} for glyosidic linkage (C-O-C) in polysaccrides, and 1375 cm^{-1} for C-H deformation in various polysaccrides (Faix 1991; Pandey & Pitman 2003). The distinct peaks at 1734 cm^{-1} and 1060 cm^{-1} are also used as reference for polysaccrides in many studies, but not in this study. The peak at 1735 cm^{-1} arises from C = O stretch, typically in hemicellulose (xylan). The intensity of this peak decreased substantially during leaf litter decomposition, which could indicate the degradation of hemicellulose (Fig. 3.1). However, it can also arise from ester C = O in suberin (Ferreira *et al.,* 2013), which is abundant in fine roots (Riederer *et al.,* 1993). The strong band at 1060 cm^{-1} typically arises from C-O stretch in polysaccharides. However, Si-O in clay minerals also contribute to this band. Because the decomposed samples in this study contained highly variant ash concentrations, it is difficult to interpret the intensity for this peak.

Fine roots exhibited different spectra than leaf litter, with a stronger lignin peak at 1511 cm^{-1}, and generally weaker carbohydrate peaks (Fig. 3.1). The spectra of the undecomposed roots were similar with that of decomposed roots at three years, while the comparison of spectra for undecomposed and decomposed leaf litter indicates considerable chemical shifts during decomposition (Fig. 3.1), mostly due to the decreases of carbohydrate peaks. We estimated the preservation of lignin relative to polysaccharides during decomposition by computing the lignin/carbohydrate peak intensity ratios ($\Delta I_{L/C}$) for decomposed material minus those of initial litters before decomposition. The positive values for all $\Delta I_{L/C}$ indicated that lignin was preferentially preserved relative to polysaccharides for both leaf litter and fine roots during three years of decomposition (Table 3.5). Generally, N additions increased $\Delta I_{L/C}$ values (Table 3.5). Fine roots decomposed under simulated N deposition consistently had significantly higher $\Delta I_{1511/1158}$, $\Delta I_{1511/1370,}$

$\Delta I_{1511/\Sigma}$ across sites ($P < 0.034$), while the responses of leaf litter were more variable among sites (Table 3.5). Simulated N deposition generally increased leaf litter $\Delta I_{1511/1370}$, whether decomposed at the forest floor or at O/A interface, while the increases were the strongest at site A (Site × N: $F = 11.25$, $P < 0.001$, forest floor; $F = 15.74$, $P < 0.001$, O/A interface). Simulated N deposition also tends to increase leaf litter $\Delta I_{1511/\Sigma}$ at all sites but not at site C for leaf litter decomposed at O/A interface (Site × N: $F = 4.18$, $P = 0.023$).

All lignin/carbohydrate FT-IR peak ratios showed significant correlation with AIF and AIF/ASF ratios determined using gravimetric methods ($P < 0.001$, Table 3.6). Among four lignin/carbohydrate peak ratios, the values of $I_{1511/\Sigma}$ exhibited the highest correlation with both AIF ($R = 0.866$, $P < 0.001$) and AIF/ASF ratios ($R = 0.896$, $P < 0.001$, Table 3.6).

3.5 Discussion

In Chapter 2, we reported that simulated nitrogen (N) deposition significantly decreased fine root mass loss in the late-stages of decomposition. In this chapter, we sought to understand what specific chemical fractions within fine root material were affected by simulated N deposition or if simulated N deposition slows litter decomposition in a relative non-specific way. Our measurements of both the acid-insoluble fraction (AIF, also termed Klason lignin) and the lignin/carbohydrate reference FT-IR peak ratios provide evidence that simulated N deposition selectively preserved lignin. Also, lignin/carbohydrate FT-IR peak ratios were highly correlated with AIF concentrations and AIF/acid-soluble fraction ratios ($R > 0.866$, $P < 0.001$, Table 3.6). As AIF is the dominant component of fine root tissue (see Table 1.2 in Chapter 1), we estimated that the reduced degradation of AIF comprised nearly three thirds of the additional root mass remaining at our sites.

Our observations of leaf litter decomposition may help identify the mechanism underlying the contrasting effects of N additions on different litter materials. Knorr *et al.*, (2005) conducted a meta-analysis on the effects of N additions leaf litter decomposition and found that N additions tend to inhibit decomposition when litter was lignin-rich, but stimulated or had neutral effects when litter was

low in lignin. In this study, simulated N deposition tended to decrease lignin degradation in leaf litter (Table 3.1, Table 3.5). However, such effect only occurred in the early stage of decomposition, and did not increase overall mass retention (Table 3.1), probably because that leaf litter was low in AIF (14.4 – 16.8 %, see Table 1.2 in Chapter 1), and the small increase in AIF can be easily offset by increased decomposition of other chemical classes. We observed that N additions significantly stimulated the degradation of non-structural carbohydrates in leaf litter (Table 3.1). Nitrogen additions have also been observed to enhanced cellulose-degrading enzyme activity in other ecosystems (*e.g*. Carreiro *et al.,* 2000; Sun *et al.,* 2016). Accordingly, although simulated N deposition tends to inhibit lignin degradation, the ability of this effect to meaningfully increase the amount of litter mass remaining (and thus cause the accumulation of soil organic matter) apparently depends on the quantity of lignin within the initial substrates.

The inhibition of lignin degradation by simulated N deposition is consistent with evidence that N additions suppress lignin-degrading oxidative enzyme activity observed by many studies. Under laboratory conditions, higher external N availability repressed lignin-degradation activity of certain white-rot fungal species (Fenn *et al.,* 1981; Boominathan *et al.,* 1990; Van der Woude *et al.,* 1993), while N limitation stimulated lignin-degrading activity (Reid *et al.,* 1983). Previous work at our study sites observed that simulated N deposition suppressed the activity of the lignin-degrading phenol oxidase and peroxidase extracellular enzymes (DeForest *et al.,* 2004), and down-regulated expression of the *lcc* gene that is responsible for synthesis of laccase (phenol oxidase, Edward *et al.,* 2011). One proposed mechanism for this effect is "microbial N mining": N limitation can increase litter decomposition as microorganisms tend to use labile substrate to decompose and then acquire N from more recalcitrant organic fraction; while high N availability can relieve the pressure of N acquisition (*sensu* Craine *et al.,* 2007).

Simulated N deposition also caused a slower loss of condensed tannins (CTs) and soluble proteins in both leaf litter and fine roots (Table 3.1, Table 3.2). Notably, the oxidative enzymes involved in lignin decomposition and repressed by N additions can also degrade CTs. Hernandez *et*

al. (2005) cultured 11 fungal strains on tannins, and found that while hydrolysable tannins were degraded by tannase (a hydrolase), the fungi used laccase and peroxidase to utilize condensed tannins. The fraction of protein remaining was positively and significantly correlated that of condensed tannins (CTs) in both leaf litter and fine roots (Table 3.4). It is known that CTs can form recalcitrant complexes with proteins (Bravo 1998). The greater fraction of CTs remaining may help stabilize proteins in the decomposing litter, leading to a greater fraction of proteins remaining in the decomposing litter. Because CTs can be toxic and inhibit soil enzyme activity (Ushio *et al.,* 2013), the slower degradation of CTs in soils may contribute to the general reduction of microbial biomass and heterotrophic respiration that have been observed in many N additions studies (*e.g*., Treseder 2008; Janssens *et al.,* 2010).

Lignin/carbohydrates reference FT-IR peak ratios exhibited high correlations with AIF and AIF/ASF (Table 3.6). AIF and ASF obtained through sequential extraction and measured gravimetrically have conventionally been thought as the counterpart of lignin and structural polysaccrides (*e.g*. Taylor *et al.,* 1989; Berg 2000). The recalcitrance of AIF has been demonstrated by a number of reports that higher initial AIF contents or AIF/N ratio resulted in slower litter decomposition (Taylor *et al.,* 1989; Berg 2000; Preston *et al.,* 2000; Adair *et al.,* 2008). However, the ability of AIF to accurately predict lignin concentration is often questioned, because AIF is not purely lignin, but a mixture of lignin and other complex substrates such as cutin, suberins, and CT-protein complexes (Preston *et al.,* 1997). In this study, AIF is highly associated with lignin/carbohydrate characteristics band ratios, and the AIF/ASF ratio explained 80.3 % of variation in $I_{1511/\Sigma}$ values. The strength of these relationships supports the idea that AIF is a good predictor of lignin in plant chemistry and litter decomposition studies.

3.6 References

Adair EC, Parton WJ, Del Grosso SJ, Silver WL, Harmon ME, Hall SA, Burke IC, Hart SC. 2008. Simple three-pool model accurately describes patterns of long-term litter decomposition in diverse climates. *Global Chang Biology* 14: 2636-2660.

Berg B. 2000. Litter decomposition and organic matter turnover in northern forest soils. *Forest Ecology and Management* 133: 13-22.

Bligh EG, Dyer WJ. 1959. A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology* 37: 911-917.

Booker FL, Anttonen S, Heagle AS. 1996. Catechin, proanthocyanidin, and lignin contents of loblolly pine (*Pinus taeda*) needles after chronic exposure to ozone. *New Phytologist* 132: 483-492.

Boominathan K, Reddy CA. 1992. cAMP-mediated differential regulation of lignin peroxidase and manganese-dependent peroxidase production in the white-rot basidiomycete Phanerochaete chrysosporium. *Proceedings of the National Academy of Sciences* 89: 5586-5590.

Bowman WD, Cleveland CC, Halada Ĺ, Hreško J, Baron JS. 2008. Negative impact of nitrogen deposition on soil buffering capacity. *Nature Geoscience* 1: 767-770.

Bravo L. 1998. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition reviews* 56: 317-333.

Broadbent F. 1965. Effect of fertilizer nitrogen on the release of soil nitrogen. *Soil Science Society of America Journal* 29: 692-696.

Carreiro MM, Sinsabaugh RL, Repert DA, Parkhurst DF. 2000. Microbial enzyme shifts explain litter decay responses to simulated nitrogen deposition. *Ecology* 81: 2359-2365.

Chapin III FS, Chapin MC, Matson PA, Vitousek P. 2011. *Principles of terrestrial ecosystem ecology*. New York, USA: Springer.

Chow PS, Landhäusser SM. 2004. A method for routine measurements of total sugar and starch content in woody plant tissues. *Tree physiology* 24: 1129-1136.

Ciais P, Sabine C, Bala G, Bopp L, Brovkin V, Canadell J, Chhabra A, DeFries R, Galloway J, Heimann M, Jones C. Carbon and other biogeochemical cycles. 2014. In *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge University Press.

Craine JM, Morrow C, Fierer N. 2007. Microbial nitrogen limitation increases decomposition. *Ecology* 88: 2105-2113.

DeForest JL, Zak DR, Pregitzer KS, Burton AJ. 2004. Nitrate deposition and the microbial degradation of cellulose and lignin in a northern hardwood forest. *Soil Biology & Biochemistry* 36: 965-971.

Edwards IP, Zak DR, Kellner H, Eisenlord SD, Pregitzer KS. 2011. Simulated atmospheric N deposition alters fungal community composition and suppresses lignocellulolytic gene expression in forest floor of a northern hardwood forest. *PLoS One* 6: e20421.

Fabiyi JS, McDonald AG, Morrell JJ, Freitag C. 2011. Effects of wood species on durability and chemical changes of fungal decayed wood plastic composites. Composites Part A: *Applied Science and Manufacturing* 42: 501-510.

Faix O. 1991. Classification of lignins from different botanical origins by FT-IR spectroscopy. *Holzforschung-International Journal of the Biology, Chemistry, Physics and Technology of Wood* 45: 21-28.

Fenn P, Kirk TK. 1981. Relationship of nitrogen to the onset and suppression of ligninolytic activity and secondary metabolism in Phanerochaete chrysosporium. *Archives of Microbiology* 130: 59-65.

Ferreira R, Garcia H, Sousa AF, Freire CS, Silvestre AJ, Rebelo LP, Pereira CS. 2013. Isolation of suberin from birch outer bark and cork using ionic liquids: a new source of macromonomers. *Industrial Crops and Products* 44: 520-527.

Frey SD, Ollinger S, Nadelhoffer K, Bowden R, Brzostek E, Burton A, Caldwell BA, Crow S, Goodale CL, Grandy AS, Finzi A. 2014. Chronic nitrogen additions suppress decomposition and sequester soil carbon in temperate forests. *Biogeochemistry* 121: 305-316.

Gruber N, Galloway JN. 2008. An Earth-system perspective of the global nitrogen cycle. *Nature* 451: 293-296.

Gundersen P, Rasmussen L. 1990. Nitrification in forest soils: effects from nitrogen deposition on soil acidification and aluminum release. In *Reviews of Environmental Contamination and Toxicology: p*1-45. Springer.

Högberg P. 2007. Environmental science: nitrogen impacts on forest carbon. *Nature* 447: 781-782.

Hyvönen R, Persson T, Andersson S, Olsson B, Ågren GI, Linder S. 2008. Impact of long-term nitrogen addition on carbon stocks in trees and soils in northern Europe. *Biogeochemistry* 89: 121-137.

Janssens IA, Dieleman W, Luyssaert S, Subke JA, Reichstein M, Ceulemans R, Ciais P, Dolman AJ, Grace J, Matteucci G, Papale D. 2010. Reduction of forest soil respiration in response to nitrogen deposition. *Nature Geoscience* 3: e15-322.

Knorr M, Frey S, Curtis P. 2005. Nitrogen additions and litter decomposition: a meta-analysis. *Ecology* 86: 3252-3257.

LeBauer DS, Treseder KK. 2008. Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. *Ecology* 89: 371-379

Li C, Trombley JD, Schmidt MA, Hagerman AE. 2010. Preparation of an acid butanol standard from fresh apples. *Journal of chemical ecology* 36: 453-460.

Little RC, Milliken GA, Stroup WW, Wolfinger RD, Schabenberger O. 2006. *SAS for mixed models.* Cary, US: SAS Institute Inc.

Liu X, Zhang Y, Han W, Tang A, Shen J, Cui Z, Vitousek P, Erisman JW, Goulding K, Christie P, Fangmeier A. 2013. Enhanced nitrogen deposition over China. *Nature* 494: 459-462.

Magill AH, Aber JD. 1998. Long-term effects of experimental nitrogen additions on foliar litter decay and humus formation in forest ecosystems. *Plant and Soil* 203: 301-311.

Pandey K, Pitman A. 2003. FTIR studies of the changes in wood chemistry following decay by brown-rot and white-rot fungi. *International biodeterioration & biodegradation* 52: 151-160.

Pandey K, Pitman A. 2004. Examination of the lignin content in a softwood and a hardwood decayed by a brown-rot fungus with the acetyl bromide method and Fourier transform infrared spectroscopy. *Journal of Polymer Science Part A: Polymer Chemistry* 42: 2340-2346.

Pregitzer KS, Burton AJ, Zak DR, Talhelm AF. 2008. Simulated chronic N deposition increases carbon storage in northern temperate forests. *Global Change Biology* 14: 142–153.

Preston CM, Trofymow JA, Working Group TC. 2000. Variability in litter quality and its relationship to litter decay in Canadian forests. *Canadian Journal of Botany* 78: 1269-1287.

Rodrigues J, Faix O, Pereira H. 1998. Determination of lignin content of Eucalyptus globulus wood using FTIR spectroscopy. *Holzforschung-International Journal of the Biology, Chemistry, Physics and Technology of Wood* 52: 46-50.

Sun T, Dong L, Wang Z, Lü X, Mao Z. 2016. Effects of long-term nitrogen deposition on fine root decomposition and its extracellular enzyme activities in temperate forests. *Soil Biology & Biochemistry* 93: 50-59.

Sutton MA, Simpson D, Levy PE, Smith RI, Reis S, Van Oijen M, De Vries WI. 2008. Uncertainties in the relationship between atmospheric nitrogen deposition and forest carbon sequestration. *Global Change Biology* 14: 2057-2063.

Taylor BR, Parkinson D, Parsons WF. 1989. Nitrogen and lignin content as predictors of litter decay rates: a microcosm test. *Ecology* 70: 97-104.

Thomas RQ, Canham CD, Weathers KC, Goodale CL. 2010. Increased tree carbon storage in response to nitrogen deposition in the US. *Nature Geoscience* 3(1): 13-7.

Treseder KK. 2008. Nitrogen additions and microbial biomass: A meta-analysis of ecosystem studies. *Ecology Letters* 11: 1111-1120.

Ushio M, Balser TC, Kitayama K. 2013. Effects of condensed tannins in conifer leaves on the composition and activity of the soil microbial community in a tropical montane forest. *Plant and soil* 365: 157-170.

Van der Woude MW, Boominathan K, Reddy CA. 1993. Nitrogen regulation of lignin peroxidase and manganese-dependent peroxidase production is independent of carbon and manganese regulation in Phanerochaete chrysosporium. *Archives of Microbiology* 160: 1-4.

Vitousek PM, Aber JD, Howarth RW, Likens GE, Matson PA, Schindler DW, Schlesinger WH, Tilman DG. 1997. Human alteration of the global nitrogen cycle: sources and consequences. *Ecol. Appl.* 7: 737–750.

Xia M, Talhelm AF, Pregitzer KS. 2015. Fine roots are the dominant source of recalcitrant plant litter in sugar maple-dominated northern hardwood forests. *New Phytologist* 208: 715-726.

Zak DR, Holmes WE, Burton AJ, Pregitzer KS, Talhelm AF. 2008. Simulated atmospheric NO_3^- deposition increases soil organic matter by slowing decomposition. *Ecol. Appl.*18: 2016-2027.

Table 3.1 Fraction remaining (%) of original mass and amount of chemical classes in leaf litter decomposed in the forest floor and at O/A interface after three months, one year, and three years of decomposition under simulated N deposition across four northern hardwood forests

Litter traits	Treatment	Forest floor			O/A interface		
		3 mon	1 yr	3 yr	3 mon	1 yr	3 yr
Mass	Ambient	64.9 (2.7)	45.7 (2.9)	24.6 (3.6)	52.3 (4.5)	30.2 (2.9)	15.0 (4.2)
	NO_3^-	64.6 (3.3)	45.3 (3.9)	25.4 (5.2)	51.9 (3.1)	30.1 (4.0)	15.1 (3.3)
AIF	Ambient	145.8 (11.2)	113.0 (9.7)	65.9 (12.1)	127.5 (18.5)	76.5 (7.0)	37.0 (11.5)
	NO_3^-	156.4** (6.6)	116.6 (11.2)	69.9 (11.1)	136.3 (15.6)	82.3 (12.3)	41.2 (10.2)
ASF	Ambient	62.6 (5.2)	43.6 (5.2)	22.8 (3.1)	51.4 (5.2)	29.3 (5.1)	14.8 (3.9)
	NO_3^-	59.8 (5.0)	46.0‡ (5.4)	24.8 (6.6)	50.0‡ (6.2)	28.8 (4.8)	14.2 (4.9)
PHE	Ambient	20.0 (4.3)	6.0 (2.0)	1.4 (0.5)	7.8 (2.4)	2.03 (0.6)	0.7 (0.2)
	NO_3^-	20.3 (5.6)	6.0 (2.1)	1.8 (0.8)	8.6 (2.0)	2.37 (0.4)	0.9 (0.3)
CTs	Ambient	27.5 (5.8)	14.3 (8.3)	4.8 (4.0)	14.7 (7.1)	5.1 (2.4)	2.8 (1.6)
	NO_3^-	41.1** (14.3)	16.5 (7.3)	5.4‡ (7.1)	20.9**(6.2)	7.3 (6.0)	3.1 (1.3)
NSCs	Ambient	16.7 (3.4)	10.3 (2.5)	4.6 (1.1)	11.1 (1.8)	5.8 (1.1)	3.1 (1.1)
	NO_3^-	13.1** (2.5)	7.8*** (2.5)	4.3 (1.1)	9.0**‡ (2.0)	4.5** (1.1)	2.4 (1.0)
Lipids	Ambient	52.2 (5.7)	31.2 (5.0)	14.9 (2.7)	38.7 (5.1)	20.7 (3.0)	9.3 (3.5)
	NO_3^-	53.2 (5.5)	30.0 (7.2)	16.9 (3.4)	38.8 (4.2)	19.6 (4.4)	10.4 (4.2)
Proteins	Ambient	105.8 (30.1)	101.4 (25.8)	59.2 (15.9)	93.4 (23.6)	73.4 (21.8)	43.9 (11.7)
	NO_3^-	122.9**‡ (30.2)	103.8 (25.0)	67.6 (15.8)	98.4‡ (23.1)	69.1‡ (13.2)	45.8 (16.3)
Nitrogen	Ambient	111.4 (21.0)	112.2 (11.1)	78.2 (13.0)	99.0 (17.2)	76.3 (12.3)	49.2 (13.0)
	NO_3^-	101.8 (10.2)	100.0**‡ (16.0)	69.2 (13.7)	87.6***‡ (7.8)	65.6***‡ (10.5)	40.0 (7.5)

Values are shown as means (SD) of fraction remaining of mass and chemical classes in leaf litter decomposed in each plot of ambient or N-amended across four study sites (n = 12). *, **, *** denote significant main effects of simulated N deposition at $P < 0.05$, $P < 0.01$, $P < 0.001$. ‡ refers to a significant interaction of site and NO_3^- at $P < 0.05$. The details of the interaction effects were explained in the results. AIF: acid-insoluble fraction; ASF: acid-soluble fraction; PHE: solubel phenolics; CTs: condesned tannins; NSCs: non-structural carbohydrates.

Table 3.2 Fraction remaining (%) of original mass and amount of chemical classes in fine roots after three months, one year, and three years of decomposition under simulated N deposition across four northern hardwood forests

Litter traits	Treatment	Fraction remaining (%) in fine roots		
		3 mon	1 yr	3 yr
Mass	Ambient	82.6 (3.0)	70.9 (4.1)	51.3 (2.5)
	NO_3^-	83.4 (1.8)	72.8 (4.4)	56.4*** (3.2)
AIF	Ambient	99.5 (4.9)	82.4 (7.9)	60.1 (4.6)
	NO_3^-	99.3 (4.8)	84.1‡ (6.2)	69.3*** (4.4)
ASF	Ambient	64.1 (5.4)	61.6 (5.8)	45.9 (3.3)
	NO_3^-	64.9 (6.6)	61.1 (3.6)	46.4 (4.3)
PHE	Ambient	54.6 (5.4)	40.1 (8.8)	24.1 (5.2)
	NO_3^-	56.0 (9.8)	42.4‡ (5.7)	30.0 (10.8)
CTs	Ambient	49.7 (5.9)	35.5 (6.0)	26.3 (4.6)
	NO_3^-	56.7** (8.4)	39.5 (5.1)	30.7** (5.5)
NSCs	Ambient	42.9 (9.6)	36.5 (7.8)	29.4 (3.8)
	NO_3^-	44.4 (7.0)	36.0 (10.0)	33.4 (7.1)
Lipids	Ambient	71.3 (23.6)	59.6 (16.7)	43.8 (8.8)
	NO_3^-	77.3 (13.0)	52.7 (19.5)	50.3 (7.6)
Proteins	Ambient	49.0 (4.5)	51.1 (7.2)	43.8 (4.8)
	NO_3^-	55.2* (5.4)	57.4* (8.4)	52.3** (5.4)
Nitrogen	Ambient	89.8 (5.3)	84.8 (9.0)	69.1 (6.8)
	NO_3^-	86.3 (4.2)	80.9 (5.8)	72.3 (5.9)

Values are shown as means (SD) of fraction remaining of mass and chemical classes in fine roots decomposed in each plot of ambient or N-amended across four study sites (n = 12). *, **, *** denote significant main effects of simulated N deposition at $P < 0.05$, $P < 0.01$, $P < 0.001$. ‡ refers to a significant interaction of site and NO_3^- at $P < 0.05$. The details of the interaction effects were explained in the results. AIF: acid-insoluble fraction; ASF: acid-soluble fraction; PHE: solubel phenolics; CTs: condesned tannins; NSCs: non-structural carbohydrates:

Table 3.3. Mixed linear model analysis on a factorial split-plot design testing the differences of AIF concentrations and fraction remaining (%) in fine roots at three years of decomposition among study sites, external N availability, and substrate source (fine roots collected from ambient vs. NO_3^- amended plots)

Source of variance		*AIF concentration*		*AIF fraction remaining*	
	n/m	*F*	*P*	*F*	*P*
Site	3/16	1.07	0.390	2.14	0.135
External N Availability	1/16	49.10	**<.001**	33.16	**<.001**
Site × N	3/16	0.71	0.559	0.62	0.613
Substrate Source	1/16	0.02	0.886	1.01	0.329
Site × Source	3/16	0.41	0.750	5.01	**0.012**
N × Source	1/16	0.62	0.443	2.59	0.127
Site × N × Source	3/16	2.18	0.130	1.32	0.304

The degrees of freedom, numerator *df* / denominator *df*, are shown as "n/m".

Table 3.4 Partial Spearman rank correlation matrix for fraction remaining of chemical classes in leaf litter and fine roots during three years of decomposition.

	AIF	ASF	PHE	CTs	LIP	PRO	NSC
Leaf litter (forest floor)							
ASF	-0.053						
PHE	0.009	0.139					
CTs	0.148	0.046	0.451***				
LIP	0.096	0.119	0.127	0.233*			
PRO	0.345	0.121	-0.220*	0.207*	0.230*		
NSC	-0.139	0.006	0.095	0.095	0.061	-0.242**	
N	0.397	-0.031	-0.103	-0.103	0.081	0.355***	0.038
Leaf litter (O/A)							
ASF	-0.117						
PHE	0.180	0.111					
CTs	0.105	-0.039	0.540***				
LIP	0.228*	0.052	0.240**	0.202*			
PRO	0.208*	0.070	-0.250**	0.121	0.186*		
NSC	-0.142	0.236*	0.254**	0.164	0.172	-0.162	
N	0.137	-0.068	-0.115	-0.046	0.008	0.337***	0.197*
Fine roots							
ASF	-0.395***						
PHE	0.196**	-0.108					
CTs	0.088	-0.259***	0.521***				
LIP	0.050	-0.036	-0.009	0.177**			
PRO	-0.042	-0.161*	0.438***	0.400***	0.003		
NSC	0.001	-0.066	0.149*	0.014	0.092	0.330***	
N	0.392***	0.096	0.211**	-0.142*	-0.073	0.084	0.082

Partial Spearman rank correlation coefficients were shown here, because preliminary examination revealed that many relationships between two variables in this table were non-linear. We used chemical data of leaf litter and fine roots from all five harvest time points (from 1 months to 3 years of decomposition) to conduct this correlation analysis (n = 240 for fine roots, n = 128 for leaf litter). AIF: Acid-insoluble fraction; ASF: acid-soluble fraction; PHE, soluble phenolics; CTs, condensed tannins; LIP, lipids; PRO, soluble proteins; NSCs, non-structural carbohydrates; N, nitrogen.

Table 3.5. The differences of ratios of lignin/carbohydrate reference peaks ($\Delta I_{L/C}$) between decomposed (3 years) and initial litters under simulated N deposition across four norther forests.

Litter type	Treatment	$\Delta I_{1511/898}$	$\Delta I_{1511/1158}$	$\Delta I_{1511/1370}$	$\Delta I_{1511/\Sigma}$
Leaf litter	Ambient	2.95 (1.36)	0.805 (0.663)	0.595 (0.123)	0.331 (0.104)
(forest floor)	NO_3^-	3.69 (1.67)	0.709 (0.255)	$0.726^{**‡}$ (0.165)	0.349 (0.055)
Leaf litter	Ambient	1.70 (1.48)	0.267 (0.442)	0.691 (0.148)	0.258 (0.097)
(O/A interface)	NO_3^-	2.09 (1.43)	0.489 (0.358)	$0.816^{**‡}$ (0.182)	$0.326^{*‡}$ (0.084)
Fine roots	Ambient	2.49 (2.0)	0.374 (0.292)	0.412 (0.181)	0.185 (0.071)
	NO_3^-	3.24 (2.2)	0.560^{*}(0.230)	0.704^{*} (0.394)	0.294^{**} (0.120)

Values are shown as means (SD) of fraction remaining of mass and chemical classes decomposed in each of ambient or NO_3^- amended plots across four study sites (n = 12). The calculation of $\Delta I_{L/C}$ is shown in Materials and Methods. Generally, higher values of $\Delta I_{L/C}$ imply that more lignin is preserved relative to carbohydrates during litter decomposition. *, ** denote significant main effects of simulated N deposition at $P < 0.05$, $P < 0.01$. ‡ refers to a significant interaction of site and N treatment at $P < 0.05$. The sum of intensity at 898 cm^{-1}, 1158 cm^{-1}, and 1370 cm^{-1} was used as the carbohydrate reference intensity when calculating $\Delta I_{1511/\Sigma}$ values.

Table 3.6. Pearson's correlation coefficient matrix for lignin/carbohydrates FTIR peak intensity ratios, AIF and AIF/ASF ratio.

	AIF	AIF/ASF	$I_{1511/898}$	$I_{1511/1158}$	$I_{1511/1370}$
AIF/ASF	0.945				
$I_{1511/898}$	0.638	0.693			
$I_{1511/1158}$	0.673	0.706	0.565		
$I_{1511/1370}$	0.821	0.845	0.718	0.597	
$I_{1511/\Sigma}$	0.866	0.896	0.808	0.792	0.950

All correlation coefficients were significant at $P < 0.001$. We used initial leaf litter and fine roots, and decomposed litters at the end of study to generate correlation coefficients (n = 128).

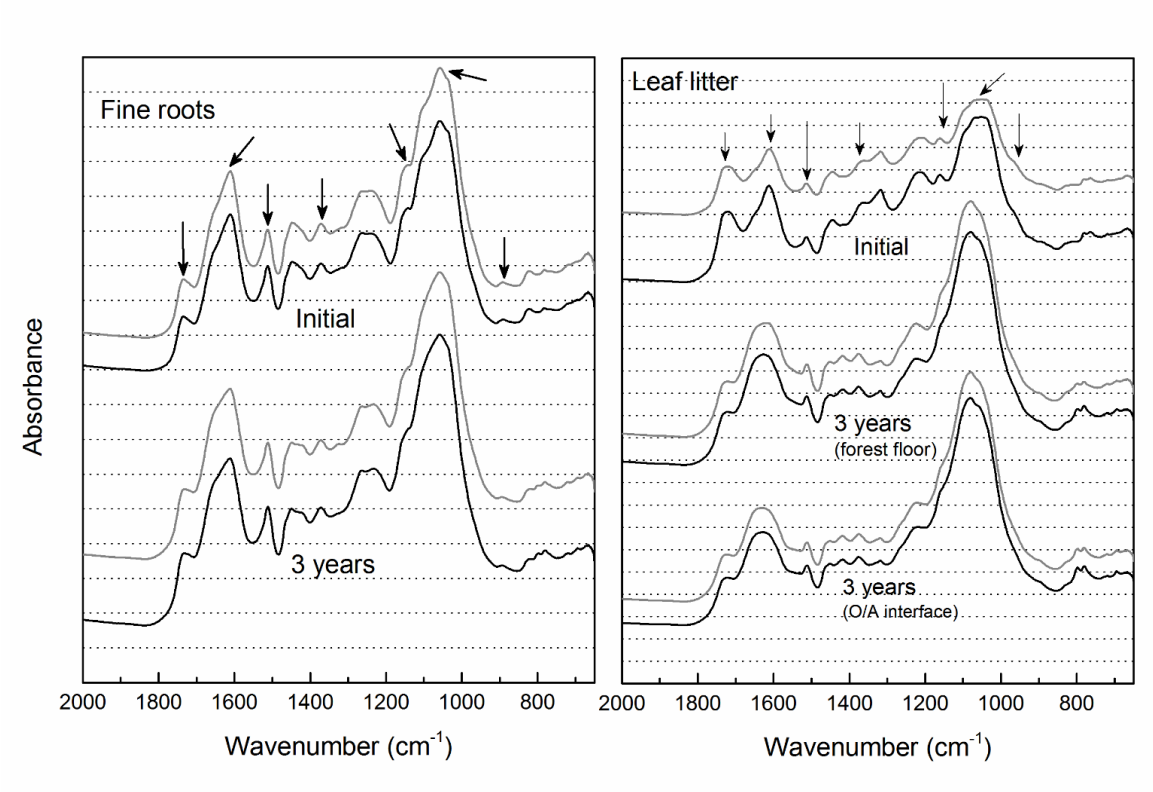

Fig. 3.1 FTIR spectra of fine roots and leaf litter before decomposition and after three years of decomposition under ambient conditions (grey line) and simulated N deposition (dark line). Spectra were shifted along the y-axis. Each spectrum represents the average of three replicates of a N treatment across four northern hardwoods forests (n = 12). Arrows indicate the peaks at 1740 cm^{-1}, 1620 cm^{-1}, 1511 cm^{-1}, 1370 cm^{-1}, 1158 cm^{-1}; 1060 cm^{-1} and 898^{-1} respectively for both leaf litter and fine root spectra.

Appendix A: A letter from New Phytologist Managing Editor

From: New Phytologist Managing Editor <np-managinged@lancaster.ac.uk>

Sent: Apr 13, 2016 8:14 AM

To: Xia, Mengxue (xia7836@vandals.uidaho.edu)

Subject: RE: Permission to use published article in Ph.D. dissertation, thanks!

Dear Mengxue,

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Dr FIONA TOOKE

Peer Review Manager, New Phytologist

New Phytologist Central Office, Bailrigg House, Lancaster University, Lancaster, LA1 4YE, UK

Tel: + 44 1524 592918 Fax: + 44 1524 594696 Email: f.tooke@lancaster.ac.uk

Website: www.newphytologist.org Twitter: @NewPhyt Facebook: fb.com/NewPhytologist

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2014 Impact factor 7.672

From: Xia, Mengxue (xia7836@vandals.uidaho.edu) [mailto:xia7836@vandals.uidaho.edu]

Sent: 13 April 2016 09:24

To: New Phytologist

Subject: Permission to use published article in Ph.D. dissertation, thanks!

Dear New Phytologist Trust office,

My colleagues and I had the honor to publish an article "Xia M, Talhelm AF, Pregitzer KS. 2015. Fine roots are the dominant source of recalcitrant plant litter in sugar maple-dominated northern hardwood forests. *New Phytologist* 208(3):715-726" in *New Phytologist* last year. I am graduating this May. I would like to include this paper in my Ph.D. dissertation. According to the policy of my university, I should include a copy of a letter from the publisher of this article to indicate that it is permissible to include this article in my dissertation.

I am the first author of this article. My major professor Dr. Kurt Pregitzer is the corresponding author of the article. I'd like to know how to proceed so that you can send me a permission letter.

Thanks!

I included this article in the email.

Mengxue Xia

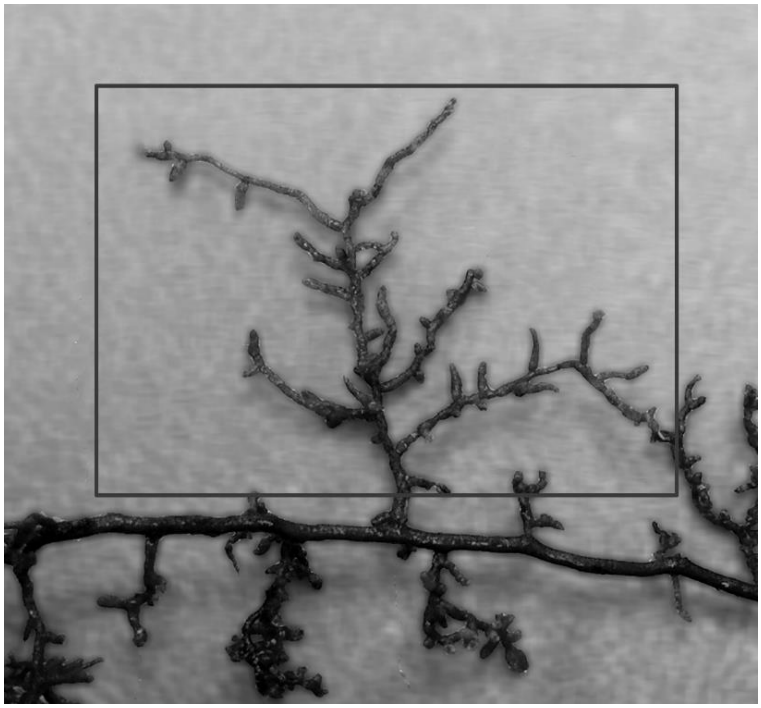
College of Natural Resources

University of Idaho

USA

Phone: +1 208-596-0261

Appendix B: First three orders of the branching fine root system of maple (*Acer*) collected from the hardwood forest study sites.



Appendix C: Diameter (mm) distribution for the first three orders of maple (*Acer*) roots

Root order	Root diameter	
	Average	n
Order 1	0.26 (0.08)	1517
Order 2	0.26 (0.05)	1507
Order 3	0.30 (0.11)	383

The average diameter (SD) of each order were derived from root segments in three ambient plots in each of the four study sites. For each plot, approximately five intact large root branches were randomly selected when roots were excavated according to the procedure described from the Methods and Materials. For each large root branch, five to six root segments of Order 3 and about 20 segments of Order 1 and 2 were carefully dissected from their mother branches according to the procedures of Pregitzer *et al.* (2002) and measured for diameter with a dissecting microscope (Zeiss, Oberkochen, Germany).

Reference

Pregitzer KS, DeForest JL, Burton AJ, Allen MF, Ruess RW, Hendrick RL. 2002. Fine root architecture of nine North American trees. *Ecological Monographs* 72: 293-309.

Appendix D: Sequential extraction for the extractive-free fraction

Extractive-free fraction was obtained with a sequential extraction procedure (Friend, 1992; Booker *et al.,* 1996). Extractive-free tissue was prepared by washing 25 mg samples with 50% methanol (3×), methanol : chloroform : water (2.0 : 1.0 : 0. 8) (3×), phenol : acetic acid : water (PAW, 2.0 : 1.0 : 0. 9) (3×, with an overnight incubation during the second wash), and ethanol (5×), with centrifugations (1400 g, 5 min) between washings. PAW washes were used to remove bulk proteins from the residue (Laird *et al.,* 1976; Friend, 1992). Samples were dried at 70 °C and weighed as the mass of extractive-free fraction.

Reference

Booker FL, Anttonen S, Heagle AS. 1996. Catechin, proanthocyanidin, and lignin contents of loblolly pine (Pinus taeda) needles after chronic exposure to ozone. *New Phytologist* 132: 483-492.

Friend J. 1992. Lignin and associated phenolic acids in cell walls. In: Gurr SJ, McPherson MJ, eds. *Molecular Plant Pathology: A Practical Approach*. Oxford: IRL Press.

Laird WM, Mbadiwe EI, Synge RL. 1976. A simplified procedure for fractionating plant materials. *Journal of the Science of Food and Agriculture* 27:127-130.

Appendix E: Mixed linear model analysis of biochemical traits among study sites, nitrogen (N) deposition treatments, and tissue type in a split-plot design

Chemical	Whole-plot						Within-plot							
	Site		N deposition		Site × N		Tissue		Tissue × site		Tissue × N		Tissue × site ×	
	df = 3 / 16		*df* = 1 / 16		*df* = 3 / 16		*df* =1 / 16		*df* =3 / 16		*df* =1 / 16		*df* =3 / 16	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Cell-wall fraction	2.54	0.093	4.88	**0.042**	4.1	**0.024**	188.3	**<0.001**	2.76	0.076	7.61	**0.014**	4.8	**0.014**
AIF	4.28	**0.021**	6.55	**0.021**	0.8	0.496	8435	**<0.001**	3.25	0.050	14.6	**0.002**	5.6	**0.008**
Hemicellulose	0.38	0.771	0.11	0.745	0.1	0.924	18.85	**<0.001**	1.29	0.311	0.03	0.874	0.5	0.630
Cellulose	1.00	0.418	2.75	0.117	5.0	**0.012**	216.1	**<0.001**	3.45	**0.042**	5.73	**0.029**	2.5	0.091
Extractive fraction	1.66	0.214	1.94	0.183	3.7	**0.034**	314.9	**<0.001**	3.22	0.051	7.62	**0.014**	4.5	**0.018**
Soluble phenolics	3.15	0.054	0.21	0.655	0.9	0.456	940.6	**<0.001**	10.6	**<0.001**	1.70	0.210	3.4	**0.042**
Condensed	1.36	0.289	5.71	**0.030**	0.7	0.520	161.3	**<0.001**	2.88	0.068	1.14	0.301	2.7	0.075
NSCs	5.51	**0.009**	0.90	0.358	1.0	0.403	514.5	**<0.001**	2.38	0.108	2.37	0.143	1.1	0.361
Lipids	5.48	**0.009**	0.74	0.403	1.3	0.290	670.1	**<0.001**	0.42	0.740	0.62	0.443	0.4	0.695
Soluble proteins	1.40	0.278	6.93	**0.018**	0.1	0.942	730.7	**<0.001**	0.61	0.621	0.32	0.582	3.8	**0.031**
Unidentified	1.14	0.362	3.61	0.076	4.1	**0.024**	30.26	**<0.001**	4.13	**0.024**	6.04	**0.026**	6.3	**0.005**
N	7.63	**0.002**	20.4	**<0.001**	2.0	0.149	946.1	**<0.001**	7.82	**0.002**	4.06	0.061	1.4	0.277
AIF : N	4.16	**0.023**	26.6	**<0.001**	2.5	0.093	114.8	**<0.001**	3.29	**0.048**	14.0	**0.002**	2.5	0.093
C : N	10.1	**<0.001**	19.6	**<0.001**	2.1	0.135	790.5	**<0.001**	7.53	**0.002**	7.99	**0.012**	2.1	0.134
Lignocellulose	0.36	0.783	0.49	0.492	3.3	**0.045**	3643	**<0.001**	1.72	0.204	0.02	0.901	2.1	0.130

The mixed linear models included fixed effects shown in the table and random effects of plots nested (n = 3) in (site × N treatment). The degrees of freedom are shown as 'numerator *df* / denominator *df*'. Bold numbers: $P < 0.05$. AIF: acid-insoluble fraction; NSCs: non-structural carbohydrates.

Appendix F: Major biochemical components and three litter quality indices of leaf litter and fine roots at each of the four hardwood forest study sites receiving simulated nitrogen (N) deposition

Chemical characteristics	Site A				Site B			
	Leaf litter		Fine roots		Leaf litter		Fine roots	
	Ambient	N deposition	Ambient	N deposition	Ambient	N deposition	Ambient	N deposition
Cell-wall fraction (%)	70.64 (14.33)	55.59 (1.92)*	82.49 (1.18)	81.91 (0.51)	69.99 (5.77)	64.07 (3.33)	84.32 (0.50)	86.04 (0.22)
AIF	15.54 (0.99)	12.80 (0.24)**	42.71 (1.09)	45.49 (1.76)	15.84 (1.28)	14.91 (0.47)	45.34 (0.49)	45.72 (0.81)
Hemicellulose	15.09 (3.64)	13.91 (0.56)	14.78 (0.99)	15.43 (0.54)	13.60 (1.24)	13.43 (1.39)	16.70 (1.24)	16.02 (1.02)
Cellulose	40.01 (11.26)	28.88 (1.67)	25.01 (0.18)	21.00 (1.11)	40.55 (4.47)	35.73 (2.14)	22.28 (1.99)	24.30 (1.73)
Extractable fraction (%)	29.36 (14.33)	44.41 (1.92)*	17.51 (1.18)	18.09 (0.51)	30.01 (5.77)	35.93 (3.33)	15.68 (0.50)	13.96 (0.22)
Soluble phenolics	12.86 (2.54)	14.55 (0.89)	3.88 (0.21)	4.03 (0.11)	13.26 (2.52)	12.43 (1.25)	3.24 (0.24)	2.99 (0.34)
Condensed tannins	7.53 (2.58)	3.48 (0.73)	14.83 (0.77)	15.82 (1.13)	6.05 (4.17)	4.92 (2.55)	11.70 (1.25)	10.49 (0.50)
Extractable fraction	6.80 (2.55)	3.17 (0.70)	11.75 (0.61)	12.89 (1.27)	5.51 (4.06)	4.46 (2.47)	9.11 (1.18)	7.97 (0.67)
Bound fraction	0.73 (0.15)	0.31 (0.07)*	3.08 (0.16)	2.93 (0.17)	0.53 (0.11)	0.46 (0.10)	2.59 (0.19)	2.52 (0.20)
NSCs	4.81 (0.67)	6.04 (0.59)	2.09 (0.08)	2.10 (0.32)	4.31 (0.24)	4.46 (0.17)	1.73 (0.24)	1.71 (0.10)
Lipids	8.98 (1.50)	8.21 (0.27)	3.72 (0.29)	3.54 (0.33)	7.47 (1.33)	7.41 (0.50)	3.72 (0.21)	3.18 (0.43)
Soluble proteins	1.28 (0.22)	0.91 (0.18)	3.20 (0.07)	3.30 (0.26)	1.06 (0.30)	1.02 (0.16)	3.24 (0.25)	2.61 (0.28)
Unidentified	4.18 (7.24)	14.70 (0.56)*	4.61 (0.94)	5.13 (0.37)	3.90 (1.75)	10.62 (1.80)	3.75 (0.47)	3.47 (0.84)
N (%)	0.63 (0.04)	0.92 (0.27)*	1.35 (0.02)	1.55 (0.06)	0.70 (0.02)	0.94 (0.07)	1.74 (0.11)	1.72 (0.06)
Litter quality indices (ratio)								
AIF : N	24.61 (1.87)	14.73 (4.18)**	31.73 (0.68)	29.42 (1.44)	22.47 (1.21)	15.96 (0.78)(*)	26.30 (1.92)	26.63 (1.31)
C : N	78.22 (3.66)	55.38 (15.01)*	37.38 (0.59)	33.66 (1.43)	67.56 (1.94)	50.81 (4.07) (*)	29.05 (1.80)	29.32 (1.02)
Lignocellulose index	0.23 (0.04)	0.23 (0.004)	0.52 (0.01)	0.56 (0.02)	0.23 (0.02)	0.23 (0.02)	0.54 (0.01)	0.53 (0.01)

(continued)

Chemical characteristics	Site C				Site D			
	Leaf litter		Fine roots		Leaf litter		Fine roots	
	Ambient	N deposition	Ambient	N deposition	Ambient	N deposition	Ambient	N deposition
Cell-wall fraction (%)	67.29 (5.79)	54.76 (1.90) (*)	84.10 (1.71)	86.24 (1.11)	64.67 (3.17)	73.72 (2.84)	83.42 (0.90)	83.32 (2.35)
AIF	14.49 (0.71)	13.57 (0.71)	45.90 (1.37)	46.72 (0.72)	14.81 (0.94)	14.81 (0.52)	46.42 (2.36)	45.16 (1.27)
Hemicellulose	13.67 (0.53)	13.04 (1.14)	15.71 (0.62)	15.82 (1.96)	13.96 (1.14)	14.99 (1.54)	16.09 (2.02)	15.64 (1.10)
Cellulose	39.13 (5.00)	28.15 (1.78)	22.49 (1.00)	23.70 (2.36)	35.90 (1.45)	43.92 (1.15)	20.91 (4.96)	22.53 (0.82)
Extractable fraction (%)	32.71 (5.79)	45.24 (1.90)(*)	15.90 (1.71)	13.76 (1.11)	35.33 (3.17)	26.28 (2.84)	16.58 (0.90)	16.68 (2.35)
Soluble phenolics	10.92 (2.32)	13.44 (1.09)	4.11 (0.73)	3.16 (0.28)	11.55 (1.48)	9.71 (0.87)	4.57 (0.47)	4.79 (0.96)
Condensed tannins	4.80 (1.27)	5.73 (0.63)	13.02 (2.54)	9.90 (1.50)	4.50 (0.70)	2.95 (0.93)	14.82 (1.02)	13.36 (2.42)
Extractable fraction	4.29 (1.28)	5.21 (0.66)	10.54 (2.72)	7.79 (1.35)	3.84 (0.72)	2.42 (0.80)	11.75 (1.12)	10.73 (2.34)
Bound fraction	0.52 (0.06)	0.51 (0.09)	2.48 (0.37)	2.11 (0.17)	0.66 (0.13)	0.53 (0.13)	3.07 (0.74)	2.62 (0.13)
NSCs	4.25 (0.68)	5.47 (1.29)	1.74 (0.07)	1.66 (0.29)	4.23 (0.61)	3.79 (0.38)	1.94 (0.23)	1.92 (0.52)
Lipids	7.05 (1.09)	7.56 (0.63)	3.07 (0.56)	3.31 (0.32)	8.24 (0.16)	8.23 (0.76)	3.89 (0.27)	3.60 (0.07)
Soluble proteins	1.06 (0.11)	1.19 (0.13)	3.58 (0.72)	2.80 (0.28)	1.03 (0.10)	0.87 (0.22)	3.12 (0.34)	3.08 (0.39)
Unidentified	9.42 (2.32)	17.58 (3.70)	3.40 (0.35)	2.83 (0.35)	10.28 (1.38)	4.10 (3.64)	3.07 (0.81)	3.30 (0.50)
N (%)	0.61 (0.07)	0.66 (0.05)	1.66 (0.05)	1.78 (0.04)	0.64 (0.04)	0.71 (0.07)	1.46 (0.15)	1.51 (0.12)
Litter quality indices (ratio)								
AIF : N	24.06 (4.04)	20.52 (2.36)	27.84 (1.61)	26.36 (0.27)	23.25 (1.39)	21.02 (1.19)	32.32 (4.78)	30.19 (3.20)
C : N	80.11 (6.51)	76.40 (6.56)	31.33 (1.01)	29.06 (0.63)	77.79 (6.12)	70.46 (5.32)	35.75 (3.99)	34.59 (2.98)
Lignocellulose index	0.22 (0.02)	0.25 (0.02)	0.55 (0.01)	0.54 (0.01)	0.23 (0.004)	0.20 (0.001)	0.56 (0.03)	0.54 (0.01)

Values are means (SD) for each treatment in three plots from each site (n = 3). (*) denotes significant N effects at $P < 0.1$, * at $P < 0.05$, and ** at $P < 0.01$. AIF: acid-insoluble fraction; NSCs: non-structural carbohydrates.

Appendix G: Major biochemical components and three litter quality indices of spring and autumn fine roots across the four hardwood forest study sites receiving simulated nitrogen (N) deposition

Chemical characteristics	Spring		Autumn		Main effects
	Ambient	N deposition	Ambient	N deposition	
Cell-wall fraction (%)	84.5^{b} (1.0)	84.4^{b} (2.4)	82.6^{a} (1.8)	84.4^{b} (2.8)	Season, Site
AIF	45.8^{b} (2.4)	46.1^{b} (1.5)	44.3^{a} (2.2)	45.4^{a} (2.2)	Season
Hemicellulose	16.4^{a} (1.3)	15.9^{a} (1.5)	15.3^{b} (1.7)	15.5^{b} (1.4)	Season
Cellulose	22.3^{a} (3.0)	22.4^{a} (1.9)	23.0^{a} (2.9)	23.4^{a} (3.4)	
Extractable fraction (%)	15.5^{a} (1.0)	15.6^{a} (2.4)	17.4^{b} (1.8)	15.6^{a} (2.8)	Season, Site
Soluble phenolics	3.91^{a} (0.70)	3.82^{a} (1.29)	3.99^{a} (0.63)	3.66^{a} (0.75)	Site
Condensed tannins†	13.2^{a} (1.5)	12.2^{a} (2.1)	14.0^{a} (2.7)	12.5^{a} (3.9)	Site, N
NSCs	1.38^{a} (0.21)	1.45^{a} (0.44)	2.37^{b} (0.38)	2.24^{b} (0.39)	Season
Lipids	3.31^{ab} (0.53)	3.11^{a} (0.33)	3.89^{c} (0.71)	3.70^{bc} (0.48)	Season
Soluble proteins	3.12^{ab} (1.31)	2.86^{a} (0.50)	3.45^{b} (0.55)	3.03^{ab} (0.47)	Season, N
Unidentified‡	3.76^{b} (0.98)	4.37^{b} (0.87)	3.66^{ab} (1.24)	2.99^{a} (1.54)	Season, Site
N (%)	1.49^{a} (0.15)	1.58^{ab} (0.14)	1.61^{bc} (0.24)	1.70^{c} (0.04)	Season, Site, N
Litter quality indices (ratio)					
AIF : N	31.1^{b} (3.4)	29.5^{b} (2.9)	28.0^{ab} (4.1)	26.8^{a} (2.4)	Season, Site
C : N	33.9^{b} (3.0)	32.5^{ab} (3.5)	32.8^{ab} (5.34)	30.8^{a} (2.0)	Season, Site
Lignocellulose index	0.54^{a} (0.03)	0.55^{a} (0.01)	0.54^{a} (0.02)	0.54^{a} (0.03)	

Values are means (SD) with three replicated plots for each treatment at each of four sites (n = 12). Different letters in the same row indicate significant differences ($P < 0.05$). Significant main effects are shown ($P < 0.05$). AIF: acid-insoluble fraction; NSCs: non-structural carbohydrates. †Condensed tannins (CTs) are a subset of plant phenolics. There is no generally-accepted CT standard for the acid-butanol assays used to determine CTs. Thus, the CT concentrations reported here should be interpreted more as relative comparisons between fine roots and leaf litter than absolute quantification. Bound tannins could be double-counted in AIF in this table, however, bound CTs only represented 11.8 % and 20.9 % of total CTs by average in fine roots and leaf litter respectively (Table S3). ‡Unidentified portion is the difference between extractable fraction and the sum of soluble phenolics, non-structural carbohydrates, lipids, and soluble proteins.

Appendix H: Analysis of two-way ANOVA (site × simulated N deposition) on the annual litter production of leaf litter, fine roots, and total litter at the four hardwood forest study sites

Source of variation	*df*	Leaf litter		Fine roots		Total litter production	
		F	*P*	*F*	*P*	*F*	*P*
Study site	3	29.52	**<0.001**	19.59	**<0.001**	19.48	**<0.001**
N deposition	1	1.68	0.213	0.60	0.450	0.05	0.825
Site × N	3	0.69	0.573	1.41	0.276	1.24	0.326
Error	16						

Bold numbers: $P < 0.05$.

Appendix I: Mixed linear model analysis of biochemical fluxes among study sites, nitrogen deposition treatments, and tissue types in a split-plot design

Chemical characteristics	Whole-plot						Within-plot							
	Site		N deposition		Site × N		Tissue		Tissue × Site		Tissue × N		Tissue × Site × N	
	df = 3 / 16		*df* = 1 / 16		*df* = 3 / 16		*df* =1 / 16		*df* =3 / 16		*df* =1 / 16		*df* =3 / 16	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
AIF	15.43	**<0.001**	1.29	0.272	1.03	0.405	538.20	**<0.001**	19.09	**<0.001**	0.02	0.890	2.74	0.078
Hemicellulose	10.29	**<0.001**	0.19	0.669	0.88	0.473	3.26	0.090	9.35	**<0.001**	0.51	0.487	1.07	0.391
Cellulose	10.80	**<0.001**	2.07	0.169	3.38	**0.044**	356.97	**<0.001**	25.16	**<0.001**	0.86	0.368	2.39	0.107
Soluble phenolics	11.61	**<0.001**	0.27	0.610	1.22	0.333	557.3	**<0.001**	15.78	**<0.001**	2.42	0.139	2.61	0.087
Condensed tannins	3.62	**0.036**	5.27	**0.036**	0.27	0.848	80.47	**<0.001**	10.56	**<0.001**	0.25	0.627	3.65	**0.035**
NSCs	13.94	**<0.001**	0.25	0.627	1.21	0.338	504.86	**<0.001**	18.79	**<0.001**	4.24	0.056	1.44	0.268
Lipids	17.86	**<0.001**	0.59	0.455	0.07	0.977	387.13	**<0.001**	11.79	**<0.001**	1.61	0.223	0.81	0.507
Soluble proteins	4.90	**0.013**	4.32	0.054	0.20	0.894	224.78	**<0.001**	12.54	**<0.001**	0.54	0.473	3.98	**0.027**
Nitrogen	10.31	**<0.001**	7.59	**0.014**	3.21	0.051	179.52	**<0.001**	7.19	**0.003**	7.00	**0.018**	0.35	0.792

The mixed linear models included fixed effects shown in the table and random effects of plots nested (n = 3) in (site × N treatment). The degree of freedom is shown as 'numerator *df* / denominator *df*'. Bold numbers: $P < 0.05$. AIF: acid-insoluble fraction; NSCs: non-structural carbohydrates.

Appendix J: Analysis two-way ANOVA (site × simulated N deposition) on the combined fluxes of leaf litter and fine root biochemical fluxes at the four hardwood forest study sites

Sum fluxes	Study site		N deposition		Site × N	
	F	*P*	*F*	*P*	*F*	*P*
AIF	15.37	**<0.001**	0.41	0.529	1.37	0.287
Hemicellulose	10.05	**<0.001**	0.14	0.709	0.71	0.563
Cellulose	9.22	**<0.001**	2.99	0.103	5.16	**0.011**
Soluble Phenolics	1.19	0.346	0.43	0.522	0.99	0.421
Condensed tannins	11.78	**<0.001**	3.56	0.077	0.43	0.723
NSCs	3.61	**0.036**	3.65	0.074	1.54	0.242
Lipids	21.32	**<0.001**	0.04	0.841	0.04	0.988
Soluble proteins	10.20	**<0.001**	3.19	0.093	1.07	0.391
Nitrogen	13.30	**<0.001**	4.18	0.058	3.47	**0.041**

Bold numbers: $P < 0.05$. AIF: acid-insoluble fraction; NSCs: non-structural carbohydrates.