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

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Introduction

Plant litter decomposition drives major flows of carbon (C) in soil systems: C mineralization and C storage (Chapin et al., 2011). As litter decomposes, C is returned to the atmosphere via respiration (Raich & Schlesinger, 1992); the remaining C provides a source of heterogeneous soil organic C that accounts for approximately two-thirds of terrestrial C (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 C 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 C 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 break down 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 Huy sen et al., 2013; but see Oster tag & 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 C retention than leaf litter in a temperate forest (Bird & Torn, 2006; Bird et al., 2008) and an acidic tundra soil (Loya et al., 2004). In temperate deciduous forest soils, isotopic measurements estimated that root-derived C represented > 60% of microbial biomass after 3 yr of litter addition (Kramer et al., 2010). Rasse et al. (2005) found that roots contributed an average of two-fold more to soil organic C than

Summary

• 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 the concentrations of eight biochemical fractions and nitrogen (N) 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 N deposition.
• Compared with leaf litter, fine roots contained 2.9-fold higher acid-insoluble fraction (AIF) and 2.3-fold more condensed tannins; both are relatively difficult to decompose. Comparatively, leaf litter had greater quantities of more labile components: nonstructural 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.

Key words: acid-insoluble fraction, chemical recalcitrance, fine roots, leaf litter, lignin, litter input, litter quality, nitrogen (N) deposition.
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 C was more abundant than leaf-derived C 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., 2014), decomposer community composition (Wickings et al., 2012) and interactions with soil particles (Six et al., 2002), and endogenous factors, such as tissue chemistry (Mellilo 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 C 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 (N) 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; Mellilo 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 nonstructural carbohydrates (Waldrop & Firestone, 2004) and simple phenolics (Hättenschwiler et al., 2011). We also calculated litter quality indices including the ratios of C and N (C : N), AIF and N (AIF : N), and AIF and the sum of AIF + 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 15N that was allocated to old leaves, but increased the 15N 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.

Materials and Methods

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). These sites are heavily dominated by sugar maple (Acer saccharum M.) and similar in stand composition, age and soil properties (Table 1). The Oe/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). Soils are sandy (Kalkaska series, Typic haploarthrod) 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 NaNO₃ in six equal increments across the growing season) similar to rates of N deposition occurring in some areas of Europe (Holland et al., 2005).

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
Table 1 Location, climate and edaphic characteristics of the four northern hardwood forest study sites

<table>
<thead>
<tr>
<th>Site characteristic</th>
<th>Site A</th>
<th>Site B</th>
<th>Site C</th>
<th>Site D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latitude (N)</td>
<td>46°52'</td>
<td>45°33'</td>
<td>44°23'</td>
<td>43°40'</td>
</tr>
<tr>
<td>Longitude (W)</td>
<td>88°53'</td>
<td>84°51'</td>
<td>85°50'</td>
<td>86°09'</td>
</tr>
<tr>
<td>Mean annual precipitation (mm)*</td>
<td>873</td>
<td>871</td>
<td>888</td>
<td>812</td>
</tr>
<tr>
<td>Mean annual temperature (°C)*</td>
<td>4.7</td>
<td>6.0</td>
<td>6.9</td>
<td>7.6</td>
</tr>
<tr>
<td>Ambient wet + dry N deposition (g N m⁻² yr⁻¹)†</td>
<td>0.68</td>
<td>0.91</td>
<td>1.17</td>
<td>1.18</td>
</tr>
<tr>
<td>Growing season length (d)*</td>
<td>134</td>
<td>150</td>
<td>154</td>
<td>157</td>
</tr>
<tr>
<td>Total basal area (m² ha⁻¹)‡</td>
<td>34</td>
<td>31</td>
<td>32</td>
<td>33</td>
</tr>
<tr>
<td>Sugar maple basal area (%)§</td>
<td>86</td>
<td>86</td>
<td>83</td>
<td>75</td>
</tr>
<tr>
<td>Overstory age</td>
<td>108</td>
<td>102</td>
<td>103</td>
<td>107</td>
</tr>
<tr>
<td>Ambient soil carbon content (0–70 cm, g m⁻²)*</td>
<td>8341</td>
<td>9259</td>
<td>7841</td>
<td>7470</td>
</tr>
<tr>
<td>Oe+a 0–10 cm soil depth</td>
<td>350</td>
<td>625</td>
<td>720</td>
<td>640</td>
</tr>
<tr>
<td>Soil texture, 0–10 cm depth (% sand-% silt-% clay)*</td>
<td>75-22-3</td>
<td>89-9-2</td>
<td>89-9-2</td>
<td>87-10-3</td>
</tr>
<tr>
<td>Soil texture, 10–70 cm depth (% sand-% silt-% clay)*</td>
<td>84-11-5</td>
<td>88-7-5</td>
<td>91-6-3</td>
<td>92-5-3</td>
</tr>
</tbody>
</table>

*Pregitzer et al. (2008).
†MacDonald et al. (1992)
‡Burton et al. (2000).
§Burton et al. (2000).

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 N₂. They were then packed with solid CO₂ 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). 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 had mean diameters < 0.30 mm (Supporting Information Table S1), similar to the mean diameter observed in minirhizotrons at our sites (c. 0.31 mm, Burton et al., 2000). Approximately 2 g DW of roots for each plot were used for the chemical analyses presented here.

Substrate biochemistry

We analyzed plant tissues for total C and N, nonstructural 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, CA, USA). For NSCs, samples were extracted with 80% ethanol and analyzed for sugars using phenol-sulfuric acid (Chow & Landhäusser, 2004). The residues were digested with a mixture of α-amylase/amiloglucoisidase 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). 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 heteropolysphotungstes-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 may also contribute to this reducing capacity. After digestion, the glucose hydrolyzates were measured colorimetrically with a peroxidase-glucose oxidase-o-dianisidine reagent (Chow & Landhäusser, 2004). 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 heteropolysphotungstes-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 may also contribute to this reducing capacity.
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, IL, USA) with the addition of diluted polyvinylpyrrolidone (Fisher BioReagent, Pittsburgh, PA, USA) to minimize the interference by brown quinones (Jones et al., 1989). Bovine serum albumin (Thermo Fisher Scientific) was used to construct the standard curve. For total lipids, samples were homogenized using methanol/chloroform/water, adapted from 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 (Methods S1). 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 nonpolar extractives in this study is referred to as the ‘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 fractions (AIF) using a two-phase H$_2$SO$_4$ hydrolysis adapted from Booker et al. (1996). The acid-soluble fraction, consisting of dominantly cell-wall polysaccharides, along with other compounds, for example phenolics and lipids, linked to cell wall via ester bonds (Iiyama et al., 1994; Preston et al., 2000), was hydrolyzed by H$_2$SO$_4$ incubation. The remaining residues were dried and weighed to determine the AIF. For hemicellulose, the pellets remaining after tannins extraction were incubated with 10% KOH for 24 h at 30°C (Dickson, 1979; Chapman et al., 2005). The extracts were mixed with 4 M acetic acid in ice-cold ethanol for 24 h. 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; these were 7.6 ± 1.3% in leaf litter and 3.6 ± 1.3% in fine roots. All concentrations were expressed on an ash-free dry mass basis.

### Annual litter flux

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

\[ I_a = P_a \times C_a \]

($I_a$ annual input of an individual biochemical class; $P_a$ annual litter production of leaf litter or fine roots; $C_a$ concentration of the 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 that 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 (c. 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 (c. 0.30 mm, Table S1). 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 predominantly focused on major C fractions rather than element concentrations, such as phosphorus, sodium, and potassium that vary strongly by soil depth.

### 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, Littell et al., 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, whereas 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 before being analyzed in SAS 9.3 (SAS Institute Inc., Cary, NC, USA) to reduce the effects of variations increasing with means.

Results

Biochemical composition and nitrogen

We investigated the abundance of eight major biochemical fractions (representing c. 90% of substrate dry mass; Table 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 vs fine roots) resulted in a considerably greater variance than both site and simulated N deposition for all biochemical traits (Tables 2, S2, S3). Leaf litter had substantially higher concentrations of nonstructural carbohydrates (NSCs) and lipids (P < 0.001, Table 2). Leaf litter also exhibited higher concentrations of cellulose and soluble phenolics than fine roots in general (Table 2), but the magnitude of difference varied among sites (tissue × site, P < 0.05, Tables S2, S3). 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 S2, S3).

By contrast, fine roots contained a greater acid-insoluble fraction (AIF), and more condensed tannins (CTs) and N than leaf litter (P < 0.001, Table 2). Fine roots averaged c. ×2.9 higher AIF concentrations and c. ×2.3 greater concentrations of CTs than those in leaf litter across four sites (Table 2). AIF was the most abundant of all eight biochemical fractions in fine root tissue, whereas cell wall polysaccharides (cellulose + hemicellulose) were the dominant constituent of leaf litter (Table 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, Tables S2, S3). The AIF : N ratio and lignocellulose index (LCI) were higher in fine roots than leaf litter (P < 0.001, Table 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, Table S4). The most striking difference between spring and autumn roots was in the concentration of NSCs (Table S4): sugar increased from 10.1 ± 0.7 mg g⁻¹ in spring roots to 15.7 ± 0.8 mg g⁻¹ in autumn roots, whereas starch was about twice as abundant in autumn roots (data not shown).

Table 2 Major biochemical components and litter quality indices of leaf litter and fine roots averaged across the four forest study sites receiving simulated nitrogen (N) deposition

<table>
<thead>
<tr>
<th>Chemical characteristics</th>
<th>Leaf litter</th>
<th>Fine roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-wall fraction (%)</td>
<td>Ambient</td>
<td>N deposition</td>
</tr>
<tr>
<td></td>
<td>68.2 b (7.6)</td>
<td>62.0 a (8.3)</td>
</tr>
<tr>
<td></td>
<td>15.2 b (1.0)</td>
<td>14.0 a (1.0)</td>
</tr>
<tr>
<td></td>
<td>14.1 b (1.8)</td>
<td>13.8 a (1.3)</td>
</tr>
<tr>
<td></td>
<td>38.9 b (9.9)</td>
<td>34.2 a (6.8)</td>
</tr>
<tr>
<td>Extractable fraction (%)</td>
<td>31.8 b (7.6)</td>
<td>38.0 b (8.3)</td>
</tr>
<tr>
<td>Soluble phenolics</td>
<td>12.1 b (2.2)</td>
<td>12.5 b (2.1)</td>
</tr>
<tr>
<td>Condensed tannins¹</td>
<td>5.7 a (2.5)</td>
<td>4.2 a (1.7)</td>
</tr>
<tr>
<td>NSCs</td>
<td>4.40 b (0.55)</td>
<td>4.94 a (1.11)</td>
</tr>
<tr>
<td>Lipids</td>
<td>7.94 b (1.24)</td>
<td>7.85 b (0.62)</td>
</tr>
<tr>
<td>Soluble proteins</td>
<td>1.11 a (0.20)</td>
<td>1.00 b (0.20)</td>
</tr>
<tr>
<td>Unidentified²</td>
<td>6.25 a (4.55)</td>
<td>11.75 b (5.79)</td>
</tr>
<tr>
<td>N (%)</td>
<td>0.65 a (0.05)</td>
<td>0.81 a (0.18)</td>
</tr>
<tr>
<td>Litter quality indices (ratio)</td>
<td>AIF : N</td>
<td>23.6 b (2.2)</td>
</tr>
<tr>
<td></td>
<td>C : N</td>
<td>75.9 b (6.6)</td>
</tr>
<tr>
<td></td>
<td>Lignocellulose index</td>
<td>0.22 a (0.02)</td>
</tr>
</tbody>
</table>

AIF, acid-insoluble fraction; NSCs, nonstructural carbohydrates.

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 Supporting Information Table S2.

¹Condensed 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 Table S3. 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.

²Unidentified portion is the difference between extractable fraction and the sum of soluble phenolics, NSCs, lipids and soluble proteins.
Biochemical fluxes

Leaf litter production across the four sites ranged from 324.0 to 447.1 g m$^{-2}$ yr$^{-1}$, whereas fine root litter production ranged from 175.1 to 420.1 g m$^{-2}$ yr$^{-1}$ (Table 3). These two types of litter production together made up 89 ± 4% of total litter production at these sites (Table 3). Simulated N deposition did not affect fine root, leaf or total litter production ($P > 0.1$, Table S5).

Because the litter production of leaves and fine roots both varied among sites (Table 3), the magnitude of differences in each biochemical flux between leaf litter and fine roots also varied among sites (site x tissue; $P < 0.01$, Table S6). However, there were considerable differences between the two tissues in all biochemical fluxes except hemicellulose (Tables 4, S6). 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 (c. 71% of the total flux) and CTs (c. 68%) across the four sites (Table 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 4). By contrast, leaf litter contributed considerably more cellulose, NSCs, soluble phenolics and lipids to the soil ($P < 0.001$, Table 4).

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 2, S2). 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 2), but these effects were not consistent across either tissues, sites or their interactions (Tables S2, S3). 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 x 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 x N x site; $P < 0.02$ for each; Tables S2, S3). Within

### Table 3 Litter production across four northern hardwood forest study sites

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

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 nitrogen (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 ($P > 0.05$), whereas leaf litter, fine root and total litter production varied among sites ($P < 0.001$, Table S5).

### Table 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

<table>
<thead>
<tr>
<th>Biochemical class</th>
<th>Leaf litter flux</th>
<th>Fine root flux</th>
<th>Sum</th>
<th>Fine root (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ambient</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf litter</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIF</td>
<td>56.7$^*$ (5.6)</td>
<td>54.5$^*$ (9.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemicellulose</td>
<td>52.7$^*$ (7.9)</td>
<td>53.7$^*$ (9.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellulose</td>
<td>145.6$^*$ (23.2)</td>
<td>134.3$^*$ (41.5)</td>
<td>280.1$^*$ (64.7)</td>
<td>314.9$^*$ (86.2)</td>
</tr>
<tr>
<td>Soluble phenolics</td>
<td>45.3$^*$ (7.9)</td>
<td>47.7$^*$ (9.0)</td>
<td>93.0$^*$ (16.9)</td>
<td>105.6$^*$ (20.8)</td>
</tr>
<tr>
<td>Condensed tannins$^1$</td>
<td>20.9$^*$ (7.8)</td>
<td>16.4$^*$ (6.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonstructural carbohydrates</td>
<td>16.5$^*$ (2.6)</td>
<td>18.7$^*$ (2.7)</td>
<td>33.2$^*$ (5.3)</td>
<td>40.2$^*$ (6.6)</td>
</tr>
<tr>
<td>Lipids</td>
<td>29.6$^*$ (4.5)</td>
<td>30.3$^*$ (4.3)</td>
<td>60.0$^*$ (8.6)</td>
<td>70.6$^*$ (12.9)</td>
</tr>
<tr>
<td>Soluble proteins</td>
<td>4.1$^*$ (0.6)</td>
<td>3.9$^*$ (0.9)</td>
<td>8.0$^*$ (1.5)</td>
<td>11.9$^*$ (2.4)</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>2.4$^*$ (0.3)</td>
<td>3.1$^*$ (0.5)</td>
<td>5.5$^*$ (1.0)</td>
<td>8.6$^*$ (1.7)</td>
</tr>
</tbody>
</table>

AIF, acid-insoluble fraction. 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 $P < 0.1$ (Table S7) are denoted with (*).

$^1$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 ($P = 0.036$, Table S6).
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 (Tables 2, S3).

Other effects of simulated N deposition were more idiosyncratic. Soluble protein concentrations were generally lower under simulated N deposition (\( P = 0.018 \), Table 2), but this decrease did not occur at site C for leaf litter and site A for fine roots (tissue × N × site: \( P = 0.031 \), Tables S2, S3). Soluble phenolics of leaf litter and fine roots showed both positive and negative responses to N deposition that depended on site (tissue × N × site: \( P = 0.042 \), Tables S2, S3).

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 4, S6). Simulated N deposition generally decreased the fluxes of CTs (\( P = 0.036 \), Tables 4, S6), but this effect was not observed for fine roots at site A and for leaf litter at site C (site × tissue × N: \( P = 0.035 \), Table S6). 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: \( P = 0.027 \), Table S6). When fluxes through leaf litter and fine roots were combined, simulated N deposition marginally decreased the total fluxes of CTs and soluble proteins, and increased those of NSCs and N (\( P < 0.1 \), Table 4), yet this increase of N was not apparent at site C (site × N: \( P = 0.041 \), Table S7). Simulated N deposition increased the cellulose flux at site D, but decreased this flux elsewhere (site × N: \( P = 0.011 \), Table S7).

**Discussion**

**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: for example, 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. Although exogenous factors also influence retention of detrital C 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 C (SOC).

The most striking difference between leaf litter and fine roots was that fine roots had considerably higher concentrations of acid-insoluble fraction (AIF) than leaf litter (Table 2). The AIF has often been referred to as lignin, an aromatic heteropolymer that is difficult to degrade because its complex structure limits degradation to only nonspecific 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-yr decomposition study, AIF defined the recalcitrant litter pool, whereas 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.

In order 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 fractions across a number of boreal and temperate forests (Fig. 1), 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, whereas leaf litter often had more extractive materials (Fig. 1). 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 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 4).

Although leaf litter contained lower concentrations of CTs than fine roots, leaf litter contained higher concentrations of
soluble phenolics (Table 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 used to assess CTs provides a relative – not absolute – quantification of these compounds (see the Materials and Methods section). Aside from CTs, total phenolics also include hydrolyzable tannins (HTs) and low-molecular-weight phenolics, which we did not quantitatively separate in this study. Soil microorganisms often utilize these non-CT phenolics as labile C sources (Schimel et al., 1998; Nietop 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. By 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 suggest 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 which suggest that no nutrient resorption occurs (Nambiar, 1987; Aerts, 1990; Gordon & Jackson, 2000). 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.

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 (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 4; Fig. 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 C in pools associated with soil aggregates or mineral particles (Marschner et al., 2008). At the timescale 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 C 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 that 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.

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 C 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 (Oe/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 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 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 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 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 arbucular 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 solely by analyzing leaf litter.

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**Supporting Information**

Additional supporting information may be found in the online version of this article.

**Table S1** Diameter distribution for the first three orders of maple (*Acer*) roots

**Table S2** Mixed linear model analysis of biochemical traits among study sites, nitrogen (N) deposition treatments and tissue type in a split-plot design

**Table S3** Major biochemical components and three litter quality indices of leaf litter and fine roots at each of the four forest sites receiving simulated nitrogen (N) deposition

**Table S4** Major biochemical components and three litter quality indices of spring and autumn fine roots across the four forest sites receiving simulated nitrogen (N) deposition

**Table S5** Analysis of two-way ANOVA (site × N deposition) on the annual litter production of leaf litter, fine roots and total litter at the four northern hardwood forest study sites

**Table S6** Mixed linear model analysis of biochemical fluxes among study sites, nitrogen deposition treatments and tissue types in a split-plot design

**Table S7** Analysis two-way ANOVA (site × N deposition) on the combined fluxes of leaf litter and fine root biochemical fluxes at the four northern hardwood forest study sites

**Methods S1** Sequential extraction for the extractive-free fraction.

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