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**Long-term doubling of litter inputs accelerates soil organic matter degradation and
reduces soil carbon stocks**

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Abstract

Soil organic matter (SOM) constitutes more than two-thirds of terrestrial carbon stocks yet there are many uncertainties about the fate of soil carbon reserves with global environmental change. Moisture, altered nutrient cycles, species shifts, growing season length or rising temperatures may alter forest primary productivity and the proportions of above and belowground biomass entering soil. We investigated SOM composition using molecular-level techniques after 20 years of Detrital Input and Removal Treatment (DIRT) at the Harvard Forest. SOM biomarkers (solvent extraction, base hydrolysis and cupric (II) oxide oxidation) and nuclear magnetic resonance (NMR) spectroscopy were used to quantify changes in SOM composition and microbial activity and community composition was assessed using phospholipid fatty acid (PLFA) analysis. Doubling aboveground litter inputs decreased soil carbon content, increased the degradation of labile SOM and enhanced the sequestration of aliphatic compounds in soil. The exclusion of belowground inputs resulted in a decrease in root-derived components and enhanced the degradation of stable SOM components such as leaf-derived aliphatic structures (cutin). The DIRT manipulations resulted in soil microbial community changes that were attributed to the accelerated processing of specific SOM components. These results collectively reveal that a detailed molecular-level characterization of SOM can provide information on SOM compositional changes and transformations after 20 years of input manipulation in a temperate forest.

Keywords: Detrital input and removal treatment, organic matter biomarkers, lipids, lignin, cutin, suberin, phospholipid fatty acids (PLFAs), and nuclear magnetic resonance (NMR).

Introduction

Both above and belowground litter quality and quantity are currently recognized as major determinants of soil organic matter (SOM) composition and turnover (Cotrufo et al. 2013). Globally, forests store as much as ~45 % of terrestrial carbon (Bonan 2008) in SOM and have the potential to temporarily sequester large amounts of carbon in tree biomass (Bonan 2008), which may partially offset increased atmospheric CO₂ emissions with soil warming. Another aspect of higher mean annual temperatures is the potential increase in above and belowground carbon inputs to soil (King et al. 1997; Norby et al. 2005) which are likely to also alter carbon flux in forests (Litton & Giardina 2008). However, it is unclear if shifts in both above and belowground carbon will be linearly related to carbon storage in soil because soils may have finite capacities to store additional carbon inputs (Bowden et al. 2014; Crow et al. 2009a; Lajtha et al. 2014; Sulzman et al. 2005). Furthermore, the quantity and quality of detrital inputs has been directly linked to SOM composition in forest soils (Crow et al. 2009a; 2009b). SOM is derived from aboveground (fine litter such as leaves, twigs and seeds) and belowground (fine roots and exudates) inputs (Nadelhoffer et al. 2004). Although the importance of SOM in forests and the global carbon cycle is widely recognized (Bonan 2008; Schmidt et al. 2011), the fundamental processes controlling SOM stabilization are still unclear (Cotrufo et al. 2013; Schmidt et al. 2011).

Global environmental change (e.g., rising temperature, atmospheric CO₂ and nitrogen deposition) is expected to alter ecosystem net primary productivity and allocation of resources between above and belowground productivity in forests (King et al. 1997; Melillo et al. 1993; Norby et al. 2005). Small changes in the quality and quantity of litter inputs may alter the net accumulation or loss of SOM (Boone et al. 1998). The molecular composition of above and

belowground plant tissues varies (Kögel-Knabner 2002) and variations in soil inputs between above and belowground sources may impart differences in the SOM composition resulting in SOM components with different stability. For example, root-derived (suberin) and leaf-derived (cutin) aliphatic compounds have been hypothesized to be environmentally persistent in soil (Feng et al. 2008; Lorenz et al. 2007) because of their chemical recalcitrance and strong associations with clay mineral surfaces. However, the relative contribution of plant roots to soil organic carbon stocks may be larger than that of plant leaves (Rasse et al. 2005). Therefore, natural changes in forest productivity and shifts between above and belowground allocation of resources may alter SOM stabilization and degradation patterns (Crow et al. 2009b).

To assess how variations in the detrital chemistry and quantity control SOM accumulation and stabilization, a long-term Detrital Input and Removal Treatment (DIRT) experiment was established in 1990 at the Harvard Forest (Nadelhoffer et al. 2004). The experimental design is derived from a project started in 1957 in forest and grassland ecosystems at the University of Wisconsin Arboretum (Nielsen & Hole 1963) and exists now as an international network of ongoing experiments at various sites (Nadelhoffer et al. 2004). The DIRT experiments at the Harvard Forest consist of chronically altering above and belowground litter inputs to permanent plots in a temperate forest. Litter manipulations include: Control (normal above and belowground inputs), Double Litter (aboveground inputs are doubled annually), No Litter (aboveground inputs are excluded annually), No Roots (roots are excluded by inserting impenetrable barriers in backfilled trenches), No Inputs (aboveground and root inputs are excluded) and O/A Less (organic and A horizons are replaced with B horizon soil with normal inputs thereafter). To date, studies have examined soil and root responses to detrital input manipulations (Boone et al. 1998; Bowden et al. 1993), shifts in the soil microbial community

(Brant et al. 2006), and in soil carbon cycling (Crow et al. 2009a; 2009b; Leff et al. 2012). A recent study by Lajtha et al. (2014) examined soils from the DIRT experimental plots at the Harvard Forest after 20 years of manipulation. A number of analyses were performed including: measurement of C and N content, soil respiration of incubated soils, density fractionation, acid hydrolysis, and enzyme (β -glucosidase and phosphomonoesterase) measurements (Lajtha et al. 2014). Interestingly, 20 years of doubling aboveground litter inputs did not increase carbon in soil mineral horizons (0-10 cm). Surprisingly, all treatments, decreased soil C content in the mineral soil suggesting that the long-term variation of above- and below-ground litter did not enhance soil C storage (Lajtha et al. 2014). The exclusion of leaf litter decreased soil C by 18% whereas root exclusion resulted in a 9% decline in soil C. Soil density fractionation only detected a small decline in the light fraction with doubling of litter inputs, possibly due to increased biodegradation of these additional litter inputs. This coincides with the observed increases in enzyme concentrations with double litter inputs which suggest enhanced microbial activity but warrants further examination with more sensitive, molecular-level techniques that can provide direct and detailed insight into SOM chemistry (Simpson & Simpson 2012).

Based on the results reported in Lajtha et al. (2014), we developed the following hypotheses: 1) variation in above and belowground litter has accelerated SOM decomposition and decreased labile carbon in soil, 2) microbial activity and community structure is altered with litter manipulation, and 3) degradation of more stable components, such as cutin, suberin and lignin is likely accelerated due to increased microbial activity and shifts in microbial community structure. To test these hypotheses, mineral horizon soil samples (0-10 cm) were collected from the Harvard Forest DIRT experimental plots and subjected to sequential biomarker extractions (solvent extraction, base hydrolysis and cupric (II) oxide (CuO) oxidation) for the identification

of free compounds of plant and microbial origin, ester-bound lipids from leaf-derived cutin and root-derived suberin, and lignin-derived phenols, respectively (Otto & Simpson 2007). Phospholipid fatty acid (PLFA) analysis was performed to examine the microbial community composition and differences in activity (Feng & Simpson 2011). Solid-state and solution-state NMR analyses were also employed to characterize SOM in DIRT samples. The combination of both biomarker and NMR methods provides detailed insight into SOM composition and biogeochemistry (Feng & Simpson 2011).

Materials and Methods

Experimental design and soil sampling

Samples were collected in October 2010 from the Long-Term Ecological Research DIRT site at the Harvard Forest in Petersham, Massachusetts, USA (42° 29' N, 72° 11' W). The site is located in a mixed hardwood forest which is dominated by northern red oak (*Quercus rubra*), paper birch (*Betula papyrifera*) with some red maple (*Acer rubrum*). The soils at the Harvard Forest are moderately well-drained stony, fine sandy loam Inceptisols from the Charlton series that average about 3 m in depth. The forest floor depth ranges from 3 to 8 cm and has a thin Oa horizon (1-3 cm thick). The mean annual air temperature ranges from -7 °C in January to 20 °C in July and the mean annual precipitation is 1100 mm distributed evenly throughout the year. The DIRT manipulations began in 1990 and include five input/exclusion treatments (Double Litter, No Litter, No Roots, No Inputs, O/A Less) and a Control, each replicated three times ($n = 3$). The plots are 3x3 m and none include trees or saplings. The distance of trees from each of the experimental plots varies. The No Roots and No Inputs treatments include the installation of a barrier to prevent the encroachment of roots from nearby trees. The O and A horizons in the

O/A Les plots were removed and replaced with B horizon material at the beginning of the experiment. For the last 20 years, these plots have received inputs from both above and belowground litter. Forest floor soil horizons were collected from the three Control plots as 20x20 rectangular volumes and analyzed to establish baseline SOM composition. Fine roots were hand-picked from the forest floor samples prior to analyses. Two 0-10 cm mineral soil cores were collected from each plot with a diamond bit corer mounted on a power auger. Soil samples were passed through a 2mm sieve, kept at 4 °C during transport and then air-dried. Samples were then freeze-dried and ground for analysis. Samples from each of the two cores from each plot were combined into one composite sample for molecular-level analyses because preliminary analyses and carbon measurements did not show any significant differences between the core samples (data not shown).

Soil organic matter biomarkers and microbial phospholipid fatty acids

Sequential chemical extractions (solvent extraction, base hydrolysis and CuO oxidation) were performed in duplicate for each composite sample (Otto & Simpson 2007). This method provides information about lipids and small molecules, cutin- and suberin-derived compounds, and lignin-derived phenols respectively (Pisani et al. 2015). For example, solvent extraction isolates unbound SOM components including aliphatic and cyclic lipids and carbohydrates. Aliphatic lipids are useful biomarkers for SOM source assessments because long-chain compounds ($\geq C_{20}$) are characteristic of the epicuticular waxes from leaves (Simoneit 2005) whereas short-chain compounds ($< C_{20}$) originate from soil microbes such as fungi and bacteria (Lichtfouse et al. 1995). The cyclic lipids include diterpenoids, triterpenoids and steroids which stem from both plants and microbes. Diterpenoids of the abietane, pimarane and isopimarane

152 classes are common constituents of conifers and pine needles (Otto & Wilde 2001) while
153 triterpenoids of the oleanane and ursane type are typical biomarkers for angiosperms (Bianchi
154 1995). Steroids are common constituents of plants (Bianchi 1995), but some structures can
155 originate from non-plant sources such as fungi (Ruzicka et al. 2000). Carbohydrates are not
156 source-specific because they originate from both plants and microbes (Feng et al. 2008). Ester-
157 bound lipids are cleaved from SOM using base hydrolysis and these compounds are attributed to
158 cutin (short-chain mid-chain hydroxy acids, C₁₆ mono- and dihydroxy acids and dioic acids),
159 suberin (long-chain ω -hydroxyalkanoic and dioic acids, and 9,10-epoxy- ω -hydroxy C₁₈ acid) or
160 both polymers (C₁₆ ω -hydroxyalkanoic acid, C₁₈ di- and trihydroxy acids, C₁₆ and C₁₈ dioic
161 acids; Kolattukudy 1980; Otto & Simpson 2006b). The degradation of cutin-derived compounds
162 can be evaluated by determining the changes in the relative abundance of ω -hydroxyalkanoic
163 acids, expressed as ω -C₁₆/ Σ C₁₆ and ω -C₁₈/ Σ C₁₈. These ratios have been shown to increase with
164 progressive cutin degradation in soil (Otto & Simpson 2006b). The ratio of mid-chain-substituted
165 acids to the total suberin and cutin acids (Σ Mid/ Σ SC) has also been used to assess cutin
166 degradation in soils and decreasing values are indicative of enhanced cutin degradation (Otto &
167 Simpson 2006b). Lignin-derived phenols are components of the lignin biopolymer and are
168 released from SOM through CuO oxidation. The composition of these phenols is characteristic of
169 major plant groups and can be used to assess the botanical sources of lignin (Hedges & Mann
170 1979). Lignin-derived phenols are also used to assess lignin degradation in soils because
171 progressing lignin degradation is reflected by elevated ratios of phenolic acids to their
172 corresponding aldehydes for both syringyl and vanillyl units (Ad/Al; Hedges & Mann 1979; Otto
173 & Simpson 2006a). To complement this biomarker approach, we also extracted PLFAs from
174 freeze-dried soil samples (0.75-1.5 g) by a modified Bligh-Dyer method (Frostegård & Bååth

1996). Total PLFAs are used to determine the total microbial activity in soil because they are only found in viable cells and are characteristic biomarkers for living microbes (Frostegård & Bååth 1996). Microbial activity was calculated by summing the PLFAs specific to fungi (18:2 ω 6,9c), actinomycetes (10Me18:0), gram-negative bacteria (16:1 ω 7c, *cy*17:0, 18:1 ω 7c and *cy*19:0) and gram-positive bacteria (*i*14:0, *a*16:0, *i*15:0, *a*15:0, *i*16:0, *i*17:0 and *a*17:0). PLFAs can also assess the physiological state of soil bacteria because these microbes alter their membrane fatty acid composition in response to external environmental stress such as substrate availability, water limitations and temperature (Bossio & Scow 1998; Feng & Simpson 2009). For example, cyclopropane PLFAs are produced when a bacterial community runs out of easily degradable carbon substrate, indicating that increased ratios of cyclopropane PLFAs to their monoenoic precursors (*cy*17:0/16:1 ω 7c and *cy*19:0/18:1 ω 7c) are indicators of microbial starvation (Bossio & Scow 1998). A decreasing ratio of monoenoic to saturated PLFAs (mono/sat) is typically observed when gram-negative bacteria are starved (Kieft et al. 1994). Changes in the microbial community composition were assessed using the ratios of fungal to bacterial PLFAs (the sum of gram-negative and gram-positive bacterial PLFAs) and gram-negative to gram-positive PLFAs. All biomarker and PLFA compounds were derivatized prior to identification and quantification by gas chromatography – mass spectrometry (Pisani et al. 2015). Details about the biomarker extraction methods and quantification are provided in the Supplementary Material.

Nuclear magnetic resonance (NMR) spectroscopy

Both solid- and solution-state NMR techniques were employed to examine the composition of SOM with DIRT manipulation. One composite sample from each DIRT

manipulation was prepared and extracted for NMR analyses (Supplementary Material). Solid-state ^{13}C NMR spectra provide information about the overall SOM composition and are integrated into four chemical shift regions corresponding to: alkyl carbon (0-50 ppm) from cutin and suberin from plants, lipids and amino acid side chains; *O*-alkyl carbon (50-110 ppm) including *O*- and *N*-substituted aliphatic constituents that arise from carbohydrates, peptides and the methoxyl carbon in lignin; aromatic and phenolic carbon (110-165 ppm) from lignin and amino acids found in peptides; carboxyl and carbonyl carbon (165-215 ppm) from fatty acids and peptides (Baldock et al. 1992; Clemente et al. 2012; Simpson et al. 2007). The alkyl/*O*-alkyl ratio is used to estimate the relative degree of SOM degradation (Baldock & Preston 1995) because *O*-alkyl components are preferentially degraded compared to alkyl components resulting in increased values with increased SOM degradation. One-dimensional and two-dimensional solution-state NMR techniques provide more detailed information about molecular structure and bond connectivity of base-extractable SOM (Clemente et al. 2012; Pisani et al. 2015; Simpson et al. 2004). The one-dimensional solution-state ^1H NMR spectra were integrated into seven chemical shift regions corresponding to different structural components including: aliphatic structures from lipids, waxes, cutin and suberin (0.6-1.3 ppm); *O*- and *N*-substituted aliphatic structures from lipids and amino acids (1.3-2.9 ppm); *O*-alkyl structures from lignin, carbohydrates and proteins (2.9-4.1 ppm); the ^1H on an α -C from peptides (4.1-4.8 ppm); the ^1H in anomeric structures of carbohydrates (4.8-5.2 ppm); aromatic structures attributed to lignin and peptide side chains (6.2-7.8 ppm); amide structures from peptides (7.8-8.4 ppm; Clemente et al. 2012; Simpson et al. 2007). SOM constituents that exhibit a high degree of motion (self-diffusion) are edited out of DE ^1H NMR spectra which highlight proton signals that arise from large, macromolecular and rigid compounds (Simpson et al. 2007). DE ^1H NMR may distinguish

between plant and microbial inputs to SOM because a large abundance of the methylene (CH₂)_n signal (1.2 ppm) relative to the terminal CH₃ signal (0.8 ppm) is consistent with plant-derived components. In soils with high protein contents, the CH₃ signal is much larger due to the abundance of methyl-rich side chains (Clemente et al. 2012). A signal at ~1.8 ppm arises from the *N*-acetyl group in peptidoglycan which is a major component of bacterial cell walls and comprises up to 50 % by weight of gram-positive bacteria (Kögel-Knabner 2002). This material may be protected from degradation after cell death and has been shown to add to the refractory SOM pool (Kögel-Knabner 2002; Liang et al. 2011).

Statistical analyses

A two-factor one-way analysis of variance (ANOVA) with a Bonferroni post hoc test was used to compare the concentration of SOM biomarkers between the mineral horizon Control and the experimental treatments (Double Litter, No Litter, No Roots, No Inputs and O/A Less). Treatments (n=3) were the fixed factor and analytical replication (n=2 per treatment sample) was the random factor used in the two-factor ANOVA. A difference was considered significant at the level of $p \leq 0.05$. Statistical analyses were performed using SPSS Statistics (version 17.0).

Results

SOM composition of the forest floor and Control mineral soil samples

The solvent extracts of the forest floor and mineral horizon Control soil samples contained a series of aliphatic lipids including: *n*-alkanes, *n*-alkanols, *n*-alkanoic acids, ω -hydroxyalkanoic acids, *n*-alkane- α,ω -dioic acids and *n*-alkanones (Supplementary Table S1). Long-chain aliphatic lipids, derived primarily from the epicuticular waxes of leaves, were the

most abundant solvent-extractable compounds of the forest floor and mineral horizon Control soils, whereas short-chain lipids that are derived from microbial biomass were minor components (Table 1). Carbohydrates were also observed in the mineral soil and forest floor (Table 1). Some plant-derived cyclic compounds, including diterpenoids, triterpenoids and steroids were present in the forest floor (Supplementary Table S1).

The ester-bound hydrolysable lipids of the forest floor and mineral horizon Control soils consisted of a series of aliphatic lipids including: *n*-alkanols, *n*-alkanoic acids, branched alkanoic acids, *n*-alkane- α,ω -dioic acids, ω -hydroxyalkanoic acids, α -hydroxyalkanoic acids, mid-chain hydroxy and epoxy acids (Table 1; Supplementary Table S2). The forest floor contained similar amounts of cutin- and suberin-derived compounds and was dominated by aliphatic lipids that originate from both polymers (Table 1). The suberin- and cutin-derived hydrolysable lipids were more abundant in the mineral horizon Control soils which were dominated by suberin-derived compounds. The ω -C₁₆/ Σ C₁₆ ratio increased from the forest floor to the mineral horizon Control soil indicating enhanced cutin degradation with soil depth. The ω -C₁₈/ Σ C₁₈ ratio did not change (Table 1). The Σ Mid/ Σ SC ratio decreased with soil depth (Table 1) suggesting enhanced cutin degradation.

The concentration of lignin-derived phenols was higher in the forest floor compared to the mineral Control soil (Table 1) indicating direct lignin inputs to the forest floor and lignin degradation with soil depth. Consistent with the dominance of gymnosperm vegetation at the site, vanillyl-type monomers were the most abundant lignin monomers in both the forest floor and mineral Control soil (Table 1). The Ad/Al ratios were higher in the mineral horizon Control soil compared to the forest floor, suggesting enhanced lignin oxidation with soil depth (Table 1). The total PLFAs were higher in the forest floor compared to the mineral horizon Control soil

(Fig. 1a) indicating a larger active microbial community in the forest floor. Both the forest floor and mineral horizon Control soil contained fungi, actinomycetes, gram-positive and gram-negative bacteria, but were dominated by gram-negative bacteria (Fig. 1b). The cy19:0/18:1 ω 7c ratio was lower in the mineral horizon Control soil (Fig. 1c) while the cy17:0/16:1 ω 7c and mono/sat ratios were not different between the two horizons. The forest floor soil is dominated by fungi relative to bacteria as indicated by an elevated fungi/bacteria ratio (Fig. 1d) while the mineral Control soil is dominated by bacteria.

The forest floor soil ^{13}C NMR spectrum is dominated by *O*-alkyl carbon (Table 2) from carbohydrates, peptides and lignin. Alkyl components from cutin and suberin of plants, lipids and amino acid side chains were also abundant in the forest floor. Aromatic and phenolic carbons from lignin and aromatic amino acids, and carboxyl and carbonyl carbons from fatty acids and peptides were minor components of the forest floor SOM (Table 2). The mineral horizon Control soil ^{13}C NMR spectrum (Fig. 2a, Table 2) contained similar relative amounts of alkyl and *O*-alkyl carbon, but had a more intense signal originating from carboxyl and carbonyl carbon compared to the forest floor. The alkyl/*O*-alkyl ratio increased from the forest floor to the mineral horizon Control soil (Table 2) suggesting enhanced SOM degradation with soil depth.

The solution-state ^1H NMR integration results (Table 2) indicate that the forest floor base-soluble SOM is also dominated by alkyl and *O*-alkyl components while the mineral horizon Control soil (Fig. 2b) is dominated by *O*- or *N*-substituted aliphatic constituents from lipids and amino acids. The base-soluble SOM of the forest floor and mineral horizon Control soils also contained minor signals from α - and anomeric protons from peptides, aromatic protons from lignin and peptides, and amide protons from peptides. The greater abundance of the methylene $(\text{CH}_2)_n$ signal relative to the terminal CH_3 signal in the DE ^1H NMR spectrum of the mineral

Control soil (Fig. 2c) is more consistent with plant-derived than microbial-derived components. The intensity of the peak originating from the *N*-acetyl group in peptidoglycan was higher in the mineral Control DE ¹H NMR spectrum compared to the forest floor (Table 2), likely due to the accumulation of dead microbial cells in the mineral horizon Control soil (Fig. 2a). The intensity of the *O*-alkyl signal in the forest floor and mineral horizon Control soils increased in the DE ¹H NMR spectra (Fig. 2c; Table 2), suggesting that this signal arises from large SOM structures such as lignin, carbohydrates or peptides.

Variation in SOM quantity and quality with detrital input and removal treatments

The molecular-level SOM composition of the mineral soils varied with the DIRT manipulations (Table 1) relative to the Control samples. Solvent-extractable short- and long-chain aliphatic lipids decreased with the removal of aboveground and with removal of both above and belowground inputs (No Litter and No Inputs). Plant-derived cyclic compounds such as triterpenoids and steroids, increased in the plots receiving aboveground inputs (Double Litter and No Roots) while carbohydrates decreased in the No Litter and No Roots plots. All solvent-extractable compounds decreased significantly ($p \leq 0.05$) in the O/A Less plots.

Aboveground litter additions (Double Litter) increased both cutin-derived and suberin-derived compounds although the increase in cutin-derived compounds was not significant ($p \leq 0.05$; Table 1). The removal of both above and belowground inputs (No Inputs) resulted in a significant decrease in suberin-derived hydrolysable lipids ($p \leq 0.05$; Table 1). The exclusion of roots caused a significant decrease in suberin-derived compounds ($p \leq 0.05$) which also decreased in the O/A Less plots. Enhanced cutin degradation (increase in the ω -C₁₆/ΣC₁₆ ratio) was observed in the No Litter and No Roots Plots ($p \leq 0.05$) but not observed in the Double

Litter and O/A Less plots. The ω -C₁₈/ΣC₁₈ ratio did not show a similar trend and decreased in the No Roots and No Inputs plots ($p \leq 0.05$). However, similar to the ω -C₁₆/ΣC₁₆ ratio, the ω -C₁₈/ΣC₁₈ ratio decreased in the O/A Less plot ($p \leq 0.05$). Finally, the ΣMid/ΣSC ratio increased in the plots that are still receiving aboveground litter inputs (Double Litter and No Roots), confirming the contribution of leaf-derived material to these mineral soils.

Lignin-derived phenols did not vary significantly with DIRT (Table 1). Aboveground litter additions (Double Litter) resulted in a small increase in extractable lignin-derived phenols. Vanillyl-type monomers decreased with the removal of above and belowground inputs (No Litter, No Roots and No Inputs) and were lower in the O/A Less plot. Elevated Ad/Al values were observed with all the treatments except in the No Litter plot suggesting progressive lignin oxidation although these were not statistically significant.

The microbial activity increased in all plots relative to the Control except for the O/A Less treatment (Fig. 1a) which decreased significantly ($p \leq 0.05$). Although not statistically significant, the increase in PLFA concentrations in the Double Litter plots is likely due to an increase in leaf litter inputs and associated labile carbon substrates which promoted and sustained an active microbial community (Fig. 1a). The addition of aboveground litter (Double Litter) caused a significant increase in the amount of actinomycetes, and both gram-negative and gram-positive bacteria ($p \leq 0.05$; Fig. 1b). The amount of actinomycetes also increased significantly with the exclusion of roots (No Roots and No Inputs). Fungal PLFAs increased with No litter ($p \leq 0.05$; Fig. 1b). The cy17:0/16:1 ω 7c ratio increased significantly only in the O/A Less plots ($p \leq 0.05$; Fig. 1c) relative to the Control, likely due to lower root inputs and the lack of an organic horizon to sustain an active microbial population. The cy19:0/18:1 ω 7c ratio increased significantly in the plots receiving no aboveground or belowground inputs (No Litter,

No Roots and No Inputs), suggesting substrate-limited stress to the bacterial community. The mono/sat ratio decreased in the Double Litter and No Input plots. All the DIRT manipulations caused a significant shift in the soil microbial community towards fungi as shown by elevated fungal/bacterial PLFA ratios (Fig. 1d). The removal of the organic and A horizons (O/A Less) resulted in a higher gram-negative to gram-positive bacterial PLFA ratio compared to the Control (Fig. 1d), indicating a shift towards gram-negative bacteria. In the No Litter plots, there was a significant shift in microbial community towards gram-positive bacteria.

The solid-state ^{13}C NMR integration results (Table 2) showed a relative increase in the amount of alkyl carbon with all the DIRT manipulations that removed above and belowground inputs (No Litter, No Roots and No Inputs) and in the O/A Less plots. This relative increase in alkyl constituents was accompanied by a decrease in *O*-alkyl components, resulting in elevated alkyl/*O*-alkyl ratios with all the treatments (Fig. 3). The alkyl/*O*-alkyl ratio was highest in the plots that did not receive belowground inputs (No Roots and No Inputs), suggesting that the SOM is at a more advanced stage of degradation compared to the Control. The aromatic and phenolic carbon signal showed a slight relative increase with the addition of aboveground litter (Double Litter) and a slight decrease with the removal of belowground inputs (No Roots). The carboxyl and carbonyl carbon signal showed a slight relative increase in the Double Litter, No Litter, No Inputs and O/A Less plots.

The solution-state ^1H NMR integration results (Table 2) showed a relative increase in the amount of alkyl components in the base-soluble SOM in response to all the detrital input and removal treatments. This relative increase was accompanied by a decrease in the amount of *O*- or *N*-substituted aliphatic and *O*-alkyl components. A decrease was also observed in the relative intensity of the signal originating from the α -protons in peptides. The alkyl and the *O*- or *N*-

substituted aliphatic signals of the DE ^1H NMR spectra were attenuated (Table 2), indicating that these components were mostly small molecules with large amounts of motion or diffusion. The signal for the *O*-alkyl components increased in the DE ^1H NMR spectra, suggesting a contribution from large, macromolecular components. The intensity of the terminal CH_3 peak increased with all the treatments and was greatest in the Double Litter plot. The intensity of the $(\text{CH}_2)_n$ peak was greatest in the No Inputs plot (Table 2), suggesting the prevalence of macromolecular long-chain aliphatic structures relative to other aliphatic compounds in soil. The peak intensity from the *N*-acetyl signal was higher in the Double Litter, No Litter and No Roots plots than that observed in the Control sample (Table 2).

Discussion

After twenty years of variation in plant inputs at the Harvard Forest, differences in both the amount of soil carbon stored (Lajtha et al. 2014) as well as the molecular-level composition of SOM were observed. The addition of aboveground litter increased the amount of plant-derived cuticular compounds in mineral soil horizons. The amount of solvent-extractable and lignin-derived compounds did not change significantly with the doubling of litter inputs, likely as a result of higher rates of litter decomposition and microbial respiration from soil priming. Soil priming is the accelerated decomposition of organic carbon after the addition of easily-decomposable organic substrates to the soil (Kuzyakov 2002). This priming has also been observed at other temperate and tropical forests undergoing detrital input manipulations (Crow et al. 2009a; 2009b; Leff et al. 2012; Sayer et al. 2011). Lajtha et al. (2014) also found that long-term doubling of litter inputs did not increase labile carbon in SOM and suggested that increased microbial degradation, likely depleted any gains in labile carbon from additional litter inputs.

Our observations of increased litter decomposition is likely driven by the observed increase in microbial activity (as observed with PLFA profiling) and shift in the soil microbial community towards fungi. These results are consistent with increased enzyme activity also reported by Lajtha et al. (2014). The increased presence of fungi, the main decomposers of lignin in forest soils (Thevenot et al. 2010), and the lack of a significant change in the lignin phenol concentrations and oxidation (Table 1), suggests that there may be other environmental factors involved in lignin stabilization and decomposition in these temperate soils.

The exclusion of belowground inputs (No Roots and No Inputs) caused a decrease in the total amount of plant-derived hydrolysable lipids and resulted in enhanced cutin degradation in mineral soils. This is also consistent with a decrease in labile SOM (light fraction) from these samples as reported by Lajtha et al. (2014). Several studies have reported that the radiocarbon age of roots varies (Gaudinski et al. 2001; Sah et al. 2010; Solly et al. 2013; Vargas et al. 2009) but consistently suggest that any roots present at the time of barrier placement were likely mineralized before 20 years (the time of sampling). This is also reflected by the significant decrease in suberin-derived biomarkers ($p \leq 0.05$, Table 1) in the No Inputs and No Roots plots. The significant decrease relative to the Control plots suggests that root-derived carbon has declined five times more in the No Inputs and No Roots plots within the 20 year period. Lajtha et al. (2014) also reported considerably lower root biomass in the O horizons of these plots further supporting the experimental approach to limit root inputs over the 20 year period. The exclusion of roots in the DIRT mineral soils resulted in a microbial community composition shift towards fungi and an increase in actinomycetes. Actinomycetes are filamentous bacteria that have traditionally been associated with the degradation of more recalcitrant carbon compounds (Brant et al. 2006; McCarthy & Williams 1992). This shift in microbial community structure

reflects microbial adaptation to changes in substrate quality and quantity. This resulted in the accumulation of more degraded SOM, as observed by degradation parameters (ω -C₁₆/ΣC₁₆ and Ad/Al ratios) and NMR results (alkyl/*O*-alkyl ratio). Our results indicate that the exclusion of roots and litter also favored degradation of cutin compounds that are believed to be stable SOM components (Lorenz et al. 2007; Riederer et al. 1993). Although a shift was observed in the microbial community composition towards fungi, root exclusion did not significantly increase lignin oxidation, suggesting that above and belowground litter manipulation did not alter lignin stabilization in soils within the time frame of the experiment (20 years).

Plots that had the organic and mineral A horizons removed (O/A Less plots) 20 years prior to our sampling showed significantly lower amounts of solvent-extractable compounds and of hydrolysable lipids, suggesting that soils require longer than two decades to recover, even with ambient above and belowground plant litter inputs. The low microbial biomass in the O/A Less plots compared to the Control suggests that the exclusion of roots and an organic horizon cannot sustain a large active microbial population and that microbial function in these plots has not fully recovered even after twenty years of inputs. Lajtha et al. (2014) also reported that CO₂ respiration and root mass from the O/A Less plots was lower relative to the Control, further suggesting that microbial and rhizospheric functions in these plots are not yet restored. Bioavailability of litter may have been lower than in the other plots because of plant-derived OM sorption to clay mineral surfaces (Feng et al. 2005) which may have reduced microbial substrate availability (Sollins et al. 2009). Consequently, as mineral surfaces become saturated, more OM may be available for microbial degradation.

Global changes in temperature, precipitation, growing season length, and atmospheric CO₂ that are predicted in the coming decades will likely increase the net primary productivity in

terrestrial ecosystems (King et al. 1997; Melillo et al. 1993; Norby et al. 2005). The increase in plant productivity and associated litter and root inputs to soils may result in enhanced microbial respiration, releasing some of the newly added carbon as well as some of the stored and more stable SOM components. Consistent with our hypotheses, this priming occurred in the Double Litter plots at the Harvard Forest and has also been observed at other DIRT sites (Crow et al. 2009b; 2009b; Sulzman et al. 2005) but within shorter time frames. These results reveal that enhanced primary production may lead to the accelerated processing of stored soil carbon in a mixed temperate forest and is consistent with reports of warming-mediated priming leading to the decomposition of old carbon (Hopkins et al. 2012). Even cuticle-derived compounds were observed to be in a more advanced stage of decomposition despite their presumed recalcitrance and long-term stability. Root exclusion may also favor the degradation of SOM through changes in the soil microbial community structure. Our results suggest that the quantity of plant litter inputs may have a direct influence on the molecular-level composition of SOM and this relationship is likely to be ecosystem-dependent and warrants further investigation. For example, doubling litterfall inputs in a wet tropical forest resulted in an increase in soil carbon concentration (Leff et al. 2012). The Harvard Forest is a temperate ecosystem where increasing plant litter inputs did not increase soil carbon and instead, enhanced SOM degradation. Consequently, different ecosystems may exhibit varying responses to global environmental change that lead to increased plant productivity and plant inputs to soils. However, our study suggests that increased litterfall is unlikely to enhance soil carbon storage over the long-term in temperate forests. This decrease in carbon storage, which is a direct result of enhanced microbial activity and shifts in microbial community structure, is also problematic from a SOM quality perspective. Consistent with Lajtha et al. (2014), we observed a decline in labile carbon stocks

but our molecular-level analysis also provides clear evidence for microbial degradation of cutin and suberin. Interestingly, lignin degradation was not significantly different after 20 years of above and belowground manipulation. In another study at Harvard Forest, accelerated lignin degradation was observed after 4 years of soil warming (Pisani et al. 2015) indicating that lignin is indeed susceptible to microbial degradation. Analysis of samples from other DIRT sites, which include the addition of aboveground woody tissues, will help elucidate the role of litter manipulation on lignin biogeochemistry in soil.

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Table 1. Chemical composition and organic matter degradation parameters of the forest floor and mineral horizon soils from the Harvard Forest DIRT plots. All biomarker values are reported as average $\pm$ standard error and values in bold font are significantly different from the Control ($p \leq 0.05$). Details regarding individual biomarker concentrations are provided in the Supplementary Material.

	Forest Floor	Mineral Horizon					
		Control	Double Litter	No Litter	No Roots	No Inputs	O/A Less
C (%)	32.5 $\pm$ 1.1	7.24 $\pm$ 0.67	6.85 $\pm$ 0.19	5.80 $\pm$ 0.75	6.75 $\pm$ 0.76	6.16 $\pm$ 0.50	2.69 $\pm$ 0.40
<i>Solvent-extractable compounds (mg g⁻¹ soil)</i>							
Short-chain aliphatics	0.31 $\pm$ 0.03	0.05 $\pm$ 0.002	0.05 $\pm$ 0.001	0.03$\pm$0.001	0.04$\pm$0.001	0.03$\pm$0.001	0.01$\pm$0.001
Long-chain aliphatics	1.72 $\pm$ 0.11	0.33 $\pm$ 0.008	0.36 $\pm$ 0.03	0.20$\pm$0.05	0.32 $\pm$ 0.007	0.22$\pm$0.01	0.05$\pm$0.003
Plant cyclics	1.22 $\pm$ 0.28	0.05 $\pm$ 0.001	0.07$\pm$0.01	0.04 $\pm$ 0.001	0.06$\pm$0.001	0.06$\pm$0.001	0.02$\pm$0.001
Simple carbohydrates	1.49 $\pm$ 0.28	0.03 $\pm$ 0.001	0.03 $\pm$ 0.001	0.01 $\pm$ 0.0004	0.02 $\pm$ 0.001	0.03 $\pm$ 0.01	0.01 $\pm$ 0.003
<i>Cutin- and Suberin-derived hydrolysable lipids (mg g⁻¹ soil)</i>							
Cutin-derived	1.57 $\pm$ 0.25	4.24 $\pm$ 0.16	7.39 $\pm$ 0.38	5.76 $\pm$ 0.49	3.26 $\pm$ 1.17	0.71 $\pm$ 0.0005	6.40 $\pm$ 1.05
Suberin-derived	1.67 $\pm$ 0.10	6.62 $\pm$ 0.29	10.02$\pm$0.58	7.89 $\pm$ 0.15	1.21$\pm$0.16	1.18$\pm$0.43	1.99$\pm$0.07
Suberin- or cutin-derived	2.58 $\pm$ 0.64	7.74 $\pm$ 0.70	14.53$\pm$1.12	8.07 $\pm$ 0.20	1.94$\pm$0.14	2.14$\pm$0.0001	3.15$\pm$0.28
Suberin- and cutin-derived	5.82 $\pm$ 1.62	18.61 $\pm$ 1.22	31.94$\pm$2.05	18.58 $\pm$ 0.33	3.88$\pm$0.47	3.53$\pm$1.29	7.14$\pm$0.25
ω -C ₁₆ / Σ C ₁₆	0.15 $\pm$ 0.001	0.46 $\pm$ 0.01	0.37$\pm$0.004	0.65$\pm$0.003	0.74$\pm$0.001	0.50 $\pm$ 0.01	0.33$\pm$0.01
ω -C ₁₈ / Σ C ₁₈	0.029 $\pm$ 0.0003	0.029 $\pm$ 0.001	0.025 $\pm$ 0.001	0.029 $\pm$ 0.0002	0.023$\pm$0.001	0.020$\pm$0.001	0.021$\pm$0.002
Σ Mid/ Σ SC	0.52 $\pm$ 0.04	0.47 $\pm$ 0.005	0.52$\pm$0.005	0.48 $\pm$ 0.004	0.51$\pm$0.004	0.50$\pm$0.002	0.49 $\pm$ 0.01
<i>Lignin-derived phenols</i>							
Vanillyls	1.39 $\pm$ 0.26	0.10 $\pm$ 0.006	0.13 $\pm$ 0.03	0.08 $\pm$ 0.01	0.09 $\pm$ 0.02	0.08 $\pm$ 0.01	0.05 $\pm$ 0.01
Syringyls	0.53 $\pm$ 0.09	0.03 $\pm$ 0.002	0.04 $\pm$ 0.01	0.02 $\pm$ 0.003	0.03 $\pm$ 0.004	0.02 $\pm$ 0.002	0.01 $\pm$ 0.002
Cinnamyls	0.25 $\pm$ 0.07	0.02 $\pm$ 0.001	0.03 $\pm$ 0.01	0.02 $\pm$ 0.003	0.02 $\pm$ 0.003	0.02 $\pm$ 0.003	0.01 $\pm$ 0.01
VSC	2.17 $\pm$ 0.42	0.15 $\pm$ 0.01	0.20 $\pm$ 0.05	0.12 $\pm$ 0.02	0.14 $\pm$ 0.02	0.12 $\pm$ 0.01	0.07 $\pm$ 0.01
(Ad/Al)s	0.89 $\pm$ 0.23	1.13 $\pm$ 0.35	1.13 $\pm$ 0.30	0.95 $\pm$ 0.11	1.24 $\pm$ 0.19	1.38 $\pm$ 0.43	1.07 $\pm$ 0.26
(Ad/Al)v	1.37 $\pm$ 0.34	1.71 $\pm$ 0.26	1.85 $\pm$ 0.54	1.61 $\pm$ 0.13	2.23 $\pm$ 0.36	2.58 $\pm$ 0.65	1.60 $\pm$ 0.39

Carbon contents from: Lajtha et al. (2014). Cutin- and suberin-derived biomarkers are identified based on Otto and Simpson (2006b) and include: cutin-derived compounds (C₁₆ mono- and dihydroxy acids and dioic acids), suberin-derived compounds (long-chain ($\geq$ C₂₀) ω -hydroxyalkanoic acids and α,ω -dioic acids) and suberin- or cutin-derived compounds (C₁₆ ω -hydroxyacids, 9,10-epoxy- ω -hydroxy C₁₈, and C₁₆, C₁₈ α,ω -dioic acids). The VSC is the sum of all vanillyl, syringyl and cinnamyl phenols and the (Ad/Al) is the acid to aldehyde ratio of syringyl and vanillyl phenols.

Table 2. Integration results showing the relative percent (%) of different structural units for solid-state ^{13}C Cross Polarization with Magic Angle Spinning (CP-MAS), solution-state ^1H and diffusion edited (DE) ^1H Nuclear Magnetic Resonance (NMR) Spectroscopy of the forest floor and mineral horizon soils from the Harvard Forest Detrital Input and Removal Treatment (DIRT) experimental plots.

	Chemical Shift (ppm)	Forest Floor	Mineral Horizon					
			Control	Double Litter	No Litter	No Roots	No Inputs	O/A Less
<i>Solid-State ¹³C CP-MAS NMR</i>								
	¹³ C							
Alkyl	0-50	30	38	39	43	47	45	42
O-Alkyl	50-110	52	39	36	33	32	31	34
Aromatic + Phenolic	110-165	14	14	15	14	12	14	14
Carboxyl + Carbonyl	165-215	4	9	10	10	9	10	10
Alkyl/O-Alkyl		0.58	0.97	1.08	1.30	1.47	1.45	1.24
<i>Solution-State ¹H NMR</i>								
	¹ H							
Alkyl	0.6-1.3	29	19	25	30	30	35	25
O- or N-Aliphatic	1.3-2.9	23	38	36	37	34	32	40
O-Alkyl	2.9-4.1	28	29	25	24	25	22	27
α-H in peptides	4.1-4.8	4	7	6	5	5	4	4
Anomeric	4.8-5.2	5	3	3	1	2	3	1
Aromatic	6.2-7.8	8	3	4	2	3	3	2
Amide	7.8-8.4	3	1	1	1	1	1	1
<i>Solution-State DE ¹H NMR</i>								
	¹ H							
Alkyl	0.6-1.3	15	11	17	18	18	22	16
O- or N-Aliphatic	1.3-2.9	17	20	25	26	28	21	23
O-Alkyl	2.9-4.1	45	47	38	37	36	35	44
α-H in peptides	4.1-4.8	10	10	9	9	9	10	9
Anomeric	4.8-5.2	5	4	3	2	2	4	2
Aromatic	6.2-7.8	6	5	6	6	5	5	4
Amide	7.8-8.4	2	3	2	2	2	3	2

Figure Captions

Figure 1. PLFA results for DIRT experimental plots. A) Total PLFAs in the forest floor and mineral soil horizons; B) Microbial community structure; C) Microbial stress ratios; and D) Microbial composition ratios for the forest floor and mineral horizon soils from the Harvard Forest. Error bars are standard errors of both field and analytical replicates ($n = 6$). Data labeled with an asterisk are significantly different from the Control ($p \leq 0.05$).

Figure 2. NMR spectra for the mineral horizon Control soils: A) Solid-state ^{13}C CP-MAS NMR spectrum highlighting the major carbon functional groups; B) solution-state ^1H NMR spectrum and structural assignments of the base-soluble extracts; C) diffusion edited (DE) ^1H NMR spectrum showing signals from large/rigid molecules; and D) ^1H - ^{13}C Heteronuclear Quantum Coherence (HSQC) NMR which is used to confirm signals observed in one-dimensional experiments. Details regarding the structural assignments are provided in the text. Integration results for major structural classes are listed in Table 2.

Figure 3. Ratio of alkyl C (0-50ppm) to *O*-alkyl C (50-110ppm) from solid-state ^{13}C NMR analysis. The alkyl/*O*-alkyl ratio increases with progressive soil organic matter degradation.

Figure 1

Figure 2

Figure 3