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The Path From Litter to Soil: Insights Into Soil C Cycling From Long-Term Input Manipulation and High-Resolution Mass Spectrometry

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Key Points:
- Twenty years of litter input manipulation caused changes not only in the size but also in the chemical composition of soil C pools
- Litter exclusion led to a 30% loss of soil C from nonmineral-associated C
- Though litter addition did not change C content, there was a change in the chemical composition of the finest mineral-associated fraction

Supporting Information:
- Supporting Information S1

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Abstract The path of carbon (C) from plant litter to soil organic matter (SOM) is key to understanding how soil C stocks and microbial decomposition will respond to climate change and whether soil C sinks can be enhanced. Long-term ecosystem-scale litter manipulations and molecular characterization of SOM provide a unique opportunity to explore these issues. We incubated soils from a 20-year litter input experiment for 525 days and asked how litter quantity and source (i.e., roots versus aboveground litter) affected C cycling, microbial function, and the size and molecular composition of C pools. Input exclusion led to a 30% loss of soil C, attributable largely to the nonmineral-associated C fraction, and to declines in soil C decomposition. The absence of roots caused a shift in the microbial catabolic profile, though there was little evidence that root litter was preferentially stabilized. Although C pool size did not change with litter additions, Fourier transform ion cyclotron resonance mass spectrometry analysis of the finest mineral fraction revealed dramatic changes to the chemical composition of carbon. Lipid content increased proportionally with input addition and was subsequently mineralized during incubation, indicating that this fraction was metabolically active. Moreover, nonmetric dimensional scaling showed that both litter treatments and incubation caused the molecular composition of SOM to change. We conclude that the path of C from litter to soil may involve labile pools and root-driven microbial activity associated directly with SOM in the soil mineral matrix otherwise previously hypothesized to be stable.

1. Introduction

Soils play a key role in regulating climate by storing carbon (C) as soil organic matter (SOM) (Houghton, 2005; Jobbagy & Jackson, 2000) and releasing C into the atmosphere via soil respiration, one of the largest terrestrial C fluxes (Raich & Schlesinger, 1992). Global warming is expected to perturb the balance between SOM storage and soil respiration by altering net primary productivity, and thus plant-litter inputs, while increasing SOM decomposition (Anav et al., 2013; Friedlingstein et al., 2006). Meanwhile, there is speculation that soils may offer a means to offset the rise in atmospheric CO_2 by enhancing natural C accumulation mechanisms thereby storing additional carbon (Canadell et al., 2007; Dungait et al., 2012; Lal, 2005; Smith & Fang, 2010).

Soil C research, however, is riddled with apparent paradoxes (see Dungait et al., 2012), in part because the mechanisms driving C cycling dynamics are poorly understood (Schmidt et al., 2011; von Lützow et al., 2007, 2008). Model projections of soil responses to climate range widely in both magnitude and direction (Anav et al., 2013; Friedlingstein et al., 2006). Experiments simulating climate change have reported a variety of soil responses (Rustad et al., 2001; Wu et al., 2011) indicative of underlying complexities. Importantly, some studies showed that decomposition of older SOM did not have a higher-temperature sensitivity (Dalias et al., 2001; Giardina & Ryan, 2000; Liski et al., 1999), contradicting assumptions that older SOM was more chemically complex (Conant et al., 2011). Some of this apparent complexity may be due to our poor understanding of the nature of SOM. It is not clear how plant litter quantity and quality and source (i.e., roots versus aboveground litter) are linked to C retention in soils (Dungait et al., 2012; Lajtha, Bowden, & Nadelhoffer, 2014; Lajtha, Townsend, et al., 2014). Thus, the potential to sequester additional C within soils through management remains speculative (Dungait et al., 2012), especially as C addition may potentially "prime" decomposition of

native C stocks (Bowden et al., 2014; Kuzyakov, 2002; Lajtha, Bowden, & Nadelhoffer, 2014; Lajtha, Townsend, et al., 2014). To predict, and possibly manipulate, soil-climate feedbacks, we must understand the pathways C takes through soil, from the incorporation and transformation of litter inputs to its ultimate mineralization.

Our understanding of the nature of SOM and its stabilization has evolved rapidly in recent years. Historically, SOM was thought to become stabilized in soils as it became increasingly chemically complex during decomposition leading to organic compounds that were resistant to microbial degradation (see Kleber & Johnson, 2010). However, direct investigations of the biochemical nature of SOM with high-resolution imaging techniques revealed it to be a complex amalgam of interacting, low molecular weight organic molecules in a matrix of minerals (Lehmann et al., 2008; Schmidt et al., 2011). Furthermore, SOM that has typically been considered to be chemically labile (i.e., readily utilized by microbial decomposers) can have turnover times on the order of centuries (Kleber et al., 2011). Thus, the hypothesis that SOM decomposition is determined by chemical complexity alone has been challenged in favor of spatial and soil-matrix-mediated controls on availability (Ekschmitt et al., 2005; Kleber, 2010; Schmidt et al., 2011). SOM age, and thus "stability," tends to increase with its degree of mineral association, with free, particulate organic matter being the youngest and most plant like and organic matter occluded within microaggregates and adsorbed onto the surface of minerals being older and primarily microbial in origin (Sollins et al., 2006). Organic matter becomes incorporated into these pools due to microbial activity in close association with mineral particles (Kleber & Johnson, 2010); chemical complexity per se is not the sole determinant of SOM stability. However, the turnover rates of these mineral-associated C pools are largely unknown (Conant et al., 2011). Although dissolved organic matter chemistry controls C adsorption on mineral particles and thus stabilization (Kleber et al., 2007), it is not clear how this may determine turnover rates within these pools. Moreover, the pathway by which organic matter enters C pools with the longest residence time is not well known, nor is the role of litter C quantity and quality, particularly the role of roots versus aboveground litter (Bird et al., 2008; Dungait et al., 2012; Hatton et al., 2015; Lajtha, Bowden, & Nadelhoffer, 2014; Prescott, 2010).

The Detritus Input and Removal Treatment (DIRT) sites were designed to directly test the effects of chronic input manipulation on SOM decomposition and accumulation on decadal time scales (Nadelhoffer et al., 2004). Thus far, these experiments have revealed indirect and nonlinear responses (Bowden et al., 2014; Lajtha, Bowden, & Nadelhoffer, 2014; Lajtha, Townsend, et al., 2014) in total C and mineral-associated SOM pools, including evidence for context-specific dynamics and priming. Overall, the mineral-associated fractions are reported to be less vulnerable to decomposition and less responsive to C accumulation from aboveground inputs, indicating that direct litter addition does not necessarily result in rapid or direct accumulation of additional C in the putatively "stable" pools (Bowden et al., 2014; Lajtha, Bowden, & Nadelhoffer, 2014; Lajtha, Townsend, et al., 2014) even when soils are not "carbon saturated" (Mayzelle et al., 2014). Moreover, the variability of responses among sites may be due to differences in litter decomposition rate as controlled by climate (Fekete et al., 2014), litter quality (Bowden et al., 2014), and soil mineralogy that in turn drive the litter-to-soil pathway.

It has been proposed that root C may be preferentially incorporated into soils and stabilized (Schmidt et al., 2011), possibly because of its proximity to the mineral matrix or due to the layer of suberin, which slows decomposition (Rasse et al., 2005). However, Hatton et al. (2015) reported that while root C contributed more particulate matter to the free, light fraction, likely due to its slower decomposition, leaf C contributed more to the mineral-associated and potentially more stable fraction in a coniferous forest. Thus, labile inputs such as foliar litter may actually contribute more than roots to mineral-associated SOM pools and be preferentially stabilized as they stimulate microbial activity and exudation of microbial products (Cotrufo et al., 2013, 2015). This is in contrast to the DIRT findings that there is little evidence of C accumulation in the mineral fractions due to aboveground litter addition. It is possible that the foliar C that is deposited in the mineral-associated fractions may remain active as there is evidence for multiple turnover rates within this C pool (Torn et al., 2013). Additionally, the relative importance of root versus aboveground litter C may vary with forest type; for example, though there was no overall loss of C due to root exclusion, Crow et al. (2009) reported preferential stabilization of foliar-derived aliphatics in a coniferous forest and root-derived aliphatics in a deciduous forest. Meanwhile, root exclusion has been variously shown to cause C losses that are greater (Fekete et al., 2014), lower (Bowden et al., 2014; Lajtha, Bowden, & Nadelhoffer, 2014), or not different (Lajtha, Bowden, & Nadelhoffer, 2014; grassland sites) from C losses due to aboveground litter exclusion, indicating that the response may be context specific and not consistent across ecosystem types.

We asked how C cycles through terrestrial soils by combining a 20-yearlong chronic root and litter input manipulation in a northeastern deciduous forest with a long-term laboratory incubation and comparing whole-soil responses with C pools defined by mineral association. We asked if litter input manipulation changed C quantity and which C pools were most vulnerable to change. We also took a unique opportunity to directly examine the molecular nature of C in the fine mineral fraction, demonstrated to be the oldest and thus considered the most stable pool of C (Christensen, 2001; Sollins et al., 2006), and under extremes of litter manipulation to directly test its chemical complexity and putative stability.

2. Methods

2.1. Site Description and Soil Collection

Soils were collected from the Detritus Input and Removal Treatment (DIRT) plots located in the Bousson Experimental Forest (41°36′N, 80°3′W, 381 m), Pennsylvania, USA. Details about the site and experiment can be found in Bowden et al. (2014). Average annual precipitation is 1,050 mm/yr and daily temperatures average −4°C in January and 21°C in August. Soils are classified as coarse loamy mixed superactive mesic Oxyaquic Fragiudalfs (Cambridge series) derived from glacial till overlying shale and sandstone (USDA-SCS, 1979) with a fragipan present at 60 cm and with a pH of 4.0. Black cherry (*Prunus serotina*) and sugar maple (*Acer saccharum*) are the dominant tree species. The Bousson Forest DIRT plots were initiated in 1991 and consist of three 3 × 3-m replicate plots under five litter manipulation treatments: no inputs (NI; no roots or litter), no litter (NL), no roots (NR), control conditions (CO), or double litter (DL). Screens are used to exclude aboveground litterfall, which is transferred to the DL treatment plots. Roots were meant to be excluded from the NR and NI treatments via impermeable plastic barriers buried from the surface to the C horizon (~1.4 m), but some roots have entered the plots from below the barriers and the litter treatment also affected root biomass (Bowden et al., 2014). Root biomass from 0 to 10 cm depth was 129%, 90%, 51%, and 10% of the control in the DL, NL, NR, and NI treatments, respectively, at the time of sampling.

In fall 2011, two cores from 0 to 10 cm depth were collected and bulked per plot using a gas-powered 9.62-cm diameter diamond-bit stainless steel soil corer (Earthquake, 9800B). All samples were passed through a 2-mm sieve and sorted for rocks, roots, and other debris then subsampled and stored field moist at 4°C. Following review of the results of Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) analysis of the soils (see below), leaf litter was collected Fall 2014 and air dried for further analysis.

2.2. Incubations

Incubations began January 2012 and continued for 525 days. Total C prior to incubation was determined on dried, ground soil with a Costech Analytical Technologies 4010 elemental combustion analyzer (Valencia, CA, USA). Soil pH was determined with a 1:1 by volume slurry with distilled water, and soil moisture was determined by drying ~10 g of soil at 60°C for 48 hr. The soils' water holding capacity and water content at 60% of saturation were determined according to the standard methodology (Klute, 1986). Approximately 20 g dry-weight equivalent of mineral soil was weighed into 120-mL serum bottles and moisture content adjusted to 60% of saturation with distilled water. Moisture content was monitored by weight and water applied when necessary.

Soils were incubated at 35°C. Soil respiration was measured every day for the first week, each week for the first month, and every other month thereafter. To measure the soil respiration rate, each serum bottle was sealed, overpressurized by injecting 5 mL of air, and the headspace CO_2 concentration measured via direct injection with a LI-7000 infrared gas analyzer or a LI-6400 plumbed for direct injection (LICOR Inc., Lincoln, NE), then measured again after 3 hr; a CO_2 standard calibration curve was determined on each measurement day.

2.3. Catabolic Profiles

The substrate-induced respiration technique MicroResp™ (Aberdeen, Scotland) was used to assess the each soil's microbial community's ability to utilize substrates of varying quality, as demonstrated by Campbell et al. (2003). Following the MicroResp™ protocol, in January 2012 we measured the response from each DIRT treatment to 18 carbon substrates (supporting information Table 1), including simple sugars, amino acids, carboxylic acids, and complex structural carbohydrates. Briefly, soils with equivalent soil moisture contents were loaded into 96 well microtiter plates, inoculated with 25 μL of 30 mg substrate g^{-1} soil water, and incubated for 6 hr at 25°C. The detector plate, filled with a creosol red-agar solution, was analyzed with an

Infinite 200 PRO® plate reader (Tecan, Switzerland) at 570 nm to determine the amount of CO_2 respired during the incubation. CO_2 standards were run in a similar manner.

2.4. Density Fractionation

Soils were separated by density fractionation with sodium polytungstate using methods modified from Crow et al. (2006). Approximately 5 g of soil from each litter treatment from day 0 and day 525 of the incubation were mixed with 20 ml of 1.6 g/cm^{-3} sodium polytungstate and reciprocally shaken for 24 hr. The floating fraction (hereafter the light fraction or LF) was aspirated off, triple rinsed with DI, dried at 60°C for 48 hr, and weighed. The heavy fraction (HF) was dried at 60°C and sieved, with the fine HF (fHF; $<$53 μm) consisting of silt and clay and the remaining HF ($\geq$53 μm) consisting of sand and aggregates. The remaining HF was then mixed with 20 mL of 5% hexametaphosphate and reciprocally shaken for 24 hr to disperse aggregates. It was then vacuum filtered and triple rinsed with DI water to remove the hexametaphosphate, dried at 60°C, and fractionated again with sodium polytungstate as described above. The floating fraction derived from dispersed aggregates was defined as the occluded LF (OLF). The HF remaining was defined as coarse HF (cHF; $\geq$53 μm) consisting of sand and the clay and silt released from the dispersed aggregates. All fractions were dried at 60°C for 48 hr, weighed, and total C and N determined as described above for bulk soil.

2.5. FTICR-MS

Fine HF soil from the NI, CO, and DL treatments from day 0 and day 525 of the incubation and leaf litter (collected Fall 2014 for comparison to the soil samples) were processed and analyzed with Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS), using a 12 T Bruker SolariX FTICR mass spectrometer at the Environmental Molecular Sciences Laboratory, a Department of Energy national user facility in Richland, WA, as described in Tfaily et al. (2015). Briefly, 100 mg of dried soil or litter was extracted with 1 ml of methanol. Tfaily et al. (2015) determined that methanol preferentially captures organic matter with low O/C ratios (O/C $<$ 0.6), particularly lipids and lignin-like compounds in addition to unsaturated hydrocarbons especially for soils with low % C as the ones used in this study. Thus, while our results do not represent a complete inventory of OM in the fHF and leaf litter, they do represent a consistent chemical footprint across the various treatments. As this was a developing method (Tfaily et al., 2015), we were unable to determine how much of the total C from our samples was extracted and thus the values presented are relative and proportional only; however, the relative differences should be representative of the samples.

To interpret the large data set, the assigned compounds were visualized on van Krevelen diagrams. The compounds were plotted on the van Krevelen diagram on the basis of their molar H/C ratios (*y* axis) and molar O/C ratios (*x* axis; Fenn et al., 1990; Kim et al., 2003). Van Krevelen diagrams provide a means to compare the average properties of OM and enable identification of the major biochemical classes. For this study, the chemical compounds were grouped into the eight main families: condensed aromatic compounds, unsaturated hydrocarbon, tannins, lignin, lipids, protein, amino sugars, and carbohydrate derived. From the formula assignment, the average (by number-weighted) abundance of each class was calculated and compared between samples.

3. Analysis

3.1. C Concentration of Bulk Soil and Density Fractions

Data were assessed and transformed as necessary to meet the assumptions of each analysis. Soil responses were analyzed with one-way ANOVA followed by Tukey's post hoc tests. Differences in C concentration in density fractions between incubation days were tested with repeated-measures analysis of variance (ANOVA) to account for dependence through time, followed by ANOVAs within day and *t* tests within treatment when there was a significant interaction. All ANOVAs and regressions were performed using SPSS version 19.0 (IBM SPSS Statistics for Windows, 2010, Armonk, NY).

3.2. Cumulative Soil Respiration Modeling

Cumulative C respired was calculated by smoothly incrementing the respiration rate g C^{-1} (i.e., the C turnover rate) and g dw soil^{-1} across nonmeasurement days. Treatment differences for total cumulative C respired were analyzed as described above. Cumulative soil respiration was fitted against a one-pool model,

$$X_t = X_0 \left(1 - e^{-kt}\right) \quad (1)$$

Table 1
Mean (se) C Concentration, Cumulative C Respired, and Decomposition Model Coefficients

Parameter treatment		
C concentration	**Mg CO_2-C g dw soil^{-1}**	
***p* value**	0.016	
NI	36.0 (3.2)^{a}	
NL	44.7 (3.6)^{b}	
NR	47.1 (3.5)^{b}	
CO	51.3 (1.3)^{b}	
DL	50.7 (0.30)^{b}	
Cumulative C respired	**Mg CO_2-C g dw soil^{-1}**	**Mg CO_2-C gC^{-1}**
***p* value**	<0.0001	<0.0001
NI	2.36 (0.13)^{a}	70.7 (2.83)^{a}
NL	3.99 (0.14)^{b}	98.9 (7.19)^{b}
NR	4.01 (0.39)^{b}	92.9 (4.00)^{b}
CO	5.76 (0.18)^{c}	126.3 (2.33)^{c}
DL	6.19 (0.13)^{c}	139.0 (3.18)^{c}
X_0		
***p* value**	<0.0001	0.01
NI	4.14 (0.099)^{a}	139.7 (12.09)^{a}
NL	4.59 (0.014)^{a}	121.1 (11.36)^{a}
NR	5.56 (0.049)^{ab}	143.6 (6.61)^{ab}
CO	6.53 (0.018) ^{bc}	153.7 (4.8)^{ab}
DL	7.66 (0.11)^{c}	194.8 (16.97)^{b}
k		
p-Value	0.007	0.001
NI	0.0016 (0.00001)^{a}	0.0013 (0.00021)^{a}
NL	0.0032 (0.00001)^{c}	0.0029 (0.00014)^{c}
NR	0.0023 (0.00002)^{ab}	0.0019 (0.00006)^{ab}
CO	0.0035 (0.000003)^{c}	0.0029 (0.0001)^{c}
DL	0.0029 (0.00002)^{bc}	0.0023 (0.00031)^{bc}

Note. Different small letters indicate significant differences at $p < 0.05$.

where X_t is the total C respired, X_0 is the mineralizable C pool, k is the mineralization rate, and t is day. Nonlinear regressions were fitted to the cumulative respiration data using Levenberg-Marquardt and sequential quadratic programming nonlinear regression parameter estimation procedures in SPSS version 19.0. Global stability of fitted constants was confirmed by increasing or decreasing each by 50% and confirming that the model converged on the original value. A two-pool model was also attempted but never stabilized (data not shown). Treatment differences for X_0 and k were analyzed as for C concentration described above.

3.3. Catabolic Profile

The response to each substrate was standardized by subtracting the baseline soil respiration rate and dividing by the average response for each treatment (Campbell et al., 2003). Treatment differences in the response to each substrate were analyzed as for C concentration described above. We assessed the overall response of each treatment to the full suite of substrates with nonmetric multidimensional scaling (NMS) (McCune & Grace, 2002) on a Bray-Curtis dissimilarity matrix and then tested for treatment differences with permutational multivariate analysis of variance (PERMANOVA). Both analyses were performed using the package vegan (Oksanen et al., 2012) in R v. 3.2.0.

3.4. FTICR-MS

We tested for differences in the proportion of each biochemical class detected in the fHF due to input treatment and incubation as described above. Most biochemical compounds (all except carbohydrates, proteins, and "other") had a significant interaction between incubation day and litter treatment in the repeated measures ANOVA; thus, the p values for treatment differences are from ANOVAs within each incubation day and differences due to incubation are from t tests within treatments. Changes in biochemical content between input treatments and leaf litter was also compared visually using van Krevelen diagrams (Kim et al., 2003). We visualized changes in biochemical classes with nonmetric multidimensional scaling (NMS) performed on a Bray-Curtis dissimilarity matrix and tested for differences among treatments and between incubation days within this nonparametric space with PERMANOVA. Both NMS and PERMANOVA were performed using the package vegan in R v. 3.2.0.

4. Results

4.1. C Concentration

C concentration was reduced in the NI treatment versus all other treatments (Table 1; $p = 0.016$), with a 30% depletion versus the control. There was also a nonsignificant 13% and 8% reduction in C concentration in the NL (pairwise comparison: $p = 0.47$) and NR (pairwise comparison: $p = 0.80$) treatments, respectively.

4.2. Cumulative C Respired and Model Coefficients

Cumulative C respired was reduced by input exclusion, g dw^{-1} by 30–59%, and g C^{-1} by 21–26%, but largely unaffected by input addition. However, the DL treatment respired somewhat more than CO treatment, while litter and root exclusion appeared equivalent. Similar results occurred regardless of whether results were expressed per g soil or as a turnover rate of the soil C pool (Table 1). The modeled size of the mineralizable pool, X_0, per g soil was significantly reduced in the NI and NL treatments versus the CO and DL treatments, while X_0 of the NR treatment was only different from the DL treatment. The overall trend for X_o was similar when expressed as a C turnover rate, but only the NI and NL treatments were significantly reduced versus the DL treatment. The mineralization rate, k, was significantly reduced in the NI treatment versus the NL, CO, and DL treatments. The k of the NR treatment was also lower than the NL and CO treatments.

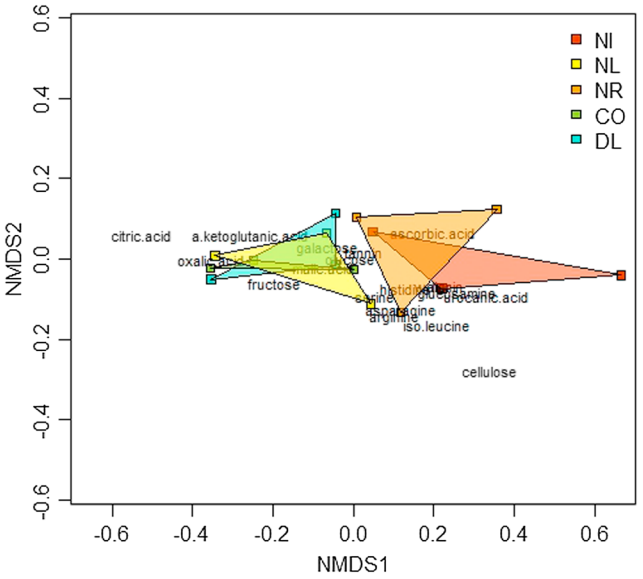


Figure 1. Nonmetric dimensional scaling of substrate-induced respiration for catabolic profile of DIRT soil. NI = no inputs, NL = no litter, NR = no roots, CO = control, DL = double litter. See supporting information Table 1 for substrates.

4.3. Catabolic Profile

In general, the NI and NR treatments respired less in response to substrate addition than the NL, CO, and DL treatments, with the exception of urocanic acid, isoleucine, and glucosamine. However, there was a significant treatment effect for only 6 out of 18 substrates (supporting information Table 1). NMS explained 99.6% of the variance in soil respiration due to substrate addition in the catabolic profile analysis and showed that the NI and NR treatments separated from the NL, CO, and DL treatments (Figure 1; stress = 0.064; $p = 0.047$) due to differences in their response to cellulose versus sugars, and some carboxylic and amino acids.

4.4. Density Fractionation

After 20 years of litter input manipulation, the C concentration in the light fraction (LF) was reduced by 25% in the NL treatment, 35% in the NR treatment, and 49% in the NI treatment versus the CO treatment, though only the NI treatment was significantly different (Figure 2; $p = 0.049$). The C concentration in the OLF was reduced in the NI treatment versus the NL, NR, and CO treatments, though this was only significant versus the NR treatment (ANOVA: $p = 0.021$) and marginally different from the CO treatment (pairwise comparison: $p = 0.077$). In the fHF, C concentration was significantly higher in the NI treatment versus CO ($p = 0.031$) and marginally higher than the NR treatment (pairwise comparison: $p = 0.085$). In the cHF, the NR treatment had a significantly higher C concentration than all other treatments ($p = 0.001$). The NI treatment was marginally reduced versus the NL treatment (pairwise comparison: $p = 0.067$) and the CO treatment (pairwise comparison: $p = 0.054$). The soil C concentration of the DL treatment was 19% lower than the control in the LF, 70% lower in the OLF, and 29% lower in the cHF, though these differences were not significant (pairwise comparisons: $p \geq 0.10$).

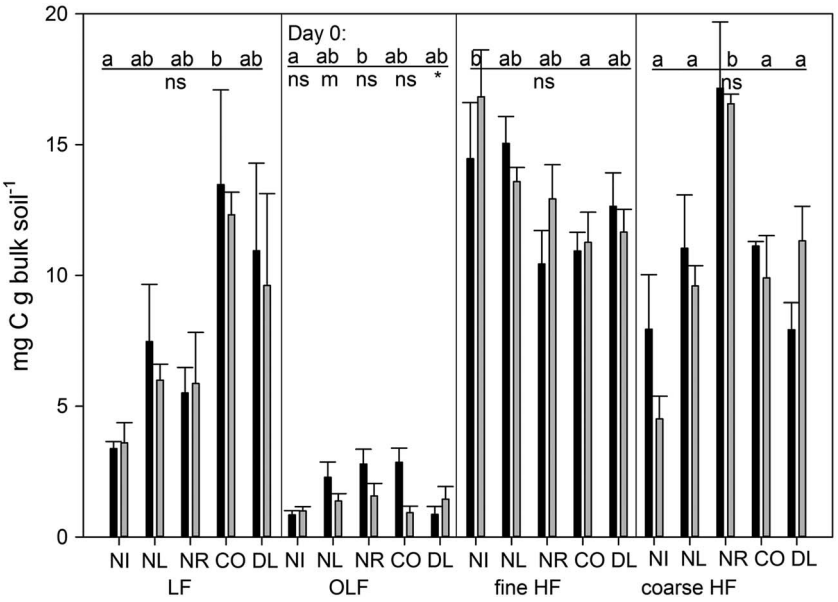


Figure 2. Mean (se) mg C g bulk soil^{-1} in density fractions for DIRT soil at the beginning (day 0, black bars) and end (day 525, gray bars) of incubation. Treatment differences are shown by different small letters and incubation differences by * = significant ($p < 0,05$), m = marginal ($0.05 < p < 0.01$), and ns = nonsignificant ($p \geq 0.1$). NI = no inputs, NL = no litter, NR = no roots, CO = control, DL = double litter. Treatment effects were broken out by day if there was a significant treatment *x* incubation interaction. LF = light fraction, OLF = occluded light fraction, fine HF = fine heavy fraction, coarse HF = coarse heavy fraction.

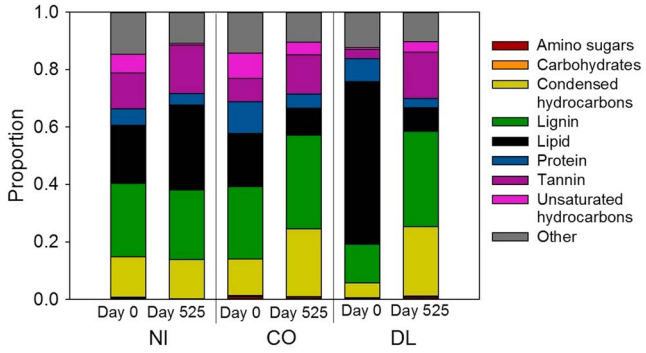


Figure 3. Proportion of each biochemical class detected with FTICR-MS in the fine heavy fraction (fHF) of no inputs (NI), control (CO), and double litter (DL) treatments at the beginning (day 0) and end (day 525) of incubation.

During incubation, the concentration of C in the OLF decreased in the NL, NR, and CO treatments, though only the NL treatment was even marginally significant (p = 0.099). The C concentration in the OLF of the DL treatment increased during incubation (p = 0.026). The difference in C concentration in the OLF in the NI treatment versus the NL, NR, and CO treatments was lost by day 525 (ANOVA: p = 0.20).

4.5. FTICR-MS

There were substantial changes due to input manipulation and incubation in the classes of biochemical compounds in the fHF detected by FTICR-MS (Figure 3). NMS explained 78.8% of the variance in biochemical composition and showed separation on both treatment (p = 0.012) and incubation day (p = 0.001), while treatments also differed between days (p = 0.001) (Figure 4; stress = 0.046). After 20 years of litter input manipulation, the NI and CO treatments differed from the DL treatment due to the lipid enrichment in the latter (p = 0.001). After 525 days of incubation, the DL treatment had become more similar to the CO treatment as both lost lipids and became relatively more enriched with tannins and lignin (p = 0.015).

ANOVA was used to determine which specific chemical classes drove the overall patterns revealed by NMS above. Considering the effect of litter input manipulation first, the NI treatment was very similar to the CO treatment, differing only by a marginally significant 5.1% absolute loss of proteins (Figure 3 day 0; statistics in supporting information Table 2; repeated measures ANOVA: p = 0.068 for DIRT treatment; day x treatment interaction: p = 0.23; pairwise comparison: p = 0.072), though these treatments diverged during incubation (see below). In contrast, the DL treatment differed from the control treatment, with a much greater proportion of lipids (56.6% versus 18.6%, respectively; day 0 ANOVA: $p < 0.0001$) and a lower proportion of amino sugars (−0.7%, day 0 ANOVA: p = 0.054), condensed hydrocarbons (−7.3%, day 0 ANOVA: p = 0.023), lignin (−11.9%, day 0 ANOVA: p = 0.035), tannins (−4.8%, day 0 ANOVA: p = 0.009), and unsaturated hydrocarbons (−8.2%, day 0 ANOVA: p = 0.001).

The proportion of the classes of biochemical compounds changed very little over the course of the incubation in the NI treatment, with small but significant losses of amino sugars (Figure 3; −0.3% absolute percentage change, p = 0.076), proteins (−2%, p = 0.001), unsaturated hydrocarbons (−6.1%, p = 0.017), and other unidentified compounds (−3.9%, p = 0.039). However, though they started very similar, the biochemical composition of the CO treatment diverged from the NI treatment over the course of the incubation, with the CO treatment showing substantial decreases in lipids (−9.3%, p = 0.024), proteins (−5.9%, p = 0.001), unsaturated hydrocarbons (−4.4%, p = 0.019), and other compounds (those that did not correspond to the main biogeochemical groups; −3.8%, p = 0.039), and increases in condensed hydrocarbons (+10.9%, p = 0.001), lignins (+7.6%, p = 0.035), and tannins (+5.4%, p = 0.052). The chemical signature of the DL treatment became more similar to the CO treatment over the incubation: The majority of the large lipid pool disappeared over the incubation (−48.3%, p = 0.013), other compounds also declined (−2%, p = 0.039), accompanied by an enrichment in amino sugars (+0.5%, p = 0.012), condensed hydrocarbons (+19%, p = 0.006), lignin (+19.8%, p = 0.031), tannins (+12.7%, p = 0.023), and unsaturated hydrocarbons (+3%, p = 0.041). There was no change in the proportion of carbohydrates (p = 0.36), which remained small, though it should be noted that this method is not optimal for detecting carbohydrates (see Tfaily et al., 2015).

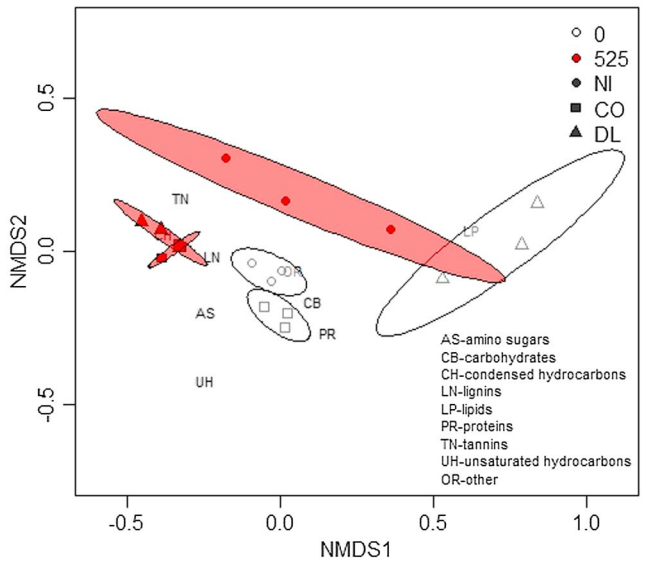


Figure 4. Nonmetric dimensional scaling of biochemical classes detected with FTICR-MS in the fine heavy fraction (fHF) of no inputs (NI), control (CO), and double litter (DL) treatments at the beginning (day 0) and end (day 525) of incubation.

The lipids detected in the DL treatment were compared against those from the dominant foliar components of the aboveground litter using a van Krevelen diagram (Figure 5), revealing that the lipids, which

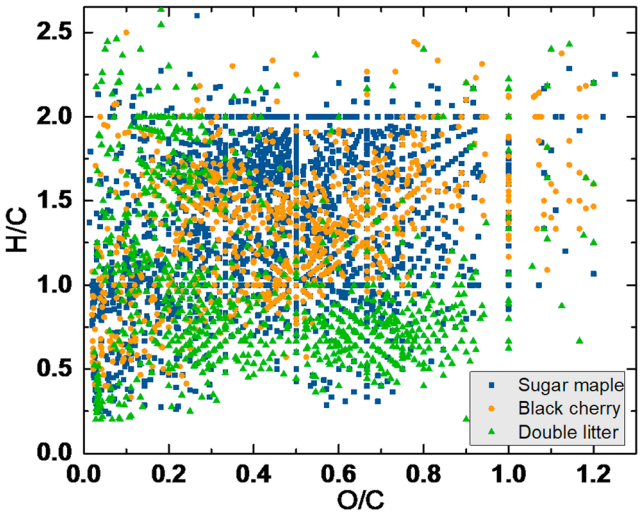


Figure 5. Van Krevelen diagram of biochemical compounds detected with FTICR-MS in the fine heavy fraction of the double litter treatment and the leaf litter of the dominant tree species showing that lipids, defined as H/C $\geq$ 1.55, O/C $\leq$ 0.3, differed between these samples.

enriched the fHF of the DL treatment, were not the same as those in the leaf litter and thus not directly translocated from the litter to this mineral fraction.

5. Discussion

Herein, we add to the rapidly evolving understanding of soil C cycling (Conant et al., 2011; Dungait et al., 2012; Schmidt et al., 2011) by using a unique combination of methods, including a long-term input manipulation experiment and advanced molecular techniques, to directly examine the nature of soil C, and report surprising trends in the path of C from litter to soil.

Two decades of input exclusion revealed trends in C cycling consistent with those reported by other long-term DIRT studies (Bowden et al., 2014; Lajtha, Townsend, et al., 2014), whereby C associated with the soil mineral matrix was least vulnerable to decomposition. This supports the hypothesis that the mineral matrix facilitates the stabilization of C through organo-mineral associations and aggregation (Conant et al., 2011; Dungait et al., 2012), whereas particulate organic matter (i.e., light fraction C) is more available and turns over more rapidly, and thus, its decomposition dynamics are dictated more strongly by chemical quality (Conant et al., 2011; von Lützow et al., 2007). However, examination of the molecular constituents of the mineral-associated C pool with FTICR-MS revealed potentially active turnover of C within this putatively stable fraction, with an enrichment of lipids during field manipulation of inputs, and subsequent decomposition of this pool during long-term incubation, complicating the supposition that increased litter had no effect on soil C and that mineral-fractions are inert. Additionally, DIRT studies and others have reported that though roots are often slower to decompose (i.e., lower in quality) and in closer contact with the mineral matrix, their component carbon is not necessarily preferentially stabilized compared to aboveground litter inputs and may be, as we report here, apparently equivalent in their contribution to soil C. At the same time, we also observed a fundamental shift in microbial catabolism with the exclusion of roots, indicating that though gross C content was unchanged, there may be shifts in the microbial community's function and thus overall C cycling that are not otherwise apparent.

5.1. The Stability of C Pools Under Litter Input Exclusion

The 30% in situ loss of soil C in the no inputs treatment was largely attributable to decreases in light fraction C (Figure 2), representing 13%, 8%, and 10% reductions, respectively, of the total C recovered from density fractionation in the no inputs, no litter, and no roots treatments compared to the control treatment. Litter and root exclusion also resulted in a change in microbial community function, with a reduction in both cumulative respiration and mineralizable C (X_o; Table 1), as well as a shift in the catabolic profile for the no inputs and no roots treatments (Figure 1). This may indicate a decrease in both availability and quality of the remaining carbon as light fraction C was depleted. However, the interpretation of the light fraction C as being most vulnerable to decomposition is complicated by our finding of no decrease in the C concentration of the light fraction over the 525-day incubation, despite an overall 6–12% loss of total soil C, indicating that other pools were preferentially utilized.

The response of the mineral-associated C fractions to litter exclusion was variable, and there was some evidence for turnover, consistent with a complex and active mineral C pool (Torn et al., 2013). The occluded light fraction of the no litter, no roots, and control treatments showed a decline in C concentration during incubation, indicating that this fraction is actively turning over on a relatively rapid time scale (i.e., 525 days). There was also a small, marginally significant (Figure 2) decrease of occluded light fraction C in the no inputs treatment versus the control treatment, representing 3% of the total soil C recovered, indicating that there may be greater losses from this fraction as the DIRT manipulations at Bousson Forest continue. This is consistent with losses from the "intermediate density" C pools roughly equivalent to our occluded light fraction (i.e., not the heaviest clay and silt fraction) reported for other DIRT sites (see Lajtha, Bowden, & Nadelhoffer, 2014; Lajtha, Townsend, et al., 2014).

5.2. The Role of Roots in the Stability of Soil C Pools

Our results provide little support for the hypothesis that roots contribute disproportionately to soil C stocks (Rasse et al., 2005). There was no difference in whole-soil C losses under root versus aboveground litter exclusion or cumulative C respired (Table 1), and previous studies from the Bousson Forest reported no change in C quality under root versus aboveground litter exclusion as determined by pyrolysis (Bowden et al., 2014) and mineralization rate during incubation (Crow et al., 2009). And though Hatton et al. (2015) found that roots contributed more to the free light fraction than to the mineral-associated fractions, we found no evidence that exclusion of roots alone led to a loss of light fraction C.

Roots and their associated microbial communities drive soil aggregation and thus protection of soil C (Six et al., 2004), and there is evidence that root exclusion resulted in movement of C into the nonaggregated mineral fractions, potentially due to a decrease in aggregation. The soil C concentration of the no inputs treatment fine, heavy fraction increased versus the control treatment (Figure 2), possibly due to a decrease in soil aggregation (not measured in this study) as observed by Mayzelle et al. (2014) in the same soil, though this was not apparent in the no roots treatment. There was also an increase in the C concentration of the coarse heavy fraction in the no roots treatment, though this was not apparent in the no inputs treatment (Figure 2). Why C accumulated in the fine heavy fraction under no inputs, and in the coarse heavy fraction under no roots, while there was no response under no aboveground litter, is unclear but illustrates the potential importance of roots in determining how soil C stabilizes.

Even if roots are not preferentially stabilized, they may have a greater influence than aboveground litter over soil C decomposition dynamics and microbial catabolism. The long-term mineralization model suggests that root exclusion left a larger mineralizable pool (X_o) that decayed at a slower rate (k) than litter exclusion (Table 1, only k significant). Moreover, root exclusion caused a substantially different catabolic profile than the control, whereas the litter exclusion did not (Figure 1), indicating that the loss of roots may cause a shift in community function that is not otherwise apparent and which may have profound consequences for soil C cycling.

5.3. Litter Addition and the Stabilization of Soil C

The potential to manipulate the stable C pools to offset ongoing climate change has driven extensive debate (Dungait et al., 2012), but the evidence thus far indicates that adding C to the most stable pools would not be straightforward (Bowden et al., 2014). Our results largely support this conclusion, as increasing aboveground input quantity for 20 years had no effect on soil C quantity, as had been previously reported for this site (Bowden et al., 2014; Crow et al., 2009; Mayzelle et al., 2014). There was also no indication that C concentration increased in any density fraction (Figure 2), similar to results reported after 12 years of litter addition (Crow et al., 2009), or that the microbial community's catabolic profile was altered (Figure 1). The C content of the occluded light fraction did increase in the double litter treatment over the course of the incubation (Figure 2), but there is no clear biological explanation for this aside from a change in microbial activity within the incubation jars, which increased aggregation. As we did not measure aggregation before or after incubation, this is only speculation, but it is consistent with the understanding that microbial activity may vary with ecological context.

5.4. Investigating the Chemical Nature of a Putatively Stable Soil C Pool

The fine heavy fraction is widely hypothesized to be the oldest and most stable soil C pool (Sollins et al., 2009). Consistent with that hypothesis, we found little evidence of change in the C concentration of the fine heavy fraction over the course of the incubation or under the double litter treatment. However, we also examined the biochemical content with FTICR-MS of the fine heavy fraction before and after incubation for the no inputs, control, and double litter treatments (Figures 3 and 4 and supporting information Table 2), speculating that these extreme treatments would be mostly likely to show differences. We recognize that the scope of this investigation is limited as we only examined three treatments (no inputs, control, and double litter), one density fraction (fine heavy fraction), and used only one extractant, which removed an unquantified proportion of the organic C and preferentially extracted lipids (Tfaily et al., 2015). However, there is no reason to believe that these biomarkers should not be biased to any treatment.

Our results indicate that although this mineral-associated C fraction was stable in terms of total C concentration, it was metabolically active. The fine heavy fraction of the double litter treatment was enriched with lipids

over 20 years (Figure 3). We propose that they are likely of microbial origin, not directly translocated from either aboveground litter or roots, and deposited in the fine heavy fraction as the result of active decomposition stimulated by litter addition. The molecular nature of the lipids detected in the fine heavy fraction of the double litter treatment differed (i.e., are not shared in common) from that of the dominant leaf litter, as shown in a van Krevelen diagram (Figure 5), and though roots were detected in somewhat higher amounts under double litter (see section 2), there was no lipid enrichment in the fine heavy fraction of the control versus the no inputs treatment, leaving the addition of aboveground litter as the most probable cause.

We posit that this is a typical pathway that is amplified under an excess of aboveground litter, as the control soils also contained a lipid pool that was reduced during incubation (Figure 3). We propose that as aboveground litter decomposed in the soil, a portion of the microbial by-products was preferentially captured in the mineral fraction. However, as there was no concurrent increase in total C concentration, these lipids may have resulted in turnover of other SOM compounds by competing for adsorption sites on mineral surfaces (Kleber et al., 2007; Mitchell & Simpson, 2013).

These results support a C stabilization pathway proposed in Cotrufo et al. (2013) and subsequently tested in Cotrufo et al. (2015), in which labile inputs stimulate microbial activity and their by-products are then preferentially deposited on mineral surfaces, where SOM can reach extreme age (Kleber et al., 2011). Moreover, "stabilization" may be relative as these lipids were rapidly (compared to 20 years of input addition) depleted over the course of a 525-day incubation (Figure 3). This indicates either that this pool is constantly turning over or that the microbial community is choosing to utilize other pools of C in the presence of fresh inputs in situ. This is consistent with reports that there are multiple pools with differing turnover times within the mineral fractions (Torn et al., 2013) and that labile inputs, such as leaves, may contribute more to the mineral fractions than "lower quality" inputs such as roots (Hatton et al., 2015).

6. Conclusions

Twenty years of input exclusion revealed patterns in the turnover of decades old C that are consistent with mineral association acting as the primary soil C stabilization mechanism. Overall, shifts in the C concentration of the mineral-associated fractions were either relatively small or inconsistent with input quantity, indicating that these fractions were relatively stable in terms of C concentration on a decadal time scale. However, FTICR-MS revealed shifts in the biochemical composition of the fine heavy density fraction in response to both input exclusion and addition. Our results are consistent with reports that mineral-associated C contains an active pool (Torn et al., 2013) that may play a role in C uptake from fresh litter (Cotrufo et al., 2013, 2015). We also present evidence consistent with previously published results that the pathway from litter to soil C is complex, neither linear nor direct (Bowden et al., 2014), and may include continued biochemical transformations. Importantly, many of the trends we report here were not apparent at the scale of gross C content or operationally defined density fractions. We propose that understanding the controls over C decomposition and stabilization will require pairing traditional methods such as incubations and fractionations with observations at molecular scales. Our findings shed new light on the path that C takes from litter to soil C pools and the role of litter source and quantity in maintaining C stocks and lability.

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