DISSERTATION

MOVING BEYOND MASS LOSS: ADVANCING UNDERSTANDING ABOUT THE FATE OF DECOMPOSING LEAF LITTER AND PYROGENIC ORGANIC MATTER IN THE MINERAL SOIL

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ABSTRACT

MOVING BEYOND MASS LOSS: ADVANCING UNDERSTANDING ABOUT THE FATE OF DECOMPOSING LEAF LITTER AND PYROGENIC ORGANIC MATTER IN THE MINERAL SOIL

Leaf litter decomposition recycles the energy and nutrients fixed by plants during net primary productivity back to the soil and atmosphere from where they came. Traditionally, leaf litter decomposition studies have focused on litter mass loss rates, without consideration for where that mass ends up in the ecosystem. However, during litter decomposition by soil microbes a fraction of the litter mass lost is truly lost to the ecosystem as respired CO_2, while another fraction remains in the ecosystem stored in the soil as soil organic matter (SOM). SOM is heterogeneous in composition, with various SOM pools remaining stored in the soil for time spans ranging from days to millennia depending on their biochemical and physical properties. Pyrogenic organic matter (py-OM) is the partially combusted plant residue left behind by fires, and has been found to contribute to long term SOM pools. SOM accounts for the largest terrestrial pool of carbon (C) in the global C cycle and stores nitrogen (N) and other nutrients for plant productivity. Therefore the formation of SOM during litter decomposition is critical to terrestrial C and N cycling and its feedback to global biogeochemical cycles.

The focus of my dissertation is the study of leaf litter and py-OM decomposition, and quantitatively tracing how much decomposing litter and py-OM is used by soil microbes, how much is lost as CO_2, and how much remains in the soil and contributes to SOM formation under different conditions. In order to best address my research questions, I first studied the methods

of leaching of dissolved organic carbon (DOC) and ^{13}C and ^{15}N isotope labeling of plant material in the laboratory. Then, I conducted a laboratory incubation where I found that the amount of hot water extractable C and the lignocellulose index (Lignin/(lignin+cellulose)) can be used to predict DOM leaching, and the partitioning of C loss between DOC and CO_2 from leaves and py-OM during decomposition. I also conducted two field studies using ^{13}C and ^{15}N labeled *Andropogon gerardii* leaf litter and py-OM to trace the fate of C and N losses during their decomposition in a fire affected tallgrass prairie, and understand the role of soil microarthropods in this process. I found that soil microarthropods increase the amount of leaf litter C that contributes to stabilized SOM formation during litter decomposition, by increasing litter inputs to the soil where they can be utilized by soil microbes. Finally, I found that frequent inputs of py-OM, rather than litter, due to annual burning of the tallgrass prairie alters the SOM formation process by removing relatively labile litter inputs to the soil and replacing it with py-OM that is unusable by soil microbes.

Overall, my dissertation has focused on taking a mechanistic approach to understanding the process of litter and py-OM decomposition, and how their decomposition contributes to SOM formation and ecosystem CO_2 fluxes. My results have helped to improve our understanding of terrestrial biogeochemistry, and the processes that control SOM formation during litter decomposition.

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Chapter 1: Introduction

Litter decomposition is the process that returns the energy and nutrients fixed by plants during net primary productivity (NPP) back to the soil and atmosphere from where they came. Traditionally, litter decomposition studies have focused on litter mass loss rates, without consideration for where that mass ends up (Berg and McClaugherty 2003; Zhang et al. 2008). During litter decomposition, mass loss occurs through the processes of catabolism to CO_2, fragmentation and leaching (Swift et al. 1979). One of these processes (catabolism) results in an immediate loss of C from the terrestrial biosphere back to the atmosphere, while the other two (fragmentation and leaching) contribute to the formation of soil organic matter (SOM) (Cotrufo et al. 2009). SOM accounts for the largest terrestrial reservoir of C in the global C cycle (Schlesinger and Bernhardt 2013) and stores C for time periods ranging from days to millennia depending on its biochemical and physical properties (Trumbore and Zheng 1996; Six et al. 2002). Due to the rapid pace of human induced alterations of the global C and climate cycles (IPCC 2013), understanding the mechanisms behind how SOM is formed during litter decomposition, and what characteristics of ecosystems dictate how much C is stored as SOM with various mean residence times (MRT) in the soil, is critical to understanding the role of soils in global biogeochemical cycles.

Litter decomposition studies using stable isotopes have estimated that during the first year or two of decomposition between 20-33% of litter C mass lost during litter decomposition contributes to bulk SOM formation (Bird and Torn 2006; Rubino et al. 2010). During the initial, fast phase of litter decomposition, C and N enters the soil through leaching of dissolved organic

matter (DOM) (Klotzbücher et al. 2013). This soluble material can either sorb directly to mineral soil particles (Kalbitz et al. 2005) or be used with high efficiency by soil microbes, who deposit biochemically transformed litter derived products into the soil where they become associated with silt and clay sized minerals in the soil (Grandy and Neff 2008; Cotrufo et al. 2013). Litter fragments also enter the soil through either abiotic weathering and pedoturbation, or physical breakdown and bioturbation by soil fauna (Hattenschwiler et al. 2005). These litter fragments are readily used as a food source by soil microbes and have a shorter MRT in the soil (Trumbore and Zheng 1996), thus contributing less to soil C sequestration. Many properties of the soil structure, litter chemistry and climate control how litter is decomposed into SOM (Schmidt et al. 2011; Cotrufo et al. 2013; Zhang et al. 2008; Kogel-Knabner et al. 2008) mainly due to their influence on the soil microbial community responsible for litter decomposition into SOM (Paul 2007).

Bacteria and fungi in the soil are the main agents of litter decomposition, SOM formation and the release of CO_2 to the atmosphere from soils (Paul 2007). Their activity and community composition controls ecosystem functioning due to their variable rates of storage or release of C and nutrients in the soil (Wallenstein and Hall 2012) and is critical to understanding global soil C cycling patterns (Wieder et al. 2013). Soil mesofauna are an often overlooked group of soil organisms who may control microbial community activity and composition through top-down controls on their activity (Hattenschwiler et al. 2005) and whose incorporation into biogeochemical models could improve their predictions of C and N cycling (de Vries et al. 2013; Garcia-Palacios et al. 2013).

Grasslands cover nearly one fifth of the Earth's land surface (Grieser et al. 2006) and contain an estimated 30% of the world's total soil carbon (Anderson 1991). Fire is a common

land management strategy in grasslands (Collins and Wallace 1990), which globally accounts for a significant flux of C to the atmosphere (Hall and Scurlock 1991), and leaves behind partially combusted pyrogenic organic matter (py-OM). The removal of aboveground biomass that would otherwise contribute to SOM formation through litter decomposition, and its replacement with py-OM, which is biochemically recalcitrant (Lehmann et al. 2006; Knicker et al. 2013) and remains in the soil relatively untransformed by microbes (Kuzyakov et al. 2014; Kuzyakov et al. 2009), could thus alter SOM formation and C and N cycling in frequently burned grassland ecosystems (Knicker et al. 2012).

In order to accurately and quantitatively study the fate of decomposing litter and py-OM in the soil we first need to optimize the methods to do so. During my dissertation, I specifically tested methods of leaching and ^{13}C and ^{15}N isotope labeling of plant material. As a PhD student, my goal has been to learn how best to study these processes and which methods are best suited to answering our specific research questions.

The objective of my dissertation is to improve understanding of how decomposing plant material and py-OM contribute to SOM formation and the storage and cycling of C and N in terrestrial ecosystems, and to develop the best methods to study this. I address the following specific questions

1. How do litter chemistry, fragmentation, and laboratory methods for leaching dissolved organic matter from litter compare in terms of the quantity and quality of dissolved organic matter leached?
2. Does aboveground litter quality determine the amount of dissolved organic carbon and carbon dioxide lost during litter decomposition?

3. Can we produce ^{13}C and ^{15}N labeled plant material that is either uniformly or differentially labeled in its metabolic and structural components using a continuous isotope-labeling chamber?
4. Do soil microarthropods increase litter contributions to stabilized soil organic matter formation through top-down controls on microbial activity?
5. How does fire affect soil organic matter formation through the conversion of aboveground litter to pyrogenic organic matter as an input to soil organic matter formation?

In the following chapters I address each of these questions through laboratory studies, a greenhouse study and field studies in a tallgrass prairie ecosystem.

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Chapter 2: Quantifying and characterizing dissolved organic carbon and total dissolved nitrogen leaching from litter: a comparison of methods across litter types[1]

Introduction

Aboveground plant litter decomposition is one of the main processes by which organic carbon (C) and nitrogen (N) from plant biomass enter the soil, where they can be stored long term as soil organic matter or decomposed completely back to their mineral forms. Litter decomposition occurs through: i) catabolism of litter C and N to CO_2 and NH_4^+, ii) litter fragmentation, when litter is shredded into smaller pieces, which then are moved down the soil by bio- or pedo-turbation, and iii) leaching of soluble litter and microbial components to the soil with water (Swift 1979). The leaching of dissolved organic matter (DOM) from leaf litter during precipitation events plays a significant role in terrestrial biogeochemistry (Neff and Asner 2001). Estimates of litter dissolved organic carbon (DOC) leaching range from 6% to 39% of litter C losses during decomposition (Qualls and Haines 1991; Magill and Aber 2000; Don and Kalbitz 2005). Most of the leaf litter soluble components are leached out soon after abscission, with anywhere from 0.08-2.11% of initial dry biomass lost in a single leaching event (Cleveland et al. 2004). Thus, the initial fast phase of litter decomposition is likely dominated by leaching losses (Gimenes et al. 2013; Magill and Aber 2000; Cheever et al. 2013).

The fate of this leachate depends on the quantity and chemical composition of the DOM, as well as on the soil matrix (Cotrufo et al. 2013). Litter quality is often used as a predictor of decomposability (Aber et al. 1990; Adair et al. 2008), and could control DOM leaching and

[1] In Revision in *Plant and Soil* with F.J. Calderon, J. Betzen and M.F. Cotrufo. Submitted January 2014

composition. Leaves from different plant species have been found to leach DOM in different amounts and with different DOM chemical quality for biogeochemical reactivity in the soil (Wieder et al. 2008; Cuss and Gueguen 2013; Cotrufo et al. 2013). The functional group chemistry of DOM can be used to estimate potential sorption to soil minerals, with carboxyl and phenolic functional groups exhibiting high sorption capacity and polysaccharide groups exhibiting low sorption capacity (Oren and Chefetz 2012). Temperature, UV exposure and nitrogen inputs also affect the concentration and characteristics of DOM leaching (Fellman et al. 2013; Dieter et al. 2013; Cheever et al. 2013; Lu et al. 2013).

While the importance of DOM leaching is becoming more widely recognized, we still lack a thorough evaluation of the methods used to quantify it. We believe that, in order to better understand the controlling factors of litter DOM leaching, it is necessary to know how the methods used to leach DOM from litter affect the quantification and characterization of leachate across litter species. Various litter leaching methods have been used and are currently published, but thus far none have been directly compared in the literature. In order to advance the study of DOM leaching from litter and to compare results across studies it is important to understand whether leaching methods affect DOM quantity and composition. Most commonly, litter leachate collection occurs in the laboratory due to the challenges of *in situ* field collection and the rapid decomposition of DOM generated in the litter layer (Cleveland et al. 2004; Corrigan and Oelbermann 2013). In the laboratory, DOM can be leached from litter by (1) soaking of cut (e.g., (Cleveland et al. 2004; Magill and Aber 2000) and whole (Don and Kalbitz 2005; Nykvist 1962) leaves in water, (2) gentle shaking of litter in water (e.g., Wallenstein et al. 2010; Bowen et al. 2009; Fellman et al. 2013) , or (3) dripping water over litter samples, in an attempt to better simulate leaching during a precipitation event (e.g., Hansson et al. 2010) . Hot water can be used

to extract more soluble components from organic matter, but these extracts are not directly comparable to DOM leaching and therefore are not considered in this study (Landgraf et al. 2006; Nkhili et al. 2012).

Soaking, shaking and dripping water over leaves to leach DOM may affect not only the quantity of DOM leached, but also the components of litter released as DOM. The amount of time that the litter is in contact with the water, the abrasiveness of that contact as well as the litter-to-water ratio could affect the DOM concentration and its suitability for chemical charactarization. Additionally, whether whole (Don and Kalbitz 2005) or cut (Magill and Aber 2000; Cleveland et al. 2004) leaves are used for leaching may affect what components are released into the water for some types of litter due to the increased surface area available for leaching (Nykvist 1962). The leaching method of choice in any one study may reflect either the need for overall high DOM concentrations for spectral or other concentration dependent analyses, or the characterization of DOM to mimic field conditions and DOM-soil interactions. An understanding of how the methods of DOM leaching in the laboratory affects the concentration and composition of DOM leachate will help in cross comparisons of litter leachate studies.

In this study, we quantify and characterize DOM leached by different methods. DOM quantity is measured as DOC and total dissolved nitrogen (TDN) concentration in the leachate, while composition is assessed based on the functional groups of the compounds leached using fourier transformed infrared (FTIR) analysis. FTIR is a sensitive and inexpensive method for analyzing the chemistry of organic matter, and has been used successfully to study the functional group distribution in decomposing litter (Gallo et al. 2005), and extensively on freeze-dried aqueous extracts of soils and litters (He et al. 2011a; He et al. 2011b; He et al. 2012; Kaiser and

Ellerbrock 2005; Kaiser et al. 2007; Peltre et al. 2011). FTIR is particularly useful to gain information regarding the aromaticity of DOM (Strobel et al. 2001), or in detecting spectral features in the extracts such as 3400 cm^{-1} O–H/N–H stretching, 2936 cm^{-1} aliphatic C–H stretching, the 1605 cm^{-1} band for aromatic C=C vibrations, COO− stretching, and/or H-bonded C=O in conjugated ketones, and the peak at 1070 cm^{-1} for polysaccharide-like absorbance (He et al. 2009). Water extracts are thought to contain the labile organics from environmental samples, so the FTIR data from this fraction has been used to observe changes in aliphatic and proteinaceous functional groups during composting (He et al. 2011a). FTIR analysis thus provides an informative characterization of the composition and reactivity of litter leachate, and can improve our understanding of how the leaching method, cutting of litter and litter species affects the functional composition of DOM leached.

My main research question was: how do the leaching method, cutting and litter quality affect DOM leaching quantity and composition across a range of litter types? We tested four leaching methods, on both cut or whole litter samples, from five plant species ranging in C:N ratios. By comparing dripping water over litter *vs*. soaking litter in water, we test the effect of time of water and litter contact. By comparing soaking litter in water *vs*. shaking litter in water, we test the effect of abrasion. We test the effect of litter-to-water ratio by shaking the litter in two different volumes of water. Finally, we test the effect of cutting by leaching whole and cut leaves in three of the four methods. We hypothesize that, 1) the leaching method and cutting of litter affects the amount of DOM leached from the litter, with shaking and cutting dissolving more components than dripping and whole leaf leaching, 2) litter with a higher C:N ratio will leach less DOM overall but contain more C-rich compounds, and 3) the leaching methods with

better extraction efficiency will result in a more concentrated extract that in turn will result in FTIR spectra with better band definition and spectral quality.

Methods

Litter samples

For this study we used five litter types: alfalfa (*Medicago sativa)*, ash (*Fraxinus excelsior)*, big bluestem grass *(Andropogon gerardii)*, oak (*Quercus macrocarpa)* and pine (*Pinus ponderosa)*, representing a range of litter quality in terms of %N, C:N ratios and % lignin (Table 1). We collected the leaf and needle litters in the fall of 2011 as freshly senesced litter that had not hit the ground and had not been rained on since abscission. However, mature alfalfa leaves were standing and still green when collected. Abscised ash leaves and pine needles were collected from separate raised litter traps. Senesced, standing big bluestem was hand cut from a native tallgrass prairie. Senesced oak leaves were shaken off of a tree and collected in a litter trap. We removed all stems and petioles from the litter. We pooled the litter samples by species and air-dried them. We ground three subsamples from each litter pool for elemental analysis as described below.

Leaching methods

We tested four leaching methods, each on four replicate sub-samples of the air-dried litter samples, for all litter types, with either whole or cut litter. All cut litter samples were cut into 1 cm x 1 cm pieces, or 1 cm lengths (pine needles and bluestem grass blades), and homogenized. A blank, with no litter, was also added to each method to account for any background C and N on our equipment. For the ‘Soak’ method, 1 g of cut or whole litter was soaked in 70 ml of

deionized water in an acid washed 250 ml beaker for one hour at 4°C. For the 'Shake 70 ml' and 'Shake 30 ml' methods, 1 g of cut or whole litter was placed in an acid washed 250 ml beaker with 30 or 70 ml deionized water and shaken for one hour on an orbital shaker at 1 rpm. The DOM from the Soak, Shake 70 ml and Shake 30 ml methods were collected by filtering the samples over a 20 µm ash free (Whatman #41) filter and freezing it at -5°C. For the 'Drip' method, 1 g of cut or whole litter was placed on an acid washed funnel fitted with a 20 µm ash free (Whatman #41) filter and 70 ml of deionized water was slowly dripped evenly over the entire sample at a rate of 23 ml/minute. The DOM from the drip method was also frozen at -5°C until further analysis. In these four methods, we tested how the length of time of litter and water contact (Drip *vs.* Soak), the abrasiveness of contact (Soak *vs.* Shake) and the litter to water ratio (Shake 30 ml *vs.* Shake 70 ml) affected DOM leaching. Additionally for the Soak, Shake 70 ml and Drip methods we compared DOM leaching of both whole and cut litters for all litter species, except alfalfa, whose leaves are already approximately 1 cm x 1 cm. Only cut (other than whole alfalfa leaves) litter was used in the Shake 30 ml method because this volume of water was not great enough to cover the entire whole leaf samples.

Chemical analysis

We analyzed all initial litter samples for %C and %N on a solid-state elemental analyzer (LECO Tru-SPEC, St. Joseph, MI). We measured % lignin content of the litters as the mass that was resistant to digestion in 73% sulfuric acid, according to the Vansoest and Wine (1968) acid detergent fiber digestion method. All leachate samples were thawed and analyzed on a Shimadzu TOC analyzer (Shimadzu TOC 5000) for DOC and TDN. Leachates were prepared for Mid-infrared spectroscopy (FTIR) analysis by adding 0.5 g KBr to 250 microliters of

leachate, then freeze-drying the mixture. The dried leachates, in KBr, were scanned using a Digilab FTS 7000 spectrometer (Varian, Inc., Palo Alto, CA) with a Pike AutoDIFF sampler (Pike Technologies, Madison, WI). The scans were done on the mid-infrared (FTIR) from 4000 to 400 cm^{-1}, 4 cm^{-1} resolution, and each spectrum was the result of 64 co-added scans.

Data analysis

We tested the effect of leaching method, cutting and litter type on the concentration of C (DOC) and N (TDN) in the leachate by means of a generalized linear mixed model. We included leaching method, cutting, litter type and all interactions as categorical fixed effects. Due to the large variation in DOC and TDN concentrations between litter types and leaching methods, we applied a log-transformation to the data to homogenize variance and make pairwise comparisons. We checked for normality of the data and applied the Tukey-Kramer method for multiple comparisons of pairwise differences. In all cases, we used Type III tests of fixed effects. A direct comparison of Drip *vs.* Soak, Soak *vs.* Shake 70 ml, and Shake 70 ml *vs.* Shake 30 ml methods was done using a paired t-test by pairwise comparison of the methods within each litter type and cutting status. We carried out all the above analyses using SAS® software version 9.3.

We used GRAMS version 9.1 software with the GRAMS IQ package (Thermo Fisher, Woburn, MA) to perform the spectral averaging and Principal Components Analysis (PCA). We centered all means before the PCA analyses. The Pearson correlation coefficient (R) between the spectral data and total C and N was obtained using GRAMS IQ. We tested the effect of leaching method, cutting and litter type on the relative contribution of different FTIR peaks by examining the ratios of bands at 3350, 2920, 1605 and 1070 cm^{-1}. We included all combinations of these ratios to determine overall DOM chemistry differences by means of a multivariate generalized

linear mixed model. We used the Wilks' Lambda multivariate measures and Tukey's HSD post-hoc testing for pairwise comparisons. The data met our tests for homogeneity of variance.

Results

Method effects on DOM leaching from litter

The leaching method, cutting and litter type all had significant main effects and significant interactive effects on DOC and TDN concentrations in the leachate (Figure 1, Table 2). The leaching method affected the total concentration of DOC and TDN obtained from all of the different litter types (Figure 1): the Shake 70 ml method resulted in the highest DOC and TDN leaching across all litter types, followed by the Soak, Shake 30 ml, and Drip treatments. The Drip treatment leached significantly less DOM than the other methods, across all litter types.

Paired t-tests show the differences in the DOC and TDN leached according to litter type and cutting status (Table 3). We tested the effect of time of water contact in the Drip *vs.* Soak comparison. Soaking the litter for one hour in water leached significantly more DOM from all of the litter types than dripping the same amount of water over the litter ($p < 0.0001$), indicating that the amount of time the litter is in contact with the water does affect the quantity of DOM leached. We tested the abrasiveness of water contact in the Soak *vs.* Shake 70 ml comparison. The Shake 70 ml method had significantly higher DOC values ($p = 0.0046$) but not significantly different TDN values ($p = 0.0963$). This indicates that abrasion had a stronger effect on DOC leaching than TDN leaching, likely due to the higher amount of C leached. We tested the effect of the water to litter ratio in the Shake 70 ml *vs.* Shake 30 ml comparison. The Shake 70 ml method yielded significantly more DOC and TDN from the cut litter than the Shake 30 ml method ($p < 0.0001$), demonstrating that we leached more DOM with more water. However, the

Shake 30 ml treatment, with a higher litter to water ratio, produced a more concentrated leachate solution, which was better for a clear FTIR analysis (Figure 2).

The average spectra for the uncut samples from the four leaching methods are shown in Figure 2. The 30 mL shake had higher quality spectra than the rest of the leaching methods, while the Drip treatment had less spectral quality in agreement with its low extraction capacity (Figures 1 and 2). The spectra from the Drip treatment show that overall absorbance is low, with relatively little spectral information present in the extracts from all litter species. The 30 ml shake had pronounced spectral features at 3320-3120 2950-2870, 1600, and 1070 cm^{-1} bands (Figure 2). These bands are assigned to polysaccharides and cellulose-like compounds (~1070 cm^{-1}), amide C=O stretch, aromatic C=C stretch, carboxylate C-O stretch and/or conjugated ketone C=O stretch (~1605 cm^{-1}), aliphatic C-H stretch (~2950-2870 cm^{-1}), and OH/NH stretch (~3320 cm^{-1}) (Stewart 1996; Socrates 1994). The alfalfa extract, with its higher C and N leaching (Figure 1) produced better quality spectra with the Soak and 70 mL shake methods relative to the other species. The Drip spectra do not resolve the alfalfa leachate from the ash and pine leachates (Figure 2), even though the alfalfa leachate had much higher DOM (Figure 1).

PCA analysis was used as a dimension reduction technique to identify the spectral differences between the extraction methods (Figure 3a) and the litter species (Figure 3b). Litter species was the main source of variation between the spectral data, with extraction method having a secondary influence. Component 1 explains 85.2 % of the variation in the spectral data between the samples and shows the differences between the Alfalfa and the rest of the plant species. Component 2 explains 10.7 % of the variation and helps to discern between the Drip, and the rest of the extraction procedures (Figure 3c). The Shake 30 ml and 70 ml treatments have a tendency for low component 2 scores, and are mostly separated from the Drip treatment.

Loadings indicate that this is due to higher absorbance in the Shake 30 ml and 70 ml treatments at 1067, 1607, 2950-2870, and 3320 cm^{-1} (Figure 2). The highest combined component 1 and component 2 scores fall in the Drip treatment (Figure 3a), and loading values (Figure 3c) are consistent with reduced organic absorbance bands due to the lower concentration of DOM in the Drip leachates (Figure 1). The DOM FTIR band ratios of 3350:2920, 3350:1070, 2920:1070, 2920:1605, 1605:1070, 3350:1605 cm^{-1} showed no significant multivariate or pairwise differences between leaching method or cutting ($p > 0.1$).

Cutting effects on DOM leaching from litter

Cutting has a significant main effect on DOC and TDN concentrations in the leachate (Table 2, Figure 1). In general, cutting of the litter increased DOC and TDN concentrations, except in bluestem, which saw no effect of cutting on DOM concentrations. A comparison of the cut and uncut Shake 70 ml extracts showed that the cut extracts had increased absorbance at the 3320, 1605, and 1070 cm^{-1} spectral bands (data not shown).

Litter species effects on DOM leaching

Litter type had the most significant effect on DOC and TDN concentrations in the leachate (Table 2), and generally reflects the C:N of the source litters, with litter with low C:N (e.g., alfalfa) having higher DOC and TDN concentrations overall than litters with higher C:N (Table 1, Figure 1). The % lignin contents of the litters did not correlate with DOC or TDN of the leachate from any of the methods ($R^2 < 0.1$). PCA analysis of the FTIR spectra according to plant species separates the alfalfa spectra from the rest of the species (Figure 3b), in agreement with the DOC and TDN data (Figure 1). Species treatment averages from the Shake 30 ml

treatment (Figure 2) confirm the PCA results (Figure 3b and c), showing that the organic spectral bands at 3320, 1605, and 1070 cm^{-1} are strongest in the alfalfa DOM, and least in the Pine. The DOM FTIR band ratios of 3350:2920, 3350:1070, 2920:1070, 1605:1070, 3350:1605 cm^{-1} showed no significant multivariate or pairwise differences between litter types ($p > 0.1$)

Correlation of DOC and TDN concentration and mid infrared spectral data

We determined the correlation coefficients across the mid infrared spectral range for the C and N concentration of the DOM from the Shake 70 ml treatment (Figure 4). The Shake 70 ml method was chosen for this analysis given that it has relatively high extraction efficiency (Figure 1), as well as a full complement of cut and whole samples resulting in a higher number of available data points to build a correlation compared to the Shake 30 ml method. The bands that correlated better with the C and N include all the main bands in the litter spectra, namely 3330, 2900, 1600, and 1067 cm^{-1}. Absorbance at 3010 and 3110 cm^{-1} form inverted peaks in the correlation spectra. These bands fall within the aromatic C-H stretch region and our analysis indicates that they are less related to C and N extraction than the four main spectral bands.

Discussion

Leaching method and cutting

Our results demonstrate the importance of considering the leaching method, cutting of the litter, and litter species when interpreting the results of laboratory litter leaching studies. Consistent with our first hypothesis, our results show that the leaching method of choice significantly affects the amount of DOM released when leaching plant litter (Table 2). Since soaking, shaking and dripping have all been used to attempt to quantify DOM availability, but

none actually measures DOM leaching *in situ*, estimates of DOM leaching using these methods in the laboratory must be considered in the context of how much DOM each method leaches. For example, the relatively high DOC concentrations reported by Don and Kalbitz (2005) and Wieder et al. (2008) were obtained from litter soaked for 24 hours, rather than the one hour soaking used in this study and by Cleveland et al. (2004). Although this length of time was not tested in our experiment, we did find time of litter and water contact to have a statistically significant impact on the amount of DOM leached, and this could help to explain differences in DOM availability across these studies. Magill and Aber (2000) report relatively low DOM concentrations in their leaching study, but they also were attempting to simulate rainfall by dripping water through their litter samples. Our results show that the difference between their DOM quantities and others can be mechanistically explained by the difference in leaching methods applied. Additionally, the litter to water ratio differs across this study and others, and must be taken into consideration when comparing DOM estimates across studies.

Also confirming our first hypothesis, cutting of the litter had a confounding effect on the amount of DOM leached by each method (Table 2). Cutting of the litter likely increases DOM leaching due to the increased surface area available for water-litter contact when the litter is cut. Cutting may not have affected the bluestem grass blades as strongly as the other litter species because they are long and thin compared to the broad leaves of oak and ash, so cutting may have a weaker effect on increasing surface area on grasses. In their study, Don and Kalbitz (2005) report that sycamore maple (*Acer pseudoplatanus* L.) had the highest DOC leachate concentration out of the five species in their study, however these were also the only leaves that were cut prior to leaching while all of the other litter species were leached whole. Although litter species differ in their DOM availability (see below), our results demonstrate that the

comparability of DOM availability across litter types depends on whether whole or cut leaves are leached. Our results suggest that DOM extractions of cut and uncut litters should not be directly compared. However, cutting of the litter provided a more concentrated solution for spectral analysis.

Confirming hypothesis 3, our results indicate that studies focused on optimizing spectral resolution by employing a leaching strategy, such as using cutting and shaking, do not misrepresent DOM characterizations as compared to less aggressive techniques such as dripping or soaking (Fellman et al. 2013; Wallenstein et al. 2010). The Drip method produced a relatively dilute leachate that results in limited resolution of organic spectral bands. The Drip treatment, however, had the advantage that it mimics field conditions in which rainfall drips through the litter layer and mobilizes the DOM into the soil. The methods that include litter-water contact for one-hour (Soak, Shake 70 ml and Shake 30 ml) result in spectra with more defined spectral bands that can be related to important quality components. Regardless, the resulting spectra are relatively simple with only 4 major spectral features. These results confirm those of Gressel et al. (1995) who found that infrared spectra of pine litter leachate had the most defined peaks at 3350, 1610, 1410, and 1070 cm^{-1}. The relatively simplified nature of the FTIR characterization of the leachate is a result of the leaching process, which fractionates the complex leaf litter chemistry into the limited set of soluble components.

The FTIR spectra from litter leachate in a similar study (He et al. 2009) show similarities to those in ours, with five main spectral features: 3400, ~2936, 1605, 1400, and 1074 cm^{-1}. These bands are representative of the soluble components of plant materials that are active in the mid-infrared region including O–H/N–H bonds, aliphatic C–H, aromatic C=C, and polysaccharides. In addition, other minor spectral features at 1260 cm^{-1} (C-O stretching) and 1678 cm^{-1}

(aromatics) showed up as shoulders of the more prominent spectral bands. Across all methods, our FTIR analysis shows higher absorbance at four major bands across most of the leachate samples at 3320 (OH/NH stretch), 2950-2870 (aliphatic C-H stretch), 1605 (amide C=O stretch, aromatic C=C stretch, carboxylate C-O stretch and/or conjugated ketone C=O stretch), and 1070 cm^{-1} (cellulose-like compounds) (Stewart 1996; Socrates 1994). A previous study has related absorbance near 1070, 1605, and 2950-2870 cm^{-1} to changes in litter quality in decomposing northern deciduous forest litter under different N levels (Gallo et al. 2005). The peak at 1400 cm^{-1} can be explained by several functional groups including CH_3 bending modes of methyl groups, stretching C-N, deformation N-H, and deformation C-H (Movasaghi et al. 2008). This band at 1400 cm^{-1} has also been observed in the aqueous extracts of pine litter and assigned to COO- stretching, aliphatic CH_2 and CH_3 deformation, and C-O stretching of phenolic OH (Gressel et al. 1995). However this peak also appears in the spectra from our blanks, indicating that it is partly an artifactual peak and not entirely of litter origin.

FTIR band ratios can be used as a semi-quantitative indicator of differences in organic matter quality based on different proportional contributions of the various functional groups to the leachate chemistry (Calderon et al. 2006; Gressel et al. 1995). Although the same major bands were found in all of the DOM samples, we wanted to know whether the methods and cutting leached out different relative amounts of each functional group due to the mechanisms of shaking, soaking, dripping and cutting. In examining all of the band ratios between peaks 3350, 2950-2870, 1605 and 1070 cm^{-1}, we did not find any statistically significant differences between the leaching methods or cutting. This further demonstrates that although the leaching methods and cutting are mechanistically different in how much DOM they leach from litter, they do not differ in the functional group chemistry of leachate.

Litter quality and DOM composition

In this study, the C:N of the source litter was a strong inverse predictor of DOM availability ($p < 0.001$), as we predicted in our second hypothesis. However, in another study by Cleveland et al. (2004), C:N of the litter did not predict DOC and TDN leaching. These confounding results indicate that the predictability of DOM leaching across different litter species may be controlled by something more complex than C to N stoichiometry, such as litter structural composition. Lignin content has been shown be an important regulator of DOM leaching from litter in previous studies (Kalbitz et al. 2006; Klotzbucher et al. 2011), but we did not find any correlation between % lignin content and DOC or TDN leaching from any of the methods ($R^2 < 0.1$). However, the objective of our study was to compare the DOM leaching from fresh litters whereas lignin has been shown to be more important for controlling DOM leaching during the later phases of decomposition (Klotzbucher et al. 2011).

PCA analysis of the FTIR spectra according to plant species separates the alfalfa spectra from the rest of the species (Figure 3b). This separation between litter species is mainly due to the strengths of the organic spectral bands at 3320, 1605, and 1070 cm^{-1}, which are strongest in the alfalfa DOM, and least in the Pine. However, Figure 2 shows that the same four bands, namely 3330, 2900, 1600, and 1067 cm^{-1}, appear across all litter species and leaching methods, but are subdued in the Drip treatment. These bands should be regarded as the main features of soluble organics in litter. C and N followed very similar patterns of R scores, which reached 0.84 for TDN at 1600 cm^{-1} (Figure 4). The higher R score for TDN at 1600 cm^{-1} suggests that this peak might be due in part to amide I absorbance caused by proteinaceous material in the DOM extract. This band has been partly attributed to amide I in studies involving forest litter extracts (Gressel et al. 1995). In contrast, correlation at 3330 cm^{-1} was higher for the DOC than

the TDN, indicating that this band could be proportionately more due to phenolic OH than to NH. Absorbance in the OH/NH region between 3690-3300 cm^{-1} is a common feature of DOM extracts and the light fraction of soil (Gressel et al. 1995; Strobel et al. 2001; Gallo et al. 2005; Calderon et al. 2011). Our correlation analysis suggests that the ratio of 3300/1600 cm^{-1} in litter leachate can be a good predictor of DOM C:N. This could be helpful in studies relating litter quality to decomposition dynamics and C incorporation into SOM.

The best correlations include the major peaks in the spectra of the litter leachate (Figure 2), suggesting that all spectral features in the leachate are of organic origin. Previous studies of DOM leachate have identified silicate bands, possibly because of the presence of soil minerals in the leachate (Gallo et al., 2005). In our study however, efforts were taken to avoid soil contamination in the litters before the assay. There was a relatively high R at 2900 cm^{-1} (aliphatic CH; Figure 4) even though the Shake 70 ml data (Figure 2) shows that this is not a particularly strong band in the average spectra. The peak near 1400 cm^{-1} present in the DOM spectrum of the Shake 30 ml treatment forms a negative peak in the correlation spectrum, further supporting that this may be a band unrelated to the extractable organics in litter, and that it is instead a product of the extraction or sample preparation.

The five different litter types ranged in litter quality in terms of % N and % lignin content. However, the band ratios between peaks 3350, 2950-2870, 1605 and 1070 cm^{-1}, were not significantly different between litter types. This demonstrates that the soluble components of fresh litters contribute the same relative amounts to DOM across a broad range of litter types. The ratios of these different functional groups are likely to change with decomposition stage (Calderon et al. 2006). However, our results help to confirm the fact that differences in litter-to-soil DOM inputs between plant species is more dependent on differences in the quantity of DOM

inputs rather than differences in composition or quality (Strobel et al. 2001). The identification of this rather simplified DOM functional group chemical composition can thus be used to further understand how the initial flush of DOM from fresh litter to the soil may interact with the mineral soil and soil microbes to form SOM.

Conclusions

We found that the litter to water ratio, the time of contact between the litter and water, and to a lesser extent the abrasiveness of litter and water contact all had statistically significant effects on the amount of DOM leached from the five litter types tested. Additionally, cutting and litter species also had statistically significant main and interactive effects on the amount, but not the composition, of DOM leached from fresh litter. These results provide a mechanistic explanation for why leaching method, cutting of the litter, and litter species must be taken into consideration when comparing estimates of DOM availability within and across laboratory studies. The relationships between DOM availability, lignin content and litter C:N found here may not always apply at all stages of litter decomposition, and is an important area for future research. Furthermore, FTIR spectroscopy revealed that the same four components were leached out across all methods and litter types, but the amount of the components depends on litter species and leaching method. Based on this and other studies it becomes apparent that the functional groups associated with wn's 3330, 2900, 1600 and 1067 cm^{-1} are the main soluble features of undecomposed litters across a range of litter types.

Table 2.1. Initial litter carbon, nitrogen and lignin concentrations. Values are means of three laboratory replicates, with standard errors in parentheses.

Litter Type	%C	%N	C:N	% Lignin
Alfalfa (*Medicago sativa*)	44.1 (0.026)	4.09 (0.012)	10.8 (0.035)	5.63 (0.345)
Ash (*Fraxinus excelsior)*	46.5 (0.608)	0.884 (0.012)	52.6 (0.139)	10.03 (0.109)
Bluestem *(Andropogon gerardii)*	44.1 (0.049)	0.478 (0.003)	92.2 (0.637)	8.42 (0.522)
Oak (*Quercus macrocarpa)*	47.6 (0.038)	1.32 (0.010)	34.0 (0.286)	18.80 (0.219)
Pine (*Pinus ponderosa)*	52.3 (0.085)	0.413 (0.007)	126.9 (2.120)	24.39 (0.302)

Table 2.2. Results of the generalized linear mixed models of the effects of litter type, cutting, leaching method, and all their interactions on dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) concentrations in the leachate.

Effect	d.f.	DOC		TDN	
		F-value	p-value	F-value	p-value
Litter	4	110.88	<0.001	364.27	<0.001
Cutting	1	47.48	<0.001	37.36	<0.001
Method	3	272.01	<0.001	250.71	<0.001
Litter x Cutting	3	15.84	<0.001	13.54	<0.001
Litter x Method	11	5.00	<0.001	11.25	<0.001
Cutting x Method	2	4.66	0.0118	8.99	0.0003
Litter x Cutting x Method	6	2.93	0.0114	6.30	<0.001

Table 2.3. Results from the paired t-tests of leaching methods on dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) concentrations.

Effect	d.f.	DOC		TDN	
		t-value	p-value	t-value	p-value
Drip x Soak	35	-4.91	<0.0001	-2.75	0.0094
Soak x Shake 70 ml	35	-3.03	0.0046	-1.71	0.0963
Shake 70 ml x Shake 30 ml	19	16.01	<0.0001	19.62	<0.0001

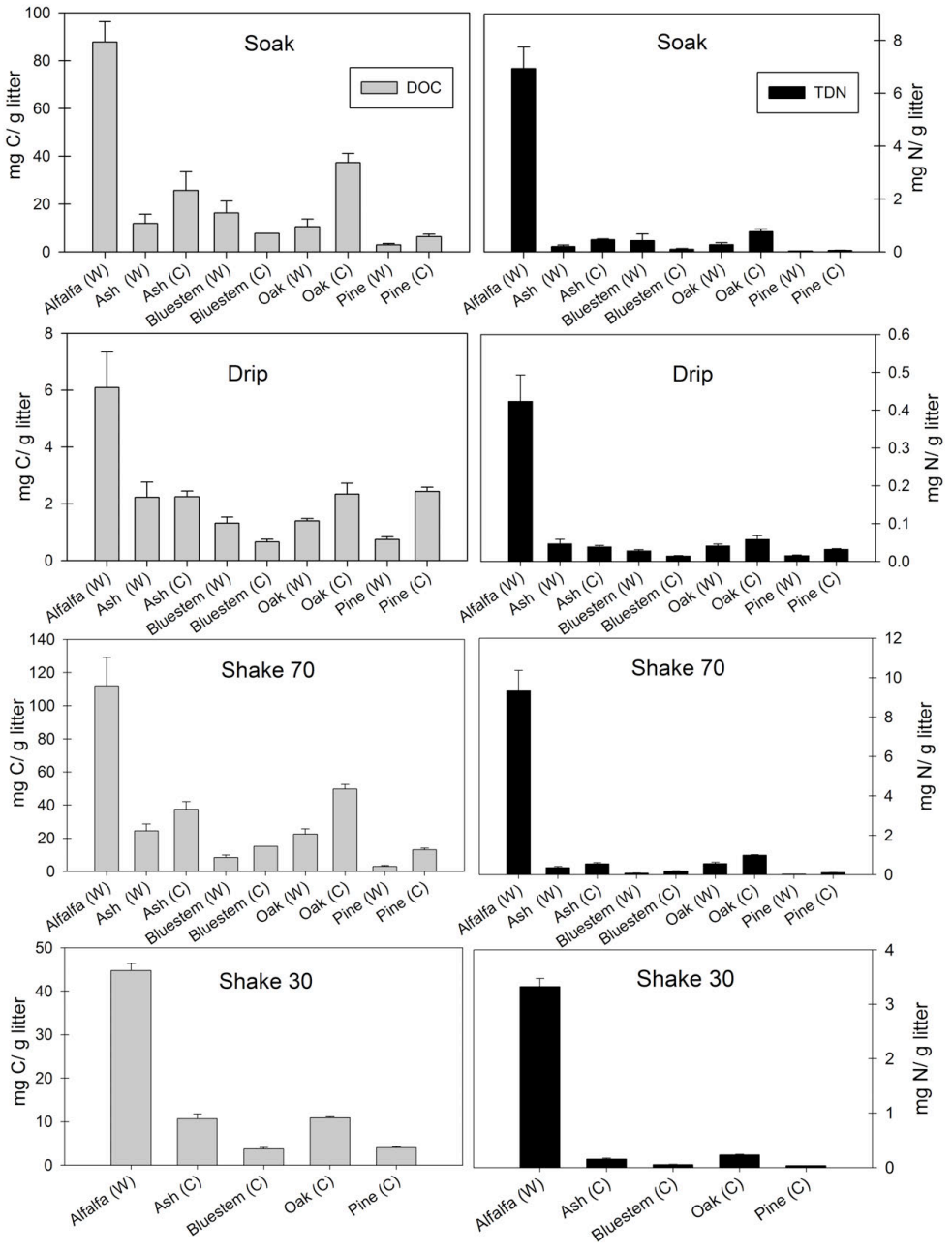

Figure 2.1. Dissolved organic carbon (DOC, left axis) and total dissolved nitrogen (TDN, right axis) in leachates from the Soak, Shake 70 ml, Drip, and Shake 30 ml leaching methods, for the five plant litter species. (W) indicates whole leaf treatments and (C) indicates cut leaf treatments. Error bars are standard error (n=4). Only cut leaves were used in the Shake 30 ml method, due to the fact that the 30 ml volume of water did not cover the entire whole leaf samples.

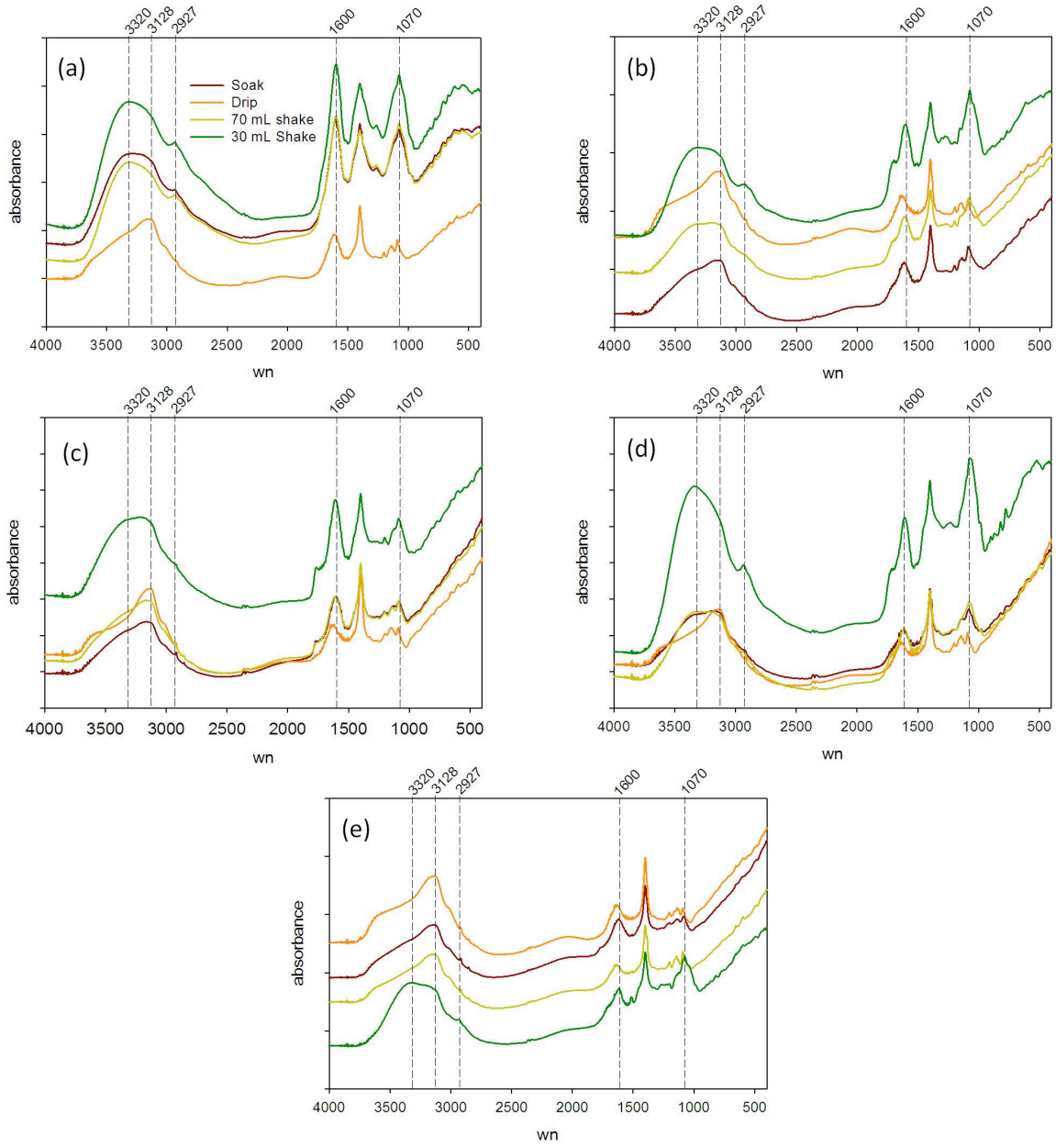

Figure 2.2. Spectral averages of the four dissolved organic matter (DOM) leaching methods for each plant litter species a) alfalfa, b) ash, c) bluestem, d) oak, and e) pine. Whole samples only, no controls. Only the uncut samples were used for the averages. Note that absorbance is unit-less, and spectra were stacked when necessary to improve the visualization of the spectral differences between the averages.

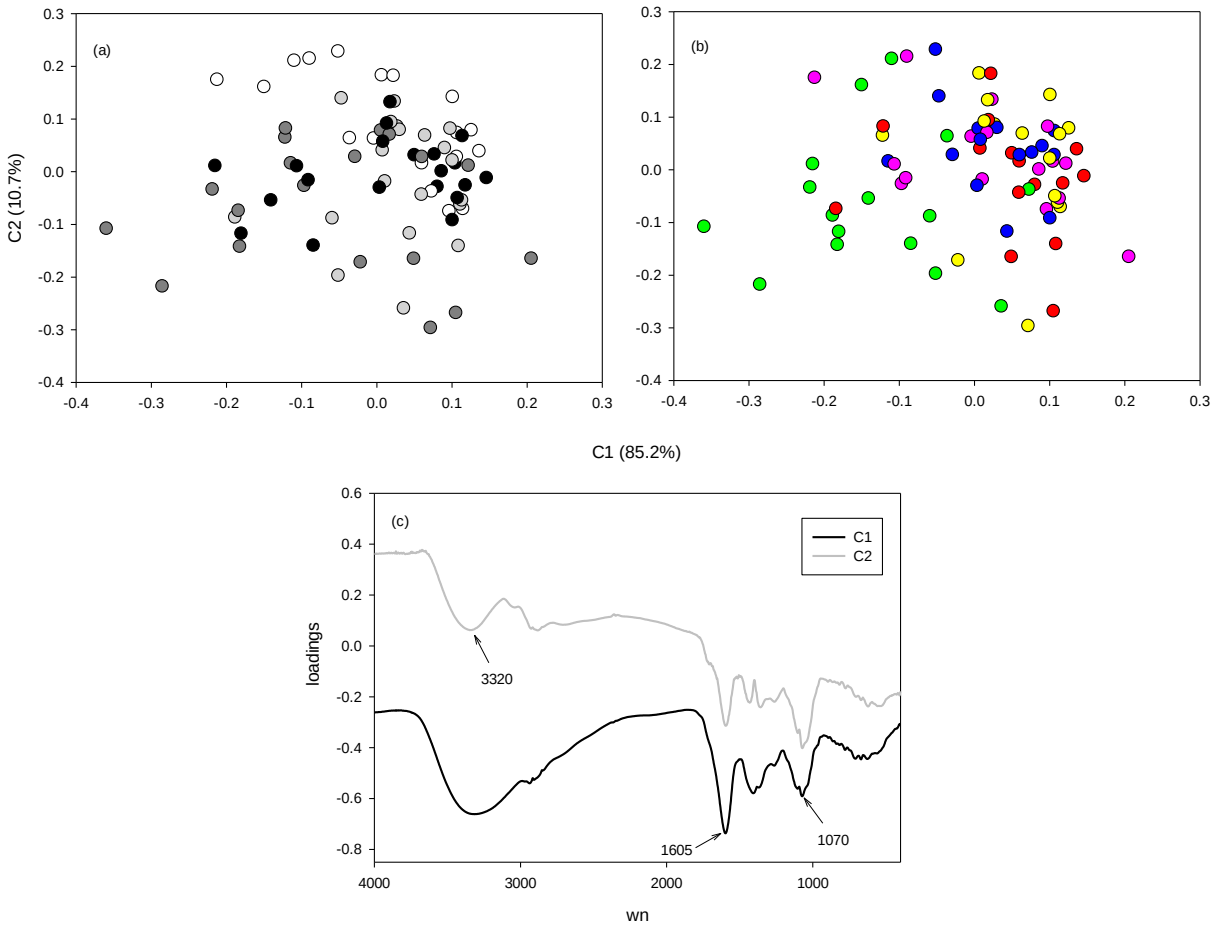

Figure 2.3. Principal Component Analyses (PCA) of the FTIR spectral data from the litter leachates, only whole litter were included, no controls. Data are the same for the two upper panels: (a) Coded by extraction method, white is Drip, light gray is Shake 70 ml, black is Soak, and dark gray is Shake 30 ml. (b) Coded by plant species, yellow is oak, red is ash, pink is pine, blue is bluestem and green is alfalfa. Panel (c) reports component loadings for the PCA.

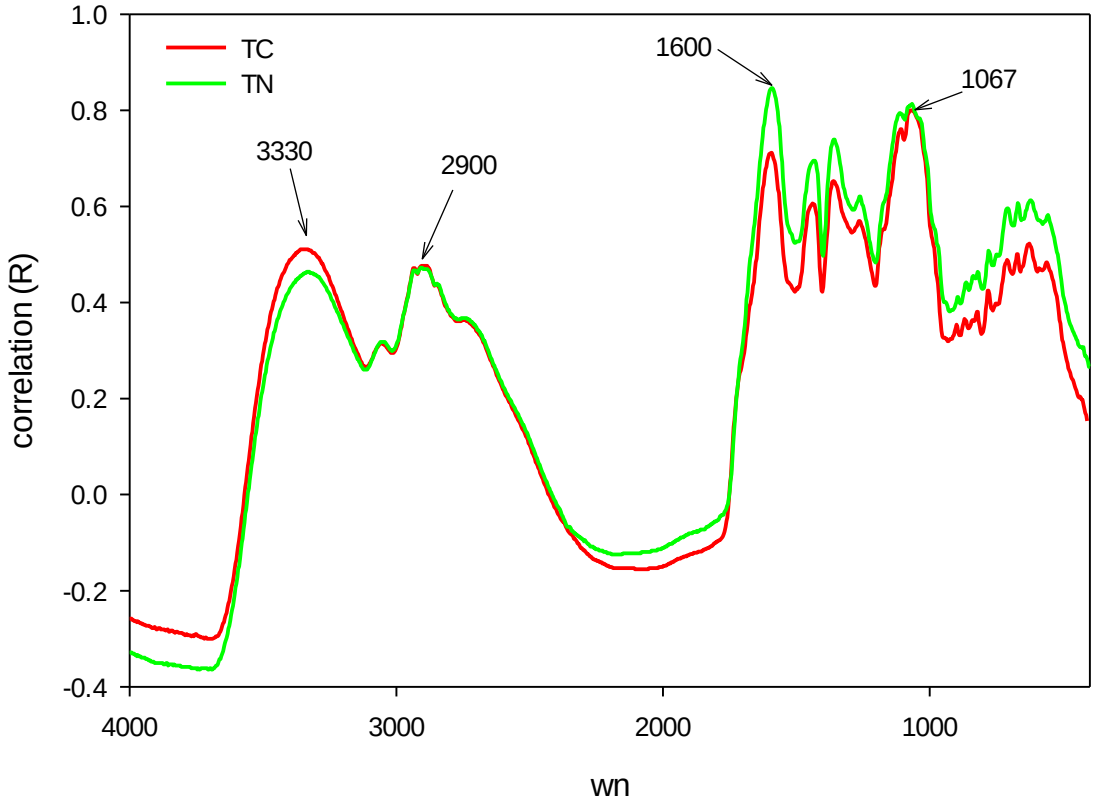

Figure 2.4. Correlation coefficients for the FTIR absorbance data and total C and N in the DOM of the Shake 70 ml treatment.

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Chapter 3: Chemical traits of fresh or pyrolyzed aboveground plant inputs to soil determine the amount and quality of dissolved organic matter leaching during decomposition[2]

Introduction

Aboveground plant litter decomposition is a key component of carbon (C) and nutrient cycling in terrestrial ecosystems, generating products such as CO_2 and microbially processed or partially decomposed organic matter (OM) that connects aboveground plant production to both the atmosphere and soils. Traditionally, studies of litter decomposition have focused on measurements of litter mass loss rates using litter bags (Bocock and Gilbert 1957), or on CO_2 production in laboratory incubations (e.g. Coûteaux et al. 1991; Li et al. 2011), assuming that litter C lost during decomposition is oxidized to CO_2 (Cotrufo et al. 2009). Within this framework, litter mass is generally lost until it reaches a non-zero asymptote, with the indecomposable solid mass remaining providing the main litter C contribution to soil organic matter (SOM) formation (Berg and McClaugherty 2003). This framework does not take into account the estimates of 6 to 39% of C loss during litter decay that can enter the soil in the form of dissolved organic C (DOC), in particular during the early phase of decomposition (Qualls and Haines 1991; Magill and Aber 2000; Don and Kalbitz 2005). New evidence suggests that dissolved organic matter (DOM) input from surface litter to the soil plays a significant role in SOM formation and long-term soil C sequestration (Kaiser and Kalbitz 2012; Cotrufo et al. 2013). Yet, to our knowledge, the proportion of litter C truly lost to the atmosphere as respired

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CO_2 during decomposition *versus* the proportion of litter C entering into the mineral soil through DOC leaching remains unknown.

The ability to predict the partitioning of DOC *versus* CO_2 losses during litter decomposition would greatly improve our ability to predict aboveground litter contributions to SOM formation across different ecosystems and under future global change scenarios. What controls this partitioning? Beside climate, litter chemistry is the main controlling factor of litter mass loss rates (Aber et al. 1990; Li et al. 2011) and CO_2 efflux from litter decomposition (Hessen et al. 2004). In particular, lignin and nitrogen (N) content have long been shown to be good predictors of litter mass loss rates (Melillo et al. 1982; Berg and Matzner 1997) and carbon use efficiency (CUE) (Manzoni et al. 2012). More recently, the Lignocellulose index (LCI) [lignin/(lignin+cellulose)] has been suggested as a chemical control of mass loss (Osono and Takeda 2005), as well as proposed to be a major predictor of microbial CUE (Moorhead et al. 2013). These studies suggest LCI could control microbial-derived DOC leaching during litter decomposition, but thus far there is limited work explicitly assessing how litter chemistry controls CO_2 efflux *versus* DOC efflux partitioning with litter mass losses.

Positive relationships between soil DOC concentrations and microbial biomass or CO_2 respiration have been found in mineral soils (Marschner and Bredow 2002; Fang and Moncrieff 2005), however not in all cases (Lundquist et al. 1999). Aboveground leaf litter inputs to soil can broadly be categorized into a soluble fraction, cellulose/hemicellulose and lignin (Berg and McClaugherty 2003). Litter DOM may be comprised of both soluble plant components that have bypassed microbial processing-generally more dominant in early stages of litter decomposition-and waste products from microbial activity as litter decomposition processes progress. Thus, plant litter chemistry could dictate the availability of leachable soluble litter material as well as

microbial C availability and CUE during litter decomposition. Plant litter chemistry could, therefore, control both the chemical composition of DOM and the proportion of mass lost to CO_2 *versus* DOC (Klotzbucher et al. 2011).

Although lignin is known to control litter decomposition, it is still not fully understood whether lignin is selectively preserved (Berg and McClaugherty 2003), continuously degraded (Preston et al. 2009a) or preferentially degraded (Klotzbucher et al. 2011) during the decomposition process. Lignin has been shown to control DOM production in the later phase of decomposition for some litter types, but not in the early phase when labile components are still available (Kalbitz et al. 2006; Klotzbucher et al. 2011). Understanding whether or not lignin is selectively preserved during decomposition therefore could have strong implications for understanding the plant input chemistry controls on DOM leaching.

Beyond lignin, other chemical characteristics of plant residues may affect C dynamics during decomposition. Fourier transformed mid-infrared (FTIR) spectroscopy can be used to identify the functional group chemistry of fresh or pyrolyzed plant material and DOM, and thus provide a sensitive and inexpensive method for analyzing the chemical changes of litter and DOM over the course of decomposition (Lammers et al. 2009; Strobel et al. 2001; Gallo et al. 2005). FTIR has been used to detect several absorbance bands in litter extracts including: 3400 cm^{-1} for O–H or N–H, 2850-2930 cm^{-1} for aliphatic C–H, 1605 cm^{-1} for aromatic C=C, and at 1070 cm^{-1} for polysaccharides (He et al. 2009). FTIR analysis of forest floor litter, litter ADF and litter lignin fractions showed the following informative bands: carbonyl absorption bands at 1724 cm^{-1} (esters) and 1659 cm^{-1} (proteins), as well as the 1603 cm^{-1} band assigned to lignin from aromatic skeletal vibrations (Galletti et al. 1993). Thus, FTIR functional group chemistry can be used to study differences in plant input chemistry between species and over the course of decomposition.

If litter chemistry does control the partitioning of C losses to CO_2 *versus* DOC leaching to the soil, we can expect large changes in litter-soil C fluxes to occur as a consequence of global changes, for example as plant type ranges shift with climate and land use change (Thomas 2010; Kelly and Goulden 2008). The projected increases in the frequency and severity of fire (Stocks et al. 1998; Flannigan et al. 2000) will also change the chemical composition of the plant matter inputs to the soil surface, with larger inputs in the form of pyrogenic organic matter (py-OM). Py-OM is partially combusted plant material, and is generally considered a chemically recalcitrant substance (Knicker 2011) with overall turnover times on centennial scales (Singh et al. 2012). However, py-OM has a measureable soluble component that can leach into the soil (Novak et al. 2009). DOM leaching from leaf litter and py-OM (which here we collectively refer to as aboveground plant inputs – API - to soil) may therefore both be controlled by similar chemistry changes in substrates as decomposition progresses.

The objectives of this study were: (1) to identify whether API chemical traits can be used to predict how much mass is lost as DOC leaching during decomposition, (2) to identify a proportional relationship between DOC leaching to the soil and CO_2 respiration to the atmosphere over the course of API decomposition, and (3) to identify how differences in both initial chemistry among API types and chemistry changes over time within a API type affect the chemical composition of DOM leached from decomposing litter. The study was designed to specifically test two hypotheses. (I) DOM leaching represents a significant fraction of mass loss, which it is not directly proportional to CO_2 production but rather is controlled by API chemistry. We predicted that APIs with a large fraction of structural components (e.g., cellulose and lignin) would decompose more slowly due to the extra metabolic activity required for their breakdown, and thus lose more C as CO_2, while APIs with a large fraction of soluble, non-structural,

components would decompose quickly and, due to a higher proportion of water soluble compounds, lose more C as DOC. (II) DOM chemical composition changes over the course of decomposition, reflecting sequential leaching of different soluble plant and microbial products as decomposition progresses. We hypothesize that initial DOM leaching reflects API soluble material, and then shifts toward partially broken down cellulosic and structural materials and microbial products. Additionally, since leaching only involves water-soluble compounds, we expect this compound class to be chemically similar across different litter types, and differences in DOM chemistry to reflect different stages of decomposition rather than differences in initial litter chemistries.

To test these hypotheses we incubated six API types, one py-OM and five leaf litters that ranged in initial chemistry, and measured DOC and CO_2 losses during their decomposition. We characterized API chemistry based on C:N, % structural material as % acid soluble fraction (ASF) and % acid unydrolyzable residue (AUR), % non-structural material (total mass-neutral detergent fiber (NDF) mass), % hot water extractable C (HWE-C) and FTIR spectroscopy. We characterized DOM throughout the incubation based on DOC, total dissolved nitrogen (TDN), NH_4^+, NO_3^- and FTIR spectroscopy.

Methods

Aboveground plant inputs (API) samples

For this study we used 5 API types: 4 above ground leaf litters and one py-OM sample. We collected ash (*Fraxinus excelsior),* big bluestem *(Andropogon gerardii),* oak (*Quercus macrocarpa)* and pine (*Pinus ponderosa)* leaf litter in the fall of 2011 as freshly senesced litter that had neither hit the ground nor been rained on since abscission. Alfalfa (*Medicago sativa)*

leaves were collected standing green at crop maturity. Plant types were selected to cover a range of chemical characteristics such as C-to-N ratio (C:N) and LCI. Abscised ash leaves and pine needles were collected from separate raised litter traps. Senesced oak leaves were shaken off of a tree and collected in a litter trap. Senesced, standing big bluestem grass blades were hand cut from a native tallgrass prairie. We removed all stems and petioles from the leaves, and pooled them all by species. We air dried all litter samples, cut them into 1 cm x 1 cm pieces or 1 cm lengths, and homogenized them. We did not cut the alfalfa litter, whose leaves were already approximately 1 cm x 1 cm. To produce a py-OM sample, we placed ground big bluestem grass into porcelain crucibles, which were heated at 300ºC for four hours in a muffle furnace with ultra-high purity nitrogen flow as described by Rutherford et al. (2012). Three subsamples from each litter and py-OM pool were oven dried at 105ºC for dry weight correction, ground, and used for elemental and chemical analysis as described below.

Laboratory incubation

Three replicates of each API type, i.e. all five litter types and the py-OM, were incubated for one year (365 days) under optimal laboratory conditions. We incubated an additional set of three replicates per each API type for 95 days, for destruction at an intermediate time point. For each sample, 2 g of litter or py-OM was placed in leaching cups, 3 cm in diameter (CellSmart Vaccum Filtration Systems, Argos Technologies) fitted on the bottom with a 20 µm nylon filter. Initially, we leached the samples with deionized water on day 0, and then inoculated them with 1 ml of microbial inoculum. We created the inoculum by mixing 1 g of a partially decomposed mixture of grass and deciduous tree litter, collected from a nearby creek bank, with 100 ml deionized water for two hours. The mixture was left to settle, filtered, and then 1 ml of the

filtrate inoculum was added to each sample. We had three blank samples consisting of a leaching cup that was inoculated, with which we quantified any potential microbial degradation of the plastics in the leaching cup, and subtracted this from all DOC and TDN results. Each leaching cup was sealed in an airtight 3.8 l jar fitted with a rubber septum for gas sampling throughout the entirety of the incubation. The jars were periodically checked to ensure air tightness. A thin layer of water at the bottom of the jars maintained constant humidity, and the jars were incubated at a constant temperature of 25°C in the dark.

We measured CO_2 and DOM leaching periodically throughout the incubation. We used a gas tight syringe to sample air in the headspace of the jars through the septum, and analyzed it on an infrared gas analyzer (IRGA, Li-Cor 800, Li-Cor, NE) for CO_2 concentration (Stewart et al. 2013a). After CO_2 collection, we opened the jars and placed the leaching cups on a 250 ml flask for leachate collection. We dispensed 30 ml of deionized water evenly over the litter samples and allowed the leachate to drain through the leaching cup filter and into the collection flask. After twenty minutes of leaching, we used a light vacuum line to drain out any remaining leachate from the cups. We subsampled the leachate for FTIR analysis as described below, then immediately froze them until further analysis.

After leachate collection we placed each leaching cup back in the airtight jars and flushed them with soda lime scrubbed air to minimize initial CO_2 concentrations. We measured this initial CO_2 concentration to accurately quantify the amount of CO_2 produced during each incubation period. Jars were then returned to the constant temperature room until the following sampling. We wanted to prevent CO_2 from building up to toxic levels in the jars (>2%), so sampling frequency was determined by the maximum rate of CO_2 production, with more frequent initial sampling tapering off through the duration of the experiment.

Chemical analysis

We characterized three replicates of initial (day 0), day 95 and day 365 API chemistry based on mass remaining, % C, % N, % acid-unhydrolyzable residue (AUR), % acid soluble fiber (ASF) and their FTIR spectral properties. For some of the day 365 API samples, not enough mass remained for replicate analysis so the remaining API mass was aggregated and analyzed in one replicate sample. All API samples were analyzed for % C and % N on an elemental analyzer (LECO tru-SPEC, Leco Corp., St. Joseph, MI). Percent ASF and AUR were determined using the acid detergent fiber (ADF) digestion method (Vansoest and Wine 1968). In brief, an initial heated digestion in Cetyl trimethylammonium bromide (CTAB) and sulfuric acid removes hemicellulose and other non-structural carbohydrates and lipids, and then the samples are digested in 73% sulfuric acid. The ASF from this 73% sulfuric acid digestion is a proximate estimate of cellulose content and the acid unhydrolysable fraction (AUR) is a proximate estimate of lignin content (Rowland and Roberts 1994), with both fractions corrected for ash content. Using this proximate analysis, LCI is calculated as [AUR/(AUR+ASF)]. Additionally, we characterized the initial % non-structural material on three replicates of the day 0 API samples using a neutral detergent fiber (NDF) digestion (Van Soest et al. 1991). We also characterized the initial API based on hot water extractable C (HWE-C) on three replicates of the day 0 API samples, following Tappi (1981). All three replicate samples of the dried, ground API samples from day 0, 95 and 365 were also scanned using a Digilab FTS 7000 spectrometer (Varian, Inc., Palo Alto, CA) with a Pike AutoDIFF sampler (Pike Technologies, Madison, WI) for FTIR spectral analysis. The spectrometer had a deuterated, Peltier-cooled, triglycine sulfate detector and potassium bromide (KBr) beam splitter. KBr was used as background. Data were

obtained as pseudo-absorbance (log [1/Reflectance]). Each spectrum was collected at 4 cm^{-1} resolution, with 64 co-added scans from 4000 to 400 cm^{-1}.

All leachate samples were thawed and analyzed for total organic carbon (TOC) and total dissolved nitrogen (TDN) on a TOC analyzer (Shimadzu TOC 5000). Inorganic NH_4^+ and NO_3^- of the leachate were measured on days 0, 1, 4, 7, 10, 15, 20, 28, 39, 64, 76, 95, and 284 using an Alpkem Flow Solution IV Automated wet chemistry system (O.I. Analytical, College Station, TX). We also analyzed the leachate chemistry on days 0, 1, 4, 7, 10, 95, 118, 228 and 365 by FTIR analysis. An aliquot (0.5 ml) of leachate was added to 250 µg of KBr and freeze-dried. The dried KBr-leachate samples were scanned using the same spectrometer and settings as the litters.

Data analysis

We tested the effect of API type and incubation time on mass loss, changes in C:N ratio, %ASF and %AUR by means of a generalized linear mixed model. We included API type, time and their interaction as categorical fixed effects. The effects of API chemistry on CO_2 efflux, DOC leaching and the DOC-to-CO_2 ratio over time was tested by means of a repeated measures test within a generalized linear mixed model containing incubation day, API type and their interaction as categorical fixed effects. Individual samples were treated as random effects within each API type. These analyses were carried out using the SAS® software version 9.3. The data passed tests for normality and homogeneity of variances of the residuals. In all cases we used type III tests of fixed effects.

We performed spectral averaging of the FTIR data using GRAMS version 9.1 software with the GRAMS IQ package (Thermo Fisher, Woburn, MA). All spectra were mean-centered

before statistical analysis. We utilized a distance-based redundancy analysis (dbRDA) for statistical assessment of the overall differences in FTIR spectral chemistry among API and leachate composition over time using the R: Vegan package (Oksanen 2013). Briefly, we chose the dbRDA analysis over other multivariate statistical approaches due to its non-linear distance-metric options, which have robust multi-dimensional resolution to assess categorical variables. Distance based RDA is a three step ordination technique that tests the effects of response parameters (i.e. wn absorbance) on defined groups (i.e. litter or leachate type at a given incubation time). First, a dissimilarity or distance matrix is calculated for the different litter/leachate types. We chose the Bray-Curtis dissimilarity (non-linear) measure to model the species matrix as suggested by Legendre and Anderson (1999b). For steps two and three of the dbRDA, a principal coordinate analysis (PCoA) is calculated based on the distance matrix, from which the eigenvalues (obtained in the PCoA) were applied to a redundancy analysis (RDA).

Results

Mass loss, DOC and CO_2 dynamics

At the two destructive harvests on day 95 and 365, alfalfa had lost 77 and 83% of its initial mass respectively; ash 44 and 62%, oak 45 and 58%, bluestem 21 and 46% and pine 18 and 28% (Table 1). The py-OM lost 4% of its mass by the end of the incubation and DOC and CO_2 fluxes were detected, however due to high variance this change in mass was not statistically significant (Figure 1, $p>0.05$). We used our measured DOC and CO_2 values to calculate cumulative DOC and CO_2-C losses, relative to the initial litter C basis. At the end of the 365 day incubation, 15% of the initial C from alfalfa was lost as DOC, 16% for ash, 10% for bluestem, 8% for oak, 3% for pine, and 1.57% for the py-OM. For alfalfa, 37% of the initial C was lost as

CO_2, 25% for ash, 18% for bluestem, 35% for oak, 15% for pine, and 2.59% for py-OM (Table 1).

We also tracked the dynamics of API C loss to DOC *versus* CO_2 over the course of the incubation (Figure 1). Overall at the end of the experiment the DOC:CO_2 ratio ranged between 0.20 and 0.65 (Figure 1). As expected, most of the DOC and CO_2 fluxes occurred during the initial period of the incubation, with alfalfa decomposing much more rapidly than the other litter types. Ash and bluestem litters continued to leach some DOC after the initial period in contrast to the other litter types, which tended to level off in DOC leaching after approximately day 15 (Figure 1).

Three phases of DOC versus CO_2 dynamics

Three distinct phases of decomposition were observed across all APIs, on the basis of the DOC:CO_2 dynamics. The early phase, up to day 15, was characterized by very high DOC losses (~70% of total DOC lost) and high DOC:CO_2 ratio (average= 2.49, standard error= 0.830), a second or mid phase (16-65 days) was characterized by declining DOC:CO_2 and a third or late phase, where very little DOC was produced, was characterized by a constant DOC:CO_2 ratio (average= 0.370, standard error= 0.165).

In examining the early decomposition phase, we found a highly significant correlation between the % HWE-C of the initial API and the total amount of DOC leached in the first 15 days of the incubation ($p= 0.0017$, Figure 2a). The % non-structural material also correlated strongly with the amount of initial DOC leaching in days 0-15 for all litter types and the py-OM, with the exception of pine litter (R^2=0.912 without pine, R^2=0.767 with pine).

For the mid and late stages of the incubation (days 16-64 and days 65-365) we tested several initial chemical characteristics against DOC *versus* CO_2 partitioning (Table 2). We found the strongest correlation between initial LCI- with the exception of day 95 LCI for alfalfa- and the ratio of DOC:CO_2 losses during these phases (Table 2, Figure 2b). We assumed that the day 95 LCI for the alfalfa would better characterize the litter structural composition starting at day 16 due to its rapid decomposition, which reached its asymptote for CO_2 and DOC fluxes by day 15. The initial LCI still fit for the other litter types who had not reached their asymptote for CO_2 and DOC fluxes by day 16. We divided the second stage of the incubation into mid and late phases of decomposition due to the different offsets of the DOC:CO_2 vs. LCI curves for these two phases (Figure 2b). We omitted the py-OM from the second stage DOC:CO_2 dynamics because we did not have enough sample for the day 0 AUR and ASF determination.

Litter and py-OM chemistry

The five litter types and py-OM studied spanned a broad range of initial API chemistries based on C:N, % ASF, % AUR, % non-structural material and % HWE-C (Table 1). Alfalfa represented the most labile litter type due to its high N content, low AUR content and low LCI of 0.24. Oak and pine had a high initial LCI of 0.46 and 0.51 respectively, while ash and bluestem had low LCI's of 0.34 and 0.20 respectively. The initial API's also ranged in their initial % non-structural composition, from 73.62% (alfalfa) to 11.46% (py-OM) (Table 1). The initial % HWE-C varied proportionally to the % non-structural composition (R^2= 0.99, $p<0.0001$; Table 1).

The C:N of alfalfa and py-OM did not change significantly throughout the incubation, while the C:N of ash, bluestem and oak significantly decreased between day 0 and 95 but then

remained constant between day 95 and 365. The only litter type whose C:N continued to decrease from day 0 to 95 and day 95 to 365 was pine ($p<0.01$).

Results from the ADF digestion of the litter at all three time points revealed a relative increase in the AUR fraction over time (Table 1). Alfalfa was the only litter type whose absolute amount of the AUR did not change over time. For ash, bluestem, oak and pine the amount of AUR increased on average by 190% from day 0 to 95, and then decreased by on average 69% from day 95 to 365. The ASF fraction decreased over time, both in relative (Table 1) and absolute amounts for all API types. The three replicates of the pine sample had to be aggregated for the day 365 ADF digestion, so there was no statistical power to the increase in ASF for pine from day 95 to day 365 (Table 1). There was not enough day 0 py-OM for initial AUR and ASF determination, but from day 95 to 365 the amount of ASF decreased by 50%, while the amount of AUR decreased by 3%.

Results from the FTIR analysis of litter and py-OM at day 0, day 95 and day 365 revealed shifts in chemical composition among the API types and over time. Initially, the py-OM samples differed significantly in chemical composition from the five litter types ($p<0.05$, Figure 3). When we removed the py-OM from our initial sample dbRDA analysis, there was still a statistically significant difference between the five remaining litter types ($p<0.05$). This difference was mainly driven by the differences between the alfalfa and bluestem litters. At time zero, the litters had defined bands at ~3400, 2950-2850, 1650-1580, 1435, 1100, and 897 cm^{-1} (Figure 3). The region between 3600-2850 was very similar between the litters, except for the more pronounced absorbance of the pine litter at the aliphatic CH band (2950-2850 cm^{-1}), possibly because of the presence of resinous material. The py-OM had reduced absorbance at ~3400 cm due to the loss of OH and NH groups during pyrolysis. Oak and pine had the most

marked carbonyl peaks at 1740 cm^{-1}. The peak between 1650-1580 narrowed towards 1610 in the py-OM and the pine indicating higher aromatic skeletal vibrations, whereas the alfalfa had a peak towards 1655 consistent with esters in proteins (Galletti et al. 1993). Between 1500 cm^{-1} and1510 cm^{-1}, the lignin peak (White et al. 2011) was absent in the alfalfa spectrum, consistent with the high non-structural character of this litter (Table 1, Figure 3). Multivariate analysis showed that for all of the API types, except for pine, there was a loss in absorbance during the incubation at ~3470, 2925-2850, 1740 and 1080 cm^{-1}, consistent with the loss of carbonyl-containing moieties and aliphatic CH (data not shown). Pine, in contrast, showed a reduction in absorbance at 3570 and 2940-2850 cm^{-1} during incubation. The litters tended to gain absorbance at 1680 and 1545 cm^{-1} during the incubation, which suggests an increase of amide and aromatic C=C during decomposition (Calderon et al. 2006).

API chemistry controls on DOM composition over time

In addition to quantifying the API chemistry controls on the amount of DOC leaching over time, we also measured changes in DOM DOC:TDN, organic and inorganic N composition and functional group chemistry over time. Initially, the DOC:TDN of the DOM significantly correlated to the C:N of the initial litter (R^2=0.7967, p= 0.017). All of the DOC:TDN ratios of the DOM decreased exponentially until they reached a constant level, with the DOC:TDN of alfalfa DOM leveling off at 2.9 by day 4, oak at 18.9 by day 20, ash at 30.2 by day 64, bluestem at 66.2 by day 64, py-OM at 15.1 by day 64 and pine at 31.0 by day 64 (Figure 4a). These ultimate DOC:TDN values of the DOM also correlate strongly with initial litter C:N (R^2=0.9906), when the pine litter is excluded (Figure 4b). Throughout the incubation over 80%

of the TDN in the DOM was organic, i.e. not NH_4^+ or NO_3^-, except for alfalfa, whose DOM contained 40-60% inorganic N from day 28 to 365 (data not shown).

We analyzed the FTIR spectral data from nine of the leaching events during the incubation, on days 0, 1, 4, 7, 10, 95, 118, 228 and 365. When we compiled the leachate FTIR data by API type over time, we found no significant differences in the overall composition of the leachate between the different API types ($p=0.074$). The leachates showed absorbance peaks at 330, 2926, 1590, and 1060 cm^{-1} (data not shown). Instead, across all API types (excluding the py-OM), multivariate analysis showed that there was a statistically significant shift in the chemical composition of the leachate over time (Figure 5, $p=0.005$). A more in depth analysis of the FTIR spectra data shows that the absorbance at 3400 (OH/NH), 2070 and 1622 cm^{-1} (aromatic, carboxylate, conjugated ketone C=O) decreased from days 0 and 4 to days 7 and 10. Absorbance at wn 2926 (aliphatic CH), 1590 (amide II) and 1060 cm^{-1} (polysaccharide) decreased later in the incubation. The day 1 leachate resembled the day 7 and 10 leachate more closely than the day 0 and day 4 leachates (Figure 5). This is likely due to the presence of excess inoculum-derived DOM at day 1, which was added after the initial day 0 leaching event, being leached out along with the day 1 litter leachate.

Discussion

DOM production and DOM versus CO_2 partitioning during aboveground plant input decomposition

After 365 days of incubation, we found that a significant fraction of the mass lost from API was lost as DOM. Total DOC losses ranged from 2% to 16% of the initial C, and did not correlate with rates of mass loss. This range of DOC leaching falls at the lower end of those

found in previous laboratory leaching studies (Magill and Aber 2000; Kiikkila et al. 2012). It is important to note, however, that laboratory litter leaching estimates may differ based on the method of leaching employed (Soong et al. In Revision), and provide a measure of potential DOC leaching rather than a measure of actual DOC leaching *in situ*. Confirming our first hypothesis, DOC leaching rates did not correlate with CO_2 production rates when examined over the entire course of the incubation (Figure 1). Similar results were found by Klotzbucher et al. (2011), who proposed that excess soluble C in the early stage accounts for the disconnect between DOC leaching *versus* C mineralization and that soluble C availability limits CO_2 production in the later stages of decomposition. We also found that the direct leaching of soluble C in the early decomposition stage accounts for the disconnect between DOC and CO_2 production, but we propose that API chemistry, in particular the LCI and potentially N availability, control the relationship between DOC leaching and C mineralization in the later stages of decomposition.

The results of our incubation suggest that API chemistry controls the amount of litter-C lost to DOC leaching and CO_2 efflux during decomposition. We selected a three-phase decomposition model that accounted for the initial fast phase of decomposition from day 0-15, characterized by high DOC and CO_2 fluxes, and the mid and late slow phases from day 16-64 and 65-365, characterized by lower DOC and CO_2 fluxes (Figure 1), as a framework to clarify these changes in litter chemistry controls (Figure 6). Our results suggest these three decomposition phases are controlled first by initial leaching of plant soluble material during the early phase (day 0-15) and then subsequently by microbial breakdown of cellulose and lignin (day 16-365) (Berg and McClaugherty 2003) (Figure 6). Our theory for the control of changing plant residue chemistry on DOC leaching over time, shown in Figure 6 and discussed in the

subsequent paragraphs, demonstrates how lignin is continuously decomposed in plant material with a high LCI but is selectively preserved in litters with a low LCI.

During the early phase (day 0-15) of the incubation the amount of HWE-C, or non-structural material, in the initial API predicted DOC leaching (Figure 2a). On average, 22% of the HWE-C was leached out in the early phase. HWE-C is a measure of the water-soluble fraction of litter and has been suggested to be readily mineralizable, and consisting mainly of soluble carbohydrates (Landgraf et al. 2006). HWE-C may be a more applicable metric than % non-structural material as measured by the NDF procedure for estimating the early phase DOC leaching, due to the wide range of NDF procedure modifications throughout the literature and the inclusion of lipids in the NDF fraction (Van Soest et al. 1991). In our study, %HWE-C did not correlate as well with CO_2 mineralization during the initial phase (R^2=0.67), and so should be considered a better predictor of leachable C rather than readily mineralizable C in fresh litter samples. The lack of correlation between DOC and CO_2 during the early phase of decomposition suggests that soluble plant materials leach from litter without requiring microbial transformation (Berg and McClaugherty 2003). The reflection of initial litter C:N in the initial high DOC:TDN ratios provides further evidence that in this early phase DOM consists of soluble plant material, not microbial products (Figure 4).

During the mid and late phases of decomposition (day 16-64 and day 65-365), the initial litter LCI controlled partitioning of C loss to DOC and CO_2 fluxes (Figure 2b). The LCI has historically been a good predictor of litter mass loss rates (Preston et al. 2009b), but to our knowledge this is the first time it has been shown to control the proportion of mass lost as CO_2 *versus* DOC. We propose that the higher DOM leaching from litters, such as bluestem and ash with a low LCI, is mainly due to leaching from unprotected cellulose, whereas the higher CO_2

production from litters, such as pine and oak with high LCI, is due to lignin protection of cellulose decomposition. The mid and late phases showed different DOC:CO_2 losses, with higher DOC:CO_2 losses during the mid phase (Figure 2b). The higher intercept of the curve for the mid phase (days 16-64) is likely due to the availability of unprotected cellulose, which has a high CUE leading to more DOC production (Moorhead et al. 2013) (Figure 6). During the late phase (days 65-365) there is more CO_2 production relative to DOC than the mid phase due to the lower energy yield from the decomposition of remaining lignin and lignin-encrusted cellulose (Moorhead et al. 2013) (Figure 6). The similar slope of the relationship between LCI and DOC:CO_2 losses during the mid and late phases demonstrates the LCI controls on DOC *versus* CO_2 partitioning once the initial plant soluble fraction has been leached (Figure 2b, Figure 6). For low LCI material, as free cellulose is leached during the mid-phase the remaining residue begins to resemble the high LCI material, so CUE is lower during the late phase as lignin and lignin protected cellulose are degraded resulting in higher CO_2 losses (Figure 6).

Our results are consistent with the Moorhead et al. (2013) model of the relationship between LCI and CUE during litter decomposition. In accordance with their model, our results show that at a higher LCI there is a higher energetic cost to the breakdown of the litter structural material and more C is lost as CO_2, whereas at lower LCI the excess energy from unprotected cellulose breakdown causes more C to be leached as DOC. Ash and bluestem, both characterized by low LCI's, continued to have high DOC leaching throughout the mid and late phases of the incubation, likely due to the high availability of unprotected cellulose (Figure 1; Figure 6). The alfalfa litter was the one exception to this trend; when using the initial LCI value, alfalfa did not fit the DOC:CO_2 trend exhibited by other litters in later stages of decomposition, however alfalfa did fit this trend when its day 95 LCI was used (Figure 2b, Table 2). This can be explained by

the fact that alfalfa was the only litter type in our study that is not N limited (Table 1). Abundant N availability can reduce DOC leaching due to higher C immobilization in microbial residues (e.g., higher microbial CUE), leading to a lower DOC leaching than would be expected based on LCI (Sinsabaugh et al. 2013; Manzoni et al. 2012). For N-limited material such as ash, bluestem, oak and pine, N did not limit DOC leaching, rather LCI controlled CUE and the partitioning between DOC and CO_2 fluxes. Under field conditions external N inputs could add N to decomposing plant material, and thus the interaction between N availability and LCI controls on DOC leaching *in situ* is an important area of future research.

Approximately 30% of the C lost from all of the litter types in our incubation was not accounted for as DOC or CO_2. This could be due to a leaking of unaccounted for CO_2 from the jars, or possibly from the emission of unmeasured volatile organic compounds from the decomposing litter (Gray and Fierer 2012). We could not confirm that all of the missing C was lost as CO_2, so did not include it in our measurements of CO_2 flux, but when added to the CO_2 flux we found the same significant LCI control over the ratio of DOC:CO_2 losses during the mid and late phases (R^2=0.85 for day 16-64, and R^2=0.96 for day 65-365).

DOC and CO_2 fluxes from pyrogenic organic matter

Depending on feedstock and pyrolysis conditions, chemical properties and biodegradability of py-OM may differ significantly (Baldock and Smernik 2002). During our incubation of py-OM produced from pyrolysis of *Andropogon gerardii* at 300°C for 4 hours, we found an overall non-statistically significant mass loss of 4%. Of the initial C in the py-OM, 1.57% was lost as DOC and 2.59% was lost as CO_2. These values are similar to the 1% DOC and 2.2% CO_2 losses found in a field study from py-OM leaching in a savanna Oxisol (Major et

al. 2010). Similarly to non-pyrolyzed litters, nearly all the DOC leached from py-OM in the early phase of decomposition (day 0-15). This points toward the need for a two-phase model of py-OM decomposition with a fast early stage and a much slower later stage (Foereid et al. 2011). The implications of py-OM DOM inputs to the soil after a fire depend on complex DOM-soil interactions, but could increase soil fertility in some circumstances (Novak et al. 2009). HWE-C is a good measure of the leachable fraction of py-OM, with 75% of the total DOC leaching from py-OM occurring in the early phase, accounting for 20% of the HWE-C, similar to the litter dynamics (Figure 2a). By day 95, the LCI of the py-OM was 0.97, with CO_2 fluxes greatly outweighing DOC fluxes (Figure 1). Although py-OM decomposition is slow, this study confirms the idea that, in soils, py-OM may behave similarly to non-pyrolyzed litter (Knicker 2011). Thus, py-OM chemistry could be used to predict the proportional loss of py-OM to leaching or respiration, and may help explain the variable estimates of py-OM mean residence times in soils (Schmidt et al. 2011), as well as better inform projections of fire impacts on ecosystem C cycling.

Residues and DOM chemistry changes during decomposition

The ASF and AUR chemistry of the different APIs changed progressively throughout the incubation (Table 1). ASF content declined throughout the incubation, while AUR content increased from day 0-95 then decreased from day 95-365 (Table 1). Our observations of increasing absolute AUR amounts are consistent with the concept of AUR as a measurement that contains not only lignin but also secondary compounds, or microbial products, produced during decomposition (Preston et al. 1997; Johansson et al. 1986). We took efforts to ensure that our litter samples had not touched the ground or been rained on prior to collection, so the initial AUR

values used to calculate the initial LCI are a good estimate of initial lignin content. The increase in AUR at day 95 (Table 1) could be due to the production of secondary compounds from high microbial activity (i.e. CO_2 flux) during the early and mid-phases of decomposition (Figure 6). While the AUR fraction cannot be used to infer lignin dynamics during decomposition (Cotrufo et al. 2013), our results suggest that this recalcitrant fraction is a key regulator of DOC *versus* CO_2 losses from decomposing plant material, and that the AUR fraction is relevant to calculating LCI and predicting whether lignin will be degraded or preferentially preserved.

The leachate chemistry changed during the course of the incubation, reflecting the progressive loss of non-structural plant material followed by cellulose decay products (Figure 5). The DOC:TDN reflected the C:N of the litter and py-OM initially, but decreased exponentially to reach a constant value, which also correlated with the initial C:N of the litters and py-OM, except for pine (Figure 4b). The asymptote DOC:TDN for pine was lower than to be expected by its initial litter C:N (Figure 4a). However, negligible amount of DOM were leached from the pine residue at this stage. The C:N of the bluestem was high at its asymptote, reflecting the relatively high DOC flux from the continual leaching of products of cellulose degradation, from this low LCI litter type.

The high C:N of the initial leachates suggests that the initial leachates are not labile and likely not readily taken up by microbes in the soil. The reflection of the initial litter C:N in the leachate throughout the experiment also indicates that soluble leaf components were being cleaved and leached out during the course of decomposition (Figure 4b). Although microbial products were being produced during the incubation, as indicated by the accumulation of the AUR fraction, these low C:N microbial products (Cleveland and Liptzin 2007) did not appear to be the main contributors to DOM. The leachate from all API's except alfalfa contained mainly

organic N with very little NH_4^+ and NO_3^-. The low C:N of the alfalfa leachate, along with the presence of NH_4^+ and NO_3^-, provide evidence that the all of the API's except alfalfa were N limited, and thus fit the proposed LCI model (Figure 6). Over time, all of the litters except pine lost absorbance at ~3400, 2925-2850, 1740 and 1080 cm^{-1}, consistent with the loss of carbohydrates, aliphatic CH, and progression of decomposition state (Haberhauer et al. 1998).

The functional group chemistry of the DOM reflects the sequential changes in API residue chemistry over time (hypothesis 2). Across all API types, DOM functional group chemistry changed significantly with time, but not between API types. DOM functional group chemistry became more similar over time (Figure 5), indicating that the effect of initial API chemistry (Figure 3) on DOM composition decreases as litter chemistry converges with increasing decomposition (Preston et al. 2009b). Initially (day 0-10), the DOM showed high absorbance of OH/NH (3400 cm^{-1}), aromatic, carboxylate and conjugated ketone C=O (2070 and 1622 cm^{-1}) functional groups (Figure 5). This is also when most of the leaching occurred. Carbohydrates (-OH) in this initial DOM have limited sorption potential to mineral components in the soil (Oren and Chefetz 2012). Carboxylic groups at 1622 cm^{-1}, however have been shown to play a major role in the binding of OM to metal oxides, and could be directly stabilized onto the minerals in the soil, especially under high pH conditions (Oren and Chefetz 2012; Kaiser and Guggenberger 2000). Later (day 95-365), the DOM showed absorbance indicative of mainly aliphatic CH (2926 cm^{-1}), amide II (1590 cm^{-1}) and polysaccharide (C-O, 1060 cm^{-1}) functional groups (Figure 5). These DOM functional groups may be indicative of later stage decomposition products such as waxes (2926 cm^{-1}, (White et al. 2011)), lignin degradation products (1590 cm^{-1}, (Calderon et al. 2006)) and polysaccharides with limited mineral sorption potential (1060 cm^{-1}, (Oren and Chefetz 2012)). Therefore leaching of aromatic structures and carboxyl groups (1622

cm^{-1}) in the early phase of API decomposition may contribute significantly to soil C stabilization through ligand exchange reactions with metal oxide surfaces in the soil (Kaiser and Guggenberger 2000). Since HWE-C correlates strongly with early phase DOC leaching, HWE-C could be a good metric for estimating litter contributions to SOM formation across different plant species.

Implications and conclusions

During our 365 day incubation, we found that up to 16% of litter mass was lost as DOC, up to 37% was lost as CO_2, and that litter chemistry controlled this partitioning (hypothesis 1). The large amount of DOM leaching from decomposing fresh and pyrolyzed aboveground plant inputs to soil can be used to help estimate aboveground plant inputs to SOM formation and to explain the increasing age of SOM with soil depth (Rubino et al. 2010; Cotrufo et al. 2013; Kaiser and Kalbitz 2012). Our results are in line with the Microbial Efficiency Matrix Stabilization (MEMS) hypothesis (Cotrufo et al. 2013) that labile litter (i.e. large HWE-C fraction) contributes more to long term soil C sequestration. However, the MEMS framework suggests that this is due to high microbial use of the initial non-structural litter material, whereas we found that the DOM leaching in the early phase came directly from the plant material, is recalcitrant and can sorb directly to the mineral soil. A sequential change in litter chemistry during decomposition appears to control the composition of DOM leaching from litter over time (hypothesis 2). Non-microbial transformed soluble plant components from the partial breakdown of cellulose and lignin appear to leach out even during the later phases of decomposition. The shift in functional group chemistry of DOM over the course of

decomposition has important implications for its fate once in the soil (Oren and Chefetz, 2012), and is an important area of future research.

Litters with a high amount of non-structural material leached more DOC initially during the high leaching period, but litters with a low LCI leached more DOC relative to CO_2 during the later lower leaching period. These relationships help to confirm our hypothesis that litter chemistry can be used to predict the fate of litter mass loss. Under field conditions, climate largely controls litter decomposition rates (Aerts 1997) and we did not test the role of climate in the DOC and CO_2 partitioning. However the specific litter chemical mechanism controlling DOC and CO_2 fluxes found here can be used to start building and testing models of litter decomposition and API-soil-atmosphere C cycling under field conditions. Although mass loss cannot be used to predict DOC leaching, the % HWE-C of plant inputs to soil can be used to predict initial DOC leaching, while the LCI of the plant material can be used to predict the partitioning of CO_2 emissions *versus* DOC leaching from decomposing material in later phases of decomposition. We propose that these highly significant litter chemistry controls on DOC leaching should be incorporated into models of ecosystem C cycling to account for the large flux of DOC that can leach from aboveground plant material into the soil.

Table 3.1. Average values of litter chemistry at three experimental time points (n=3), standard error is in parentheses. DOC and CO_2 are reported as total cumulative values.

Litter	Time	Mass (g)	%C	%N	DOC (mg)	CO_2 (mg)	%AUR ±	%ASF+	%Non-structural	%HWE-C
Alfalfa	*Day 0*	2.00 (<0.001)	44.1 (0.026)	4.09 (0.012)	NA	NA	4.66 (0.432)	14.2 (0.418)	74.7 (0.308)	28.3 (0.676)
	Day 95	0.466 (0.040)	42.5 (0.218)	4.49 (0.086)	128 (4.04)	285 (23.5)	17.8 (1.18)	15.9 (0.651)		
	Day 365	0.333 (0.013)	38.0 (0.508)	4.21 (0.171)	1.49 (0.288)	41.5 (2.13)	23.2 (NA)	15.1 (NA)		
Ash	*Day 0*	2.00 (<0.001)	46.5 (0.608)	0.88 (0.012)	NA	NA	9.81 (0.188)	19.1 (0.256)	69.0 (0.344)	25.4 (0.213)
	Day 95	1.13 (0.015)	50.6 (0.120)	1.55 (0.086)	131 (3.83)	137 (9.15)	33.7 (2.77)	20.9 (3.485)		
	Day 365	0.753 (0.048)	47.4 (0.797)	1.85 (0.134)	18.4 (6.79)	98.5 (7.31)	36.5 (NA)	17.2 (NA)		
Bluestem	*Day 0*	2.00 (<0.001)	44.1 (0.049)	0.478 (0.003)	NA	NA	8.42 (0.522)	33.4 (0.567)	32.9 (0.266)	11.2 (0.348)
	Day 95	1.59 (0.066)	47.0 (0.263)	0.593 0.025)	66.2 (3.83)	94.0 (11.7)	19.9 (1.14)	32.9 (0.289)		
	Day 365	1.09 (0.075)	43.2 (0.097)	0.487 (0.025)	25.6 (4.30)	62.3 (12.9)	19.9 (NA)	32.3 (NA)		
Py-OM	*Day 0*	2.00 (<0.001)	59.2 (1.021)	2.11 (0.043)	NA	NA	NA	NA	11.5 (0.713)	2.10 (NA)
	Day 95	2.00 ($8x10^{-4}$)	64.7 (0.443)	2.46 (0.185)	15.6 (0.206)	13.3 (0.500)	93.7 (5.07)	NA		
	Day 365	1.93 (0.004)	62.0 (0.309)	2.07 (0.119)	2.99 (0.130)	17.4 (4.81)	89.1 (NA)	1.60 (NA)		
Oak	*Day 0*	2.00 (<0.001)	47.6 (0.038)	1.32 (0.010)	NA	NA	18.8 (0.259)	22.4 (0.297)	53.1 (0.591)	18.7 (2.68)
	Day 95	1.10 (0.017)	51.1 (0.165)	2.16 (0.096)	70.8 (2.89)	227 (3.19)	52.9 (2.28)	18.8 (1.893)		
	Day 365	0.833 (0.017)	45.7 (0.323)	2.36 (0.049)	6.66 (0.604)	103 (8.80)	53.0 (NA)	20.5 (NA)		
Pine	*Day 0*	2.00 (<0.001)	52.3 (0.049)	0.413 (0.007)	NA	NA	24.4 (0.302)	22.9 (0.327)	48.3 (0.723)	9.28 (2.07)
	Day 95	1.64 (0.022)	56.6 (0.130)	0.613 (0.007)	31.3 (1.48)	92.1 (2.33)	68.1 (6.52)	11.9 (5.405)		
	Day 365	1.44 (0.018)	51.0 (0.185)	0.490 (0.059)	2.73 (0.069)	70.2 (9.97)	45.0 (NA)	29.0 (NA)		

*NA indicates that either there was not enough sample remaining to do the analysis, or that there was only enough sample for one aggregated analysis, so no standard error can be reported. For Bluestem, Pine and py-OM one composite sample of all three replicates was used for the day 365 lignin and cellulose determination.

+AUR= acid unhydrolyzable residue, a proximate value for lignin; ASF= acid soluble fraction, a proxmiate value for cellulose

Table 3.2. Regression results from the average Day 16-365 proportion of C lost as DOC vs. CO_2 against various initial litter chemistry parameters on three replicates.

	R^2	F-value	p-value
Day 0 LCI, with day 95 LCI for alfalfa*	0.9724	105.626	0.001964
Day 0 % Cellulose	0.6172	4.837048	0.115244
Day 0 % non-structural	0.3479	1.600248	0.295198
Day 0 LCI	0.317	1.392538	0.323027
Day 0 %N	0.3066	1.326557	0.332878
Day 0 % Lignin	0.1186	0.403701	0.570334
Day 0 Lignin:N	0.0128	0.039007	0.856059

*This is the regression shown in Figure 2b.

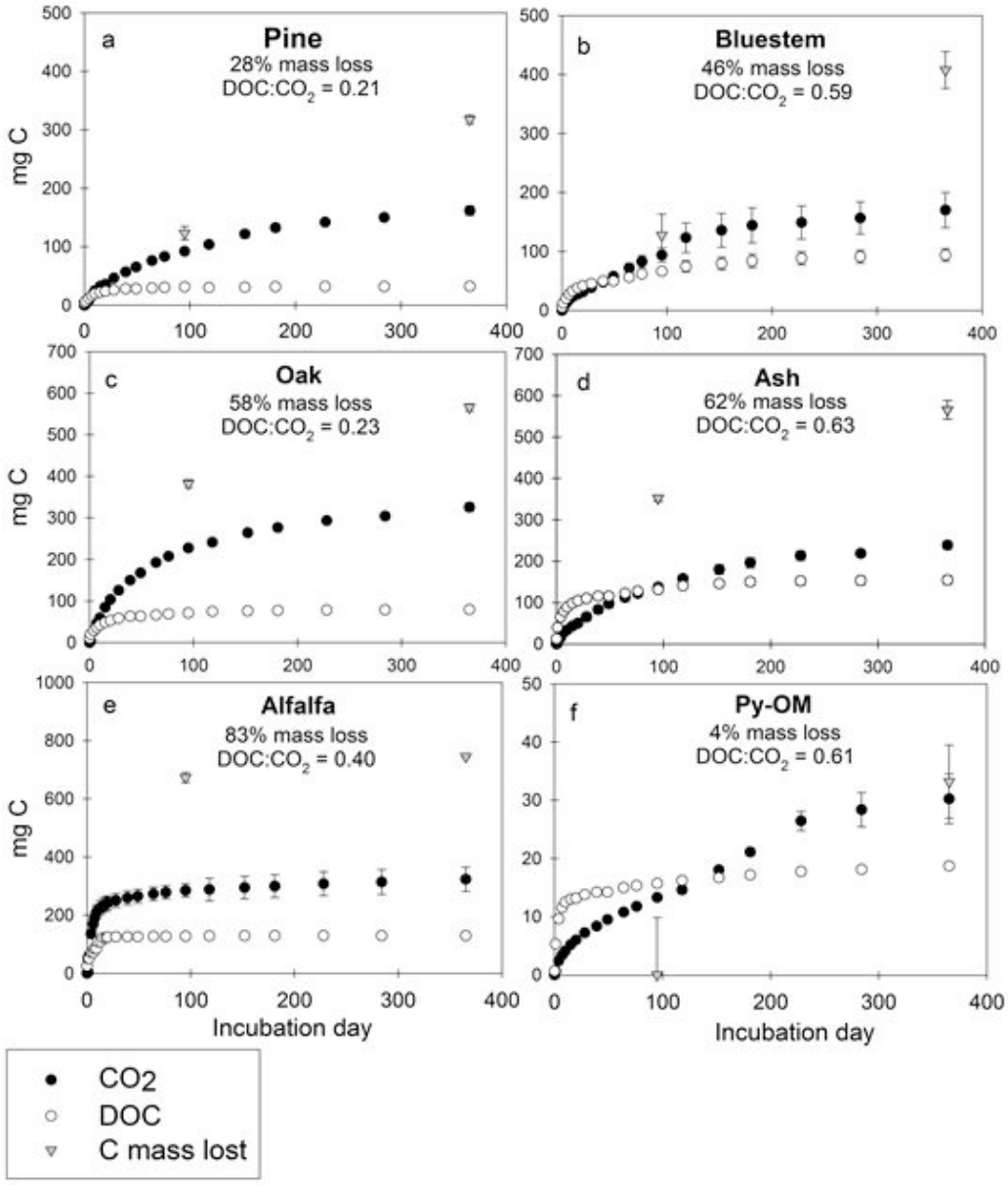

Figure 3.1. Residue carbon losses, DOC leaching and CO_2 efflux dynamics over the course of the 365 day incubation of five above ground leaf litters and one pyrogenic organic matter (py-OM). Black circles are CO_2, open circles are DOC, and grey inverted triangles are residue C lost. N=3, error bars are standard error.

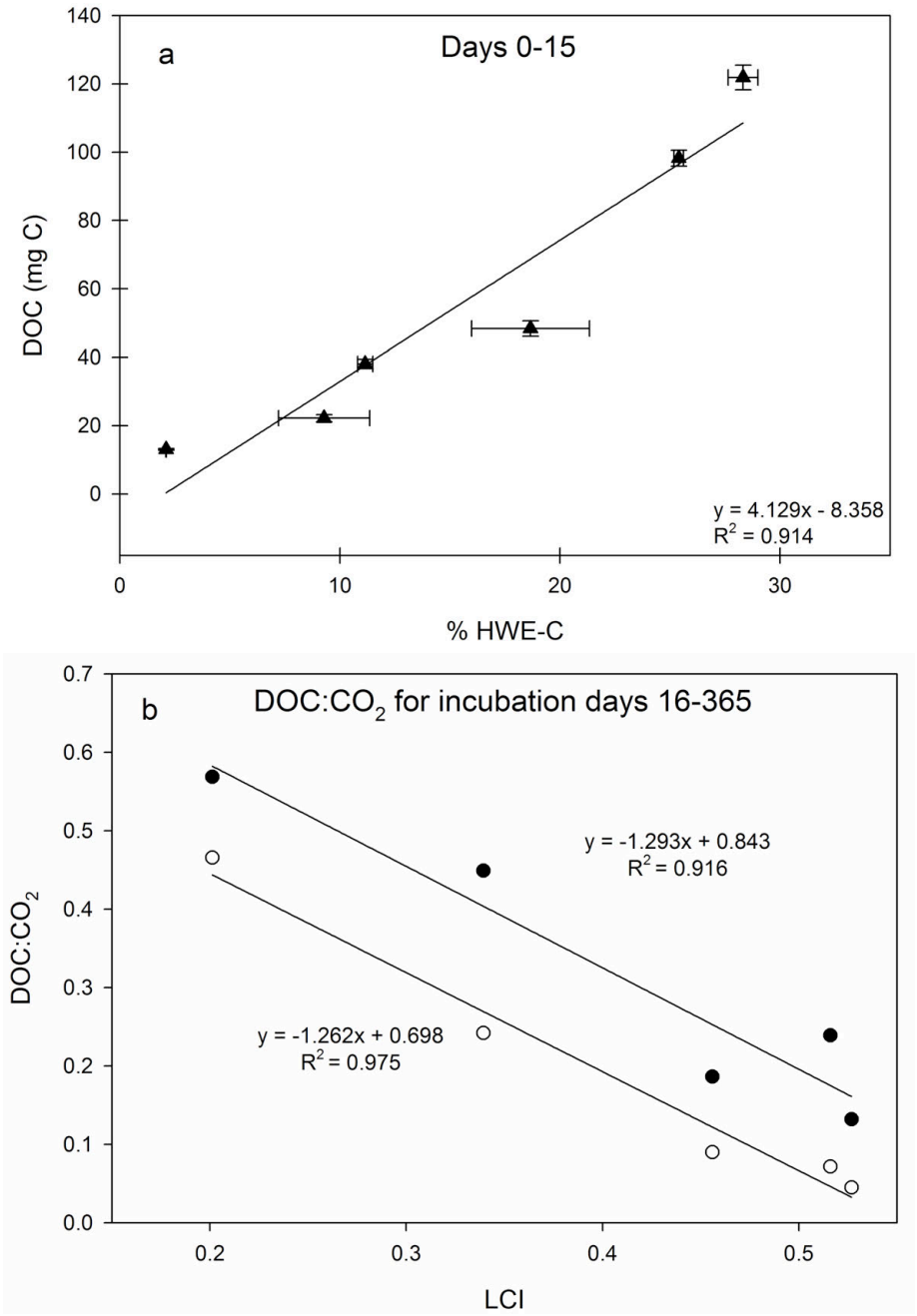

Figure 3.2. a) Average total DOC from the early phase leaching events versus % hot water extractable carbon (HWE-C) for all five litter types and py-OM. Bars are standard errors (n=6 for DOC, n=3 for HWE). b) Average DOC:CO_2 ratio vs. initial litter LCI for all litter types, except alfalfa, where we used the day 95 LCI (n=3). Closed circles are average DOC:CO_2 for days 16-64 (mid phase), open circles are average DOC:CO_2 for days 65-365 (late phase).

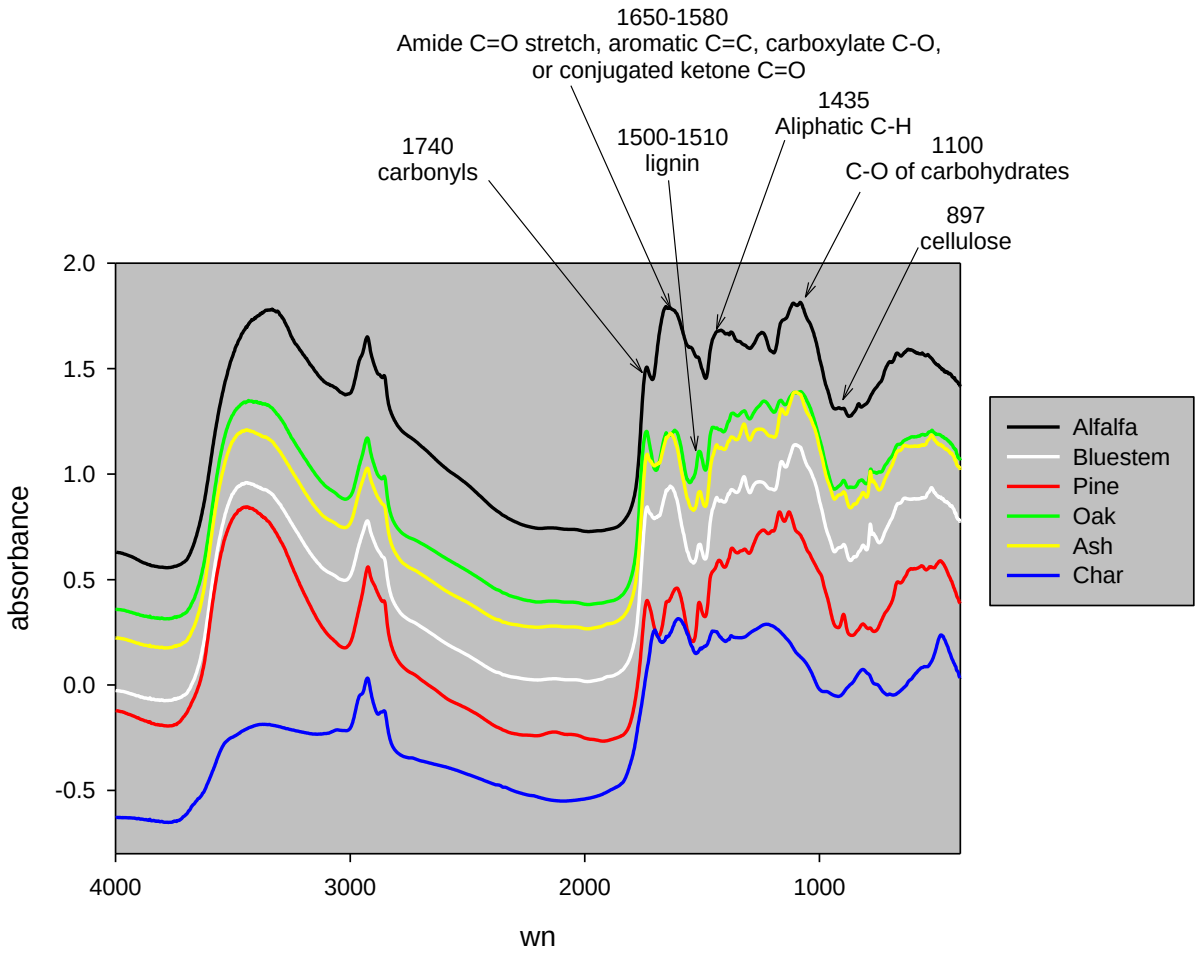

Figure 3.3. FTIR spectra of the initial litters and py-OM before the incubation. Absorbance is unitless and the spectra were stacked for better visualization.

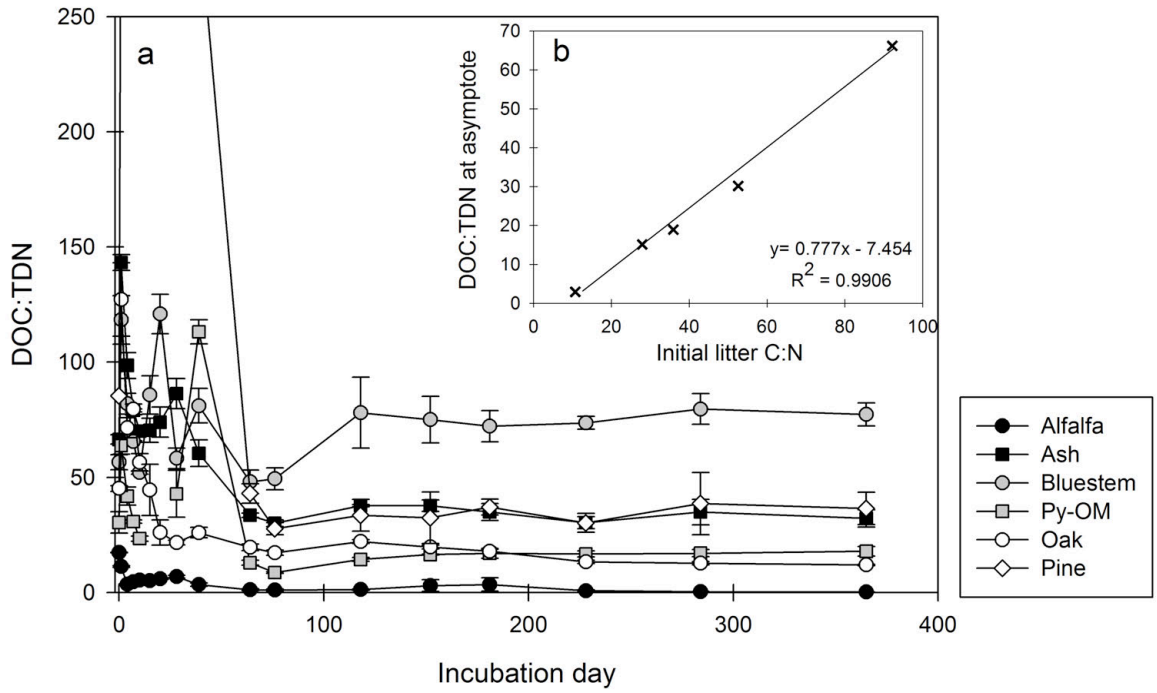

Figure 3.4. a) DOC:TDN ratio for all aboveground plant input types over the course of the incubation. Bars are standard error bars, N=6 for days 0-95 and N=3 for days 96-365. Y-axis scale is reduced to better display the DOC:TDN dynamics, cutting of the initial phase very high DOC:TDN of the pine. b) Regression of initial litter C:N versus average DOC:TDN of the asymptote for all aboveground plant input types except pine.

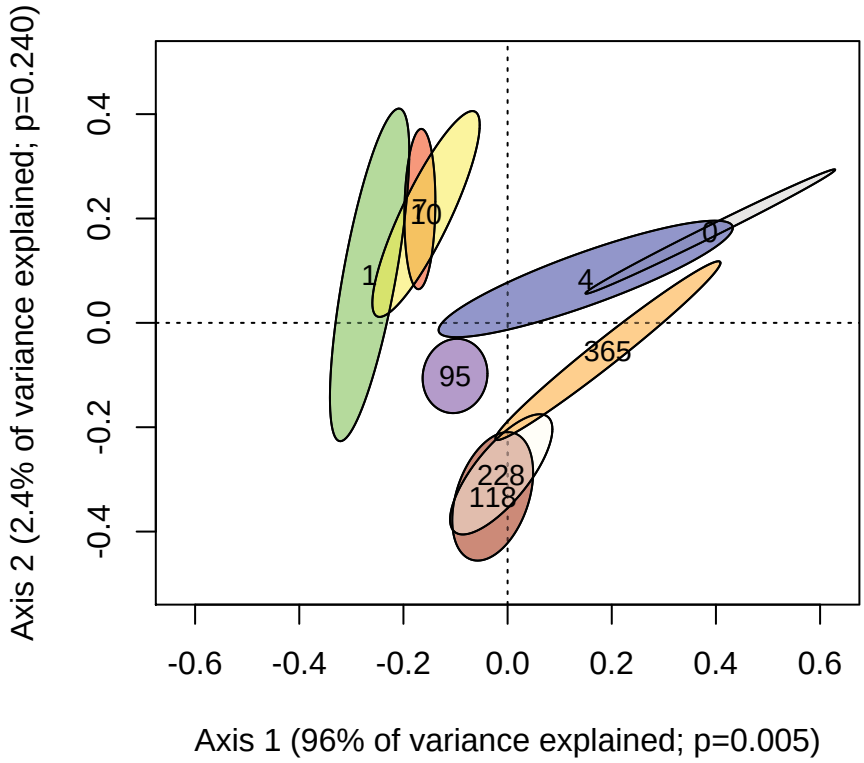

Figure 3.5. DB-RDA results of FTIR functional group chemistry of all litter DOM leachates over time.

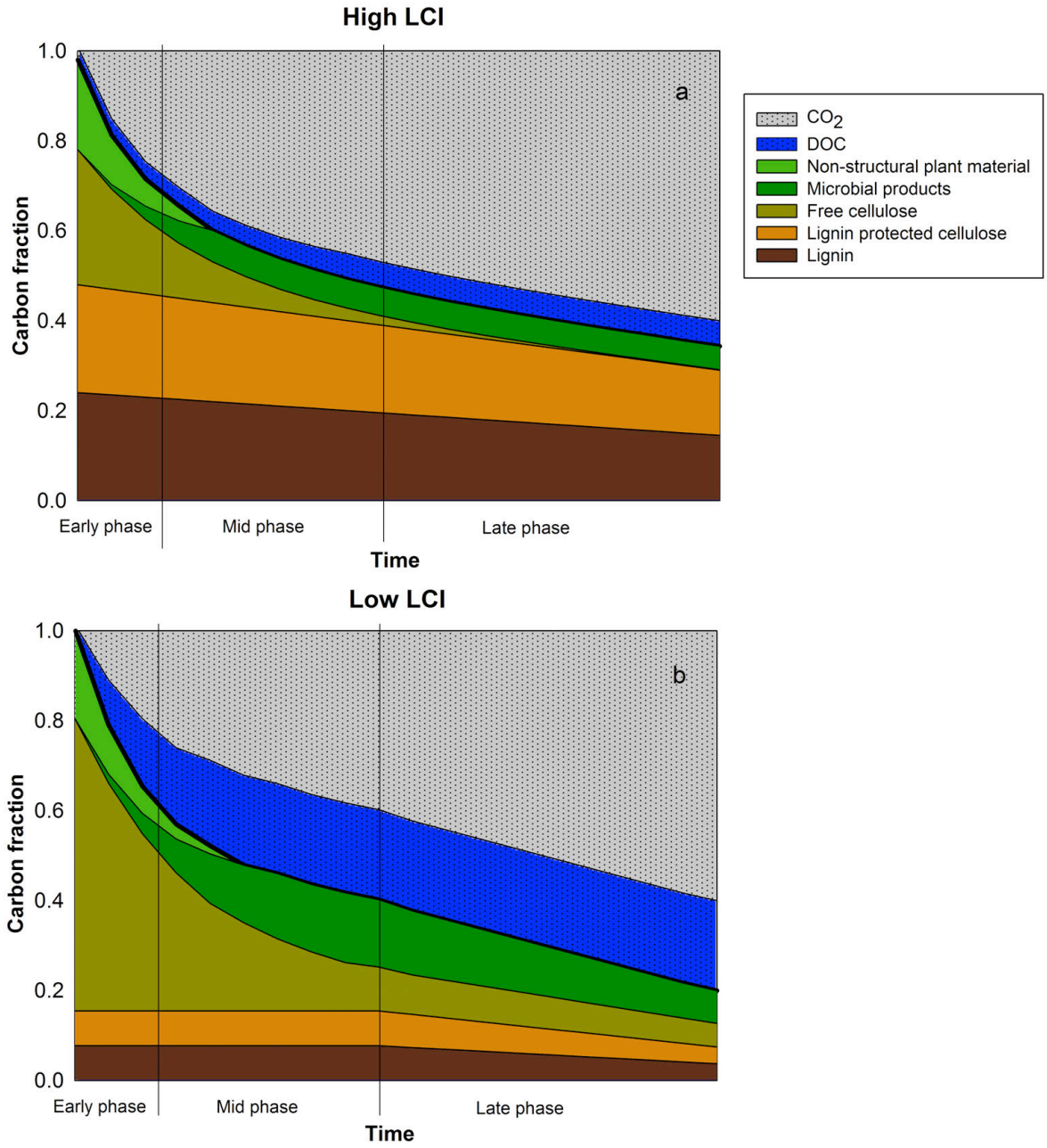

Figure 3.6. Conceptual model of the relative distribution of carbon in decomposing litter components (non-structural compounds, microbial products produced during decomposition, free cellulose, lignin protected cellulose, lignin), dissolved organic C (DOC) and CO_2, over time during decomposition of aboveground plant inputs to soil with (a) high lignocellulose index (LCI) or (b) low LCI. The carbon fraction remaining in litter components and DOC add to soil carbon stocks, while the carbon fraction in CO_2 is lost to the atmosphere.

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Chapter 4: Design and operation of a continuous ^{13}C and ^{15}N labeling chamber for uniform or differential, metabolic and structural, plant tissue isotope labeling [3]

Introduction

Understanding the dynamics of plant-soil-atmosphere processes is critical for accurately predicting how the global carbon (C) and nitrogen (N) cycles function under current and future environmental conditions. Stable isotopes are powerful tools in quantitative studies of plant-soil-atmosphere C and N cycling. Tracing rare stable isotopes from plant material through the ecosystem provides sensitive information in studies of biogeochemical cycling, from CO_2 fluxes and soil organic matter formation to small-scale stable-isotope biomarker probing [e.g.,](Denef et al. 2009; Bird et al. 2008; Rubino et al. 2010). Combining ^{13}C labeling with ^{15}N labeling, or other stable isotopes such as ^{2}H or ^{18}O in plant tissue provides a high-detection, traceable, yet complex substrate for use in coupled studies of plant and soil biochemistry. The ability to uniformly or differentially label structural and metabolic plant material adds further ability to address complex questions about C and N cycling through ecosystems. The benefit of using isotope labeled plant material in quantitative studies of C and N accounting, however, depends on the ability to produce ^{13}C and ^{15}N labeled material that is either uniformly or differentially labeled.

Isotope labeling has been used in studies addressing plant C and N assimilation(Andresen et al. 2008), allocation(Horwath et al. 1994) and rhizodeposition(Denef et al. 2007). Uniformly

[3] Soong J.L., Reuss D., Pinney C., Boyack T., Haddix M.L., Stewart C.E., Cotrufo M.F. Journal of Visualized Experiments (83) e51117 DOI 10.3791/51117 (2014). Corresponding video publication can be found at: https://www.jove.com/video/51117/design-operation-continuous-13c-15n-labeling-chamber-for-uniform-or

^{13}C and ^{15}N labeled plant material provides a complex labeled substrate for studies of litter decomposition(Rubino et al. 2010; Bird and Torn 2006), soil organic matter formation(Bird et al. 2003; Mambelli et al. 2011), soil CO_2 emissions(Rubino et al. 2010), soil food web studies(Pollierer et al. 2007), and studies of soil C residence times(Bird et al. 2003; Stewart and Metherell 1999). Studies utilizing ^{13}C labeled biochar from labeled plant material are also beginning to reveal new information about formally overlooked soil char pools(Santos et al. 2012). While ^{15}N, ^{2}H and ^{18}O labeling are relatively easy to achieve through water and fertilizer treatment, the challenge exists in producing uniformly ^{13}C labeled plant material through ^{13}C-CO_2 fixation.

Continuous isotope labeling from seedling to maturity in a sealed chamber produces uniform isotope labeling throughout the plant. Other methods such as repeated pulse labeling(Bromand et al. 2001) and foliar application or wicking(Putz et al. 2011; Wichern et al. 2010) do not produce uniformly isotope labeled plant material, nor clear differential labeling of specific C-compounds (e.g., metabolic vs. structural)(Fahey et al. 2011). An important consideration in isotope labeling is labeling efficiency, due to the high cost of rare isotope enriched compounds used in labeling. Although continuous ^{13}C labeling has been used in the past $^{e.g.,}$(Bird et al. 2003; Stewart et al. 2008; Rubino et al. 2010; Denef and Six 2006), there is not to our knowledge a published detailed technical description of a continuous labeling chamber with evidence of high labeling efficiency and accurate control of the amount and uniformity of isotope labeling.

On the forefront of litter decomposition and soil organic matter formation research is the concept that metabolic plant material (i.e. leachable, labile, low molecular weight compounds) and structural plant material (i.e. lignin, cellulose, hemicellulose) are processed differently in

terms of microbial use efficiency, soil organic matter formation, and long term soil C storage (Cotrufo et al. 2013; Prescott 2010; Mambelli et al. 2011). Plant material that is differentially labeled in its structural and metabolic components, therefore, is a useful tool in advancing litter decomposition and soil organic matter formation research. Differential labeling with dual isotopes allows tracing of structural and metabolic components separately through the ecosystem using a multiple-pool isotope technique(Fry 2006).

Continuous isotope labeling with ^{13}C and other isotopes in a sealed chamber requires careful attention to plant physiological conditions to maximize plant productivity and isotope labeling efficiency. Daytime temperature spikes must be controlled to prevent plant damage when growing in an airtight chamber. An optimal range of humidity and temperature are required to maintain open plant stomata and CO_2 uptake (Nippert et al. 2009). High levels of humidity cause fogging of the chamber walls, which minimizes light availability and may damage the chamber structure. Careful consideration to isotope labeling efficiency by eliminating natural abundance isotopes from the chamber (e.g., coming from potting with soil organic matter) and preventing exposure to external air is important when working with expensive heavy-isotope labeled compounds.

Here, we present a method for building and operating a continuous dual ^{13}C and ^{15}N isotope labeling chamber for the production of plant material that is either uniformly labeled or has its structural and metabolic components labeled at distinct levels. ^{13}C labeling is controlled at the chamber level, while fertilization and ^{15}N labeling is controlled at the individual pot level. Representative results are shown to demonstrate the ability of this method to control temperature, humidity and CO_2 concentration throughout the growing season. Results from growing *Andropogon gerardii* Kaw also demonstrate this method's ability to produce uniformly or

differentially labeled plant material. The specific chamber design and operation scheme described can be modified to grow different plant species, as well as to accommodate ^{2}H or ^{18}O labeling.

Protocol

1. Chamber construction

1.1) Construct the labeling chamber in a greenhouse to allow for maximum natural light potential for plant growth. Make sure that adequate power supply is available to power all chamber components.

1.2) Construct the labeling chamber by mounting 3.175 mm thick transparent acrylic walls (polycarbonate would also be suitable) and a 6.35 mm thick transparent acrylic ceiling on an aluminum frame with a white-painted steel floor to maximize solar reflectance. The dimensions of the chamber can be tailored to suit individual research needs.

1.3) Mount the chamber on ¾ inch (19 mm) plywood on cinder blocks.

1.4) Drill holes in the acrylic glass, aluminum frame and steel floor and use screws to fasten all components together.

1.5) Seal all seams with silicone to insure an airtight seal.

1.6) Construct a door by mounting one section of the acrylic paneling on long screws, which can be screwed down using removable wing nuts.

1.7) Seal the door with weather stripping to prevent air leakage.

1.8) Select an area directly adjacent to the chamber as the control center to mount all temperature, humidity, CO_2 controls and monitoring equipment.

1.9) Carefully drill small holes in the chamber wall adjacent to the control center for all electrical wires and gas tubing. Use silicone to seal the holes around all wires and tubing to prevent air leakage.

1.10) Test the chamber for air leakage by filling it with a high level of CO_2 (e.g., 800 ppm) and letting it sit overnight. If the CO_2 concentration in the chamber is maintained at its original level then it is airtight. If the concentration drops overnight then all seams should be examined and re-sealed with silicone until an airtight seal is achieved.

1.11) For some plants adapted to high light conditions, add lights connected to a timer to the immediate exterior of the chamber to increase light penetration and plant productivity.

2. Temperature and humidity controls

2.1) Regulate chamber temperature by installing a commercial split type air conditioner with the cooling (evaporator) coils located inside the chamber and the compressor and condenser coils located outside the greenhouse to dissipate the heat. Set the air conditioner to maintain the desired temperature.

2.2) Use a small room dehumidifier to control humidity in the chamber.

2.2.1) Drill a drainage hole through the floor of the chamber adjacent to the dehumidifier.

2.2.2) Remove the condensate collector from the dehumidifier, and connect a drainage tubefrom the dehumidifier through a drain hole in the floor of the chamber.

2.2.3) Underneath the chamber, place an open jar filled with water for the dehumidifier to drain directly into. This creates an airtight seal but also allow for pressure equilibration.

2.3. Install a controller, such as the Omega iSeries controller, in the control center with a humidity sensor inside the chamber.

2.3.1) Connect the controller to the dehumidifying system with a solid-state relay and set the humidity controller with a high alarm and swing to maintain optimal growing conditions in the chamber. Optimal temperature and humidity conditions will differ for different plant species.

3. CO_2 controls

3.1) The ^{13}C-CO_2 enrichment is achieved using two pure CO_2 gas tanks, one of 10 atom% ^{13}C-CO_2 or higher and one of 1.1 atom% ^{13}C-CO_2 (natural abundance).

3.2) Monitor CO_2 concentration by having a diaphragm pump continuously draw chamber air through an Infrared Gas Analyzer (IRGA) and then pump the air back into the chamber, thus maintaining a closed system (Figure 1).

3.3) Set a low alarm and dead band on the IRGA software to maintain CO_2 concentrations within a desired range. Here, we use a low alarm of 360 ppm with a 40 ppm dead band to maintain CO_2 concentrations between 360-400 ppm.

3.4) Connect a metering valve to each tank and carefully adjust them to achieve the target ^{13}C enrichment level. Set the tank regulators to 20 PSI.

3.5) Insert a solenoid valve between the metering valve and the regulator of each tank. Join the outlets of the two metering valves together and pipe them into the center of the chamber. Wire a solid-state relay to the IRGA output to control the solenoid valves (Figure 1).

4. Web-based remote monitoring system

4.1) Monitor CO_2 concentrations from the IRGA software by logging it to a local file once every 30 seconds.

4.2) Create a custom utility (e.g., in perl or another programming language) to pick up entries from the local CO_2 logging file, along with the current laptop timestamp, and upload them to a back-end web application.

4.3) Set the web application to query the temperature and humidity sensor data.

4.4) Use a monitoring system to check the status of the temperature in the chamber every five minutes to prevent potential temperature spikes that would destroy the plants if the air conditioning system failed.

4.5) Monitor the CO_2, temperature and humidity data on any standard web browser so that any unexpected temperature spikes or CO_2 drops can be immediately attended to.

5. Irrigation system

5.1) Drill one small hole in the wall of the acrylic glass chamber per pot.

5.2) Use irrigation tubing to create one drip irrigation ring per pot and feed the irrigation tubing through the chamber wall to the exterior.

5.3) Seal the holes around the irrigation tubing with silicone to prevent air leakage.

5.4) On the exterior of the chamber, connect the irrigation tubing to the tubing for a peristaltic pump.

5.5) Use a small hose clamp to close off all irrigation tubing to prevent air leakage between watering.

6. Potting plants

6.1) Select a pot size appropriate for the plants being grown. Here, we used 40, 15 liter pots.

6.2) Create a soil-free potting mix by mixing sand, vermiculite, and profile porous ceramic.

6.3) Test the water holding capacity of the potting mix by weighing a filled pot dry, soaking the pot with water and allowing it to drain completely, and weighing the pot wet. Use this maximum water holding capacity to ensure that excess labeled fertilizer and water does not leak from the pots during watering.

6.4) Germinate seeds in potting soil prior to planting them in the pots. This ensures that only successfully germinated seeds are started in the labeling chamber.

6.5) Inoculate the seedlings with a fresh soil slurry to introduce beneficial microbes.

6.6) Once the seeds have germinated, carefully transplant seedlings to the pots, in the desired number.

6.7) Once potted, move the pots into the chamber and assemble each pot with an individual irrigation hose.

7. Sealing the chamber

7.1) When first sealing the door to the chamber a large mass of external air fills the chamber space. Scrub out this external CO_2 by connecting a soda lime scrubber to the air pump to scrub the CO_2 concentration down to at least 200-250 ppm before filling the chamber back up to 400 ppm using the ^{13}C-CO_2 tank mixture.

7.2) Try to keep the chamber closed through the duration of the growing season to minimize natural abundance CO_2 contamination.

7.3) Monitor plant growth visually and adjust fertilization and irrigation according to demand.

8. Fertilization and irrigation

8.1) Use a fertilizer solution, such as a modified Hoagland's solution(Hoagland 1950), to fertilize the plants through the irrigation system.

8.2) Label the fertilizer with ^{15}N by using a ^{15}N-KNO_3 sub-solution at the targeted atom% ^{15}N level by mixing 98 atom% ^{15}N-KNO_3 with natural abundance ^{15}N-KNO_3 (0.37 atom% ^{15}N).

8.3) Mix up enough of the fertilizer solution upon each fertilization event for the entire chamber, based on the water holding capacity of the potting mix. Place the fertilizer amount required to fertilize one pot into a glass jar, and prepare as many jars as the number of pots.

8.4) Unclamp the irrigation hoses and place each of them in a jar with the fertilizer solution, and then connect them to the peristaltic pump.

8.5) Water plants by pumping water through the peristaltic pump through the individual drip irrigation hoses as regularly as the plants need it.

8.6) Fertilization with Hoagland's solution should follow the plant demand or experimental design, with increasing nutrient demand as the plant productivity increases to maximize productivity.

8.7) First, pump the Hoagland's solution through the irrigation hose then pump a water rinse through to minimize algal and bacterial growth in the tubes.

8.8) Re-clamp all hoses after fertilization and irrigation to eliminate chamber air leakage.

9. Uniform and differential labeling

9.1) For differential labeling of structural and metabolic components, remove plants from labeling chamber 1-3 weeks prior to harvest. Plants that are to be uniformly labeled can remain in the sealed labeling chamber continuously until harvest and irrigated with ^{15}N-Hoagland's solution.

9.2) Keep the removed plants in the greenhouse during this time so that they receive adequate light and CO_2 at natural abundance ^{13}C.

9.3) For differential ^{15}N labeling, continue to fertilize and irrigate the plants as usual, but use natural abundance ^{15}N-KNO_3 in the Hoagland's fertilizer solution.

10. Harvest

10.1) Stop watering plants 1 week prior to harvest so plants begin to senesce and potting medium dries out.

10.2) Open the chamber and move the pots out for immediate clipping and harvest

10.3) Pour out the potting mix and roots over a coarse screen.

10.4) Use the screen to separate out the roots from the potting mix. Shake the roots free of the potting mix and place them in a paper bag.

10.5) Place the roots on a 2 mm sieve and rinse them with water to remove any remaining potting material. Use tweezers to remove any vermiculite that may cling to the roots.

10.6) Allow roots to air-dry in preparation for future experiments.

11. Litter Chemistry

11.1) Weigh air-dry plant material to determine labeling chamber biomass.

11.2) Grind a subsample for chemical analysis.

11.3) Place 2.0 g of oven dried (60°C) litter in a 125 mL acid washed flask and add 50 mL of de-ionized water.

11.4) Place the sample on a pre-heated (60°C) stirrer plate and place a stirrer bar in the flask. Set the stirring for 200 rpm and allow the sample to heat for 30 minutes.

11.5) After 30 minutes, filter the litter solution through a 20µm nylon mesh on a vacuum filtration system.

11.6) Transfer the extract to a pre-weighed acid washed tube and freeze.

11.7) Transfer the solid litter residue to a pre-weighed aluminum pan and dry at 60°C. Weigh pan and litter after drying to determine hot water residue mass.

11.8) Freeze dry the hot water extract and weigh to determine the hot-water extract mass.

11.9) Analyze oven-dried (60°C) litter, freeze-dried hot water extract, and oven-dried hot water residue in an Elemental Analyzer - Isotope Ratio Mass Spectrometer (EA-IRMS).

Representative results

Our labeling chamber is 1.2x2.4x3.6 meters in size and holds 40, 15 L pots (Figure 1). The computerized IRGA control system maintained CO_2 concentrations between our set values of 360 and 400 ppm during the photosynthetically active period of the day (Figure 2a). The low CO_2 alarm feature on the IRGA triggered solenoid valves to allow CO_2 from the ^{13}C enriched and natural abundance tanks into the chamber when the concentration dropped below the minimum threshold (e.g., 360 ppm). The dead band feature stopped the

flow when the concentration reached the upper set point (e.g., 400 ppm). The iSeries temperature and humidity monitoring system connected to the air conditioner and dehumidifier held climate conditions within the set parameters throughout the growing season (Figure 2b). We used a one-ton (3.5 kW) air conditioning unit to keep the chamber cool.

The remote monitoring system allowed the logged data to be viewed at any time by a standard web browser. The CO_2 concentrations, temperature and humidity values were down sampled by the web application to display graphs over the past 24-240 hours, in 24 hour increments. This created a quick visual to confirm that the daily fluctuations were within the expected limits. Viewing the web interface also showed the current chamber status, as well as provided alerts to potential problems such as not receiving recent data. At any time the complete dataset could also be downloaded from the web interface.

We measured photosynthetically active radiation (PAR) in the immediate interior and exterior of the chamber at four points with and without the lights on in the middle of the summer and the middle of the day using a quantum sensor (Apogee Instruments, Logan, UT). The PAR in the chamber was 31.5% lower than the exterior when the chamber lights were off and 22% lower than the exterior when the lights were on. Thus, the chamber lights help to significantly increase PAR penetration within the chamber by 9.5% ($P<0.05$).

Our continuous labeling system was able to produce 2759 g of *A. gerardii* biomass, 37% of which was aboveground biomass and 63% of which was belowground biomass. We achieved a 4.4 atom% ^{13}C whole plant label in our uniform plant material by setting the solenoid valves on the two CO_2 tanks accordingly (Figure 1, Table 1). We achieved a 6.7 atom% ^{15}N whole plant label in our uniform plant material by mixing 98 atom% ^{15}N-KNO_3 with 0.37 atom% ^{15}N-KNO_3

in the KNO_3 sub-solution of a modified Hoagland's solution(Hoagland 1950) (Table 1). We watered the *A. gerardii* weekly with 750 ml total fluid (water plus Hoagland's solution) throughout the growing season. We fertilized with 200-500 ml of ^{15}N labeled Hoagland's solution per week depending on plant productivity.

We utilized the hot water extraction method to determine if there were isotopic differences between the uniform and differentially labeled plant material. For the differentially labeled plants, upon harvest we removed any leaves that were completely dead and handled these separately as they were likely not differentially labeled. When looking at ^{13}C content all four incorporation days were significantly different from each other for the whole plant and the hot water extract, but for the hot water residue day 14 and 22 were not significantly different from each other (Table 1). When comparing the plant tissue fractions within each day, the hot water extract and residue were significantly different from each other for all four days and by day 22 the whole plant, extract, and residue were all significantly different from each other (Table 1). For the ^{15}N incorporation into plant components there were differences between days of incorporation and plant tissue fractions. For the hot water extract all four of the incorporation days were significantly different from each other for ^{15}N, and for the whole plant and the hot water residue the shorter days of incorporation were significantly different than the longer days of incorporation (Table 1). The plant tissue fractions in the uniform plants were not significantly different from each other in ^{15}N, but the hot water extract and residue were significantly different from each other in ^{15}N for the differentially labeled litter.

All isotopic values are reported using the atom percent (atom %) notation (eq. 1), which is a more accurate notation than ‰ to use at high levels of heavy isotope enrichment (Fry 2006). For example:

$$Atom\ \%\ ^{13}C = \frac{^{13}C}{^{13}C + ^{12}C} \times 100 \quad (1)$$

For this study, we ran statistical analyses using SAS version 9.2 (SAS Institute, Cary, NC, USA). We tested differences between the chamber interior and exterior light levels, using a paired t-test. We tested differences between ^{13}C and ^{15}N labeling of hot water extracts and hot water residues using one-way analysis of variance (ANOVA) in PROC ANOVA. We used Duncan's multiple range test for multiple comparisons analysis. Significance was accepted at a P- level of 0.05. We used a Wilcoxon rank sum test to test that the data met the assumptions of the analysis.

Discussion

This design for a continuous isotope labeling chamber was used to produce uniformly and differentially ^{13}C and ^{15}N labeled *A. gerardii* for subsequent field and laboratory experiments. During three growing seasons of operation, the chamber has successfully maintained temperature, humidity, and CO_2 concentrations within the set parameters (Figure 2). The reliability of the temperature control system is critical during the peak of the summer when high solar radiation can cause overheating in the air-tight chamber. Eliminating excess humidity caused by transpiration from the growing plants ensures that plant stomata remain open for photosynthetic uptake (Nippert et al. 2009) and that water condensation does not inhibit light penetration or damage the structure of the chamber.

The near continuous monitoring of CO_2 concentration by the IRGA software maintained continuous ^{13}C labeling of the plants while they were growing in the chamber. Due to the high photosynthetic activity of *A. gerardii* growing in this chamber, CO_2 was injected into the system frequently during daylight hours of the peak growing season when photosynthetic activity drew

CO_2 concentrations down to 360 ppm, approximately every 15-20 minutes. The metering of the enriched and natural abundance ^{13}C-CO_2 tanks allowed for a controlled 4.4 atom% ^{13}C atmosphere through the growing season for uniform plant tissue labeling. ^{13}C-CO_2 production can also be achieved by mixing ^{13}C-sodium bicarbonate or ^{13}C-sodium carbonate and hydrochloric acid, however this type of system is more complicated and requires more monitoring and maintenance, so we recommend using ^{13}C-CO_2 gas. An important consideration for monitoring CO_2 concentrations using an IRGA is that infrared analyzers lose two thirds of their sensitivity when measuring ^{13}C-CO_2. This underestimation of approximately 2.9% for our 4.4% ^{13}C-CO_2 mixture was not of great concern to us, but could become a more significant issue when labeling at higher ^{13}C levels[27].

A. gerardii is a warm season perennial tallgrass prairie graminoid species. The design of this chamber was optimized for *A. gerardii* production (Figure 1). The size and height of the chamber were chosen in consideration for the maximum height productivity of *A. gerardii* in the field, as well as for the desired plant biomass production for future experiments. *A. gerardii* is known to be light limited in the field (Lett and Knapp 2003; Knapp and Seastedt 1986). PAR within a greenhouse can be diminished by 30-47% as compared to exterior levels(Ting and Giacomelli 1987). Since our plants were grown in an acrylic glass chamber inside a greenhouse, PAR limitation was a concern. When turned on, the fluorescent lights increased PAR within the chamber by 9.5%, which may have helped to increase productivity in this light sensitive species. When using this chamber design to grow other types of plants specific physiological needs such as size, lighting, nutrient demands, temperature sensitivity, and soil moisture should be carefully tailored to maintain optimal growing conditions for the plants.

When working with expensive isotopically labeled compounds, such as 10 atom% ^{13}C-CO_2 and 98 atom% ^{15}N-KNO_3, efficiency of labeling is an important consideration. This chamber design optimizes ^{13}C labeling by making all efforts to seal the chamber and minimize air leakage into and out of the chamber. If this chamber is never opened during the growing season, then none of the ^{13}C labeled CO_2 from the chamber is leaked out to the atmosphere. The CO_2 build up during nighttime respiration does not appear to damage the growing plants and is quickly taken up after sunrise (Figure 2). During differential labeling, the chamber was briefly opened to remove the differentially labeled pots but this did not appear to dilute the targeted 4.4 atom% ^{13}C labeling of the continuously labeled plants (Table 1). ^{13}C labeling was also optimized by scrubbing out the initial atmospheric air trapped in the chamber upon sealing. During preliminary tests on the chamber without an initial scrub of atmospheric CO_2, plants were measured to have a diluted ^{13}C level in the first leaves produced than in the later leaves produced. The initial scrub of atmospheric CO_2 upon chamber closure appears to eliminate this issue by allowing for continuous ^{13}C labeling from seedling to maturity. Maintaining a soil-free potting mixture of sand, vermiculite and clay also eliminates natural abundance CO_2 contamination from soil respiration. The elimination of soil from the system does require careful fertilization and inoculation considerations, which may be unique to different plant species. ^{15}N labeling through the targeted 7 atom% ^{15}N Hoegland's solution produced highly labeled plant material at 6.7 atom% ^{15}N (Table 1). A slight dilution from the targeted ^{15}N label may be caused by some natural abundance N in the potting mix or from the native soil inoculation.

During biosynthesis of compounds, natural discrimination of ^{13}C (or ^{15}N) occurs as a result of kinetic fractionation and non-statistical isotope distribution in the synthesized compounds. Thus, in the case of C, secondary products (e.g., lipids, phenol compounds) are

generally depleted in ^{13}C as compared to primary products (carbohydrates). This natural ^{13}C discrimination appears to persist also when plants are grown in an enriched ^{13}C atmosphere, as can be seen in the slight difference in atom% ^{13}C of the hot water extracts and hot water residues of the uniformly labeled plants (Table 1). This natural kinetic fractionation is very small compared to the enrichment and does not compromise the uniformity of the labeling.

Differential labeling of structural and metabolic plant tissues is a novel technique with potential for advanced studies in litter decomposition, microbial ecology and soil organic matter formation. The difference in ^{13}C and ^{15}N of the hot water extracts and hot water residues indicates a significant dilution of ^{13}C and ^{15}N in the leachable, low molecular weight compounds (hot water extracts) from the structural plant material (hot water residues) after 7, 14 and 22 days of differential labeling ($P<0.005$). This differential labeling of plant tissues can be used to track the fate of structural and metabolic components separately through an ecosystem. The ^{13}C differential labeling was more extreme than ^{15}N differential labeling. This may be due to the immediacy of ^{13}C dilution when removing the plants from the ^{13}C-CO_2 labeled atmosphere, while the ^{15}N label still remains in the potting mix for some time and ^{15}N dilution occurs more slowly. For more drastic differential labeling of ^{15}N, one may consider flushing the pots with water prior to natural abundance fertilization during the final weeks of growth outside the chamber.

The design and operation of this continuous ^{13}C and ^{15}N labeling system for uniform or differential, metabolic and structural, plant tissue labeling provides a novel method for producing isotopically labeled plant material for advanced research. The design and operational details of this chamber have been chosen for 4.4 atom% ^{13}C and 7 atom% ^{15}N labeling of *A. gerardii*, but can be tailored to other plant types and isotope labeling levels. The growing conditions

described here should be tailored to suit the size, temperature, humidity, light, water and nutrient demands of the particular plant species of interest. Labeling with ^{18}O or ^{2}H can also be achieved by labeling the water used in the irrigation system. The system described here addresses many of the challenges of uniform and differential ^{13}C labeling of plant material. This basic chamber design can be used by other research groups to produce highly labeled plant material for advanced studies in ecosystem biogeochemistry.

Table 4.1. Isotopic composition and litter chemistry for uniform and differentially labeled litter. Days of differential labeling outside the chamber are in parentheses. Comparisons between days of incorporation are in capital letters (across rows) and between litter fractions are in lower case letters (down columns) for each variable.

		Uniform (0)	**Differential (7)**	**Differential (14)**	**Differential (22)**
Whole Litter:	^{13}C Atom %	4.46±0.02 Aab	3.93±0.05 Ba	3.64±0.03 Ca	3.35±0.06 Db
	^{15}N Atom %	6.69±0.07 Aa	6.72±0.01 Aa	6.33±0.06 Ba	6.41±0.07 Ba
Hot Water Extract:	^{13}C Atom %	4.59±0.04 Aa	3.35±0.06 Bb	2.79±0.06 Cb	2.37±0.03 Dc
	^{15}N Atom %	6.69±0.03 Aa	6.43±0.01 Bb	5.89±0.07 Db	6.16±0.05 Cb
Hot Water Residue:	^{13}C Atom %	4.37±0.06 Ab	4.1±0.03 Ba	3.79±0.10 Ca	3.66±0.05 Ca
	^{15}N Atom %	6.57±0.04 Ba	6.71±0.02 Aa	6.45±0.02 Ca	6.44±0.03 Ca

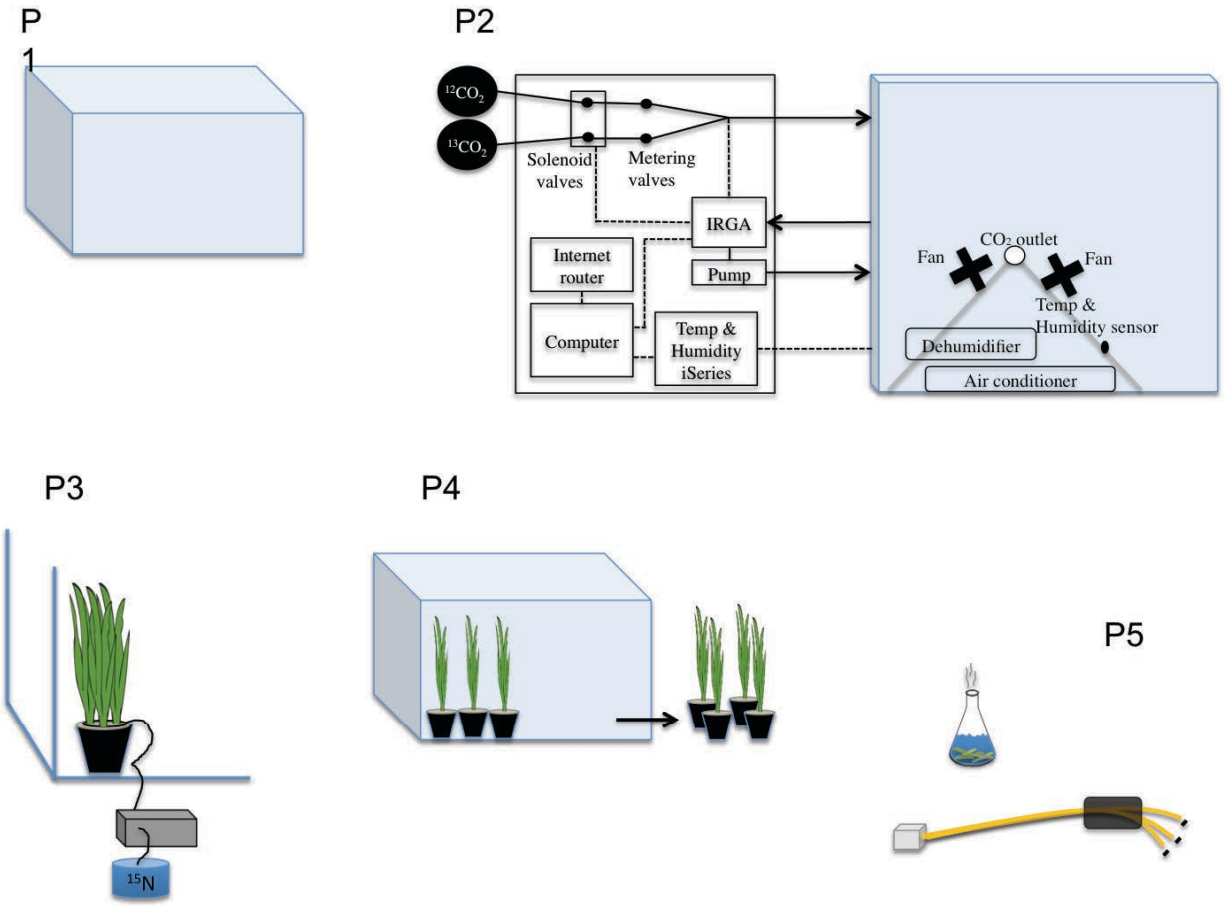

Figure 4.1. JoVE labeling chamber schematic diagram

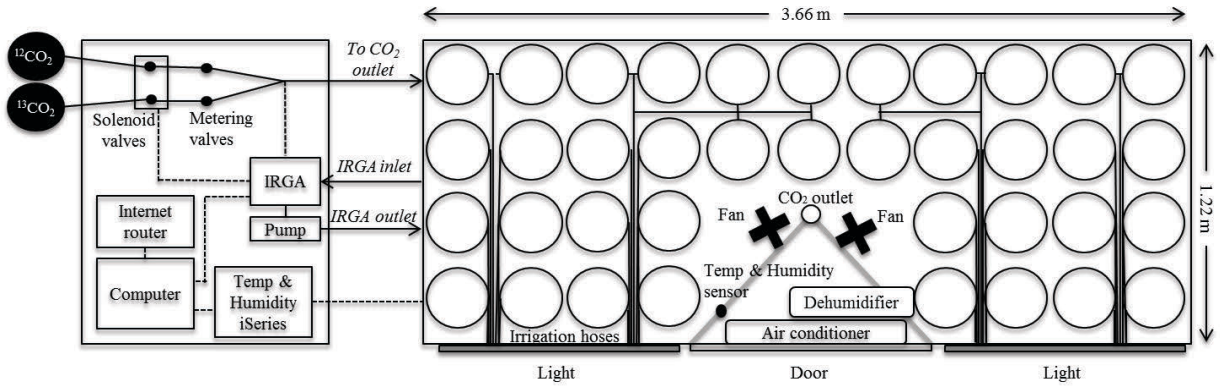

Figure 4.2. Schematic diagram of the 40 pot capacity continuous multi-isotope labeling chamber from a bird's eye view. Dotted lines represent electrical wiring, while solid lines represent gas or water tubing.

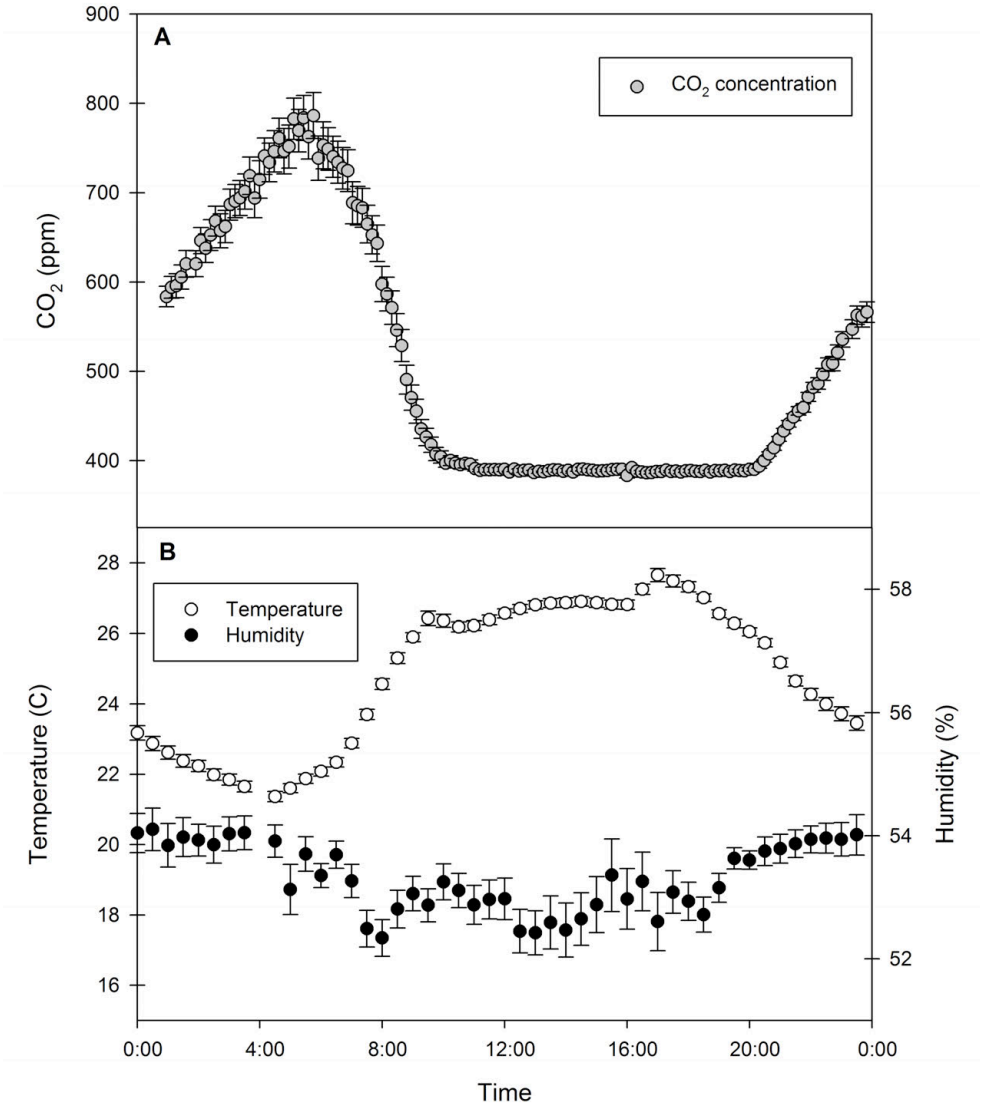

Figure 4.3. A) Average CO_2 concentration (ppm) (+/- SE) over a twenty-four hour period for an entire growing season. B) Average temperature (ºC), open circles, and humidity (%), closed circles (+/- SE) over a twenty-four hour period for an entire growing season.

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Chapter 5: Microarthropods increase stable soil organic matter formation from the decomposition of aboveground litter in a fine-textured grassland soil[4]

Introduction

In terrestrial ecosystems, litter decomposition is the main process by which carbon (C) fixed from the atmosphere by plants though photosynthesis is transformed into soil organic matter (SOM), where it can be stored for tens to thousands of years (Wardle et al. 2004; Trumbore 1993). In a world with increasing atmospheric CO_2 concentrations (IPCC 2013), accurately estimating how much C is stored in the mineral soil from plant litter decomposition, and what mechanisms control it, remains one of the most pressing issues in ecosystem sciences today (Schmidt et al. 2011; Cotrufo et al. 2013). Although much is known about how climate and litter chemistry affect litter decomposition rates (Berg and McClaugherty 2003), estimates of SOM formation and ecosystem C cycling can be much improved by the incorporation of biological mechanisms, which represent the biological processes underlying decomposition and SOM formation (Wieder et al. 2013). Soil fauna have long been recognized to play a major role in litter decomposition rates directly through physical fragmentation and mobilization, and indirectly through top down controls on microbial activity (Hattenschwiler et al. 2005). The impact of soil fauna on not just litter decomposition rates, but also on stabilized SOM formation during litter decomposition remains a largely unexplored area of research with important implications on biogeochemical cycling under changing climate and land use scenarios.

[4] In preparation for submission to Ecology Letters with Karolien Denef, Andrew J. Horton, Diana H. Wall, William Parton and M. Francesca Cotrufo

The long-term soil C sequestration potential of SOM formed during litter decomposition depends on both the physical-chemical state of the SOM (Trumbore 1993) and its environmental conditions (Schmidt et al. 2011). Primary organomineral complexes isolated from fully dispersed soils account for the primary level of SOM stabilization (Christensen 2001). The light fraction (LF) of SOM (<1.85 g/cm^3) is comprised of litter fragments, which are more labile and have shorter mean residence times (MRTs) than the bulk soil (Trumbore and Zheng 1996). Within the heavy fraction of SOM (>1.85 g/cm^3), silt (2-53 μm) and clay (<2 μm) associated OM have longer MRTs due to chemical adsorption on mineral surfaces (Wiseman and Puttmann 2005; Schulten and Leinweber 2000). The free particulate organic matter, or sand sized heavy fraction of SOM (>53 μm), likely has an intermediate MRT due to the fact that is microbially transformed but not protected from decomposition by mineral association (Christensen 2001). The processes controlling how much decomposing litter C enters these four SOM pools, therefore affects how long it will remain stored in the soil.

Bacteria and fungi are the primary decomposers responsible for the transformation of litter into SOM. Decomposing litter fragments enter the soil in the LF through fragmentation, while soluble substrates leach into the soil with water (Swift et al. 1979). Microbial transformation of fresh litter inputs in the soil are now thought to be the main precursors to long-term stabilized SOM on soil minerals (Cotrufo et al. 2013). The microbial community is comprised of a diversity of organisms with variable physiologies and capabilities (Paul 2007). A shift to a microbial community more dominated by gram-positive bacteria and fungi, capable of degrading recalcitrant litter substrates, over gram-negative bacteria for example, could alter the rate and products of litter decomposition (Rubino et al. 2010). Conditions affecting the activity

and community composition of soil microbial decomposers could thus impact the transformation of litter material into stabilized SOM.

Soil mesofauna, such as microarthropods, are known to increase litter decomposition rates in temperate grasslands (Wall et al. 2008), and stimulate nutrient mineralization through their top-down soil food web regulation of the microbial community (Cole et al. 2004). The inclusion of soil fauna and soil food web dynamics into models of ecosystem functioning has be argued to be a major missing link in our understanding of soil processes (de Vries et al. 2013; Garcia-Palacios et al. 2013). If soil fauna affect the formation of stabilized SOM during litter decomposition, either directly by increasing litter fragmentation or indirectly through mediation of the microbial community, then alterations in soil food web dynamics due to environmental changes (Birkhofer et al. 2011; Kardol et al. 2011) could have major implications for soil C sequestration.

Quantifying the effects of soil microarthropods on stabilized SOM formation during litter decomposition, will help to resolve the debate over whether soil fauna should be incorporated into models of ecosystem functioning (Hunt and Wall 2002; de Vries et al. 2013; Garcia-Palacios et al. 2013; Kampichler and Bruckner 2009). If microarthropods merely accelerate litter decomposition processes without fundamentally altering it, then their role may be relatively inconsequential to terrestrial biogeochemistry. However if microarthropods inherently alter the decomposition process, by stimulating increased microbial activity and the formation of mineral stabilized SOM residues, then they would prove to play a significant role in terrestrial C cycling.

The major question our research addressed is: do soil microarthropod controls on leaf litter decomposition result in a net increase of SOM storage? We hypothesized that microarthropods promote C sequestration (i.e. the allocation of decomposing litter C to stable

forms in the soil) by enhancing litter fragmentation and its incorporation to depth in the soil in the form of LF, where it promotes soil aggregation and SOM stabilization. Additionally, we hypothesized that soil microarthropods stimulate microbial turnover, thus promoting the transfer of microbial metabolites belowground. This would result in greater sequestration of litter derived C in free POM and mineral associated SOM fractions. Finally, we hypothesized that soil microarthropods alter microbial community composition, enhancing the gram-positive: gram-negative ratio. This change in microbial community composition will facilitate the later stages of decomposition further enhancing litter decomposition and C flow belowground.

In order to test these three hypotheses, we incubated isotopically labeled *Andropogon gerardii* leaf litter in the field at a tallgrass prairie site and tracked its decomposition to CO_2 and into SOM fractions and microbial biomarkers for three years. We applied a naphthalene treatment to repel microarthropods in order to test the effect of microarthropod suppression versus a control treatment on litter decomposition and SOM formation.

Materials and Methods

Isotopically labeled litter production and analyses

Uniformly 3.4 atom % ^{13}C labeled *A. gerardii* Kaw was grown in a ^{13}C continuous labeling chamber as described in Soong et al. (2014). *A. gerardii* Kaw 2" seedlings purchased from a nursery (Fort Collins nursery, Fort Collins, CO, USA) were clipped down to the crown, washed free of all potting soil and grown in the labeling chamber in a sand-soil-vermiculite media to maturity for 15 weeks. At harvest, the plants were removed from the chamber and the aboveground partially senesced biomass (litter) was harvested by cutting at the crown, pooled in one homogeneous pool, and air-dried.

Three replicates of the initial litter were analyzed for % C, % N and $\delta^{13}C$ on an elemental analyzer connected to an isotope ratio mass spectrometer (EA-IRMS, Carlo Erba NA 1500 coupled to a VG Isochrom continuous flow IRMS, Isoprime inc.), and % hemicellulose, % cellulose and % lignin using the neutral detergent fiber (NDF) and acid detergent fiber (ADF) methods (Van Soest et al. 1991). In brief, the NDF procedure removes all starch, leaving behind hemicellulose, cellulose, lignin and ash by boiling the sample for one hour in a neutral detergent solution plus a heat stable amylase. For the ADF method, an initial heated digestion in Cetyl trimethylammonium bromide (CTAB) and sulfuric acid removes hemicellulose and other non-structural carbohydrates and lipids, leaving behind cellulose, lignin and ash, then the samples are further digested in 73% sulfuric acid, which removes cellulose. The final lignin and ash residues are combusted in a muffle oven to determine the ash fraction, and the hemicellulose, cellulose and lignin fractions are determined by gravimetric weight loss at each step. Native, senesced, partially decomposed *A. gerardii* litter from the field site was collected in June 2010 and similarly characterized for % C, % N, $\delta^{13}C$, % hemicellulose, % cellulose and % lignin.

Experimental site and design

The experiment was conducted at Konza Prairie long-term ecological research station in Kansas, USA. This is a tallgrass prairie, dominated by *A. gerardii*. Climate at the site is temperate-continental, with average annual precipitation of 835 mm and a mean annual temperature of 12.8ºC. The soils are a silty clay Mollisol, and present characteristics of the footslope soils at the site (Knapp et al. 1998a). The experimental area used for this study was burned annually from 1972-2000, when burning treatments ceased, except for one wild fire in 2008. A detailed description of the site can be found in Knapp et al. (1998a).

Site preparation and the soil microarthropod suppression (MS) treatment began in June of 2010. At that time we installed 20 cm diameter PVC collars at the site to a depth of 5 cm. We removed the native litter layer from within the collars and applied 4 ml of glyphosate (Roundup®) to deter plant growth within the collars. We pooled all of the collected native litter, air-dried it and used it for chemical analyses and field incubation as described below. We tested four replicates of the soil down to 20 cm for inorganic C by pressure transducer following acid addition and found no inorganic C at the site (data not shown). Additionally we collected four replicates of the 0-2, 2-5, 5-10 and 10-20 soil samples in a 5 cm diameter core to measure bulk density. The soil MS treatment consisted of monthly additions to the soil surface of 477 g/m^2 of naphthalene to repel micro-arthropods. Naphthalene, a polycyclic aromatic hydrocarbon ($C_{10}H_8$) is a common chemical additive used to suppress soil micro-arthropod abundances in field decomposition studies, with minimal non-targeted effects on soil microbes (Seastedt and Crossley 1983; Cotrufo et al. 2014). The MS treatment and its effects on the soil fauna are reported in Cotrufo et al. (2014). Overall, the abundance of oribatid mites, predatory mite and springtails in the soil was reduced in abundance by -45%, -52% and -49% respectively with the MS treatment. The MS treatment did not affect nematode, bacteria or fungal abundances (Cotrufo et al. 2014).

The litter decomposition experiment began on September 29, 2010, when 18.4 g of ^{13}C labeled *A. gerardii* litter was added to the PVC collars, and lasted for three years. Five destructive soil and litter harvests occurred at 5, 12, 18, 24 and 36 months after the addition of the labeled litter in the field. Harvest times were selected to capture more frequent early stage litter decomposition dynamics, and spaced further out later on as decomposition rates slowed. An additional set of PVC collars with 18.4 g of native *A. gerardii* litter collected from the site

were similarly treated and followed to compare our chamber produced litter decomposition rates to that of the native litter from the site.

The litter decomposition experiment consisted of a split-split-plot fully randomized complete block design with 4 replicate blocks. Within each replicate block, 5 whole plots (6 m^2) were randomly assigned to one of the five sampling dates. Each whole plot was split in half by naphthalene treatment (split-plots) so that in one half three subplots were treated with naphthalene (MS) while three subplots were not (Control). Each subplot consisted of three PVC collars, one with the native litter, with the labeled *A. gerardii* litter addition, and one which was left as bare soil for use in the isotope mixing model as described below. For each PVC collar, soil was sampled at different depths (sub-subplots). The experiment is thus treated as a split-split plot on a randomized complete block design, with sampling times assigned to whole plots, naphthalene treatment assigned to subplots and depth segment assigned to sub-subplots.

Soil CO_2 efflux and litter contribution

We measured soil CO_2 efflux and its isotopic composition periodically (intervals between CO_2 sampling ranged from 14 days initially to longer periods during the winter and as decomposition progressed) from September 2010 through September 2013 using a LI-8100 portable infrared gas analyzer (IRGA; LI-COR, Lincoln, NE) with a 20 cm diameter survey chamber (LI-8100, LI-COR, Lincoln, NE). We determined the rate of CO_2 efflux using the LI-8100 software, which fits a linear equation to the CO_2 concentration increase measured during the first 130 seconds of chamber closure. The chamber remained closed for a total of 600 seconds in order to achieve a CO_2 concentration range sufficient for application of the Keeling plot method (Keeling 1958) for estimating $\delta^{13}C$ of soil CO_2 efflux following Cotrufo et al.

(2014). Briefly, an atmospheric sample, as well as two gas samples at approximately 200 and 570 seconds during chamber closure were collected and used as three points in the Keeling plot calculation (Pataki et al. 2003).

We recorded the CO_2 concentration of the gas samples from the LI-8100 software upon gas collection in the field. We collected the gas samples using a two needle flow diversion into pre-evacuated 12 ml septum-capped glass sampling vials (Labco, UK). On the same day of gas sampling, we filled additional gas vials with a reference gas of known CO_2 concentration and $\delta^{13}C$. Within 24 h of collection, the gas sampling vials were brought back to the laboratory and $\delta^{13}C$ was measured on a Delta V isotope ratio mass spectrometer (IRMS) coupled to a GC-isolink unit with a pre-concentrator (Thermo Scientific, Waltham, MA, USA), within one week of sampling. Each day of $\delta^{13}C$ analysis, two reference gas samples were also analyzed for $\delta^{13}C$ and CO_2 concentration (LI-COR, LI-6252, Lincoln, NE, USA) and samples $\delta^{13}C$ and CO_2 values adjusted if necessary, following Tu et al. (2001).

Litter and soil sampling

On May 1, 2011 (7 months), October 8, 2011 (12 months), April 13, 2012 (18 months), September 29, 2012 (24 months), and September 25, 2013 (36 months), soil and litter samples were collected in each of the four replicates of each treatment. First, the litter was collected by hand and stored in plastic bags. Then an intact soil core (6 cm diameter) was collected to 20 cm depth and separated in the field into three depth segments (0-5, 5-10 and 10-20 cm) for microarthropod extraction. The remaining soil within the collar was then gently excavated with the use of hand shovels by incremental depths (0-2, 2-5, 5-10 and 10-20 cm) and the soil collected from each layer was stored separately in pre-labeled plastic bags. The corer and

equipment were sterilized with 70% ethanol before each sample to prevent isotopic cross contamination. All soil and litter samples were stored with ice in coolers before being brought to the laboratory the following day. There they were stored at 4°C until they were processed within two weeks of collection.

Bulk soil and litter

The litter was picked clean of any non-A. *gerardii* leaves, roots and soil, then weighed at field moisture. A subsample of the litter was oven dried at 65ºC for analysis of gravimetric water content, and another subsample was combusted at 660°C in a muffle oven to determine ash content. The bulk soil was sieved to 2 mm and then analyzed for gravimetric water content by mass loss after drying at 105ºC. All oven dry samples were then ground and analyzed for % C, % N, $\delta^{13}C$ and $\delta^{15}N$ on an elemental analyzer coupled to an isotope ratio mass spectrometer (EA-IRMS, All samples were analyzed on a Carlo Erba NA 1500 elemental analyzer and VG Isochrom continuous flow IRMS, Isoprime Inc., Manchester, UK except the 36 month soil and litter samples, which were analyzed on a Costech ECS 4010Costech Analytical Technologies, Valencia CA USA, coupled to a Delta V Advantage IRMS, Thermo-Fisher Bremen, Germany, which was cross calibrated with the Carlo Erba EA-IRMS).

Soil organic matter fractionation

In order to separate the primary soil organo-mineral fractions (Christensen 1992) we employed a physical soil organic matter (SOM) fractionation scheme modified from Denef et al. (2013). Briefly, a 5 g subsample of oven dried bulk soil from the 0-2 and 2-5 cm soil layers collected from the bare soil and labeled litter collars was dispersed with glass beads in 25 ml of

1.85 g cm^{-3} sodium polytungstate (SPT) to break up all aggregates. Then, the samples were centrifuged and the light fraction (LF <1.85 g cm^{-3}) was aspirated off of the sample and rinsed clean of SPT. The remaining heavy fraction was rinsed thoroughly of SPT then sieved through a 53 μm screen to separate the sand-sized (>53 μm) fraction from the silt and clay. This was further fractionated via centrifugation in water to separate the silt sized (>2 μm) from the clay (<2 μm) fraction based on Stockes Law. All fractions were oven dried at 105ºC prior to weighing and analysis of %C, %N, $\delta^{13}C$ and $\delta^{15}N$ on an EA-IRMS. The total fractions mass recovery was within +/- 5% of the initial mass.

PLFAs extractions and ^{13}C-PLFA measurements

A sub sample of the sieved bulk soils from the 0-2 and 2-5 cm depth layer collected from the bare soil and enriched litter collars was picked clean of all visible roots, frozen (-20ºC) and lyophilized for 48 h prior to PLFA extraction. PLFAs were extracted on these samples using conventional methods (Bligh and Dyer 1959; Denef et al. 2007). In brief, for the extraction, 6 g of freeze-dried soil were mixed with a 0.1 M potassium phosphate buffer:chloroform:methanol solution (0.8:1:2 ratio volume, ml g^{-1} of soil). Neutral, clyco- and phospholipids were separated over SPE silica columns eluting with chloroform, acetone and methanol, respectively. Phospholipids were saponified to obtain free fatty acids, which were subsequently methylated using 0.2 M methanolic KOH to form fatty acid methyl esters (FAMEs). FAMEs were quantified and analyzed for ^{13}C by capillary gas chromatography combustion isotope ratio mass spectrometry (GC-c-IRMS, Trace GC Ultra, GC Isolink and DeltaV IRMS, Thermo Scientific). A capillary GC column type DB-5 was used for FAME separation (length 30 m, i.d. 0.25 mm, film thickness 0.25 μm; Agilent). The GC temperature program proceeded at 60ºC with a 0.10

min hold, followed by a heating rate of 10ºC min^{-1} to 150ºC (2 min hold), 3ºC min^{-1} to 220ºC, 2ºC min^{-1} to 255ºC, and 10ºC min^{-1} to 280ºC with a final hold of 1 min. Individual fatty acids were identified based on relative retention times to an internal standard (12:0), which was added to the FAME extract prior to gas chromatography, and cross referenced with several standards in a mixture of 37 FAMEs (37 component FAME Mix, 47885-U, Sigma-Aldrich, USA). FAME identification was verified by analyzing a few samples on a capillary GC-mass spectrometer (Shimadzu QP-2010SE) with a SHRIX-5ms column (30 m length x 0.25 mm i.d., 0.25 μm film thickness) using the NIST 2011 mass spectral library.

Quantification was performed using relative response factors (RRF) relative to an internal standard (19:0), added to the FAME extract prior to GC analysis. RRFs were determined in advance by using a dilution series of the 37 component FAME mix, to which the 19:0 standard was also added. The abundance of individual PLFAs was calculated in absolute C amounts (ng PLFA-C g^{-1} soil) based on the PLFA-C concentrations in the liquid extracts.

The biomarker PLFAs analyzed within this dataset included: 18:1ω9c and 18:2ω6,9c (indicative of saprophytic fungi), 16:1ω5 (indicative of arbuscular mycorrhizal fungi-AMF), i15:0, a15:0, i16:0, i17:0, a17:0 (indicative of Gram-positive bacteria), cy17:0, cy19:0, 16:1ω7c and 18:1ω7c (indicative of Gram-negative bacteria), 14:0, 15:0, and 18:0 (indicative of non-specific bacteria, or bacteria (ns)) and 10Me PLFAs (indicative of Actinobacteria) (Kroppenstedt 1985; Olsson et al. 1995; Zelles 1997). ^{13}C values were corrected using the working standards (12:0 and 19:0) calibrated on an EA-IRMS. To obtain $\delta^{13}C$ values of the PLFAs, measured $\delta^{13}C$ FAME values were corrected individually for the addition of the methyl group during transesterification by simple mass balance (Denef et al. 2007).

Data analysis

The litter contribution to the CO_2, soil, and PLFA-C was assessed for the litter added plots as compared to the bare soil plots within each subplot. The isotopic mixing model was applied as follows:

$$f_{litter} = \frac{\delta_S - \delta_B}{\delta_{litter} - \delta_B}$$

where f_{litter} is the litter derived C fraction of bulk soil, SOM or PLFA sample, δ_S and δ_B is the $\delta^{13}C$ of the specific bulk soil, SOM or PLFA sample from the litter treatment collar (δ_S) and the back ground soil (δ_B), obtained from averaging across all bare collars (n=20; Table 2); and δ_{litter} is the $\delta^{13}C$ (or $\delta^{15}N$) of the initial litter. Litter derived-C pools in CO_2, bulk soil, SOM or PLFA sample were obtained by multiplying the f_{litter} values to corresponding C pool sizes. Background soil C pools (Table 2) were calculated for each soil depth and SOM fraction from the overall average (n=40) values of % C, % N, $\delta^{13}C$, % fraction and using bulk density values measured at the site.

We tested the effect of the MS treatment, sampling time and soil depth on the amount of litter incorporation into the bulk soil, SOM fractions and PLFAs using a general linear mixed model, including MS treatment, time of sampling, soil depth and all interactions as fixed effects and replicate block, block x sampling time and block x sampling time x MS treatment as random effects with standard variance components. Due to the decreasing correlation assumed between horizontal soil layers that are further apart, the depth effect for the bulk soil samples was modeled as a random effect with a first order autoregressive covariance structure with the levels of the block x sampling time x MS treatment term as subjects. The approximation by Kenward and Roger (1997) was used to compute the denominator degrees of freedom. Significance of all pairwise differences was determined using the Tukey-Kramer method for multiple comparisons.

We checked for normality of the data and homogeneity of variances of the residuals, and applied a log-transformation when necessary. We analyzed all general linear mixed models using SAS® software version 9.3. In all cases, we used type III tests of fixed effects.

To test the effect of the MS treatment and time on the overall microbial community based on the relative contribution of all of the individual PLFAs to the entire extractable PLFA pool (mol %), we utilized a distance-based redundancy analysis (dbRDA) using the R: Vegan package (Oksanen 2013), and following (Bell et al. 2014). Briefly, we chose the dbRDA analysis over other multivariate statistical approaches due to its non-linear distance-metric options, which have robust multi-dimensional resolution to assess categorical variables. Distance based RDA is a three step ordination technique that tests the effects of response parameters (i.e. mol %) on defined groups (i.e. MS treatment or time). First, a dissimilarity or distance matrix is calculated for the different treatments. We chose the Bray-Curtis dissimilarity (non-linear) measure to model the species matrix as suggested by Legendre and Anderson (1999a). For steps two and three of the dbRDA, a principal coordinate analysis (PCoA) is calculated based on the distance matrix, from which the eigenvalues (obtained in the PCoA) were applied to a redundancy analysis (RDA).

Results

Initial litter and site characteristics

The ^{13}C ($\delta^{13}C$= 2113 ‰) labeled *A. gerardii* leaf litter that we produced in our labeling chamber is isotopically very distinct from the soil (Table 2) at the site. The ^{13}C labeled *A. gerardii* leaf litter had a C:N ratio of 30, lower than that of *A. gerardii* collected from the site (C:N=74) due to its higher N content (Table 1). The ^{13}C labeled *A. gerardii* leaf litter also had a

lower % cellulose and lignin content, but a similar % hemicellulose content, compared to the native *A. gerardii* collected from the site (Table 1).

Microarthropod suppression effect on litter mass loss

After 36 months of decomposition in the field, the labeled *A. gerardii* litter had lost 98% of its initial mass (Figure 1). Sampling time ($p<0.0001$) and MS treatment ($p=0.0215$) had significant effects on the amount of litter mass remaining during the experiment. At 7, 12 and 18 months the control treatment had less mass remaining than the MS treatment, however there was no difference in the mass remaining after 24 and 36 months of field incubation (Figure 1). The native litter (NL) reached 81% mass loss by the end of the experiment. The MS treatment did not affect mass loss rates of the NL as compared to the Control ($p=0.8134$; Figure 1).

Microarthropod suppression effect on litter CO_2 efflux

Litter derived CO_2 flux was partitioned from total soil CO_2 efflux periodically throughout the experiment (Figure 4). Across all sampling events, the MS treatment did not differ from the Control treatment in terms of litter derived CO_2 flux rates ($p=0.9471$), however soil CO_2 flux rates were slightly higher with the MS treatment as compared to the control ($p=0.0261$). Soil CO_2 flux followed soil temperature and precipitation patterns, while litter derived CO_2 fluxes mainly followed precipitation events, with fluxes on some dry summer days equivalent to those on dry winter days (Figure 4).

Micro-arthropod suppression effect on litter inputs to the soil

Litter derived C was recovered down to 20 cm in the soil (Figure 2). The largest recovery of litter derived C was in the top 0-2 cm soil layer. At the 24 and 36-month soil harvests we sampled further down to 50 cm, but did not detect any litter derived C below 20 cm (data not shown). As litter decomposition progressed over time, the total amount of litter derived C in the soil increased (Figure 2). The MS treatment did not differ significantly from the control treatment in the amount of litter derived C that was recovered in the bulk soil down to 20 cm ($p=0.3146$), however the Control treatment bulk soils had a tendency for higher litter derived C throughout the experiment (Figure 2).

Although there was not a statistically significant higher amount of litter derived C in the bulk soils of the control treatment than the MS treatment, increasing the resolution of detecting pool changes by physically fractionating the SOM, showed greater amount of litter derived C in the sand sized ($p=0.0181$, Figure 3b), silt sized ($p=0.0007$, Figure 3c) and clay ($p=0.0051$, Figure 3d) fractions of the 0-2 cm and 2-5 cm layers in the Control than in the MS treatment. The control also had a greater amount of litter derived C then the MS in the light fraction at the 12 and 18 month harvests, however this difference was not statistically significant throughout the entire experiment ($p=0.0982$, Figure 3a).

Micro-arthropod suppression effect on microbial decomposition of litter

We quantified the ng of litter derived PLFA-C g^{-1} of soil by applying the isotope mixing model to our PLFA extractions from the labeled litter and bare soil plots. All of the microbial groups identified had incorporated litter derived C throughout the 36-month incubation, with decreasing amounts over time (Figure 4, a-e). PLFA incorporation of litter C decreased with

depth from the 0-2 cm soil layer to the 2-5 cm soil layer ($p<0.0001$). The MS treated plots had a significantly lower amount of litter C incorporation into all of the PLFAs than the Control plots over the first 24 months of the experiment ($p=0.0002$, Figure 4, a-d). By the 36 months harvest, overall PLFA litter C incorporation was low and there was no difference in the amount of litter C uptake between the PLFAs of the MS and Control treatments (Figure 4e). There was no effect of MS treatment on the overall abundance of PLFAs ($p=0.2710$).

We calculated the microbial uptake efficiency of litter derived C by dividing the amount of litter derived C found in the PLFAs by the amount of litter derived C found in the bulk soil of the same layer at each sampling time (Figure 5). Microbial uptake efficiency of the 0-2 cm soil depth was lower than the 2-5 cm depth ($p<0.0001$), and decreased over time ($p<0.0001$). The MS treatment did not differ significantly from the Control treatment in microbial uptake efficiency ($p=0.2774$).

The dbRDA analysis of the soil PLFA community revealed a shift in the microbial community composition both over time and by MS treatment ($p<0.0005$, $F=199$; Figure 6). At the earliest sampling date, 7 months, the i15:0, a15:0 (gram-positive bacteria) and 16:1ω7c (gram-negative bacteria) PLFAs were more prevalent then at the later sampling dates (Figure 6, Axis 1). The ratio of total fungal PLFA-C vs. total bacterial PLFA-C increased over time ($p<0.0001$). The MS treatment (red) had a higher prevalence of the 10Me18:0 (actinobacteria), 17:0cy, 18:1ω9c and 16:1ω7c (gram negative bacteria) PLFAs whereas the control treatment (blue) had a higher prevalence of the cy19:0 (gram negative bacteria), i15:0 (gram positive bacteria) and 10Me16:0 (actinobacteria) PLFAs (Axis 2, Figure 6). The ratio of gram-positive to gram-negative litter derived PLFA C g^{-1} soil decreased significantly with MS treatment (0.762) as compared to the Control (0.912) ($p<0.001$). The ratio of fungi to bacteria litter derived PLFA

C g^{-1} soil was not affected by the MS treatment (MS ratio= 0.202, Control ratio= 0.204; p=0.8954), however it did increase with time for both the MS and Control treatments ($p<0.0001$).

Discussion

The role of soil fauna, such as microarthropods, is one of the most understudied aspects of the terrestrial decomposition process. Naphthalene addition is a commonly used method to suppress soil microarthropods in litter decomposition studies without effecting soil microbial activity or soil C dynamics (Cotrufo et al. 2014; Wall et al. 2008), and was used here to investigate the role of microarthropods in SOM formation during litter decomposition in a tallgrass prairie. Confirming what has been seen in other studies (Garcia-Palacios et al. 2013; Wall et al. 2008), the suppression of microarthropods decreased the ^{13}C labeled *A. gerardii* litter mass loss rates during the first 18 months of decomposition (~70% mass loss, Figure 1). However by 24 months, the MS and Control treatments had converged in their rates of mass loss (Figure 1). Similarly, the MS treatment did not affect the decay rate of the more decomposed NL (Figure 1). The long-term nature of our experiment demonstrates that the promotion of litter decomposition by microarthropods may be limited to the early stages of decomposition, confirming patterns seen in previous studies (Garcia-Palacios et al. 2013; Smith and Bradford 2003). It is important to note that the *A. gerardii* litter we produced in our ^{13}C labeling chamber had not been exposed to field conditions and was more labile, i.e. had a higher %N and a lower %ASF and %AUR, then the native *A. gerardii* litter layer from the site (Table 1), further highlighting the early stage limitation of the MS effect on litter decomposition.

Although the acceleration of litter decomposition by microarthropods was limited to the first 18 months of decomposition (Figure 1), we found that microarthropods increased the allocation of litter C to stable SOM forms, which persisted throughout the 36-month experiment, confirming our first hypothesis (Figure 3). When examining the bulk soil, the Control treatment showed a non-statistically significant trend of increased litter derived C (Figure 2). However, fractionating SOM based on density and size has proven to provide more accurate estimations of SOM formation, soil C sequestration and turnover times (Trumbore 1993; Christensen 2001). The increased litter C contribution to the silt sized and clay fractions in the Control treatment vs. the MS was evident at the first (7-month) sampling, and persisted relatively unchanged for 36 months (Figure 3). Although the MS treatment did not have an overall effect on LF incorporation ($p=0.0982$), a posteriori tests reveal that at the 12 and 18-month harvests there was significantly more litter LF in the Control soils than the MS ($p<0.0001$).

We initiated our litter decomposition experiment in the fall, concurrent with natural senescence, so the initial 7 months of decay occurred over the winter months. The immediate contribution and persistence of litter derived C to the silt and clay fraction over the winter, prior to major contribution to the LF (Figure 3), indicates leaching of dissolved organic carbon (DOC) as a likely mechanism for the contribution of litter derived C to the mineral soil fractions (Soong et al. Submitted; Kalbitz et al. 2005). *A. gerardii* is known to have a large fraction of leachable C in its tissue (Soong et al. Submitted). At the initial 7-month harvest there was not a significant effect of the MS treatment on LF contributions to the soil, but it is likely that the microarthropods in the Control treatment had already began fragmenting the litter, exposing increased surface area for DOC leaching (Soong et al. In Revision), although the LF did not enter

the soil until after the summer (between 7-12 months) when the soils visibly cracked open due to drying.

After 36 months of decomposition in the field, the majority of the litter derived C in the soil was in the 0-2 cm LF (Figure 3). The accumulation of this fraction during our incubation inversely follows the trend of litter mass loss, particularly for the MS treatment (Figure 1). Although the LF accumulates with mass loss, it is known to have a short MRT of <10 years (Bird et al. 2008), so we expect that in a follow up harvest (planned for 2020) to find that the litter derived LF has been fully decomposed and the silt and clay fractions to retain the ^{13}C litter C (Trumbore and Zheng 1996). Thus, we predict that the input of litter C to the silt and clay fractions during the early phase of decomposition from leaching to comprise the long term stabilized SOM pool in the soil, and the microarthropod influence on the early phase of litter decomposition to influence the size of this stabilized fraction.

We found no discernable difference in the CO_2 losses of litter C due to the MS treatment (Figure 4); therefore the increased litter decomposition rates in the Control treatment (with abundant microarthropods) resulted in increased litter inputs to the soil, not increased C losses due to respiration. Microathropods are not known to have significant direct effects on C mineralization, and their role in ecosystem energy flows and C cycling is generally through their effects on the microbial activity (Hattenschwiler et al. 2005; Ruf et al. 2006). However in one microcosm experiment, microbial respiration was found to be enhanced by the presence of oribatid mites (Wickings and Grandy 2011). The lack of an MS effect on the overall PLFA abundances indicates that our MS treatment did not affect microbial population size, merely their incorporation of litter derived C, similar to what Gan et al. (2013) found. Our lack of daily CO_2 flux sampling capabilities, however, limited our ability to make any strong conclusions about the

effect of our MS treatment on litter CO_2 fluxes. We captured only a few days of high litter CO_2 fluxes, following precipitation events (Figure 1), but missed many other post-precipitation days when CO_2 flux was likely also high. Modeled estimates of CO_2 mineralization from litter decomposition that are based on soil models i.e. Century (Parton et al. 1988) overestimate CO_2 fluxes from litter (data not shown). Better calibration of models of CO_2 efflux from the surface litter layer is thus an area of ecosystem studies in need of improvement.

We hypothesized that in addition to increasing litter C inputs to the soil, microarthropods would also increase microbial turnover and incorporation of litter C during decomposition. Although we did see a significantly higher total amount of litter C incorporation (f_{litter}*PLFA abundance) into the PLFAs of the Control treatment *versus* the MS (Figure 5, p=0.0002), the amount of PLFA incorporation relative to the amount of litter derived C in the 0-2 or 2-5 cm bulk soils was the same for the MS and Control treatments (Figure 6, p=0.2774). Thus, rather than having a direct effect on microbial activity and decomposition of litter C, microarthropods increase microbial decomposition of litter C mainly by increasing the fragmentation, leaching and litter input to the soil (Figure 3). Since microbial byproducts are thought to have long MRTs in the soil (Cotrufo et al. 2013), the increase in litter C input to the soil is an indirect mechanism by which microarthropods stimulate stabilized soil C formation. Total litter derived PLFA-C incorporation declined with time, even as litter-C in the soil accumulated (Figure 5). We suggest this is due to the higher microbial use efficiency of labile litter components, which would enter the soil in the early stage of decomposition most likely through DOM leaching (Cotrufo et al. 2009). The litter-C entering the soil at later stages of decomposition (18-36 months) was mostly as litter fragments contributing to the SOM LF, and reveals declining uptake efficiencies not seen in shorter term decomposition studies (Williams et al. 2006).

As we hypothesized, the suppression of microarthropods affected the microbial community composition by decreasing the amount of litter derived C in the gram-positive vs. the gram-negative bacteria (Figure 7; Axis 2), however the fungi:bacteria ratio was not affected by the MS treatment. Litterbag soil fauna manipulation studies, based on variable mesh sizes have found both increases (Wilkinson et al. 2002) and decreases (Carrillo et al. 2011) in the gram-positive: gram-negative ratio with decreased fauna access. The results of these litterbag studies are difficult to interpret, however, due to the alteration of litter microclimate and fragmentation due to litterbags of different mesh sizes (Kampichler and Bruckner 2009). The decrease in gram-positive to gram-negative community composition due to the MS treatment that we found could help to explain the decreased silt and clay stabilized litter C, due to the fact that gram-negative bacteria are less capable of breaking down more complex substrates (Fanin et al. 2014). The fact that we did not see an effect of the MS on the fungi: bacteria ratio, as seen in other studies (Wilkinson et al. 2002; Anderson and Ineson 1984; Scholle et al. 1992), could be due to the fact that our site is bacteria dominated, with fungi only making up 18% of the total PLFA biomass (Knapp et al. 1998a). The increase in fungi:bacteria uptake of litter C over time is consistent with other findings that bacteria are more involved with the decomposition of initial, labile litter, while fungi are more involved with the decomposition of later stage, more recalcitrant litter substrates (Moore-Kucera and Dick 2008; McMahon et al. 2005).

Conclusions

In conclusion, the results from our MS experiment demonstrate how microarthropods increase stabilized SOM formation during litter decomposition due to their role in increasing litter fragmentation and litter inputs to the soil, where they can be processed by soil microbes

into minerally associated forms in a fine-textured grassland soil. Although microarthropods accelerated only the initial 70% of litter mass loss, our results indicate that they actively alter the decomposition process through increasing physical fragmentation, leaching and microbial access to litter C in the soil. An examination of physically defined SOM fractions along with microbial PLFA analysis helped to reveal the complex direct and indirect effects of microarthropods on SOM formation during three years of ^{13}C labeled *A. gerardii* litter decomposition in a tallgrass prairie.

Table 5.1. Initial ^{13}C and ^{15}N labeled *A. gerardii* leaf litter and native litter, collected from the litter layer, chemical characteristics.

	%C	%N	Atom % ^{13}C	% Hemicellulose	% Cellulose	% Lignin
^{13}C labeled *A. gerardii*	44.3	1.47	3.3804	25	29	3.9
Native *A. gerardii*	44.0	0.599	1.0977	20	43	12

Table 5.2. Bare soil values for the 0-2, 2-5, 5-10 and 10-20 cm depth layers and SOM fractions (0-2 and 2-5 cm layers only) for use in the isotope mixing model and determination of the amount of litter derived C in each soil sample. Data are average across all 4 replicates, 2 treatments and all 5 sampling dates, n=40.

	%C	%N	$\delta^{13}C$	Bulk density	% of total soil
0-2 cm Bulk	5.21 (0.075)	0.393 (0.006)	-16.2 (0.319)	0.702 (0.050)	100
0-2 cm LF	27.3 (0.510)	1.43 (0.023)	-18.4 (0.311)	NA	4.46 (0.169)
0-2 cm Sand sized	6.15 (0.413)	0.399 (0.024)	-16.4 (0.181)	NA	10.1 (0.399)
0-2 cm Silt sized	2.60 (0.149)	0.251 (0.017)	-15.3 (0.109)	NA	70.6 (0.477)
0-2 cm Clay	4.13 (0.173)	0.422 (0.027)	-15.3 (0.313)	NA	14.8 (0.353)
2-5 cm Bulk	4.41 (0.055)	0.340 (0.004)	-14.9 (0.098)	0.922 (0.037)	100
2-5 cm LF	25.9 (0.772)	1.23 (0.025)	-17.7 (0.193)	NA	2.74 (0.114)
2-5 cm Sand sized	5.05 (0.310)	0.356 (0.020)	-15.8 (0.134)	NA	9.11 (0.301)
2-5 cm Silt sized	2.60 (0.149)	0.232 (0.018)	-13.8 (0.514)	NA	73.3 (0.391)
2-5 cm Clay	4.25 (0.126)	0.474 (0.034)	-14.3 (0.079)	NA	14.9 (0.310)
5-10 cm Bulk	3.76 (0.052)	0.300 (0.004)	-14.0 (0.067)	1.11 (0.024)	100
10-20 cm Bulk	3.27 (0.042)	0.271 (0.004)	-13.3 (0.074)	1.28 (0.011)	100

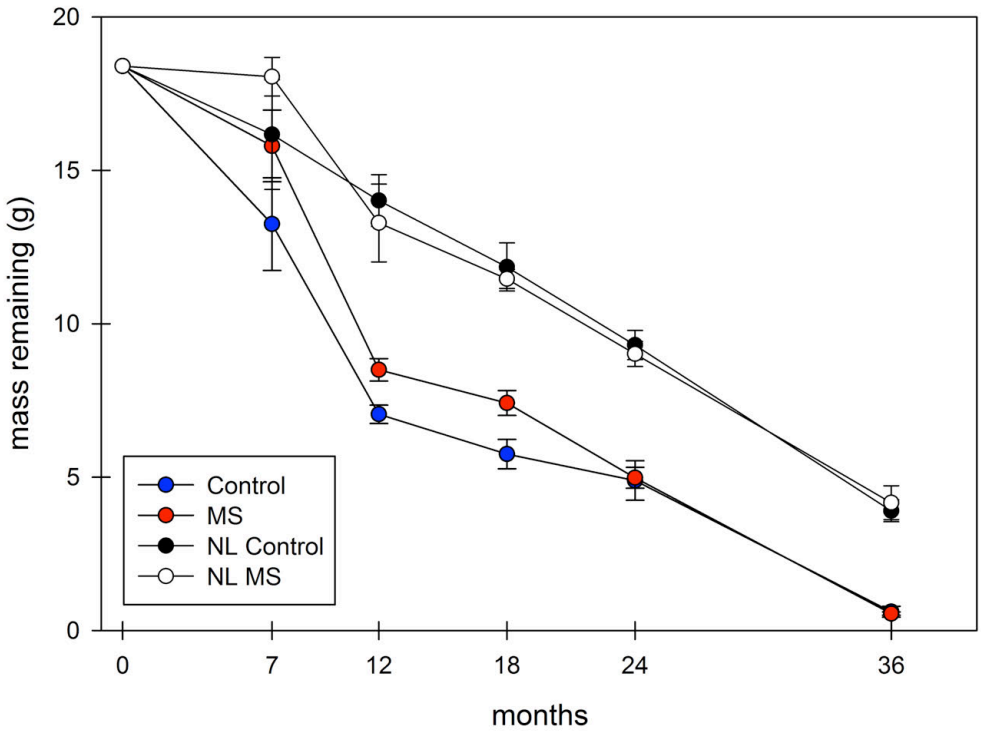


Figure 5.1. Ash-free litter dry mass remaining during 36 months of decomposition in the field for the Control (blue) and Microarthropod Suppressed (MS, red) treatments of the ^{13}C labeled *A. gerardii* litter, and the native *A. gerardii* litter collected from the site (NL). Values are averages, N=4, bars are standard error.

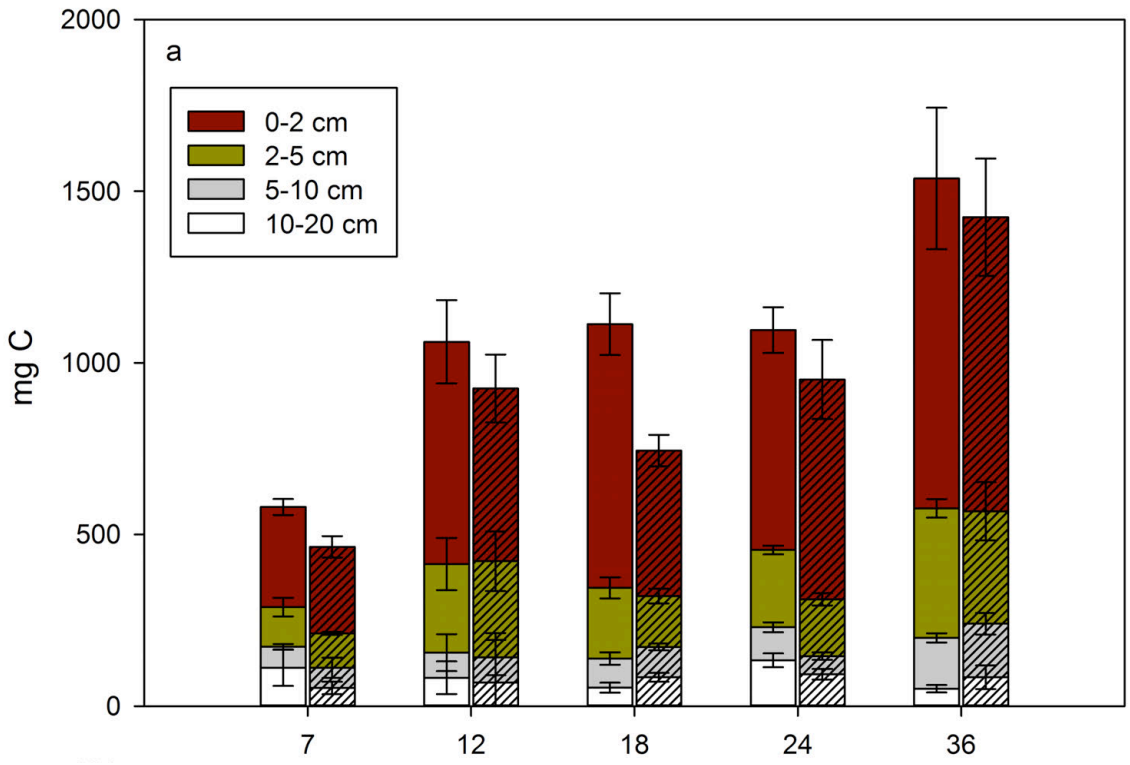


Figure 5.2. Litter derived C recovery in the bulk soil within the 0-20 cm layer beneath the soil collar (i.e., 20cm diameter). Open bars are the Control treatment and hashed bars are the Microarthropod suppressed (MS) treatment. N=4, bars are standard error.

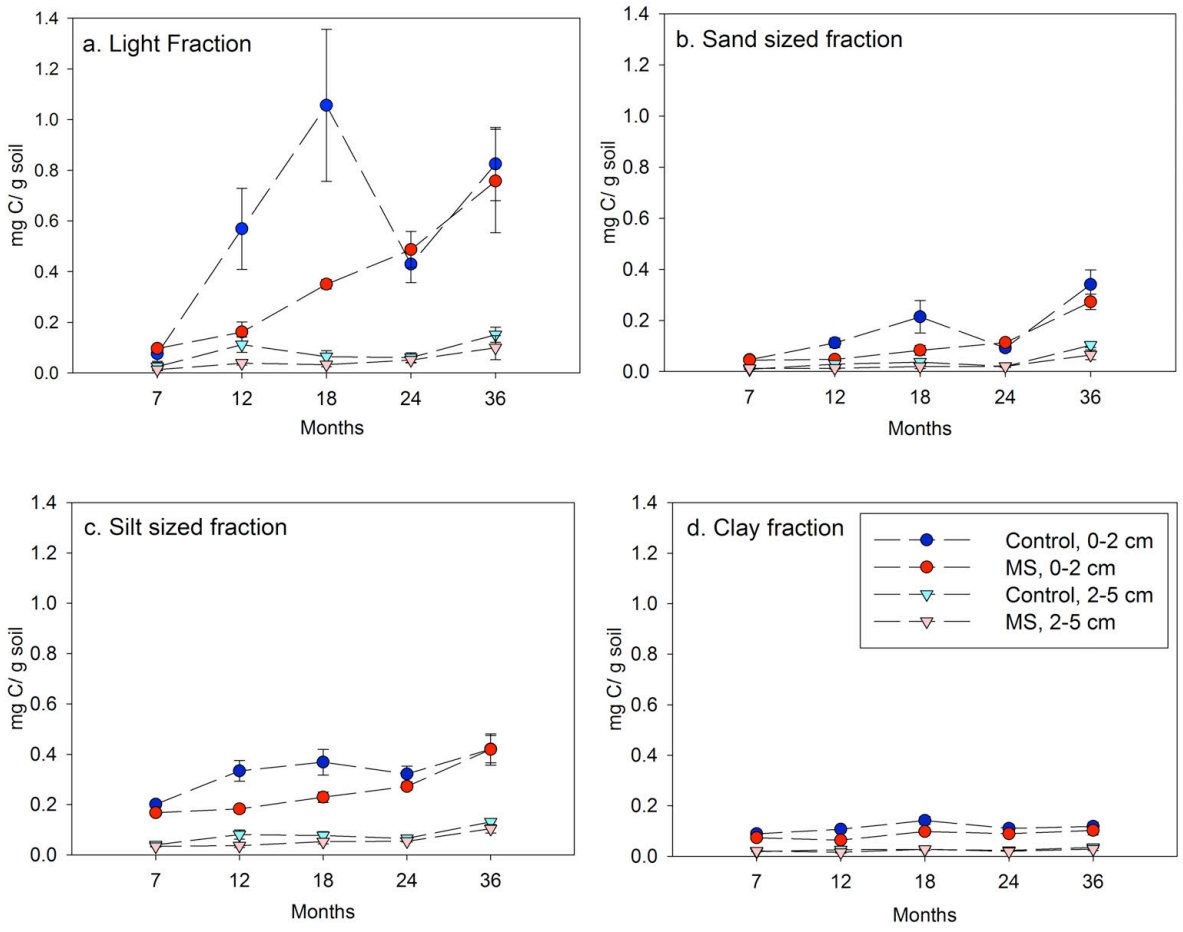


Figure 5.3. Concentration of litter derived C in the (a) light fraction, (b) sand sized fraction, (c) silt sized fraction, and (d) clay fraction of the soil organic matter in the 0-2 cm soil depth (circles) and 2-5 cm soil depth (triangles) for the Control (blue) and Micrarthropod Suppressed (MS) treatments (red). Values are averages, N=4, error bars are standard error.

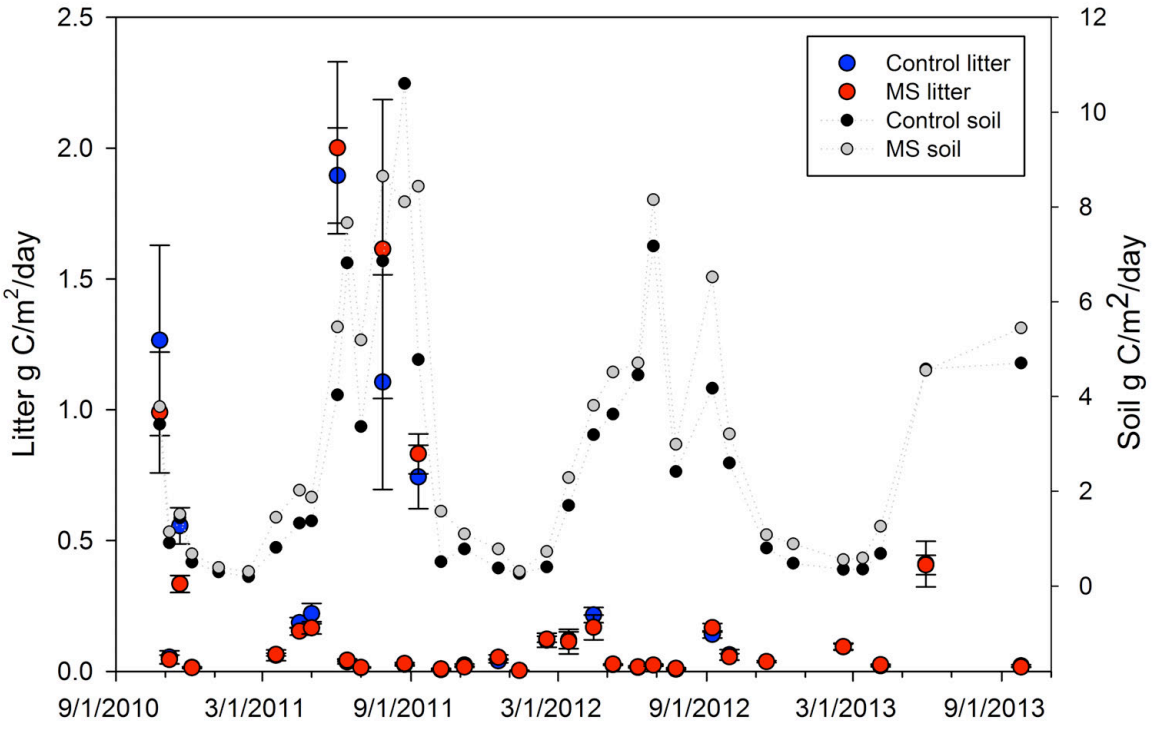
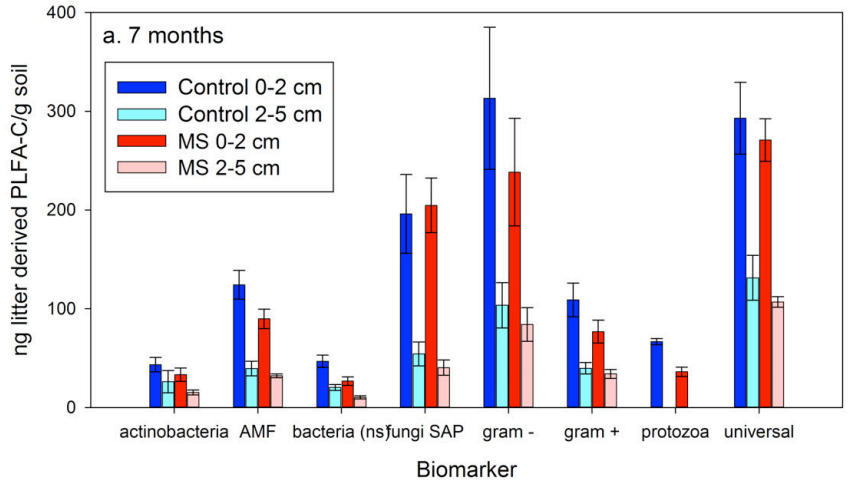
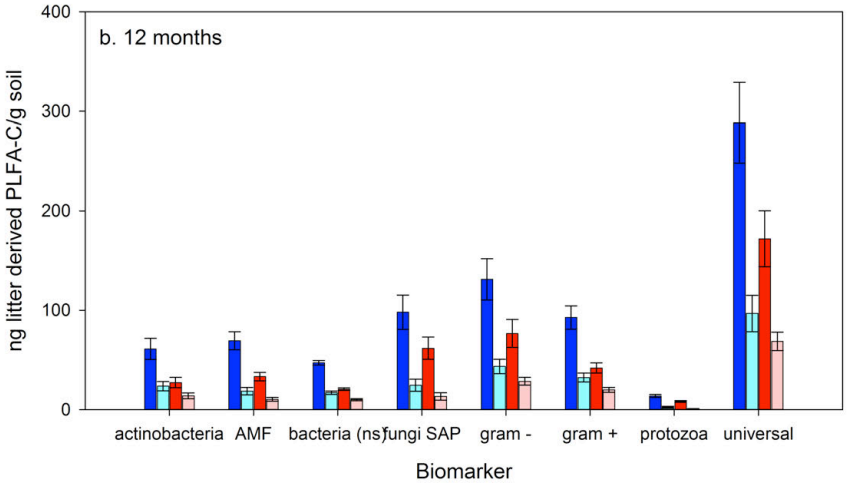
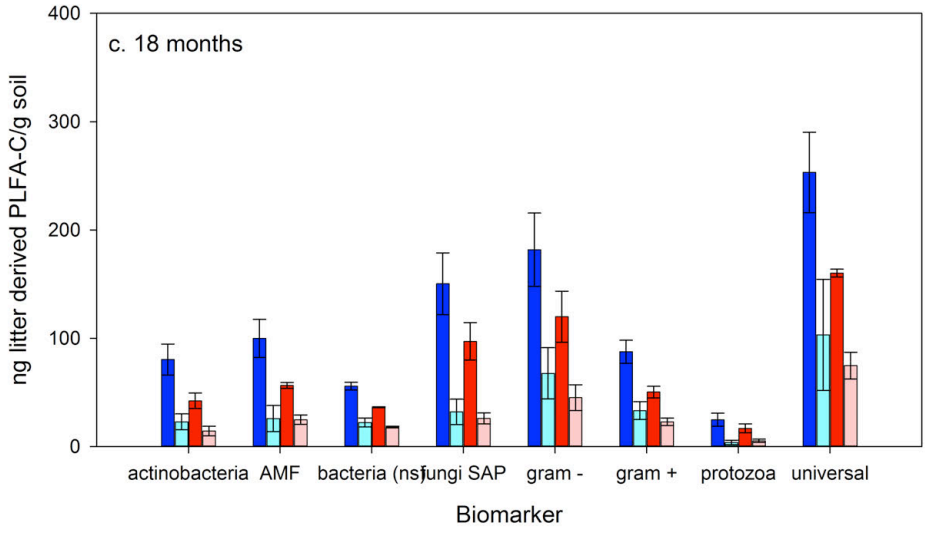
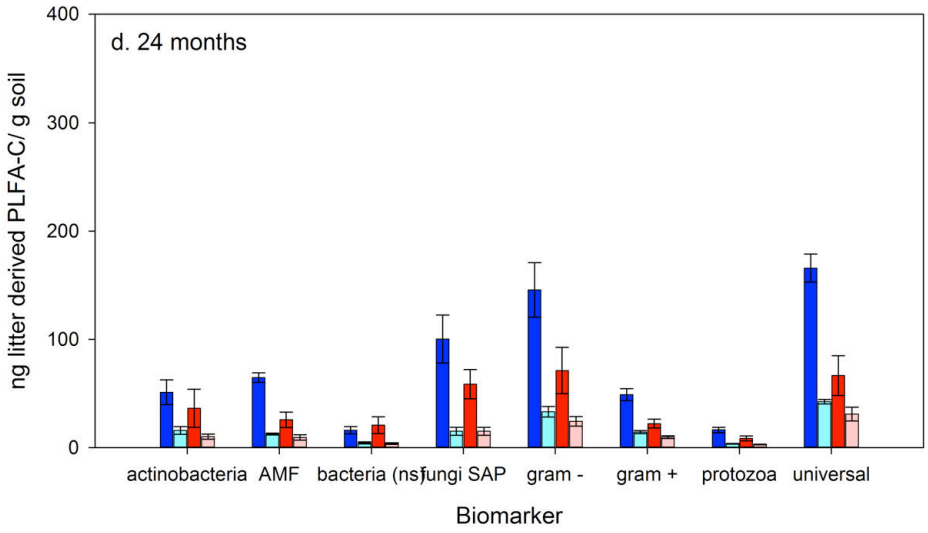


Figure 5.4. ^{13}C labeled *A. gerardii* litter derived CO_2 flux for the Control (blue) and MS (red) treatments scaled on the left axis. Total soil CO_2 efflux for the Control (black) and MS (grey) treatments scaled on the right axis. Values are averages, n=4, bars are standard error.


a. 7 months
Control 0-2 cm
Control 2-5 cm
MS 0-2 cm
MS 2-5 cm
ng litter derived PLFA-C/g soil
400
300
200
100
0
actinobacteria
AMF
bacteria (ns)
fungi SAP
gram -
gram +
protozoa
universal
Biomarker


b. 12 months
ng litter derived PLFA-C/g soil
400
300
200
100
0
actinobacteria
AMF
bacteria (ns)
fungi SAP
gram -
gram +
protozoa
universal
Biomarker


c. 18 months
ng litter derived PLFA-C/g soil
400
300
200
100
0
actinobacteria
AMF
bacteria (nsf
fungi SAP
gram -
gram +
protozoa
universal
Biomarker


d. 24 months
ng litter derived PLFA-C/ g soil
400
300
200
100
0
actinobacteria
AMF
bacteria (nsf
fungi SAP
gram -
gram +
protozoa
universal
Biomarker

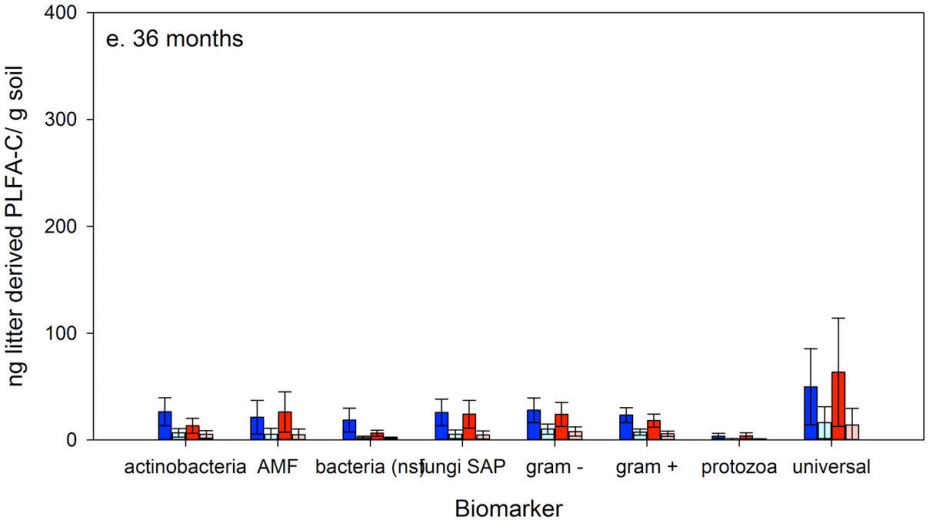

e. 36 months
ng litter derived PLFA-C/ g soil
400
300
200
100
0
actinobacteria
AMF
bacteria (nsf
fungi SAP
gram -
gram +
protozoa
universal
Biomarker

Figure 5.5. PLFA incorporation of litter derived C at 7 (a), 12 (b), 18 (c), 24 (f) and 36 (e) months of litter decomposition. Blue bars are the control treatment and red bars are the MS treatment. Darker bars are the 0-2 cm soil layer and lighter bars are the 2-5 cm soil layer. Values are averages, N=4, error bars are standard error.

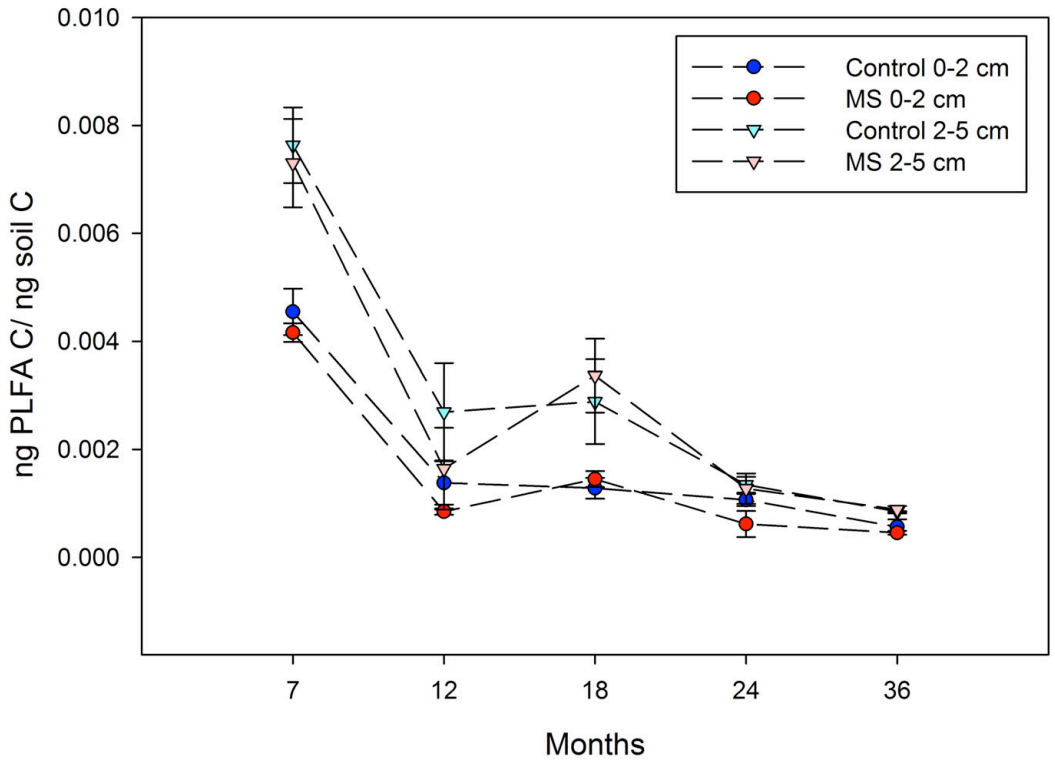


Figure 5.6. Microbial uptake efficiency calculated as the ng of litter derived PLFA C ng^{-1} of litter derived C in the soil of the 0-2 cm (circles) and 2-5 cm (triangles) soil depth for the Control (blue) and Microarthropod Suppressed-MS (red) treatments. Values are averages, N=4, error bars are standard error.

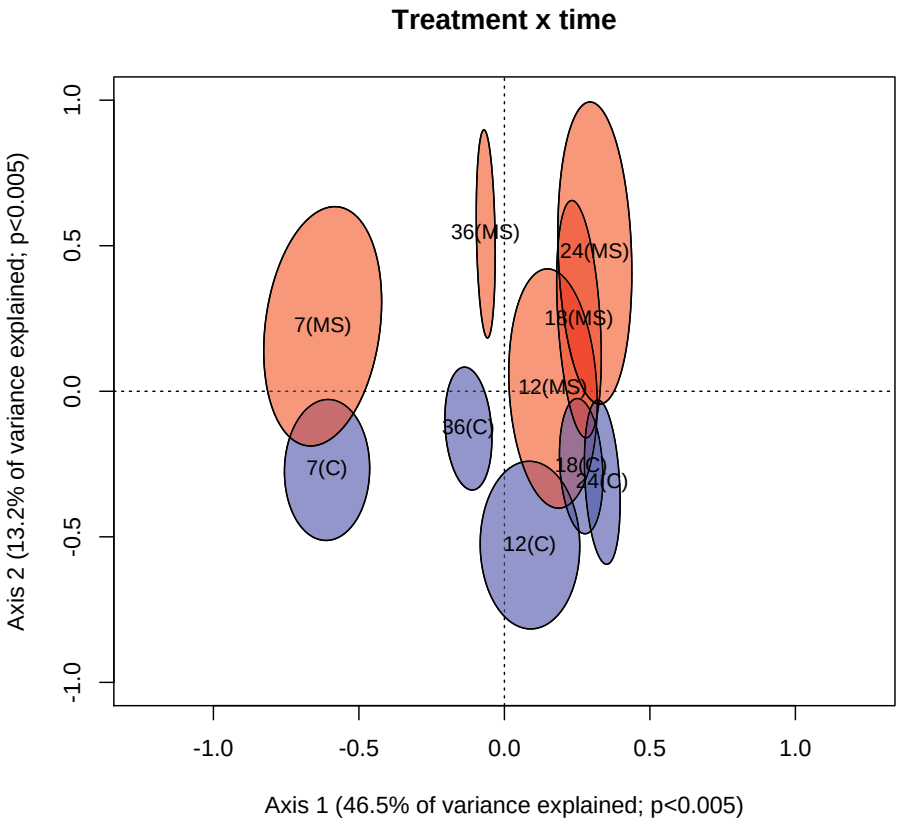


Figure 5.7. Distance based redundancy analysis on relative abundances of PLFAs across time (7, 12, 18, 24 and 36 months) and MS treatment (MS in red, Control in blue)

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Chapter 6: Frequent burning of a tallgrass prairie inhibits microbial C and N cycling in soil, increasing recalcitrant pyrogenic organic matter storage while reducing N availability[5]

Introduction

Fire is a critical driver of ecosystem structure and functioning in grassland ecosystems (Collins and Wallace 1990), which cover nearly one fifth of the Earth's land surface and store an estimated 30% of the total world soil carbon (C) (Grieser et al. 2006; Anderson 1991). The removal of dead aboveground biomass in fires alters the C and nutrient balance of grassland ecosystems, while alleviating energy constraints of a dense litter layer (Collins and Wallace 1990; Knapp and Seastedt 1986). Fire is thus used as a regular management practice to promote grassland productivity worldwide, in both natural and grazed ecosystems (Hall and Scurlock 1991; Mouillot and Field 2005). Additionally, the frequency and intensity of wild fires is predicted to increase with climate change (Stocks et al. 1998; Flannigan et al. 2000). While much of the aboveground C and nitrogen (N) in biomass burned during fire gets combusted and removed from the ecosystem, a fraction of the biomass remains, thermally transformed by fire into pyrogenic organic matter (py-OM) (Knicker et al. 2012). Although many of the effects of fire on grassland productivity and ecosystem C and N cycling have been explored (Collins and Wallace 1990; Ojima et al. 1994; Johnson and Matchett 2001; Knapp et al. 1998b; Blair 1997), the effect of frequent fire on soil organic matter (SOM) formation through the removal of litter inputs and the deposition of py-OM (or black carbon) has received little attention. Soils are the largest terrestrial reservoir of C with the potential to store or release more C to the atmosphere

[5] In preparation for submission to Global Change Biology with M. Francesca Cotrufo

due to climate and land management changes (Schlesinger and Bernhardt 2013). N is often the most limiting nutrient in temperate terrestrial ecosystems (Schlesinger and Bernhardt 2013), and the N mineralized during litter decomposition is critical to ecosystem productivity as high C:N plant material is converted into lower C:N SOM (Paul 2007). How grassland fires alter SOM formation through the removal of the litter layer and the deposition of py-OM is thus critical to understanding soil biogeochemical cycling and its feedback to global biogeochemical cycles.

Fire can have both direct and indirect effects on soil physical, chemical and biological properties. The removal of the litter layer may increase nutrient infiltration to the soil, increase soil surface temperatures, reduce soil moisture and promote topsoil erosion (Blair 1997; Knapp and Seastedt 1986). Annual inputs of py-OM can also induce a shift in the soil microbial community composition responsible for the formation of stabilized SOM (Anderson et al. 2011) and their N use efficiency (NUE) (Ojima et al. 1994). Frequently burned grassland soils typically have higher C mineralization and lower N mineralization rates than unburned soils (Ojima et al. 1994; Johnson and Matchett 2001). By combusting aboveground biomass, fire removes the organic matter (OM) that would otherwise be decomposed by bacteria and fungi and contribute to SOM formation (Paul 2007). A history of frequent fire, and frequent py-OM inputs, could thus offset the stoichiometric balance of soils mediated through microbial decomposition of non-pyrolized OM inputs.

The formation of SOM through litter decomposition accounts for the recycling of nutrients and the transformation of C fixed through aboveground net primary productivity (ANPP) into SOM. This is a heterogeneous pool, and to understand the contribution of litter derived C and N to its formation and persistence, better insights can be obtained by fractionating it into its primary organo-mineral fractions, such as the light fraction (LF, >1.85 g/cm^3), and the sand, silt

and clay fractions (Christensen 2001). These physically defined fractions have different mean residence times (MRT) ranging from a few years (LF) (Trumbore and Zheng 1996; Bird et al. 2008) to hundreds of years (silt and clay stabilized fractions) (Wiseman and Puttmann 2005; Schulten and Leinweber 2000), and are believed to be formed by the progressive transformation of plant residues (LF) by microbes, with the silt and clay associated OM being predominantly of microbial origin (Grandy and Neff 2008). Py-OM deposits in the soil have a long MRT, similar to or longer than the most stabilized SOM fractions formed through microbial decomposition of non-pyrolized OM (Schmidt et al. 2011). Although an initial labile fraction of py-OM has been seen to contribute to dissolved organic matter (DOM) leaching and CO_2 efflux (Foereid et al. 2011; Soong et al. Submitted), py-OM is largely resistant to microbial decomposition due to its highly aromatic chemical structure (Masiello 2004; Knicker 2011), and remains in the soil minimally transformed by microbes. The addition of py-OM from fires, thus adds OM with a long MRT to the soil but circumvents the microbial process that typically forms it (Kuzyakov et al. 2009; Grandy and Neff 2008; Anders et al. 2013). Organic C and N are used as energy and nutrient sources by soil microbes, which mineralize both elements in maintenance of a specific stoichiometric balance (Cleveland and Liptzin 2007; Fanin et al. 2014; Manzoni et al. 2012). Annual deposits of py-OM from annual burning can thus alter the SOM formation process and de-couple the C and N cycles mediated by microbes in the soil, altering ecosystem C and N availability. Disentangling the effects of fire on C and N cycling through SOM formation from its effects on ANPP responses (Knapp et al. 1998b; Blair 1997; Kitchen et al. 2009) will help to inform predictions of the impact of fires on global biogeochemical processes. A comparison of py-OM and litter inputs on soil C and N cycling in soils with either a history of annual fire and

py-OM inputs or a history of infrequent fire and annual litter inputs will help to differentiate the effects of py-OM inputs *versus* fire history on soil C and N cycling.

The objective of our research is to understand how fire in a tallgrass prairie effects SOM formation through the removal of the litter layer and the input of partially combusted py-OM residues. We will specifically address the questions of (1) how does the input of py-OM rather than litter affect SOM formation, microbial activity and soil CO_2 fluxes? And (2) does a history of frequent burning (i.e. annual removal of litter and input of py-OM) alter soil biochemical properties in ways that affect the cycling of C and N as compared to unburned soils? The first question addresses our hypothesis that py-OM remains undecomposed by microbes in the soil contributing to soil C sequestration while litter is decomposed by microbes, with much of its biomass being lost to the ecosystem as CO_2 and a small fraction being stored in minerally stabilized SOM fractions. The second question addresses our hypothesis that a history of annual inputs of non-decomposable py-OM rather than decomposable litter inputs affects soil microbial nutrient and energy balance in a way that decouples C and N cycling in the soil. Microbes in annually, or frequently burned soils, thus are starved of the labile C and N from litter inputs, which helps to explain the decreased N mineralization rates seen in annually burned grasslands (Johnson and Matchett 2001; Ojima et al. 1994).

To address these hypotheses, we conducted a field experiment incubating ^{13}C and ^{15}N labeled *Andropogon gerardii* above-ground litter and py-OM produced from the partial combustion of the same litter in a frequently burned (FB) and infrequently burned (IF) tallgrass prairie site for 11 months. Using the ^{13}C and ^{15}N label, we traced litter and py-OM decomposition into CO_2 fluxes, the bulk soil down to 40 cm, SOM fractions, and microbial phospholipid fatty acids (PLFA) biomarkers.

Materials and methods

^{13}C and ^{15}N labeled litter and pyrogenic organic matter

Dual and uniformly 4 atom % ^{13}C and 7 atom % ^{15}N labeled *Andropogon gerardii* Kaw was grown in a ^{13}C and ^{15}N continuous labeling chamber as described in Soong et al. (2014). *A. gereardii* was started from seeds and grown in the labeling chamber to maturity for 15 weeks. Then the plants were removed from the chamber and the aboveground senesced biomass (litter) was harvested by cutting at the crown, and air-dried. Half of the labeled *A. gerardii* litter was pyrolyzed for four hours at 400ºC in a muffle furnace with ultra-high purity nitrogen flow, as described by Rutherford et al. (2012) to produce our labeled py-OM samples. Three replicates of the initial litter and py-OM were analyzed for %C, %N, $\delta^{13}C$ and $\delta^{15}N$ on an elemental analyzer connected to an isotope ratio mass spectrometer (EA-IRMS, Carlo Erba NA 1500 coupled to a VG Isochrom continuous flow IRMS, Isoprime inc.). The py-OM was analyzed for pH in water (1:10). The py-OM O, H, ash and volatile matter concentrations were determined using proximate and ultimate analyses by Hazen Research, Inc. (Golden, CO, USA).

Experimental site and design

The study was conducted at the Konza Prairie long-term ecological research station in Kansas, USA. This is a tallgrass prairie, dominated by *A. gerardii*. Climate at the site is temperate-continental, with average annual precipitation of 835 mm and a mean annual temperature of 12.8ºC. Two sites were chosen for this study. The infrequently burned (IB) site was burned annually from 1972 to 2000, when burning treatments ceased, and was then unburned from 2000 onwards, except for one wild fire in 2008. This site was chosen as the IB site since it did not have a recent history of fire, but due to its previous fire regime *A. gerardii*

still dominates the system. The frequently burned (FB) site has been burned annually in the springtime from 1972 until the present. Both sites are on topographically located on footslopes, with silty-clay textured Mollisol soils (Knapp et al. 1998a). Daily total precipitation and mean soil temperature were recorded at the Konza Headquarters site and downloaded from the LTER CLIMDB/HYDRODB database (Figure 1; http://climhy.lternet.edu/).

The litter and py-OM experiment consisted of two treatments: a site treatment with two levels, FB and IB, and a soil surface amendment treatment with also two levels, litter and py-OM. At each of the two sites, the surface amendment treatments were replicated 4 times in a randomized block design. At the IB site a time treatment was added, with two sampling times 4 and 11 months, while at the FB site we only had one 11-month sampling. The experimental unit was a PVC collar (20 cm diameter and 10 cm tall) inserted in the ground to 5 cm. All collars were inserted 24 hours prior to the start of the experiment, when the native litter was removed. The ^{13}C and ^{15}N labeled *A. gerardii* litter was added to the collars at the rate of 400 g/m^2, which is the estimated above ground net primary productivity (ANPP) at the site (Knapp et al. 1998a). The py-OM was added at the rate of 132 g/m^2, corresponding to a 30% burning recovery of the ANPP. Additionally, bare soil collars (with no above ground inputs) were established in 4 replicates per each sampling time and site, to serve as the natural soil end member for the isotope-mixing model (See Data Analysis). The litter was placed on the surface of the soil in the collars and the py-OM was sprinkled on the surface of the soil and tilled in to 2 cm.

Within each replicate block, there were three PVC collars (plots), one with the labeled litter, one with the labeled py-OM and one bare soil plot. For each PVC collar, soil was sampled at 0-2, 2-5, 5-10, 10-20, and 20-40 cm depths (sub plots). The experiment is thus treated as a split plot on a randomized complete block design.

Initial soil characterization

Four replicate soil samples from the 0-2, 2-5, 5-10, 10-20 and 20-40 cm soil layers were collected just outside of the four replicate blocks at both the IB and FB sites for initial soil characterization. We measured bulk density gravimetrically for each soil layer. Initial differences in the FB and IB soil properties were compared based on %C and %N (Elemental analysis, LECO corp., St. Joseph, MI) phospholipid fatty acid (PLFA) extraction (as described below) and benzene poly-carboxylic acid (BPCA) analysis.

BPCAs are biomarkers of py-OM and were determined on four replicates of 0-5 cm time 0 soil from both the IB and FB sites using HPLC (HPLC-DAD/ELSD, Agilent Technologies, Alpharetta, GA) equipped with a photo diode array detector as described by (Wiedemeier et al. 2013; Boot et al. Submitted). For the BPCA analysis, 50-150 mg of finely ground, oven dried sample of each soil was digested with 70% nitric acid for 8 hours at 170°C. The solution was filtered with ashless cellulose filters, an internal reference standard of phthalic acid was added to the solution, and the filtrate was cleaned by cation exchange resin and freeze-dried. The freeze-dried sample was re-dissolved in HPLC grade water. The re-dissolved solution containing the BPCAs was separated by a reversed stationary phase (Waters X-Bridge C18, 3.5 um particle size, 2.1 x 150 mm) using a gradient method consisting of mobile phase A: HPLC grade water with 25 mL/L 85% orthophosphoric acid buffered with monosodium phosphate to pH 1.2, and mobile phase B: acetonitrile. Gradient separation was 0-5 min 0.5% mobile phase B, at 0.2 mL/min 5 to 25.9 min 30% mobile phase B, 25.9 to 26 min, 95% mobile phase B followed by column re-equilibration. Individual BPCAs were quantified by using a 5 point calibration from standard solutions of benzenetricarboxylic acids (1,2,3-B3CA/hemimellitic acid, 1,2,4-B3CA/trimellitic acid, 1,3,5-B3CA/trimesic acid), benzenetetracarboxylic acid (1,2,4,5-

B4CA/pyromellictic acid), benzenepentacarboxylic acid (B5CA), and benzenehexacarboxylic acid (B6CA/mellitic acid). The B4CA standards that are not commercially available (1,2,3,4-B4CA/prehenitic acid, and 1,2,3,5-B4CA/mellophanic acid) were identified by their ultraviolet adsorption spectra (Yarnes et al. 2011) and quantified using the calibration for 1,2,4,5-B4CA. Total BPCAs C was calculated from the some of the individual BPCAs.

Soil CO_2 efflux and ^{13}C-CO_2 measurements

We measured soil CO_2 efflux and its isotopic composition periodically (intervals between CO_2 sampling ranged from 2 days immediately after incubation to 60 days during the winter) from May 8, 2012 through April 4, 2013 using a LI-8100 portable infrared gas analyzer (IRGA; LI-COR, Lincoln, NE) with a 20 cm diameter survey chamber (LI-8100, LI-COR, Lincoln, NE). We determined the rate of CO_2 efflux using the LI-8100 software, which fits a linear equation to the CO_2 concentration increase measured during the first 132 s of chamber closure. The chamber remained closed for a total of 600 s in order to achieve a CO_2 concentration range sufficient for application of the Keeling plot method (Pataki et al. 2003) for estimating $\delta^{13}C$ of soil CO_2 efflux following Cotrufo et al. (2014). Briefly, an atmospheric sample, as well as two gas samples at approximately 200 and 570 s during chamber closure were collected and used as three points in the Keeling plot calculation.

We read the CO_2 concentration of the gas samples from the LI-8100 software upon gas collection in the field. The gas samples were collected using a two needle flow diversion into pre-evacuated 12 ml septum-capped glass sampling vials (Labco, UK), following Cotrufo et al. (2014). On the same day of gas sampling, additional vials were filled with a reference gas of known CO_2 concentration and $\delta^{13}C$. Within 24 h of collection, the gas sampling vials were

brought back to the laboratory and $\delta^{13}C$ was measured on a Delta V isotope ratio mass spectrometer (IRMS) coupled to a GC-isolink unit with a pre-concentrator (Thermo Scientific), within one week of sampling. On each day of $\delta^{13}C$ analysis, two reference gas samples were also analyzed for $\delta^{13}C$ and CO_2 concentration (LI-COR, LI-6252, Lincoln, NE) and samples $\delta^{13}C$ and CO_2 values adjusted if necessary, following Tu et al. (2001).

Soil sampling

On September 8, 2012 (IB site only, 4 months) and April 4, 2013 (FB and IB sites, 11 months), soil and litter samples were collected from each of the four replicate collars of each treatment. First, where present, the litter was collected by hand and stored in plastic bags. Then, the soil within the collar was gently excavated with the use of hand shovel by incremental depths of 0-2, 2-5, 5-10, 10-20 cm (also 20-40 cm for the 11 months harvest only) and stored in plastic bags. All soil and litter samples were stored with ice in coolers before being brought to the laboratory the following day. There they were stored at 4ºC until they were processed within two weeks of collection.

Bulk soil, roots and litter analyses

The litter was picked clean of any non-A. *gerardii* leaves, roots and soil, then weighed at field moisture. A subsample of the litter and roots was oven dried at 65ºC for analysis of gravimetric water content, and another subsample was combusted at 660°C in a muffle oven to determine ash content. The soil samples were sieved to 2 mm and all large roots remaining on the sieve were collected. The bulk soil was also analyzed for gravimetric water content by mass loss after drying at 105ºC. All oven dry samples were then ground and analyzed for %C, %N,

$\delta^{13}C$ and $\delta^{15}N$ on an elemental analyzer coupled to an isotope ratio mass spectrometer (EA-IRMS, Carlo Erba NA 1500 elemental analyzer and VG Isochrom continuous flow IRMS, Isoprime Inc., Manchester, UK).

Soil organic matter fractionation

In order to separate the primary soil organo-mineral fractions (Christensen 1992) we employed a physical soil organic matter (SOM) fractionation scheme modified from Denef et al. (2013). Briefly, a 5 g subsample of oven dried bulk soil from the 0-2 and 2-5 cm soil layers was dispersed with glass beads in 25 ml of 1.85 g cm^{-3} sodium polytungstate (SPT) to break up all aggregates. Then, the samples were centrifuged and the light fraction (LF <1.85 g cm^{-3}) was aspirated off of the sample and rinsed clean of SPT. The remaining heavy fraction was rinsed thoroughly of SPT then sieved through a 53 μm screen to separate the sand-sized (>53 μm) fraction from the silt and clay. The remaining silt and clay were further fractionated via centrifugation in water to separate the silt sized (>2 μm) from the clay (<2 μm) fraction based on Stockes Law. All fractions were oven dried at 105ºC prior to weighing and analysis of %C, %N, $\delta^{13}C$ and $\delta^{15}N$ on an EA-IRMS. The total fractions mass recovery was within +/- 5% of the initial mass.

PLFAs extractions and ^{13}C-PLFA measurements

A sub sample of the sieved soils from the 0-2 and 2-5 cm depth layer was picked clean of all visible roots, frozen (-20ºC) and lyophilized for 48 h prior to PLFA extraction. PLFAs were extracted on these samples using conventional methods (Bligh and Dyer 1959; Denef et al. 2007). In brief, for the extraction, 6 g of freeze-dried soil were mixed with a 0.1 M potassium

phosphate buffer:chloroform:methanol solution (0.8:1:2 ratio volume, ml g^{-1} of soil). Neutral, clyco- and phospholipids were separated over SPE silica columns eluting respectively with chloroform, acetone and methanol. Phospholipids were saponified to obtain free fatty acids, which were subsequently methylated using 0.2 M methanolic KOH to form fatty acid methyl esters (FAMEs). FAMEs were quantified and analyzed for ^{13}C by capillary gas chromatography combustion isotope ratio mass spectrometry (GC-c-IRMS, Trace GC Ultra, GC Isolink and DeltaV IRMS, Thermo Scientific). A capillary GC column type DB-5 was used for FAME separation (length 30 m, i.d. 0.25 mm, film thickness 0.25 μm; Agilent). The GC temperature program proceeded at 60ºC with a 0.10 min hold, followed by a heating rate of 10ºC min^{-1} to 150ºC (2 min hold), 3ºC min^{-1} to 220ºC, 2ºC min^{-1} to 255ºC, and 10ºC min^{-1} to 280ºC with a final hold of 1 min. Individual fatty acids were identified based on relative retention times to an internal standard (12:0), which was added to the FAME extract prior to gas chromatography, and cross referenced with several standards: a mixture of 37 FAMEs (37 component FAME Mix, 47885-U, Sigma-Aldrich, USA). FAME identification was verified by analyzing a few samples on a capillary GC-mass spectrometer (Shimadzu QP-2010SE) with a SHRIX-5ms column (30 m length x 0.25 mm i.d., 0.25 μm film thickness) using the NIST 2011 mass spectral library.

Quantification was performed using relative response factors (RRF) relative to an internal standard (19:0), added to the FAME extract prior to GC analysis. RRFs were determined in advance by using a dilution series of the 37-component FAME mix, to which the 19:0 standard was added. The abundance of individual PLFAs was calculated in absolute C amounts (ng PLFA-C g^{-1} soil) based on the PLFA-C concentrations in the liquid extracts.

The biomarker PLFAs analyzed within this dataset included: 18:1ω9c and 18:2ω6,9c (indicative of saprophytic fungi), 16:1ω5 (indicative of arbuscular mycorrhizal fungi-AMF),

i15:0, a15:0, i16:0, i17:0, a17:0 (indicative of Gram-positive bacteria), cy17:0, cy19:0, 16:1ω7c and 18:1ω7c (indicative of Gram-negative bacteria), 14:0, 15:0, and 18:0 (indicative of non-specific bacteria, or bacteria (ns)) and 10Me PLFAs (indicative of Actinobacteria) (Kroppenstedt 1985; Olsson et al. 1995; Zelles 1997). Total fungi, bacteria, gram-positive bacteria and gram-negative bacteria PLFA quantities were calculated as the sum of all PLFA-C from each biomarker group. ^{13}C values were corrected using the working standards (12:0 and 19:0) calibrated on an EA-IRMS. To obtain $\delta^{13}C$ values of the PLFAs, measured $\delta^{13}C$ FAME values were corrected individually for the addition of the methyl group during transesterification by simple mass balance (Denef et al. 2007).

Data analysis

The litter and py-OM contribution to the soil CO_2 efflux, bulk soil, SOM fractions and PLFAs was assessed for the litter and py-OM treated plots as compared to the bare soil plots. The isotopic mixing model was applied as follows:

$$f_{blue} = \frac{\delta_S - \delta_B}{\delta_{blue} - \delta_B}$$

where f_{blue} is the litter or py-OM derived C (or N) fraction of a CO_2 efflux, bulk soil, SOM or PLFA sample, δ_S and δ_B is the $\delta^{13}C$ (or $\delta^{15}N$) of the specific CO_2 efflux, bulk soil, SOM or PLFA sample from a litter or py-OM treatment plot (δ_S) and the corresponding bare (δ_B) plot; and δ_{blue} is the $\delta^{13}C$ (or $\delta^{15}N$) of the initial litter or py-OM. First, the size of the CO_2, bulk soil, SOM or PLFA pool was determined for the litter or py-OM amended plots, then it was multiplied by the f_{blue} to quantify the amount of litter or py-OM derived C or N in each pool.

We tested the effect of litter or py-OM input and site over time on soil CO_2 efflux and the $\delta^{13}C$-CO_2 values using a repeated measures general linear mixed model with input and site as

categorical fixed effects and block, block x site and block x time as categorical random effects. We tested the effect of litter or py-OM input at the IB site on the amount of input incorporation into the bulk soil, SOM fractions and PLFAs (H1) using a general linear mixed model with input (py-OM or litter), depth, time and their interactions as categorical fixed effects, with block and the interaction between block and time as categorical random effects. We tested the effect of fire history between the IB and FB sites at the 11 month sampling on the amount of py-OM and litter incorporation into the bulk soil, SOM fractions and PLFAs (H2) using a general linear mixed model with site (IB or FB), input, depth and their interactions as categorical fixed effects, with block and the interaction between block and site as categorical random effects. We used the SAS software, version 9.3 and used type III tests of fixed effects. We checked for normality of the data and homogeneity of variances of the residuals and used a log transformation when necessary.

To test the effect of litter or py-OM input , site (11 month harvest only) and time (IB site only) on the overall microbial community based on the relative contribution of all of the individual PLFAs to the entire extractable PLFA pool (mol %), we utilized a distance-based redundancy analysis (dbRDA) using the R: Vegan package (Oksanen 2013), following the approach described in Bell et al. (2014). Briefly, we chose the dbRDA analysis over other multivariate statistical approaches due to its non-linear distance-metric options, which have robust multi-dimensional resolution to assess categorical variables. Distance based RDA is a three step ordination technique that tests the effects of response parameters (i.e. mol %) on defined groups (i.e. input, site or time). First, a dissimilarity or distance matrix is calculated for the different treatments. We chose the Bray-Curtis dissimilarity (non-linear) measure to model the species matrix as suggested by Legendre and Anderson (1999a). For steps two and three of

the dbRDA, a principal coordinate analysis (PCoA) is calculated based on the distance matrix, from which the eigenvalues (obtained in the PCoA) were applied to a redundancy analysis (RDA).

Results

Initial litter, py-OM and site properties

Our chamber produced litter had a C:N ratio of 40 (Table 1), lower than that of *A. gerardii* collected wild at the site (C:N = 92,(Soong et al. Submitted)). After pyrolysis, the py-OM produced had a C:N of 34 and a pH resembling py-OM collected after a wildfire at the site in the summer of 2011 (pH=10). In terms of the initial IB and FB site properties (Table 2), the FB site had a significantly higher %C and %N concentration in the top 0-2 and 2-5 cm layers ($p<0.005$), but in the deeper depths there was no difference in the %C and %N of the two sites. The bulk density of the soils increased from 0.22-2.67 g/cm^3 from the 0-2 to the 20-40 cm depths, and did not differ significantly between the two sites. The FB site had a significantly higher concentration of BPCAs ($p<0.0001$) and total PLFAs ($p<0.0001$). The two sites did not differ in their fungi:bacteria ratio ($p=0.4309$), however the FB site had a significantly higher ratio of gram-positive to gram-negative bacteria ($p=0.0040$) (Table 2). A dbRDA analysis of the total microbial PLFA community composition reveals a significantly different relative community composition between the IB and the FB sites ($p=0.038$), with the FB site having a higher relative contribution from the a15:0, i16:0 PLFAs (gram negative bacteria), and the IB site having a higher relative contribution from the 16:1ω7c (gram negative bacteria) and 16:1ω5 (AMF) PLFAs (Figure 6b).

Litter and py-OM contributions to CO_2 efflux

We measured total soil CO_2 flux and quantified py-OM and litter contributions to total soil CO_2 efflux using the keeling plot method with our ^{13}C labeled py-OM and litter at both the IB and FB sites for the entire 11-month study period (Figure 1). Across all of our sampling dates, CO_2 fluxes from litter decomposition were greater than those from py-OM decomposition by an order of magnitude ($p<0.0001$). However, some days of minimal litter derived CO_2 flux were within the range seen of py-OM derived fluxes. Py-OM CO_2 fluxes declined exponentially with time and did not appear to be affected by precipitation or temperature (Figure 1a and 1b). By fitting an exponential decay curve through our measured data points ($y=\text{day 1 flux}*e^{(-0.18*\text{ day n flux})}$), we estimate that approximately 229 mg py-OM C was lost as CO_2 during the entire course of our experiment. This corresponds to 3% of the initial py-OM, significantly less than the 17% measured as volatile matter in our py-OM (Table 1). Litter derived CO_2 fluxes were generally low, but were high on days immediately following precipitation events (Figure 1c and 1d). Unfortunately soil CO_2 efflux models fail to capture the fast responses of surface litter to precipitation events (W. Parton pers. comm.), therefore we did not attempt to estimate the cumulative CO_2 losses from the litter during the 11 month field incubation.

Total soil CO_2 flux rates were higher at the FB site than the IB site throughout the 11-month study ($p<0.0001$). The addition of litter or py-OM did not affect overall soil CO_2 flux rates at either the IB or the FB site ($p=0.1853$). CO_2 flux rates from py-OM were similar at both sites, but CO_2 flux from litter decomposition was higher at the FB site than the IB site ($p=0.0375$). Neither the litter nor the py-OM made a substantial contribution to the total soil respiration, which followed precipitation and soil temperature dynamics (Figure 1e and 1f).

Mass recovery of litter and py-OM

Using the ^{13}C and ^{15}N isotopes, we were able to quantify the contribution of the litter and py-OM inputs to the bulk soil (Figure 2). All of the py-OM that remained was recovered in the soil while the litter C and N remained in both the litter layer and the soil. After 11 months of decomposition in the field the litter had lost 55% of its initial mass at both the IB and FB sites. However at the IB site, 50% of the initial litter C, and 59% of the initial litter N, remained in the litter layer and the soil (down to 40 cm), while at the FB site 47% of the initial litter C and 98% of the initial litter N remained in the litter layer and the soil (down to 40 cm) (Figure 2). After 11 months in the field 67% and 56% of the initial py-OM C and N, respectively, remained in the soil (down to 40 cm) in the IB site, while 75% of the initial py-OM C and 62% of the initial py-OM N remained in the soil (down to 40 cm) in the FB site (Figure 2).

There was significantly more py-OM derived C and N recovered in the 0-2 cm surface soil layer than litter derived C and N at both the IB and FB sites ($p<0.0001$). Within the 0-2 cm layer, the FB soils retained more of the initial py-OM N ($p=0.0034$) and litter N ($p=0.073$) than the IB soils. The FB site also retained more py-OM C in the 0-2 cm layer than the IB site ($p<0.0001$), but there was no difference in litter derived C retention between the sites ($p=0.6700$). Below 2 cm, an equivalent amount of the applied py-OM and litter derived C and N was recovered and there was no effect of site ($p>0.05$).

Litter and py-OM contributions to soil organic matter fractions

We recovered py-OM and litter derived ^{13}C and ^{15}N in the light fraction (LF), sand sized, silt sized and clay SOM fractions of the 0-2 and 2-5 cm soils at the 4 months at the IB site and at 11 months at both the IB and the FB site (Figure 3, Appendix 1). The distribution of py-OM

versus litter C and N among the SOM fractions differed significantly ($p<0.005$) (Figure 3, Appendix 1). Approximately 100 times more py-OM was recovered in the LF fraction than any of the other fractions (Figure 3a vs 3b,c and d), whereas less litter C and N were distributed within the same scale among all four fractions (Figure 3e, f, g, h). Depth was a significant factor ($p<0.05$) for litter and py-OM recovery in all of the SOM fractions, with more py-OM and litter C and N recovery in the SOM fractions in the 0-2 cm than the 2-5 cm depth.

At the IB site, time did not have a significant effect on the amount of py-OM recovered in any of the fractions ($p>0.05$), however py-OM was recovered primarily in the LF at both 4 and 11 months (Figure 3, Appendix 1). From 4 to 11 months, however, time had a significant effect on the litter derived OM in all of the SOM fractions, except for the silt sized fraction, which remained constant over time ($p=0.0834$ for C, $p=0.3739$ for N). The amount of litter derived C in the LF and sand sized fractions remained stable over time ($p=0.9623$ and $p=0.1783$, respectively) while the amount of N declined ($p=0.0007$ and $p<0.0001$, respectively), and the amount of litter derived C and N in the clay fraction increased over time ($p<0.0001$ and $p=0046$, respectively).

Between the IB and FB sites at 11 months, the total amount of py-OM C and N recovery across the four SOM fractions was similar, except the FB site had higher amount of py-OM N in the LF ($p=0.0011$). The total amount of C and N litter recovery between the IB and FB sites at 11 months was similar in the LF and clay fractions, but the FB site had more C and N in the silt and sand sized fractions ($p<0.05$; Figure 3).

The C:N ratio of the py-OM in the LF was 33 on average, which decreased with depth from the 0-2 to 2-5 cm layer, but was not significantly different over time at the IB site or between sites after 11 months. The C:N ratio of the litter-derived OM in the LF varied greatly

with time, depth and site (Figure 4). The C:N ratio of the litter-derived OM in the sand sized, silt sized and clay fractions was lower (~11) than that of the py-OM and remained consistent over time at the IB site (Figure 4).

Litter and py-OM uptake by microbial phospholipid fatty acids

We recovered py-OM and litter derived ^{13}C in all of the PLFAs examined, however it was not distributed equally across all groups (Figure 5). The magnitude of PLFA uptake of litter C far exceeded that of py-OM C by over 50 fold (Figure 5). The gram-negative bacteria were the group responsible for the greatest uptake of both litter and py-OM C (Figure 5). However the ratio of input derived C in the gram-positive versus gram-negative bacteria was significantly higher for litter C (0.449) than for py-OM C (0.048) at the IB site ($p<0.0001$). There was no significant difference in the ratio of input derived C in fungi:bacteria PLFAs between litter (0.166) and py-OM (3.75) at the IB site ($p=0.4617$). Time did not have a significant effect on the overall amount of litter or py-OM derived C taken up into any of the PLFAs in the IB site ($p>0.05$). At 11 months, there was significantly more litter C incorporated into the PLFAs of the FB site than the IB site ($p<0.05$, Figure 5a), while site did not have a significant effect on PLFA incorporation of the py-OM C ($p>0.05$, Figure 5b).

Input (litter or py-OM) and site (IB or FB) had significant effects ($p<0.05$) on the microbial community composition, as assessed by the dbRDA analysis of the relative contribution of the individual PLFAs (Figure 6). The soils with the py-OM addition differed from the litter added and bare soils by the lower prevalence of the 18:1ω9c, 18:2ω6,9c (gram-negative bacteria) and cy19:0 (gram-negative bacteria). The IB site had a higher predominance

of the cy19:0 (gram-negative bacteria), while the UB site had a higher predominance of the a15:0 (gram- positive bacteria) PLFAs.

Discussion

Prescribed grassland fires produce py-OM with a fairly consistent chemical composition that can be well represented by model py-OM produced through pyrolysis from the respective vegetation cover (Knicker et al. 2008). The results from our study demonstrate how pyrolized aboveground OM input to SOM formation from annual burning of grasslands alters SOM formation and C and N cycling in frequently burned grassland soils through the replacement of fresh litter inputs with inputs of recalcitrant py-OM. The accumulation of py-OM in frequently burned soils was evident by the abundance of BPCAs in the FB soils *versus* IB soils at our sites (Table 2).

Litter versus pyrogenic organic matter decomposition

Losses of C through respiration were higher for litter than py-OM inputs (Figure 1). Litter CO_2 efflux followed precipitation events rather than an exponential decay as the CO_2 efflux from py-OM (Figure 1). This indicates that litter CO_2 efflux is primarily limited by moisture, as was previously observed for total soil respiration in this grassland (Knapp et al. 1998b). Litter respiration is expected to persist throughout the decomposition of *A. gerardii* litter as seen in the three year *A. gerardii* decomposition experiment presented in Chapter 5. On the contrary, py-OM CO_2 flux was primarily controlled by its chemistry and only the initial labile fraction of py-OM appeared to be susceptible to microbial decomposition, while the remaining fraction persists unused, similar to results found other studies (Foereid et al. 2011; Knicker et al. 2013). Our

approximation of 3% of the initial py-OM C lost as CO_2 flux is equivalent to that found in laboratory incubations (Stewart et al. 2013b; Soong et al. Submitted; Major et al. 2010). However, this fraction is much lower than the total % volatile matter of our py-OM (Table 1), which has been found to correlate well with total CO_2 losses in other laboratory studies (Zimmerman 2010; Stewart et al. 2013b). We believe that the lower than expected measured CO_2 flux, and overall recovery, from py-OM can be explained by losses through wind erosion. Nearly 40% of the initial py-OM that we added to the soils was lost within the first 4 months of the experiment. Topsoil erosion is known to be a major issue in this windswept Central Great Plains prairie (United States Department of Agriculture 2013), making the lightweight py-OM we added to the top 2 cm of the soil susceptible to loss. Natural py-OM depositions after fires in the tallgrass prairie are also likely to erode and move down wind or down slope, and to contribute to the dissolved organic carbon export from the system (Ding et al. 2013). Clearly, fully understanding py-OM dynamics in an ecosystem requires landscape scale studies of where py-OM is produced and where it is transported (Foereid et al. 2011; Major et al. 2010).

Due to the erosion of py-OM during our experiment, the total recovery of py-OM and litter inputs was relatively similar after 11-months in the tallgrass prairie (Figure 2). In fact, below the top 2 cm of the soil, there was no effect of input or site on the amount of initial input C or N recovered. However, the majority of the py-OM remaining was recovered in the top 0-2 cm of soil, whereas the majority of the litter OM remained in the litter layer after 11 months (Figure 2). In this system we determined that it takes three years to reach the complete decomposition of aboveground litter of similar chemistry as that used here (Chapter 5). Nonetheless after 11 months in situ, a sizeable amount of the py-OM and litter derived OM, respectively, was found to 40cm depth (Figure 2). Leaching of dissolved organic matter is a major flux in the initial

decomposition phase for both litter and py-OM (Hilscher and Knicker 2011; Hockaday et al. 2007; Soong et al. Submitted; Klotzbücher et al. 2013), which along with fragmentation and soil fauna mobilization (Lehmann et al. 2011; Paul 2007), could account for much of their movement to depth in this system.

In examining how the litter and py-OM was distributed among the different SOM fractions in the 0-2 and 2-5 cm soil layers, we found that over 95% of the py-OM remained in the LF (Figure 3a). Additionally, at the IB site the amount of py-OM C and N contribution to the four SOM fractions remained unchanged over time (Figure 3a,b,c,d). This indicates that the py-OM allocation to different fractions is stable over time and more a function of its physical-chemical properties then of microbial transformations. Py-OM is characterized by low density (Lehmann and Joseph 2009) and commonly recovered preferentially in the LF. However it can chemically interacts with minerals, and smaller amounts are found associated to heavy fractions (Singh et al. 2014). By contrast, the litter-derived C and N was more evenly distributed among all of the SOM fractions and appeared to increase in the clay fraction over time (Figure 3e,f,g,h), indicating a progression in the decomposition of litter, with DOM and microbial products contributing to the accumulation of litter decomposition products in the clay fraction over time (Cotrufo et al. 2013). The decrease in the amount of litter derived N in the LF and sand sized fraction over time could imply a more open N cycle with active microbial utilization and mineralization of N as litter decomposition progressed at the IB site.

The input derived C:N ratio in SOM indicates its degree of microbial processing, with a C:N closer to the input material indicating less microbial processing and a C:N closer to that of microbial biomass (C:N of ~4-8) indicating microbially transformed OM (Paul 2007). A C:N of ~10 for the litter derived OM in the sand sized, silt sized, clay and subsoil LF fractions clearly

indicates microbial transformation of the litter material in the soil (Figure 4). The high C:N in the surface layer LF at 11 months could come from non-transformed litter fragments that entered the surface soil between 4 and 11 months and had not yet been utilized by the microbes over this winter period (Figure 4). The high C:N ratio of the py-OM material in all of the SOM fractions further confirms that although some py-OM is distributed among all of the SOM fractions, it is minimally transformed (Figure 4). These results from our SOM analysis demonstrate that the litter material in the soil is biochemically transformed over time by microbes, decreasing the input derived C:N and increasing its contribution to the mineral soil fraction (Grandy and Neff 2008), while py-OM inputs to the soil remains unchanged over time. This inaccessibility of py-OM to microbial decomposition is what gives py-OM its long MRT in the soil and contributes to the accumulation of C and N in frequently burned soils (Lehmann et al. 2011).

Further evidence for this comes from examining the PLFA incorporation of py-OM and litter C during our study. Approximately 50 times more litter C was incorporated into PLFAs than py-OM C (Figure 5). Although use of isotope labeling reveals that a small fraction of lipids and polysaccharides from fresh py-OM can be taken up by microbes into PLFAs, the extremely low rates of utilization compared to litter and SOM reaffirm the overall low microbial use of py-OM as a C source (Kuzyakov et al. 2014; Singh et al. 2014). The gram-negative bacteria were the most dominant users of both litter and py-OM C (Figure 5), but the ratio of C uptake by gram-positive to gram-negative bacteria was lower for py-OM inputs than litter inputs at the IB sites ($p<0.0001$), indicating that gram-negative bacteria are more dominant in py-OM decomposition than litter decomposition. Some laboratory studies using ^{13}C incorporation into PLFAs have similarly found gram-negative bacteria to preferentially utilize py-OM additions to the soil (Gomez et al. 2014) while in contrast others have found gram-positive bacteria to be

preferentially stimulated (Santos et al. 2012). Based on the PLFAs we could detect, our soils had an approximately equal ratio of gram-positive to gram-negative bacteria in contrast to the soils in the Santos et al. (2012) study, which were gram-positive dominated, and this difference in the soil microbial community composition could impact which groups preferentially incorporate py-OM C. The ratio of fungi to bacterial PLFA uptake of litter and py-OM C did not differ in our study, however these soils are dominated by bacteria rather than fungi (Knapp et al. 1998a).

The results from our CO_2 flux, SOM and microbial PLFA analysis clearly demonstrate the contrast between the biochemical transformation of *A. gerardii* litter inputs to SOM formation *versus* the minimal biological transformation of py-OM inputs that remain after a fire. In frequently burned grasslands, this chronic removal of a large input of biologically available OM to the soil and its replacement with largely bio-unavailable py-OM in the soil alters the C and N cycling and increases N demand as demonstrated in our comparison of the FB and IB sites in our study.

Fire history effects on soil organic matter formation through litter and py-OM decomposition

Annual burning of the tallgrass prairie has been seen to increase soil respiration rates over unburned or infrequently burned prairie (Knapp et al. 1998b; Johnson and Matchett 2001). The main mechanism proposed for this increase in CO_2 flux from the soils is through the increased ANPP, belowground allocation and C:N ratios of autotrophs along with soil warming (Johnson and Matchett 2001; Knapp et al. 1998a), however the contribution of autotrophic *versus* heterotrophic respiration to this increased flux has not been determined. Over the course of our 11-month experiment, we similarly found higher soil respiration rates from the FB soils than the IB soils (Figure 1). By partitioning our ^{13}C labeled litter CO_2 flux from the soil CO_2 flux, we

saw that both the soil and the litter respired more CO_2 at the FB site than the IB site (Figure 1), somewhat contrasting the hypothesis that the higher soil respiration in the annual burned areas is mainly of autotrophic origin (Johnson and Matchett 2001; Knapp et al. 1998b). It is estimated that 60% of total soil respiration in the tallgrass prairie is from microbial decomposition processes, while 40% comes from root respiration (Kucera and Kirkham 1971). Considering the high N demand observed at our FB site, the higher soil respiration at the FB site could be a result of microbial acceleration of SOM turnover to scavenge for N, possibly primed by increased root exudation (Dijkstra et al. 2013). Similarly, increased respiration of litter inputs may result from the increased N limitation and thus lower C use efficiency in FB soils (Manzoni et al. 2012).

Frequent burning of grasslands is known to decrease N mineralization and decrease N availability in grassland soils (Turner et al. 1997; Johnson and Matchett 2001). The results of our experiment provide mechanistic evidence for why the accumulation of inert py-OM alters N cycling in frequently burned soils as compared to infrequently burned soils, which receive actively cycled litter OM inputs. Litter decomposition rates and py-OM recovery were not affected by burning history, but the FB soils retained much more of the decomposing litter N, while losing more litter C to CO_2, than the IB soils (Figure 2 and 1). The FB soils have a higher C and N content than the IB soils (Table 2). Since py-OM inputs build up unused in the soil, the annual contribution of recalcitrant py-OM inputs to the FB soils explains the buildup of microbially inaccessible C and N at this site (Johnson and Matchett 2001; Turner et al. 1997). However, when labile litter material is added to these soils it is quickly utilized and the C respired as the N is tightly conserved (Ojima et al. 1994). This result indicates that the biota in the FB soils have not lost the ability to decompose fresh OM inputs, but that the OM in the soil is

inaccessible to them, and they are in fact starved of fresh inputs and readily use it if provided the opportunity.

Evidence from the microbial PLFA incorporation of py-OM and litter reveals the biological mechanism behind litter decomposition, and the lack there of for py-OM decomposition. Although we hypothesized that the microbial community in FB soils would be more adapted to py-OM inputs and thus more able to decompose it, we saw no difference in the py-OM use by the microbes at the FB site *versus* the IB site (Figure 5a). Incorporation of litter C into PLFAs however, was higher at the FB site than the IB site (Figure 5b) and this increased microbial use of litter C could explain the higher CO_2 flux from the decomposing litter at the FB site (Figure 1). Although we saw an effect of py-OM input on the preferential uptake of C by gram-negative bacteria, and the dbRDA analysis revealed significant differences in the microbial community composition at the two sites (Figure 6b), we did not see any site effects on the gram-positive to gram-negative ratio or the fungi to bacteria ratio. PLFAs are a coarse biomarker of microbial community composition, however and we cannot make any strong conclusions about how the FB and IB microbial communities differ.

The frequency of burning clearly affects C and N cycling in tallgrass prairie soils due to annual input of either litter or py-OM in IB or FB soils, respectively. Soil decomposers breakdown litter OM releasing C and N in mineralized forms, which can be lost to the atmosphere or taken up by plants continuing to cycle biogeochemically. In contrast, when py-OM replaces aboveground litter as a source of SOM input it remains in the soil relatively unused and unaltered by decomposers. Thus this SOM becomes removed from biogeochemical cycling and its C and N remains locked up in the soil, increasing C and N stocks, but limiting their bioavailability in the ecosystem. Thus FB soils cycle fresh litter N inputs much more tightly and

conserve the N within the system where it is limited in comparison to IB sites, which have abundant bioavailable N and more open N cycling.

This effect of fire on SOM formation through py-OM inputs provides a mechanism for the indirect effects of fire on tallgrass prairie productivity. For example, fire has been seen to reduce N mineralization and its availability for plant uptake, but not alter (Kitchen et al. 2009) or reduce total soil N content (Fynn et al. 2003). The reduction in N cycling is likely due to the lack of bioavailable N in py-OM inputs, and the overall balance between N loss during combustion and indecomposable N remaining as SOM in the soil may depend on fire intensity and erosion. Although fire removes bioavailable N during combustion, the N remaining in py-OM builds up in the soil but is unusable by soil biota and thus unavailable for ANPP. Thus the C:N ratio of roots is higher in burned grasslands, and biomass is preferentially allocated belowground, leading to higher CO_2 flux rates in these N limited soils (Johnson and Matchett 2001; Kitchen et al. 2009). The initial pulse in N mineralization seen immediately after a late spring fire has been explained by the increase in soil temperatures during a period of high moisture, with microbes using bioavailable root litter as a C and N source (Ojima et al. 1994). However, fire has been seen to reduce overall N mineralization rates and increase NUE in annually burned prairie (Ojima et al. 1994). This is in contrast to ungulate grazed prairie, where the aboveground biomass is similarly removed, but SOM formation is not altered by py-OM inputs so N availability is high due to biological decomposition of SOM (Johnson and Matchett 2001). The reduced bioavailability of SOM formed by py-OM in burned grasslands helps to explain the difference in N cycling in burned and grazed grasslands. Finally, unburned sites that are burned in a single fire event are seen to be even more productive than annually burned sites (Blair 1997). Blair (1997) explains this pulse in productivity after a single fire in an unburned prairie as due to

the simultaneous maximization of N availability in an unburned prairie and the removal of energy limitation of the litter layer in a single fire. Our research provides more insight into how the N available SOM formed in an unburned grassland provides the nutrients to support this pulse in productivity, whereas the SOM largely formed through py-OM in an annually burned grassland does not have SOM that is able to be decomposed and released as mineralized nutrients for plant uptake and maximum productivity even though it may have a similar or higher soil N content. Many studies have focused on the effects of fire on N mineralization and ANPP rates in grassland (Blair 1997; Johnson and Matchett 2001; Ojima et al. 1994; Collins and Wallace 1990), but examining SOM formation and decomposition may help to provide more insight into the effect of fire on grassland ecosystems overall.

Conclusions

Our incubation of ^{13}C and ^{15}N labeled *A. gerardii* litter and py-OM in the tallgrass prairie at both an IB and a FB site reveal the different mechanisms behind their contribution to SOM formation and the implications this has on C and N cycling. The microbial decomposition of *A. gerardii* litter releases both C and N into mineral forms, and microbially transformed litter material, some of which are lost to the soil system as SOM is formed and accumulated. The microbial community at the IB site received annual inputs of bioavailable litter C and N, so has a rather open N cycle leading to the loss of N through mineralization and eventually plant uptake or volatilization. In contrast, microbes do not actively decompose py-OM inputs to the soil, so any major loss of C and N is abiotic and what remains in the soil is unprocessed and mostly unavailable to the soil decomposers. Annual inputs of bio-unavailable py-OM infer limited N and C availability to soil microbes at the FB site, so that an input of bioavailable litter is rapidly

decomposed by microbes and its N is tightly conserved. The overall effect of long term py-OM inputs to the soil from fire on soil C and N stocks depends on how much C and N is lost in combustion and erosion, however we believe that the py-OM that remains in the soil will have a long MRT due to its resistance to biological decomposition. These results also highlight the importance of N-fixing bacteria in maintaining N availability in chronically burned systems (Blair et al. 1998; Ojima et al. 1990). Our study demonstrates how when fire removes aboveground litter inputs to SOM formation and replaces it with py-OM the biological process of SOM formation is fundamentally altered, changing C and N cycles in annually burned grassland soils.

Table 6.1. Initial *A. gerardii* litter and pyrogenic organic matter (py-OM) characteristics

	Py-OM	Litter
% C	58.17	41.92
% N	1.73	1.06
C:N	34	40
$\delta^{13}C$	3084	3045
$\delta^{15}N$	17729	19253
% O	39.21	na
% H	1.94	na
% Ash	12.1	24.2
pH	10.0	na
% Volatile matter	17.36	na

Table 6.2. Initial soil characteristics of the infrequently burned (IB) and frequently burned (FB) sites for the top 0-5 cm. Values are averages with standard error in parentheses, n=4.

	Infrequently burned (IB)	Frequently burned (FB)
%C	4.16 (0.199)	5.22 (0.546)
%N	0.334 (0.014)	0.395 (0.0357)
C:N	12.5	13.2
BPCA (ng C/g soil)	994 (95.7)	1539 (121)
Total PLFAs (ng C/g soil)	20,590 (1646)	17,221 (1004)
Fungi:Bacteria PLFAs	0.181 ($4.8x10^{-3}$)	0.176 ($3.9x10^{-3}$)
Gram+:Gram- PLFAs	0.737 ($1.40x10^{-2}$)	0.836 ($1.67x10^{-2}$)

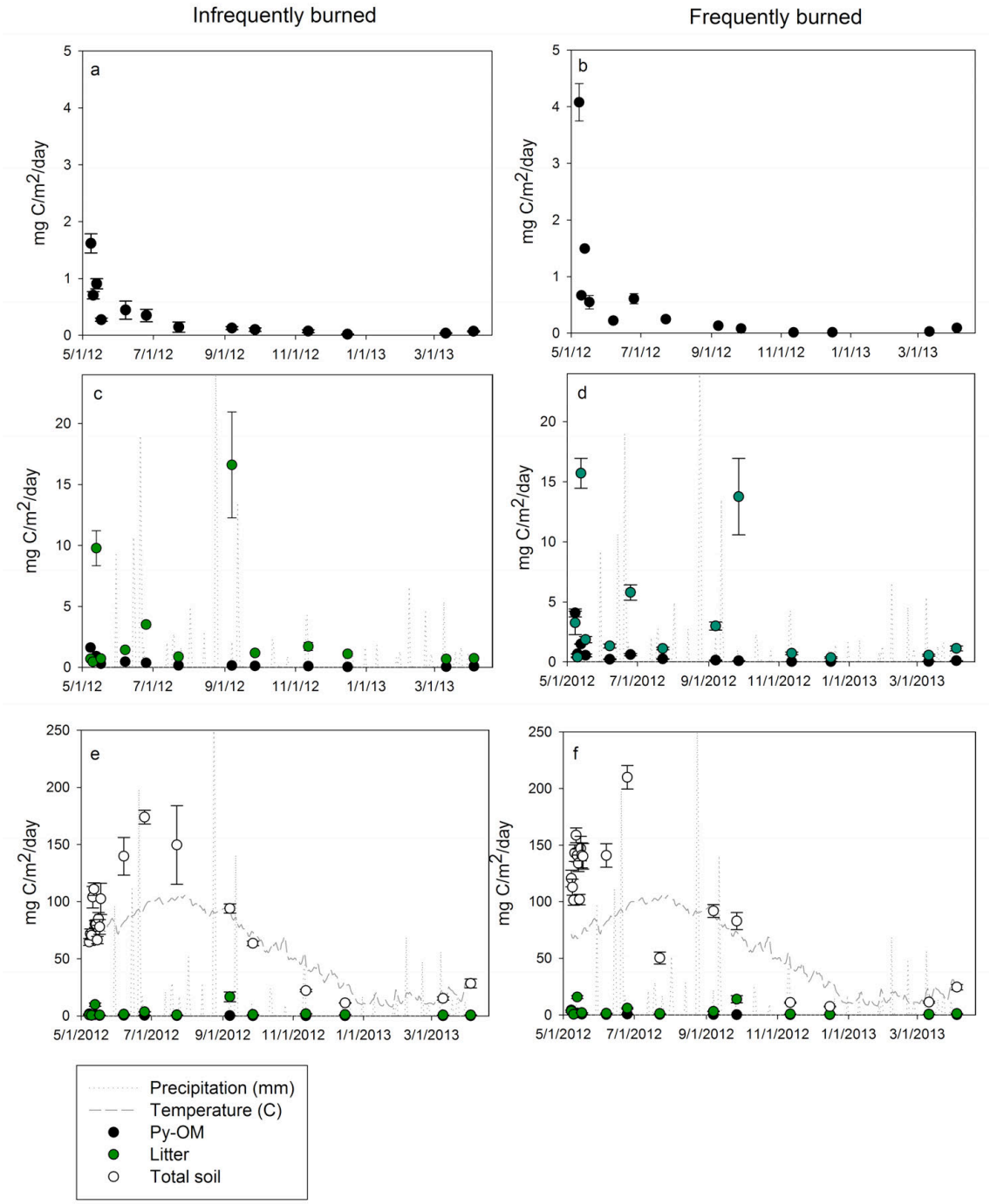


Figure 6.1. CO_2 fluxes from py-OM (a,b), litter (c,d) and total soil (e,f) at the infrequently burned (a,c,e) and frequently burned (b,d,f) sites. Error bars are standard error, n=4. Daily total precipitation (mm) and mean daily soil temperature (°C) are plotted as grey lines on a scale from 0-60.

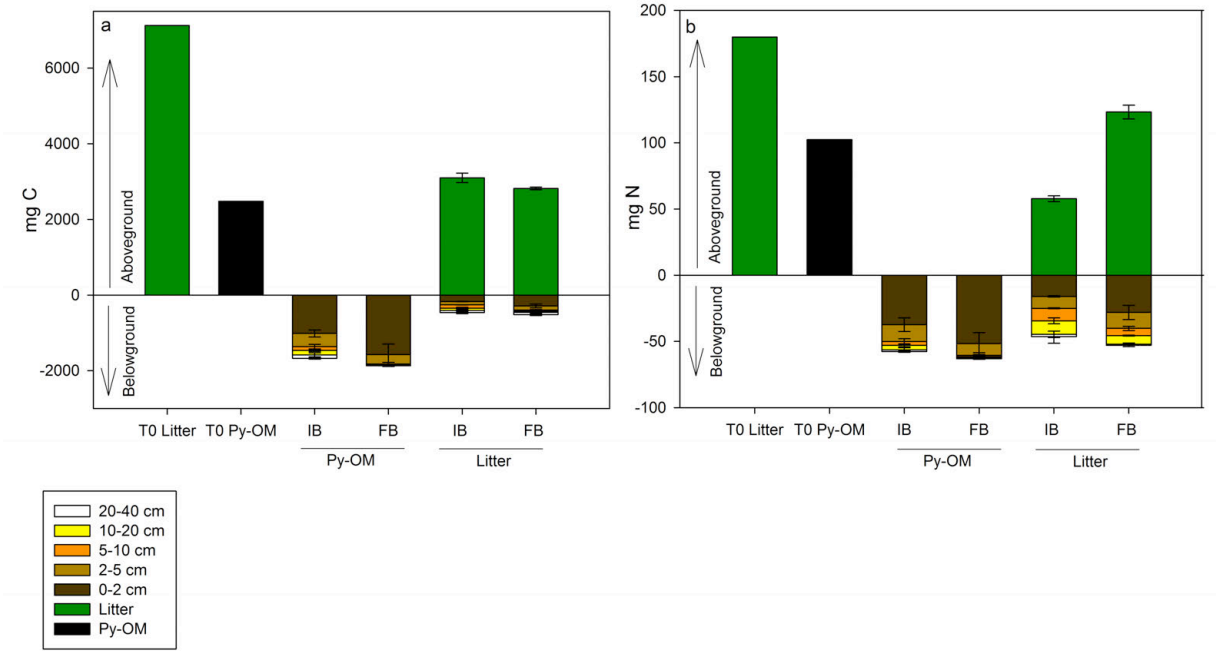


Figure 6.2. Carbon (a) and nitrogen (b) recovery in litter and py-OM (aboveground) and soil (belowground) after 11 months of incubation in the field. IB= infrequently burned site, FB= frequently burned site. Error bars are standard error, n=4.

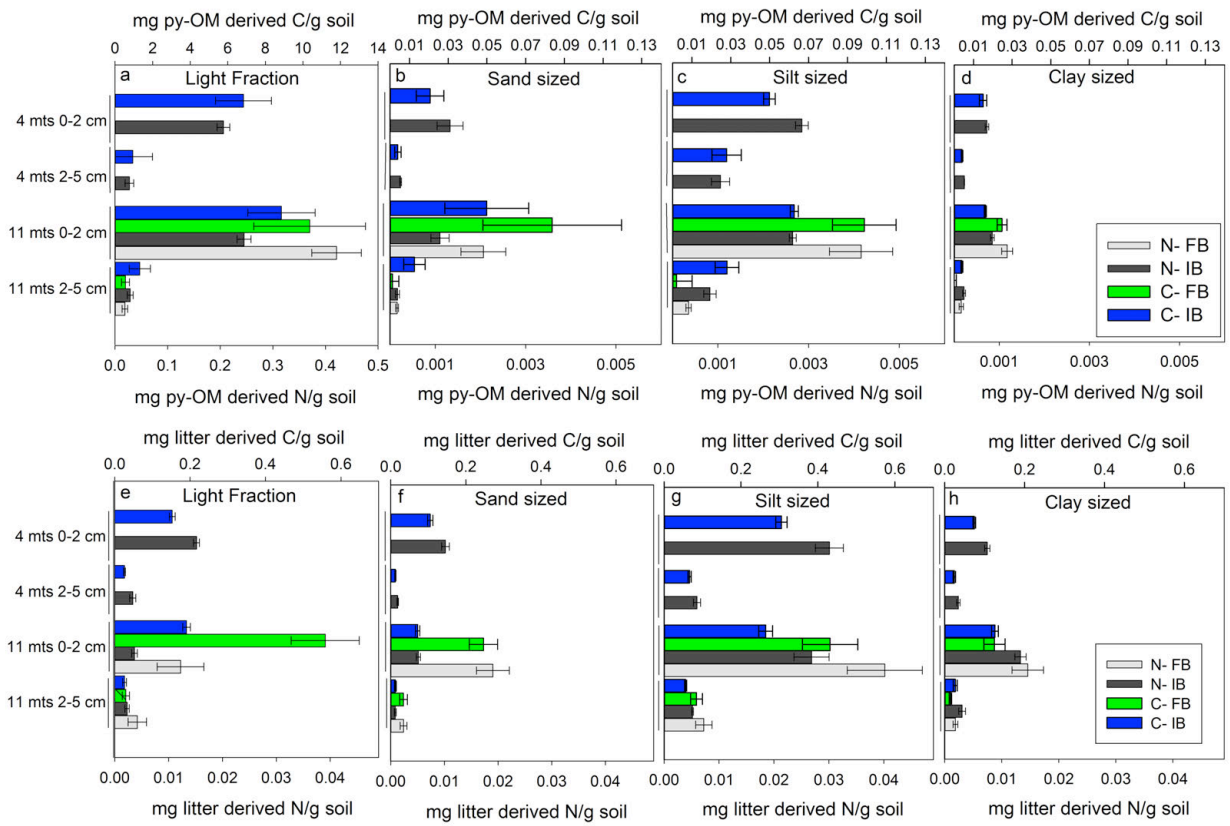


Figure 6.3. Concentration of py-OM (a,b,c,d) and litter (e,f,g,h) derived C (hashed bars) and N (open bars) recovered in the light fraction (a,e), sand sized (b,f), silt (c,g), and clay (d,h) soil organic matter fractions of the Infrequently Burned (IB) and Frequently Burned (FB) sites at two soil depths and two time points (IB only). Error bars= standard error, n=4.

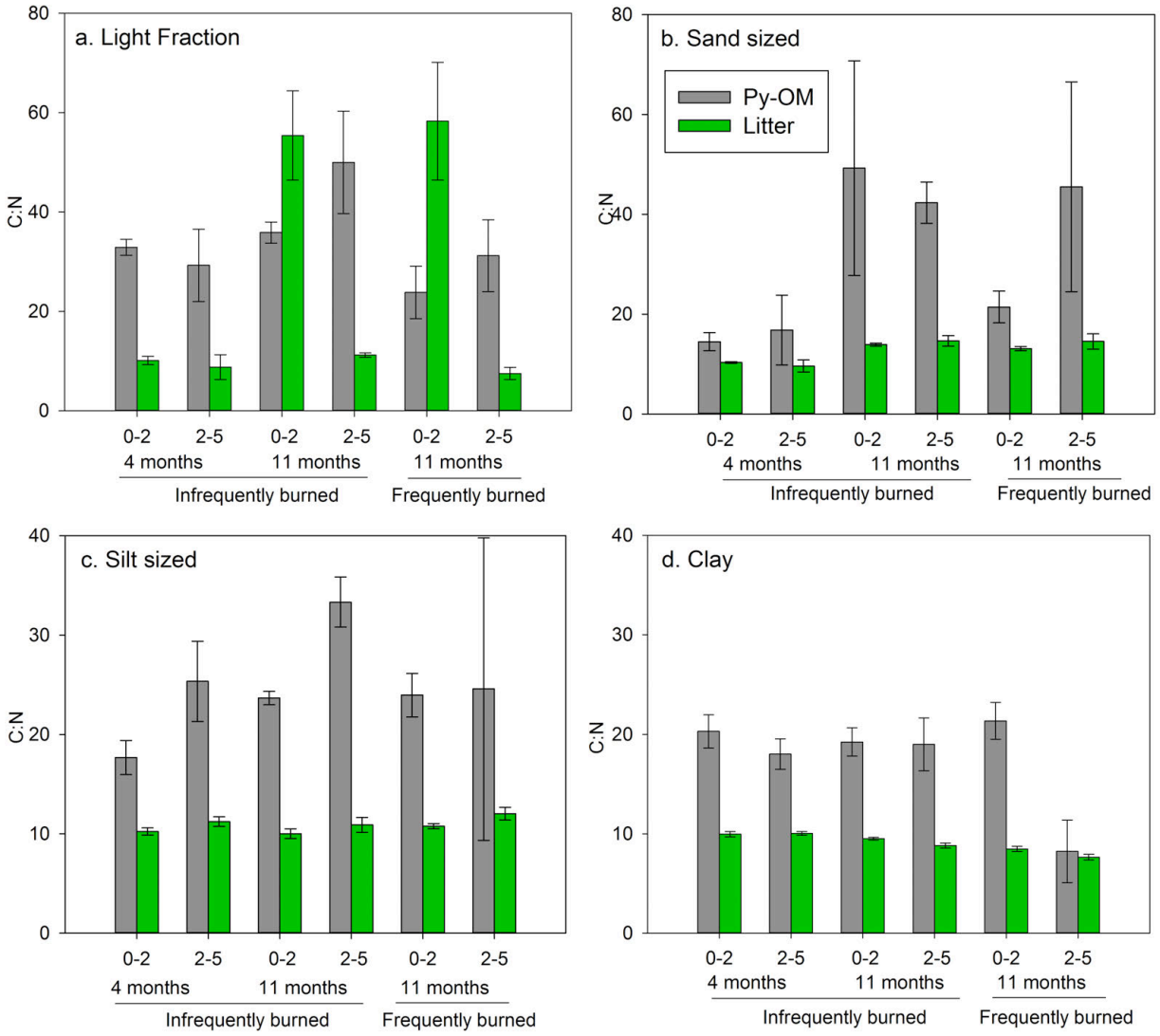


Figure 6.4. C:N ratio of the litter and py-OM derived organic matter in the physically defined SOM fractions, a) light fraction, b) sand sized fraction, c) silt sized fraction and d) clay fraction. Values are averages, n=4, bars are standard error.

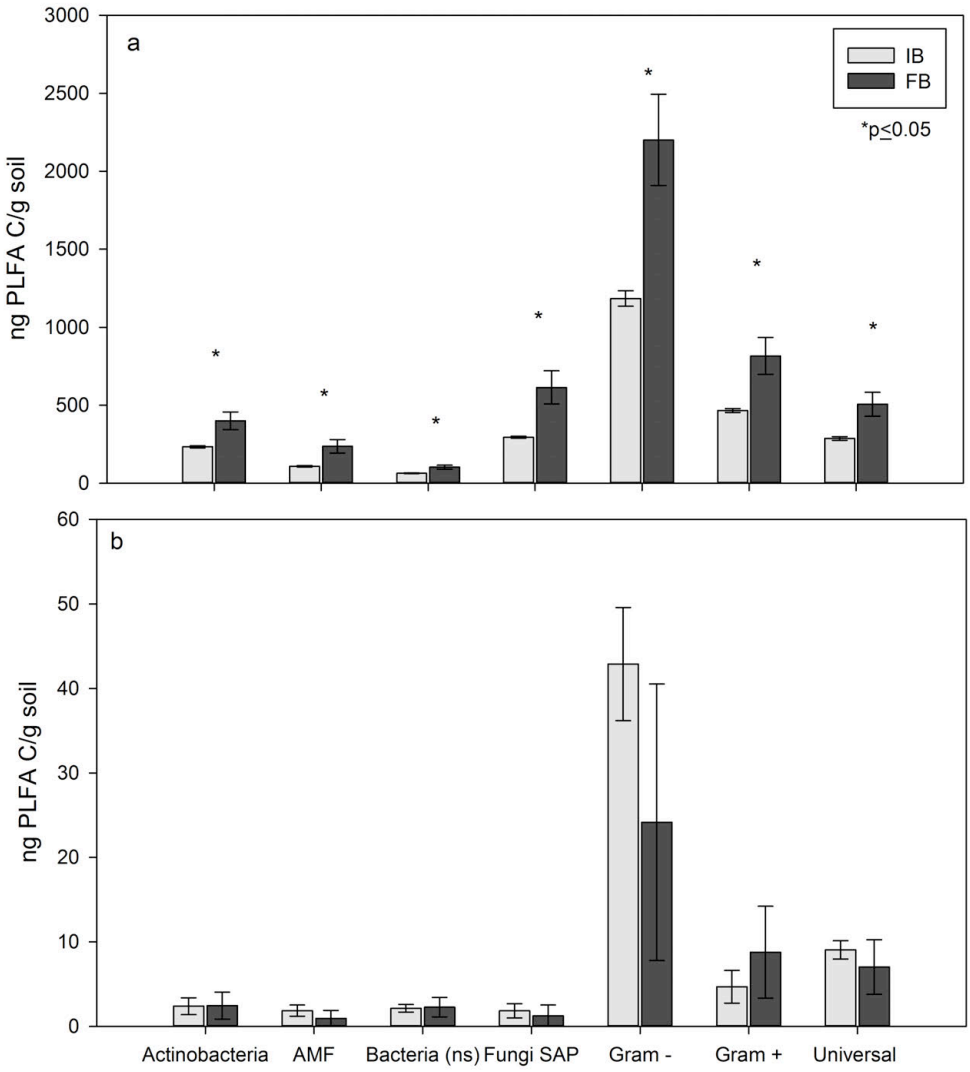
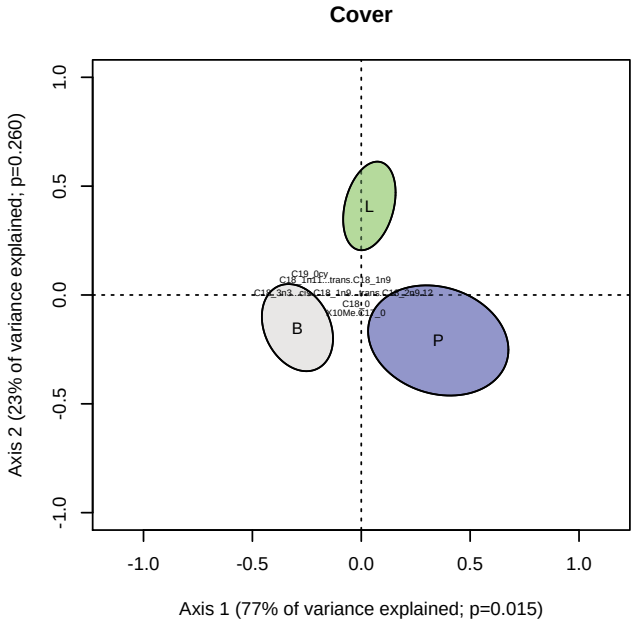


Figure 6.5. Incorporation of a) litter derived C and b) py-OM derived C in the microbial phospholipid fatty acid (PLFA) at 11 months of incubation in the 0-2 cm soil. PLFAs biomarkers were summed by microbial group, error bars are standard error, n=4.

a



b

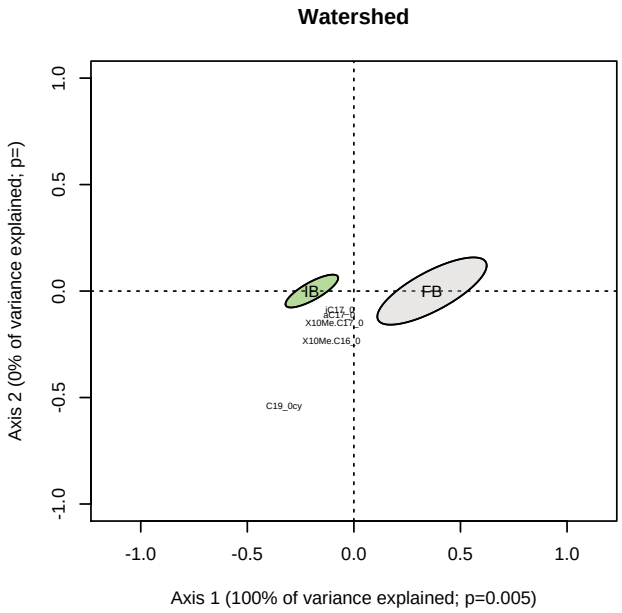


Figure 6.6. DbRDA results of the microbial community composition using relative abundance of the individual PLFAs analyzed by (a) cover where B= bare soil, L= litter and P= py-OM, and (b) site, IF= infrequently burned, and FB= frequently burned.

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Chapter 7: Summary and Conclusions

The primary objective of my dissertation was to improve understanding of how decomposing plant material and py-OM contribute to SOM formation and the storage and cycling of C and N in terrestrial ecosystems, and to develop the best methods to study this. My major questions were

1. How do litter chemistry, fragmentation, and laboratory methods for analyzing dissolved organic matter compare in terms of the quantity and quality of dissolved organic matter leachate from litter?
2. Does aboveground litter quality determine the amount of dissolved organic carbon and carbon dioxide lost during litter decomposition?
3. Can we produce ^{13}C and ^{15}N labeled plant material that is either uniformly or differentially labeled in its metabolic and structural components using a continuous isotope-labeling chamber?
4. Do soil microarthropods increase litter contributions to stabilized soil organic matter formation through top-down controls on microbial activity?
5. How does fire affect soil organic matter formation through the conversion of aboveground litter to pyrogenic organic matter as an input to soil organic matter formation?

In the laboratory experiment presented in chapter 2, I addressed the first question and found that cutting, litter type and leaching method all significantly affected the amount, but not the functional group chemistry, of the dissolved organic matter (DOM) leached. Based on

fourier-transformed infrared spectroscopy (FTIR), the functional group chemistry of all five of the fresh litter types tested were all relatively similar in composition. These results provide one of the first FTIR cross comparison of five different fresh litter types, and provides a basis for comparing litter leaching results across various studies in the literature.

In chapter three I continued my investigation of DOM leaching to answer question 2. I found that litter and py-OM chemistry, the amount of hot water extractable carbon and the lignocellulose index in particular, control the amount of dissolved organic carbon (DOC) leached during decomposition and that the lignocellulose index (lignin/(lignin+cellulose)) can be used to predict the ratio of C lost to DOC:CO_2 during the later phases of decomposition. Additionally FTIR leachate chemistry changed more with time over the course of decomposition than with litter type. These results provide a description of how litter and py-OM initial characterization can be used to predict DOC inputs to the soil and their chemical composition, and can be used to model C fluxes during decomposition.

The third question was addressed in chapter four, where I described how to build a dual ^{13}C and ^{15}N continuous isotope labeling chamber for uniform or differential labeling. The chamber design can now be used and adapted by others to produce dual labeled plant material for various ecological experiments. Our design has been used to build at least two other isotope labeling chambers, and has continued to be used at Colorado State University to answer various questions about litter and py-OM decomposition.

Using the isotope labeled plant material we produced, I found that microarthropods increase the formation of stabilized SOC in the silt and clay fractions of the soil in a fine textured tallgrass prairie. These results highlight the impact of soil biodiversity and food web dynamics on SOM formation and reveal the mechanisms behind how they impact SOM formation. These

results can be used to inform models of SOM formation that want to include soil fauna to improve biogeochemistry modeling.

Finally, again utilizing the dual isotope labeled plant material, I answered question five in the field study of litter and py-OM decomposition in a frequently burned and infrequently burned tallgrass prairie site. I found that the input of py-OM from frequent burning circumvents the biological pathway of litter decomposition, which alters C and in particular N cycling in the frequently burned site. This study reveals the mechanisms behind how the process of SOM formation by litter or py-OM inputs is fundamentally different, and affects ecosystem functioning.

My dissertation research has focused on advancing studies in litter decomposition by using laboratory incubations and isotope labeled plant material in field studies to track the fate of decomposing leaf litter and py-OM into the mineral soil. Using the methods I developed in chapters one and three, I have been able to quantify fluxes of C and N from decomposing materials into losses to the ecosystem (i.e. CO_2) and inputs to the soil through leaching, fragmentation and microbial uptake. Rather than describing the specific functioning of a single ecosystem (i.e. the tallgrass prairie), I have taken a mechanistic approach in an attempt to reveal the underlying biological, chemical and physical processes controlling C and N cycling during decomposition. By using this approach I hope to have presented results that can be used to improve our understanding of biogeochemistry and ecosystem functioning in tallgrass prairie soils and under laboratory conditions, which should be similarly investigated in other ecosystems and can be used to improve predictions for how these processes may be altered and feedback to local and global change scenarios in the future.