DISSERTATION

PATHWAYS OF SOIL ORGANIC MATTER FORMATION IN AGROECOSYSTEMS AS INFLUENCED BY LITTER CHEMISTRY, ROOT DEPTH AND AGGREGATION

Submitted by

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ABSTRACT

PATHWAYS OF SOIL ORGANIC MATTER FORMATION IN AGROECOSYSTEMS AS INFLUENCED BY LITTER CHEMISTRY, ROOT DEPTH AND AGGREGATION

Soils contain more carbon (C) than any other terrestrial reservoir, and the increase of these C stocks has been targeted as a potential climate solution globally. Agroecosystems play a critical role in our ability to provide these climate solutions through increasing soil organic matter (SOM). There is significant potential for SOM accrual in agroecosystems due to the degradation of SOM typically observed in these systems. One promising approach to increasing soil C sequestration is through the selection of deep-rooted crops, such as *Sorghum bicolor*. However, significant questions remain about root inputs' ability to contribute to SOM in order to balance the greenhouse gas (GHG) lifecycle of a bioenergy feedstock. My dissertation aims to answer some of these questions as well as to propose a framework to integrate the study of SOM formation from crop inputs with soil aggregate structure.

Bioenergy has the potential to emit fewer GHGs than other fuel sources, such as fossil fuels, yet there are some emissions during the transportation production of bioenergy feedstocks and fuels that could be offset by soil C sequestration. However, in annual bioenergy systems, aboveground biomass is typically removed from the system, meaning roots are the primary source of OM available to return to the soil. However, roots and shoots may differ significantly in their ability to contribute to SOM due to differences in litter chemistry. In Chapter 2, I conducted a field incubation to understand how sorghum root versus leaf litter, as influenced by their contrasting chemistry, affect the formation and stabilization of SOM. Using unique soil-

biomass microcosms to incubate root or leaf litter in topsoil (0-30 cm) for 19 months in the field, I traced the fate of litter decomposition products by combining stable ^{13}C and ^{15}N isotope labeling with extensive separation of physical soil fractions, free or within different aggregate structures. I found that roots, which were lower quality than leaves, decomposed more slowly but contributed more efficiently to total SOM formation than leaves. However, leaves contributed more to the stable SOM pool (i.e. associated to minerals) while roots contributed more to less stable fractions (i.e. light particulate organic matter).

Additionally, sorghum is known to produce roots to a depth of 2 meters. There is limited understanding of how roots deeper in the soil (e.g., below 30 cm) lead to SOM formation and stabilization. In Chapter 3, I used the same microcosm approach as in Chapter 2, with roots that were incubated up to a 90 cm depth to better understand how depth influences the ability of roots to contribute to the formation of SOM and what role aggregates play in this process. Results of this study showed that differences in root decomposition dynamics with depth resulted in greater accrual of root litter C in more stable mineral associated SOM pools in the surface depth while there was slower decomposition and greater accrual in the less stable particulate organic matter fractions in the deep soil. Interestingly, most of the stable fraction was recovered within soil aggregates, particularly microaggregates.

The results of these experiments emphasized the important role of microaggregates in modulating SOM dynamics. In Chapter 4, I used the information gleaned from Chapters 2 and 3 as well as advances in the SOM research community to speculate on the role of aggregation, specifically microaggregates, in moderating SOM formation by presenting a conceptual framework that integrates aggregates within our current understanding of particulate and mineral associate SOM dynamics.

Overall, my dissertation addresses fundamental questions about our ability to increase SOM levels and resulting soil C accrual through the production of a deep-rooted crop through a field incubation. At the same time, I have connected these relevant results to the broader SOM research community by presenting a novel conceptual model that advances our current SOM framework. My hope is that this will be a valuable contribution to the field and spark discussion and future research.

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CHAPTER 1- INTRODCUTION

Soils contain more carbon (C) than any other terrestrial reservoir, and the increase of these C stocks has been targeted as a potential climate solution globally. Agroecosystems can play a critical role in providing climate change solutions through regenerative practices and management decisions that can increase soil organic matter (SOM), and therefore soil C (Lal, 2020; Paustian et al., 2016; Prairie et al., 2023). Given the degradation of SOM typically observed in agroecosystems (Sanderman et al., 2017), there is significant potential for SOM accrual. This C deficit is likely due to soil disturbance and breakdown of aggregate protection from conventional management practices, such as tillage and reliance on inorganic nutrient inputs (Bailey et al., 2019; Jastrow et al., 2007). One promising approach to increasing soil C sequestration potential is through increasing the production and depth distribution of roots through selective crop breeding (Heinemann et al., 2023; Kell, 2011, 2012; Paustian et al., 2019), and through feedstock selection of deep-rooted crops, such as *Sorghum bicolor*.

Sorghum is a varied bioenergy feedstock due to genetic diversity leading to a range of biomass yields, grain production, and sugar content within the stalks (Bean et al., 2007; Mullet et al., 2014; Olson et al., 2013). In fact, some varieties produce root systems up to 2 m in depth leading to large amounts of biomass (up to 7 Mg ha^{-1}) (Lamb et al., 2022; Meki et al., 2013; Monti & Zatta, 2009; Sainju et al., 2005). In annual bioenergy systems, roots are often the main source of organic matter (OM) available to return to the soil, since most of the aboveground biomass, like grain and stalks, is harvested for ethanol production (Lal, 2009). However, roots and leaves may differ significantly in their ability to contribute to SOM formation.

When evaluating potential differences in SOM formation, measuring total soil C is not adequate to understand the likely fate of decomposed litter, because it does not indicate the soil C pool that was formed. Increasing SOM levels can simultaneously improve C accrual or storage *and* soil health and function (Lehmann et al., 2020; Moinet et al., 2023) by contributing to two functionally distinct SOM pools, mineral associated (MAOM) and particulate organic matter (POM), respectively (Angst et al., 2023; Lavallee et al., 2020). These two soil pools are also understood to form via two pathways (Cotrufo et al., 2015) and have different stabilization mechanisms, in which MAOM is on average more stable with longer mean residence times in the soil (Cotrufo et al., 2019; Lavallee et al., 2020; Lutzow et al., 2006). Moreover, MAOM and POM can exist free in the soil or occluded within soil aggregates. Aggregation, particularly macroaggregates, are critical to the incorporation of plant inputs to the soil (Jastrow et al., 2007; Six et al., 2000), while microaggregates are considered important stabilizers of POM (Lehmann & Kleber, 2015).

Litter chemistry is a determinant in the differential contribution of root or shoot derived litter to SOM and the likelihood that it will contribute to the stable SOM pool, MAOM (Bird & Torn, 2006; Lavallee et al., 2018). Litters characterized as "high quality" or low biochemical recalcitrance, such as with low C:N and acid unhydrolyzable fibers, are expected to be efficiently transformed by microbes and preferentially contribute to MAOM (Cotrufo et al., 2013; Kallenbach et al., 2015). Conversely, biochemically recalcitrant inputs generally contribute more to POM formation (Castellano et al., 2015). This led to my first research question:

1. How does above versus belowground litter, as influenced by different litter chemistry, impact litter decomposition and subsequent formation of functionally different C and N SOM pools?

There is limited understanding of how roots deeper in the soil (e.g., below 30 cm) lead to SOM formation and stabilization (Harper & Tibbett, 2013; Rumpel & Kögel-Knabner, 2010). The concentration of organic C in the soil decreases with increasing depth, yet globally over 50% of soil C is stored in the deep soil (Jobbágy & Jackson, 2000) with longer mean residence times than surface soil C (Fontaine et al., 2007; Rumpel & Kögel-Knabner, 2010; Schmidt et al., 2011). Roots, their exudates, and associated microbes are the primary contributor to SOM, especially in the deep soil (Angst et al., 2018; Gross & Harrison, 2019; Jackson et al., 2017; Kong & Six, 2010; Rasse et al., 2005). Deep soil C pools are buffered from environmental conditions, such as seasonally fluctuating precipitation and temperature (Gulser & Ekberli, 2004), microbial activity decreases with depth (Blume et al., 2002; Liang et al., 2018), and they are relatively stable unless the conditions under which they formed change (Gross & Harrison, 2019). These dynamics of roots in the deep soil led to my second research question:

2. How does soil depth impact the ability of root litter to contribute to the formation of MAOM and POM particularly in the deep soil where there is more potential for C accrual? What role do aggregates play in this process?

I explore these two questions in detail through the experiments described in Chapters 2 and 3. Using novel soil-biomass microcosms to incubate root or leaf litter in the 0-30 cm depth and root litter at three depths up to 90 cm, I traced the fate of litter decomposition products by combining stable ^{13}C and ^{15}N isotope labeling of *Sorghum bicolor* root and leaf litter with extensive separation of primary and secondary soil fractions by size and density in order to understand the litter contribution to MAOM and POM and the likely fate of the newly formed SOM. The results of these experiments emphasized the important role of microaggregates in POM and MAOM dynamics which led me to my third question:

3. Does aggregation, specifically microaggregates, play a role in moderating the transformation of POM and the formation of stable MAOM?

The advances in our understanding of SOM dynamics over the last 20 years have been tremendous (Rocci et al., in review). However, the role of aggregates has been largely left out of these recent conceptual frameworks. Macroaggregate incorporation of POM leads to the formation of microaggregates within them (Gale et al., 2000; Jastrow et al., 2007; Oades, 1984); as POM decomposes it is encrusted with primary soil particles, forming organo-mineral complexes (i.e., MAOM). Thus, MAOM and POM are inherently linked in proximity within microaggregates. In Chapter 4, I explore this last research question through a perspective paper that presents a SOM framework to advance current POM and MAOM frameworks to include aggregates as moderators of POM transformation and MAOM formation.

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CHAPTER 2- PATHWAYS OF SOIL ORGANIC MATTER FORMATION FROM ABOVE AND BELOWGROUND INPUTS IN A *SORGHUM BICOLOR* BIOENERGY CROP[1]

Introduction

The sustainability of bioenergy feedstocks has long been debated (Fargione et al., 2008; Karp & Shield, 2008; Searchinger et al., 2008) prompting extensive research to focus on the ability of specific bioenergy feedstocks to sequester carbon (C) in the soil in order to offset the production of greenhouse gases throughout the lifecycle of the feedstock (Heaton et al., 2008; Lemus & Lal, 2005; Nocentini et al., 2017) *Sorghum bicolor* has been identified as an important bioenergy feedstock due to the combination of several unique characteristics, such as drought tolerance, high water use efficiency, low nutrient requirements, vast genetic diversity, deep root systems, and tremendous yield potential (up to 30 T ha^{-1}) (Bean et al., 2007; Energy, 2016; Mullet et al., 2014; Olson et al., 2013). Given the vast genetic diversity of sorghum, it can contribute to bioenergy production in three ways: sorghum grains from grain varieties for first generation biofuel production; crop residues remaining after grain harvest for cellulosic biofuel production; and as a dedicated energy crop – high biomass varieties or high sugar varieties' aboveground materials – for cellulosic biofuel production (US Department of Energy, 2016). In cellulosic biofuel production, aboveground residues are harvested. Thus, above-ground residue C and N are exported out of the system which may be detrimental to soil C stocks and recycling of nutrients in these annual cropping systems (Blanco-Canqui & Lal, 2007; Lal, 2005, 2008). There is a need

[1] Fulton-Smith, S.E. and Cotrufo, M.F. Pathways of soil organic matter formation from above and belowground inputs in a Sorghum bicolor bioenergy crop, GCB Bioenergy, 11, 971-987, 2019. https://doi.org/10.1038/s41467-022-31540-9

to understand the ability of cellulosic biofuel crops to contribute to C sequestration while supporting nutrient cycling through the decomposition of below ground organic matter.

Crop research has begun to focus on the enhancement of root systems, such as increases in total root production or specific root length (Kell, 2011, 2012) as a strategy to improve soil health, increase drought tolerance, and as a C sequestration strategy through the contribution of root biomass to SOM formation (Paustian et al., 2016). Sorghum is a crop of particular interest in this context, because cultivars produce large deep root systems, to 1-2m in depth (Monti & Zatta, 2009; Sainju, Singh, et al., 2005; Schittenhelm & Schroetter, 2013; Stone et al., 2001). Additionally, it is unique as an actively researched bioenergy feedstock, because it is grown as an annual crop, thus the entire root system is left to decompose every year. Sorghum root biomass estimates range from ~ 0.5 to 6.1 Mg dry matter ha^{-1} based on field measurements, to 7 Mg dry matter ha^{-1} based on modeled projections (Meki et al., 2013; Monti & Zatta, 2009; Sainju, Whitehead, et al., 2005; Schittenhelm & Schroetter, 2013).

The ability of crop residues to contribute to SOM formation has traditionally focused on aboveground biomass, or shoots. However, live and dead roots are major actors in the formation, stabilization, and destabilization of SOM (Sokol et al., 2018), and their contribution to SOM formation has started to receive significant attention (Balesdent & Balabane, 1996; Kong & Six, 2010; Lavallee et al., 2018; Rasse et al., 2006; Schmidt et al., 2011) . Yet, we lack a complete understanding of how root *versus* shoot detritus form SOM into differently protected physical fractions and contribute to soil C and N storage and/or recycling. While the point of entry of plant inputs may significantly determine their fate (Mitchell et al., 2016, 2018; Sokol et al., 2019), differences in litter chemistry between roots and shoots may be the most important

determining factor in their contribution to SOM and the likelihood that this new SOM will persist in the soil (Bird & Torn, 2006; Lavallee et al., 2018).

Plant inputs have been proposed to contribute to SOM formation via two main pathways (Cotrufo et al., 2015). Leaching of water-soluble compounds happens early in the decomposition process and leads to the formation of mineral associated organic matter (MAOM) either through direct association to minerals or after being metabolized by microbes (Liang et al., 2017). Later in the decomposition process, the remaining residue structural components fragment and contribute to SOM mostly in the form of light organic matter (LF) (Haddix et al., 2016). Further decomposition of plant residues or LF in the soil may also contribute to sand-sized heavy particulate organic matter (POM) formation (Grandy & Neff, 2008). LF, heavy POM and MAOM can be considered primary SOM fractions that may be found in soil free or occluded in aggregates of different size classes (Christensen, 2001). Aggregates are composite dynamic structures (Jastrow, 1996; Poeplau et al., 2018; Six, Elliott, et al., 2000). Micro-aggregates (250-53 μm) have been consistently found to resist disturbance, thus are believed to offer protection from further mineralization to the occluded primary SOM fractions (Six & Paustian, 2014). The role of organic matter in the formation of aggregates is well studied, but much less is known regarding the role of aggregates in the formation of new SOM and its distribution between different primary fractions (Jastrow, 1996; Six, Elliott, et al., 2000).

Mineral association and physical occlusion are believed to be the main drivers of long-term SOM persistence (Lehmann & Kleber, 2015), along with environmental inhibition of microbial activity, while biochemical recalcitrance has been dismissed from this role (Dungait et al., 2012; Marschner et al., 2008; Schmidt et al., 2011). Although biochemical recalcitrance may not correlate to long-term SOM persistence (Kleber & Johnson, 2010; Klotzbücher et al., 2011;

Preston et al., 2009), plant input chemistry determines annual-scale litter decomposition rates (Adair et al., 2008; Preston et al., 2009) and the pathways and efficiency of SOM formation (Cotrufo et al., 2015; Lavallee et al., 2018). Measures of litter chemistry such as hot water extractable C (%HWE-C), lignin content (Melillo et al., 1982), C:N ratio (Manzoni et al., 2012), and lignocellulose index (LCI) [lignin/ (lignin + a-cellulose)] (Osono & Takeda, 2005) are good indicators of dissolved organic C production (Soong et al., 2015), litter mass loss rates, and microbial carbon use efficiency (Manzoni et al., 2012; Moorhead et al., 2013). According to the Microbial Efficiency-Matrix Stabilization framework plant inputs characterized by low biochemical recalcitrance (i.e., high %HWE and low C:N and LCI) are expected to contribute to MAOM formation more than biochemically recalcitrant plant inputs (Cotrufo et al., 2013). As an extension, biochemically recalcitrant inputs would preferentially accumulate as POM (Castellano et al., 2015). While this hypothesis has been tested and often confirmed in the laboratory (Córdova et al., 2018; Haddix et al., 2016; Lavallee et al., 2018) field experiments are still limited and largely performed in natural forest or grassland systems (Bird et al., 2008; Bird & Torn, 2006; Sokol et al., 2018; Soong et al., 2016). Understanding how differences in litter chemistry impact the decomposition and resultant SOM formation of root *vs* shoots is going to be critical to informing agricultural management, particularly in bioenergy production systems.

We designed a field experiment to address the knowledge gaps highlighted above. Specifically, we compared differences in litter chemistry between *Sorghum bicolor* roots and leaves to study: (1) how they form SOM into differently protected physical fractions and contribute to soil C and N storage and/or recycling to depth, and (2) the role of aggregates in SOM formation. We hypothesize that the root residues, because of their higher biochemical recalcitrance will decompose slower, and will contribute more to LF than leaves, which

conversely would decompose faster and form relatively more MAOM. Due to the greater LF contribution from roots we expect relatively more N to be released from root than leaf litter over time, and overall, relatively more residue-derived N to be accumulated at depth than C from either litter type. Moreover, we expect aggregates to promote MAOM formation, particularly in microaggregates through high physical proximity of the new organic matter to soil minerals.

To test these hypotheses, we incubated ^{13}C and ^{15}N enriched sorghum roots or leaves in unique soil-biomass microcosms *in situ* for 19 months. To test the effect of litter chemistry both litter types were incubated within the same soil volume (i.e., top 0-30cm). We depicted differences in the chemistry of the litters by C:N, % hemicellulose, % α-cellulose, % acid unhydrolyzable residue (AUR), and % HWE-C.

Incubated soils were destructively harvested and separated into light (LF), heavy POM, and MAOM, as found free or occluded in macro and micro-aggregates.

Materials and Methods

Site description

The study was conducted at the Agricultural Research Development and Education Center (ARDEC), Colorado State University, Fort Collins, Colorado (40°39′ N, 104°59′ W; 1554 m above sea level). The field site has a history of mixed-use irrigated agriculture. An area of approximately 400 m^2 was tilled and planted with *Sorghum bicolor v BTx 623* in 2013 and 2014 and irrigated during the growing season for the purpose of this experiment. Average annual precipitation for the area is 330 mm and mean monthly temperature ranges from 0 °C in January to 22 °C in July. The clay loam soil is classified as a mixed, superactive, mesic, Aridic Haplustalf. Average soil bulk density (n=4) is 1.07, 1.09, 1.13 g cm^{-1} at 0-30 cm, 30-60 cm, and 60-90 cm depths, respectively. Soil texture across the profile is on average (n=3) 34.34% sand,

34.22% silt and 30.75% clay (percentage sand increased by 52.23% with depth from 29.43% at 0-30 cm to 44.81% at 60-90 cm, but the soil remains within clay loam classification throughout its profile).

Soil collection

In October 2013, after sorghum harvest, 36 soil cores up to 90 cm were extracted using a truck mounted hydraulic soil probe (Giddings Machine Company Inc., Windsor, Colorado) approximately 1 m apart throughout the experimental area. A PVC tube was inserted in each hole, as a placeholder, until microcosms were incubated in the field as described below. Each core was divided into three depths: 0-30 cm, 30-60 cm, and 60-90 cm. Soils from each depth were carefully 8mm sieved to avoid breaking up soil aggregates, with large rocks and roots removed. The sieved soil was combined and homogenized in large bins according to depth and kept moist at room temperature for two weeks until used for the incubation as described below.

Isotopically labeled Sorghum litter production

Sorghum bicolor v. BTx 623 was grown in a ^{13}C and ^{15}N continuous labeling chamber as described in Soong *et al*. (2014). After 22 weeks of growth in the chamber, the plants were harvested. Aboveground plant material was cut at the base of the plant ~3 cm from the soil surface, separated into leaves and stalks by stripping the leaves from the stalks by hand, and air-dried. Belowground plant material was removed from the potting mixture (sand, vermiculite, and profile porous ceramic) by dumping the whole pots over a 6mm sieve and gently shaking the roots loose. Roots were rinsed thoroughly with deionized water and air-dried. While Sorghum stalks are likely the dominant type of above ground residue after grain is harvested, leaves were used in our study. During preliminary analysis of the litter, leaves and stalks did not differ significantly in their C:N, LCI, and % AUR, but both did differ from roots. HWE-mass was

higher for stalks (44.10%; se = 0.97) than leaves (27.01%; se = 0.17) but both were greater than roots (14.69%; se = 0.27) (n=3). These analyses were repeated for leaves and roots only as reported in the results (Table 2.2). We concluded that leaves were a good representative of aboveground materials from sorghum in regard to litter chemistry, contrasted well with the litter chemistry of roots, and could be incorporated into the soil more similarly to roots than stalks allowing for a more direct comparison of the two different litters in the sorghum production system. Fine roots, less than 2mm in diameter, or leaves, cut into ~2 cm long pieces, were homogenized. Three replicate sub-samples of initial root and leaf litter were analyzed for water content by drying in an oven at 65 °C, and subsequently analyzed for their isotopic composition, %C and %N on an elemental analyzer - isotope ratio mass spectrometer (EA-IRMS: Costech ECS 4010 elemental analyzer, Italy coupled to a Thermo-Fisher Delta V Advantage isotope ratio mass spectrometer). Roots were enriched at 4.55 ^{13}C atom % and 5.94 ^{15}N atom % while leaves were enriched at 4.60 ^{13}C atom% and 6.35 ^{15}N atom%. Additional measures of litter chemistry, such as % hemicellulose, % α-cellulose and % acid un-hydrolyzable residue (AUR) were determined using the neutral detergent fiber (NDF) and acid detergent fiber (ADF) methods (Soest et al., 1991), and % hot water extractable (HWE) mass was determined using the hot water extract method (Tappi, 1981).

Field experiment

To study litter decomposition in the field and track litter derived C and N in SOM and along the soil profile a microcosm approach was used, using 1m long, 5.08 cm diameter PVC tubes. To allow root ingrowth 18 circular holes (3.2 cm diameter) were cut along each of the PVC tubes. Nylon mesh (1.6 mm) was sewn into 1m columns with heavy-duty nylon thread and placed inside the PVC tubes. Collected soils were then placed at their corresponding field depth

Table 2.1. Carbon (C) and nitrogen (N) concentration and their isotopic composition in bulk soil, native litter, and all the SOM physical fractions isolated in this study, as well as the relative contribution of each fraction to the bulk soil, for the 0-30cm depth layer. C and N concentrations are averaged across all litter types, harvests and replicates, n=36 with ± standard error in parentheses. ^{13}C and ^{15}N isotope delta values are averaged across four replicates and three harvests from the control microcosms, n=12 with ± standard error in parentheses.

Fraction	Abbreviation	% of total soil	% C	% N	$\delta^{13}C$	$\delta^{15}N$
Bulk Soil		100	2.09 (0.02)	0.17 (0.003)	-14.17 (0.11)	6.07 (0.15)
Litter residues		0.03 (0.01)	35.76 (0.84)	1.21 (0.07)	-16.42 (1.71)	8.47 (2.39)
Macroaggregates	M	63.00 (1.01)	2.29 (0.01)	0.19 (0.001)	-14.51 (0.07)	6.05 (0.13)
Free microaggregates	m	30.06 (0.82)	1.53 (0.03	0.12 (0.002)	-14.27 (0.20)	5.61 (0.19)
Free MAOM	MAOM	6.55 (0.22)	2.37 (0.02)	6.51 (0.28)	-13.05 (0.16)	6.51 (0.28)
Occluded coarse POM	Coarse POM	3.11 (0.12)	1.21 (0.05)	0.08 (0.004)	-18.02 (0.92)	5.28 (0.74)
Occluded microaggregates	M_m	36.07 (0.74)	2.16 (0.05)	0.19 (0.004)	-15.34 (0.08)	6.04 (0.26)
Occluded MAOM	M_MAOM	23.82 (0.37)	2.38 (0.02)	0.18 (0.002)	-13.38 (0.11)	6.67 (0.26)
Free microaggregate occluded LF	m_LF	0.07 (0.02)	35.67 (0.80)	2.60 (0.06)	-23.82 (0.70)	9.24 (1.43)
Free microaggregate occluded POM	m_ heavy POM	12.90 (0.37)	0.38 (0.01)	0.019 (0.001)	-11.81 (0.32)	1.66 (0.22)
Free microaggregate occluded MAOM	m_MAOM	16.86 (0.53)	1.98 (0.03)	0.19 (0.002)	-16.15 (0.19)	6.60 (0.09)
Occluded microaggregate LF	Mm_LF	0.13 (0.02)	32.18 (0.10)	2.47 (0.09)	-23.07 (0.09)	6.18 (0.53)
Occluded microaggregate POM	Mm_heavy POM	10.44 (0.25)	0.87 (0.02)	0.07 (0.002)	-17.49 (0.20)	3.30 (0.15)
Occluded microaggregate MAOM	Mm_MAOM	23.68 (0.78)	2.20 (0.02)	0.23 (0.003)	-16.36 (0.13)	6.68 (0.09)

(0-30 cm, 30-60 cm or 60-90 cm) within one continuous soil column. The appropriate weight of soil was used at each depth in order to recreate field site bulk density. In order to study the effect

of root and leaf residue chemistry on their decomposition and resultant SOM formation, isotopically enriched sorghum root or leaf residues (~1 g) were mixed with the 0-30 cm soil depth prior to being funneled into the mesh sock inside the PVC column. Soil without litter was placed at the 30-60 and 60-90 cm depth. Control columns did not receive any litter addition, throughout the depth layers. The mesh column was closed at both ends with a zip tie.

The *in-situ* decomposition study began on November 9, 2013, when 36 soil-biomass microcosms were returned to the field and placed vertically 0-90 cm below the surface of the soil with 10 cm protruding above the soil surface. The microcosms were placed in the holes remaining from the initial soil collection and the sides were packed with soil to minimize atmospheric interaction.

The experimental design was a split plot randomized complete block design with four replicate blocks. Within each block, harvest time (7, 13 or 19 months) was randomly assigned to one of three rows and litter types (root, leaf, or no residue control) and randomly assigned within rows. Three destructive harvests of 12 microcosms (4 replicate * 3 litter types) occurred on June 2nd, 2014, December 1st, 2014, and June 1st, 2015.

Litter and bulk soil processing

At harvest, microcosms were removed from the field and placed in a 4°C refrigerator until they were deconstructed the following day. They were cut into three pieces by depth (0-30 cm, 30-60 cm, and 60-90 cm) with a circular saw. Each piece was removed from the PVC placed intact into a sealed plastic bag and returned to the 4 °C refrigerator until being processed within two weeks from harvest. All processing and analyses happened individually per tube and depth layer.

Harvested soils were removed from the mesh sock, gently broken to pass through an 8mm sieve. Roots or leaves greater than 8mm in length were removed with tweezers and saved as litter mass remaining, while the soil was homogenized and weighed at field moisture. Bulk soil subsamples of ~20 g were dried at 105°C for % moisture and bulk density (BD) determination. The remaining soil was air dried for 3-4 days. The recovered litter was rinsed with deionized water over a 250 μm sieve to remove soil particles and then oven dried at 65 °C. Oven-dry soil and leaf or root litter were finely ground and analyzed for %C, %N, $\delta^{13}C$ and $\delta^{15}N$ on the EA-IRMS.

Soil organic matter fractionation

Macro- and micro- aggregates and free MAOM were isolated by size, and aggregates further fractionated, in order to separate occluded micro aggregates and primary SOM pools within aggregates: light fraction (LF), heavy POM, and MAOM.

First, a 75 g bulk soil subsample was wet sieved to separate into three aggregate size classes, macroaggregates (M: >250μm), free microaggregates (m: 250-53 μm), and free silt and clay-sized organic matter (MAOM: <53 μm), modified from (Six, Paustian, et al., 2000). We did not isolate large macroaggregates (>2000 μm) as preliminary testing showed they comprised less than 5% of the total soil, likely due to the history of tillage at the site. All fractions were oven dried at 65°C, subsamples of M and m were set aside for further fractionation, and the remainder was finely ground and stored at room temperature in glass jars.

Subsamples of 20g of the M fraction were dispersed and separated into coarse particulate organic matter (Coarse POM: >250 μm), occluded microaggregate (Mm: 53–250 μm), and macroaggregate occluded MAOM (M_MAOM: <53 μm), using a microaggregate isolator as described in Six *et al*. (2000a) and Galdo *et al*. (2003) . All fractions were oven dried at 65°C,

finely ground and stored in glass jars at room temperature. Subsamples of Mm were set aside for further fractionation.

Subsamples of m and Mm were further fractionated by density and size as described in Soong & Cotrufo (2015) into a light fraction (mLF or MmLF: < 1.85g cm^{-3}) and a heavy fraction. The heavy fraction was separated by size into microaggregate (m and Mm) occluded heavy POM (>53 μm) and microaggregate (m and Mm) occluded MAOM (<53 μm). POM and MAOM fractions were oven dried at 65°C, finely ground, and stored in glass jars. LF fractions were too small to be analyzed individually, thus four unground replicates were composited for further analysis. Subsamples from all fractions were analyzed for %C, %N, $\delta^{13}C$ and $\delta^{15}N$ on the EA-IRMS. A full characterization in terms of C and N distribution across the different physical fractions of the soil from the control microcosms is provided in Table 2.1.

Data analysis

Litter-C and N contribution to the bulk soil, recovered litter residue, and SOM fractions were assessed for the litter-added plots as compared to the control (no litter-added) plots, using the isotopic mixing model as follows:

$$f_{litter} = \frac{\delta_s - \delta_c}{\delta_{litter} - \delta_c}$$

where f_{litter} is the litter derived C or N fraction of bulk soil, recovered litter, or SOM fraction, δ_s is the $\delta^{13}C$ or $\delta^{15}N$ of the specific C or N pool (bulk soil, recovered litter or SOM fractions), δ_c is the $\delta^{13}C$ or $\delta^{15}N$ of the corresponding control pool, averaged by block across harvests (Table 2.1), and δ_{litter} is the $\delta^{13}C$ or $\delta^{15}N$ of the initial litter. The percentage of litter-derived C (LDC) and N (LDN) in these pools were obtained by multiplying the f_{litter} values to corresponding C or N pool size of the fraction, then dividing by the amount of litter C or N added to the soil. The LDC and LDN pool in the SOM fractions was calculated for individual samples. Percentage LDC and

LDN are used to report the results of this study rather than mg-C or N g-soil^{-1} in order to be able to directly compare the two litter types with differing initial %C and %N (Table 2.2).

R (version 3.0.2) was used for statistical analysis, including specific packages lme4, lmerTest and lsmeans. Separate linear mixed models (fit by REML t-tests and Satterthwaite approximations to degrees of freedom) were considered for the following C and N pools: bulk soil, recovered litter, primary and secondary SOM fractions, and total SOM, POM and MAOM recovered (sum of all three depths). Response variables were % LDC and LDN in each pool and % fraction in total soil. Separate models were fit for each fraction, due to the different scales of response variables across fractions. The model included litter type, sampling time and their interactions as fixed effects and block and block x sampling time as random effects. We checked for normality and homogeneity of variances of the residuals and applied a log-transformation when necessary.

Results

Litter chemistry

Sorghum bicolor root and leaves had significantly different initial litter chemistry (Table 2.2), with the leaves characterized, as expected, by a lower biochemical recalcitrance, as shown by the significantly higher HWE (by 62%), and lower C:N (by 29%) and LCI (by 69%) than root litter. Both litters showed relatively low biochemical recalcitrance with the AUR fraction being less than 10% in both litter types.

Litter decay and recovery of litter derived C and N along the soil profile

We measured litter decay by applying the isotopic mixing model to all litter collected from the microcosms at each harvest. This litter also contained new root ingrowth, but isotopic labeling allowed us to accurately trace the fate of the remaining isotopically enriched leaf or root

Table 2.2. Initial *Sorghum bicolor* roots and leaves chemistries. Values are laboratory replicate means ± standard error in parentheses; n=3 for %Carbon, %Nitrogen, C:N and n=4 for % hemicellulose, % α-cellulose, % AUR (acid unhydrolyzable residue, a proxy for lignin or suberin), LCI (lignocellulose index) and % HWE (hot water extractables). * P < 0.05; ** P < 0.01; *** P < 0.001.

	Carbon (%)	Nitrogen (%)	C:N	hemi-cellulose (%)	α-cellulose (%)	AUR (%)	LCI	HWE (%)
Roots	46.66*** (0.10)	1.04*** (0.60)	45.02*** (0.56)	22.48 (0.34)	35.57 (0.23)	8.27*** (0.07)	0.188*** (0.002)	14.49*** (0.01)
Leaves	48.81*** (0.23)	1.45*** (0.02)	33.68*** (0.42)	21.20 (1.43)	37.45 (1.26)	3.80*** (0.22)	0.092*** (0.005)	27.50*** (0.01)

treatment. Our isotopically enriched *Sorghum bicolor* leaf and root litter lost over 99.99% of initial C content and 99.99% of initial N content, by 19 months of incubation (Figure 2.1). Exponential decay was observed for C and N loss dynamics from both litter types. Leaf litter C and N had faster rates of loss than root litter C and N:

- Leaf C = $100.00e^{-0.0117x}$, $r^2 = 0.9370$
- Leaf N = $100.00e^{-0.131x}$, $r^2 = 0.9855$
- Root C = $100.00e^{-0.0113x}$, $r^2 = 0.8112$
- Root N = $100.00e^{-0.0121x}$, $r^2 = 0.9107$

By 19 months of incubation, 19.27% LDC from leaf litter and 26.68% LDC from root litter was recovered in total SOM ($p = 0.0109$); 42.48% LDN from leaf litter and 40.21% LDN from root litter was recovered in total SOM. These LDC and LDN recoveries are cumulative across the incubation depth (0-30cm) and the deeper soil (30-60cm and 60-90cm) for both litter types (Figure 2.1).

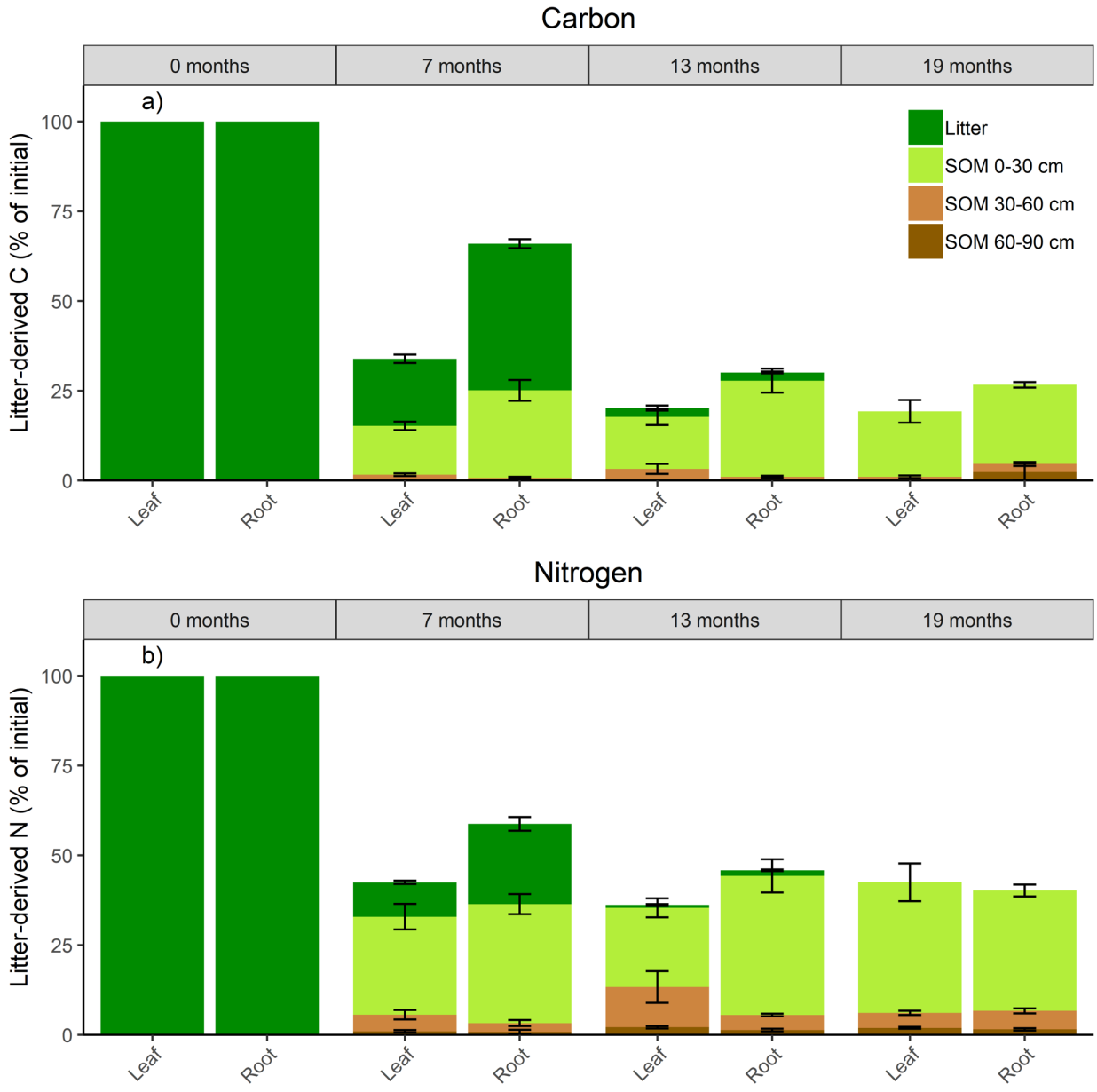

Figure 2.1: Sorghum bicolor leaf- and root-derived (a) Carbon and (b) Nitrogen recovered in the litter residue or bulk soil organic matter (SOM) within the depth layer where the litter was incubated (0-30cm) or in those below (30-60; 60-90cm), for the three time harvests during the 19 months of field incubation. Amounts are given in % of the initial represented as the litter residue added at time 0. Data are average with standard errors (n=4).

Most of the LDC and LDN were recovered in the incubation depth, though more N moved to the deeper depths than C (Figure 2.1a-1d). Litter type had a main effect on LDC in total SOM

(sum of all three depths), in which leaves contributed significantly less to bulk SOM than roots (Table 2.3, Figure 2.1a & 1b). There was no main effect of time and no significant interaction between time and litter type for LDC recovered in bulk SOM; there was no main effect of time or litter type and no interactive effect on LDN in bulk SOM (Table 2.3).

Recovery of litter derived C and N in free and aggregate occluded SOM fractions

After initial fractionation of the 0-30cm bulk soil into M, free m and free MAOM, most of the LDC and LDN was recovered in M, macroaggregate structures (Figure 2.2). After only 7 months of incubation, 7.65% LDC and 13.91% LDN were recovered from leaf litter and 11.25% LDC and 14.20% LDN were recovered from root litter in the M fraction. Litter type had a main effect on LDC but not on LDN recovered in M. Time had a main effect on LDC and LDN recovered in M, but there was no interaction between the two for LDC or LDN recovered in the M fraction (Table 2.3). LDC recovered in the M fraction from leaf litter was 32.69% lower than from root litter ($p = 0.0370$) at 13 months of incubation. While persisting, this difference was no longer significant ($p = 0.0516$) after 19 months of incubation. Within the leaf litter treatment, the LDC and LDN recovered in M increased over time by 67.56% ($p = 0.0461$) and 82.42% ($p= 0.0054$), respectively. Within the root litter treatment, the LDC and LDN recovered in M increased over time by 47.91% ($p = 0.0270$) and 74.63% ($p = 0.0095$), respectively (Figure 2.2). There was no main effect of litter type or time for LDC or LDN recovery in free m or free

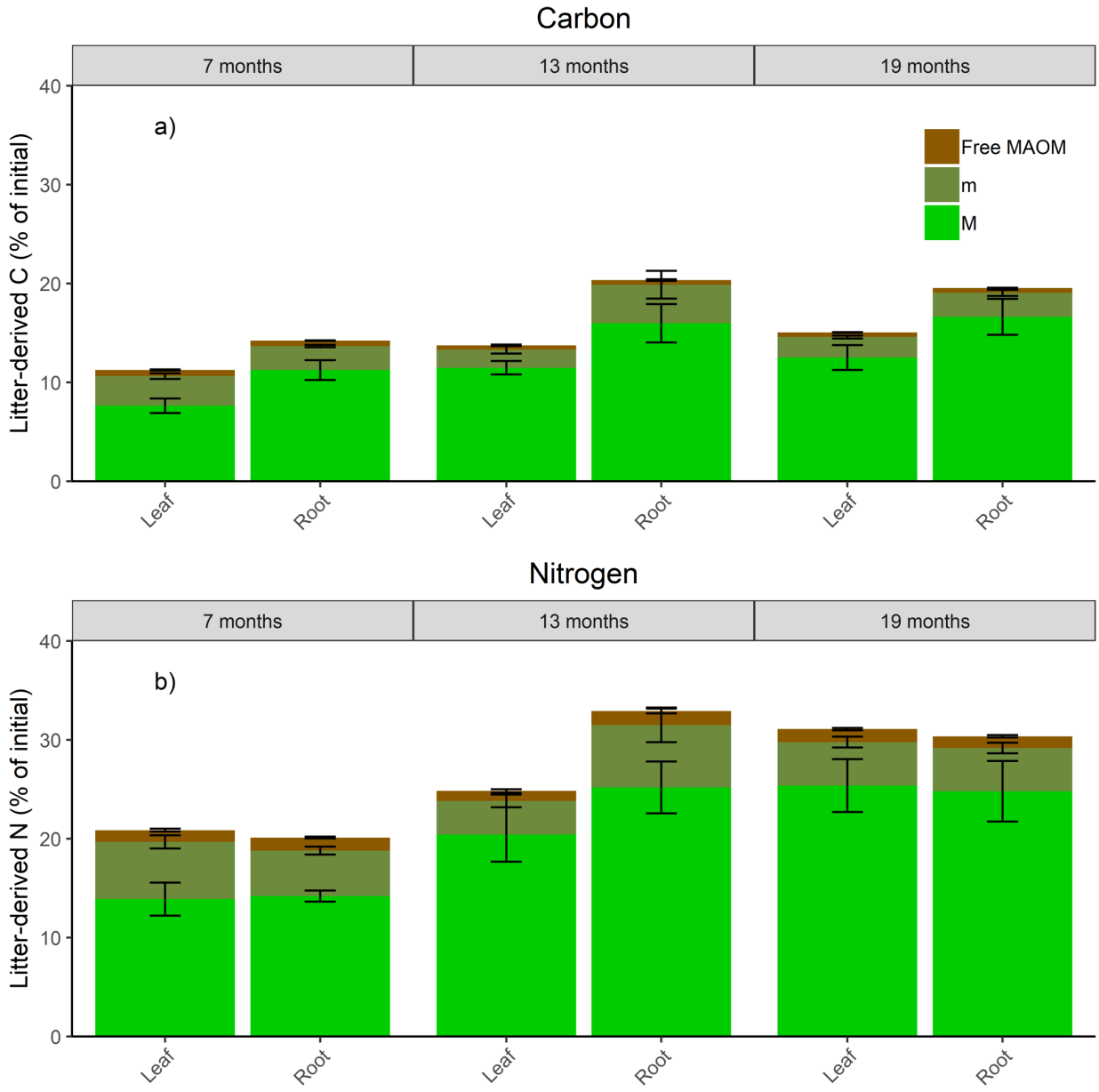

Figure 2.2: Sorghum bicolor leaf- and root-derived (a) Carbon and (b) Nitrogen recovered in the Macroaggregate (M), microaggregate (m) and free mineral-associate organic matter (MAOM) fractions within the depth layer where the litter was incubated (0-30cm), for the three time harvests during the 19 months of field incubation. Amounts are given in % of the initial litter residue at time 0. Data are average with standard errors (n=4).

Table 2.3. Results of the linear mixed model of the effect of sampling time (T), and litter type (L) and their interaction on the % litter derived C and % litter derived N on each soil and SOM fraction (d.f.: degrees of freedom (numerator, denominator)). See Table 2.1 for explanation of SOM fraction abbreviation.

	% Litter derived C				% Litter derived N		
Effect	d.f.	F	p	Effect	d.f.	F	p
Remaining in litter							
T	2, 5.82	1004.36	<0.0001	T	2, 5.82	345.02	<0.0001
L	1, 8.70	158.73	<0.0001	L	1, 8.70	71.58	<0.0001
T x L	2, 8.67	151.68	<0.0001	T x L	2, 8.67	55.76	<0.0001
SOM 0-30cm							
T	2, 6	0.19	0.8307	T	2, 6	0.90	0.4553
L	1, 9	25.92	0.0007	L	1, 9	6.14	0.0351
T x L	2, 9	2.18	0.1689	T x L	2, 9	4.57	0.0428
SOM 30-60cm							
T	2, 6	1.00	1.4213	T	2, 6	2.24	0.1873
L	1, 9	1.24	0.2942	L	1, 9	3.27	0.1041
T x L	2, 9	3.30	0.0843	T x L	2, 9	2.38	0.1482
SOM 60-90cm							
T	2, 6	1.05	0.4067	T	2, 6	3.20	0.1135
L	1, 9	1.03	0.3362	L	1, 9	2.15	0.1763
T x L	2, 9	1.13	0.3636	T x L	2, 9	0.38	0.6929
Total SOM							
T	2, 6	0.54	0.6069	T	2, 6	1.47	0.3016
L	1, 9	46.65	<0.0001	L	1, 9	3.31	0.1022
T x L	2, 9	0.42	0.6695	T x L	2, 9	3.00	0.1005
M							
T	2, 6	8.97	0.0158	T	2, 6	14.55	0.0050
L	1, 9	14.75	0.0040	L	1, 9	0.71	0.4202
T x L	2, 9	0.06	0.9431	T x L	2, 9	0.87	0.4489
Free m							
T	2, 6	0.39	0.6918	T	2, 6	0.30	0.7481
L	1, 9	2.54	0.1453	L	1, 9	1.03	0.3358
T x L	2, 9	3.97	0.0580	T x L	2, 9	4.69	0.0402
Free MAOM							
T	2, 6	2.11	0.2021	T	2, 6	0.06	0.9422
L	1, 9	0.00	0.9991	L	1, 9	1.14	0.3128
T x L	2, 9	1.55	0.2641	T x L	2, 9	1.51	0.2721
Total LF							
T	2, 6	27.33	0.0010	T	2, 6	5.54	0.0434
L	1, 9	18.09	0.0021	L	1, 9	4.50	0.0629
T x L	2, 9	12.99	0.0022	T x L	2, 9	12.79	0.0023
Total POM							
T	2, 6	20.09	0.0022	T	2, 6	22.86	0.0016
L	1, 9	11.43	0.0081	L	1, 9	1.08	0.3261
T x L	2, 9	7.00	0.0146	T x L	2, 9	5.26	0.0306
Total MAOM							
T	2, 6	16.69	0.0035	T	2, 6	14.09	0.0054
L	1, 9	1.06	0.0262	L	1, 9	2.62	0.1402
T x L	2, 9	0.24	0.7899	T x L	2, 9	1.48	0.2772

Coarse POM							
T	2, 5.87	0.41	0.6843	T	2, 5.83	0.61	0.5751
L	1, 8.55	45.57	0.0001	L	1, 8.57	40.30	0.0002
T x L	2, 8.51	2.14	0.1770	T x L	2, 8.54	1.32	0.3157
m_LF							
T	2, 5.79	8.67	0.0181	T	2, 5.79	9.96	0.0133
L	1, 8.61	0.03	0.8620	L	1, 8.65	10.60	0.0104
T x L	2, 8.58	13.23	0.0024	T x L	2, 8.62	11.26	0.0039
Mm_LF							
T	2, 6	57.43	0.0001	T	2, 6	24.87	0.0012
L	1, 9	50.79	<0.0001	L	1, 9	0.15	0.7071
T x L	2, 9	7.75	0.0110	T x L	2, 9	4.32	0.0483
m_Heavy POM							
T	2, 5.92	0.52	0.6170	T	2, 5.92	0.53	0.6138
L	1, 8.47	3.36	0.1019	L	1, 8.48	1.88	0.2053
T x L	2, 8.44	1.59	0.2592	T x L	2, 8.45	2.40	0.1496
Mm_Heavy POM							
T	2, 6	47.04	0.0002	T	2, 6	30.44	0.0007
L	1, 9	11.24	0.0085	L	1, 9	0.45	0.5170
T x L	2, 9	5.97	0.0224	T x L	2, 9	3.50	0.0750
M_MAOM							
T	2, 6	4.96	0.0535	T	2, 6	9.12	0.0152
L	1, 9	4.08	0.0741	L	1, 9	0.46	0.5161
T x L	2, 9	0.79	0.4831	T x L	2, 9	1.36	0.3046
m_MAOM							
T	2, 5.96	0.64	0.5604	T	2, 5.86	0.54	0.6075
L	1, 8.32	0.21	0.6549	L	1, 8.62	1.30	0.2842
T x L	2, 8.30	2.38	0.1528	T x L	2, 8.59	1.02	0.3993
Mm_MAOM							
T	2, 6	32.72	0.0006	T	2, 6	24.15	0.0014
L	1, 9	12.71	0.0061	L	1, 9	9.76	0.0122
T x L	2, 9	0.57	0.5853	T x L	2, 9	1.73	0.2317

MAOM. There was an interaction between time and litter type for LDN recovery in m (Table 2.3).

Aggregates (M, Mm, and m) were further separated into LF, heavy POM and MAOM. For the M fraction, LF and heavy POM are not separated but maintained within one fraction which we refer to as "Coarse POM" consistently with previous studies (Galdo et al., 2003; Six, Elliott, et al., 2000). Main effects of litter type and time varied by primary fraction. Most of the activity was found in the microaggregates occluded within macroaggregates (Mm) (Figure 2.3), with most of the LDC and LDN recovered for each litter type in the occluded MAOM fractions (Figure 2.3). Less leaf LDC was recovered in POM and LF fractions than root, particularly

within occluded microaggregates (Figure 2.3). Litter type had a main effect on LDC and LDN recovered in Coarse POM, but time did not (Table 2.3). There was a main effect of litter type and time and an interaction between the two for LDC and LDN recovered in total LF fractions (sum of m_LF and Mm_LF) (Table 2.3). While the LF pools varied over time within each treatment, by 19 months of incubation LDC and LDN recovered from leaf litter was not significantly different than those recovered from root litter for total LF ($p = 0.6606$ and $p = 0.0888$, respectively). In total LF, LDC and LDN from leaf litter tended to decrease over time, with

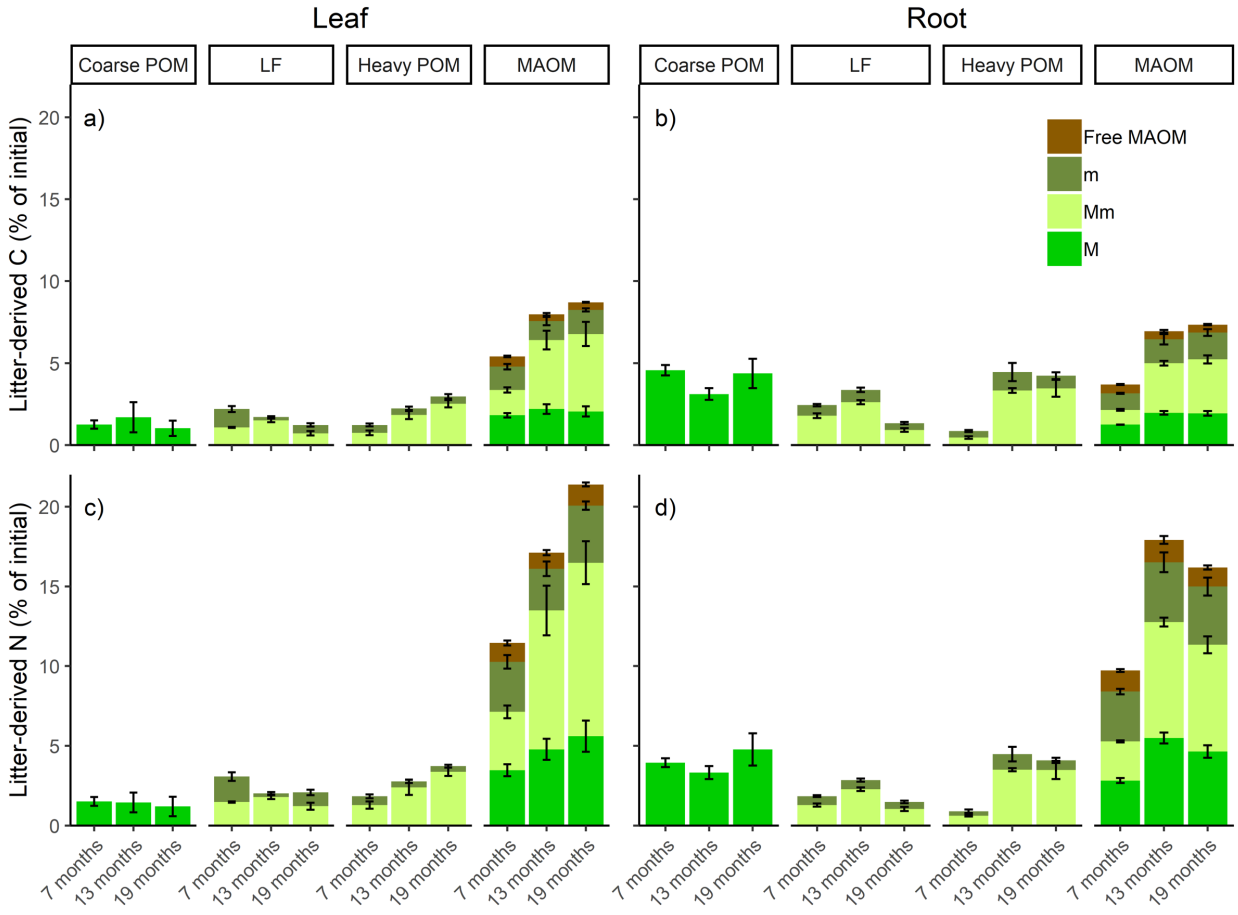

Figure 2.3: Sorghum bicolor leaf- (a, c) and root- (b, d) derived Carbon (a, b) and Nitrogen (c, d) recovered in the primary soil organic matter fractions, i.e., coarse particulate organic matter (Coarse POM), light fraction (LF), heavy POM and mineral associated organic matter (MAOM) fractions, found free or occluded in Macroaggregate (M), microaggregate (m) and microaggregate occluded within macroaggregate (Mm), in the depth layer where the litter was incubated (0-30cm), for the three time harvests during the 19 months of field incubation.

Amounts are given in % of the initial litter residue at time 0. Data are average with standard errors (n=4).

significantly less recovered from 7 to 19 months of incubation ($p = 0.0042$ and $p = 0.0160$). However, LDC and LDN recovered in total LF from root litter increased initially from 7 to 13 months of incubation ($p = 0.0017$ and 0.0062), and then decreased again from 13 to 19 months of incubation ($p < 0.0001$ and $p = 0.0016$).

There was a main effect of litter type and time on LDC recovered in total POM (sum of all POM fractions in m and Mm), and a main effect of time but not litter type on LDN recovered in total POM (Table 2.3). There was an interaction between litter type and time for LDC and LDN recovered in total POM. At 19 months of incubation, total POM LDC recovered from leaf litter was 35% less than LDC recovered from root litter ($p = 0.0364$). At 13 months of incubation total POM LDN recovered from leaf litter was 47% less than that from root litter ($p = 0.0180$) but was no longer significantly different at 19 months of incubation ($p = 0.5549$).

There was a main effect of litter type on LDC recovered in total MAOM (sum of free MAOM, m_MAOM, Mm_MAOM and M_MAOM), but not LDN (Table 2.3). In total MAOM, LDC recovery was 21.91% and LDN recovery was 14.88% greater from leaf litter than from root litter (Figure 2.3). There was a main effect of time on LDC and LDN recovered in total MAOM (Table 2.3). When looking more closely at the free and occluded MAOM fractions, there was no interaction between litter type and time on any individual MAOM fraction. However, litter type and time had main effects on LDC and LDN in Mm_MAOM (Table 2.3).

Discussion

Removal of aboveground biomass from *Sorghum bicolor* for biofuel production requires understanding differential contribution of leaf and root residue inputs to soil C and N storage and recycling, and to identify potential impacts that this removal could have on the soil. In this study

we demonstrated that the chemical composition of leaf and root *Sorghum bicolor* residues affect their contribution to SOM C and N pools along the soil profile and across different SOM physical fractions. This is consistent with the most recent frameworks suggesting higher MAOM formation from labile residues and higher POM formation from recalcitrant residues (Cotrufo et al. 2013, Castellano et al. 2015). Further we highlighted the important role of aggregates for the efficient formation of MAOM in a highly structured soil.

Our *in-situ* litter incubation experiment confirms that roots and leaves from *Sorghum bicolor* significantly differ in their inherent chemical composition and recalcitrance to decomposition. This resulted in different contribution to C and N in bulk soil, as well as different allocation of the newly formed SOM among physical fractions characterized by different mechanisms of protection. Despite being grown in a labeling chamber (Soong et al., 2014), the chemistry of our litters was similar to others reported for sorghum, with the exception of somewhat low %AUR (a lignin and suberin proxy) (Stewart et al., 2015; Zhao et al., 2009) . In every measure of litter decomposability (i.e., %HWE, C:N and LCI), sorghum leaves were of lower biochemical recalcitrance than roots (Table 2.2). However, both litter types had relatively low %AUR, and therefore overall were fairly decomposable. This was demonstrated by the rapid mass loss of both residues, which reached complete decomposition after only 19 months of incubation. In agreement with its relatively higher lability, the leaf litter decomposed faster than the root litter (Figure 2.1). Similar to other experiments conducted without confining the litter in mesh bags (e.g., Soong et al. (2016), this experiment confirmed that when left free to decompose in the soil plant residue reaches complete mass loss in a few years; subsequently, a significant fraction of the original mass is found in different forms of SOM. Moreover, the relatively fast decomposition observed in this study could have been facilitated by incubation of the litter

within the topsoil in close proximity to the soil matrix, where plant litter has been shown to decompose faster and produce SOM more efficiently than when incubated on the soil surface (Mitchell et al., 2016, 2018).

The different litter chemistry between our sorghum roots and leaves not only affected the different decay rates of the two litter types, but also the efficiency with which litter C (but surprisingly not N) was retained in bulk soil across the entire soil profile. We found that after 19 months of incubation 27% of the initial root C was retained in the bulk soil overall, *versus* 19% of the leaf litter C. This suggests that in crops where roots are inherently more recalcitrant than above ground biomass, if root production is increased this can potentially offset some of the losses from above ground input removal. This offset could be further increased by the fact that root inputs are released within the soil, at least in a no till system, while leaf litter is deposited on the soil surface where SOM formation is deemed to be less efficient (Mitchell et al., 2016, 2018; Sokol et al., 2019). Most of the LDC and LDN were found in the layer where litter was incubated, with little vertical movement (Figure 2.1), emphasizing the importance of root inputs to depth to accrue SOM in those deeper layer (Rumpel & Kögel-Knabner, 2010). Within the top 0-30cm, root and leaf litter contributed significantly different amounts not only of LDC but also of LDN to new SOM. In fact, an average of 35% of the root LDN was retained in bulk soil in the 0-30 cm *versus* an average of 28% from the leaves. This difference might have been due to the higher overall C retention from root litter, as well as the higher root C:N (Table 2.2) which potentially stimulated N immobilization in root litter-derived SOM to meet the stoichiometric needs of the microbes (Manzoni et al., 2008). Plant inputs not only provide C for SOM accrual, but through their mineralization they also recycle N to sustain productivity (Janzen, 2006). Clearly while increasing more recalcitrant root inputs can be an effective means to increase SOM

accrual, it seems less effective at providing fertility through N mineralization, with lower overall inputs due to lower %N (Table 2.2) and higher relative retention of N in soil. It is noteworthy that our experiment was short-term, and that more root LDN could be made available over-time from the mineralization of the POM fractions (Austin et al., 2017), which were higher in the root litter treatment (Figure 2.3). If above ground biomass is removed in bioenergy crops, a balanced alternative addition of organic C and N inputs needs to be implemented to sustain crop production at the same time of SOM accrual.

Examining only the bulk soil does not adequately capture SOM formation dynamics, nor allow predicting the persistence of the newly formed SOM. Aggregates are universally recognized as important SOM structures which offer physical protection to SOM from decomposition in the short term (Jastrow, 1996). Depending on the system, macroaggregates are expected to turnover within years to decades and microaggregates within decades to centuries (Lutzow et al., 2006). However, the potentially strong role that aggregates play in new SOM formation is less well understood. We demonstrated that in a highly aggregated soil, SOM formation from the decomposition of plant debris starts within aggregates. After only 7 months of litter incubation, the large majority of the LDC and LDN were found in macro- and micro-aggregate structures, increasing over time, while the LDC and LDN in free MAOM was only a minor fraction and did not significantly differ over time (Figure 2.2, Table 2.3). This finding supports the hypothesis that input of plant debris promotes aggregate formation (Six et al., 2004), and simultaneously emphasizes the dynamic, active nature of aggregates as a place where SOM, and particularly MAOM, is formed and transformed (Jastrow, 1996; Lehmann et al., 2007) over the generalized idea of aggregates as a place where decomposition is inhibited through spatial inaccessibility (Lutzow et al., 2006).

While aggregates are important SOM structures, they are composite pools and examining SOM formation only at their level prevents from identifying underlying pathways. When aggregates are disrupted and the primary SOM fractions (i.e., LF, POM, MAOM) within analyzed, then the actual SOM dynamics can be effectively studied (Six et al. 2000a, Del Galdo et al. 2003). In our study, the contribution to primary SOM fractions differed significantly between litter types: we found overall greater contribution to MAOM from the more labile leaf litter and greater contribution to particulate fractions (i.e., coarse POM, LF and heavy POM) from the more recalcitrant root litter. This observation confirmed the hypothesis that labile litter forms MAOM efficiently in soils with high matrix capacity, consistently with the Microbial Efficiency Mineral Stabilization framework (Cotrufo et al. 2013), while recalcitrant litter results in more POM formation (Castellano et al. 2015). It is also consistent with the few studies that compared SOM formation from root and shoot residues. Contribution to SOM formation from roots is often found to be much higher than from leaves (Berhongaray et al., 2019; Sokol et al., 2018), but leads to higher POM or POM like fractions (Bird et al., 2008), while leaf litters often exhibit a relatively higher contribution to MAOM or persistent SOM fractions (Hatton et al., 2015; Lavallee et al., 2018). While supporting the idea that more SOM can be formed from root residues in annual crops where they often have a higher recalcitrance than above-ground residues, these findings suggest that the mean residence time of root residues-derived SOM is not higher than that formed from above-ground residue decomposition. This conflicts with Rasse *et al*. (2005), who found that the mean residence time of root-derived C in soils is 2.4 times greater than shoot-derived C, and (Jackson et al., 2017) who suggested root inputs are 5 times as likely to be stabilized as SOM, perhaps higher in agricultural systems. Indeed, this study only investigated residues inputs, and root exudates given their inherent lability may efficiently form

persistent SOM (Cotrufo et al., 2013; Sokol et al., 2018; Strickland et al., 2012). We believe that the fractionation of SOM into physical fractions of known stabilization mechanisms can help predict the persistence of residue derived C and N in soil.

The higher MAOM formation from the leaf litter characterized by high HWE, and the higher POM formation from the root litter characterized by a higher LCI generally support the two pathways model of SOM formation (Cotrufo et al., 2015): dissolved organic matter from non-structural compounds (corresponding to high HWE-C) is available early in decomposition contributing efficiently to MAOM directly and via microbial processing (Liang et al., 2017), while POM forms through the fragmentation and partial transformation of recalcitrant structural litter components (corresponding to high LCI) (Haddix et al., 2016). However, our complete fractionation scheme and repeated sampling over time allowed identifying more subtle dynamics. First, it is important to notice that what we define as particulate structures include both little transformed plant derived light materials (LF) and heavy POM, more microbially processed and denser organic matter coating sand (Christensen, 2001). In our fractionation we have the two together inside macro-aggregates as Coarse POM, but they were separated within microaggregates in LF and heavy POM (Figure 2.3). By following the LDC in these fractions over time we can identify more specific SOM formation and transformation processes. Independent of litter types, inside micro-aggregate the decomposition of litter structural components first formed LF and then heavy POM, likely as a result of LF decomposition (Figure 2.3). This dynamic is in agreement with the general understanding of heavy sand-sized POM resulting from the microbial processing of plant derived LF (Grandy & Neff, 2008). Differently from this cascade model and in agreement with the two-pathways model, in our study MAOM formation appeared to be independent of LF and heavy POM dynamics and formed during the

active phases of litter mass loss (Figure 2.1 and 2.2). In addition to the HWE, non-lignin encrusted structural materials were abundant in both litters and must have been active contributors to the formation of MAOM. Particularly, we observed a continued increase in MAOM formation in the period between 7 and 13 months, when these structures are expected to degrade (McKee et al., 2016); MAOM remained largely stable thereafter (Figure 2.3).

Overall, MAOM is the SOM fraction deemed to be most persistent and resistant to disturbance (von Lutzow et al., 2006), and thus the fraction targeted for long-term C sequestration. Although, it is important to note that some of the new MAOM formation we observed may be the result of surface exchanges rather than net accrual (Jilling et al., 2018), *Sorghum bicolor* root and leaf residue inputs were relatively labile and had MAOM formation efficiencies of 7.4% and 9.1%, respectively. Thus, both represent a good source of persistent SOM, in particular in clay-loam highly aggregated soils. Carbon sequestration in MAOM, however, has a high N cost (Figure 2.3). While MAOM is now looked at as a significant reservoir of N (Jilling et al., 2018), mobilizing N from MOAM to sustain fertility will reduce persistent C storage, opening the well-known "soil C dilemma" (Janzen, 2006). Soil fertility must be assured without jeopardizing SOM resources through the input of N rich residues, for example through legume cover crops (Schipanski et al., 2014). These issues emphasize the importance of considering the quantity of root or shoot inputs in order to meet the competing needs for SOM pools when managing agricultural production systems.

From both litter types, we demonstrated rapid formation of MAOM within macroaggregates, thought to be an important step in the process of microaggregate formation within macroaggregates (Jastrow, 1996; Oades, 1984; Six et al., 2004). We also saw continued increases in occluded microaggregate MAOM over time (Figure 2.3), further emphasizing the dynamic

nature of aggregates and their key role in the formation of persistent organo-mineral bonding, rather than in the physical inhibition of SOM decomposition through occlusion (Lehmann et al., 2007). If increasing root inputs is pursued as a strategy to sequester C during bioenergy feedstock production, the nature and long-term persistence of the SOM accrued needs to be assessed. From this study, root residues appeared effective at forming new SOM, but relatively more in unstable particulate fractions. While stalks would constitute the majority of aboveground residue in sorghum production after grain is harvested, we used leaves in this study, since both leaves and stalks were similar in their litter chemistry and more labile than roots. Stalks had the highest %HWE-mass of the three litter types given their high sugar content, so it is likely that they would have contributed even more efficiently to MAOM production than leaves. Thus, our study may have underestimated the ability of sorghum residues to contribute to this more stable SOM. However, if aboveground materials are harvested for lignocellulosic ethanol production most of the stalks would be removed.

We also demonstrated that aggregates are a place of efficient residue decomposition and MAOM formation. The promotion of aggregation in the mineral soil through live and dead root inputs (Denef & Six, 2006) may thus be an important factor stimulating persistent SOM production in root enhanced bioenergy crops.

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CHAPTER 3- DEPTH IMPACTS ON THE AGGREGATE-MEDIATED MECHANISMS OF ROOT CARBON STABILIZATION IN SOIL: TRADE-OFF BETWEEN MAOM AND POM PATHWAYS[2]

Introduction

Agroecosystems play a critical role in providing solutions to climate change through the implementation of regenerative practices and management decisions that improve soil health and function by increasing soil organic matter (SOM) stocks. This can both enhance the resilience of these systems to climate shocks and mitigate climate change through soil carbon (C) sequestration (Keesstra et al., 2016; Lal et al., 2021; Smith et al., 2016). Conventional management practices have degraded SOM levels globally (Sanderman et al., 2017), largely by increasing disturbance and breaking down aggregate protection (Bailey et al., 2019; Jastrow et al., 2007). Increasing the production and depth distribution of belowground biomass in agricultural systems through planting deep rooted crops, such as *Sorghum bicolor*, and selective crop breeding is an exciting strategy to increase SOM inputs (Heinemann et al., 2023; Kell, 2011, 2012; Paustian et al., 2019). In crop management systems, where most of the aboveground biomass is harvested, such as with annual bioenergy crops (Lal, 2009), belowground inputs are a critical source of SOM. Root inputs typically result in more efficient SOM formation than above ground inputs (Fulton-Smith & Cotrufo, 2019; Katterer et al., 2011; Rasse et al., 2005).

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However, there is limited understanding of how the decomposition of roots deeper in the soil profile (e.g., below 30 cm) contribute to SOM formation and stabilization.

Measuring bulk soil C pools is not sufficient to capture the fate of decomposed plant litters, because it does not provide information about the likely persistence or function of the OM. The persistence of SOM for C sequestration, and the accumulation of SOM for water infiltration, water retention, and nutrient cycling are provided by functionally distinct SOM components. Mineral associated organic matter (MAOM) and particulate associated organic matter (POM) have been identified as useful pools to distinguish to connect input management to SOM changes and functions (Cotrufo & Lavallee, 2022). In fact, POM and MAOM are thought to form via two primary pathways (Cotrufo et al., 2015) and to have different mechanisms of stabilization and functional roles in the soil (Cotrufo et al., 2019; Lavallee et al., 2020). It is now largely recognized that both POM and MAOM are contributed by plant and microbial compounds (Angst et al., 2021), POM forms from their structures, while MOAM forms either by direct sorption of plant derived dissolved organic matter (DOM), or by mineral association of microbial necromass and metabolites (Cotrufo et al., 2022; C. Liang et al., 2017). Mineral associated OM is on average longer lived than POM with estimated turnover rates of decades to centuries while POM has a faster rate of turnover of years to decades (Lützow et al., 2007). While many efforts focus on the stability and sequestration of soil C, and therefore the accrual of MAOM, abundant POM is critical to healthy agroecosystem functions and crop yields (Wood et al., 2016). Thus, management practices that promote both POM and MAOM accrual should be pursued (Angst et al., 2023). Agricultural topsoils are typically characterized by lower levels of POM than other land uses (Lugato et al., 2021), most likely due to receiving low root and plant litter inputs, having fewer constraints on decomposition (Hansen et al., 2024; King et al., 2023), and

disturbance of soil aggregates through tillage (Jastrow, 1996; Six et al., 2004). A mechanistic understanding for these findings is needed to help inform agricultural management practices for the accrual of both POM and MAOM along the soil profile.

Conceptualizing SOM into POM and MAOM has significantly advanced our understanding of SOM dynamics, as proposed in Lavallee *et al*. (2020). However, this conceptualization of SOM neglects to address the role of soil aggregates. Macroaggregates, while short lived, are critical in the formation of MAOM and POM; occluded microaggregates form within macroaggregates and as macroaggregates turnover they release more stable free microaggregates, within which POM and stable MAOM are found, (Jastrow, 1996; Six, Elliott, et al., 2000; Six et al., 2004; Six & Paustian, 2014). Soil disturbances increase macroaggregate turnover, reducing incorporation of organic inputs into stable fractions (Six et al., 2000; Six & Paustian, 2014), and accelerating POM decomposition. Higher decay rates were measured for free POM *versus* aggregate occluded POM in agricultural soils (Haddix et al., 2020). Since root structural inputs can stimulate POM and aggregate formation (Even & Cotrufo, 2024; Poirier et al., 2018; Six et al., 2004), increasing root inputs at depth where decomposition and disturbance effects are reduced could result in POM accrual (Pol et al., 2022).

Although soil C concentrations decrease with depth, over 50% of soil C is stored below 20 cm (Jobbágy & Jackson, 2000) with longer mean residence of hundreds to thousands of years (Fontaine et al., 2007; Rumpel & Kögel-Knabner, 2010; Schmidt et al., 2011). Yet, the dynamics of deep soil C remain poorly understood (Harper & Tibbett, 2013; Rumpel & Kögel-Knabner, 2010). The majority of SOM, particularly in the deep soil, originates from roots, their exudates, and associated microbes (Angst et al., 2018; Gross & Harrison, 2019; Jackson et al., 2017; Kong & Six, 2010; Rasse et al., 2005), and the vertical movement of dissolved organic C through the

soil (Kaiser & Kalbitz, 2012; Sokol et al., 2019). While plant species may differ in their contribution to stable C in the deep soil (Peixoto et al., 2022), rooting depth was found to be the most important root trait affecting deep soil C storage (Poirier et al., 2018). Deep soils are influenced less by environmental conditions, such as precipitation and temperature (Gulser & Ekberli, 2004), are less sensitive to climatic fluctuations (Mathieu et al., 2015), and microbial abundance and activity decrease with depth (Blume et al., 2002; Z. Liang et al., 2018). In fact, the rate of root litter decomposition has been shown to slow with depth (Pries et al., 2018; Sanaullah et al., 2010) with few exceptions (Solly et al., 2015). As a result, deep soil C pools are considered relatively stable until the conditions under which they accumulated change (Gross & Harrison, 2019). Given the low microbial density at depth, root inputs to the deep soil are physically separated from microbial activity and therefore may persist for longer (Rumpel & Kögel-Knabner, 2010; Schmidt et al., 2011), likely as POM. Additionally, minerals in the deep soil, and agricultural soils in general, are typically far from C saturation thus have a higher potential to stabilize new MAOM when fresh litter inputs are introduced (Georgiou et al., 2022). However, the addition of fresh litter inputs in the deep soil, may also prime the decomposition of older stable C pools (Fontaine et al., 2007; Shahzad et al., 2018).

Our ability to track the fate of decomposition products from plant litter inputs into various soil fractions is critical for evaluating SOM dynamics and the impacts to soil health of new agricultural management strategies, such as increasing root production in the deep soil. Stable isotope (i.e., ^{13}C) labeling is currently the most effective method to quantitatively track the fate of plant material in the soil. In this experiment, we assess the impact of soil depth on root decomposition and SOM formation in *Sorghum bicolor*. Sorghum is a unique genetically diverse agricultural crop used as feed, forage, fiber, and bioenergy feedstock due to a wide range of

aboveground yields, sugar content, and grain production (Bean et al., 2007; Mullet et al., 2014; Olson et al., 2013). Bioenergy varieties of sorghum produce extensive root systems up to 2 m in depth and 7 Mg/ ha (Lamb et al., 2022; Meki et al., 2013; Monti & Zatta, 2009; Sainju et al., 2005).

To advance the understanding of managing for deep root contribution to SOM regeneration, we examined the fate of ^{13}C labeled *Sorghum bicolor* fine roots in unique soil-biomass microcosms at three depths (0-30 cm, 30-60 cm, and 60-90 cm) in an irrigated sorghum field and traced the products of root-litter decomposition into POM and MAOM, free and protected inside micro- and macro-aggregates. We hypothesized that (i) root litter decomposition slows with depth, (ii) MAOM forms during the initial stages of decomposition, when leaching is known to dominate mass loss processes, and increases over time as products of fragmentation (POM) breakdown further (iii) rapid decomposition of root litter in the topsoil (0-30 cm) leads to more accumulation of MAOM while in the deep soil (60-90 cm), known to be depleted in microbial biomass, decomposition is expected to proceed mostly through physical fragmentation leading to more POM accumulation at depth, and that (iv) microaggregates are a primary location for the formation of microbially-derived MAOM.

Materials and Methods

Site description

The study was conducted in an irrigated field at the Agricultural Research Development and Education Center (ARDEC), Colorado State University, Fort Collins, Colorado (40°39′ N, 104°59′ W; 1554 m above sea level), as described in Chapter 2. At the site, mean monthly temperatures ranged from 0 °C in January to 22 °C in July with an average annual precipitation of 330 mm. After tillage, a 400 m^2 area was planted with *Sorghum bicolor v BTx 623* in June

2013 and June 2014 and sprinkler irrigated throughout the growing season, as prescribed for optimal crop growth. The soil was classified as a mixed, superactive, mesic, Aridic Haplustalf with an average soil bulk density (n=4) of 1.07, 1.09, and 1.13 g cm^{-1} at 0-30 cm, 30-60 cm, and 60-90 cm depths, respectively. Soil texture at all three depths was classified as clay loam; averaged across all three depths the soil was (n=3) 34.34% sand, 34.22% silt and 30.75% clay. The percentage of sand increased with depth from 29.43% at 0-30 cm to 44.81% at 60-90 cm but remained within the clay loam classification at each depth.

Soil collection

In October 2013, forty-eight 90 cm deep soil cores spaced approximately 1 m apart were collected using a truck-mounted hydraulic soil probe (Giddings Machine Company Inc., Windsor, Colorado). Cylinders made from PVC, 5.08 cm in diameter and 1 m in length, were placed into each hole created from the extracted soil cores until microcosms were returned to the field for incubation. Soil cores were separated by depth (0-30 cm, 30-60 cm, and 60-90 cm), sieved to < 8 mm gently by hand to avoid disrupting soil aggregates, and large rocks and roots were discarded. The 8 mm sieved soil was homogenized by depth in large containers, covered, stored at room temperature for two weeks, and then placed in microcosms for field incubation, as described below.

Isotopically labeled Sorghum litter production

Sorghum bicolor v. BTx 623 plant litters isotopically enriched with ^{13}C were produced in a continuous labeling chamber (Soong et al., 2014) for 22 weeks, as previously described (Fulton-Smith & Cotrufo, 2019). Plants were harvested, stalks and leaves removed, and belowground root litters were separated from the potting mixture (sand, vermiculite, and profile porous ceramic) by pouring the entire contents of the pot over a 6 mm sieve and gently shaking. Root

litters were carefully rinsed with deionized water and air-dried. To ensure similar litter quality for all incubated root litters, fine roots, less than 2 mm in diameter were used for this study, cut into ~2 cm long pieces and homogenized before placement in the microcosms. Three lab replicate samples of the fine roots were dried in an oven at 65 °C and analyzed for water content, and afterward finely ground and analyzed for isotopic (^{13}C) composition and % C and % N on an elemental analyzer - isotope ratio mass spectrometer (EA-IRMS: Costech ECS 4010 elemental analyzer, Italy coupled to a Thermo-Fisher Delta V Advantage isotope ratio mass spectrometer). Root litters had an enrichment of 4.55 ^{13}C atom %, additional measures of root litter chemistry are reported in Chapter 2 and Fulton-Smith and Cotrufo (2019).

Field experiment

A microcosm approach was used to study the impact of depth on root litter decomposition in the field and to track decomposition products (litter derived C) into SOM fractions to a depth of 90 cm, as described in Chapter 2 and Fulton-Smith and Cotrufo (2019). Microcosms consisted of PVC collars (5.08 cm diameter and 1m length) with 18 circular holes (3.2 cm diameter) evenly distributed along the length of the collars to allow for root ingrowth from the crop and lateral flow of water and nutrients. One of three depth treatments was assigned to each microcosm: 0-30 cm, 30-60 cm, and 60-90 cm (Figure 3.1). The isotopically enriched root litters (~1 g) were mixed with the collected soil from one of three soil depth treatments (0-30, 30-60 and 60-90 cm) corresponding to field depth. While the majority of sorghum root production is in the top 30 cm of the soil and is shown to decline with depth (Lamb et al., 2022), the same amount of roots were incubated at each depth in order to be able to compare depth treatments independent of root input amounts. Soils were funneled into a 1.6 mm mesh column, within the PVC collar according to

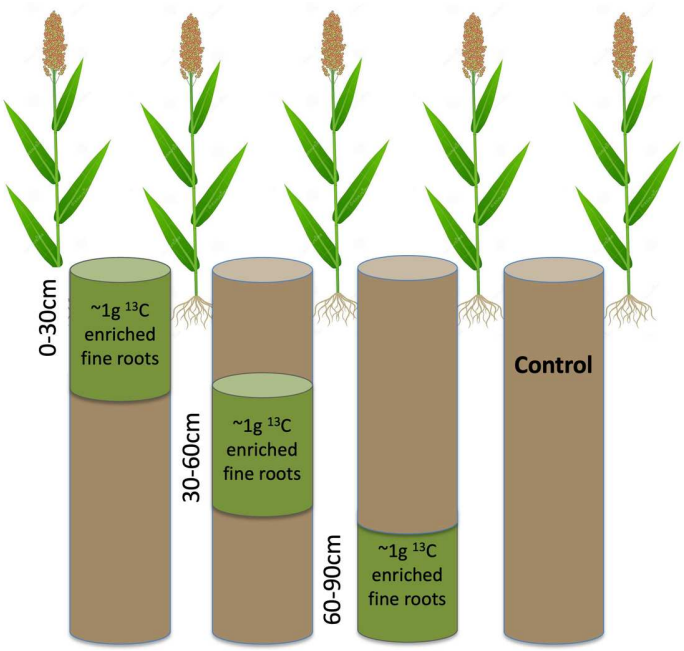

Figure 3.1. A diagram of soil-biomass microcosms depicting ^{13}C root litter incubation at one of three treatment depths, 0-30, 30-60, and 60-90 cm, and a control with no enriched litter added at any depth.

their field depth, i.e., soil that was originally collected from the 0-30 cm depth in the field was placed at the 0-30 cm depth within the microcosm. The weight of the soil used at each depth was appropriate to recreate field site bulk density. In each microcosm, the enriched root litters mixed with soil were only placed at one treatment depth, and the other two depths contained soil from the corresponding field depth without any litters added (Figure 3.1). The mesh columns were sealed with a zip tie at both ends. Control microcosms did not receive litter additions at any depth and were used as the natural abundance end member in the isotope mixing model described below.

On November 9, 2013, 48 soil-root litter microcosms were placed in the field for the *in-situ* decomposition study in the holes created during the initial soil collection. They were placed vertically to a depth of 90 cm with 10 cm of the PVC remaining aboveground; the sides were backfilled with soil to avoid any air gap and assure a moisture continuum between the soil inside

and outside the microcosms. *Sorghum bicolor BTx 623* was planted in the field among the microcosms in June 2014.

The split-plot randomized complete block design had four replicate blocks. Within each block, harvest time (7, 13 or 19 months) was assigned randomly to one of three rows and depth treatment (0-30 cm, 30-60 cm, 60-90 cm, or no root control) was assigned randomly within rows. On June 2, 2014, December 1, 2014, and June 1, 2015, 16 microcosms (4 replicates * 4 treatments (3 depths + 1 control)) were harvested, to assess decomposition of root litters and incorporation into SOM after winter, and after one growing season; the third harvest was conducted sooner than originally planned given the rapid rate of litter mass loss measured after the first two harvests.

Litter and bulk soil processing

At the assigned harvest time, microcosms were extracted from the field and stored in a 4 °C walk-in refrigerator until deconstruction the next day. As described in Chapter 2 and Fulton-Smith and Cotrufo (2019), the microcosms were cut into three pieces by depth (0-30, 30-60, and 60-90 cm) the day after harvest using a circular saw. Each section of soil still contained within the mesh sock was removed from the PVC collar, placed whole into a sealed plastic bag, labeled, and returned to the 4 °C refrigerator until further processing occurred within two weeks from initial harvest.

The mesh sock was cut away from the harvested soils and discarded. The soil was then carefully broken up to passthrough an 8 mm sieve. Root litters greater than 8 mm in length were removed with tweezers and collected as litter mass remaining. The remaining bulk soil was homogenized, weighed at field moisture, and then subsamples of ~20 g were dried at 105 °C to calculate % field moisture and bulk density (BD). The remainder of the soil was air-dried for 3-4 days, and

then used for fractionation. Litter mass remaining was rinsed with deionized water to eliminate soil particles over a 250 μm sieve and oven dried at 65 °C. Oven-dried bulk soil and litter mass remaining were finely ground by hand with a mortar and pestle and analyzed for % C, % N, and $\delta^{13}C$ on the EA-IRMS (Table 3.1).

Soil organic matter fractionation.

Soils were sequentially fractionated by size and density to isolate macroaggregates, free and occluded microaggregates (M, m, and Mm, respectively) and further separated from aggregates into primary SOM pools (light particulate OM, OM isolated with the heavy coarse soil fraction, and mineral associated OM) as described in Chapter 2 and Fulton-Smith and Cotrufo (2019), (Figure 3.2).

Initially, a subsample of bulk soil was wet sieved to isolate three aggregate size classes: macroaggregates (M: > 250 μm), free microaggregates (m: 250-53 μm), and free silt and clay-sized OM (MAOM: < 53 μm), modified from (Six, Paustian, et al., 2000). Large macroaggregates (> 2000 μm) comprised less than 5% of the soil so were not isolated separately. Fractions were oven dried at 65 °C and subsamples collected for further fractionation. Using a microaggregate isolator as described in Six *et al*. (2000a) and (Galdo et al., 2003)), M fractions were dispersed and separated into coarse POM (Coarse POM: > 250 μm), macroaggregate occluded microaggregate (Mm: 53–250 μm), and macroaggregate occluded MAOM (M_MAOM: < 53 μm; Figure 3.2).

Table 3.1. Carbon concentration and ^{13}C isotopic composition at each of three incubation depths for bulk soil, recovered root litter and SOM fractions, and the relative contribution of each fraction to the bulk soil. C and N concentration, and % fraction of total soil is averaged across control and treatment at each depth for three harvests and four replicates, *n*=24 with ± standard error in parentheses. ^{13}C values are averaged across four replicates of three depth treatments and one control, *n* = 12 with ± standard error in parentheses.

		0-30 cm				**30-60 cm**				**60-90 cm**			
Fraction	**Abbr.**	**% C**	**% N**	**$\delta^{13}C$**	**% fraction**	**% C**	**% N**	**$\delta^{13}C$**	**% fraction**	**% C**	**% N**	**$\delta^{13}C$**	**% fraction**
Bulk	Bulk	2.09 (0.03)	0.17 (0.00)	-14.17 (0.11)	100	1.67 (0.03)	0.11 (0.00)	-10.88 (0.11)	100	1.32 (0.03)	0.08 (0.00)	-8.62 (0.08)	100
Litter	Litter	34.78 (0.87)	1.28 (0.09)	-16.42 (1.71)	---	35.63 (1.63)	1.08 (0.10)	-16.41 (2.25)	---	37.46 (1.98)	1.03 (0.10)	-17.57 (2.17)	---
Macroaggregates	M	2.31 (0.01)	0.19 (0.00)	-14.51 (0.07)	62.21 (1.27)	1.88 (0.02)	0.13 (0.00)	-11.35 (0.09)	61.47 (1.08)	1.48 (0.04)	0.09 (0.00)	-10.09 (0.16)	31.94 (1.60)
Free microaggregates	m	1.53 (0.03)	0.12 (0.00)	-14.26 (0.20)	30.81 (1.01)	1.27 (0.02)	0.08 (0.00)	-10.58 (0.140)	31.06 (0.90)	1.15 (0.02)	0.06 (0.00)	-8.46 (0.14)	58.55 (1.43)
Occluded microaggregates	Mm	2.16 (0.08)	0.19 (0.01)	-15.34 (0.08)	35.88 (0.93)	1.66 (0.06)	0.12 (0.00)	-12.00 (0.079)	35.13 (0.92)	1.28 (0.07)	0.09 (0.01)	-10.82 (0.20)	17.19 (0.97)
Free MAOM	MAOM	2.36 (0.03)	0.17 (0.00)	-13.05 (0.16)	6.68 (0.30)	2.02 (0.03)	0.12 (0.00)	-10.92 (0.313)	7.35 (0.28)	1.97 (0.03)	0.10 (0.00)	-8.59 (0.217)	9.46 (0.37)
Macroaggregate coarse POM	M_POM + M_hcOM	1.23 (0.07)	0.08 (0.01)	-18.02 (0.91)	2.90 (0.11)	0.67 (0.06)	0.04 (0.01)	-12.98 (1.16)	2.22 (0.15)	0.60 (0.07)	0.03 (0.01)	-8.61 (2.35)	3.29 (0.12)
Macroaggregate MAOM	M_MAOM	2.40 (0.04)	0.18 (0.00)	-13.38 (0.11)	23.55 (0.50)	1.95 (0.07)	0.13 (0.01)	-10.73 (0.116)	24.12 (0.47)	1.85 (0.03)	0.12 (0.00)	-10.11 (0.19)	11.43 (0.68)
Free microaggregate Light POM	m_POM	36.21 (0.27)	2.64 (0.03)	-23.82 (0.35)	0.07 (0.01)	35.96 (0.76)	2.22 (0.05)	-21.56 (0.443)	0.03 (0.00)	31.30 (2.01)	2.09 (0.12)	-13.55 (2.59)	0.09 (0.05)
Free microaggregate hcOM	m_hcOM	0.39 (0.01)	0.02 (0.00)	-11.81 (0.32)	13.29 (0.47)	0.36 (0.05)	0.02 (0.00)	-7.51 (0.250)	13.02 (0.36)	0.32 (0.01)	0.01 (0.00)	-6.98 (0.77)	28.56 (0.97)
Free microaggregate MAOM	m_MAOM	2.01 (0.03)	0.19 (0.00)	-16.15 (0.19)	17.20 (0.66)	1.58 (0.04)	0.13 (0.00)	-12.81 (0.296)	17.74 (0.60)	1.39 (0.05)	0.11 (0.01)	-11.12 (0.28)	29.90 (0.91)
Occluded microaggregate Light POM	Mm_POM	32.61 (0.34)	2.49 (0.03)	-23.07 (0.05)	0.12 (0.01)	31.41 (0.67)	2.31 (0.05)	-20.58 (0.06)	0.08 (0.01)	29.86 (0.69)	2.30 (0.05)	-19.60 (0.12)	0.03 (0.00)
Occluded microaggregate hcOM	Mm_hcOM	0.89 (0.02)	0.07 (0.00)	-17.48 (0.20)	10.31 (0.34)	0.48 (0.02)	0.03 (0.00)	-11.41 (0.39)	10.82 (0.43)	0.42 (0.02)	0.02 (0.00)	-8.71 (0.22)	8.06 (0.60)
Occluded microaggregate MAOM	Mm_MAOM	2.23 (0.02)	0.22 (0.01)	-16.36 (0.13)	22.71 (1.03)	1.91 (0.04)	0.17 (0.00)	-13.61 (0.10)	24.25 (0.84)	1.70 (0.05)	0.16 (0.00)	-13.26 (0.11)	10.66 (1.44)

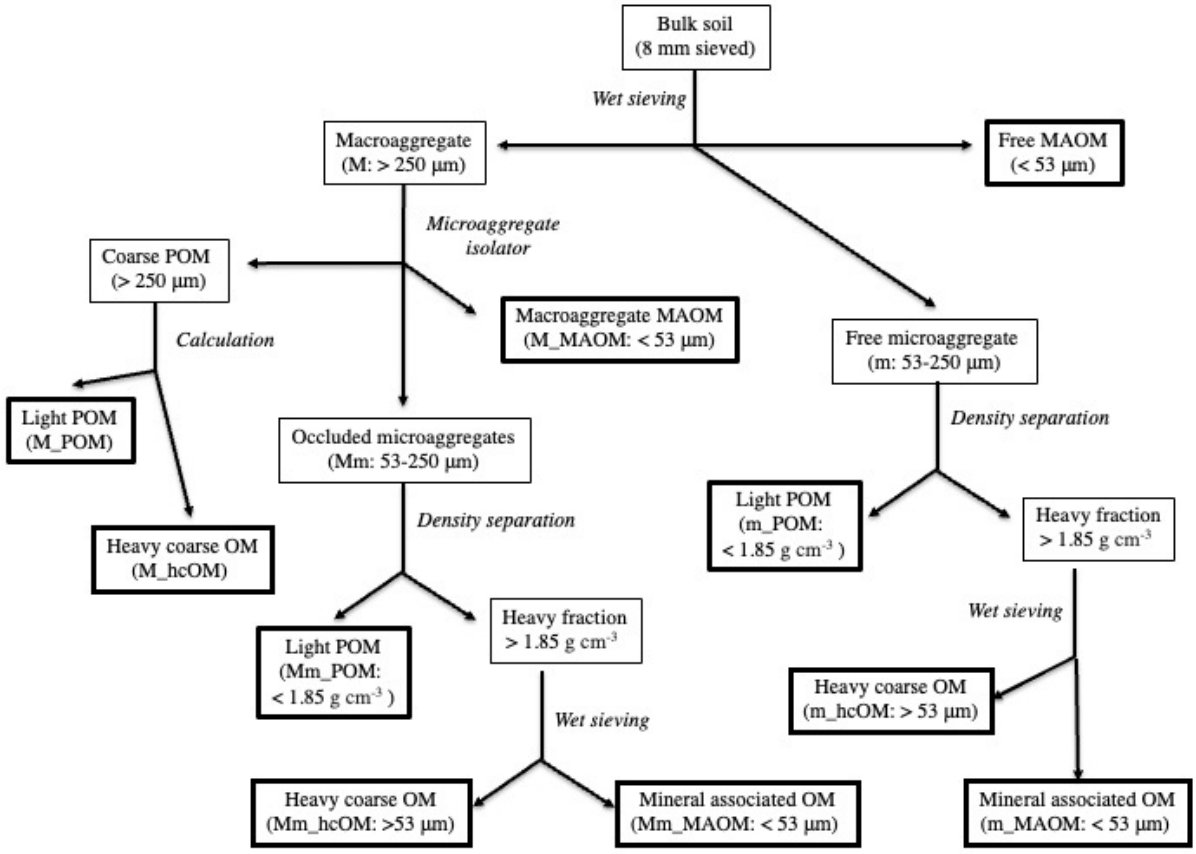

Figure 3.2. A soil fractionation scheme to isolate primary soil organic matter fractions, light particulate organic matter (POM), organic matter associated to the heavy coarse soil fraction (hcOM), and mineral associated organic matter (MAOM) found free or into different aggregate size classes: macroaggregates (M), microaggregates (m), and macroaggregate occluded microaggregates (Mm).

Further, m and Mm were fractionated by density into a light POM (m_POM or Mm_POM: < 1.85 g cm^{-3}) and a heavy fraction (> 1.85 g cm^{-3}) as described in (Soong & Cotrufo, 2015). The heavy fraction was 53 µm sieved to separate by size into free or occluded microaggregate heavy coarse OM (m_hcOM or Mm_hcOM: > 53 µm) and free or occluded microaggregate MAOM (m_MAOM or Mm_MAOM: < 53 µm; Figure 3.2). The light POM samples were not large enough for individual analysis so four replicate light POM samples each for m or Mm were composited for these analyses. Mass recovery of the overall fractionation procedures was

97.80%. Subsamples of all fractions were oven dried at 65 °C, finely ground, and analyzed on the EA-IRMS for % C, % N, and $\delta^{13}C$, as described above.

Data analysis

Isotopically enriched root litter contribution to the bulk soil, litter mass remaining, and SOM fractions were evaluated using the isotopic mixing model with the isotopically labeled litter and the control (no litter-added) samples as the two end members, as described in Chapter 2 and Fulton-Smith and Cotrufo (2019), following Equation 1:

$$f_{root} = \frac{\delta_s - \delta_c}{\delta_{root} - \delta_c}$$

where f_{root} is the root litter derived C fraction of bulk soil, litter mass remaining, or SOM fraction, δ_s is the $\delta^{13}C$ of the specific C pool (bulk soil, litter mass remaining or SOM fractions), δ_c is the $\delta^{13}C$ of the corresponding control pool, averaged by block across harvests (Table 3.1), and δ_{root} is the $\delta^{13}C$ of the initial root litter.

The percentage of root litter-derived C (LDC) recovered in the various C pools was calculated for individual samples by multiplying f_{root} to the corresponding C pool size of the fraction, and then dividing by the quantity of litter C initially added to the soil. To directly compare the three depths which had differing initial bulk density, we report LDC as a percentage of the initial litter C added. As the isolated Coarse POM fraction is made of both POM and hcOM (namely: M_POM and M_hcOM), we calculated the LDC in the M_hcOM by assuming that M_hcOM had the same f_{root} as the Mm_hcOM and that the total mass of the Coarse POM was contributed by M_hcOM (i.e., that the light M_POM mass was negligible). We calculated the LDC in the M_POM as the difference between the LDC measured for the Coarse POM and that calculated for the M_hcOM.

R (version 4.3.1) was used for statistical analysis, including specific packages *lme4, lmerTest* and *emmeans*. Separate linear mixed models fit by REML t-tests and Satterthwaite approximations to degrees of freedom were used for the following analyses: the effects of depth, sampling time and their interactions on LDC in bulk soil, remaining litter, and primary and secondary SOM fractions with block and block x sampling time as random effects (separate models were fit for each fraction, due to the different scales of response variables across fractions); the effect of depth on the cumulative % LDC recovered in all fractions of each pool (POM, hcOM, or MAOM) at 19 months of incubation with block as a random effect; the effects of sampling time, MAOM fraction, and their interaction on C:N using a linear mixed model with block and block x sampling time as random effects. Linear regression was fit to the litter mass remaining using R packages *lme4* and *lmerTest* and was used to extract the slope and intercept. We checked for normality and homogeneity of variances of the residuals and applied a log-transformation when necessary.

Results

Litter decay and litter derived C in total soil

We measured *Sorghum bicolor* root litter decay by collecting the litter remaining in the microcosms at each of three depths (0-30 cm, 30-60 cm, and 60-90 cm) over time to determine litter mass loss and LDC in bulk soil and fractions.

Decomposition dynamics of the added roots best fit a linear equation for all three depths from the beginning of the incubation (time 0) to the final harvest (19 months), with equations being as follows:

- 0-30 cm: $y = -0.18x + 89$, $R^2 = 0.8948$
- 30-60 cm: $y = -0.17x + 90$, $R^2 = 0.8999$

- 60-90 cm: $y = -0.17x + 89$, $R^2 = 0.9226$

While the overall rate of decomposition was similar for all depths following a linear fit, the pattern of decay dynamics differed with depth (Figure 3.3a). There were no significant differences between depths at the first harvest (7 months of incubation), but at the second harvest (13 months) significantly less litter mass was remaining in the 0-30 cm depth than the 30-60 ($p < 0.0001$) and 60-90 cm depths ($p < 0.0001$) (Figure 3.3a) with the 0-30 cm depth achieving 97% mass loss by 13 months of incubation compared to 77% and 81% in the 30-60 and 60-90 cm depths, respectively. Despite these differences, litter at all three incubation depths (0-30 cm, 30-60 cm, and 60-90 cm) showed complete decomposition by the end (19 months) of incubation, with at least 99.9% of the initial C lost (Figure 3.3a).

After only 7 months of incubation, on average 24% (+/- SE), 25% and 25% of LDC was recovered in bulk SOC for the 0-30, 30-60 and 60-90 cm incubation depths, respectively (Figure 3.3b). No statistical changes were detected over time or across depth, or their interaction on % LDC in bulk soil (Table 3.2).

Average bulk density of the harvested microcosms increased with depth with 0.92, 0.98, and 1.19 g cm^{-1} at the 0-30, 30-60, and 60-90 cm depths, respectively. Field moisture at the time of sampling decreased with depth and fluctuated over time (Table 3.3).

Litter derived C in primary soil fractions

To better understand the contribution to functionally different SOM pools over time, bulk soil at each incubation depth from three destructive harvests was fractionated by size and density to isolate primary soil fractions (light POM, hcOM, and MAOM) found free in the soil or within macroaggregates (M), free microaggregates (m) and macroaggregate occluded microaggregates (Mm), as depicted in Figure 3.2. The majority of LDC was recovered in fractions that originated

from microaggregates inside macroaggregates, Mm, particularly in heavy coarse OM (Mm_hcOM) and MAOM (Mm_MAOM; Figure 4). High LDC recovery was found also in light M_POM (Figure 4). However, for the M_POM our procedure does not enable us to distinguish the free from the macroaggregate-occluded POM fraction (Figure 3.2).

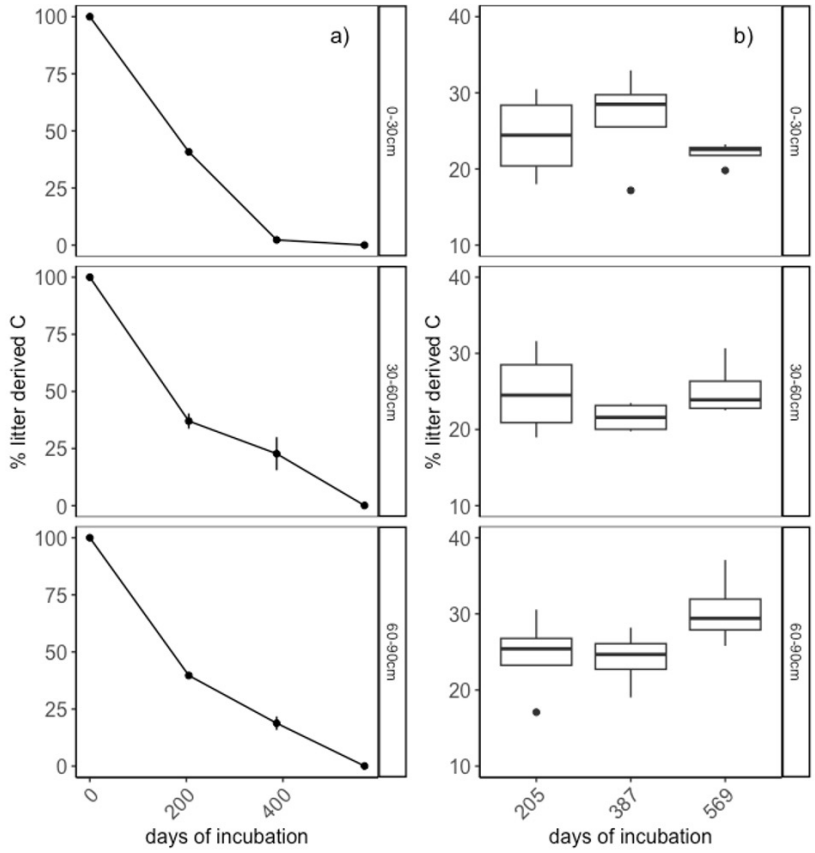

Figure 3.3. Root litter derived C recovered in (a) litter residue or (b) bulk soil organic matter (SOM) within the depth layer where the litter was incubated (0–30, 30-60, or 60-90 cm) for three harvests over the 19 months of field incubation. Amounts are given in % of the initial root litter C added at time 0. (a) Plotted points are measured replicate values at each time point. (b) Box plots indicate the medians (horizontal lines), 1st and 3rd quartiles (boxes), and 1.5× interquartile range (whiskers) *(n* = 4).

Table 3.2. Results of the linear mixed model of the effect of sampling time (T), depth treatment (D), and their interaction on the % litter derived C of each soil and litter, and results of the linear mixed model of the effect of aggregate fraction (F), depth treatment (D), and their interaction on the C:N of MAOM (d.f.: degrees of freedom [numerator, denominator]). See Table 3.1 for explanation of SOM fraction abbreviations.

% Litter derived C

Effect	d.f.	F	p	Effect	d.f.	F	p
Bulk Soil				*Litter*			
T	2, 27	0.4342	0.6522	T	2, 9	577.722	<0.0001
D	2, 27	0.9368	0.4043	D	2, 17	42.053	<0.0001
T x D	4, 27	1.7516	0.1679	T x D	4, 17	10.598	0.0002
M_POM				*M_hcOM*			
T	2, 23	7.5956	0.0029	T	2, 26	31.652	<0.0001
D	2, 23	8.644	0.0016	D	2, 26	9.263	0.0009
T x D	2, 23	2.8767	0.0455	T x D	4, 26	4.443	0.0072
m_POM				*m_hcOM*			
T	2, 21	57.968	<0.0001	T	2, 21	6.707	0.00539
D	2,21	68.074	<0.0001	D	2, 20	4.834	0.019
T x D	4, 21	41.942	<0.0001	T x D	4, 20	1.017	0.4215
Mm_POM				*Mm_hcOM*			
T	2, 9	26.074	0.0002	T	2, 7	41.75	<0.0001
D	2, 17	2.57	0.1048	D	2, 17	25.269	<0.0001
T x D	2, 17	13.37	<0.0001	T x D	4, 17	8.1	<0.0001
Free MAOM				*m_MAOM*			
T	2, 27	1.6437	0.212	T	2, 22	3.087	0.0653
D	2, 27	13.8938	<0.0001	D	2, 22	14.066	0.0001
T x D	4, 27	1.6227	0.1973	T x D	4, 22	0.51	0.7287
M_MAOM				*Mm_MAOM*			
T	2, 9	5.341	0.0296	T	2, 26	48.791	<0.0001
D	2, 18	18.9597	<0.0001	D	2, 26	62.068	<0.0001
T x D	4, 18	4.748	0.0086	T x D	4, 26	13.11	<0.0001

C:N

Effect	d.f.	F	p
MAOM			
F	3, 33	103.801	<0.0001
D	2, 33	25.609	<0.0001
F x D	6, 33	3.866	0.005

Table 3.3. Field moisture at the time of sampling averaged across control and treatment at each depth for three harvests and four replicates, *n*=24 with ± standard error in parentheses.

	Field moisture
June 1, 2014	
0-30 cm	23.31% (1.06)
30-60 cm	23.50% (0.29)
60-90 cm	20.66% (0.59)
December 1, 2014	
0-30 cm	20.29% (0.34)
30-60 cm	18.67% (0.55)
60-90 cm	16.23% (0.30)
June 2, 2015	
0-30 cm	21.96% (0.22)
30-60 cm	20.94% (0.21)
60-90 cm	19.80% (0.76)

While there were no significant differences in LDC recovered in the bulk soil across time or depth, both time and depth were significant moderators of LDC recovered in the primary fractions (Table 3.2). For most light POM and hcOM fractions, there were main effects of time, depth, and an effect of their interaction (Table 3.2). M_POM contained the most LDC and fluctuated significantly but did not change over time from 7 to 19 months. In m_POM, there was no fluctuation over time for the 0-30 cm or 30-60 cm depths (Figure 4), but for the 60-90 cm depth, % LDC in the m_POM was more dynamic; while it did not change from the first to last harvest there was a significant increase from 7 to 13 months ($p < 0.0001$) and then a decrease from 13 to 19 months ($p < 0.0001$; Figure 4). Litter-derived C recovered in Mm_POM, fluctuated less than the other light POM fractions, decreasing over time in the 0-30 and 30-60 cm depths from 7 to 19 months of incubation ($p = 0.0077$ and $p = 0.0240$, respectively).

Heavy coarse OM (hcOM) LDC started forming after the first year of incubation and was found primarily in the Mm fraction, decreasing with depth. At 19 months, there was significantly more

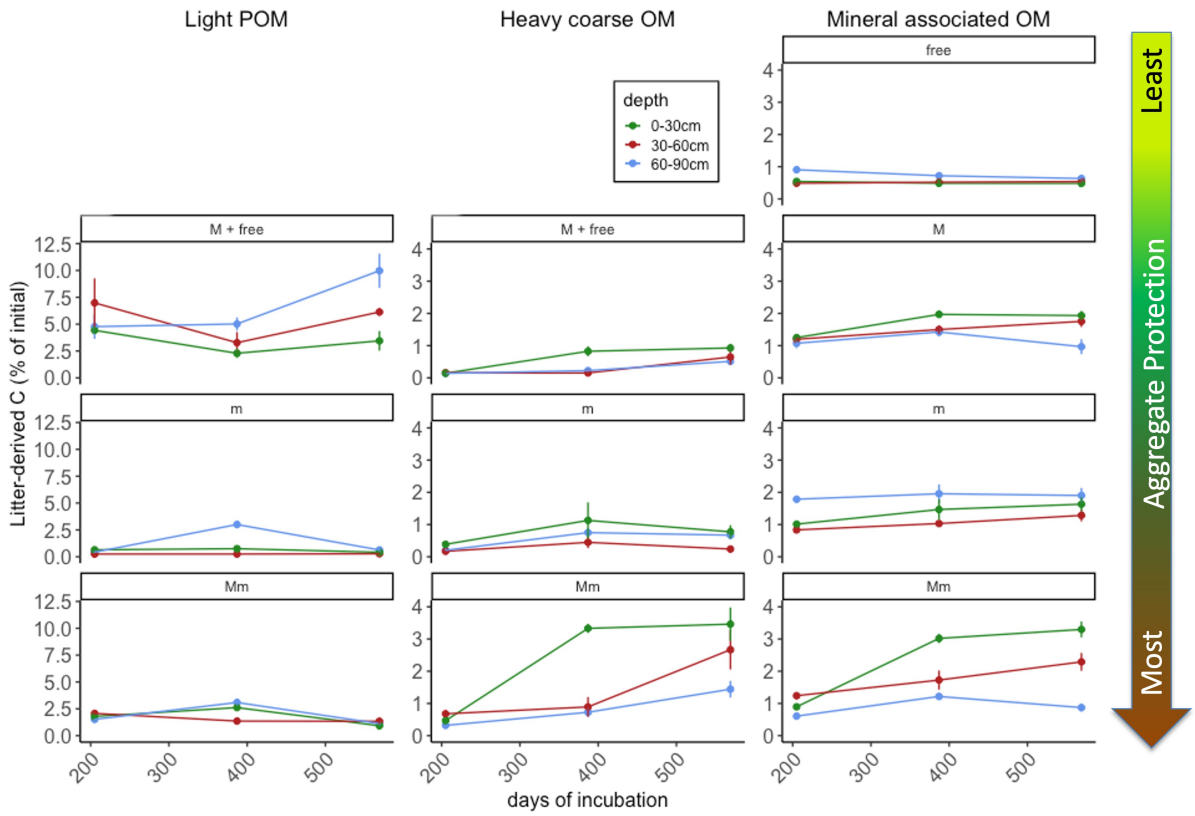

Figure 3.4. Root litter derived carbon recovered in the primary soil organic matter fractions light particulate organic matter (light POM), heavy coarse organic matter (hcOM), and mineral associated organic matter (MAOM) fractions, found free or within macroaggregate (M), microaggregate (m), and microaggregate occluded within macroaggregate (Mm), in the depth layer where the litter was incubated (0–30 cm, 30-60 cm or 60-90 cm), for the three time harvests during the 19 months of field incubation. Amounts are given in % of the root litter carbon at time 0. Data are average with standard errors (n = 4).

LDC in Mm_hcOM at the 0-30 cm (p = 0.0045) and 30-60 cm (p = 0.0023) depths than in the 60-90 cm depth (Figure 4). Averaged across depths, % LDC in M_hcOM and Mm_hcOM increased over time (p = 0.0007; p = 0.0080), but m_hcOM was relatively stable over time within each depth (Figure 4).

Decomposition products were rapidly incorporated into MAOM at all depths (Figure 4; Table 3.2). Percent LDC in free MAOM and m_MAOM remained relatively stable over time during the 19 months of incubation (Figure 4; Table 3.2). MAOM from macroaggregate structures,

M_MAOM and Mm_MAOM, was more dynamic with a main effect of time and an interactive effect between depth and time (Table 3.2). Within the M_MAOM and Mm_MAOM fractions, % LDC increased over time from 7 to 19 months in the 0-30 cm (M_MAOM $p = 0.0089$; Mm_MAOM $p < 0.0001$) and 30-60 cm depths (M_MAOM $p = 0.0332$; Mm_MAOM $p= 0.0005$), but not in the 60-90 cm depth. Overall, there was not a consistent pattern between the time dynamics of light POM and those of hcOM or MAOM.

Accumulation of litter derived C in SOM pools

By the end of the incubation, the distribution and accumulation of LDC was affected by depth. The majority of LDC accumulated in the M and Mm aggregate fractions, particularly as M_POM in the 30-60 and 60-90 cm depth, and Mm_hcOM and Mm_MAOM in the 0-30 cm depth (Figure 3.5). A depth effect was also observed when primary fractions (i.e., light POM, hcOM, or MAOM) were analyzed across aggregate structures, with LDC decreasing along the depth profile in hcOM and MAOM, with 96% more total hcOM and 68% more total MAOM recovered in the 0-30 cm depth than the 60-90 cm depth. Concurrently, LDC recovered in light POM increased with depth with 145% more light POM in the 60-90 cm depth than the 0-30 cm depth (Figure 3.5).

Carbon to Nitrogen ratio of MAOM fractions

To understand if there were inherent differences in the degree of microbial transformation of the MAOM pools found in different aggregate structures along the soil profile we investigated the total C:N of MAOM fractions free and within the different aggregate structures (Figure 3.6). There was a main effect of depth and aggregates and their interaction on the C:N ratio of MAOM fractions (Table 3.2). C:N ratio decreased as level of aggregate protection increased (Figure 3.6).

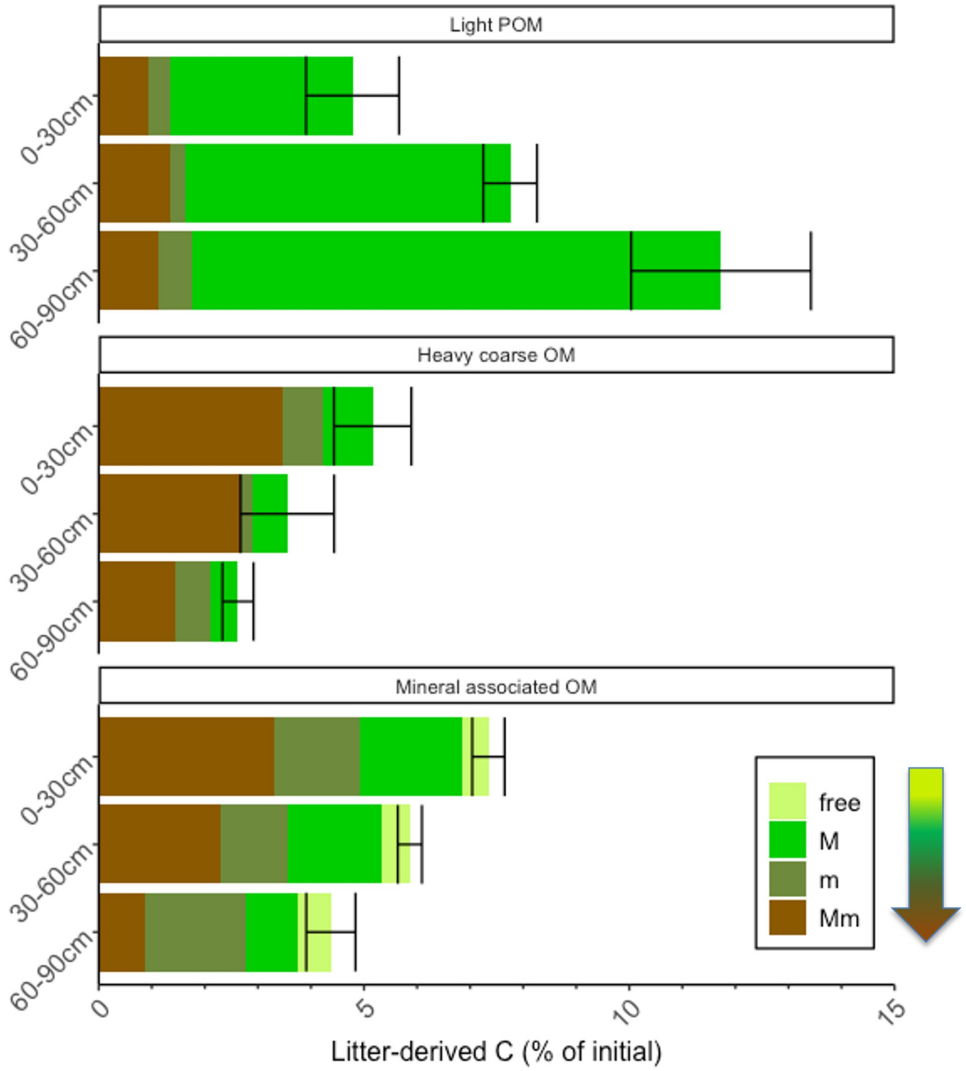

Figure 3.5. Cumulative root litter derived carbon recovered for the final harvest at 19 months of field incubation in the primary soil organic matter fractions light particulate organic matter (light POM), heavy coarse organic matter (hcOM) and mineral associated organic matter (MAOM) fractions, found free or within macroaggregate (M), microaggregate (m), and microaggregate occluded within macroaggregate (Mm), in the depth layer where the root litter was incubated (0–30 cm, 30-60 cm or 60-90 cm). Amounts are given in % of the root litter carbon at time 0. Data are average with standard error of the cumulative recovered in each pool: light POM, hcOM, or MAOM (n = 4).

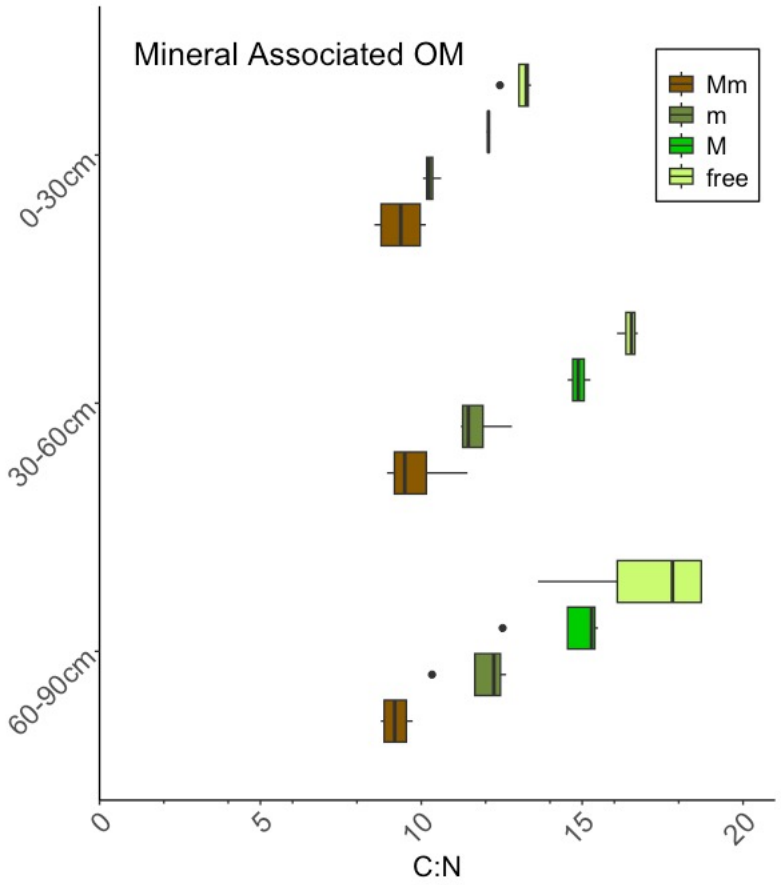

Figure 3.6. Carbon to nitrogen (C:N) ratio of mineral associated organic matter (MAOM) fractions found free or within macroaggregate (M), microaggregate (m), and microaggregate occluded within macroaggregate (Mm), in the depth layer where the root litter was incubated (0–30 cm, 30-60 cm or 60-90 cm). Box plots indicate the medians (vertical lines), 1st and 3rd quartiles (boxes), and 1.5× interquartile range (whiskers) (*n*=4).

Averaged across depths, the lower the level of protection the higher the C:N with significant differences between each soil fraction:

Free MAOM	>	M_MAOM	>	m_MAOM	>	Mm_MAOM
15.52		13.88		11.30		9.46

Averaged across MAOM fractions, C:N was lower in the 0-30 cm depth than the 30-60 cm ($p < 0.0001$) and 60-90 cm depth ($p < 0.0001$), but not different between the two deeper depths.

Discussion

Fractionation into primary and secondary soil fractions (Christensen, 2001) at three depths, despite being a cumbersome and time-consuming method of separating SOM (Poeplau et al., 2018) allowed us to elucidate the role of depth, aggregation and aggregate protection in the transformation of fresh root litter into POM and MAOM and to assess differences in the pathways of POM and MAOM formation inside and outside microaggregates. These various aggregates and primary fractions represent a spectrum of physical protection, whereby free primary fractions, i.e., not occluded within aggregates, are the least physically protected, followed by M, free m, and then Mm (Lützow et al., 2007; Six et al., 2004).

Pattern of root litter decay dynamics changed with depth but not overall decay rates

Contrary to our hypothesis, decomposition rates were only marginally affected by depth (Figure 3.3a). Root litter at all three depths reached complete mass loss by 19 months (Figure 3.3). This was more rapid than studies in grassland and forest soils using litter bags or keeping roots in bunches within the soil (Pries et al., 2018; Sanaullah et al., 2010). This may be due to our method of mixing the roots into the soil before incubation to simulate a more realistic environment of soil-biomass contact, as well as the lack of substrate available in agricultural soils due to POM depletion (Lugato et al., 2021). Also, we incubated fine roots less than 2 mm in diameter which may have contributed to rapid mass loss (Graaff et al., 2013; Silver & Miya, 2001; Zhang & Wang, 2015), although, root litter chemistry may play a larger role in decomposition rate than root diameter (Silver & Miya, 2001); we used the same root substrate at all depths. Plant species and cultivar impacts root litter decomposition rates (Graaff et al., 2013), thus our observed dynamics cannot be generalized, and we invite more studies using our approach on a variety of root litter chemistries.

Though decomposition rates were not significantly affected by depth, different patterns of decay dynamics were observed along the soil profile, especially during later stages of decomposition (Fig. 3a). These differences may have been the result of seasonal dynamics, as topsoils are more susceptible to temperature and climatic fluctuations (Gulser & Ekberli, 2004). While we did not measure soil temperature in our study, it is possible that decomposition is delayed at depth due to differing temperature and moisture; soil profile temperature and moisture data (unpublished) at a nearby site suggest environmental factors were not a major driver of the observed differences in litter decomposition. At all depths, root litter reached around 60% mass loss after the first 7 months of incubation which happened over winter. During winter months, decomposition may be primarily a result of leaching and fragmentation when microbial activity is lower. At our study site, freeze/ thaw cycles occur that particularly impact the topsoil. We measured decreased field moisture at all depths during the second harvest in December (Table 3.3), which we posit correlated to lower microbial activity at all depths. In contrast, during the growing season (7-13 months) the topsoil (0-30 cm) had a faster rate of decomposition than the deeper layers (30-60 cm and 60-90 cm), reaching almost complete mass loss by the second harvest (13 months; Fig. 3a). In croplands, microbial activity is typically enhanced in the topsoil during the growing season when soil temperatures and moisture are higher while crops are irrigated (Franzluebbers et al., 1994; Ullah et al., 2012) as for our field where Sorghum was grown during this period.

Given the numerous biotic, abiotic, and environmental variables that change with depth, it is difficult to isolate the cause of a depth effect on decomposition. The overall lower field moisture measured in the 60-90 cm depth (Table 3.3) may also contribute to lower rate of root decomposition at this depth. Soil moisture affects microbial community structure and enzyme

activity (Borowik & Wyszkowska, 2016; Brockett et al., 2012) and the ability of microbes to access OM substrates. As soil moisture decreases, so does diffusion of soluble OM, and decomposer activity (Manzoni et al., 2012). Pries et al. (2018) also observed slower decomposition with depth and declining microbial biomass C, but soil temperature, water and oxygen content did not change considerably with depth in their study, so the effect of soil moisture must be considered in the context of other characteristics that change with depth.

Comparison across multiple sites indicated that differences in climate and parent material impact root decomposition more than depth (Solly et al., 2015). In our study, the percentage of sand increased significantly with depth. Soil texture influences decomposition of litter inputs, which may be due to the impact of soil texture on water filled pore space, with lower microbial respiration observed in sandy soils (Angst, Pokorný, et al., 2021; Scott et al., 1996). Additionally, decomposition of fine roots is positively correlated with C:N (Solly et al., 2014). Our soil C:N also changed with depth increasing from 12.3 at the 0-30 cm depth to 16.5 in the 60-90 cm depth (Table 3.1). The combination of these factors point to higher decomposition in the topsoil with lower % sand, higher field moisture, and lower C:N all influencing microbial activity, abundance and efficiency. We speculate that the fast decomposition during this time in the topsoil was due to microbial catabolic and anabolic reactions, as confirmed by the resulting formation of MAOM (see below).

Despite these differences in seasonal decay patterns, LDC recovered in bulk soil did not change with depth or time. On average 25% LDC was recovered in bulk soil after complete litter mass loss (Figure 3.3b); this is similar to efficiencies of SOC formation from other litter incubation studies (Bird et al., 2008; Castellano et al., 2015; Chapter 2 and Fulton-Smith & Cotrufo, 2019; Gentile et al., 2011; Pries et al., 2018; Sanaullah et al., 2010) and no changes in

total soil C were observed (Table 3.1). There may be some translocation of LDC below the depth in which the roots were incubated; this was not possible to measure in the depths below the microcosms, but translocation to lower depths was less than 5% of the root litter added to the 0-30 cm depth as reported in Chapter 2 and Fulton-Smith and Cotrufo (2019). These results confirm that the analysis of the bulk soil does not provide sufficient detail to assess the fate and stabilization of the root LDC at different soil depths, further stressing the value of separating SOM physical fractions for accurate studies of SOM dynamics.

Distinct pathways of SOM formation with depth

While depth did not significantly affect overall root decay rates or the absolute amount of SOM formed from root decomposition, it did affect the pattern of decay dynamics and pathways of SOM formation. Litter derived C was rapidly incorporated into all primary fractions and secondary structures at all three depths, similar to other studies using stable isotopes to trace litter decomposition products (Even & Cotrufo, 2024; Kong & Six, 2010; Soong et al., 2016), highlighting the dynamic nature of OM cycling.

We expected to see more MAOM form initially due to the rapid root decomposition, but differences varied by MAOM fraction and with depth. Free MAOM and m_MAOM formed early and stayed relatively consistent over time and across depth, suggesting that the LDC associated with these fractions was relatively stable during the 19 months of this incubation (Figure 4; Table 3.2). These findings support the hypothesis that MAOM is formed early in the decomposition process, either through direct sorption of DOM or efficient transformation by microbes from labile plant materials (Cotrufo et al., 2013, 2015; Cotrufo & Lavallee, 2022; Haddix et al., 2016), and that free MAOM is stabilized by mineral association (Kleber et al., 2015). In this case, we propose initial MAOM formation was due to direct sorption of DOM

from leaching rather than microbial processing due to lower microbial activity in winter months (Schnecker et al., 2023). Moreover, the quick incorporation of LDC into m_MAOM and Mm_MAOM could be due to direct diffusion of DOC into the microaggregate structures. During the growing season (7-12 months of incubation), rapid decomposition of added root litter in the topsoil was accompanied by decreases in M_POM and increases in MAOM and hcOM suggesting microbial transformation of decomposing litter. Conversely, delayed decomposition at depth may indicate a different pathway with less microbial processing and greater physical fragmentation and POM formation (Pries et al., 2018) as is seen with slow decomposing litters due to higher recalcitrance which are preferentially incorporated into POM fractions (Fulton-Smith & Cotrufo, 2019; Kong & Six, 2010; Lavallee et al., 2018). These different pathways may be influenced by differing environmental, biotic, and abiotic conditions with depth as discussed above. Further research is required to understand the fate of POM that accumulates due to delayed decomposition at depth and whether or not this will persist longer than in topsoils or eventually undergo microbial transformation and depolymerization to contribute to MAOM formation. Moreover, further investigation of pathways of MAOM formation within microaggregates are necessary, particularly to understand whether this process is dominated by DOM, transformation of OM, or turnover of microbial biomass. These results highlight a trade-off of differing decay dynamics with MAOM formation in the topsoil where decomposition is dominated by microbial processing and POM formation in the deep soil where decomposition appears to be primarily a result of litter fragmentation.

MAOM accumulates in the topsoil while POM accumulates in the subsoil

This pattern of SOM formation in primary fractions across soil depth became clear by looking at the cumulative of all MAOM fractions (total MAOM), hcOM fractions (total hcOM)

and light POM fractions (total POM) at 19 months of incubation (Figure 3.5). More stable, more protected MAOM accumulated in the 0-30 cm depth, and less stable, less protected POM accumulated in the 60-90 cm depth; the 30-60 cm depth falls neatly in between. In this study, the sand-sized fraction, or heavy coarse OM, which has also been classified as POM or coarse MAOM in some studies, more closely followed the dynamics of the MAOM than the POM (Leuthold et al., 2022; Samson et al., 2020), confirming the recent observation that size fractionation methods which isolate hcOM together with the POM fraction introduce a bias (Leuthold et al., 2024). Moreover, percent sand increases considerably with depth; the 0-30 and 30-60 cm depths had about 30% sand while the 60-90 cm depth had 45% sand. While this means there is less total silt and clay available at depth for MAOM formation the minerals available may be less saturated in C as the % C decreases with depth across all MAOM fractions (Table 3.1). With a few notable exceptions (Sleutel et al., 2010, 2011; Urbanski et al., 2023), sandier soils have shown less efficient microbial utilization of litter than soils with higher % of silt and clay resulting in less retention of LDC (Angst, Pokorný, et al., 2021). This is corroborated by the lower amounts of MAOM accumulation observed in the deep soil (Figure 3.5).

Microaggregates are critical locations for the formation of microbially-derived MAOM.

Mineral associated OM overwhelmingly originated from aggregate structures with very little LDC recovered in free MAOM (Figure 3.5) and increased over time (Figure 3.5) supporting hypotheses that aggregates play an important role in the formation and stabilization of MAOM (Jastrow, 1996; Jastrow & Miller, 1997; Six et al., 2004). Macroaggregate formation and cycling fosters the rapid incorporation (Plante & McGill, 2002) and decomposition of OM (Bach & Hofmockel, 2016) with estimated turn over times of months to years (Six & Jastrow, 2002)(Lutzow et al., 2006), or even days to weeks by some estimates (Gryze et al., 2006). The

formation of new microaggregates within macroaggregates around a POM core encrusted with soil minerals (Jastrow et al., 2007; Oades, 1984; Six et al., 2004) facilitates the association of fresh residues with soil minerals to form hcOM and MAOM, and the protection of POM within microaggregates. In our study, an interesting dynamic emerged later in decomposition from the formation of both hcOM and MAOM in the Mm, where there was small initial incorporation of LDC. Contrary to our hypothesis, there was not a clear pattern of decreases in POM leading to increases in microaggregate hcOM or MAOM consistently across depths, rather m_POM and Mm_POM constitute a small percentage of total POM and remain relatively stable over time. Light POM was primarily recovered as M_POM which may be free or within macroaggregates and fluctuated more than the other SOM fractions (Figure 4), supporting the understanding that this is an actively cycling SOM pool. However, those fluctuations were too small and relatively inconsistent across depths and time to fully explain the large accumulation of LDC observed in the Mm_hcOM and Mm_MAOM, in the topsoil.

The percentage of macroaggregates was lower at the 60-90 cm depth (Table 3.1) which should have reduced POM physical protection, but we observed higher litter-derived POM accumulation at depth suggesting there were other constraints to decomposition at this depth. Longer term studies will be required to understand the eventual fate of POM that accumulates at depth. We speculate that further transformation of POM within microaggregates stimulates microbial turnover and results in preferential necromass accumulation in the Mm, where pore size is conducive to efficient MAOM formation (Kravchenko et al., 2019)), and MAOM and POM are in close proximity in the soil. This is supported by work demonstrating that microaggregates within macroaggregates are of biological origin (Six & Paustian, 2014), and the

OM associated with minerals inside microaggregates is microbially derived (Lehmann et al., 2007).

In order to better understand the degree of microbial transformation of the MAOM pool free and within aggregates, we examined the C:N ratio of these fractions. The free MAOM, which is the least protected was the least microbially processed fraction with the highest C:N (16.98), suggesting that a higher proportion of free MAOM is formed from the direct sorption of plant derived DOM (with a higher C:N). More work is required to confirm this potential mechanism and to investigate whether free MAOM, being richer in sorbed DOM, may also be the least stable form of MAOM, experiencing more sorption-desorption dynamics (Cotrufo et al., 2013). On the other hand, the fractions most physically protected, m_MAOM and Mm_MAOM, were also the most microbially transformed with C:N ratios < 12 (Figure 3.5), approaching that of soil microbial communities (Robertson & Groffman, 2007). This again supports the hypothesis that aggregates encourage microbial transformation of OM inputs to the soil and promote necromass-derived MAOM, which may be the more stable form of MAOM.

Conclusions

Understanding the fate of root litter decomposition products is critical to assessing the sustainability of crops and their ability to contribute to soil C sequestration, particularly in the deep soil when considering deep rooted crops. Isolating POM and MAOM from the bulk soil rather than from different aggregate size classes may be more efficient to inform changes in soil C over time, less expensive and less time consuming. However, detailed fractionations such as these combined with the decomposition of ^{13}C enriched litter give us a glimpse of the complexity of the SOC cycle and the role that aggregates play in the formation of POM and MAOM in the soil and how this might differ with depth.

The accumulation of MAOM in the topsoil and POM in the deep soil highlights an inherent trade-off between agronomic practices or plant breeding efforts to increase SOM levels in soils for C sequestration attributed to stable MAOM versus improving soil health for other ecosystem benefits such as nutrient cycling, water retention, and aggregation attributed to POM. If POM is critical for aggregate formation (Jastrow & Miller, 1997) and aggregates are a site of MAOM formation, efforts to increase soil C sequestration cannot focus solely on the addition of minerally stable C, but also to POM to promote aggregation and overall soil health and function; these dynamics are exacerbated with depth.

Differential decomposition dynamics at depth highlight the potential challenges in increasing stable MAOM in the deep soil where there is potential to increase mineral soil C pools that are less saturated, but decomposition is slower and POM preferentially accrued. Longer term studies, possibly exploring in situ deep root turnover, are needed to better understand the fate of POM additions at depth, in particular to isolate the role of environmental, biological and chemical factors that change with depth. If the introduction of fresh litter inputs at depth leads primarily to the formation of POM, it is important to understand whether this root-derived POM may lead to increased aggregation at depth and thus persist as occluded POM, eventually be transformed into MAOM, or will represent an available source of C priming the decomposition of older stable C pools (Fontaine et al., 2007)

Given the observation of C rich free MAOM and N rich microaggregate MAOM (Figure 3.6), our findings allude to different formation pathways and potential differences in the nature of MAOM inside versus outside microaggregates whereby the stable necromass-derived MAOM would be formed preferentially inside microaggregates, while the more dynamic exchangeable DOM-derived MAOM would be formed preferentially outside of microaggregates. We invite

future research to test these potential mechanisms, that are very relevant to the accumulation and persistence of C in soil.

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CHAPTER 4 – BACK TO THE FUTURE: MERGING THE CONCEPTS OF AGGREGATE HIERARCHY WITH MECHANISMS OF POM AND MAOM FORMATION, TRANSFORMATION, AND STABILIZATION[3]

Introduction

There is increasing interest in the role of soils as a terrestrial carbon (C) sink to mitigate climate change, particularly through the adoption of regenerative management practices in agricultural soils that may be depleted in soil organic matter (SOM) (Lal, 2020; Lal et al., 2021; Paustian et al., 2006). This emphasis on soil C sequestration has led to vast improvements in our understanding of the complex processes and mechanisms that lead to SOM formation and stability. In particular, there has been an increase in the study of primary soil particles *sensu* Christensen (2001), such as mineral-associated (MAOM) and particulate organic matter (POM), in relation to their differential function, stability and pathways of formation in the soil (Angst et al., 2023; Cotrufo et al., 2021; Cotrufo & Lavallee, 2022; Lavallee et al., 2020). However, over the last decade, SOM research has shifted away from the role of secondary structures, or aggregates, in this complex SOM cycle, largely because they are composite units and their isolation increases methodological efforts without necessarily providing additional clarity in the interpretation of the turnover and drivers of soil C stocks (Lavallee et al., 2020; Poeplau et al., 2018). The past two decades have significantly advanced our understanding of SOM dynamics (Rocci et al., in review), and focusing on POM and MAOM has brought considerable advancements in the understanding of the mechanisms and controls of C storage and accrual.

[3] This manuscript is in preparation: Fulton-Smith, S.E., Cotrufo, M.F. Back to the Future: Merging the concepts of aggregate hierarchy with mechanisms of POM and MAOM formation, transformation, and stabilization.

However, significant knowledge gaps remain and the observation of differences in the MAOM fractions inside and outside of aggregates (Chapter 3 and Fulton-Smith et al., in review) suggest aggregates may require further attention.

SOM research has primarily emphasized the role of aggregates in the stabilization of SOM (Lehmann and Kleber, 2015), but has not incorporated the potential role of aggregates in influencing mechanisms of POM transformation and MAOM formation within this framework. Since both these fractions can be present free in the soil or occluded inside aggregates, studying the effect of aggregation on their dynamics appears essential to "multi-pool" management efforts designed to promote both soil C accrual and soil health (Angst et al., 2023; Lehmann, Bossio, et al., 2020; Moinet et al., 2023). The multi-pool approach to soil C sequestration advocates for increasing both POM and MAOM C pools in soil. Mineral associated OM accrual appears to be constrained by plant inputs and the mineral capacity of soils (Hansen et al., 2024; King et al., 2023). Conversely, POM can theoretically accumulate indefinitely, yet it is the soil C pool that has been primarily lost following disturbance in cropland soils (Lugato et al., 2021) and is harder to regain even through regenerative management (King et al., 2024). This may be due to POM being controlled more by constraints on its decomposition than by constraints on plant inputs (Hansen et al., 2023), indicating the need for POM protection in stable aggregates to support its accrual. Microaggregate-occluded POM is more stable and protected than free POM (Haddix et al., 2020) which may be susceptible to inefficient catabolism and loss from the system.

While macroaggregates facilitate the incorporation of litter residues into the soil as POM, microaggregates stabilize POM on longer timescales. Based on aggregate hierarchical order, macroaggregates provide the conditions for microaggregate formation within them due to the encrustation of POM with primary soil particles, creating organo-mineral complexes (i.e.,

MAOM) (Oades, 1984; Six et al., 2004; Six & Paustian, 2014; Tisdall & Oades, 1982). Thus, the co-location and proximity of MAOM and POM within microaggregates is inherent to their formation and structure.

Given emerging evidence of soil aggregation modulating MAOM formation and POM stabilization pathways (Even & Cotrufo, 2024), we suggest an advancement in the evolving SOM framework (Rocci et al., in review) to incorporate the role of aggregates as moderators of POM transformation and MAOM formation (Figure 4.1). By looking back to the conceptualization of aggregate hierarchy over 20 years ago and integrating that knowledge with what we know today, we can deepen our understanding of these dynamics in the context of complex soil structure as well as inform management for multi-pool soil C accrual. The merging of these concepts can also help inform management that supports soil functionality in addition to C accrual.

Current understanding of aggregates, POM and MAOM

What are microaggregates?

As the largest structural units of the soil, macroaggregates (> 250 mm) are thought to play a critical role in the formation of microaggregates (53 to 250 mm) (Oades, 1984; Six et al., 2004; Six & Paustian, 2014; Tisdall & Oades, 1982). Macroaggregates are relatively fleeting, with estimated mean residence times of months to years (Lutzow et al., 2006; Six & Jastrow, 2002), or by some estimates of days to weeks (de Gryze et al., 2006), while microaggregates (53 to 250 mm) are relatively more stable than macroaggregates with mean residence times of years to decades (Lutzow et al., 2006). Microaggregates form within macroaggregates when a decomposing organic matter particle (i.e., POM) is encrusted with silt and clay particles, as mentioned above and described in detail by other sources (Chenu & Stotzky, 2002; Golchin et

al., 1994; Jastrow et al., 2007; Six et al., 2000; Totsche et al., 2018). Microaggregates are organo-mineral complexes comprised of pore space and different mineral components such as silt and clay particles, oxyhydroxides, carbonates, and/ or clay microstructures (< 20 um) bound to plant and microbial derived organic matter (OM), which can either be POM physically occluded among mineral particles, or OM chemically bonded to minerals (MAOM) (Jastrow et al., 2007; Totsche et al., 2018). The components of aggregates and a summary of the different concepts of aggregate formation are summarized in several reviews and will not be exhausted here (Totsche, et al., 2018, etc.).

What are POM and MAOM?

Over the last decade our conceptual understanding of POM and MAOM dynamics in the soil has strengthened, and these two pools are now well recognized for being functionally different, formed through distinctive pathways, and persisting through different stabilization mechanisms resulting in distinct average mean residence times in the soil as reviewed in Cotrufo and Lavallee (2022). Mineral associated OM may be plant or microbial derived (Angst et al., 2021) and refers to OM bound to soil minerals (< 53 mm, heavier than 1.6 or 1.84 g cm^3), such as silt and clay particles and metal oxides. Besides individual silt and clay and particles and amorphous metals, this pool is also comprised of very fine, stable (< 53 mm) microaggregates resistant to dispersal methods (Chorover et al., 2004; Ilg et al., 2008). Mineral associated OM may form by sorption to soil minerals of plant-derived dissolved organic matter (DOM), products of exoenzyme depolymerization (ex-vivo pathways), or mineral association of microbial products, such as necromass and metabolites (in-vivo pathways) (Cotrufo et al., 2015, 2022; Liang et al., 2017). MAOM is generally more stable than POM with turnover times of centuries to millennia (Lützow et al., 2007), and the associated OM is less available to plants due

to association with soil minerals through chemical sorption or inaccessible to microbial decomposition via occlusion in small aggregates or micropores (Kleber et al., 2015; Kögel-Knabner et al., 2008; Totsche et al., 2018). This may be an oversimplification as recent research has indicated that a fraction of MAOM can exchange readily and is recycled on shorter timescales (Jilling et al., 2018; Kleber et al., 2021; Angst et al., 2023). From a chemical standpoint, MAOM is more labile comprised of simple lower molecular weight compounds, such as cellulose, that are often soluble in nature with a lower C:N ratio than POM.

Particulate OM (< 1.85 g cm^{-3}) is coarse and light in nature forming from the fragmentation of decomposing structural residue of plants (both above and belowground) and microbes. Particulate OM has shorter turnover times of years to decades (Lavallee et al., 2019; Lutzow et al., 2006) and is more accessible, with occlusion inside microaggregates as the primary mechanism for stabilization. However, POM is chemically more recalcitrant comprised of more complex structural materials, such as lignin, and with higher C:N ratios requiring depolymerization via microbial exoenzyme production before plant uptake of nutrients can occur (Kleber et al., 2015). These fractions can be isolated from the bulk soil or from secondary structures, macroaggregates and microaggregates. Free and occluded POM and MAOM fractions have been referred to by varied nomenclature in the literature, these definitions and functional and operational differences are addressed comprehensively in Lavallee et al. (2020) and Leuthold et al. (2022).

Merging aggregate hierarchy with POM and MAOM framework

By providing a brief overview of aggregate hierarchy and MAOM and POM formation, we want to set the stage for merging these concepts in our proposed framework (Figure 4.1). While POM may serve as hotspots for microbial activity and can drive the formation of organo-

mineral associations (Derrien et al., 2023), direct evidence for this in the bulk soil has not been clear (Leuthold et al., in review). We hypothesize that if POM depolymerization leading to MAOM formation occurs, that it is predominantly within aggregates where POM is in proximity to soil minerals and microbes, i.e., an ideal environment for the efficient binding of mostly microbially transformed P and N rich OM compounds to minerals (Spohn, 2024).

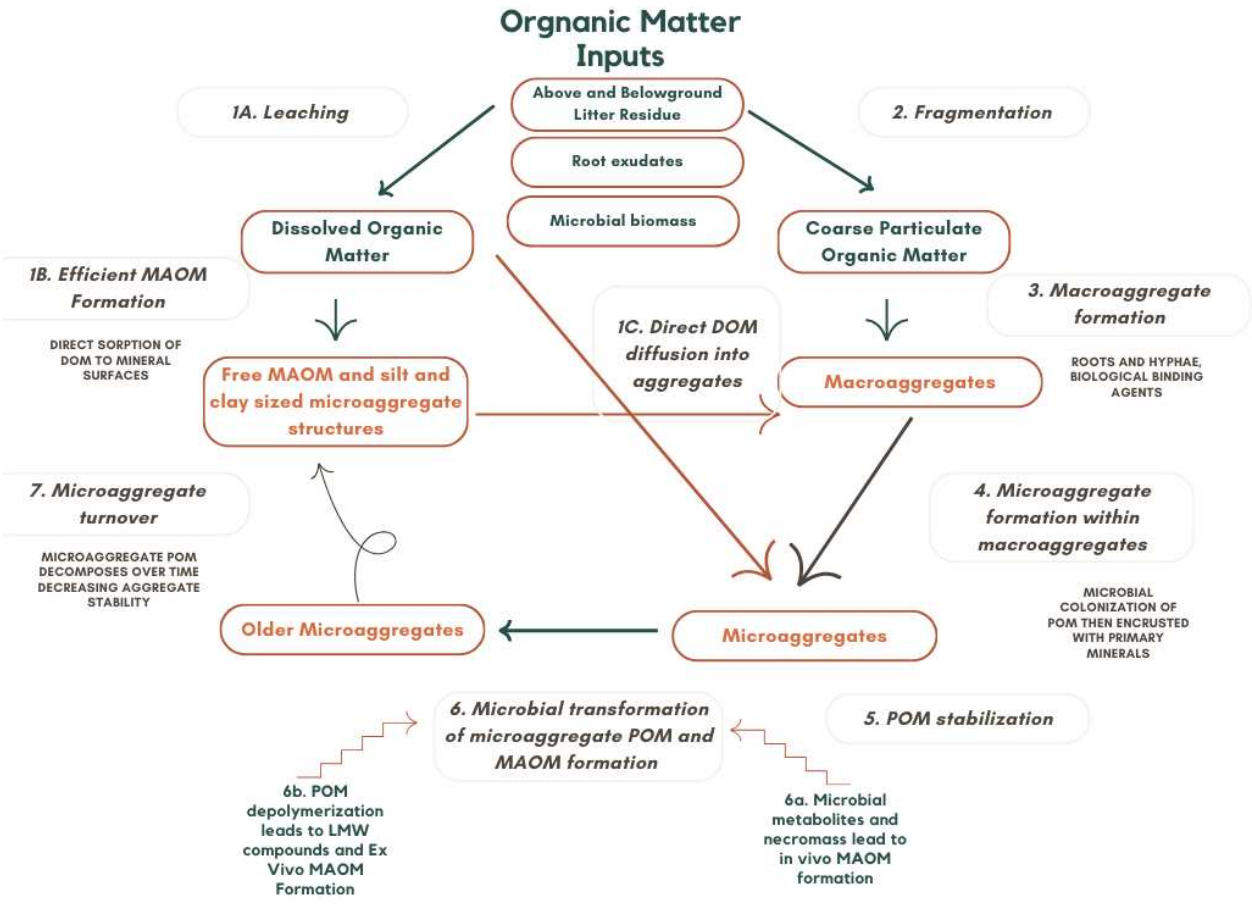

Figure 4.1. Proposed soil organic matter framework integrating aggregate structure into the dynamics of particulate and mineral associated organic matter formation, transformation and stabilization.

Litter decomposition products, root exudates, and other OM are rapidly incorporated into all aggregate size classes and primary soil OM pools, as demonstrated by the numerous studies

that trace litter residues through primary and secondary soil fractions using stable $d^{13}C$ and $d^{15}N$ isotope labeling (Denef et al., 2004; Even & Cotrufo, 2024; Fulton-Smith & Cotrufo, 2019). This confirms that aggregate structures and primary C pools are dynamic and responsive to litter and OM inputs regardless of their assumed stability in the soil.

Low molecular weight compounds, such as root exudates or DOM produced from leaching (Figure1.1a) during the initial stages of residue decomposition rapidly and efficiently form organo-mineral complexes (MAOM) through direct sorption to soil minerals (Cotrufo et al., 2022) via an ex-vivo pathway of MAOM formation (Liang et al., 2017). This ex-vivo pathway of MAOM formation from plant derived compounds may be more relevant to free MAOM, though there is evidence (Even and Cotrufo, 2023) that some DOM may diffuse into aggregates and sorb directly to charged mineral and metal surfaces (Figure1.1b).

Plant growth, decomposition of OM, and earthworm activity (or other macrofauna) produce binding agents that stimulate the formation of new macroaggregates (Figure 4.1.3) (Golchin et al., 1994; Jastrow, 1996; Six et al., 2000; Witzgall et al., 2021). As above and belowground residues are fragmented (Figure 4.1.2) they are rapidly incorporated into macroaggregates (Plante & McGill, 2002). In addition to plant residues, this POM may be of microbial origin generated from the decomposition of fungal hyphae, bacterial polymeric compounds, and other POM sources that are contained within macroaggregates (Guhra et al., 2022). This light POM is colonized by microbes then encrusted with soil minerals leading to the formation of microaggregates around a core of C rich compounds bound to minerals creating organo-mineral complexes (MAOM) – this describes the process by which microaggregates are thought to form within macroaggregates (Figure 4.1.4) (Gale et al., 2000; Golchin et al., 1994; Oades, 1984; Six et al., 2000). In addition to the formation of new microaggregates, older

microaggregates may cycle through macroaggregates numerous times as the latter are formed and turnover in relatively short timescales.

Microaggregate POM is transformed via depolymerization by microbially produced exoenzymes (Figure 4.1.6), this process is described by Liang et. al (2017) as ex vivo modification of plant residue involving the use of extracellular enzymes. If this process occurs inside microaggregates these low molecular weight plant-based compounds can readily sorb to proximate soil minerals leading to further MAOM formation within microaggregates becoming unavailable to microbes (Figure 4.1.6). Primarily, as POM within aggregates is further decomposed, released compounds will facilitate microbial processing and turnover, production of necromass and microbially derived compounds, such as metabolites that are directly sorbed to soil minerals, following an in-vivo MAOM formation pathway (Figure 4.1.6; Liang et al., 2017).

New microaggregates are thought to be more stable with fresh binding and cementing agents present, but as microaggregates age, the POM within aggregates decomposes further becoming less stable and leading to turnover (Golchin et al., 1994) (Figure 4.1.7). MAOM that is released upon microaggregate turnover is comprised of stable silt-sized (< 53mm) and clay-sized (< 2 mm) microaggregates in addition to primary mineral associated OM (Chenu & Plante, 2006; Schweizer et al., 2019; Virto et al., 2008, 2010). This soil fraction may be cycled back into new aggregates or bound to C-rich OM via weaker exchangeable bonds. POM that is released upon microaggregate turnover is susceptible to rapid catabolism and loss from the system or plant uptake.

Initial supporting evidence

Preliminary evidence from our recent research (Chapter 3 and Fulton-Smith et al. (in review); Even & Cotrufo, 2024; Fulton-Smith & Cotrufo, 2019) supports this advancement of

the MAOM and POM framework described above. While C:N decreased as aggregate protection increased (Figure 3.6), litter derived C:N of MAOM fractions was similar regardless of aggregate affiliation in the initial stages of decomposition and remained relatively unchanged over time during a 19-month microcosm experiment (Fulton-Smith (unpublished)) and a 12-month lab incubation (Even & Cotrufo, 2024). This consistent C:N of initial OM formation suggests that this OM may be of similar origin supporting the described pathway of initial sorption of DOM to free MAOM and potential diffusion directly into aggregates (ex-vivo pathways) and may indicate the chemical characteristic of the OM is the basis for chemical binding to minerals. Organic N (ON) and organic P (OP) rich compounds such as, microbial necromass and DOM have a higher affinity to bind to soil minerals than organic compounds that do not contain N or P, and these bonds are stronger and longer lasting in the soil (Spohn, 2024). Though, in other studies the amount of C that diffused into microaggregate centers was small (Chenu et al., 2001). The ability of ON and OP containing compounds to layer with N and P free compounds that are less competitive in binding directly to mineral surfaces needs to be explored further (Spohn 2024), but this OM to OM (Mitchell et al., 2018) layering could indicate a second stage of stabilization outside of aggregates.

The process by which microaggregates form within macroaggregates via colonization of POM followed by mineral encrustation is well supported, including the concept of microaggregates containing a centralized POM core (Gale et al., 2000; Golchin et al., 1994; Jastrow et al., 2007; Oades, 1984). Yet, Lehmann et al. (2007) showed through Fourier-transform infrared (FTIR) and spectroscopic visualization of whole microaggregates that C was randomly distributed within the aggregate structure. This may indicate that while microaggregates form due to the interactions of mineral surfaces and POM this does not

necessarily include the occlusion of the OM within a central core, rather distribution of C within microaggregates is heterogenous including an amalgamation of diverse OM sources at varying stages of decomposition or microbial transformation. Regardless of the mechanism of microaggregate formation, what happens to the POM once it is occluded within microaggregates in poorly understood. Microaggregate occluded POM is older (Six et al., 1998), more decomposed (Golchin et al., 1994; Kölbl et al., 2005) with a lower C:N (John et al., 2005; Chapter 3 and Fulton-Smith et al. in review) than free POM, indicating that free POM is comprised of relatively new inputs and cycles rapidly, while protected POM may persist longer, but continues to undergo microbial transformation. Higher decay rates were measured for free POM *versus* aggregate occluded POM in agricultural soils (Haddix et al., 2020), in fact once POM is released from aggregates it may be quickly catabolized and lost from the system. Golchin et al. (1994) reported that NMR analysis showed that OM associated with stable microaggregates resistant to dispersal methods was more similar in chemical composition to free POM than aggregate occluded POM released upon dispersal of less stable microaggregates. They proposed a framework in which, as microaggregates age the POM within them decomposes, eventually leading to lower microbial activity and aggregate stability and eventual turnover (Golchin et al., 1994). This supports the concept that POM continues to be transformed after it is stabilized within microaggregates.

Fulton-Smith et al. (in review) measured that protected microaggregate MAOM isolated from both free microaggregates and microaggregates within macroaggregates had similar natural abundance ^{13}C isotope signatures of about -16 $\delta^{13}C$, while the less protected free MAOM and macroaggregate MAOM had similar signatures of about -13 $\delta^{13}C$ indicating that these MAOM pools may be chemically distinct (Chapter 3; Table 3.1). Unfortunately, these soils had mixed C3

and C4 crop history, so differences in the natural abundance ^{13}C values between the two pools does not inform about the age of the MAOM-C. While C:N of added root litter was similar across MAOM fractions (Fulton-Smith, unpublished), Fulton-Smith et al. (in review) demonstrated that the C:N ratios of the MAOM fractions decreased as the level of aggregate protection increased, which may indicate that more protected aggregate associated MAOM has undergone more microbial transformation (Chapter 3; Figure 3.6). This finding combined with the distinct isotope signatures may indicate that free and occluded MAOM are chemically distinct pools supporting the hypothesis that they are formed via different pathways – free MAOM is formed primarily from ex vivo pathways while microaggregate MAOM is formed via in vivo pathways.

Reduced pore space resulting in lower rates of water and oxygen diffusion is thought to be a major factor contributing to the stability of microaggregates within macroaggregates (Totsche et al., 2018). This could lead to less microbial activity and less degradation of POM, particularly in small microaggregates (< 53 mm) which contain micropores (< 30mm). Though when POM transformation does occur in microaggregates we can expect it to be more efficiently incorporated into MAOM than in the bulk soil due to proximity to soil minerals and microbes. Macro and microaggregates have distinct microbial habitats supporting distinct microbial community distribution; in microaggregates microbial regulation resulted in the greatest concentration of MAOM, including the likely stabilization of microbial metabolites bound to soil minerals (Trivedi et al., 2017), supporting the proposed in vivo pathway as the primary pathway for MAOM formation within microaggregates. This is further supported by Lehmann et al. (2007) demonstrating that organic matter bound to mineral surfaces resembled microbial

metabolites (N and P rich compounds) more than OM of the entire microaggregates (Lehmann et al., 2007).

In addition to these specific observations, broader scale trends also point to a critical link between MAOM and POM C dynamics and aggregate structure. Higher percentages of C are found in MAOM than POM in agricultural soils, yet the percentage of C saturation of the MAOM fraction (*sensu* Georgiou et al., 2022; Six et al., 2002) in agricultural soils is typically low compared to native systems (Cotrufo & Lavallee, 2022). This is likely due to the disruption of aggregation and subsequent depletion of POM reservoirs in agriculture. Spohn (2024) hypothesized that soils low in N and P, have a reduced ability to form MAOM and have faster turnover contributing to a lower capacity to reach mineral saturation. Without sufficient POM in these systems the ability of soil minerals to reach their C saturation potential is limited, highlighting the critical role of POM and aggregates in the ability of soil C to achieve mineral saturation. After one year of incubation, soil with high capacity for aggregation had greater production and retention of MAOM from soluble plant inputs than a sandier soil with low level or capacity of aggregation (Even & Cotrufo, 2024). Additionally, the microbial transformation efficiency of plant inputs and microbial necromass formation are also found to promote C accrual in MAOM. (Kallenbach et al., 2015; Tao et al., 2023). These findings have been observed overall for the MAOM fraction, independent of it being found free or inside aggregates.

Future Research

We invite the SOM research community to investigate the POM and MAOM framework in the context of the structure microaggregates provide for moderating the formation, transformation, and stabilization of these C pools. Understanding this linkage will advance our understanding of long-term C cycling, particularly in managed ecosystems, where C

sequestration efforts are targeted, and the ecological function of soils has been degraded. Specifically, there are a few key areas that warrant further investigation based on the framework we have presented. The extent to which MAOM continues to form within microaggregates over time and whether microaggregates are preferential sites for POM transformation leading MAOM formation is little studied. If this transformation of POM does occur within microaggregates, we do not know if it will more efficiently lead to MAOM formation rather than C loss than in the bulk soil. Further investigation of the biological and chemical composition of these fractions and their resulting influence as well as high resolution imagery in a diverse set of soils would provide clarity to better understand if these pools of MAOM free or within aggregates are distinct, and if this has implications for its formation efficiency and relative stability. More research is needed to understand whether MAOM outside of aggregates that may be more layered is more exchangeable than MAOM inside aggregates that is less layered and more N and P rich.

The corollary relationship between MAOM and microaggregates needs to be expanded to other soils with varying climate, texture, and mineral capacity (Krause et al., 2018) to better understand if there is a consistent relationship between microaggregates, POM transformation, and MAOM formation in soils that demonstrate hierarchical aggregate order. Chenu et al. (2001) found that soil texture influenced microbial community composition and distribution of aggregates. Moreover, the impact of land use change to aggregation and resultant changes in MAOM and POM is influenced by soil texture (Schiebelbein et al., 2023). There is also an increasing understanding of the important role of soil pores in microbial activity and abundance and resultant influence on MAOM formation (Kravchenko et al., 2019; Kravchenko & Guber, 2017), but this needs to be considered in the context of aggregates as a key determinant of soil structure and pore sizes. Yudina and Kuzyakov (2023) propose a duality of soil structure where

pores and solids interact in groupings of varying complexity and dynamics as aggregates; they suggest that aggregates should not be artificially defined by their size as in laboratory experiment, but rather by the energy required to de-stabilize them (Yudina & Kuzyakov, 2023). Others have called for holistic approach of investigating soil architecture to include in situ study of aggregates within the context of the surrounding pore space and without artificial laboratory separation in order to fully understand the role of soil architecture on soil functioning, including carbon cycling (Vogel et al., 2022).

Advancing SOM frameworks for management

The current separation of SOM frameworks from aggregate structure has repercussions for management and achievement of C sequestration goals for agriculture. However, the concept of soil C "sequestration" can be misleading, as SOM is not "sequestered" in the soil, rather it is continuously cycled within the soil profile through biotic and abiotic processes (Lehmann, Hansel, et al., 2020; Lehmann & Kleber, 2015; Schmidt et al., 2011). It is this continuous cycling that generates soil health and fertility supporting plant production. This inherent dilemma between recycling SOM to support soil fertility (Lehmann et al., 2020b) and the desire to permanently store C in SOM (Janzen, 2006) has led to a call to refocus on building overall SOM pools as an ecosystem property (Janzen, 2014), and managing multiple SOM pools in order to unlock the potential of soils to be a C sink (Angst et al., 2023). Integrating aggregates into new proposed multi-pool management strategies that call for supporting POM and MAOM formation side by side (Angst et al., 2023) will enhance our ability to understand the relationship between these two soil C pools and efficacy of efforts to increase them in managed systems.

Soil health management principles and regenerative agriculture already provide a suite of practices that focus on the increase, restoration, or maintenance of SOM and aggregation in soils,

such as reduced tillage, cover crops, and adaptive grazing (Khangura et al., 2023; Teague & Kreuter, 2020). The benefits of practices that improve soil health go far beyond the potential increases in soil C sequestration (Lehmann, Bossio, et al., 2020). Aggregates are well understood for their role in the functioning of healthy soils, which are often attributed to healthy POM levels in soils. Soil disturbances increase macroaggregate turnover, reducing incorporation of organic inputs into stable fractions (Six et al., 2000; Six & Paustian, 2014), and accelerating POM decomposition. Changes in total soil C stocks, can take years to change after regenerative agricultural practices are implemented (Jastrow, 1996). However, a lag in soil C accrual after regenerative systems are established does not mean that these practices are not improving soil health and function. Looking beyond changes in bulk soil C is critical to our ability to assess the impact of new practices in shorter time scales. Based on this proposed framework, increasing POM stocks through increased aggregation may ultimately also increase the capacity of soils to produce MAOM within microaggregates. Practices that increase aggregation and OM inputs to soils can build POM, which in turn can build MAOM and potentially raise the ability of agricultural soils to achieve mineral saturation. We need to establish the correct metrics for measuring changes in soil structure and functioning before changes in bulk soil C may be detectable.

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CHAPTER 5- CONCLUSIONS

My dissertation brings together questions about bioenergy sustainability, litter decomposition, aggregation, and SOM formation. My overall objective was to understand how SOM formation is impacted by feedstock selection for deep root production and more broadly the role of aggregates in this process. My primary research questions were:

1. How does above versus belowground litter, as influenced by litter chemistry, impact litter decomposition and subsequent formation of SOM to functionally different C and N pools?
2. How does soil depth impact the ability of root litter to contribute to the formation of MAOM and POM particularly in the deep soil where there is more potential for C accrual? What role do aggregates play in this process?
3. Does aggregation, specifically microaggregates, play a role in moderating the transformation of POM and the formation of stable MAOM?

To address the first two questions, I created novel soil-biomass microcosms to create a more realistic environment for decomposition than traditionally used litter bags and combined this with ^{13}C and ^{15}N isotopically enriched *Sorghum bicolor* leaves and roots and extensive fractionation of primary soil particles and secondary soil structures. In Chapter 2, I found that leaves were of higher quality than roots based on multiple measures including C:N, LCI and %HWE. Roots decomposed more slowly than leaves but contributed more efficiently to total SOM formation. However, leaves were more efficient at contributing to stable MAOM while roots contributed more to less stable fractions Coarse POM and LF (referred to as light POM in Chapter 3).

In Chapter 3, I used similar methods to answer question (1), but incubated roots at 3 depths up to 90 cm within the soil-biomass microcosms. Results of this study showed that root decomposition progressed more rapidly in the 0-30 cm depth than the deeper depths (30-60 and 60-90 cm), resulting in more root litter C recovery in MAOM in the topsoil and lighter and coarser POM in the deep soil with the middle depth falling neatly in between. Interestingly, most of the MAOM was recovered within soil aggregates, particularly microaggregates, than free in the soil highlighting the potential role of microaggregates in MAOM formation.

The results of both experiments underscored that bulk soil C and N measurements are too coarse to inform about changes in total soil C in the short term and the likely fate and stability of new litter derived C and N that enters the soil. While this extensive fractionation was time consuming and cumbersome, it provided a level of detailed that allowed me to speculate more broadly on the likely role of aggregates in the formation of MAOM and transformation of POM.

The time I have spent working since I originally began my graduate studies in 2012, has given me insight and perspective as a landowner, farmer, and a conservationist working directly with farmers and ranchers to improve their agricultural practices. During this time, the SOM research community has made tremendous advancements in our understanding of POM and MAOM as functionally different SOM pools that differ in their formation and stabilization in the soil. Diving back into the literature and discussions with my co-authors between the publication of Chapters 2 and 3 gave me time to reflect on these advancements and gave me an opportunity to interpret my results in the context of this deeper understanding of the mechanisms of SOM formation and the importance of conceptualizing it into POM and MAOM. This combined with my experience in the field allowed me to understand the relevance of these results in the context of agricultural management and sustainability.

These shifts in the SOM framework, in combination with what I understood early on during my degree program about aggregate hierarchy has given me the unique opportunity as a graduate student to conceptualize the framework presented in Chapter 4 merging these two concepts. Frameworks are critical to our ability to advance scientific understanding of basic science questions; they guide and are influential to future research questions. I hope that the results of my experiments can contribute to this important body of knowledge and the continually evolving SOM frameworks, while my conceptual framework presented in Chapter 4 can prompt discussion and future research that integrates aggregates within this work moving forward.