

The role of biodegradable plastic mulches in soil organic carbon cycling

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DEDICATION

I would like to dedicate this thesis to the Biosystems Engineering and Soil Science department at the University of Tennessee- the students, the staff, the faculty. This grad school program has shaped me in so many ways. I cannot thank the people of this department enough for their kind words, advice, constant encouragement, and scientific mentorship. This department fosters a healthy sense of competition while simultaneously having so many people willing to go out of their way to help. Please keep providing students, many far away from their families for the first time, a safe place to grow into badass scientists and engineers.

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ABSTRACT

Agricultural plastic mulching improves weed management and controls the soil moisture and temperature leading to large increases in yield. Unfortunately, the rise in popularity of mulching results in more plastic pollution due to a lack of recycling options for polyethylene (PE) mulch. Biodegradable plastic mulches (BDMs) are an alternative to the traditionally used PE mulches and are recommended for tillage into the soil at the end of the season. There is evidence that by altering soil moisture and temperature, the use of mulching is tightly tied to soil carbon (C) cycling. The purpose of this study was to gain insight on the effect of BDMs on soil C storage, an important part of soil health, using field and lab studies. The field study was replicated in two diverse climates, Knoxville, TN and Mt. Vernon, WA. Multiple C pools were measured over two years (Spring 2015- Spring 2017) to monitor the effect of 7 different mulching treatments (four BDMs and three controls: PE plastic mulch, cellulosic (paper) mulch, and no-mulch). After two years, results were variable across locations, but PE treatments accumulated less soil C than the no-mulch control in each location. These differences are likely due to increased mineralization from the higher soil moistures and temperatures in the PE treatment compared to the no-mulch treatment. The use and incorporation of BDMs showed no significant declines in soil C pools compared to the no-mulch control. Season and location significantly affected C pools as well. To improve estimates of BDM fate, BDM microplastics were measured from field samples using a novel method. The second part of this study was a lab experiment to investigate how air temperature affects the biodegradation of BDMs by combining time course biodegradation measures with soil C pools, soil microbial properties, and chemical properties of the BDMs. Collectively, these results showed significantly higher BDM biodegradation at higher temperatures with amorphous polymeric regions being degraded first. The positive effects of BDMs on soil C stocks compared to PE supports their use as a substitute to PE; however more research is needed on microplastic residence times.

TABLE OF CONTENTS

I. INTRODUCTION	1
BACKGROUND	2
Plastic agricultural mulching	2
Biodegradable plastic mulches as an alternative to plastic mulching	2
BDMs alter soil physical properties	3
Effects of BDMs on soil health	4
Soil carbon and soil health	4
BDMs affect soil carbon cycling	5
BDM degradation	7
BDMs as microplastics	9
RESEARCH QUESTIONS	10
ORGANIZATION OF THESIS	12
II. CONTRIBUTION OF BIODEGRADABLE PLASTIC MULCHES TO SOIL CARBON CYCLING	13
ABSTRACT	14
INTRODUCTION	15
Soil carbon cycling and biodegradable plastic mulching	15
BDMs as a carbon input into soil	18
Research objectives and hypotheses for soil carbon cycling	19
Approach	20
MATERIALS AND METHODS	21
Experimental design	21
Carbon pools	23
Statistical analysis	31
RESULTS	32
Carbon pools	32
BDMs as a carbon input into soil	40
Mulch microclimate in two locations	42
DISCUSSION	45
APPENDIX A	52
III. THE TEMPERATURE SENSITIVITY OF BIODEGRADABLE PLASTIC MULCHES TO MICROBIAL DECOMPOSITION	58
ABSTRACT	59
INTRODUCTION	60
Background	60
Research objectives and hypotheses	61
Approach	62
MATERIALS AND METHODS	62
Jar incubation set-up	62
Carbon pool measurements	66
Microbial measurements	67
Chemical properties of the mulches	69
Statistical analysis	70
RESULTS	70
Temperature effect on carbon dioxide evolution	70
Temperature effect on carbon storage	75
Temperature effect on microbial extracellular enzyme activity	77
Temperature effect on chemical properties of BDMs	77
DISCUSSION	83
APPENDIX B	89
IV. CONCLUSIONS AND RECOMMENDATIONS	91

REFERENCES

94

VITA

107

LIST OF TABLES

Table II-1. Soil properties in Spring 2015 prior to mulch treatments.....	22
Table II-2. The effects of mulch treatment and location (TN, WA) on the change in total organic carbon (TOC), permanganate oxidizable carbon (POXC), and peroxide-resistant C from Spring 2015 to Spring 2017.....	33
Table II-3. Statistics of the effect of mulch treatment on the change in particle size fraction carbon from Spring 2015 - Spring 2017.....	35
Table II-4. Effect of mulch treatment and date on C pools in TN and WA.....	37
Table II-5. Role of particulate organic matter (POM) in the coarse sand fraction and mulch material in POM fraction.....	39
Table II-6. Mean daily average soil moisture ($\text{cm}^3 \text{ cm}^{-3}$).....	43
Table II-7. Mean daily average soil temperature ($^{\circ}\text{C}$).....	43
Table II-8. Spring 2015 C pool size ($\mu\text{g C g}^{-1}$ dry soil) for total organic carbon, peroxide-resistant carbon, permanganate oxidizable carbon, and carbon content of the particle size fractions.....	52
Table II-9. Paired t-test statistics comparing mean values of total organic carbon, peroxide-resistant carbon, permanganate oxidizable carbon in Spring 2015 and Spring 2017	53
Table II-10. Spring 2017 C pool size ($\mu\text{g C g}^{-1}$ dry soil) for total organic carbon, peroxide-resistant carbon, permanganate oxidizable carbon, and carbon content of the particle size fractions.....	54
Table II-11. Paired t-test statistics comparing mean values of carbon content of the particle size fractions in TN in Spring 2015 and Spring 2017.....	55
Table II-12. Spring 2017 C pool size ($\mu\text{g C g}^{-1}$ dry soil) of extractable organic carbon, microbial biomass carbon, and particulate organic carbon.....	56
Table II-13. Calculated and theoretical mulch loading calculations after two years of mulch incorporation.....	57
Table III-1. Pre-incubation soil physical and chemical properties.....	64
Table III-2. The effects of mulch treatments (PLA/PHA, BioAgri, no-mulch, cellulose), temperature (10, 20, 30 $^{\circ}\text{C}$) and their interaction on CO_2 evolved, the change in carbon pools, and the change in microbial properties over the incubation.....	71
Table III-3. Theoretical % biodegradation of BDMs.....	73
Table III-4. PERMANOVA results of a pairwise comparison of the effects of mulch on the change in extracellular enzyme activity.....	79
Table III-5. Composition of PLA+PHA mulch pre- and post- incubation (Nuclear magnetic resonance spectroscopic analysis).....	82
Table III-6. Change in molecular weight-related properties of PLA+PHA mulch during incubation (Gel permeation chromatography analysis).....	82
Table III-7. Soil pH before and after incubation.....	89

LIST OF FIGURES

Figure II-1. Soil C cycling under mulching.	17
Figure II-2. Field design (randomized complete block) replicated in Mt. Vernon, WA and Knoxville, TN in 2015-2016.....	22
Figure II-3. Combined particle size density fraction (PSDF) scheme created for POM and microplastic isolation.	25
Figure II-4. Change in C pools from Spring 2015- Spring 2017 under mulch treatments.	33
Figure II-5. Change in particle size fraction (PSF) C after 2 years under mulch treatments.....	35
Figure II-6. Different C pools measured from Spring 2015 – Spring 2017.....	37
Figure II-7. Spring 2017 effect of mulch on MBC.....	39
Figure II-8. Mass of microplastics (1-2 mm) per area (kg mulch ha ⁻¹) in Spring 2017 after two years of mulch incorporation.	41
Figure II-9. Soil temperature during the growing season.....	44
Figure III-1. Cumulative carbon evolved (μg CO ₂ -C g ⁻¹ dry soil) from each mulch treatment throughout the incubation (16 weeks).....	73
Figure III-2. Temperature sensitivity (Q ₁₀) of cumulative CO ₂ evolved.	74
Figure III-3. The change in δ ¹³ C in soil over the incubation.	74
Figure III-4. The change in total organic carbon over the incubation.	76
Figure III-5. The change in extractable organic carbon over the incubation.	76
Figure III-6. The change in log bacterial copy number over the incubation.....	78
Figure III-7. The change in log fungal copy number over the incubation.	78
Figure III-8. PCA plot of the change in extracellular enzyme activity over the incubation.	79
Figure III-9. TGA thermograms (<i>left</i>) and DTGs (<i>right</i>) for PLA/PHA (<i>top</i>) and BioAgri (<i>bottom</i>)	80
Figure III-10. Visual degradation of PLA/PHA mulch fragments at 20°C (left) and 30°C (right)	89
Figure III-11. Change in extracellular enzyme activity over the incubation.	90

I. INTRODUCTION

Background

Plastic agricultural mulching

Plastic mulching in agricultural systems helps growers with preventing weeds, retaining moisture, moderating soil temperatures, reducing compaction, and reducing fertilizer leaching (Espí et al., 2006; Lamont, 1993). Combining plastic mulching with drip tape irrigation has been used for decades as a successful method for increasing vegetable yields (Kasirajan & Ngouajio, 2012; Lamont, 1993). While there are extensive benefits of mulching for crop production, there is currently no feasible recycling option for these plastics after their service life. Therefore, plastic mulch, typically made from polyethylene (PE), contributes to the 150 million tons of plastic (approximately half of solid plastics produced globally) that end up in landfills (Garcia & Robertson, 2017). Finding a solution is a crucial, time-sensitive sustainability issue because the use of plastic mulch is spreading globally (Transparency Markey Research, 2018).

Biodegradable plastic mulches as an alternative to plastic mulching

The best approach for addressing the plastic waste problem could be a mulch that is tilled into the soil after harvest to biodegrade (Moore & Wszelaki, 2016). Scientists and engineers are constantly improving biodegradable plastics for multiple uses like packaging, foodwares, and agriculture (Vroman & Tighzert, 2009). Biodegradable plastics are defined by American Society for Testing and Materials (ASTM) as “a degradable plastic in which the degradation results from the action of naturally occurring microorganisms such as bacteria, fungi, and algae” (ASTM International, 2012a). Technological advances in biopolymer production provide a promising alternative to the PE mulches through a new generation of biodegradable plastic mulches (BDMs) (Miles et al., 2012; Sintim & Flury, 2017). BDMs provide the same benefits as PE and can be tilled into the field after use where they biodegrade into microbial biomass, carbon dioxide (CO₂), water, and plastic degradation by-products (Bettas Ardisson et al., 2014; Brodhagen et al., 2015). However, important questions remain such as how biodegradation rates vary across agricultural systems and how BDMs affect soil health (Goldberger et al., 2015; Sintim et al., 2019; Sintim & Flury, 2017).

BDMs alter soil physical properties

The effects of agricultural mulching on soil moisture and temperature vary based on the physiochemical properties of the mulch film and the location of deployment. Mulching creates an artificial surface cover that alters heat and water fluxes thus affecting the soil moisture and temperature (Steinmetz et al., 2016). This soil cover lowers evaporation (Lamont, 1993) which leads to increased soil moisture in mulched plots compared to no-mulch plots for depths 0-15cm (Cuello et al., 2015), 0-20cm (Li et al., 2004), and 0-90cm (Wang et al., 2016c). Mulching also protects against extreme fluctuations in soil moisture by maintaining higher moisture levels in the driest conditions and preventing water-logged roots in the wettest conditions (Snyder et al., 2015). A major incentive of mulch use is the warming of the soil above bare ground temperatures (1.6-6.2°C (Wu et al., 2017), 2-3°C (Fan et al., 2017), 0.6-1.8°C (Li et al., 2004), 2°C (Cuello et al., 2015), 1.5°C (Wang et al., 2016a), 1.7 °C-2.8 °C (Lamont, 1993)). Dark colored mulches absorb heat and transfer it directly to the soil through contact (Lamont, 2005) thereby increasing soil temperatures and encouraging earlier plant development (Kasirajan & Ngouajio, 2012) and higher crops yields (Cuello et al., 2015; Diaz-Perez et al., 2005; Ghimire et al., 2018).

The effects of BDMs on soil moisture and temperature are less studied than the effects of PE mulches. Higher water vapor permeability of the BDM films compared to “impermeable” PE films (Bilck et al., 2010; Chandra & Rustgi, 1998; Domagała-Świątkiewicz & Siwek, 2013; Martin-Closas et al., 2008; Steinmetz et al., 2016) likely leads to slightly lower temperature increases in BDMs relative to PE (Moreno & Moreno, 2008). Location also impacts how BDMs affect soil physical properties. Different trends of average daily soil temperature were observed in BDM and bare ground treatments at three different locations across the United States (Li et al., 2014b). As expected, in WA and TX, BDMs (except cellulose) had higher temperatures than bare ground; while in TN, BDMs had lower temperatures than bare ground (Li et al., 2014b). The microclimate created by BDMs is similar to PE but varies based on the material properties of the mulch and the climate of use.

Effects of BDMs on soil health

Protecting soil health, defined as “the continued capacity of the soil to function as a vital living ecosystem that sustains plants, animals and humans” (USDA, 2012), is an integral part of farming in the 21st century (USDA, 2010). Common agricultural management techniques such as intensive tillage and monocropping have led to declines in soil health (Moebius-Clune et al., 2016). As the popularity of mulching rises as a management option to increase yields and reduce water inputs, researchers are trying to untangle the effects of mulching on soil health (Sintim & Flury, 2017; Steinmetz et al., 2016). A big concern is that growers could be trading short-term agronomic benefits like yield increases for long-term negative effects on soil health (Steinmetz et al., 2016). However, existing studies on the effects of BDMs on overall soil health have found minimal impacts (Li et al., 2014a; Sintim et al., 2019). The adoption of BDMs by growers is hindered by the lack of information on potential soil health impacts (Goldberger et al., 2015).

Soil carbon and soil health

Soil organic matter (SOM) is an important indicator of soil health because of the influence it has on chemical, biological, and physical properties of the soil (Haynes, 2005; Wander, 2004). SOM provides a source of inorganic nutrients for microorganisms along with improving the buffering ability, water retention, and structure of the soil (Baldock & Skjemstad, 2000; Haynes, 2005). Microorganisms consume soil organic carbon (SOC), the C portion of SOM, as a C-energy source and in doing so increase the mineralization rates of other nutrients in the soil. Therefore SOC is constantly being decomposed by microbes and must be replaced by plant inputs to maintain the C balance of the soil. When soils have the same amount of C accumulating as is decomposed, equilibrium is reached (Haynes, 2005; Paustian et al., 1997). The amount and type of SOC that remains in the soil depends heavily on the type of inputs (Buyanovsky & Wagner, 1997). As a result, SOC consists of many (C) compounds in that range in chemical composition and decomposability (Baldock & Skjemstad, 2000; Lehmann & Kleber, 2015). Once added to the soil, mean residence time (MRT) or turnover time (the inverse of MRT) can be used to characterize the decomposability of soil C (Davidson & Janssens, 2006). Soil C with a short turnover time (days to a few years) is relatively labile and includes compounds like proteins, nucleic acids and polysaccharides that are readily accessible to microorganisms (Krull et al., 2003; Wander, 2004). On an intermediate timescale (years to decades), soil C is likely physically

protected from decomposition in some way which promotes stabilization (Baldock & Skjemstad, 2000; Wander, 2004). The longest lived soil C (decades to millenia) contains more alkyl and aromatic compounds, as well as that SOC inaccessible to microbes (Jastrow et al., 2007; Krull et al., 2003; Wander, 2004).

Since turnover times are difficult to measure *in situ*, fractionation methods are used to categorize SOC into different pools based on their kinetic properties or more commonly their physical and/or chemical properties (Lützow et al., 2007; Rodeghiero et al., 2009). The kinetic approach uses pools defined by the MRT of SOC in soils (Davidson & Janssens, 2006). Different studies and models use different terms to describe the general categories based on MRT (e.g. active, intermediate and passive). The intermediate pool is usually the largest, most difficult fraction of SOC to study (Trumbore, 1997). The passive pool can have a long MRT because of physical protection, chemical protection, or the complex nature of the compounds (Davidson & Janssens, 2006). Chemical fractionation methods attempt to divide organic matter (OM) based on molecular structure and composition (Denef et al., 2009; Essington, 2015). Physical fractionation methods divide OM into soil particle size, aggregate size, or density fractions to describe where C is being stabilized and destabilized (Christensen, 1992; Collins et al., 1997). The most common physical separation methods are dry sieving, wet sieving, sedimentation, and density fractionations (Collins et al., 1997). Interpretations of soil C dynamics can be difficult due to heterogeneity within fractions (Lützow et al., 2007; Wander, 2004). In addition, the time scales of the factors that control soil C stabilization make studying C dynamics difficult because they range from rapidly reacting microbial communities to extremely slow mineral forming reactions (Trumbore, 1997). There are many ways to study SOM depending on the purpose of the study and usually a combination of fractions are used to describe the complete SOM dynamics of a system (Collins et al., 1997; Moni et al., 2012; Schroth et al., 2003).

BDMs affect soil carbon cycling

The insulating effect of mulching presents competing effects on soil C management: higher yields increasing above (Cuello et al., 2015; Ghimire et al., 2018) and below (Li et al., 2007; Wang et al., 2016b; Zhou et al., 2011) ground C inputs versus higher mineralization rates decreasing soil C (Steinmetz et al., 2016). After two to four years of mulch use, studies have

found decreases in SOC compared to no-mulch controls indicating increased C mineralization rates (Cuello et al., 2015; Li et al., 2004). However, this decrease in soil C can be the same as the increase in C inputs from higher biomass in mulch treatments leading to no net change in soil C (Li et al., 2004; Wang et al., 2016b; Zhang et al., 2017a; Zhang et al., 2017b). BDMs are composed of polymers with C backbones (Vroman & Tighzert, 2009) and have high C contents (~ 50%). Thus, they could potentially be an additional source of C when they are tilled into the soil (Bandopadhyay et al., 2018).

Mulching has mixed effects on labile C pools which are more sensitive than SOC to the effects of mulching on soil C (Luo et al., 2015). Increases in light fraction organic carbon (LFOC) have been reported at the soil surface after two years of mulching (Zhou et al., 2011) and below the surface (20-40 cm) after four years of mulching (Luo et al., 2015). In contrast, a decrease in LFOC of 4-5% after six years of mulching was reported (Wang et al., 2016b). Studies have reported gains in microbial biomass C (MBC) under mulch treatments compared to no-mulch treatments after two to four years (Li et al., 2004; Zhou et al., 2011). Further, using mulch for a longer period of time (30 days before sowing and continually for two years) increased MBC in the soil compared to the traditional timing from sowing to harvest (Liu et al., 2013). There was no effect on the accumulation of permanganate oxidizable C (POXC) after four seasons of plastic mulch use compared to no-mulch plots (Luo et al., 2015) nor under a non-flooded system with plastic mulch compared to a traditionally flooded rice/wheat system after ten years (Tian et al., 2013).

Compared to traditional PE plastic mulch, there are fewer studies on the effects of BDMs on soil C cycling. After 18 months, no differences in SOC or MBC were found under BDMs compared to a no-mulch control (Li et al., 2014a). In agreement, a recent study comparing four different BDMs found no significant differences in %OM between BDMs and a no-mulch control after two years (Sintim et al., 2019). After two years of mulch use, higher MBC was found in BDM and no-mulch treatments compared to PE (Moreno & Moreno, 2008). When interpreting the effects of mulching on soil C, it is important to note the environment of use because mulches are used in a variety of systems ranging from semi-arid to periodically flooded. Due to the variable

effects of mulching (PE and BDMs) on soil C stocks, more studies are needed to ensure no loss of C with this agricultural management technique.

BDM degradation

Three types of degradation affect agricultural plastics: photodegradation, thermo-oxidative degradation and biodegradation (Shah et al., 2008). Photodegradation and thermo-oxidative are part of the physiochemical weathering of BDMs, the extent of which depends on mulch type and local climatic conditions (Hayes et al., 2017). After tillage, biodegradation occurs when microorganisms break down BDM material as a source of energy and produce microbial biomass, CO₂, and water in the process (Kasirajan & Ngouajio, 2012). Microbes utilize BDM C compounds once they are depolymerized to low molecular weight compounds achieved through hydrolysis and enzymatic cleavage (Hayes et al., 2017; Saadi et al., 2013; Shah et al., 2008). Soil microbes already have the catabolic pathways required for the breakdown of biodegradable polymers using extracellular and/or intracellular enzymes (Barak et al., 1991; Kyrikou & Briassoulis, 2007).

The rate of BDM biodegradation in the soil depends on many factors, mainly intrinsic mulch properties and environmental conditions. The intrinsic mulch properties that affect biodegradation rates include the starting polymer feedstock and the final physiochemical properties of the mulch film such as the molecular weight, surface area, hydrophobicity and the manufacturing process (Barragan et al., 2016; Chandra & Rustgi, 1998; Kasirajan & Ngouajio, 2012; Shah et al., 2008; Tokiwa et al., 2009). Polyesters are the most popular biodegradable polymers because they have hydrolysable ester bonds (Albertsson & Hakkarainen, 2017; Kasirajan & Ngouajio, 2012). Many mulch films are blends of polymers. For example, PLA (polylactic acid) can be blended with PHA (polyhydroxyalkanoate), a microbial by-product, to make it less brittle (Hu et al., 2008). The environmental conditions that affect biodegradation rates of BDMs in soil include abiotic factors such as soil temperature and moisture along with biotic factors like the native soil organisms (Brodhagen et al., 2015; Krueger et al., 2015; Shah et al., 2008).

In particular, the role of temperature in the biodegradation of BDMs is of interest (Brodhagen et al., 2015). Soil temperature is one of the main factors affecting SOM decomposition because of its ability to regulate biological activity in the soil (Krull et al., 2003; Manzoni et al., 2012; Yuste et al., 2007). It is well known that biochemical reactions involved in the microbial decomposition of substrates are temperature dependent (Davidson & Janssens, 2006). However, the relationship between SOM decomposition and temperature can be complex because environmental factors like soil moisture and pH can have interacting effects. In addition, different microbes (mesophilic vs. thermophilic) and their enzymes are affected differently by temperature (Voroney & Heck, 2015). Enzymes and their targeted substrates each have inherent temperature sensitivities (Davidson & Janssens, 2006; Wallenstein et al., 2011). Substrate temperature sensitivities are based on molecular structure (Davidson & Janssens, 2006) with stable soil C substrates being more temperature sensitive than labile soil C substrates (Conant et al., 2011; Fierer et al., 2005; Mikan et al., 2002; Vanhala et al., 2007). In general, SOM decomposition will increase with increasing temperature with the exception of SOM physically protected from enzymes (Wallenstein et al., 2012). Since the microbes and enzymes involved in SOM decomposition overlap with BDM biodegradation, increased temperatures would be expected to also increase BDM biodegradation. The temperature sensitivity of the BDM polymer, soil microbial activity and soil enzymes will affect BDM biodegradation rates.

One accepted method for measuring the rate of biodegradation of BDMs is CO₂ evolution (El-Din Sharabi & Bartha, 1993; Ho & Pometto III, 1999; Shah et al., 2008). The American Society for Testing and Materials (ASTM, International) test D5988 can be used to estimate biodegradability of a plastic material in ambient, aerobic soils based on CO₂ evolved or biological oxygen demand. This method should not be used to make claims of a material being “biodegradable”, but the theoretical percent biodegradation (calculated using the net gaseous C) can be reported (ASTM International, 2012b). To address the lack of standards specifically for BDMs in soil, a recent standard was developed that can be used to describe a plastic mulch film as biodegradable in ambient soil. The standard, European Committee for Standardization (CEN) EN 17033 (2018) “Plastics-Biodegradable mulch films for use in agriculture and horticulture requirements and test methods”, requires greater than or equal to 90% biodegradation (as shown by CO₂ evolution using ISO 17566) in aerobic soil conditions within two years and additionally

requires tests for physical properties, heavy metals, and ecotoxicity (Dentzman & Hayes, 2019). More information is needed on the mechanisms of BDM biodegradation to help understand the currently unpredictable breakdown (Brodhagen et al., 2017; Goldberger et al., 2015).

BDMs as microplastics

Soil biodegradation of BDMs is a time-consuming process (months to years) and during the break-down of the material there is the possibility of introducing microplastics (< 5 mm) (Alimi et al., 2017) into the soil. Currently, the amount of BDM residues that remain in the soil is unclear (Brodhagen et al., 2017) and more specifically, it is unclear if residues exist as microplastics. Measuring microplastic accumulation is a key research interest due to potential negative effects of microplastics on terrestrial environments (Horton et al., 2017; Souza Machado et al., 2018a). Microplastics can adsorb chemicals (hydrophobic organic pollutants) like organochlorine pesticides and polycyclic aromatic hydrocarbons (PAHs) (Duis & Coors, 2016; Horton et al., 2017; Rillig, 2012; Rochman et al., 2013; Steinmetz et al., 2016). Along with concentrating chemicals, the plastics themselves can leach chemicals such as plasticizers (Horton et al., 2017; Souza Machado et al., 2018a). More direct effects of microplastics on soil biota include hindering plant growth (Qi et al., 2018) and accumulation in food chains when consumed (Rillig, 2012). In addition, multiple microplastic types (polyethylene, polyesters, and polyacrylic) have been shown to decrease soil bulk density (Souza Machado et al., 2018b). In general, much is unknown about the fate of microplastics in soils due to the difficulty of isolating them from a heterogeneous medium like soil (Bläsing & Amelung, 2018; Horton et al., 2017; Rillig, 2012). Some methods focus on identifying the presence of microplastics, while others quantify their size (Elert et al., 2017). The quantification of microplastics in soil is difficult because a wide range of methods are used and there are no standard units or definitions (Bläsing & Amelung, 2018). Common microplastic isolation methods include using dense liquids, filters, sieves, or visual sorting (Hidalgo-Ruiz et al., 2012; Wang & Wang, 2018). For separating conventional plastics from sediments, it is common to use water (1.0 g cm^{-3} (density at 25°C)) or salt water (1.2 g cm^{-3} (density at 25°C)) (Claessens et al., 2011; Hidalgo-Ruiz et al., 2012; Ng & Obbard, 2006). However, the hydrophilic nature of BDMs requires the use of a denser solution.

Chemical pre-treatments (NaOH, HCl and H₂O₂) can be used in microplastic isolation to digest organic material and allow for easier identification of microplastics (Cole et al., 2014; Nuelle et al., 2014). Enzyme digestion with proteinase-K, a broad spectrum protease, is also a successful method for isolating microparticles from seawater samples rich in marine biota (Cole et al., 2014; Zhao et al., 2015). However, chemical and enzymatic pre-treatments are not always ideal because SOM consists of such a large range of C compounds (Davidson & Janssens, 2006; Lehmann & Kleber, 2015) that plastics could also be affected by digestions targeting SOM (Bläsing & Amelung, 2018). In particular, BDM microplastics are composed of starch and cellulose polymers that overlap with the chemical composition of SOM so chemicals that destroy SOM likely would have an effect on BDM microplastics. Once microparticles are isolated, spectroscopic (Raman and Fourier transform infrared) methods can be used to confirm and differentiate different types of plastics (Cole et al., 2014; Elert et al., 2017).

Research questions

In climates around the world, plastic mulches have led to higher yields of specialty crops. To prevent the large amounts of plastic waste created by the traditionally used PE mulches, BDMs were created to be tilled into the soil at the end of the season. The long-term effects of mulching, in particular BDM use, on soil C stocks are relatively unknown. Some studies have demonstrated a loss in C from increased mineralization under mulching while others show the increased C inputs from higher yields can offset C losses. In addition, biodegradation rates of different BDMs in different climates are unknown. This study has two separate goals associated with the role of BDMs in soil C cycling. First, this study aims to assess the effects of BDMs on soil C cycling by measuring soil C pools and characterizing mulches as a C input. Second, this study aims to reveal the mechanisms of temperature induced biodegradation of BDMs with a focus on C accumulation and transport.

A field experiment was used to measure the effects of mulching on C dynamics with replicate field sites in Knoxville, TN and Mt. Vernon, WA. Multiple C pools were measured under different mulch treatments over two years (Spring 2015- Spring 2017). To better understand the effect of temperature alone on biodegradation, a lab incubation study was designed. The time-

course of biodegradation was tracked using carbon dioxide evolution along with more indirect measures of biodegradation such as soil C storage pools, soil microbial properties, and chemical properties of the mulches.

Goal 1. Assess how BDMs affect soil C pool distributions after two years of deployment and incorporation into the soil using a field study at two diverse geographical locations. Goal 1 is divided into three specific objectives with corresponding hypotheses (H).

1. Compare the effects of different mulching treatments (BDMs versus three controls: PE, cellulosic (paper) mulch, and no-mulch) on soil C pools over two years.

H_{1.1} Higher soil temperatures and moistures under the BDMs and PE compared to the no-mulch treatment will lead to lower soil C stocks after two years.

H_{1.2} For the faster turnover C pools, BDMs will have lower C compared to no-mulch treatments in the fall due to higher temperatures increasing mineralization throughout the growing season under the mulch treatments.

2. Analyze changes in C pools across two diverse geographical locations to see if BDMs affect soil C differently in different climates.

H_{2.1} The soil warming effects of PE and BDMs compared to the no-mulch control will be more apparent in the cooler WA climate. This more extreme warming will lead to higher declines in soil C pools under PE and BDMs compared to no-mulch treatments in WA.

3. Characterize the BDM C input by quantifying the amount of C tilled into the soil and the amount of BDM microplastics.

H_{3.1} BDMs are composed of C polymers; so, it is expected that once tilled into the soil, BDM C will contribute a similar amount of C as crop residues annually. BDMs will have significantly more microplastics than PE and no-mulch treatments.

Goal 2. Deeply understand the mechanisms associated with BDM biodegradation at different temperatures using a lab experiment.

1. Measure the temperature sensitivity of the decomposition of BDMs with a traditional measure of biodegradation in addition to soil C storage pools, soil microbial properties and chemical properties of the mulches.

H_{1.1} Higher temperatures will increase microbial activity and lead to increased BDM material utilization. We expect the addition of BDMs to increase the temperature sensitivity of the soil-mulch system. In addition, higher temperatures and higher microbial activity will lead to higher extracellular enzyme activity and a higher bacterial and fungal abundance. We predict a decline in carbon (C) storage pools with higher temperatures and higher microbial activity.

Organization of thesis

This multi-part thesis will include an introduction (this chapter: Chapter I), two chapters written for future publication (Chapter II and III) and conclusions (Chapter IV).

II. CONTRIBUTION OF BIODEGRADABLE PLASTIC MULCHES TO SOIL CARBON CYCLING

A version of this chapter is being prepared for publication in:

Marie E. English, Sean M. Schaeffer. [More authors to be added] “Contribution of biodegradable plastic mulches to soil carbon cycling”. *Peer J*.

Abstract

The benefits of using polyethylene (PE) plastic mulches in crop production, lengthening the growing season and preventing weeds, must be weighed against their contribution to the overuse of plastics in agriculture. Biodegradable plastic mulches (BDMs) have become a viable alternative because they provide the same benefits as PE mulches. BDMs do not result in plastic waste because they are tilled into the soil at the end of the season to degrade into carbon dioxide, water, microbial biomass, and plastic degradation by-products. However, little is known about their long-term impacts on soil carbon dynamics. BDMs change soil moisture and temperature, which affects soil organic matter formation and potentially alters C cycling. We used two years of soil chemistry and microclimate data (Spring 2015- Spring 2017) under pie pumpkin production to assess changes in soil C cycling for each of 7 treatments (four BDMs and three controls: PE, cellulosic (paper) mulch, and no-mulch) at two diverse climates, Knoxville, TN and Mt. Vernon, WA. Measured pools of C ranged in turnover time and included total organic carbon (TOC), extractable organic carbon (EOC), microbial biomass carbon (MBC), permanganate-oxidizable (POXC), respired CO₂, particulate organic carbon (POC), and carbon within particle size fractions. PE was the only mulch treatment with possible negative effects on soil C. In TN, PE had lower respiration rates in Fall of 2016 and lower TOC in the silt and clay fraction compared to the bare soil treatment. In WA, PE was the only mulch treatment with a decline in TOC after two years. These differences in C pools between PE and no-mulch after two years are likely caused by higher moisture and temperature under PE. BDMs had no negative effects on C pools after two years. A method was developed to isolate and measure BDM microplastics (1-2 mm) that provided evidence of BDM microplastic accumulation amounting to a small percent of the functionally important POC pool. In order to ensure BDMs are a good alternative to PE, more research is needed on the effects of BDMs on soil C pools and BDM microplastic residence times.

Introduction

Soil carbon cycling and biodegradable plastic mulching

Soil organic matter (SOM), a key aspect of soil health, provides essential soil functions like improving the soil structure (Baldock & Skjemstad, 2000) and acting as a source of inorganic nutrients for microorganisms (Haynes, 2005). Microorganisms consume soil organic carbon (SOC) as a carbon-energy source and in doing so increase the mineralization rates of other nutrients in the soil. Given the extensive benefits for soil health, maintaining or increasing SOC levels are desirable soil management goals (Fine et al., 2017; Moebius-Clune et al., 2016). The amount of SOC is a function of carbon (C) inputs and decomposition rates (Lützow et al., 2006; Schroth et al., 2003). The stability of SOC in the soil helps prevent rapid decomposition of this important nutrient source and is controlled by three mechanisms: stabilization by intrinsic recalcitrance of the C compound, inaccessibility to microbes and enzymes, and interactions with minerals (Lützow et al., 2006; Sollins et al., 1996).

Plastic mulches are used in agriculture to reduce weed pressure and protect crops from extreme temperatures and drought conditions (Hayes et al., 2017; Lamont, 1993, 2005). Most plastic mulch is made from polyethylene (PE) and must be removed from the field after use; however, biodegradable plastic mulches (BDMs) can be tilled directly into the soil. A comprehensive review on agricultural mulching concluded that the predominant mulching conditions (increased soil moistures and temperatures) likely increase mineralization and favor the formation of labile C while destabilizing more intermediate and passive C (Steinmetz et al., 2016). As the use of plastic mulch (PE and BDMs) expands, it is important to understand the controls on soil C decomposition rates, C inputs and C stabilization mechanisms in order to maintain the balance of C in the soil. The main ways mulching (PE or BDMs) could affect soil C cycling include increasing mineralization rates of soil C (Li et al., 2014a; Li et al., 2007; Moreno & Moreno, 2008; Wang et al., 2016b) and increasing plant biomass and thus C inputs (Cuello et al., 2015; Ghimire et al., 2018; Li et al., 2007; Wang et al., 2016b; Zhou et al., 2011). Studies have shown the losses from increased mineralization under mulching can be balanced by the increased inputs (Li et al., 2004; Wang et al., 2016b; Zhang et al., 2017a; Zhang et al., 2017b). Unlike PE which is not tilled into the soil, BDMs are a direct addition of C when tilled into the soil

(Bandopadhyay et al., 2018). The amount and fate of C added from the tillage of BDMs into the soil has not been well characterized.

Using multiple C pool measurements together can serve as an early indicator of the effects of agricultural management practices (Gregorich et al., 1994). The C pools identified as important in the biogeochemical cycling of C associated with plastic mulching are illustrated in Figure II-1. Carbon pools are typically characterized by turnover times which describe the rate C leaves the soil (Davidson & Janssens, 2006). Soil C with a short turnover time (days to a few years) is relatively labile and includes compounds like proteins, nucleic acids and polysaccharides that are readily accessible to microorganisms (Krull et al., 2003; Wander, 2004). On an intermediate timescale (years to decades), soil C is likely physically protected from decomposition in some way which promotes stabilization (Baldock & Skjemstad, 2000; Wander, 2004). The longest lived soil C (decades to millenia) contains more alkyl and aromatic compounds, as well as that SOC inaccessible to microbes (Jastrow et al., 2007; Krull et al., 2003; Wander, 2004). Total organic C (TOC) is a sum of all of the organic C in the soil and includes many compounds ranging in stability and turnover times (Baldock & Skjemstad, 2000; Lehmann & Kleber, 2015). The peroxide-resistant fraction mimics oxidative processes used in microbial decomposition in order to produce a very stable C pool that represents C not available to microorganisms (Favilli et al., 2008; Jagadamma et al., 2010; Lützow et al., 2007).

Permanganate oxidizable C (POXC) is an intermediate C pool that is typically defined as having a turnover time of <10 years (Culman et al., 2012; Lützow et al., 2007). POXC is useful because it represents microbial available C (Blair et al., 1995) and is sensitive to changes in soil management (Blair et al., 1995; Culman et al., 2012). More labile than POXC, extractable organic C (EOC) (Culman et al., 2012; Tirol-Padre & Ladha, 2004) is a small percentage of total SOC (0.05-0.4%) (Haynes, 2005) but an important source of nutrients for microbes (Sanderman & Amundson, 2008). EOC consists of soluble root exudates, plant litter leachate and native SOM smaller than 0.45 μm (Blankinship & Schimel, 2018; Haynes, 2005; Neff & Asner, 2001; Silveira, 2005). Microbial biomass C (MBC) is considered an important labile and functional fraction for its role in decomposition and mineralization of nutrients (Gonzalez-Quinones et al., 2011). The amount of CO_2 that leaves the soil (CO_2 respired) is a biological indicator that

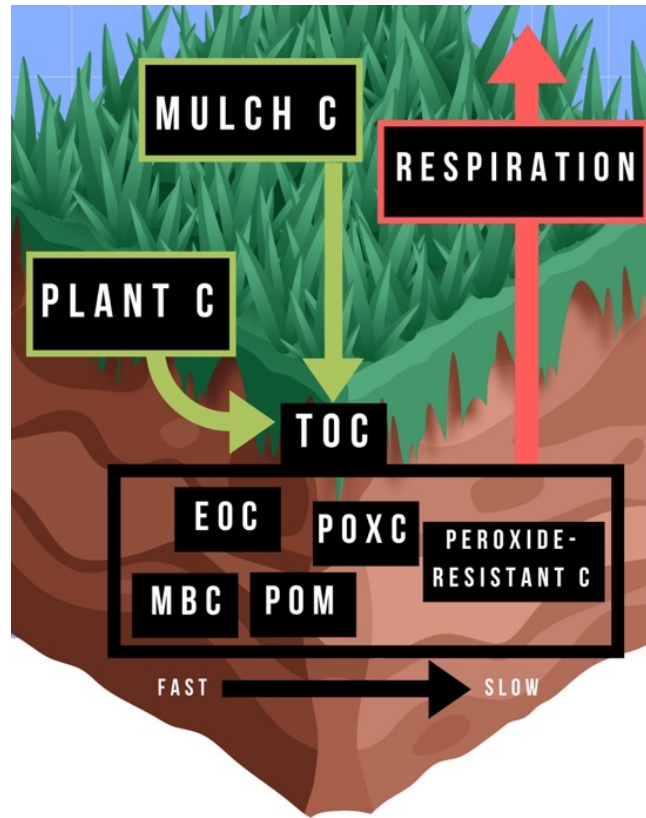


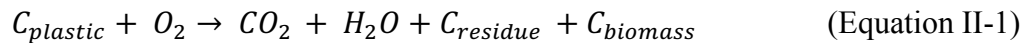
Figure II-1. Soil C cycling under mulching. Inputs include plant (not measured) and mulch C which are decomposed into different storage pools. Total organic C (TOC) is comprised of many different pools of C. In approximate order of turnover time, from faster to slower, these C pools include microbial biomass C (MBC), extractable organic C (EOC), particulate organic C (POC), permanganate oxidizable C (POXC), and peroxide-resistant C. These storage pools are then mineralized by microbes into CO₂ during respiration. *Figure created by Marie English using Canva.com*

represents aerobic microbial activity and has been shown to be useful for observing changes in soil management (Culman et al., 2013; Haney et al., 2008b). Physically fractionating soils into particle size fractions (PSF) such as sand (53- 2000 μm), silt (0.02-53 μm), and clay (<0.02 μm) can provide information on the relationship between SOM and primary particles (Schroth et al., 2003) by assuming the size of the particles matches the decomposition state (Moni et al., 2012). Carbon in the sand fraction is considered more labile, undecomposed plant litter, compared to the older, more microbially-derived C in the clay fraction (Liang et al., 2017; Lützow et al., 2007). Density fractionations often follow PSF to isolate the light fraction referred to as particulate organic matter (POM) (Moni et al., 2012). POM contains mostly plant residues, roots, and organism residues (Schroth et al., 2003) and is sensitive to land-use and management changes (Christensen, 1992; Schroth et al., 2003). Particulate organic C (POC) is the C portion of POM.

BDMs as a carbon input into soil

The amount of BDM C tilled into the soil, to our knowledge, has not been previously quantified or compared to other C pools. Due to the fact that BDMs are designed to break down in soil, and that thousands of C compounds already exist in soil (Davidson & Janssens, 2006), there is potentially some overlap in polymeric C used in BDMs and C compounds found naturally in SOM. For example, starch, cellulose, polylactic acid (PLA), and polyhydroxyalkanoate (PHA) polymers used in BDMs, occur in nature as well (Chandra & Rustgi, 1998). Because BDM feedstocks are chemically similar to SOM, BDMs decompose in a similar fashion to plant inputs (Eq. II-1).

Aerobic biodegradation of BDMs (Bettas Ardisson et al., 2014):



where: C_{plastic} = C from the plastic material

C_{residue} = By-products of plastic degradation

C_{biomass} = Microbial biomass C

O_2 = Oxygen

CO_2 = Carbon dioxide

H_2O = Water

It is unclear how much BDM C is respired as CO₂, how much enters soil C stocks and how much remains undecomposed (Bettas Ardisson et al., 2014; Chinaglia et al., 2018). Influences on BDM C fate include the composition of the material as well as the environment where it is deployed. Soil moisture, temperature, and the stoichiometry of the soil (C:N) can alter SOM decomposition rates (Davidson & Janssens, 2006; Paustian et al., 1997) and therefore the fates of BDM C.

As BDMs biodegrade at varying rates based on their composition and the environment, microplastics (<5 mm fragments) (Alimi et al., 2017) are likely introduced into the soil environment. BDMs are designed to biodegrade > 90 % after two years; however, in the short-term they could pose the same problems as other microplastics such as adsorbing chemicals (hydrophobic organic pollutants like agrochemicals) (Duis & Coors, 2016; Horton et al., 2017; Rillig, 2012; Steinmetz et al., 2016), leaching plasticizers (Horton et al., 2017; Souza Machado et al., 2018a), and hindering plant growth (Qi et al., 2018). Unfortunately, the abundance of microplastics in terrestrial environments has not been well characterized due to the difficulty of separating OM and plastics (Bläsing & Amelung, 2018; Horton et al., 2017; Rillig, 2012). This leads to gaps in knowledge on the fate of plastics in soils (Bläsing & Amelung, 2018; Steinmetz et al., 2016). One challenge with detection of BDM microplastics is that chemical pre-treatments (NaOH, HCl and H₂O₂ (Nuelle et al., 2014)) that remove OM to allow for easier identification of microplastics, would likely affect the BDM microplastics due to overlaps in chemical composition of the polymers. More information is needed on the fate of decomposed and undecomposed BDM C and promising technologies exist to track this C source.

Research objectives and hypotheses for soil carbon cycling

A field experiment was used to measure the effects of mulching on C dynamics with replicate field sites in Knoxville, TN and Mt. Vernon, WA. Multiple C pools were measured under different mulch treatments over two years (Spring 2015- Spring 2017). The main goal of the research described in this chapter is to assess how BDMs affect soil C pool distributions after two years of deployment and incorporation into the soil. This can be divided into three specific objectives with corresponding hypotheses (H).

1. Compare the effects of different mulching treatments (BDMs versus three controls: PE, cellulosic (paper) mulch, and no-mulch) on soil C pools over two years.

- H_{1.1} Higher soil temperatures and moistures under the BDMs and PE compared to the no-mulch treatment will lead to lower soil C stocks after two years.
- H_{1.2} For the faster turnover C pools, BDMs will have lower C compared to no-mulch treatments in the fall due to higher temperatures increasing mineralization throughout the growing season under the mulch treatments.
2. Analyze changes in C pools across two diverse geographical locations to see if BDMs affect soil C differently in different climates.
- H_{2.1} The soil warming effects of PE and BDMs compared to the no-mulch control will be more apparent in the cooler WA climate. This more extreme warming will lead to higher declines in soil C pools under PE and BDMs compared to no-mulch treatments in WA.
3. Characterize the BDM C input by quantifying the amount of C tilled into the soil and the amount of BDM microplastics.
- H_{3.1} BDMs are composed of C polymers; so, it is expected that once tilled into the soil, BDM C will contribute a similar amount of C as crop residues annually. BDMs will have significantly more microplastics than PE and no-mulch treatments.

Approach

To test how C cycling is affected by different mulch products, an in-depth analysis of C pools over two years was conducted. We focused on net changes in soil C caused by the combination of C inputs (crop residues and tilled in BDMs), C outputs from soil respiration and the internal cycling of soil C. This study was replicated in Knoxville, TN and Mount Vernon, WA, locations with different climates and soil types, to assess interactions between the effects of climate and mulch treatments on C cycling. Four BDMs were compared to three controls: PE, cellulosic (paper) mulch, and bare soil. Soil samples were collected bi-annually for two years (2015-2017) before planting (May) and after harvest (September-October). Soil moisture and temperature sensors were monitored throughout the season along with a meteorological station to help compare differences in location climate. A unique microplastic isolation method for soil systems was designed to isolate BDMs (1-2 mm) remaining in soil.

Materials and methods

Experimental design

Site description and mulch materials

This study was replicated in two locations, the University of Tennessee East Tennessee Research and Education Center (Plant Science Unit) in Knoxville, TN and the Washington State University Northwestern Washington Research and Extension Center at Mount Vernon in Mount Vernon, WA, with different climates and soil types (Table II-1). To test the effects of mulching on soil C cycling, three controls were compared to four BDMs. The controls included a bare ground negative control (no-mulch), a fully biodegradable mulch positive control made from cellulose called WeedGuardPlus (sunshine paper, Aurora, CO, USA), and a control of non-biodegradable polyethylene (PE) plastic mulch (Filmtech, Allentown, PA). The BDMs included BioAgri (BioBag Americas, Dunevin, FL, USA) which is a starch/ polyester blend made from feedstock Mater-Bi ® (grade M2351; starch/ polyester (PBAT) blend), Naturecycle (Custom Bioplastics, Burlington, WA) which is a starch/ polyester blend, Organix (Organix Solutions, Bloomington, MN) which is made from feedstock BASF ecovio® (grade M2351) (PBAT + PLA) and PLA/ PHA which is an experimental product made from polylactic acid/ polyhydroxyalkanoate (Ingeo® PLA / Mirel™ amorphous PHA). The field and research design of this experiment was a randomized complete block design (RCBD)-with split plot and repeated measures (Figure II-2). Mulches were laid on raised beds (15–20 cm high, 0.8m wide, 11m long). Sampling took place from Spring 2015 to Spring 2017. Pie pumpkins (*Cucurbita pepo* L) were grown in 2015 and 2016. Complete details on fertilization and field management can be found in Ghimire et al. (2018).

Soil moisture and temperature

Soil moisture and temperature sensors (5TM, Decagon Devices, Inc., Pullman, Washington, USA) were installed at two depths (10 and 20 cm) to monitor the microclimates of one replication of each treatment. The volumetric water content (VWC) and temperature (°C) were recorded hourly by dataloggers (EM50G, Decagon Devices, Inc.).

Table II-1. Soil properties in Spring 2015 prior to mulch treatments

Soil property	Knoxville	Mount Vernon
Soil type	Typic Hapludult	Typic Fluvaquent
Sand ^a	59.9%	14.2%
Silt ^a	23.5%	69.8%
Clay ^a	16.6%	16.0%
Bulk density (g cm ⁻³) ^b	1.41	1.31
pH ^b	6.03	6.24
CEC (cmol _c kg ⁻¹) ^b	7.23	9.19
Nitrate-N (mg kg ⁻¹) ^b	20.9	4.49
P (bray method) (mg kg ⁻¹) ^b	72.6	77.4
Organic matter % ^b	1.43	2.36

^aValues from Sintim et al. (2019)

^bMeasurements are mean values of all plots across the field in Spring 2015 prior to treatment

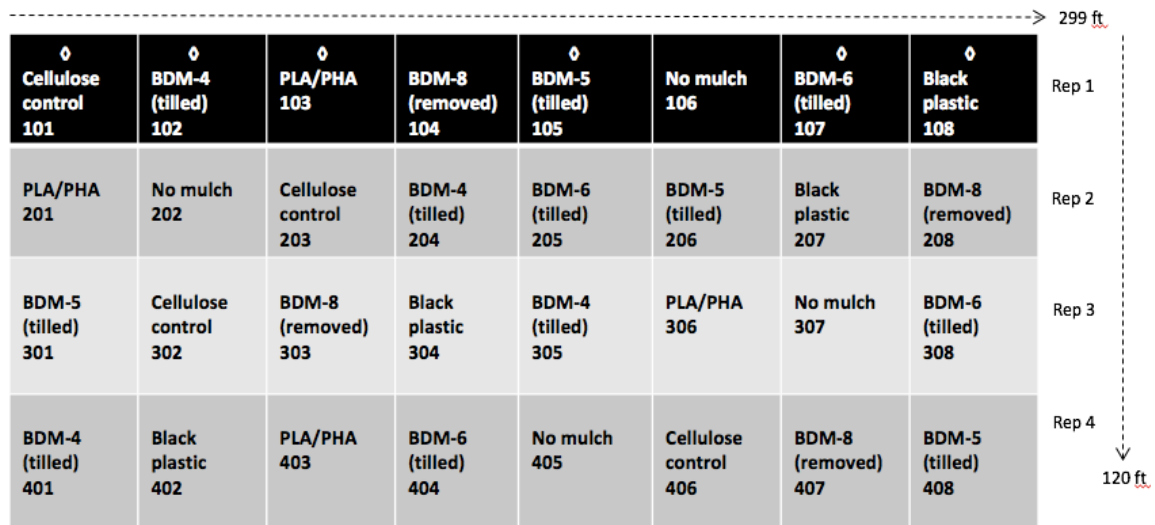


Figure II-2. Field design (randomized complete block) replicated in Mt. Vernon, WA and Knoxville, TN in 2015-2016. Abbreviations of mulch treatments: BDM-4 (BioAgri), BDM-5 (Naturecycle), BDM-6 (Organix), BDM-8 (BioAgri, removed from the field).

Soil sampling

Soil samples were collected bi-annually for two years (Spring 2015, Fall 2015, Spring 2016, Fall 2016, Spring 2017) before planting (May) and after harvest (September/ October). Multiple soil samples were collected per plot using soil cores (0-10 cm) and these subsamples were combined by hand to create a composite sample. Samples were processed (air-dried or frozen) within a week of collection and stored at 4°C before analysis of EOC/MBC.

Carbon pools

Total carbon content and isotopic ratio

Total C content (% C) and $\delta^{13}\text{C}$ of soil were measured by weighing out 80 mg of dried (105 °C), milled soil and analyzing it through a combustion module (CM-CRDS, Costech Analytical Tech Inc. Valencia, CA, USA) coupled to a Picarro cavity ring-down spectrometer (CRDS, Picarro G2121-I, Picarro Inc., Santa Clara, CA, USA). With each analysis, acetanilide and sugar standards were run to create a standard curve. The isotopic ratio (R_{sample}) which is the ratio of ^{13}C to ^{12}C in a sample was converted to units relative to the international standard (Vienna PeeDee Belamnite) (Eq. II-2).

Converting ^{13}C stable isotope ratio into units of $\delta^{13}\text{C}$:

$$\delta^{13}\text{C} = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - (1) \right) * (1000) \quad (\text{Equation II-2})$$

where: R_{sample} = Isotopic ratio ($^{13}\text{C}/^{12}\text{C}$) of the sample

R_{standard} = Isotopic ratio ($^{13}\text{C}/^{12}\text{C}$) of the Vienna PeeDee Belamnite standard

Permanganate oxidizable carbon

Permanganate oxidizable C (POXC) was measured using the methods of Weil et al. (2003) and adapted herein for use with a plate reader. In a 50 mL tube, 2.5 g of 2 mm-sieved soil (2 analytical reps of each soil), 18 mL of deionized (DI) water and 2 mL of 0.2 M KMnO_4 solution were mixed together and shaken at 120 rpm for 2 minutes on a shaker. Samples settled for 10 minutes and then 0.5 mL of sample was mixed with 49.5 mL of DI water. A 96-well plate was used to read 300 μL of each sample (3 replicates) on the plate reader. The color loss of solution is

proportional to the oxidation of labile C. The sample absorbance at 550 nm wavelength was measured using a plate reader and calibrated against known standards of differing concentrations. POXC (mg kg⁻¹ dry soil) was calculated (Eq. II-3).

$$\text{POXC} = \left(0.02 \frac{\text{mol}}{\text{L}} - (a + b * \text{abs}) \right) * \left(9000 \frac{\text{mg C}}{\text{mol}} \right) * \left(\frac{V_s}{M_s} \right) \quad (\text{Equation II-3})$$

where: a = y-int of standard curve

b = slope of standard curve of known concentrations of KMnO₄

abs = absorbance (550 nm) of samples

V_s = volume of solution (0.02 L)

M_s = mass of soil (0.0025 kg)

Peroxide-resistant carbon

The method used to isolate peroxide-resistant C was adapted from Jagadamma et al. (2010). Briefly, soils were oven dried (60 °C) and 1 g subsamples were placed into 50 mL centrifuge tubes. The soil was wetted with 10 mL of DI water for 10 min. Then 30 mL of 10 % (v/v) H₂O₂ was added to the tube and mixed. The tubes were stored at 50°C for 2-3 days or until frothing was complete. After cooling, samples were centrifuged (2500 xg for 15 minutes) and the supernatant discarded. This oxidation was repeated two additional times to ensure complete oxidation. Soils were washed (DI water/ centrifuged x 3) and then oven-dried at 60°C for 24 hours. Samples were sent off for analysis at the UT Department of Earth and Planetary sciences stable isotope facility for %C using a Costech EA (Costech Elemental Analyzer ECS4010). The values were reported per g dry soil (Eq. II-4).

Unit conversion of peroxide-resistant C to g dry soil:

$$\frac{\mu\text{g resistant C}}{\text{g dry soil}} = \frac{\mu\text{g resistant C}}{\text{g resistant soil}} \left(\frac{\text{g resistant soil}}{\text{g dry soil}} \right) \quad (\text{Equation II-4})$$

Particle size fractions

Air-dried soil (50 g) (Jiménez et al., 2011) was shaken overnight with glass beads adapted from methods in Moni et al. (2012) and Balesdent et al. (1998) (Figure II- 3). Moderate dispersion

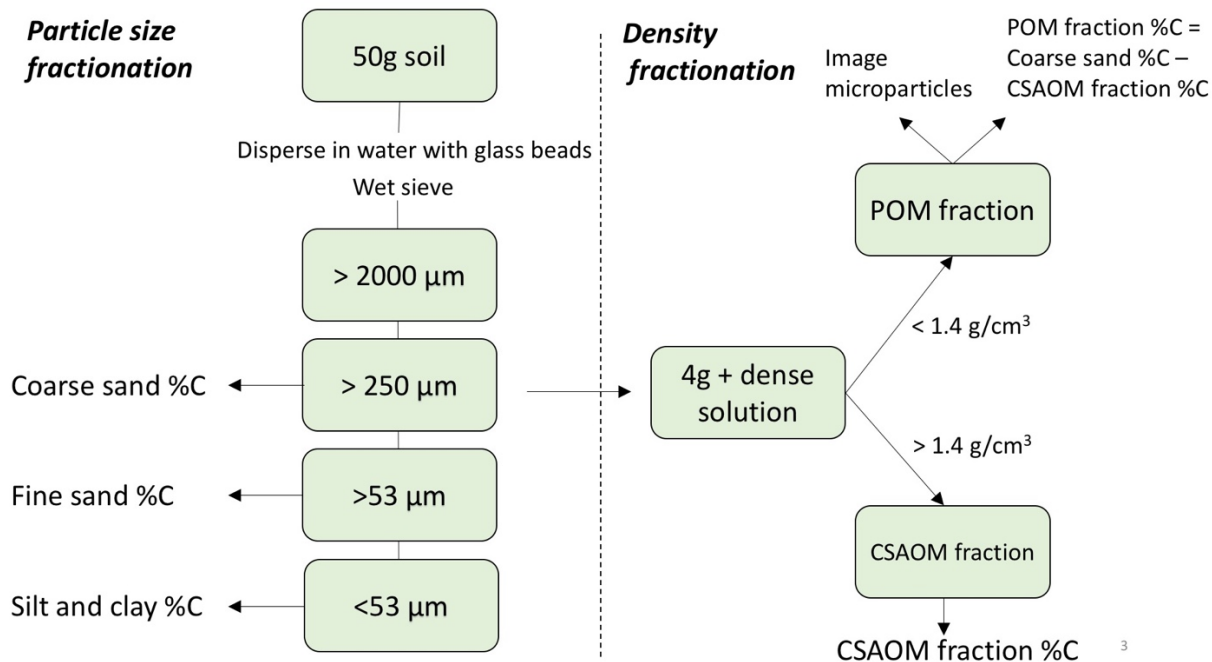


Figure II-3. Combined particle size density fraction (PSDF) scheme created for POM and microplastic isolation. The fraction lighter than 1.4 g cm^{-3} is referred to as POM (particulate organic matter) and that heavier than 1.4 g cm^{-3} is referred to as CSAOM (coarse sand associated organic matter). *Figure created by Marie English using Powerpoint.*

(140 RPM) was used to break up the aggregates without destroying POM or BDMs (Schroth et al., 2003). After dispersion, soil was wet sieved (Six et al., 2000) into particle size fractions 250 μm - 2000 μm (coarse sand), 53 μm - 250 μm (fine sand), and <53 μm (silt and clay) (Balesdent et al., 1998; Moni et al., 2012). Soils in the coarse sand and fine sand fractions were dried on the sieve at 60°C and then transferred into metal tins to dry overnight at 105°C. To settle the silt and clay (StC) fraction (<53 μm), 0.5 M CaCl_2 was added to settling bins (Balesdent et al., 1998; Jiménez et al., 2011). After 1-2 days, the plug was released, supernatant poured off and the sediment transferred to conical tubes to be rinsed three times with DI water and centrifuged (15 min at 2500 x g). The rinsed sediments were poured into tins and dried at 105°C for C analysis. Total organic C was measured on each fraction using the Picarro CRDS. For the coarse sand fractions, a coffee grinder was used to homogenize the coarse fraction before C analysis. Each fraction was converted from units per g fraction to per g dry soil using the total g dry soil recovered for each sample (Eq. II-5).

Unit conversion of PSF C concentrations:

$$\frac{\mu\text{g C}}{\text{g dry soil}} = \frac{\mu\text{g C}}{\text{g fraction}} \left(\frac{\text{g fraction}}{\text{g dry soil}} \right) \quad (\text{Equation II-5})$$

24-hour respiration

Respiration was measured as $\text{CO}_2\text{-C}$ ($\mu\text{g CO}_2\text{-C g}^{-1}$ dry soil day^{-1}) released from the soil in a 24-hr time period. Field moist soil (50 g) was placed into a 500 mL mason jar equipped with a septum after removing roots and BDM particles by hand. Headspace CO_2 concentration was measured by removing 0.5 mL gas aliquots using a syringe (2.5 mL). An infrared gas analyzer, LI-COR, 820 (LI-COR Biosciences, NE, USA) was used to measure the CO_2 concentration of the headspace air. The evolution of CO_2 was measured after 24 hours and used to calculate the 24-hr respiration rate. A standard curve of known CO_2 concentration standards was created in order to calculate $\text{CO}_2\text{-C}$ evolved ($\mu\text{g CO}_2\text{-C g}^{-1}$ dry soil). The ideal gas law was used to calculate the respiration rate (C_{24}) (Eq. II-6: II-8).

Respiration calculations:

$$C_{24} = (C_s(t=24) - C_s(t=0)) \quad (\text{Equation II-6})$$

$$C_s = (C_m * n * M_w) / M_s \quad (\text{Equation II-7})$$

$$n = PV/RT \quad (\text{Equation II-8})$$

where: C_{24} = $\mu\text{g CO}_2\text{-C g}^{-1}$ dry soil day^{-1}

C_m = CO_2 ppm

C_s = $\mu\text{g CO}_2\text{-C g}^{-1}$ dry soil

n = Number of moles in the headspace

R = The universal ideal gas law constant (8.314 L-kPa/mol-K)

T = Temperature in Kelvin (299.15)

P = Site pressure (98 kPa for Knoxville, TN)

V = Volume of headspace at 299.15 K

M_w = Molecular weight of $\text{CO}_2\text{-C}$ 12g C mol^{-1} C

M_s = Mass of soil in the jar (g dry soil)

Extractable organic carbon and microbial biomass carbon

For this study, extractable organic C was defined as organic C extractable in 0.5 M K_2SO_4 (aq.). Microbial biomass C was measured following the slurry/simultaneous chloroform extraction method (sCFE) (Schaeffer et al., 2017). Two subsamples of soil were used to measure EOC and MBC. Each sample of soil (10g, field moist) was shaken with 40 mL 0.5 M K_2SO_4 and 0.5 mL EtOH free chloroform was added to one of the two samples to fumigate. MBC was calculated as the difference between the fumigated and un-fumigated samples (Eq. II-11). Fumigated and un-fumigated extracts were frozen for analysis by persulfate digestion. Briefly, 2 mL sodium persulfate was added to 2 mL of sample in a 10 mL glass vial with a septa cap and metal crimp seal and was oxidized overnight at 80 °C (Doyle et al., 2004; Hood-Nowotny et al., 2010). After one hour of cooling, CO_2 concentration in the headspace was measured using a LICOR 820 infrared gas analyzer. To determine how much EOC was recovered from the reaction, a standard curve of known concentrations of KHP (potassium hydrogen phthalate) was measured along with the samples. A blank fumigated and un-fumigated sample were subtracted from the respective measured values as well. The extraction of MBC using the chloroform fumigation methods is not

complete; so, a correction factor (K_{EC}) was used to estimate total MBC from the extracted value (Eq. II-9: II:11). Ross (1990) suggests a common K_{EC} of 0.33 to provide a first estimate of soil microbial biomass.

EOC and MBC determination:

$$C_{KHP} = (C_{CO2} * b) + a \quad \text{(Equation II-9)}$$

$$C_{EOC} = C_{unfum} = (C_{KHP} * V) / M_s \quad \text{(Equation II-10)}$$

$$C_{MBC} = (C_{fum} - C_{unfum}) / K_{EC} \quad \text{(Equation II-11)}$$

where: C_{CO2} = CO₂ ppm (measured on IRGA)

b = slope of KHP standard curve

a = y-intercept of KHP standard curve

V = Volume of extractant (0.04 L)

M_s = Mass of soil extracted (g dry soil)

C_{KHP} = C ppm in vial

$C_{EOC} = C_{unfum}$ = mg C g⁻¹ dry soil

C_{fum} = mg C g⁻¹ dry soil

C_{MBC} = mg C g⁻¹ dry soil

K_{EC} = Extraction correction factor (0.33)

Density fractionation

POM can be isolated from the 53-2000 µm sand fraction or the coarse sand fraction 250-2000 µm (Meijboom, 1995; Schroth et al., 2003). To separate POM in this study, a density fractionation was conducted on the coarse sand fraction. A dense liquid was used to separate the coarse sand fraction into two density fractions, the lighter POM (<1.4 g cm⁻³) and the heavier CSAOM (Coarse sand associated organic matter) (>1.4 g cm⁻³) fraction (Figure II-3). Ludox™ TM-50 colloidal silica (density = 1.4 g cm⁻³ at 25°C) was used because it is the highest density silica solution available (50% wt. silica suspension in water). This density fractionation started with 4 g of soil from the coarse sand fraction. After adding Ludox™ to the soil, a plastic rod was used to briefly stir and suspend soil in the solution for 10 seconds before letting the light (<1.4 g cm⁻³), and heavy fractions (>1.4 g cm⁻³) separate for 3 minutes (Magid et al., 1996; Meijboom,

1995). After decanting the light fraction, fresh Ludox™ was added and the process repeated to ensure all POM fraction particles were extracted. The POM fraction was then rinsed over a sieve with DI water buffered to meet the pH of the Ludox™ solution to prevent crystallization. Once rinsed, the POM was transferred to a filter for viewing using a Büchner funnel system. The C content of the coarse sand fraction (part of the PSF) and the heavy (CSAOM) fraction were measured using the Picarro CRDS. Both of these values were converted to per g coarse sand fraction in order to calculate C in the POM fraction or particulate organic C (POC) (Eq. II-12: II-13). The POM fraction was not measured directly for C content because it was analyzed for microplastics.

Calculation of particulate organic carbon:

$$C_H = C_{HF} * \left(\frac{g \text{ heavy fraction}}{g \text{ coarse sand fraction}} \right) \quad (\text{Equation II-12})$$

$$C_L = (C_{2mm} - C_H) \quad (\text{Equation II-13})$$

where: C_{2mm} = TOC of coarse sand fraction ($\mu\text{g C g}^{-1}$ coarse sand fraction) (measured)

C_{HF} = Heavy fraction ($\mu\text{g C g}^{-1}$ heavy fraction) (measured)

C_H = Heavy fraction ($\mu\text{g C g}^{-1}$ coarse sand fraction)

C_L = Light fraction ($\mu\text{g C g}^{-1}$ coarse sand fraction)

BDMs as a carbon input into soil

To estimate how much C was added to the soil when the mulches were tilled in, theoretical mulch C added was calculated using the C content of the mulches. The methods for analyzing %C of the mulch materials (C_c) are described in detail in Hayes et al. (2017). The theoretical C added annually in g C m^{-2} was calculated (Eq. II-14: II-15).

Calculation of theoretical mulch C added annually:

$$M_c = M_m * C_c \quad (\text{Equation II-14})$$

$$M_m = \rho_m * SA_m \quad (\text{Equation II-15})$$

where: M_c = Concentration of mulch C tilled in annually (g mulch C m^{-2})

M_m = Mass of mulch per plot

C_C = Carbon content of mulch (g C g⁻¹ mulch)

ρ_m = Density of mulch (g m⁻²) (Hayes et al., 2017)

SA_m = Surface area of mulch per plot = 121.9cm * (914.4 cm) * 5 rows

Microplastics were isolated to calculate the amount of BDMs (1-2mm) remaining in the soil after two years (Figure II-3). Briefly, the POM fraction was decanted from the dense solution (<1.4 g cm⁻³), rinsed, filtered, and dried. The glass beads used in this method prior to wet sieving released any microplastics within aggregates (Bläsing & Amelung, 2018). Visual sorting with a headlamp, microscope and tweezers was used to differentiate BDM microplastics from POM (Hidalgo-Ruiz et al., 2012). The BDM microplastics were placed on a filter and imaged using a stereomicroscope (Nikon SMZ800N, NI-150 high intensity illuminator). Shadows were removed in paint software. Image-J software was used to size microplastics similar to the methods in Cowan et al. (2013). The surface areas measured using Image-J software were converted to units kg ha⁻¹ for comparison with standards. Calculations are similar to those in Bettas Ardisson et al. (2014). The area of the microplastics was converted to mass using the density of the mulch and this was divided by the volume of soil into which they are tilled (Eq. II-16: II-21).

Converting measured microplastic areas to kg ha⁻¹:

$$C_{kg\ ha^{-1}} = M_s * C_{m2} * \left(\frac{10,000\ m^2}{ha} \right) \quad (\text{Equation II-16})$$

$$M_s = V_s * \rho_s \quad (\text{Equation II-17})$$

$$V_s = SA_{plot} * D_t \quad (\text{Equation II-18})$$

$$C_{m2} = C_{m1} * \rho_m \quad (\text{Equation II-19})$$

$$C_{m1} = C_m * \left(\frac{M_{2mm}}{M_T} \right) \quad (\text{Equation II-20})$$

$$C_m = \frac{A_m}{M_F} \quad (\text{Equation II-21})$$

where: $C_{kg/ha}$ = Concentration of mulch tilled in after two years (kg ha⁻¹)

M_s = Mass of soil per plot (kg m⁻²)

D_t = Depth of tillage (m) = 0.15 for this study

V_s = Volume of soil per plot (m³)

SA_{plot} = 100 m²

ρ_s = Bulk density of soil (g m^{-3}) = average of bulk density of TN soils (1300 kg m^{-3})

C_{m2} = Concentration of mulch (g mulch g^{-1} dry soil)

C_{m1} = Concentration of microplastics ($\text{mm}^2 \text{ g}^{-1}$ dry soil)

C_m = Concentration of microplastics ($\text{mm}^2 \text{ g}^{-1}$ coarse sand)

A_m = Area of measured microplastics (mm^2)

$M_{2\text{mm}}$ = Mass of coarse sand fraction (g coarse sand)

M_F = Mass of coarse sand fractionated (g coarse sand) ($\sim 4\text{g}$)

M_T = Starting mass of soil fractionated = 50 g dry soil

ρ_m = Density of mulch (g m^{-2}) (Hayes et al., 2017)

Statistical analysis

All statistical analyses were conducted using RStudio for Mac desktop version 1.0.136 (Core Team, 2016; Fox & Weisberg, 2011; Grolemund & Wickham, 2011; Lenth, 2016; Luo et al., 2014; Wickham, 2007, 2017). The experimental field design was a randomized block design (RBD) with repeat measures. To analyze the effect of mulch treatment on slower turnover C pools (TOC, POXC, peroxide-resistant C), a change score (Spring 2017- Spring 2015) was used justified by high spatial variation in the field (Sintim et al., 2019) and large differences in starting C values between locations. A two-way ANOVA (lmer) (Bates et al., 2015; Kuznetsova et al., 2016) with location, mulch treatment, and the interaction as fixed variables and block as a random variable was conducted to compare treatment and location effects on the change in C pools. When the ANOVA indicated significant effects ($\alpha = 0.10$), a *post hoc* analysis with Tukey's HSD (Honest significance test) was applied to compare the means. The experimental design included four replications of each treatment within blocks. Outliers were removed using boxplots, Q-Q plots, and Cook's D. Residuals were checked for normality using the Shapiro-Wilk test ($W > 0.9$) and equal variance using Levene's test ($\alpha = 0.05$). If these conditions were not met, the data were log transformed. In addition, a two-tailed paired t-test was used to compare Spring 2015 and 2017 values. Data were checked for normality using the Shapiro-Wilk test ($W > 0.8$, $\alpha = 0.05$).

For C pools with quicker turnover times (respiration, EOC), a two-way repeated measures ANOVA with one between subject factor (mulch treatment) and one within subject factor (sampling date) was applied to these data to account for field variation and changes over time. Residuals were checked for normality using the Shapiro-Wilk test ($W > 0.9$) and equal variance using Levene's test ($\alpha = 0.05$). The results were adjusted for type 3 sums of squares because lme defaults to type 1 sums of squares which is not appropriate for unbalanced or interaction designs. If a factor was significant, Tukey's *post-hoc* ($\alpha = 0.10$) was used to test the difference of means. For additional tests to look at mulch treatment effect with no location or sampling date factor, a one-way ANOVA (lmer) (Bates et al., 2015; Kuznetsova et al., 2016) was used with treatment as a fixed variable and block as a random variable. Residuals were checked for normality using the Shapiro-Wilk test ($W > 0.9$) and equal variance using Levene's test ($\alpha = 0.05$).

Results

Carbon pools

Total organic carbon

TOC was measured on the bulk soil samples for all seven mulch treatments in both locations. The starting TOC values (mean $\pm$ SE) of the no-mulch control plots in Spring 2015 (prior to any mulch treatment) were approximately 40% lower in TN (6.24 ± 0.90 mg C g⁻¹) than WA (10.8 ± 0.27 mg C g⁻¹) (Appendix A, Table II-8). There was no effect of mulch treatment ($p = 0.18$), location ($p = 0.12$), or the interaction of location and mulch ($p = 0.86$) on the change in TOC from Spring 2015 to Spring 2017 (Table II-2). In TN, the change in TOC over two years was not significantly different from zero for any of the mulch treatments (Figure II-4). However, in WA, PE ($p = 0.0033$) had a significant change in TOC (Appendix A, Table II-9).

Permanganate oxidizable C

POXC was measured on the bulk soil samples for all seven mulch treatments in both locations. The starting POXC values of the no-mulch control plots in Spring 2015 (prior to any mulch treatment) were about 60% lower in TN (169 ± 29.4 μ g C g⁻¹) than WA (420 ± 6.43 μ g C g⁻¹) (Appendix A, Table II-8). In TN, this amount is 2.7% of the total C and in WA, POXC makes up

Table II-2. The effects of mulch treatment and location (TN, WA) on the change in total organic carbon (TOC), permanganate oxidizable carbon (POXC), and peroxide-resistant C from Spring 2015 to Spring 2017.

	TOC		POXC		Peroxide-resistant C	
	<i>p</i> -value	F stat	<i>p</i> -value	F stat	<i>p</i> -value	F stat
Location	0.12	3.24	0.058*	5.39	0.0036**	9.58
Mulch treatment	0.18	1.58	0.14	1.76	0.43	1.01
Mulch*Location	0.86	0.42	0.65	0.71	0.21	1.48

*Indicates significance at an alpha level of 0.05-0.1.

**Indicates significance at an alpha level of <0.05.

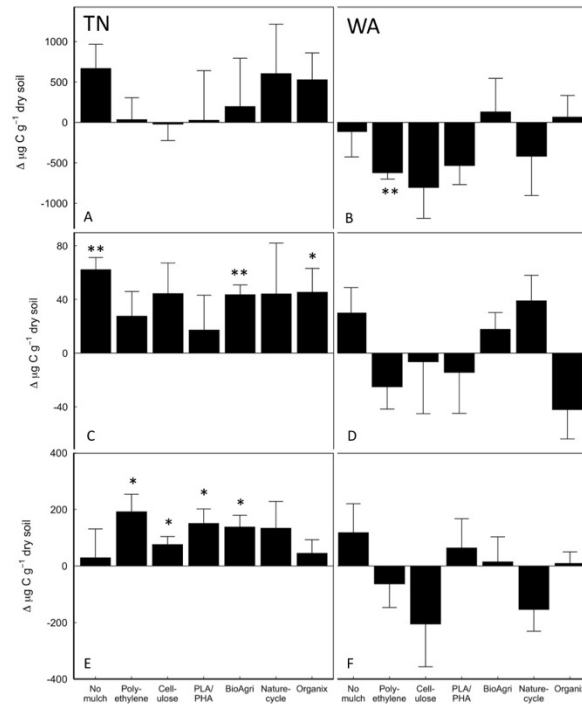


Figure II-4. Change in C pools from Spring 2015- Spring 2017 under mulch treatments. A-B) Change in total organic carbon (TOC) in TN and WA, respectively. C-D) Change in permanganate oxidizable carbon (POXC) in TN and WA, respectively. E-F) Change in peroxide-resistant C in TN and WA, respectively. Each bar represents a mean of 3-4 field reps and error bars are standard error. *Indicates a difference from zero using a two-tailed t-test at an alpha level of 0.05-0.1. **Indicates a difference from zero using a two-tailed t-test at an alpha level < 0.05 (Appendix A, Table II-9). Mulch treatments (main effect) were not statistically significant, but location (main effect) was significant for POXC and peroxide-resistant C (Table II-2).

3.9% of the total C. There was a significant effect of location on the change in POXC over two years ($p = 0.058$) (Table II-2). In TN, three treatments had a significant increase in POXC over two years: no-mulch ($p = 0.0059$), BioAgri ($p = 0.0087$), and Organix ($p = 0.080$) (Appendix A, Table II-9). In WA, the change in POXC after two years was not significantly different from zero for any of the mulch treatments (Figure II-4).

Peroxide-resistant C

Peroxide-resistant C was measured on the bulk soil samples for all seven mulch treatments in both locations. The starting peroxide-resistant C values of the no-mulch control plots in Spring 2015 were about 55% lower in TN ($560 \pm 80.0 \mu\text{g C g}^{-1}$) than WA ($1230 \pm 75.5 \mu\text{g C g}^{-1}$) (Appendix A, Table II-8). In TN, this value is about 9% of the total C and in WA it is about 11% which matches the peroxide-resistant C values (4-10%) found in the Ap1/Ap2 horizons of corn fields (Jagadamma et al., 2010). There was no effect of mulch treatment ($p = 0.43$) or the interaction of location and mulch treatment ($p = 0.21$) on the change in peroxide-resistant C over two years (Table II-2). There was a significant effect of location on the change in peroxide-resistant C ($p = 0.0036$). In TN, PE ($p = 0.051$), Cellulose ($p = 0.068$), PLA/PHA ($p = 0.056$), and BioAgri ($p = 0.079$) showed an increase in peroxide-resistant C over two years (Appendix A, Table II-9). In WA, there was no change in peroxide-resistant C over two years (Figure II-4).

Particle size fractions

The C contents of three particle size fractions (2000-250 μm , 250-53 μm , <53 μm) were measured for all seven mulch treatments for TN soils only in Spring 2015 and Spring 2017. During the particle size fractionation (PSF), only a small percentage of the soil was lost as shown by mass recovery rates of 94% in Spring 2015 and 92% in Spring 2017. The mean mass distribution of the Spring 2017 soils was $15.1 \pm 2.0\%$ for coarse sand, $49.4 \pm 1.5\%$ for fine sand, and $27.5 \pm 2.8\%$ for StC. The distribution of C in these pools can be shown by the no-mulch Spring 2017 pool size of $757 \pm 96.4 \mu\text{g C g}^{-1}$ for coarse sand, $1440 \pm 304 \mu\text{g C g}^{-1}$ for fine sand, and $3896 \pm 470 \mu\text{g C g}^{-1}$ for StC (Appendix A, Table II-10). After two years, there was no effect of mulch treatment on the coarse sand ($p = 0.72$) or fine sand ($p = 0.52$) fractions (Table II-3). There was an effect of mulch treatment on the StC fraction ($p = 0.033$) with PE and no-mulch treatments being significantly different (Figure II-5). Carbon increased in the no-mulch

Table II-3. Statistics of the effect of mulch treatment on the change in particle size fraction carbon from Spring 2015 - Spring 2017.

	Coarse sand		Fine sand		Silt and clay	
	p-value	F stat	p-value	F stat	p-value	F stat
Mulch treatment	0.72	0.62	0.52	0.90	0.033**	2.98

*Indicates significance at an alpha level of 0.05-0.1.

**Indicates significance at an alpha level of <0.05.

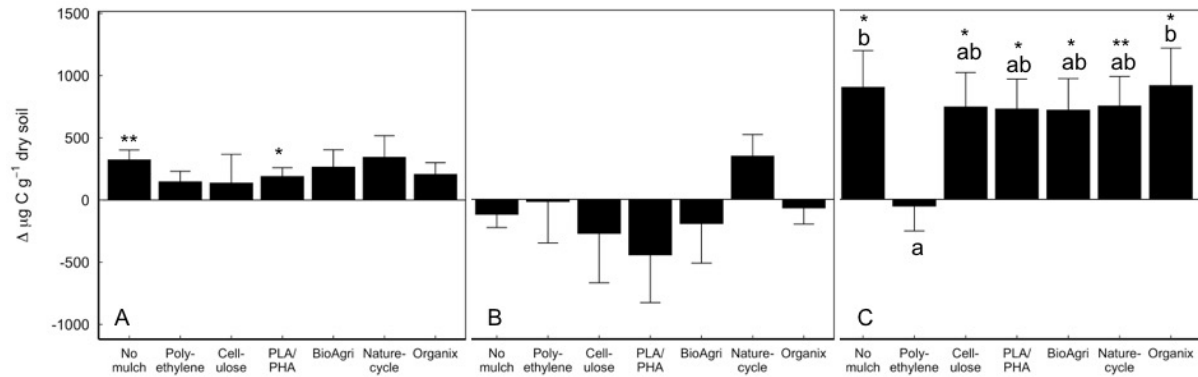


Figure II-5. Change in particle size fraction (PSF) C after 2 years under mulch treatments. A) coarse sand B) fine sand, and C) silt and clay (StC). Each bar represents a mean of 3-4 field reps and error bars are standard error. *Indicates a difference from zero using a two-tailed t-test at an alpha level of 0.05-0.1. **Indicates a difference from zero using a two-tailed t-test at an alpha level < 0.05 (Appendix A, Table II-11). The letters (Figure C) indicate an effect of mulch treatment using Tukey's HSD *post-hoc* at an alpha level < 0.05 ($p = 0.033$). No letters in Figures A and B indicate the absence of statistically significant differences in mulch treatments (Table II-3).

treatment, but not in PE, and the BDMs fell between these values. The no-mulch treatment represents the pattern of all BDM treatments which showed mean gains in C in the coarse sand fraction ($326 \pm 76.2 \mu\text{g C g}^{-1}$) and StC fraction ($901 \pm 290 \mu\text{g C g}^{-1}$) but a loss in the fine sand fraction ($-124 \pm 102 \mu\text{g C g}^{-1}$). After two years of mulch use, the no-mulch treatment showed a significant increase in coarse sand fraction C ($p = 0.023$). In the fine sand fraction, the change in C was not significantly different from zero for any of the mulch treatments. In the StC fraction, each mulch treatment had a significant increase in C except PE ($\alpha = 0.05-0.1$) (Appendix A, Table II-11).

24-hour respiration

For the more labile, quicker turnover C pools (EOC, respiration), the values were reported and analyzed for five seasons over two years (Spring 2015, Fall 2015, Spring 2016, Fall 2016, and Spring 2017) because labile pools vary so much seasonally based on environmental conditions (pH, soil moisture, soil temperature). Spring samplings mark the beginning of the season (pre-mulch application) and Fall samplings mark post-harvest before the mulches have been tilled. In TN, mulch treatment had a significant effect on respiration rates ($p = 0.040$), but not in WA ($p = 0.18$) (Table II-4). In both locations, there was a significant effect of the sampling date on respiration ($p < 0.0001$). In WA, there was no significant interaction effect ($p = 0.74$) and *post-hoc* analysis ($\alpha = 0.1$) showed Spring 2016 and Fall 2016 having similar respiration rates while all other seasons were different from each other. In WA 2015, there was a significant drop in respiration from Spring to Fall for all treatments. The values of respiration in TN ranged from $2.25 \pm 0.59 \mu\text{g C g}^{-1}\text{day}^{-1}$ in Fall 2015 (PE) to $9.11 \pm 1.01 \mu\text{g C g}^{-1}\text{day}^{-1}$ in Spring 2017 (PLA/PHA). Unlike other C pools, the values of respiration in WA were very comparable to TN and ranged from $2.06 \pm 0.33 \mu\text{g C g}^{-1}\text{day}^{-1}$ in Fall 2015 (PE) to $15.9 \pm 1.56 \mu\text{g C g}^{-1}\text{day}^{-1}$ in Spring 2015 (PE). In TN Fall 2015, mulch treatment did not have a significant effect on respiration rates ($p = 0.21$, $F = 1.58$) but in Fall 2016, mulch treatment was significant ($p = 0.029$, $F = 2.98$) and *post-hoc* analysis showed PE as significantly different. The suppression of respiration in the PE treatment in Fall 2016 is clear (Figure II-6). In WA, mulch treatment did not have a significant effect on respiration in neither Fall 2015 ($p = 0.11$, $F = 2.0$) nor Fall 2016 ($p = 0.17$, $F = 1.72$).

Table II-4. Effect of mulch treatment and date on C pools in TN and WA.

	EOC				24-hr respiration			
	TN		WA		TN		WA	
	<i>p</i> -value	F stat	<i>p</i> -value	F stat	<i>p</i> -value	F stat	<i>p</i> -value	F stat
Treatment	0.047**	2.71	0.22	1.53	0.040**	2.84	0.18	1.70
Date	0.0013**	4.93	0.15	1.83	<.0001**	11.95	<.0001**	19.89
Treatment*Date	0.63	0.88	0.60	0.88	0.047**	1.67	0.74	0.79

*Indicates significance at an alpha level of 0.05-0.1.

**Indicates significance at an alpha level of <0.05.

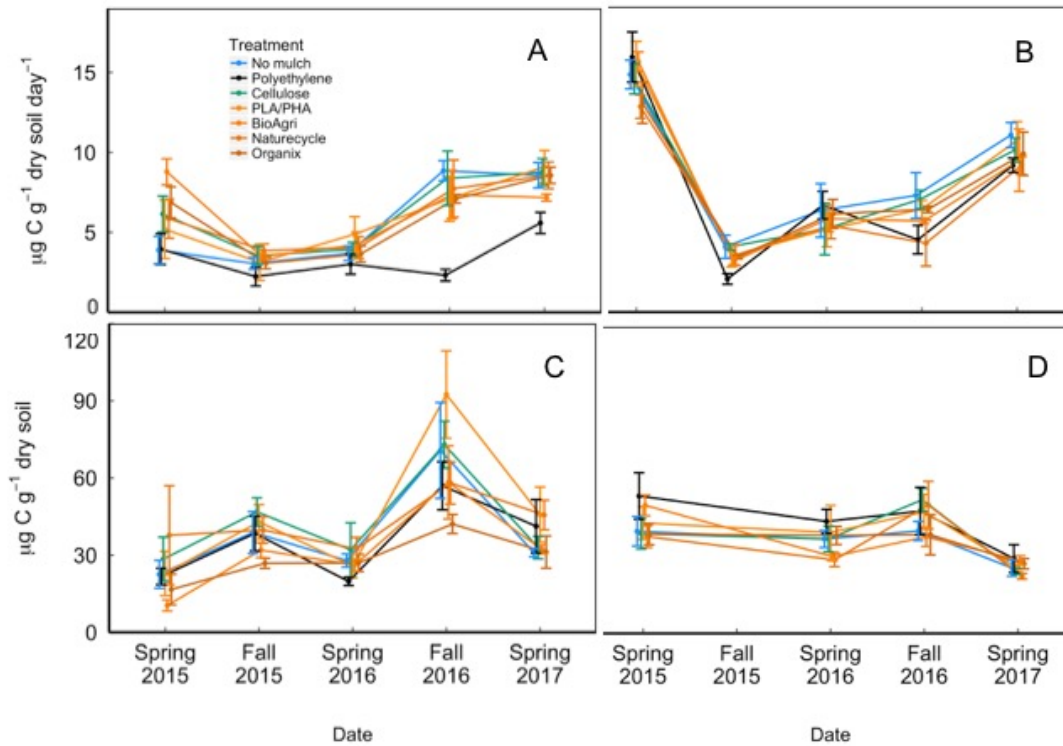


Figure II-6. Different C pools measured from Spring 2015 – Spring 2017. A-B) 24-hr respiration in TN and WA, respectively. C-D) Extractable organic carbon (EOC) in TN and WA, respectively. Each point represents a mean of 3-4 field reps and error bars are standard error. Statistics can be found in Table II-4.

Extractable organic carbon

In TN, EOC values ranged from $10.5 \pm 2.16 \mu\text{g C g}^{-1}$ in Spring 2015 (BioAgri) to $92.5 \pm 17.0 \mu\text{g C g}^{-1}$ in Fall 2016 (PLA/PHA), approximately 0.2 to 1.5% of total C. In WA, there was a tighter range with the lowest value being $22.4 \pm 1.11 \mu\text{g C g}^{-1}$ in Spring 2017 (Naturecycle) and the highest $53.8 \pm 9.11 \mu\text{g C g}^{-1}$ in Spring 2015 (PE). This is approximately 0.2 to 0.5% of total C which matches typical EOC values of 0.05-0.4% (Haynes, 2005). In TN, both mulch treatment ($p = 0.047$) and date ($p = 0.0013$) had a significant effect on EOC values while the interaction was not significant ($p = 0.63$) (Table II-4). The model outputs showed that across all seasons, Organix had the lowest EOC values and Naturecycle the highest with BioAgri, PE, no-mulch, PLA/PHA, and cellulose falling between those treatments (Figure II-6). A *post-hoc* on seasons in TN showed that EOC was significantly higher in Fall 2016 than all other seasons followed by Fall 2015 and Spring 2017 values and then Spring 2015 and 2016. In WA, there was no effect of mulch treatment ($p = 0.22$), date ($p = 0.15$), or the interaction ($p = 0.60$) on EOC.

Microbial biomass C

Spring 2017 MBC was treated as independent due to missing values and this is valid because MBC has a very fast turnover time. Mulch treatment did not have a significant effect on MBC values in TN ($p=0.47$, $F=0.97$) or WA ($p = 0.35$, $F= 1.20$) (Figure II-7). MBC values in TN in Spring 2017 ranged from $60.9 \pm 21.5 \mu\text{g C g}^{-1}$ (Naturecycle) to 129 ± 43.7 (BioAgri) (Appendix A, Table II-12). MBC values in WA in Spring 2017 ranged from $83.7 \pm 8.75 \mu\text{g C g}^{-1}$ (PE) to $142 \pm 44.0 \mu\text{g C g}^{-1}$ (PLA/PHA). Spring 2017 MBC values were on the low end of the optimum range of similar agricultural systems ($<200 \mu\text{g C g}^{-1}$) (Andrews et al., 2004).

Particulate organic carbon

POC was only measured on the TN Spring 2017 soils for all treatments except cellulose. The density fractionation used to isolate POM had a 91% mass recovery. Using no-mulch as a representative of all treatments, the POC fraction had a C content of $545 \pm 64.7 \mu\text{g C g}^{-1}$ (Appendix A, Table II-12). The percent mass and C content of the POM fraction out of the coarse sand fraction were calculated for each mulch treatment. In the no-mulch treatment, the POM fraction makes up a large majority of the C in this coarse sand fraction ($86.6\% \pm 2.43$) but $< 1\%$ of the mass ($0.88 \% \pm 0.11$) (Table II-5). There was no significant effect of mulch

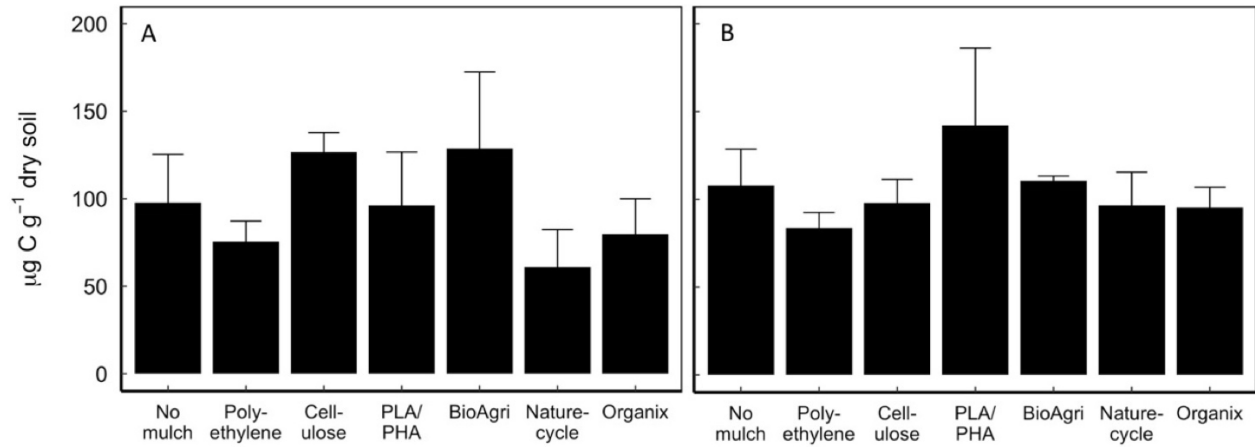


Figure II-7. Spring 2017 effect of mulch on microbial biomass carbon (MBC). A-B) 2017 MBC in TN and WA, respectively. Each bar represents a mean of 3-4 field reps and error bars are standard error. No letters in Figures A and B indicate the absence of statistically significant differences in mulch treatments in TN ($p=0.47$) and WA ($p = 0.35$).

Table II-5. Role of particulate organic matter (POM) in the coarse sand fraction and mulch material in POM fraction. Values are mean (SE).

Treatment	POM mass/ fraction (%)	POC/ fraction (%)	Mulch mass/ POM (%)	Mulch C/ POC (%)
No-mulch	0.88 (0.11)	86.6 (2.43)	0.08 (0.03)	0.09 (0.05)
Polyethylene	0.64 (0.04)	75.8 (2.47)	0.14 (0.06)	0.30 (0.14)
Cellulose	NA	NA	NA	NA
PLA/PHA	0.83 (0.22)	75.7 (8.43)	0.83 (0.21)	1.91 (1.39)
BioAgri	0.79 (0.11)	83.1 (2.42) ^a	0.38 (0.14)	0.50 (0.41) ^a
Naturecycle	0.73 (0.19)	81.8 (3.39)	0.76 (0.48)	0.68 (0.36)
Organix	1.02 (0.16)	80.9 (2.22)	0.60 (0.32)	1.36 (0.85)
Mulch treatment ^b	p -value= 0.24 F stat = 1.56	p -value= 0.37 F stat = 1.18		

^aThe BioAgri treatment had only 2 replications so these values are mean (SD) and were not included in the ANOVA.

^bMulch treatments (main effect) were not statistically significant so only p -values were displayed

treatment on % POM/ coarse sand fraction mass ($p = 0.24$, $F = 1.56$) or % POC/ coarse sand fraction C ($p = 0.37$, $F = 1.18$) (Table II-5).

BDMs as a carbon input into soil

Using the %C of un-weathered mulch material (Hayes et al., 2017), the mulch density and the amount of mulch laid on the field, the theoretical amount of C added annually from tilling in mulch was calculated for the BDMs with the following results: Cellulose ($28.4 \text{ gC m}^{-2} \text{ year}^{-1}$), Naturecycle ($8.12 \text{ gC m}^{-2} \text{ year}^{-1}$), PLA/PHA ($7.34 \text{ gC m}^{-2} \text{ year}^{-1}$), BioAgri ($7.32 \text{ gC m}^{-2} \text{ year}^{-1}$), and Organix ($5.68 \text{ gC m}^{-2} \text{ year}^{-1}$). Carbon inputs from crop residues of corn and soybean result in $448 \text{ gC m}^{-2} \text{ year}^{-1}$ and $135 \text{ gC m}^{-2} \text{ year}^{-1}$ respectively (Carvalho et al., 2017). Based on these numbers, BDM C additions were calculated to be 1.2%-6% of the C added from crop residues.

Next, microplastics (1-2 mm) in the POM fraction were measured. The effect of mulch treatment on microplastics was significant ($p = 0.060$, $F = 2.73$) (Figure II-8). No-mulch and PE had significantly lower microplastic accumulations than PLA/PHA. PLA/PHA had almost double the concentration of microplastics as the other BDM treatments. For context, due to a lack of studies on soil microplastics, our measured values were compared to calculated theoretical maxima given the following two conditions: (1) If BDMs followed their prescribed biodegradation rates (90% after two years) described by Miles et al. (2017) and (2) If none of the mulch biodegraded after two years. The results of the microplastics analysis showed evidence of microplastic accumulation in the measured BDM treatments ($16.3 \pm 9 - 36.6 \pm 16 \text{ kg ha}^{-1}$) but not above the estimated theoretical maxima with biodegradation ($76.8\text{-}107 \text{ kg ha}^{-1}$) nor without biodegradation ($221\text{-}309 \text{ kg ha}^{-1}$) (Appendix A, Table II-13). The theoretical maxima serve as an upper limit that includes BDM residues of all sizes and therefore cannot be directly compared with the microplastics from this study that were only isolated in the 1-2 mm fraction. A more direct comparison is that between microplastics and the POM fraction. The fraction of mulch material in the POM fraction was calculated along with the %C of mulch in POC (Table II-5). Recovered mulch residues were a very small contribution to the mass of the light fraction with PLA/PHA making up the largest percent at ($0.83 \pm 0.21\%$). Mulch C also makes up a very small % of POC with PLA/PHA containing the highest percent ($1.91 \pm 1.39\%$).

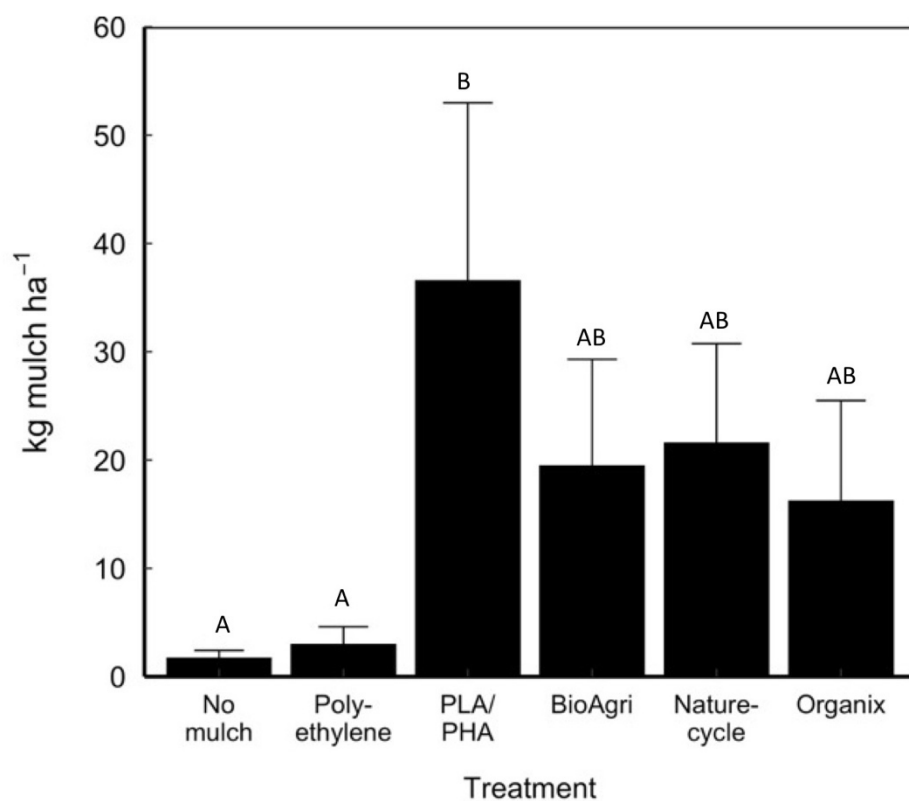


Figure II-8. Mass of microplastics (1-2 mm) per area (kg mulch ha⁻¹) in Spring 2017 after two years of mulch incorporation. Each bar represents a mean of 3-4 field reps and error bars are standard error. The uppercase letters indicate an effect of mulch treatment using Tukey's HSD *post-hoc* at an alpha level of 0.05-0.1 ($p = 0.060$).

Mulch microclimate in two locations

The physical barrier created by plastic mulch affects the interaction of soil, air, and water (Steinmetz et al., 2016) which alters the soil temperature and moisture or “microclimate” under the mulches. The extent of the microclimate created by agricultural mulching depends on the properties of the mulch film and the location of use. To measure the microclimate, soil temperature and moisture were measured hourly in one plot of each mulch treatment meaning statistical analysis could not be performed on this data. The mean daily averages at 10cm depth for 2015 and 2016 growing seasons (May-September) were calculated (Tables II-6 & II-7). Looking across both years, differences between the BDMs and the controls (no-mulch, cellulose, and PE) were observed. The expected trend was mulch films (PE and BDMs) would have more of an effect on the microclimate compared to cellulose (a paper mulch) and no-mulch treatments.

In TN, PE had the highest moisture content and cellulose had the lowest for both years and the BDMs and no-mulch fell between these two controls. Cellulose did not last through the entire season in TN making it more similar to a no-mulch plot than a mulched plot. In 2015, the no-mulch treatment (0.18 ± 0.03) (mean $\pm$ SD) actually had higher moisture than the BDMs but still lower than PE (0.25 ± 0.02). In WA, the moisture results were more variable with the controls and BDMs having a range of values. In 2016, no-mulch had a much lower moisture than all of the mulch treatments ($0.05 \text{ cm}^3 \text{ cm}^{-3}$ lower than the next lowest). In both locations, no-mulch or cellulose always had the lowest average moisture (except for WA 2015), and a BDM or PE had the highest. For soil temperature, the warming effect of BDMs and PE compared to no-mulch and cellulose controls was most clear early in the season (Figure II-9). In both locations, no-mulch or cellulose always had the lowest average temperature and PE or a BDM had the highest. PE mulch film was the highest temperature in these instances: TN, 2016 (28.31 ± 2.85) and WA, 2015 (22.91 ± 3.27). In WA in 2016, PE (19.96 ± 2.21) was a close second to BioAgri (19.97 ± 2.15). In TN, the highest mean daily averages were Organix (26.82 ± 2.32) in 2015 and PE (28.31 ± 2.85) in 2016. In WA, the highest mean daily average was PE (22.91 ± 3.27) in 2015 and BioAgri (19.97 ± 2.15) in 2016. The lowest mean daily average temperatures in TN were no-mulch (25.06 ± 2.26) in 2015 and cellulose (26.50 ± 1.36) in 2016. The lowest mean daily averages in WA were cellulose (19.72 ± 2.46) in 2015 and no-mulch (18.75 ± 2.22) in 2016.

Table II-6. Mean daily average soil moisture ($\text{cm}^3 \text{ cm}^{-3}$). Values are mean (SD). Measured at 10 cm depth during the growing season (from 17 June to 20 Sept. 2015 and 15 June to 18 Sept. 2016 in TN and 30 May to 25 Sept. 2015 and 31 May to 25 Sept. 2016 in WA). These data are the same as presented in Sintim et al. (2019) but were recalculated using different dates.

	TN		WA	
	2015	2016	2015	2016
No-mulch	0.18 (0.03)	0.13 (0.04)	0.20 (0.02)	0.16 (0.02) ^L
Polyethylene	0.25 (0.02) ^H	0.18 (0.05) ^H	0.21 (0.02)	0.22 (0.02)
Cellulose	0.09 (0.02) ^L	0.09 (0.04) ^L	0.22 (0.03)	0.21 (0.03)
PLA/PHA	0.13 (0.02)	0.15 (0.05)	0.19 (0.03) ^L	0.22 (0.03)
BioAgri	0.14 (0.03)	0.14 (0.04)	0.20 (0.03)	0.25 (0.03) ^H
Naturecycle	0.13 (0.03)	0.13 (0.03)	0.19 (0.03) ^L	0.22 (0.02)
Organix	0.13 (0.03)	0.13 (0.05)	0.26 (0.04) ^H	0.21 (0.03)

^HHighest seasonal mean daily average soil moisture

^LLowest seasonal mean daily average soil moisture

Table II-7. Mean daily average soil temperature ($^{\circ}\text{C}$). Values are mean (SD). Measured at 10 cm depth during the growing season (from 17 June to 20 Sept. 2015 and 15 June to 18 Sept. 2016 in TN and 30 May to 25 Sept. 2015 and 31 May to 25 Sept. 2016 in WA). These data are the same as presented in Sintim et al. (2019) but were recalculated using different dates.

	TN		WA	
	2015	2016	2015	2016
No-mulch	25.06 (2.26) ^L	26.89 (1.91)	19.99 (2.78)	18.75 (2.22) ^L
Polyethylene	26.07 (2.19)	28.31 (2.85) ^H	22.91 (3.27) ^H	19.96 (2.21)
Cellulose	25.24 (1.99)	26.50 (1.36) ^L	19.72 (2.46) ^L	18.85 (2.09)
PLA/PHA	26.12 (2.48)	27.92 (2.94)	21.42 (2.86)	19.66 (2.12)
BioAgri	25.73 (2.57)	27.81 (2.35)	21.62 (2.87)	19.97 (2.15) ^H
Naturecycle	25.35 (2.56)	27.88 (2.50)	21.15 (2.89)	19.90 (2.22)
Organix	26.82 (2.32) ^H	27.92 (2.34)	20.55 (2.42)	18.95 (2.13)

^HHighest seasonal mean daily average soil temperature

^LLowest seasonal mean daily average soil temperature

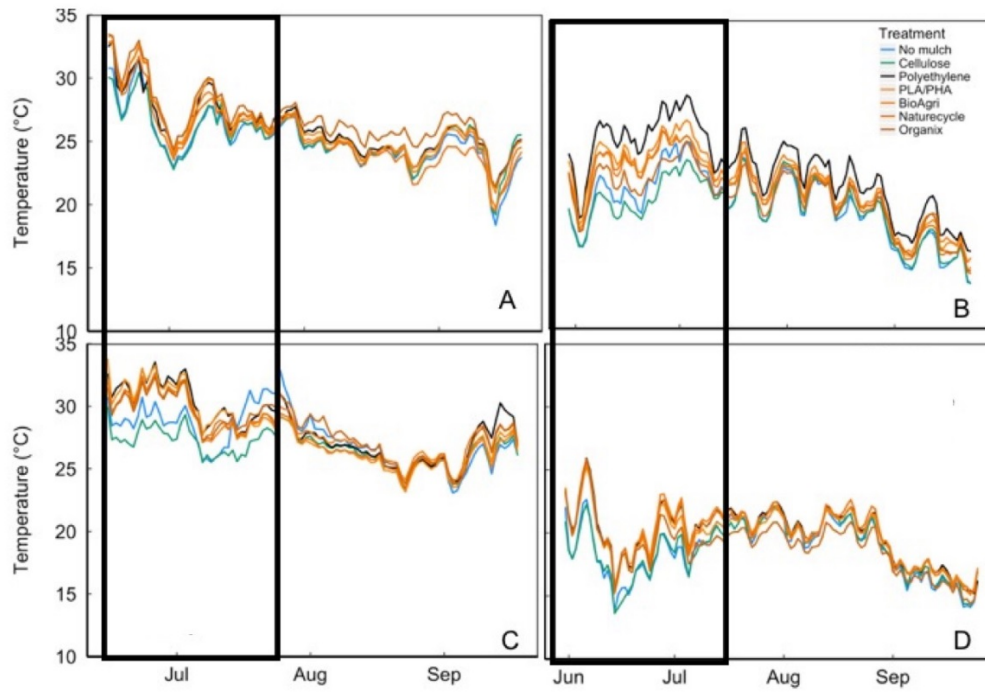


Figure II-9. Soil temperature during the growing season. A-B) Fall 2015 in TN and WA, respectively. C-D) Fall 2016 in TN and WA, respectively. The black box marks the early season before the ground was shaded by the plants when mulch treatment differences were more pronounced. These data are the same as presented in Sintim et al. (2019) but were recalculated using different dates.

Discussion

Macroclimate: The effect of location and season on soil carbon cycling

The climates in TN and WA had a large influence over C cycling as shown by the effect of location on intermediate to stable C pools and the effect of season on labile C pools. Location affects many of the environmental controls on SOM decomposition (soil temperature, moisture, and mineralogy) (Lehmann & Kleber, 2015). In addition, climate has been shown to have a strong relationship with C cycling (Kirschbaum, 1995; Post et al., 1982). Hereafter, we will refer to the collective effect of the climate and soil type in a location as the “soil climate” (Gregorich et al., 1994). TN has a warmer (4°C higher annual air temperatures), wetter (500 mm more precipitation annually) climate than WA leading to soil temperatures 5°C warmer in TN (25.06°C) than WA (19.99°C) and drier soils in TN (0.18 cm³ cm⁻³) than WA (0.20 cm³ cm⁻³) due to higher evaporation rates. This study attempted to closely control possible interacting factors across field sites (crop species, density, and tillage) (Ogle et al., 2005) in order to allow comparisons of the effects of mulching on soil moisture, temperature, and C pools across locations. Location had a significant effect on the change in C pools (peroxide-resistant and POXC) after two years likely due to the different soil climates of TN and WA (Table II-2). It was hypothesized that the cooler temperatures in WA would create less conducive C decomposition conditions than TN. Counter to the hypothesis, C in the POXC and peroxide-resistant pools increased more in TN compared to WA implying that WA was more conducive to C decomposition conditions than TN (Figure II-4).

In addition to location differences, there were very different seasonal and annual trends in C pools. Season has been shown to effect labile C pools under mulching (Tian et al., 2013) as well as other soil chemical properties under mulching (Sintim et al., 2019). In this study, respiration and EOC (TN only) were affected by sampling date (Table II-4). There was a very significant effect of sampling date on 24-hour respiration measurements in both WA and TN. In WA, there was a large drop in respiration for all mulch treatments from Spring 2015 to Fall 2015, indicating lower microbial activity in the Fall than the Spring (Figure II-6). In the higher moisture soils of WA, it is likely that cooler temperatures in the Fall compared to Spring led to the lower respiration rates (Yuste et al., 2007). The seasonal effects on respiration rates were not as clear in TN with Fall and Spring having varying effects.

Sampling date had a significant effect on EOC in TN. Both Fall 2015 and Fall 2016 had higher EOC values than Spring 2015 and Spring 2016 because warmer seasons encourage faster decomposition thereby increasing the release rate of EOC from SOM (Solinger et al., 2001). In WA, sampling date did not have a significant effect on EOC but there was a slight decrease in all mulch treatments from Fall 2016 to Spring 2017 (Figure II-4). The difference in EOC between seasons could also be due to seasonal changes in pH. Soil pH was slightly lower in the Fall seasons for both locations (Sintim et al., 2019) and lower soil pH values can correlate with higher EOC (Kemmitt et al., 2006). In Fall 2016 in TN, all mulch treatments had higher EOC values than other seasons (approximately double the Fall 2015 EOC values) likely due a severe drought that caused a decrease in TN's annual precipitation (~ 500 mm). Drought conditions can lead to higher EOC due to microbial formation of extracellular polymeric substances (EPS) during water stress that utilize biofilms to make the environment wetter for longer (Schaeffer et al., 2017; Schimel et al., 2007). Across two years, season had a significant effect on respiration in both locations and EOC in TN caused by seasonal differences in drought and/or pH.

Microclimate: The effect of mulching on soil carbon cycling

The microclimate created by mulching could have a big impact on soil C cycling through increased mineralization and storage (Steinmetz et al., 2016). The different climates (air temperature and precipitation) in TN and WA led to slightly different microclimate trends (not statistically significant) under PE and no-mulch treatments. The cooler air temperatures in WA led to a larger increase in soil temperature under PE treatments (especially during the 2015 growing season) compared to the increase in TN. In the drier TN soils, PE increased the soil moisture more clearly than in WA where moistures were more variable across all mulch treatments (Table II-6). Despite these small differences, PE performed the desired microclimate functions well across climates. Although not statistically significant, in TN, PE created the highest mean daily average soil moistures in both seasons and the warmest soil temperatures in 2016 while no-mulch had the lowest or second lowest temperatures in both seasons (Table II-7). Especially early in the season, the PE temperatures were higher than no-mulch treatments (Figure II-9). The warmer and wetter conditions created under PE compared to no-mulch in both locations likely led to the significantly different effects of PE and the no-mulch control on C pools (StC fraction C in TN, Fall 2016 respiration in TN, TOC in WA) because soil moisture and

temperature have a strong relationship with nutrient mineralization rates (Conant et al., 2011; Davidson & Janssens, 2006; Krull et al., 2003; Manzoni et al., 2012). Over two years, the no-mulch treatment promoted the accumulation of C in the StC fraction (measured only in TN) while the PE treatment showed no change (Figure II-5). The increase in C in the no-mulch treatment could be a result of the accumulated C in the coarse sand fraction being stabilized in the StC fraction. The lack of change in StC fraction C in the PE treatment over two years could be from increased moisture under PE creating a more connected soil hydrologic landscape than other mulch treatments. Higher soil moistures can make protected C in the StC fraction more accessible to microbes and enzymes leading to higher mineralization rates (Blankinship & Schimel, 2018; Lützow et al., 2007; Schimel & Schaeffer, 2012). Out of the particle size fractions, clay includes the most microbially processed, stable C because of the ability of clay particles to stabilize C within microstructures and OC-mineral interactions (Lützow et al., 2007). Mulching conditions likely destabilize C in the longer turnover pools of C such as the StC fraction C (Steinmetz et al., 2016). The StC fraction had the highest %C contribution to TOC (> 50%), so changes in this pool could indicate a future decline in TOC. PE, cellulose, PLA/PHA, and BioAgri showed an increase in peroxide-resistant C after two years in TN; however, overall mulch treatment did not have a significant effect on C in this pool (Figure II-4). The StC and the peroxide-resistant fractions are both considered stable C pools, but they could be showing different results because the peroxide-resistant C pool tends to be enriched in alkyl groups, aromatics and nitrogen-rich compounds (Favilli et al., 2008; Jagadamma et al., 2010; Righi et al., 1995) while the StC fraction is considered more microbially-derived and has a size component (Liang et al., 2017; Lützow et al., 2007). The PE treatment had significantly lower 24-hour respiration rates in Fall 2016 (Figure II-6) potentially indicating lower biological activity (Haney et al., 2008b). Soil respiration is sensitive to both temperature and water content (Davidson et al., 2002; Li et al., 2014d). The higher temperatures of the PE plots may have increased microbial activity (Alster et al., 2018) throughout the summer leading to a reduction in labile C available to microbial mineralization thereby decreasing respiration rates. Moreno & Moreno (2008) also observed lower SOM mineralization rates in PE plots.

In WA, there was more warming under the PE treatment (3°C higher than the no-mulch treatment in the 2015 season, Table II-7) although not statistically significant, and this may have

led to the loss in TOC after two years under the PE treatment. While mulch treatment did not have a significant effect on TOC, the PE treatment was the only mulch treatment with a significant change in TOC over two years which could indicate a trend of C loss. Temperature related decreases in TOC suggest higher rates of microbial decomposition of SOM under PE (Steinmetz et al., 2016). Other studies have found losses in TOC after two to four years of mulch use compared to no-mulch controls (Cuello et al., 2015; Li et al., 2007).

After two years of BDM use and tillage into the soil, BDMs had no effect on C pools in WA. In TN, a few C pools experienced an accumulation of C under BDMs. All BDM treatments showed a significant increase in C in the PSF StC fraction C after two years while PE did not (Figure II-5). For the other C pools measured as the change over two years (TOC, peroxide-resistant C, POXC), mulch treatment did not have a significant effect. BDM treatments experienced significant increases after two years in two of these pools in TN: peroxide-resistant C (cellulose and PLA/PHA along with PE) and POXC (BioAgri and Organix along with no-mulch) (Figure II-4). The increase in POXC may indicate a trend towards accumulation of C in this pool. Other studies found no effect of plastic mulching on POXC after four years (Luo et al., 2015). POXC and light fraction organic carbon (LFOC) have been recommended as the most sensitive pools of C for measuring effects of mulch use (Luo et al., 2015). Results show mulching can increase (Luo et al., 2015; Zhou et al., 2011) or decrease (Wang et al., 2016b) LFOC. LFOC is similar to POC but is separated using a denser solution (1.7 g cm^{-3}) and includes soil $< 2 \text{ mm}$ instead of $0.25\text{-}2 \text{ mm}$. This study found no effect of mulching on POC (measured only in Spring 2017). MBC is also sensitive to mulching (Luo et al., 2015) and like POC, MBC showed no treatment differences in either location indicating no lasting effects of BDMs on MBC after two years of use (Figure II-7). Any treatment effects caused by mulch microclimates may have been diminished after the eight months between the end of the growing season and the time of the Spring 2017 sampling. Other studies have reported gains in MBC under mulch treatments after two to four years of mulch use (Li et al., 2004; Zhou et al., 2011).

For C pools measured across all five seasons, there were significant effects of BDMs on EOC and respiration only in TN (Table II-4). The controls and BDMs had mixed effects on EOC in TN making interpretations difficult: BioAgri, PE, no-mulch, PLA/PHA, and Cellulose fell

between Organix (lowest EOC) and Naturecycle (highest EOC). In summary, BDMs caused no net loss in C in TN or WA. One explanation for having a more positive effect on C pools than PE is that BDMs typically possess soil moistures and temperatures between PE and no-mulch (Moreno & Moreno, 2008). BDMs have a higher water vapor permeability than PE due to intrinsic properties (Bilck et al., 2010; Martin-Closas et al., 2008). Combined with the higher likelihood of ripping and tearing, BDMs are more permeable to heat loss and evaporative water loss than PE and therefore do not heat the soil or increase C mineralization rates to the degree of PE mulch.

BDMs as a carbon input into soil

POM in this study includes a unique input (BDM material) whose fate as small particles (micro and nanoplastics) has not been well characterized. As the intermediate between litter inputs and stabilized organic matter, POM is a functionally important pool of C (Haynes, 2005). A large input of a C-rich substrate to this pool could affect C cycling by leading to a priming effect or N immobilization (Chen et al., 2014; Li et al., 2014a). However, BDM material was calculated to be a smaller input of C than hypothesized. Using the measured C content of the BDMs, the annual theoretical C input from BDM incorporation was calculated. BDM C was found to be a small annual addition of C (1 - 6%) compared to typical crop residue C inputs. Mulch microplastics were measured in POM using microplastic isolation techniques. The microplastic analysis showed that BDM mulch microplastics (1-2 mm) make up <1% of POM by mass and only ~1% of the C in the POC (Table II-5). Microplastic concentrations were also low compared to theoretical maxima (Appendix A, Table II-13). The microplastics recovered in the no-mulch treatment might be from wind blowing mulch between plots or residues from previous PE mulch use on the field. PLA/PHA had the highest accumulation of microplastics ($36.6 \pm 16.4 \text{ kg ha}^{-1}$) over two years, approximately double the residues of the other BDM mulches (Figure II-8). BDMs are produced with a range of thicknesses based on the purpose of use so it would be expected that thicker BDMs would have proportionately higher microplastics concentrations than thinner BDMs from adding more material to the soil. Naturecycle ($57 \pm 1.8 \text{ }\mu\text{m}$) and PLA/PHA ($37 \pm 1.4 \text{ }\mu\text{m}$) are slightly thicker than BioAgri ($29 \pm 1.2 \text{ }\mu\text{m}$) and Organix ($20 \pm 0.7 \text{ }\mu\text{m}$) so this could help explain why PLA/PHA accumulation is higher (Hayes et al., 2017). In addition, PLA/PHA is slower to biodegrade in ambient soil temperatures compared to other BDMs and

biodegrades more quickly at higher temperatures (40°C) (Rudnik & Briassoulis, 2011). The threshold set for polyethylene mulch residues by the Chinese standard “Limit and test method for residual quantity of agricultural mulch film” is 75 kg ha⁻¹ but concentrations as high as 502 kg ha⁻¹ have been found (Zhang et al., 2016). These values are not directly comparable because BDM microplastics are a short-term concern designed to mineralize 90% to CO₂ and microbial biomass within two years. However, while present in soil, BDM microplastics could have possible negative effects proposed for other microplastics in soil such as adsorbing chemicals (hydrophobic organic pollutants like agrochemicals) (Duis & Coors, 2016; Horton et al., 2017; Rillig, 2012; Steinmetz et al., 2016) and leaching plasticizers (Horton et al., 2017; Souza Machado et al., 2018a). Tracking accumulation of BDMs in different climates and soil types is important because manufacturers often test biodegradability only in lab conditions, with pure polymers, or in composting conditions (Brodhagen et al., 2017). The importance of *in situ* experiments was shown when residues of BDMs were measured and the degradation varied widely across climates from ~2% percentage of mulch area remaining (PMAR) in TX to 49% in TN to 89% in WA after 12 months (Li et al., 2014b). Our method is a first estimate at how much BDM remains in the soil in the form of microplastics (1-2 mm).

Limitations

Soil C pools have a wide range of turnover times and the length of this two-year study may not be long enough to capture changes caused by repeated usage of BDMs. In addition, this study is missing an important C pool in the BDM C cycle because C inputs from plant biomass were not measured. Microplastic concentrations were reported in units of kg plastic ha⁻¹ because plastic residue standards for soils report these units. However, this may not be the best representation of microplastic data. Studies have suggested reporting recovered microplastic data in units proportional to the sample size (Rios Mendoza & Balcer, 2018). In this case, it may be better to use the units mm² g⁻¹ dry soil (Appendix A, Table II-13). In addition, we used visual identification to isolate microplastics which is consistent with other microplastic protocols but verification using spectroscopy would help confirm the microplastics found in each plot were from the respective BDMs in that plot. We used visual clues to help differentiate BDM microplastics, but chemical properties of the mulches would help confirm these findings. This

verification would also help explain the source of microplastics that were recovered in the no-mulch and PE treatments.

Conclusions

The purpose of this study was to measure shifts in C pools to determine if mulching influences decomposition rates or inputs to the soil C cycle. In the first two years of mulching, the different soil climates in TN and WA affected the change in slower turnover pools (POXC, peroxide-resistant C) more than mulch treatment. In TN and WA, the higher moisture and temperature patterns under PE compared to no-mulch, could be lowering the StC fraction C in TN and TOC in WA. All mulch treatments were subject to seasonal effects in the faster turnover pools (EOC, respiration) likely due to drought and pH. The results of this study support that climate has a large influence over C cycling as shown by the effect of location on intermediate to stable C pools and the effect of season on labile C pools.

BDMs had no effects on C pools in WA. The few effects of BDMs on C pools in TN were net gains in C over two years. Soil C plays a large role in soil health and longer-term studies on the effects of mulching (BDMs and PE) on C pools are necessary for evaluating if mulching is promoting the accumulation or decomposition of C. Other studies have also found minimal effects of BDMs on soil health (Li et al., 2014a; Sintim et al., 2019). In particular, it is important to continue studying how mulching specifically affects C cycling in different climates. In this study, the effects of mulch treatment on C pool came down to location and season meaning the geography of where these mulches are used is important.

We learned that BDMs were not a significant input of C to the soil compared to crop residues and that microplastics are accumulating but make up a small percentage by mass and C content (<1%) of the functionally important POM fraction. To our knowledge, this is the first time that BDM microplastics have been quantified in soils

Appendix A

Table II-8. Spring 2015 C pool size ($\mu\text{g C g}^{-1}$ dry soil) for total organic carbon (TOC), peroxide-resistant carbon, permanganate oxidizable carbon (POXC), and carbon content of the particle size fractions (PSF). Values are the mean of 3-4 replications (SE).

	TOC		Peroxide-resistant C		POXC		PSF (TN only)		
Treatment	TN	WA	TN	WA	TN	WA	Coarse sand	Fine sand	Silt and clay
No-mulch	6240. (899)	10824 (270)	559.5 (80.0)	1230 (75.5)	168.5 (29.4)	419.6 (6.4)	431.4 (161)	1565 (305)	2995 (401)
Polyethylene	6316 (606)	10942 (291)	428.5 (47.1)	1288 (86.2)	162.2 (17.5)	424.8 (26.9)	461.7 (111)	1317 (275)	3301 (234)
Cellulose	6404 (389)	11390(93.0)	382.7 (9.0)	1363 (41.9)	155.7 (21.1)	396.9 (32.7)	524.97 (137)	1717 (393)	2885 (203)
PLA/PHA	6788 (979)	11102 (315)	440.8 (20.1)	1340.0 (54.9)	171.2 (35.8)	431.0 (26.4)	554.0 (159)	1735 (451)	3062 (405)
BioAgri	6548 (310)	11167 (135)	434.6 (25.1)	1274 (29.8)	164.0 (19.0)	400.8 (10.0)	397.2 (160)	1619 (201)	3086 (133)
Naturecycle	7401 (681)	11162 (276)	535.2 (39.0)	1426 (80.7)	216.3 (41.1)	401.4 (2.8)	534.7 (72.9)	1608 (128)	3384 (138)
Organix	6372 (314)	10724 (317)	552.8 (81.5)	1311 (47.4)	188.6 (6.1)	434.8 (28.9)	440.7 (17.6)	1645 (84.1)	3187 (294)

Table II-9. Paired t-test statistics comparing mean values of total organic carbon (TOC), peroxide-resistant carbon, permanganate oxidizable carbon (POXC) in Spring 2015 and Spring 2017

Treatment	TOC		Peroxide-resistant C		POXC	
	TN	WA	TN	WA	TN	WA
	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value
No-mulch	0.12	0.73	0.78	0.33	0.0059**	0.21
Polyethylene	0.90	0.0033**	0.051*	0.49	0.22	0.26
Cellulose	0.91	0.12	0.068*	0.26	0.14	0.88
PLA/PHA	0.96	0.104	0.056*	0.59	0.55	0.66
BioAgri	0.76	0.76	0.079*	0.87	0.0087**	0.24
Naturecycle	0.39	0.44	0.24	0.13	0.23	0.17
Organix	0.20	0.81	0.40	0.82	0.080*	0.19

*Indicates significance at an alpha level of 0.05-0.1.

**Indicates significance at an alpha level of <0.05.

Table II-10. Spring 2017 C pool size ($\mu\text{g C g}^{-1}$ dry soil) for total organic carbon (TOC), peroxide-resistant carbon, permanganate oxidizable carbon (POXC), and carbon content of the particle size fractions (PSF). Values are the mean of 3-4 replications (SE).

	TOC		Peroxide-resistant C		POXC		PSF (TN only)		
Treatment	TN	WA	TN	WA	TN	WA	Coarse sand	Fine sand	Silt and clay
No-mulch	6911 (818)	10706 (331)	589.7 (113)	1348 (122)	230.9 (21.3)	449.8 (21.4)	757.3 (96.4)	1440. (304)	3896 (470)
Polyethylene	6354 (412)	10314 (335)	620.1 (65.5)	1224 (34.8)	189.9 (11.2)	409.2 (12.2)	612.0 (50.0)	1295 (105)	3244 (205)
Cellulose	6380. (380)	10582 (297)	459.5 (18.4)	1157 (134)	200.3 (5.0)	404.5 (29.0)	664.8 (181)	1440. (232)	3630 (388)
PLA/PHA	6819 (515)	10564 (136)	592.3 (37.1)	1371 (47.8)	188.6 (17.2)	416.4 (9.6)	612.7 (157)	1286 (130)	3790 (272)
BioAgri	6749 (439)	11303 (540)	579.7 (40.1)	1289 (62.7)	207.7 (16.6)	418.8 (17.1)	774.9 (127)	1421 (219)	3803 (321)
Naturecycle	8008 (567)	10738 (226)	670.1 (128)	1272 (9.0)	260.6 (7.2)	439.0 (14.6)	832.3 (104)	1821 (152)	4136 (325)
Organix	6903 (40.7)	10793 (341)	598.8 (46.9)	1316 (27.6)	234.3 (12.7)	394.7 (10.5)	649.3 (87.4)	1574 (83.0)	4103 (33.5)

Table II-11. Paired t-test statistics comparing mean values of carbon content of the particle size fractions (PSF) in TN in Spring 2015 and Spring 2017

	PSF (TN only)		
Treatment	Coarse sand	Fine sand	Silt and clay
	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value
No-mulch	0.023**	0.31	0.053*
Polyethylene	0.16	0.95	0.79
Cellulose	0.58	0.53	0.071*
PLA/PHA	0.102	0.32	0.054*
BioAgri	0.19	0.57	0.064*
Naturecycle	0.18	0.17	0.049**
Organix	0.11	0.61	0.053*

*Indicates significance at an alpha level of 0.05-0.1.

**Indicates significance at an alpha level of <0.05.

Table II-12. Spring 2017 C pool size ($\mu\text{g C g}^{-1}$ dry soil) of extractable organic carbon (EOC), microbial biomass carbon (MBC), and particulate organic carbon (POC). Values are the mean of 3-4 replications (SE).

	EOC		MBC		POC
Treatment	TN	WA	TN	WA	TN only
No-mulch	31.0 (1.6)	25.6 (3.2)	97.7 (27.7)	108 (20.6)	545.2 (64.7)
Polyethylene	41.3 (10)	29.3 (5.4)	75.4 (11.8)	83.7 (8.7)	419.6 (23.2)
Cellulose	33.0 (4.4)	24.4 (1.2)	127 (11.1)	97.9 (13.5)	NA
PLA/PHA	45.7 (11)	25.6 (1.5)	96.2 (30.5)	142 (44.0)	450.7 (147)
BioAgri	32.2 (1.7)	28.6 (1.8)	129 (43.7)	111 (2.6)	674.1 (98.6)
Naturecycle	45.6 (5.8)	22.4 (1.1)	60.9 (21.5)	96.7 (18.8)	639.8 (108)
Organix	31.2 (6.3)	27.2 (2.0)	79.8 (20.2)	95.2 (11.7)	432.5 (52.2)

Table II-13. Calculated and theoretical mulch loading calculations after two years of mulch incorporation. Microplastics measured only in the coarse sand fraction size (0.25-2 mm) have been corrected for size based on a control (1-2 mm). The theoretical values are potential residue accumulations (mulch residues of all sizes) to show an upper limit of mulch found in the soil. Values are mean (SE).

Treatment	Mean # of particles g⁻¹ dry soil	Mean total area (mm²) of particles g⁻¹ dry soil	Recovered microplastics (1-2 mm) (kg mulch ha⁻¹)	Max theoretical residues, with biodegradation (kg mulch ha⁻¹ in the Spring)^a	Max theoretical residues, no biodegradation (kg mulch ha⁻¹)
No-mulch	0.026 (0.0088)	0.038 (0.014)	1.77 (0.65)	0.00	0.00
Polyethylene	0.028 (0.0094)	0.065 (0.035)	3.02 (1.6)	0.00	0.00
Cellulose	NA	NA	NA	NA	NA
PLA/PHA	0.26 (0.075)	0.68 (0.30)	36.6 (16)	107	309
BioAgri	0.18 (0.072)	0.44 (0.22)	19.5 (9.8)	88.3	254
Naturecycle	0.18 (0.068)	0.42 (0.18)	21.6 (9.1)	103	296
Organix	0.18 (0.085)	0.42 (0.24)	16.3 (9.2)	76.8	221

^aCalculated using biodegradation scheme laid out in Miles et al. (2017): 15% degradation during the growing season, additional 30% biodegradation after 1 year, and a total of 90% biodegradation after 2 years and these dimensions: 1.2m row width, 100m plot, 15cm tillage depth (SCRI narrative). The biodegradation scheme was adjusted for our sampling time (Spring season after two mulch incorporations).

III. THE TEMPERATURE SENSITIVITY OF BIODEGRADABLE PLASTIC MULCHES TO MICROBIAL DECOMPOSITION

A version of this chapter is being prepared for publication in:

Marie E English, Sreejata Bandopadhyay, Marife Anunciado, Dr. Hayes, Dr. DeBruyn, Dr. Larry Wadsworth, Dr. Schaeffer. “The temperature sensitivity of biodegradable plastic mulches to microbial decomposition” in *Soil Biology and Biochemistry*.

Statement on effort: Planning this experiment was a group effort by all authors. Marie English was responsible for the carbon pool measurements as well as the thesis/manuscript below. The DeBruyn lab was responsible for qPCR, enzyme measurements and PCA analysis. The Hayes lab was responsible for the physiochemical measurements of the mulches.

Abstract

Biodegradable plastic mulches (BDMs) provide a practical alternative to the polyethylene (PE) plastic ground covering widely used in agriculture for increasing crop yields. A successful BDM must remain intact during the growing season and subsequently break down completely when tilled into the soil. Due to the many biochemical reactions involved in biodegradation, temperature is a main driver of BDM break-down. This paper will focus on the role of temperature on the biodegradation of BDMs. A manipulative incubation experiment was performed to study the biodegradability of three BDMs: a polylactic acid/ polyhydroxyalkanoate (PLA/PHA) experimental product, a starch/co-polyester blend, and a cellulosic paper mulch at three different soil temperatures (10, 20, and 30°C). The evolution of carbon dioxide (CO₂) was measured biweekly for 16 weeks to track biodegradation. Soil carbon (C) pools, soil microbial properties, and chemical properties of the BDMs were measured pre- and post-incubation to help understand the underlying mechanisms of biodegradation. Higher temperatures (30°C) demonstrated higher microbial utilization of BDMs through increased cumulative CO₂ production above the no-mulch control, as well as altered chemical properties of the mulches after the soil incubation. The starch/co-polyester blend began to biodegrade at 20°C, while PLA/PHA did not biodegrade until 30°C. There was also evidence of preferential substrate use at 10°C shown by different soil EEA and isotopic signatures ($\delta^{13}\text{C}$) at this temperature. The significant influence of soil temperature on the rate and extent of biodegradation of BDMs could help determine the appropriate climates for use of BDMs.

Introduction

Background

Plastic mulches have proven effective at increasing crop yields (Diaz-Perez et al., 2005; Ghimire et al., 2018) by lengthening the growing season, decreasing evaporation, and protecting crops against weeds (Lamont, 1993; Steinmetz et al., 2016). Conventional plastic mulches are made from polyethylene (PE); therefore, the spread of plastic mulch use in agriculture contributes to the rapidly expanding use of plastics around the globe (1.5 million tons of plastic in 1950 to 348 million tons of plastic in 2017) (PlasticsEurope PERMG annual reports in (Statista, 2018)). To address the abundance of plastics and resulting plastic waste problem, researchers are developing biodegradable plastic alternatives. Biodegradable plastic mulches (BDMs) provide the same benefits as PE films without leading to plastic waste because they can be tilled into the soil, or composted, at the end of the season (Brodhagen et al., 2015; Kyrikou & Briassoulis, 2007; Steinmetz et al., 2016). The key feature of BDMs is that they are composed of biopolymers that will be biodegraded by naturally occurring bacteria (ASTM International, 2012a).

The biodegradation process of BDMs starts above the soil surface with abiotic degradation from UV and hydrolysis reactions (Hayes et al., 2017; Kasirajan & Ngouajio, 2012). Below the surface, soil microbes depolymerize the polymers and utilize the produced monomers for energy and biomass production (Brodhagen et al., 2015; Kasirajan & Ngouajio, 2012). Carbon dioxide (CO₂) is released as an end-product of this process under aerobic conditions (Six et al., 2006). The rate of biodegradation in soil varies widely based on intrinsic physiochemical properties of BDMs such as molecular weight, film additives, and the structural complexity of the polymers as well as physical access of the BDMs to microbes (Barragan et al., 2016; Kasirajan & Ngouajio, 2012). In addition, environmental factors can affect rates of the metabolic processes involved in biodegradation. Similar abiotic and biotic environmental factors such as soil temperature, moisture, and microbial community composition regulate both soil organic matter (SOM) and BDM biodegradation (Blagodatskaya & Kuzyakov, 2008; Davidson & Janssens, 2006; Kasirajan & Ngouajio, 2012; Paustian et al., 1997). However, there is a lack of studies using soil (vs. compost or a pure culture) and complete BDM films (vs. pure polymers) to better understand the effects of the aforementioned factors on biodegradation (Barragan et al., 2016; Shah et al., 2008).

Temperature, in particular, has been identified as a major factor of interest in the study of BDM biodegradation (Brodhagen et al., 2015). Higher temperatures will likely lead to increased BDM biodegradation by increasing microbial activity (Alster et al., 2018) and increasing the activity rates of degradative enzymes (Wallenstein et al., 2011). The current standards for testing biodegradability in ambient soils involve incubations that measure biodegradation rates at only one temperature (ASTM International, 2012b; Dentzman & Hayes, 2019) limiting the knowledge of the role of temperature in biodegradation of these materials in soil. One way to describe the effect of temperature on a system is to measure the temperature sensitivity of that system. A good descriptor of temperature sensitivity, Q_{10} is the extent that a rate increases with a 10 degree Celsius increase in temperature (Chen et al., 2010). The decomposition of SOM is very temperature sensitive (Davidson & Janssens, 2006; Fierer et al., 2005). This temperature sensitivity varies based on microbial access to SOM and molecular complexity of the SOM (Davidson & Janssens, 2006; Wagai et al., 2013). The temperature sensitivity of BDMs in soil is unknown but will likely be similarly affected by protection from microbial access and complexity (of the polymer).

Because the biodegradation of SOM and BDMs occurs simultaneously and relies on similar microorganisms, it is important to study the interconnectedness of the mulch and soil as one system, referred to in this study as the soil-mulch system. Existing studies and methods for measuring biodegradation do not provide a complete picture of the interaction between SOM biodegradation, BDM biodegradation and temperature. A defined temperature sensitivity of the soil-mulch system would help describe the most important mechanisms involved in biodegradation at different temperatures.

Research objectives and hypotheses

In order to determine which climates are more, or less conducive to BDM biodegradation, more research is needed on optimal soil temperature ranges (Brodhagen et al., 2015; Saadi et al., 2013; Shah et al., 2008). To address this problem, the mechanisms of BDM biodegradation in soil in addition to the effects of temperature must be better understood. The main objective of this study was to understand the temperature sensitivity of the decomposition of BDMs in soil.

We hypothesize that higher temperatures will increase microbial activity and lead to increased BDM material utilization. We expect the addition of BDMs to increase the temperature sensitivity of the soil-mulch system. In addition, we hypothesize higher temperatures and higher microbial activity will lead to higher extracellular enzyme activity and a higher bacterial and fungal abundance. We predict a decline in carbon (C) storage pools with higher temperatures and higher microbial activity.

Approach

This study combined soil C dynamics, soil microbial properties, and chemical properties of the BDMs to better describe how temperature alone affects the biodegradation of the BDMs. A jar incubation experiment was used to monitor biodegradation at three different temperatures (10, 20, 30°C). Soils were incubated with the BDMs and the biodegradability of the mulch materials was measured by tracking CO₂ evolved over 16 weeks. The Q₁₀ value was calculated to measure the temperature sensitivity of the soil-mulch system at different temperatures. Complementary C pools (total organic carbon (TOC) and extractable organic carbon (EOC)) were also measured to see how the interaction of temperature and BDMs affects C storage pathways. In addition, the $\delta^{13}\text{C}$ value of the bulk soil was measured to potentially track the transformation of mulch-derived C into TOC using stable isotope analysis. To characterize how temperature and mulch biodegradation affect decomposer activity, bacterial and fungal copy numbers were measured along with extracellular enzyme activities (EEA). Gel permeation chromatography (GPC), polydispersity index (*PDI*), thermogravimetric analysis (TGA) and nuclear magnetic resonance (NMR) spectroscopy were measured to confirm and quantify the decomposition of BDM constituents.

Materials and methods

Jar incubation set-up

A manipulative incubation experiment designed with guidance from ASTM, International D5338 was conducted to investigate *in situ* biodegradability of two types of mulch: a polylactic acid/polyhydroxyalkanoate experimental product (PLA/PHA) and a starch/co-polyester blend (BioAgri) at different temperatures (10, 20, and 30°C) (ASTM International, 2011). A no-mulch control was included with only soil along with a 100% biodegradable cellulose positive control

used to ensure soil conditions were conducive to microbial biodegradation. Soils were collected and sieved (< 2 mm) from the East Tennessee Research and Education Center (Plant Science Unit) in Knoxville, TN in the winter of 2016 (Table III-1). Soils were allowed to pre-incubate for 1 week prior to treatment and moisture content was corrected to 50% water holding capacity (WHC). Moisture content was monitored and maintained throughout the experiment by adding deionized, sterile water to a constant mass in the jar. Soil pH was measured before and after incubation (Appendix B, Table III-7). BDM material was added in 2x2 cm pieces (ASTM International, 2011) at a rate of 0.25% w/w ratio of mulch to soil. Soil (100 g at 50% WHC) and BDMs (250 mg) were combined in 500 mL mason jars. Jars were sealed with lids fitted with septa sampling ports. The mass of material added (250 mg) was approximately 20% (125 mg of C/jar) of the total organic C content of the soil (550 mg of C/ jar). This ratio created ample soil to mulch contact while maintaining a high enough substrate concentration to differentiate between CO₂ respired from mulch C and background CO₂ respired from soil (El-Din Sharabi & Bartha, 1993). We tested 0.5% w/w ratio of mulch to soil and there was extensive mulch on mulch contact so the ratio was decreased to 0.25% w/w in order to increase soil to mulch contact.

Test materials

Weathered BDMs were removed from the field after one season from the Knoxville, TN field site in Fall 2015 and were used in this incubation to represent the degradation status of the mulch before it is tilled into the soil. The BDMs were cleaned gently with brushes and water according the procedure described in Hayes et al. (2017). The weathered BDMs tested included BioAgri (BioBag Americas, Dunevin, FL, USA) a commercially available starch/ polyester blend made from feedstock: Mater-Bi ® (grade M2351; starch-polyester (PBAT) blend) and an experimental PLA/PHA product made from polylactic acid/ polyhydroxyalkanoate. The positive un-weathered cellulose (WeedGuardPlus /Sunshine Paper, Aurora, CO, USA) control was included along with a negative control of soil only (ASTM International, 2011). The starting C contents of the BDMs were $43.50 \pm 0.60\%$, $45.96 \pm 4.76\%$, and $45.98 \pm 0.90\%$ for weathered PLA/PHA, weathered BioAgri, and un-weathered cellulose respectively (Hayes et al., 2017).

Table III-1. Pre-incubation soil physical and chemical properties

Soil property	
Type	Typic Hapludult
Sand ^a	59.9%
Silt ^a	23.5%
Clay ^a	16.6%
pH ^b	5.47
CEC (cmol _c kg ⁻¹) ^b	7.40
NO ₃ (ppm) ^b	7.75
P (bray method) (mg kg ⁻¹) ^b	84.3
Total organic C content (mg C g ⁻¹) ^c	6.32
Extractable organic C (μg C g ⁻¹) ^c	49.0

^aValues from Sintim et al. (2019)

^bLab analysis on Fall 2015 soils from the field site where soils were sampled for this experiment. Mean of 4 replications (no-mulch plots).

^cLab analysis on soils collected for this experiment (Winter 2016). Mean of 2 replications (no-mulch plots).

CO₂ evolution

Evolution of CO₂ was used as a proxy for biodegradation of BDM materials (Saadi et al., 2013). An infrared gas analyzer, LI-COR, 820 (LI-COR Biosciences, Lincoln, NE, USA) was used to measure the CO₂ concentration of the headspace air. Headspace air was collected using a 2.5 mL syringe. The concentration of CO₂ was measured after 3 days, 10 days, and then every two weeks for a total of 16 weeks. During each of these samplings, a standard curve of known CO₂ concentration standards was created in order to calculate CO₂-C evolved. If the concentration of the headspace exceeded 3% CO₂, the incubation jars were opened, then resealed to prevent a build-up of CO₂ from affecting microbial activity (Fierer & Schimel, 2002). CO₂ concentration was measured before and after opening the jars to accurately calculate cumulative CO₂ evolution. The ideal gas law was used to calculate CO₂ evolved (C_s) (Eq. III-1 & III-2).

CO₂ evolution calculation:

$$C_s = (C_m * n * M_w) / M_s \quad (\text{Equation III-1})$$

$$n = PV / RT \quad (\text{Equation III-2})$$

where: C_s = µg CO₂-C g⁻¹ dry soil

C_m = CO₂ ppm

n = Number of moles in the headspace

R = The universal ideal gas law constant (8.314 L-kPa/mol-K)

T = Temperature in Kelvins (299.15)

P = Site pressure (98 kPa for Knoxville, TN)

V = Volume of headspace

M_w = Molecular weight of CO₂-C (12g C mol⁻¹ C)

M_s = Mass of soil in the jar (g dry soil)

Theoretical % biodegradation

The theoretical % biodegradation was calculated by dividing the theoretical mulch-derived CO₂ (CO₂ evolved in mulch treatments - CO₂ evolved in no-mulch control) by the total amount of C that was added in the form of mulch C (Eq. III-3).

Theoretical percent biodegradation:

$$\%bio_{theo} = \left(\frac{M_{CO_2-C, mulch} - M_{CO_2-C, no\ mulch}}{M_{C, mulch}} \right) \quad (\text{Equation III-3})$$

where: %bio_{theo} = Theoretical % biodegradation

$M_{CO_2-C, mulch}$ = CO₂-C evolved in mulch treatments (μg C g⁻¹ dry soil)

$M_{CO_2-C, no-mulch}$ = CO₂-C evolved in no-mulch treatments (μg C g⁻¹ dry soil)

$M_{C, mulch}$ = Total amount of C added in the form of mulch C (μg C g⁻¹ dry soil)

Q₁₀ calculation

Temperature sensitivity (Q₁₀) for each of the treatments was calculated from the CO₂ evolution rates. This value was calculated using the final cumulative CO₂ value. The Q₁₀ equation was from Thiessen et al. (2013) (Eq. III-4):

$$Q_{10} = \left(\frac{k_2}{k_1} \right)^{(10/(T_2 - T_1))} \quad (\text{Equation III-4})$$

where: k₂ = Rate of decomposition at the higher temperature

k₁ = Rate of decomposition at the lower temperature

T₂ = Higher temperature (°C)

T₁ = Lower temperature (°C)

Carbon pool measurements

Total carbon content and isotopic ratio

Total organic C content of the soil (% C) and δ¹³C of soil were measured by weighing out 80 mg of dried (105°C), milled soil and analyzing it on a combustion module (CM-CRDS, Costech Analytical Tech Inc. Valencia, CA, USA) coupled to a Picarro cavity ring-down spectrometer (CRDS, Picarro G2121-I, Picarro Inc., Santa Clara, CA, USA). With each analysis, acetanilide and sugar standards were run to create a standard curve. The isotopic ratio (R_{sample}) was converted to units relative to the international standard (Vienna PeeDee Belamnite) (Eq.III-5).

Converting ¹³C stable isotope ratio into units of δ¹³C:

$$\delta^{13}\text{C} = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - (1) \right) * (1000) \quad (\text{Equation III-5})$$

where: R_{sample} = Isotopic ratio of the sample

R_{standard} = Isotopic ratio of the Vienna PeeDee Belamnite standard

Extractable organic carbon

For this study, extractable organic C was defined as organic C extractable in 0.5 M K_2SO_4 (aq). Field moist soil (10 g) was shaken with 40 mL 0.5 M K_2SO_4 . Extracts were frozen for analysis by persulfate digestion. Briefly, 2 mL sodium persulfate was added to 2 mL of sample in a 10 mL glass vial with a septa cap and metal crimp seal and was oxidized overnight at 80 °C (Doyle et al., 2004; Hood-Nowotny et al., 2010). After one hour of cooling, CO_2 concentration in the headspace was measured using a LICOR 820 infrared gas analyzer. To determine how much extractable organic C was recovered from the reaction, a standard curve of known concentrations of potassium hydrogen phthalate were measured along with the samples. A blank control was prepared with no soil and the blank was subtracted from the values.

Microbial measurements

Quantitative PCR

DNA was extracted from soil before and after the 16-week incubation using MoBio PowerLyzer® PowerSoil® DNA Isolation kit using manufacturer's instructions. 100 μL of DNA was stored at -20°C till qPCRs were conducted. Quantitative PCRs (quantitative polymerase chain reaction) were conducted using Femto™ Bacterial and Fungal DNA quantification kits. Femto bacterial/fungal qPCR premixes (18 μL) were added to PCR plates. 2 μL bacterial/fungal DNA standards were added into appropriate wells. 1 μL of soil DNA were added to proper wells containing mastermix (contains primers, DNA polymerase, nucleotides). All samples were analyzed in triplicate. Blanks/No template controls (2 μL) were also added to the premix in appropriate wells. qPCR plates were sealed with an optically transparent sealing film that are compatible with the real-time/quantitative PCR instrument being used. The qPCR plates were centrifuged to eliminate bubbles and to bring any droplets to bottom of the well.

Extracellular enzyme assays

A fluorescence microplate enzyme assay was conducted using fluorescent substrates to analyze enzyme activities in soil before and after the incubation similar to the methods described in Steinweg et al. (2012). Five enzymes were assayed at room temperature (25°C), namely β -D-cellubiosidase (CB) (cellulose degradation), β -glucosidase (BG) (sugar degradation), leucine aminopeptidase (LAP) (protein degradation), N-acetyl- β -glucosaminidase (NAG) (chitin degradation) and β -xylosidase (XYL) (hemicellulose degradation). Fluorescent substrates used were 4-MUB- β -D-cellobioside, 4-MUB- β -D-glucopyranoside, L-leucine-7-amido-4-methylcoumarin hydrochloride, 4-MUB-N-acetyl- β -D-glucosaminide, 4-MUB-B-D-xylopyranoside respectively.

In this assay, soil slurries were prepared in a sodium acetate trihydrate buffer whose pH was matched closely with the soil pH. 800 μ L of soil slurry was pipetted into 96 deep-well plates. Separate plates were prepared for MUB (4-methylumbelliferone) and MUC (7-amino-4-methylcoumarin) standard curves for each sample. The standards and substrates (200 μ L) were added to respective soil slurries. The plates were sealed and inverted to mix the contents. After incubation for 3 hours (room temperature), the substrate and standard plates were centrifuged at 1500 rpm. The supernatants were then pipetted into black 96 well plates and the fluorescence was measured at excitation wavelength (365 nm) and emission wavelength (450 nm).

An esterase assay was conducted using a lipase esterase assay by Margesin et al. (2002). The protocol was standardized to a 1.5 mL microcentrifuge tube-based assay versus a test tube assay described in the paper. Saturation curves were conducted to understand the optimal substrate volume needed to add to the soil-buffer system. In short, 0.1 g of soil was added to tubes and assay was conducted in triplicate. Phosphate buffer (100 mM, 375 μ L) was added to the soil and pre-warmed in water bath at 30°C for 10 minutes. 25 μ L of substrate p-nitrophenyl butyrate (optimized using saturation curves) was added to each tube. Alongside, standard curves were prepared for each sample using p-nitrophenol. After addition of substrates and standards, the contents were mixed and tubes incubated at 30°C for exactly 10 minutes. Reaction was stopped by cooling on ice for 10 minutes. Finally, tubes were centrifuged at 2000 g at 4°C for 5 minutes. Supernatants in tubes were pipetted into 96 well plate and absorbance of the released pNP in

samples and controls were read at 400 nm. Controls without soil were also prepared and served as blanks. Standards were treated the same way as samples.

Chemical properties of the mulches

Nuclear Magnetic Resonance Spectroscopy

NMR was performed on the PLA/PHA mulches after 16-week incubation in the soil to quantify the amount of PLA and PHA and the percentage of two different monomeric units contained within PHA. Two mulch sample replications were analyzed for each temperature treatment using a 400 MHz spectrometer from Varian, implementing a pulse width of 30° for ^1H . For each sample, 15-20 mg of mulch was dissolved in 800 μL of CDCl_3 containing 1% trimethylsilane as an internal standard.

Gel Permeation Chromatography

GPC was used to determine the weight-averaged molecular weight (M_w) and polydispersity index (PDI) of chloroform-soluble components from the PLA/PHA and BioAgri mulches, initially and after 16-week incubation in the soil. Two mulch sample replications were prepared and analyzed for each temperature treatment. Filtered solution (~ 20 mg) was injected into a dual-pump HPLC system (Varian, Walnut Grove, CA, USA) equipped with model Mark III evaporative light scattering detector (WR Grace, Deerfield, IL, USA), and a 300 x 7.5 mm column purchased (ID PL Gel mixed D, Agilent (Santa Clara, CA, USA,)). Chloroform was employed as mobile phase at a flow rate of 0.8 mL/min. BDM samples (~ 20 mg) were dissolved in 5 mL of chloroform and magnetically stirred for 1 hour. The samples were centrifuged at 10,000 rpm ($6149 \times g$) for 1 min and then passed through a 1.0 μm pre-filter and 0.20 μm nylon filters to remove chloroform-insoluble materials, such as adsorbed soil. Molecular weight values are reported as polystyrene molecular weight equivalents, based on EasiVial PS-H polystyrene standards from Agilent.

Thermogravimetric Analysis (TGA)

TGA provided information on the physical and chemical properties of the mulches using different heating stages. It was carried out for PLA/PHA and BioAgri mulches, initially and after the incubation using a Discovery TGA from TA Instruments. Testing temperature starts from

room temperature (25°C) to 600°C at a heating rate of 10°C min⁻¹ in an unsealed platinum sample pan under nitrogen atmosphere.

Statistical analysis

This study used a full factorial design with mulch and temperature as factors to isolate the effects of temperature on BDM decomposition dynamics. A two-way ANOVA was conducted that examined the effect of temperature and mulch treatment using RStudio for Mac desktop version 1.0.136. When the ANOVA indicated significant effects ($p < 0.1$), a *post hoc* analysis with Tukey's HSD (Honest significance test) was applied to compare the means. The experiment design included four replications of each treatment. Data were checked for normality using the Shapiro-Wilk test ($W > 0.9$) and equal variance using Levene's test ($\alpha = 0.05$). If these conditions were not met, the data were log or rank transformed appropriately. For the C and microbial measurements, the starting value of the soil was subtracted from the measured value after 16 weeks and this change score was used for analyses. Principal component analysis (PCA) was carried out on the EEA data using Primer 7 software version 7.0.13 (PRIMER-e, Quest Research Limited). Data was normalized to a mean and standard deviation and then Euclidean distances were used to create clusters of a group average plotted in a PCA plot. PERMANOVA was used to analyze the two-way design of treatment by temperature.

Results

Temperature effect on carbon dioxide evolution

Cumulative CO₂ evolution was used to compare the biodegradation of the mulch treatments and surrounding soil. There was a strong effect of temperature ($p < 0.0001$), mulch ($p < 0.0001$), and the interaction between temperature and mulch on CO₂ evolved ($p < 0.0001$) (Table III-2).

Within mulch treatments there was a significant increase in CO₂ evolved with increasing temperature. However, the no-mulch control did not show a significant increase in CO₂ respired from 20 to 30°C. From 10 to 20°C, the percent increase of CO₂ evolved from each treatment for no-mulch, cellulose, PLA/PHA, and BioAgri was 157%, 210%, 208%, 138% respectively.

Cellulose had the highest % increase from 10 to 20°C. From 20 to 30°C, the percent increase of CO₂ evolved from each treatment for no-mulch, cellulose, PLA/PHA, and BioAgri was 42%,

Table III-2. The effects of mulch treatments (PLA/PHA, BioAgri, no-mulch, cellulose), temperature (10, 20, 30°C) and their interaction on CO₂ evolved, the change in carbon pools, and the change in microbial properties over the incubation.

	CO ₂ evolved		TOC		$\delta^{13}\text{C}$		EOC ^a		Log bacterial copy #		Log fungal copy #	
	<i>p</i> -value	F stat	<i>p</i> -value	F stat	<i>p</i> -value	F stat	<i>p</i> -value	F stat	<i>p</i> -value	F stat	<i>p</i> -value	F stat
Temperature	<0.0001**	373	<0.0001**	13.0	<0.0001**	146.5	0.48	0.53	0.69	0.37	0.063*	2.99
Mulch	<0.0001**	184	0.016**	3.95	0.76	0.39	0.18	1.88	0.057*	2.75	0.26	1.40
Temperature*Mulch	<0.0001**	32.2	<0.0001**	11.0	<0.0001**	6.66	0.16	2.06	0.60	0.77	0.50	0.91

*Indicates significance at an alpha level of 0.05-0.1.

**Indicates significance at an alpha level of <0.05.

^aStatistics were only performed on 10 and 20°C treatments

67%, 86%, 56% respectively. PLA/PHA had the highest % increase from 20 to 30°C. Within temperature treatments (10°C and 20°C), only cellulose had higher mean CO₂ respired than the control. Within the 30°C temperature treatment, all BDM treatments had significantly more CO₂ respired than the control (Figure III-1). The cumulative CO₂ of the no-mulch control was subtracted from the cumulative CO₂ of the mulch treatments to calculate theoretical % biodegradation. At 30°C, significantly more CO₂ was evolved in the mulch treatments over the control. Additional work is needed to fully quantify the proportion of respiration from mulch degradation and SOM degradation. Despite this uncertainty, theoretical % biodegradation was calculated (Table III-3). With each increase in 10°C, BioAgri showed an approximate doubling in theoretical % biodegradation (4% to 8% to 15%). For PLA/PHA from 20 to 30°C there was a much larger increase in theoretical % biodegradation (3% to 14%) than 10 to 20°C (0% to 3%). The cellulose control showed 68% biodegradation after 16 weeks at 30°C, validating that the jar incubation conditions were conducive to biodegradation.

Temperature sensitivity of the microbial community (Q₁₀)

To address the temperature sensitivity of the whole soil community with and without mulch, the Q₁₀ value was calculated using the change in respiration rates from 10-20°C, 20-30°C, and 10-30°C. A mean of four replicates was used to calculate Q₁₀ and error propagation was used to calculate the standard deviations. Although not significant, the effect of temperature led to differences in mean Q₁₀ value while the effect of mulch treatment did not have an effect on the mean Q₁₀ values. The change in temperature from 10-20°C led to a higher mean Q₁₀ value for all mulch treatments compared to that from 20-30°C. The mean (SD) no-mulch Q₁₀ value decreased from 2.57 (0.22) (10-20°C) to 1.42 (0.28) (20-30°C) and the BDM treatments showed a similar pattern. The Q₁₀ > 2 for 10-20°C means the rate of biodegradation more than doubles with this increase in temperature (Figure III-2).

Isotopic analysis of bulk soil

The change in δ¹³C of bulk soil (Figure III-3) was measured to see if mulch C with a unique signature was being incorporated into SOM. The isotopic (δ¹³C) signature of PLA/PHA (-13.0 ‰) is more enriched than the bulk soil making it a possible candidate for tracing studies that

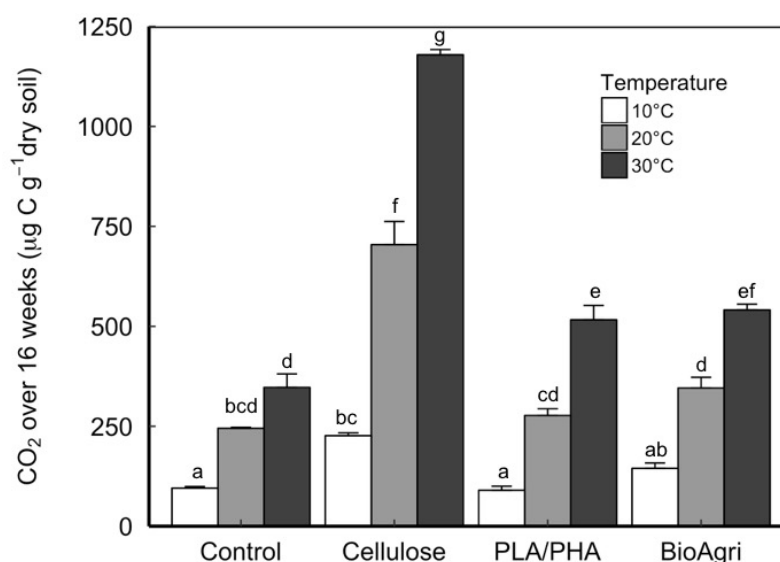


Figure III-1. Cumulative carbon evolved ($\mu\text{g CO}_2\text{-C g}^{-1}$ dry soil) from each mulch treatment throughout the incubation (16 weeks). Each bar represents a mean of 3-4 replications and error bars are standard error. Lower case letters indicate interaction effects at an alpha level < 0.05 .

Table III-3. Theoretical % biodegradation of BDMs. The second column is the amount of C that was added in the form of mulch C. The third column is the theoretical mulch CO_2 which is the amount of CO_2 from mulch C (CO_2 evolved in mulch treatments - CO_2 evolved in no-mulch control). The fourth column is theoretical % biodegradation which divides column two by column one.

Treatment	Starting mulch C ($\mu\text{g C g}^{-1}$ dry soil)	Theoretical mulch	
		$\text{CO}_2\text{-C}$ ($\mu\text{g C g}^{-1}$ dry soil)	Theoretical % biodegradation
BioAgri-10°C	1300	50	4%
BioAgri-20°C	1300	101	8%
BioAgri-30°C	1300	194	15%
Cellulose-10°C	1300	131	10%
Cellulose-20°C	1300	459	35%
Cellulose-30°C	1300	832	64%
PLA/PHA-10°C	1243	-5	0%
PLA/PHA-20°C	1243	32	3%
PLA/PHA-30°C	1243	169	14%

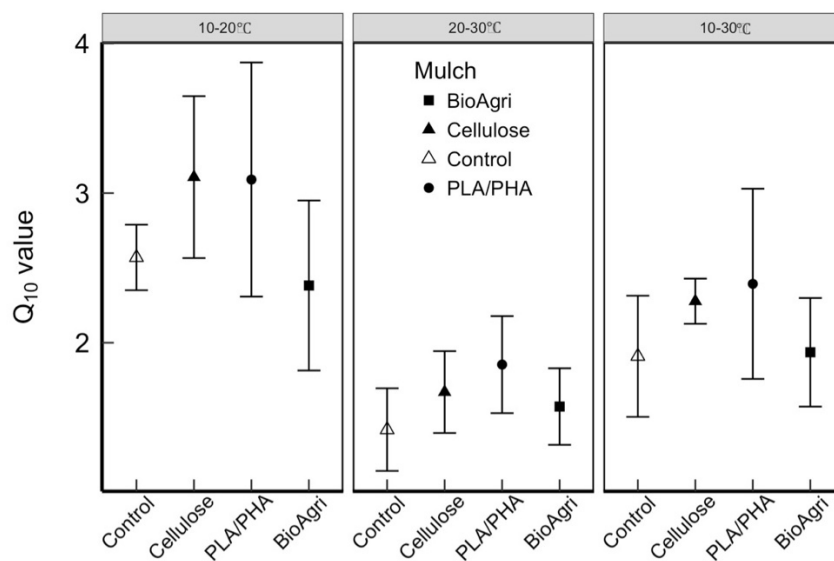


Figure III-2. Temperature sensitivity (Q_{10}) of cumulative CO_2 evolved. The Q_{10} was calculated using the final rate of cumulative CO_2 evolved over 16 weeks. Each point represents a mean of 3-4 replications and error bars are standard deviation.

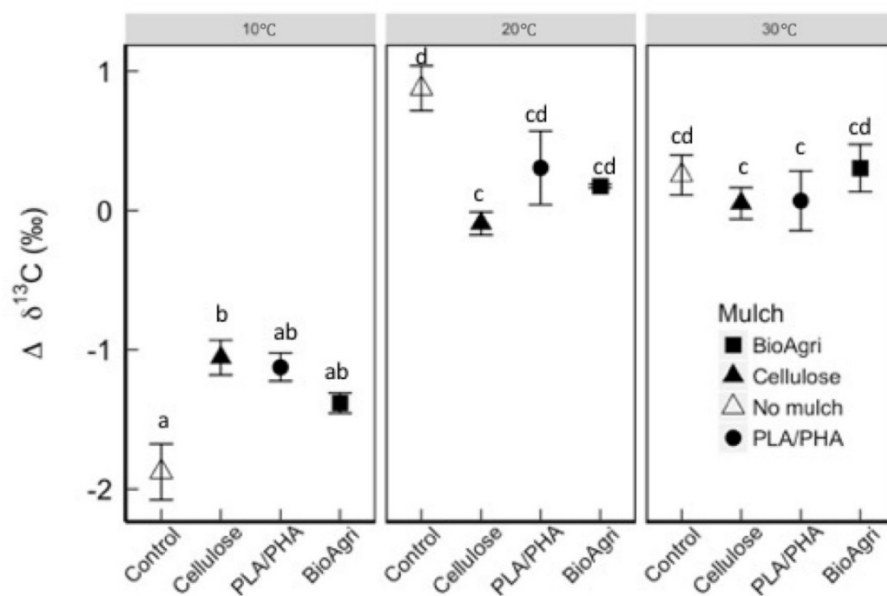


Figure III-3. The change in $\delta^{13}\text{C}$ in soil over the incubation. Each point represents a mean of 3-4 replications and error bars are standard error. Lower case letters indicate interaction effects at an alpha level < 0.05 .

could follow mulch C into different SOM pools. However, there was no effect of mulch treatment on the change in bulk soil $\delta^{13}\text{C}$ ($p = 0.76$) indicating no difference between the PLA/PHA soil and other treatments. Temperature had a significant effect on the change in bulk soil $\delta^{13}\text{C}$ ($p < 0.0001$). The interaction between temperature and mulch also had a significant effect on the change in $\delta^{13}\text{C}$ ($p < 0.0001$) (Table III-2). Within each mulch treatment, the $\delta^{13}\text{C}$ change at 10°C was significantly different from the 20°C and 30°C change. At 20°C and 30°C, there was little change in $\delta^{13}\text{C}$ over the 16 weeks as shown by the values close to zero. At 10°C, the bulk soil $\delta^{13}\text{C}$ was depleted as shown by the negative change over 16 weeks. The effect of temperature on $\delta^{13}\text{C}$ change may be an indicator that different substrates are being utilized by microbial communities at 10°C compared to 20°C and 30°C.

Temperature effect on carbon storage

Total organic carbon

There was a strong effect of temperature ($p < 0.0001$), mulch ($p = 0.016$), and the interaction of temperature and mulch ($p < 0.0001$) on the change in TOC over 16 weeks (Table III-2). A net loss in TOC was observed for all treatments except cellulose 20°C treatment which had an increase in TOC over the 16 weeks. Within mulch treatments, the no-mulch control had a significantly higher loss of TOC at 20°C compared to 10°C as would be expected based on CO_2 evolution at these temperatures. The same trend was not observed within the mulch treatments. Within the 10°C and 30°C treatments, there were no significant differences between mulch treatments (Figure III-4). However, within the 20°C treatment level, all mulch treatments had significantly lower TOC loss compared to the no-mulch control. In this study, not all TOC lost was captured as CO_2 . The mean (SE) CO_2 loss across all treatments versus the mean TOC loss was 139 ± 31.6 vs. $897 \pm 62.6 \mu\text{g C g}^{-1}$ dry soil (10°C), 393 ± 106 vs. $560 \pm 278 \mu\text{g g}^{-1}$ (20°C), and 646 ± 183 vs. $942 \pm 75.2 \mu\text{g g}^{-1}$ (30°C).

Extractable organic carbon (EOC)

Temperature ($p = 0.48$), mulch treatment ($p = 0.18$) and the interaction between mulch and temperature did not have a significant effect on the change in EOC over 16 weeks ($p = 0.16$) (Table III-2). Statistical analysis could only be performed on 10 and 20°C treatments for BioAgri, PLA/PHA and no-mulch treatments due to sample contamination (Figure III-5).

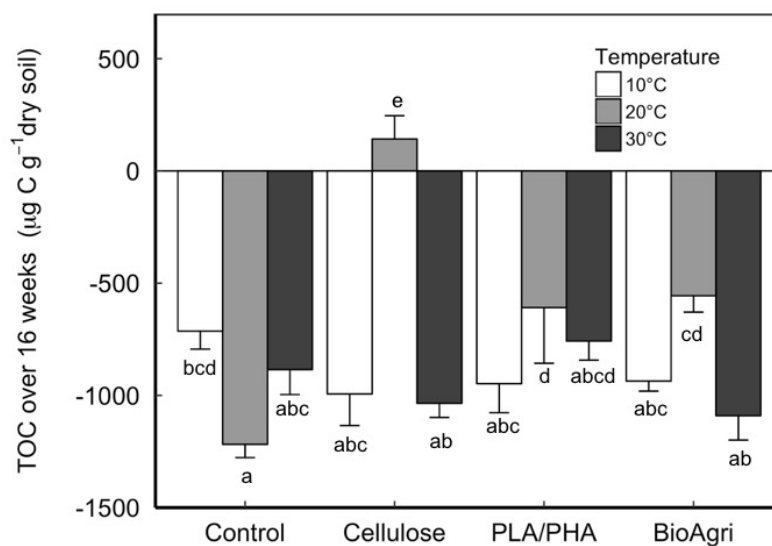


Figure III-4. The change in total organic carbon (TOC) over the incubation. Each bar represents a mean of 3-4 replications and error bars are standard error. Lower case letters indicate interaction effects at an alpha level < 0.05.

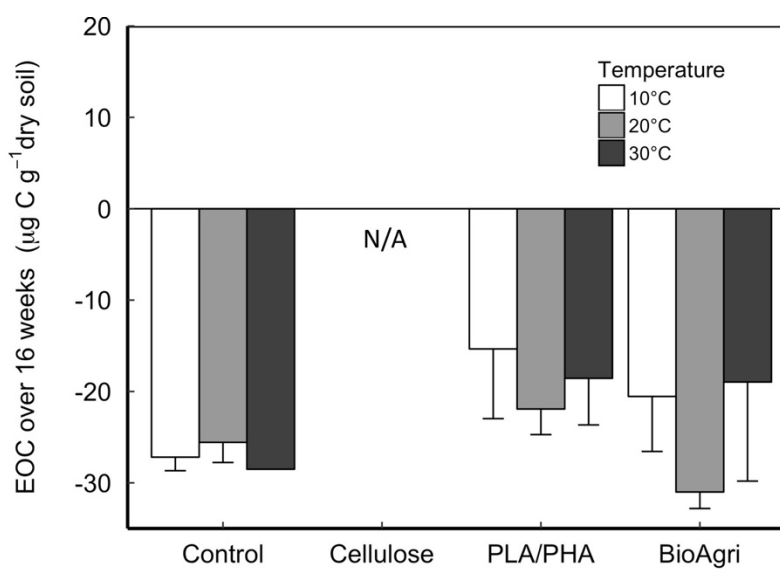


Figure III-5. The change in extractable organic carbon (EOC) over the incubation. Each bar represents a mean of 3-4 replications and error bars are standard error. Statistics were only performed on 10 and 20°C. Cellulose data were not available. No letters indicate the absence of statistically significant differences in mulch and temperature treatments (Table III-2).

Bacterial and fungal copy numbers

Bacterial and fungal copy number were measured to show how microbial abundances change with temperature. The effect of mulch treatment on bacterial copy number was significant ($p = 0.057$) while the effect of temperature ($p = 0.69$) and the interaction were not significant ($p = 0.60$) (Table III-2). Bacterial copy numbers showed a net increase over the incubation for all treatments except PLA/PHA (Figure III-6). Fungal copy numbers were more variable with the 30°C treatment showing a decrease over the incubation (Figure III-7). The effect of temperature on fungal copy number was significant ($p = 0.063$) (Table III-2) while the main effect of mulch ($p = 0.26$) and the interaction between temperature and mulch were not significant ($p = 0.50$).

Temperature effect on microbial extracellular enzyme activity

Enzyme assays were conducted on initial and final stage soil samples to test for changes in enzyme activity over the incubation. Temperature ($p = 0.036$) and mulch ($p = 0.006$) treatment had a significant effect on the change in enzymatic activity over 16 weeks while the interaction between these two factors did not ($p = 0.44$). Within each mulch treatment, there was separation with temperature (Figure III-8). No-mulch and cellulose treatments in particular were affected by temperature with the 30°C treatments clustered on the right side and the 10°C treatments on the left side of the PCA plot. *Post-hoc* analysis confirmed the change in extracellular enzyme activity (EEA) was significantly different between 10°C and 30°C ($p = 0.02$). In addition, EEA between 20°C and 30°C was close to significantly different ($p = 0.106$) while the difference between 10°C and 20°C was not ($p = 0.24$). *Post-hoc* analysis also showed cellulose had a significantly different enzymatic profile from all other mulch treatments and PLA/PHA was also significantly different from the no-mulch control (Table III-4).

Temperature effect on chemical properties of BDMs

Thermogravimetric analysis

TGA showed minor differences in soil-incubated PLA/PHA (Figure III-9, top left). DTG (derivative thermogravimetry) also showed minor differences with the most significant change occurring for 30°C and very minor changes for 10°C and 20°C (Figure III-9, top right). At a soil incubation of 30°C, the DTG peaks of PHA and PLA shifted to a lower temperature, which likely reflects a loss of molecular weight given that the crystallinity of PLA and PHA in the

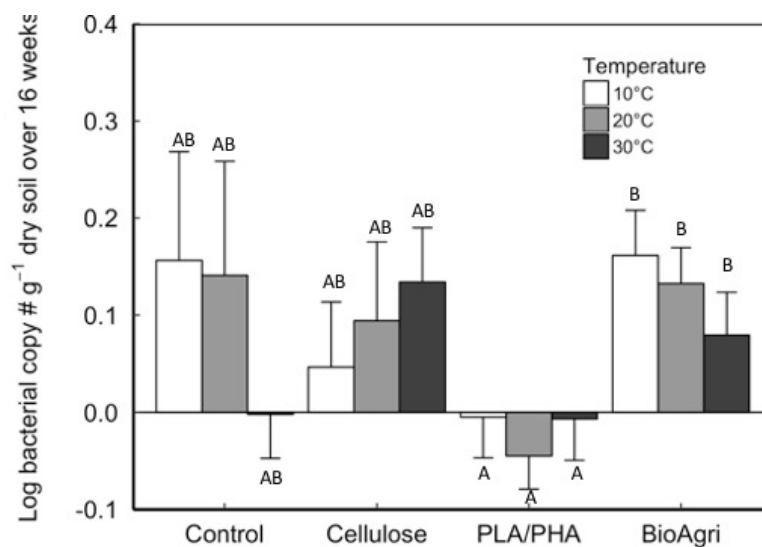


Figure III-6. The change in log bacterial copy number over the incubation. Each bar represents a mean of 3-4 replications and error bars are standard error. Uppercase letters represent a main effect of mulch treatment at an alpha level of 0.05- 0.1 ($p = 0.057$).

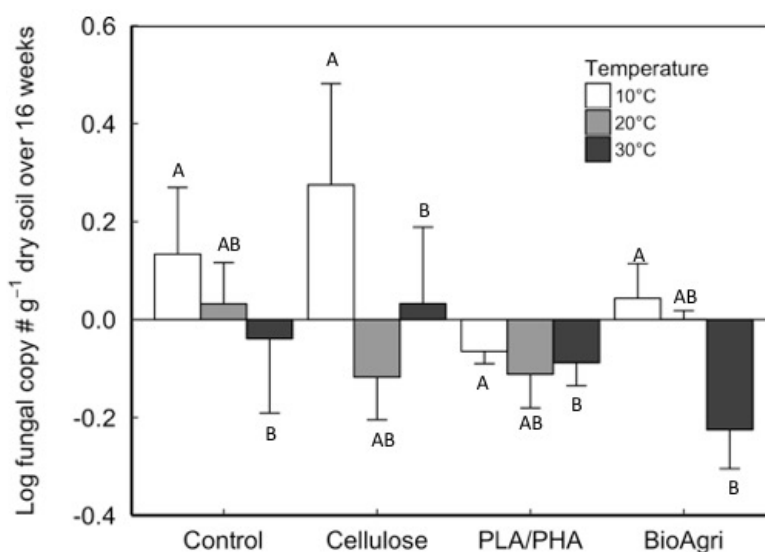


Figure III-7. The change in log fungal copy number over the incubation. Each bar represents a mean of 3-4 replications and error bars are standard error. Uppercase letters represent a main effect of temperature at an alpha level of 0.05- 0.1 ($p = 0.063$).

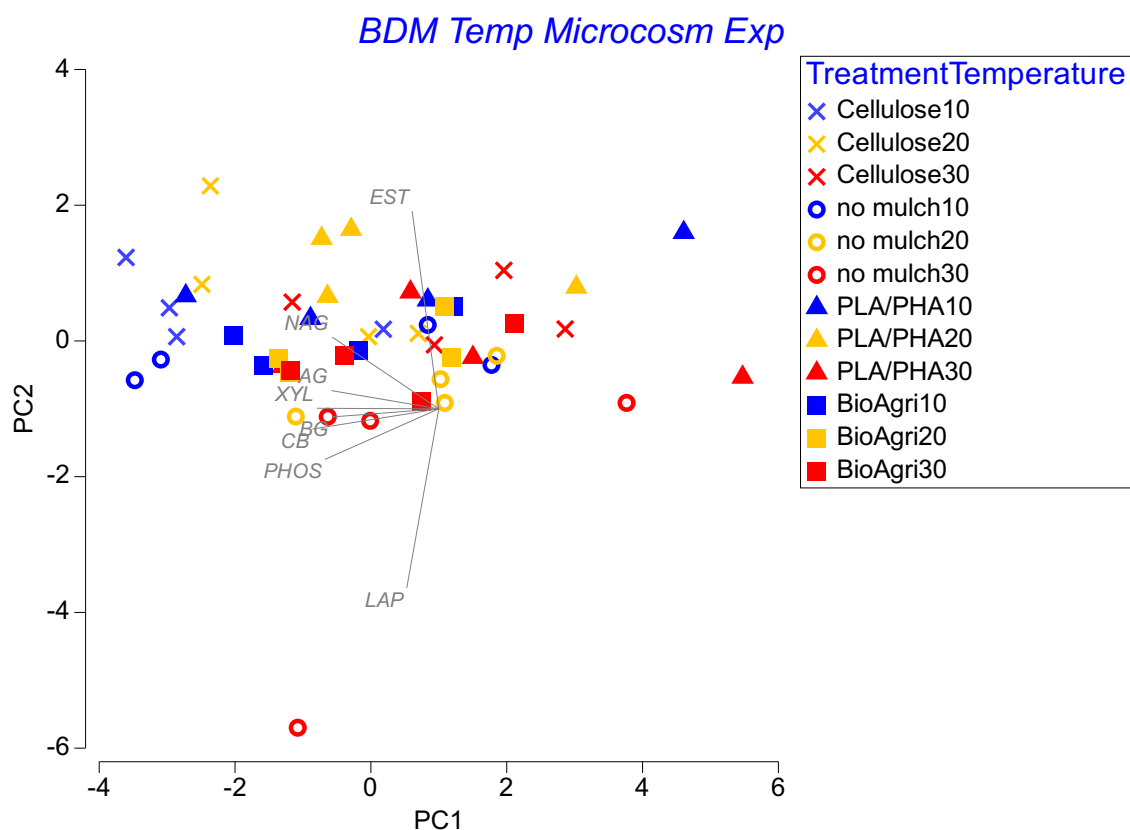


Figure III-8. PCA plot of the change in extracellular enzyme activity over the incubation.

Table III-4. PERMANOVA results of a pairwise comparison of the effects of mulch on the change in extracellular enzyme activity.

	Cellulose		PLA/PHA		BioAgri	
	<i>p</i> -value	t stat	<i>p</i> -value	t stat	<i>p</i> -value	t stat
No-mulch	0.008**	2.09	0.052*	1.67	0.107	1.39
BioAgri	0.032**	1.87	0.119	1.51		
PLA/PHA	0.037**	1.93				

*Indicates significance at an alpha level of 0.05-0.1.

**Indicates significance at an alpha level of <0.05.

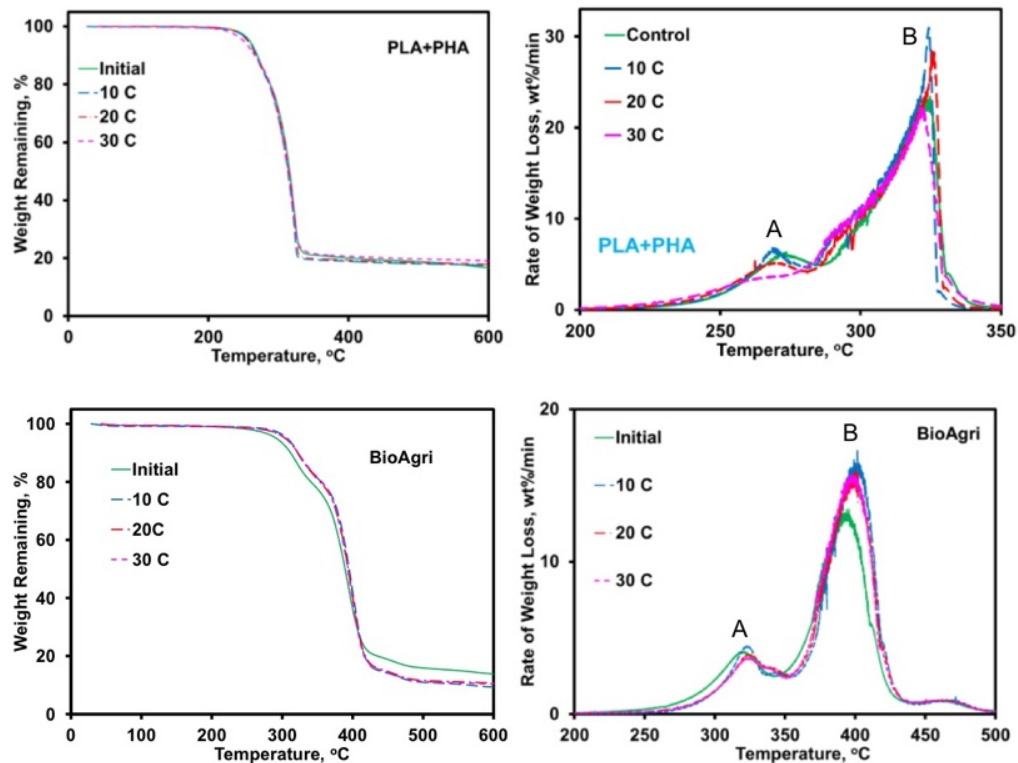


Figure III-9. TGA thermograms (*left*) and DTGs (*right*) for PLA/PHA (*top*) and BioAgri (*bottom*) (Note that time and temperature are directly proportional to each other, since the heating rate was constant, 10°C/min). For PLA/PHA, two heating stages occur (that slightly overlap), at 270°C (A) and 320°C (B), which represent PHA and PLA, respectively, while for BioAgri, heating stages A and B refer to starch and PBAT, respectively.

initial mulch are low. The curve also shows the mass fraction of PHA decreases via incubation at 30°C (Figure III-9, top right). In contrast to the trends observed in DTGs for PLA/PHA, for BioAgri, soil incubation at all three temperatures led to a shift at both heating stages (Figure III-9, bottom right). The DTG shows the proportion of PBAT in BioAgri (80% PBAT with minor starch components) increases with soil incubation (Figure III-9, bottom right, peak B). TGAs showed the weight % remaining at 600°C after soil incubation is lower than the initial mulches (Figure III-9, bottom left). It is expected that the weight remaining at 600°C would increase, due to utilization of the polymeric components as C sources for microorganisms. One explanation is that the inorganic components of the BDMs were leached into the soil, thereby explaining the decrease of weight % remaining at 600°C due to soil incubation.

Nuclear magnetic resonance

It was not until 30°C, that the weight % (Wt %) PLA increased from 72% to 83%, suggesting the preferred utilization of PHA as C source by microorganisms at this temperature (Table III-5), consistent with TGA analysis. The starting composition of PLA/PHA is roughly 70% PLA and 30% PHA.

Gel permeation chromatography

After 16 weeks of soil incubation, at 30°C, a slight decrease of weight-averaged molecular weight (M_w) and decrease of polydispersity index (PDI) was observed for PLA/PHA (although the differences may not be statistically significant) (Table III-6). For PLA/PHA, the slight increase of M_w at 10°C is attributed to microorganisms utilizing low-molecular oligomers that reside in more amorphous morphological regions. For BioAgri, M_w decreased from 10°C to 30°C and PDI decreased. The difference between 20°C and 30°C, though, is relatively low. The decrease in PDI is a counter-intuitive trend. PDI reflects the distribution of M_w values for the polymeric strands. Typically, the initial mulch's polymer will possess a low PDI , near 1.0, and as depolymerization proceeds, the PDI usually increases.

Table III-5. Composition of PLA mulch pre- and post- incubation (Nuclear magnetic resonance (NMR) spectroscopic analysis). Values are mean (SD).

Sample	Wt %PLA
Initial	72.4 (0.3)%
10°C	73.0 (0.6)%
20°C	72.5 (0.1)%
30°C	83.5 (4.2)%

Table III-6. Change in molecular weight-related properties of PLA/PHA mulch during incubation (Gel permeation chromatography (GPC) analysis). Values are mean (SD) with two replications. M_w = weight-averaged molecular weight (in kiloDaltons), PDI = polydispersity index.

Sample	PLA/PHA		BioAgri	
	M_w , kDa	PDI	M_w , kDa	PDI
Initial	184 (7)	1.78 (0.05)	273 (1)	3.69 (0.05)
10 °C	193 (5)	1.81 (0.03)	261 (3)	3.37 (0.01)
20 °C	185 (2)	1.79 (0.01)	225 (2)	3.02 (0.15)
30 °C	174 (10)	1.89 (0.04)	217 (10)	3.07 (0.04)

Discussion

A standard method of measuring biodegradability was combined with measures of soil C storage, soil microbial properties and chemical properties of the BDMs pre- and post-incubation to study the mechanisms of biodegradation at different temperatures. In addition, the temperature sensitivity of the soil-mulch system was calculated to look more closely at how temperature affects the biodegradation rates. As hypothesized, an increase in microbial utilization of BDMs was observed at 30°C for both BDMs (PLA/PHA and BioAgri) as shown by significantly higher CO₂ evolved over the no-mulch control (Figure III-1) as well as altered chemical properties of the mulch material remaining after the soil incubation (Figure III-9 and Table III-6).

Biodegradation of the soil-mulch system was sensitive to temperature possibly due to preferential substrate use at 10°C shown by different soil EEA (Figure III-8) and isotopic signatures ($\delta^{13}\text{C}$) (Figure III-3) at this temperature compared to 30°C. Surprisingly, the addition of BDMs did not appear to affect the temperature sensitivity of the system but did however affect bacterial abundance (Table III-2) and EEA (Table III-4) in the soil-mulch system.

BDM biodegradation was higher at 30°C for both BDMs (PLA/PHA and BioAgri) as shown by significantly higher CO₂ evolved over the no-mulch treatment. Consistent with the explanation that the higher CO₂ in the BDM treatments is coming from mulch material and not soil C, BDM treatments did not lose significantly more TOC than the no-mulch control at 30°C (Figure III-4). Thus, CO₂ evolved was used to calculate the theoretical % biodegradation. For PLA/PHA, there was no biodegradation observed at 10°C, 3% at 20°C, and 14% at 30°C, a result that showed the change from 20 to 30°C was important for PLA/PHA biodegradation (Appendix B, Figure. III-10). For BioAgri, there was an even doubling in theoretical % biodegradation with each 10°C increase in temperature (4 to 8 to 15%). It is important to note that while the theoretical % biodegradation increased at 20°C, the CO₂ evolved for BioAgri was not significantly higher than the control until 30°C.

Increases in biodegradation at higher temperatures were likely a result of the increased microbial activity observed with higher temperatures. Soil respiration (CO₂ evolved) is an indicator of microbial activity (Haney et al., 2008a). The optimal temperature for microbial activity (29.4°C), determined by meta-analysis (Alster et al., 2018), is where CO₂ evolved was observed to be

highest in this study (Figure III-1). Within the no-mulch control treatment, there was a significant increase in soil respiration from 10 to 20°C indicating an increase in microbial activity from temperature alone. Even though microbial activity significantly increased with temperature, bacterial copy number was not significantly affected by temperature (Table III-2). This respiration increase without an increase in microbial abundance indicates decreased C use efficiency (CUE) at higher temperatures from an increase in microbial maintenance respiration (Conant et al., 2011; Manzoni et al., 2012; Wagai et al., 2013). Consistent with a review that found fungi are more sensitive to temperature than bacteria and have a lower optimal metabolizing temperature (Alster et al., 2018), this study found significantly higher fungal copy numbers at lower temperatures (Figure III-7). This is key for cool climates because fungi are important BDM degraders that cause physical disruption of the polymers through hyphae that “penetrate” the mulch (Griffin, 1980). Different temperatures will select for particular microorganisms who can function optimally as degraders at that temperature. At higher temperatures, this study found evidence of a lowered CUE due to an increase in microbial activity accompanied by a decrease in fungal abundance and no change in bacterial abundance.

The temperature sensitivity (Q_{10}) was calculated to summarize how microbial activity, EEA, and the presence of different BDMs affected biodegradation in the soil-mulch system versus the no-mulch control. As predicted by the Arrhenius equation, the Q_{10} values in this study decreased with increasing temperature (Figure III-2) (Conant et al., 2008; Davidson & Janssens, 2006; Mikan et al., 2002; Reichstein & Janssens, 2009). The Q_{10} value for the change in temperature from 10 to 30°C across all mulch treatments was similar to the typical biological Q_{10} value of 2 (Davidson & Janssens, 2006). Although not statistically significant, the higher Q_{10} value from 10 to 20°C would suggest that microbial degradation (of mulch and/or SOM) is more sensitive at lower (10 to 20°C) than higher (20 to 30°C) temperatures. Since PLA/PHA showed greater biodegradation from 20 to 30°C, the decomposition of SOM is likely being reflected in the higher temperature sensitivity of the system from 10 to 20°C due to the concentration of mulch added being too small (only 0.25% by weight and 20% of the C in the jar) to influence whole system temperature sensitivity. This would also help explain why, contrary to our hypothesis, the addition of BDMs did not make the system more temperature sensitive. Substrate quality plays a large role in the temperature sensitivity of decomposition (Davidson & Janssens, 2006). Based

on the C temperature-quality hypothesis, lower quality litters (higher lignin:N) are more temperature sensitive (Wallenstein et al., 2012). BDMs are a low quality litter (high C:N), so it was hypothesized that BDMs would increase the temperature sensitivity of the system. However, there was no evidence that BDMs made the soil-mulch system more temperature sensitive.

EEA provides an idea of the specific substrates utilized at different temperatures by measuring the maximum potential of microorganism's extracellular enzyme activity present in a soil. Increased biodegradation at higher temperatures was complemented by a significant difference in EEA at 30°C compared to 10°C indicating microbes are targeting different substrates (BDMs or different types of SOM) at different temperatures. The mean EEA of most enzymes showed a decreasing trend with increasing temperatures (Appendix B, Figure. III-11). As soil temperatures increase and CUE declines, microbial biomass and EEA can also decline (Allison et al., 2010; Wallenstein et al., 2012). In addition, cellulose had significantly different EEA from the other mulch treatments (Table III-4). Looking at the cellulose treatment more in depth, the cellulose related EEA (β -D-cellubiosidase (CB)), hemicellulose degradation (β -Xylosidase (XYL)), and protein related EEA (leucine aminopeptidase (LAP)) at 10°C showed a mean increase in activity over the incubation compared to the decline in activity seen in other mulch treatments (Appendix B, Figure. III-11). One explanation for the increased activities of these enzymes at 10°C is that there was still cellulose material remaining in this treatment after 16 weeks. The cellulose 10°C treatment also showed the highest gain in fungal abundance over the incubation (Figure III-7) possibly because fungi are considered more effective cellulose degraders than bacteria (Horwath, 2015). These differences in the cellulose treatment compared to the other mulch treatments could indicate a shift in the functional potential of the community caused by the addition of cellulose. Given the importance of enzymatic hydrolysis of ester bonds in the biodegradability of many BDM polymers (Kim & Kim, 2008), it was surprising that higher esterase activity (EST) was not seen in the PLA/PHA nor BioAgri treatments which are composed primarily of polyesters (PLA, PHA, PBAT) and had material remaining after 16 weeks (Appendix B, Figure. III-11). Of concern, the no-mulch control had significantly different EEA than the cellulose and PLA/PHA treatments (Table III-4) indicating that background SOM degradation in the no-mulch control may not be the same as the background SOM degradation in these mulch treatments.

The stable isotope ($\delta^{13}\text{C}$) results provided additional evidence that temperature affected substrate utilization. Within each treatment, the change in $\delta^{13}\text{C}$ during the 16-week soil incubation was more negative for 10°C compared to the minor change experienced at 20 and 30°C (Figure III-3). The effect of the 10°C temperature treatment on the $\delta^{13}\text{C}$ value may be an indicator that different substrates are being utilized by microbial communities at 10°C compared to 20°C and 30°C. At 10°C, the bulk soil $\delta^{13}\text{C}$ signature was depleted (lower proportion of ^{13}C to ^{12}C) compared to the starting value of the soil. One possible explanation for this depletion is preferential decomposition of labile SOM compounds at lower temperatures (Boström et al., 2007). For example, lipids (-31.4 ‰) and lignin (-30.2 ‰) are more depleted compared to more labile polysaccharides like cellulose (-25 ‰) (Bowling et al., 2008). The lower $\delta^{13}\text{C}$ value at 10°C may be a result of stressed microbes preferentially utilizing ^{13}C enriched labile substrates resulting in a higher concentration of depleted C remaining at 10°C treatments. Combined, the $\delta^{13}\text{C}$ and EEA data suggest preferential use of labile C at lower temperatures independent of mulch treatment. However, the shift to more labile C at 10°C was not measured in one of the most labile soil C pools, EOC (Figure III-5) (Haynes, 2005). The lack of formation of EOC in the mulch treatment compared to no-mulch control treatment was likely because BDMs are a low-quality substrate (high C:N) and EOC is typically formed from higher quality substrate with a low C:N ratio (Cotrufo et al., 2013). Similar to other incubation studies, a net loss of EOC was observed over the incubation for all treatments (Galvez et al., 2012; Li et al., 2014c). Because the mulch treatments did not have significantly higher EOC, BDM C is likely entering a different C pool such as CO_2 respired or microbial biomass C.

As temperature increased, CO_2 evolution increased and TOC was expected to decrease; yet, at 20°C, PLA/PHA and cellulose lost significantly less TOC compared to 10°C (Figure III-4). Also at 20°C, all mulch treatments lost less TOC compared to the no-mulch control. The latter trend could be evidence of a negative priming effect where microbes prefer mulch C to SOM C. Priming is the short-term loss or addition of SOM caused by manipulations to soils (Kuzyakov et al., 2000). Unfortunately, there is insufficient information to prove or disprove priming in this experiment because the theoretical % biodegradation calculation does not differentiate between C respired from the mulch material and that from native soil C. Therefore, subtracting the C

respired from the background soil from that respired from the soil-mulch system does not account for the interactions that could be occurring between soil and the C-rich substrate (BDMs) such as priming (Blagodatskaya & Kuzyakov, 2008; Chen et al., 2014; El-Din Sharabi & Bartha, 1993). Microbial incorporation of mulch C can provide the most direct evidence of biodegradation but the current methods used to show microbial uptake are difficult and expensive (Chinaglia et al., 2018; Zumstein et al., 2018). Indirect methods such as changes in chemical properties of the BDMs, changes in TOC, and changes in EEA can help identify the sources of CO₂ (Chen et al., 2014).

Chemical property changes in the BDMs support the increased utilization of mulch material at higher temperatures. Similar to the theoretical % biodegradation, it was only at 30°C that there was a slight decrease in *M_w* and increase in *PDI* (Table III-6) representing depolymerization of PLA/PHA (Hayes & Wadsworth, 2016). In particular, NMR (Table III-5) and DTG (Table III-9) showed an increase in the relative % of PLA vs. PHA at 30°C indicating a preference for the PHA polymer as a substrate. PHA is used to lower the extent of crystallinity of PLA (Hu et al., 2008) because molecules of the PLA polymer reside in a tightly packed configuration that hinders microbial access at temperatures below the glass transition temperature (63.8 °C) (Vroman & Tighertz, 2009). Therefore, temperatures closer to the glass transition temperature increase the degradability of PLA. Similar to other studies, these results show soil temperatures of 30°C and higher can lead to greater biodegradation of PLA containing films (Ho & Pometto III, 1999; Rudnik & Briassoulis, 2011). For BioAgri, a change from 10°C to 20°C led to a major change of the chemical properties (*M_w*, Table III-6), while a further increase from 20°C to 30°C produced only a slightly greater extent of change. The proportion of PBAT in BioAgri increased with soil incubation at all three temperatures, demonstrating the preferred utilization of starch as a C source (Figure III-9). The decline in PHA in the PLA/PHA and starch in BioAgri reflects preferred use of lower molecular weight and lower crystalline morphological regions of polymers by microorganisms (Barragan et al., 2016; Dharmalingam et al., 2016; Shah et al., 2008).

Limitations

The length of the experiment (16 weeks) was not long enough for complete biodegradation of the BDM films which makes it difficult to create a C budget for the system. In addition, this study used a lower ratio of substrate added to soil (0.25% (w/w)) while other papers suggest a ratio of 0.5-1% (Bettas Ardisson et al., 2014; Galvez et al., 2012). The lower ratio was chosen for this study to maximize the soil to mulch contact but it may have led to SOM dynamics overshadowing the effects of mulch additions on soil C pools. Finally, it cannot be assumed that adding mulches does not influence the background CO₂ evolution (El-Din Sharabi & Bartha, 1993). Therefore, methods that use CO₂ evolved to measure % biodegradation should recognize these are an indirect measure and require further evidence of microbial substrate use.

Conclusions

Using CO₂ evolution (% theoretical biodegradation) and chemical property changes, this experiment showed that temperature can have a significant effect on microbial utilization of mulch material. Due to the higher cumulative CO₂ evolved in the mulch treatments compared to the control at 30°C and the chemical changes in the mulch materials, we conclude that BDM biodegradation was enhanced at higher temperatures. Temperature activated microbial activity led to the higher rates of biodegradation at higher temperatures. Although not significant, the higher temperature sensitivity and higher % increase in CO₂ evolved would suggest that the increase from 10 to 20°C is more important for the soil-mulch system than the 20 to 30°C increase. This could be due to different substrate use at 10 and 30°C as indicated by changes in $\delta^{13}\text{C}$ and different EEA at the two temperatures. Our results suggest that PLA/PHA and BioAgri had similar final % theoretical biodegradation, similar effects on C pools, and similar utilization of morphological regions of lower crystallinity, but large differences in biodegradation at 20°C. For BioAgri, there were chemical changes at 20 and 30°C and theoretical % biodegradation doubled with each 10°C increase in temperature while for PLA/PHA, a significant extent of biodegradation did not occur until 30°C. The wide variety of BDM compositions means different proprietary blends (feedstocks, production processes) can be affected differently by temperature. Our study shows the importance of optimizing mulches for different climates (Brodhagen et al. 2015) based on the temperature response of the BDMs.

Appendix B

Table III-7. Soil pH before and after incubation. Values are mean (SE).

Treatment	10°C	20°C	30°C
Initial (T= 0)	5.4 (0.03)		
No-mulch	6.5 (0.04)	6.1 (0.02)	6.3 (0.03)
Cellulose	6.9 (0.04)	7.0 (0.02)	6.4 (0.02)
PLA/PHA	6.7 (0.07)	6.5 (0.01)	6.6 (0.01)
BioAgri	6.9 (0.03)	6.5 (0.01)	6.3 (0.02)



Figure III-10. Visual degradation of PLA/PHA mulch fragments at 20°C (left) and 30°C (right). Remaining PLA/PHA mulch pieces after the 16-week incubation. For scale, the diameter of the tins is approximately 70 mm. More fragmentation and decomposition visible with the 30°C pieces than the 20°C mulch pieces.

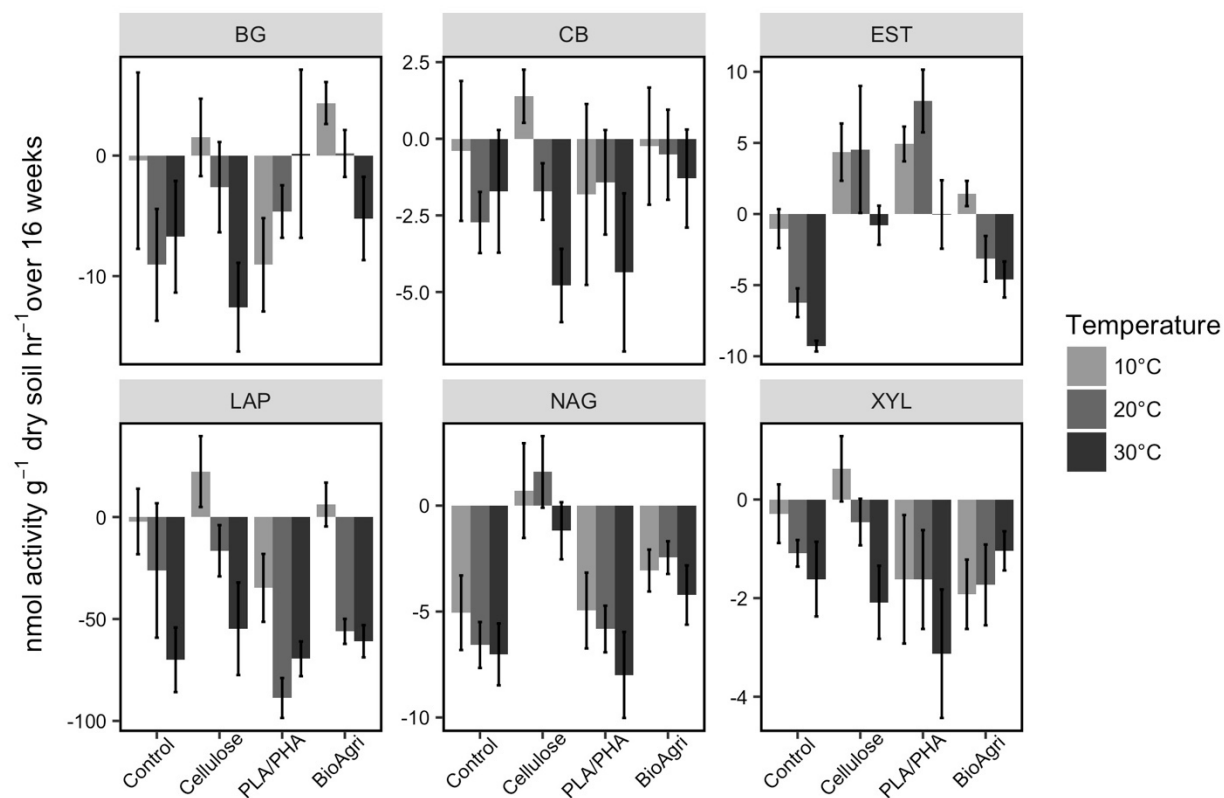


Figure III-11. Change in extracellular enzyme activity over the incubation. Six enzymes were assayed: β -Glucosidase (BG), β -D-cellubiosidase (CB), esterase (EST), leucine aminopeptidase (LAP), N-acetyl- β -Glucosaminidase (NAG) and β -Xylosidase (XYL). Each bar represents a mean of 3-4 reps and error bars are standard error.

IV. CONCLUSIONS AND RECOMMENDATIONS

Agricultural producers considering the adoption of BDMs have to consider the impact of this agricultural management technique on soil health and the fate of the BDM materials once tilled into the soil. While the crop yield benefits are clear, the long-term impacts on soil health are less clear. The first part of this study was aimed at better understanding the effects of mulching on one aspect of soil health, soil C cycling, by carrying out a field experiment with comprehensive C pool measurements in two diverse geographic locations. When assessing potentially unknown effects of a system on soil C cycling, it is important to look at the inputs, outputs, and internal cycling to determine the major controls. Since BDMs are tilled into the soil, they could provide an additional input of C; however, this study found BDM C to be a small above-ground input to the soil ecosystem. To complete a C budget associated with BDM use, a recommendation for future field studies would be to measure plant inputs. Higher yields have been shown to offset C losses from increased mineralization under mulching so plant inputs are an important input to the mulch system C cycle. Measuring POM or LFOC each Fall and Spring would complement the biomass input data to see if mulching affects the rate that these inputs are processed.

After two years, less C accumulated in the silt and clay fraction of the PE treatment compared to the no-mulch treatment in TN and was the only mulch to experience a net loss of TOC in WA. The trends we saw are likely due to increased mineralization from the higher soil moistures and temperatures in the PE treatment (especially early in the growing season) compared to the no-mulch treatments and could be indicative of longer-term negative effects of the use of PE mulch. The effects of BDMs on soil C after two years varied but showed no losses of soil C. Understanding the effects of mulching on the internal cycling of soil C was complicated by location and seasonal effects (e.g. pH and drought) having greater impacts than the effects of mulch treatment. For future studies, it is recommended to focus on one system to determine the effects of mulching on soil C balance while minimizing confounding environmental effects from climate and soil type.

This study found evidence of microplastic (1-2 mm diameter) accumulation in BDM plots. However, the measured microplastics made up a small percentage by mass and C (<1%) of the functionally important POM fraction. To our knowledge, this is the first time that BDM

microplastics have been quantified in soils. Further studies on BDM fate are important to better understand how BDM material biodegrades in different climates.

Temperature is a major control on biodegradation rates in soils. Soil temperatures vary widely across climates and seasons making it crucial to find a BDM product that breaks down in a reasonable amount of time under different temperature regimes. To address the concern of unknown biodegradation rates, the second part of this study was an interdisciplinary lab incubation experiment to determine key interactions associated with the biodegradation of BDMs at different temperatures. Biodegradability was measured by CO₂ evolution and combined with pre- and post- incubation measures of soil C pools, soil microbial properties, and chemical properties of the BDMs at different temperatures. The theoretical % biodegradation suggested that there was increased biodegradation at 30°C for PLA/PHA and BioAgri and chemical properties of the mulches confirmed this showing a preference for the more amorphous regions of the material (PHA and starch). Indirect and direct methods of measuring biodegradation in the soil-mulch system are important to help determine the source of CO₂ evolution with this method and ensure no significant priming effects.

In conclusion, the results of this study showed no significant effects of BDMs on soil C pools compared to no-mulch plots after two seasons of use and incorporation into the soil. This study provides a baseline for future, longer-term studies (4-10 years) to examine any lasting effects of BDM use on soil C pools. A method was created that combined a physical fractionation of soil with marine microplastic isolation methods to quantify BDM microplastics in soil. The presence of microplastics should be studied further based on possible negative effects of microplastics on soil health. Finally, this study examined the mechanisms of temperature induced BDM biodegradation and found evidence of microbes utilizing different substrates at 10 and 30°C in the soil-mulch system. BDM biodegradation increased with increasing temperature but the starch-polyester blend showed more biodegradation at 20°C indicating it could be a better choice for use in cooler climates than the PLA/PHA blend. Collectively, these results demonstrate BDMs are a good alternative for PE with respect to soil C pools with careful consideration of the location of use (specifically temperature). The turnover time of BDM microplastics in the soil will be important for adoption of these materials.

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