Effects of long-term use and incorporation of biodegradable plastic mulch films on soil microbial community structure and activity

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

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December 2019

DEDICATION

I want to dedicate this work to my younger self. A person who loves learning, and decided to go on a quest to do so: Thank you. To my two cats, Lolita and Cuki, for being a never-ending source of affection along this lonely journey. And to all my friends and family, for their support keeps me going.

ACKNOWLEDGEMENTS

I want to acknowledge my adviser, Dr. Jennifer DeBruyn. For believing in me since we first met in ASM 2015, and for always providing me the tools to perform my work. Needless to say that without her help, this would not have been possible. I want to give a special mention to Dr. Sreejata Bandopadhyay for being the North star during my sails across the sea of graduate research. Her help was paramount, and her company even more: ধন্যবাদ. I want to thank some of the friends I made, namely Dr. Denis Osipov, Marie English, M.Sc., Manuel Sabbagh M.Sc., Kyle Bonifer and Eric Gann. Big Caribbean thanks to my tropical buddy, Jonelle Basso: thanks for believing in me more than I believed in myself during my preliminary exam. To my friends in Puerto Rico, particularly Luis Fer and Raymond: being in Tennessee never felt so familiar. To my family, in particular my mom, dad and brother, Maruja, Ricky and Ricardo, respectively: miles de gracias por siempre ayudarme en todo, hacerme la vida muchísima más fácil y por hacerme sentir especial. Los amo y los extraño. A special thanks to The oUTsiders slow-pitch softball team, often the only company I had to go out and enjoy a nice time: Good rips. To the various funding sources that have permitted me to work: UTK-PEER, The National GEM fellowship, ASM capstone research fellowship, various departmental and graduate school travel awards, USDA-SCRI project funding, and Yale Ciencia Academy. To my undergraduate mentors, Dr. Carlos Ríos-Velázquez and Dr. Arturo Massol-Deyá, for helping me see the beauty of the microbial world. Finally, to my girlfriend, Dr. Audrey Bascoul: merci beaucoup, merci pour la patience, et pour l’amour sans distinction de distance, et pour toute votre aide, et voilà.

“Solo sé que no sé nada” // “I only know that I know nothing”

-Socrates (470—399 BC)

ABSTRACT

Plastic mulch films have become widely used in agriculture for altering the soil's microclimate, lowering water-use, suppressing weed growth, and increasing crop production. The most commonly used plastic mulch films are made from low-density polyethylene plastic (PE mulch). However, the disposal of used PE mulch has resulted in an environmental issue as they are not degradable, nor readily recyclable. Using biodegradable-plastic mulch films (BDMs) provides an alternative to PE mulches, as BDMs are made to be soil degradable or compostable. However, *in situ* film breakdown is unpredictable, and information on how BDMs influence soil health in the long-term is missing; both becoming barriers for its wide adoption. Soil microorganisms breakdown BDMs, but knowledge about how buried mulch fragments alter soil microbial community remains limited. Across two diverse locations in USA, we used and incorporated into the soil four BDMs, a PE mulch, and paper mulch treatments for four growing seasons. After the first growing season, we took weathered mulch fragments, placed them in 250 micrometer-size meshbags, and buried them 10 to 20 cm deep in our plot rows. After these four years, we sampled bulk soil and the buried mulch fragments inside meshbags for all the available treatments. To determine alterations in microbial community composition and activity, we analyzed total bacterial and fungal abundance, sequenced bacterial community 16S rRNA gene for both samples, and assayed extracellular enzyme activity on the soil samples. Our analysis indicated that mulch treatment did not alter microbial abundance, or enzyme activity of the bulk soil. However, buried mulches had a higher bacterial abundance, and a more specialized community compared to bulk soil. *Bradyrhizobium* sp. and *Nocardioides* sp. were some of the bacteria found to be more enriched on the buried mulch fragments. Our results indicate that over four years of use and till-down, BDM use did not significantly alter soil microbial community or several extracellular soil enzyme activities. In addition, the buried mulch pieces carry a specialized community with some taxa which may degrade plastic. This work lays the groundwork for other experiments that further elucidate microbial interactions with buried plastic.

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INTRODUCTION

Background

Over the millennia, agriculture has been fundamental for the development of civilization. As we further developed, so did our agricultural practices. And as with most other practices, agriculture benefited from technology. Consequently, farmers have managed to make agriculture ever more efficient. One of these technological advances was using plastic materials, a practice coined as “plasticulture”. Plasticulture thoroughly modified mulching in agriculture, which is the practice of adding a layer of material on top of soil to alter its properties. Plastic films improved mulching benefits, including reduced harvest times and increased crop yields, by altering the soil’s microclimate and suppressing weeds. Polyethylene (PE) plastic soon-after became the standard material used for plastic mulches. However, the use of PE mulches brought with itself several environmental concerns. Removing mulches from the fields leaves plastic debris in the soil. Also, sustainable and cost-effective mulch recycling methods do not exist. Although PE is the cheapest mulch material, it remains environmentally unsustainable. Biodegradable plastic mulch (BDMs) films are an alternative, intended to be plowed into the soil (or composted) and break down naturally in the environment. BDMs have performed comparably to PE mulches in several climates and crops. However, their adoption by farmers has been slow. One reason is that we do not fully understand how their incorporation might affect soil health, especially its microbial community.

In our research experiment, we used different mulch treatments in agricultural fields across two location during four seasons. The treatments were four BDMs, one conventional PE mulch, one fully-degradable paper mulch, and no mulch (i.e., bare ground). These were used to grow various specialty crops in Knoxville, Tennessee (TN) and Mount Vernon, Washington (WA), USA. These sites presented diverse climates and soil types for our experiment. Mulches were laid in early summer. After harvest, the PE mulch was removed, but all the biodegradable ones (i.e., BDMs and paper mulch) were plowed into the soil when the field was tilled. We also cut pieces of weathered mulch from all mulch treatments, placed them in a nylon meshbag and buried them in soil to assess post-burial *in situ* soil degradation. With our experimental set up, we tested for microbial community changes among (i) soils with incorporated biodegradable

mulches, and (ii) buried mulch films after three seasons. We also tested for changes in microbial function among the experimental soils.

Motivation

BDMs are laboratory-tested for 90% degradation by 2 years in soil and/or compost, but *in situ* degradation remains understudied. Early and late-breakdown *in situ* of diverse BDMs have been recorded. Understanding how *in situ* factors influence BDM degradation is critical for viability of this alternative. Plasticulture inevitably leaves plastic debris in the soil. While BDMs are intentionally mixed into the soil, PE mulch debris is left behind and inadvertently mixed into soils as well. There are large gaps in knowledge on the effects of long-term plastic incorporation into soils, particularly biodegradable plastics. Plasticulture is projected to increase, with biodegradable plastics expected to compete in the market as a sustainable alternative. Loss of arable land, and the laborious process to recover damaged soils further motivates our research. Another stimulation is the current policies which do not allow biodegradable-plastic mulch in certified organic farms, enacted largely over the concerns that incorporation of biodegradable plastics may have some adverse effect on soils.

Research questions

The questions set to be discussed in this thesis are the following:

- Does four years of incorporation of practical amounts of biodegradable mulch into soil change microbial abundance?
- Does four years of incorporation of practical amounts of biodegradable mulch into soil alter microbial community structure?
- Does four years of incorporation of practical amounts of biodegradable mulch into soil alter microbial extracellular enzymatic activity?
- What differences exists between the microbial communities associated with buried mulch films and those in soil?
- What differences exists between the microbial communities associated with BDMs and those with PE?
- Are there enriched microorganisms on buried mulch films compared to soil?

Thesis organization

The thesis will be comprised of two chapters. The first will be a review of the current state of published research in the field of plastic degradation and incorporation into the soil. The second chapter will present my research experiment, along with my findings and discussion. The thesis will wrap up with a conclusion summarizing the context and relevance of my findings, and proposing further research questions.

CHAPTER 1
LITERATURE REVIEW

Research and writing were done by Jose Enrique Liquet y Gonzalez.

Abstract

Plasticulture has been fundamental for modern agriculture. It has made many materials less expensive, while upholding their desirable characteristics. Among these, plastic mulch films have stood out. They suppress weeds, influence the soil's microclimate, reduce water consumption and increase crop production. Polyethylene (PE) mulch films are conventionally used for their durability, withstanding a whole season of weather. However, this also provides a problem as these films are generally only good for one growing season, and discarding these films is costly and unsustainable. Using biodegradable plastic mulch films (BDMs) can solve the last step, as they are made to be soil degradable or compostable. BDMs are tested to have 90% of the carbon degraded by 2 years. In the field, however, their breakdown remains unpredictable and remaining plastic fragments have worried users. Non-degradable plastic residues in the soil have an array of negative traits like potential soil mobility of organic pollutants, and detriment of desirable soil properties (e.g. water holding capacity). Thus far, BDM fragments have not been found to consistently alter soil health properties. Nevertheless, questions like how they affect the microbial community or who degrade them remain. BDM degradation has abiotic and biotic phases. Abiotic degradation of BDMs is started by weathering of the films. Heat, sunlight and moisture all influence the mulch integrity. As weathered film's surface deteriorate, biological colonization and biodegradation takes place to a greater extent. Consequently, environmental-exposed plastics have been shown to host a different microbial community than that of its adjacent environmental matrix (i.e., soil, water). Whole community sequencing of BDM-associated microbial community has been done, but meta-omics sequencing should follow so we can further understand the community composition, potential and activity.

Literature review

Agriculture and mulching

Agriculture is often defined as the practice, and science, of cultivating the soil for the growth of crops. Over the millennia, agriculture has proven to be fundamental for the development of civilization. As we further developed, so did

the agricultural practices; and like every other science, it benefited from technology. From tractors, to artificial-selection of crops, and the use of greenhouses; we have developed diverse agricultural techniques to satisfy our increasing demands. Because of advances in technology and materials, farmers have managed to make agriculture always more efficient. In the 1950s, plasticulture (i.e., the practice of using plastic materials in agriculture) became ubiquitous as Professor E.M. Emmert opted to switch the glass panels of his greenhouse for plastic films, saving money and increasing yields (Lamont 2005, Mormile et al 2017). Plasticulture also modified mulching in agriculture. Mulching is done by adding a layer of material on top of soil to alter its properties. Previously done with organic materials, like tree bark and straw; the introduction of plastic films as mulch improved mulching's benefits of reducing harvest times, extending seasons and increasing yields by altering the crop's microclimate (Lamont 1993, Lamont 2005). Polyethylene (PE) films soon-after became the standard mulch used in specialty crops.

Non-degradable plastic mulch films

Compared to non-plastic mulches, the plastic-film properties added benefits, such as: (i) reduced water evaporation and consumption, because of its high degree of impermeability paired with drip irrigation; (ii) decreased leaching of fertilizer, by repelling the excess incoming water; and (iii) CO_2 "chimney effect", its highly impervious nature focuses soil gas exchange through the holes where plants are growing (Lamont , Lamont 2005). The most commonly used PE films are low-density polyethylene (LDPE), and linear low-density polyethylene (LLDPE) film. The former is a branched homopolymer composed of ethylene monomers, while the latter is made by copolymerizing ethylene with another α-olefin (e.g., 1-butene, 1-hexene). These PE films are preferred for their low production cost, high tensile strength and stability, chemical resistance, and high durability (Kasirajan and Ngouajio 2012). Plastic films can be 8 to 50 μm-thick (Kasirajan and Ngouajio 2012, Liu et al 2014). Also, they can be made in different colors to satisfy certain needs: (i) clear, used primarily to increase the soil's temperature; (ii) black, used mainly for weed control and low reflectivity; and (iii) other colors (e.g., red, green, blue), are used mainly when the grower wants to reflect certain wavelengths (Kasirajan and Ngouajio 2012, Mormile et al 2017). Currently, a 3 foot x 2000 foot (0.9 m x 610 m) plastic-film mulch roll costs around a 100 USD.

Thanks to plastic mulch film's effectiveness, 40% of the total plastics used in agriculture during 2012 were plastic mulch films (Sintim and Flury 2017). Furthermore, the global use of plastic films is expected to almost double by 2019, from 4.4 million tons in 2012, to 7.4 million tons, and reach a market of 9.66 billion USD (Sintim and Flury 2017). Although LDPE is considered as the main PE mulch used, LLDPE mulch films are expected to become the largest segment in global agricultural films market from 2017 to 2022 (Kasirajan and Ngouajio 2012, PlusCompanyUpdates 2017). LLDPE is expected to overcome LDPE because of its high puncture resistance, and its performance to production cost ratio (Kasirajan and Ngouajio 2012, PlusCompanyUpdates 2017). In 2011, plastic films covered around 20 million hectares of the cultivation area in China, with an expected increase at a rate of 8-10% until 2024 (Liu et al 2014). Of the 7,350,000 tons of LDPE/LLDPE plastic waste USA generated during 2012, only 13.9% was recycled (EPA 2014).

After harvest, growers must remove mulch films and decide how to dispose of them. Since the early 1990s, scientists were already concerned about the unsustainable alternatives for agricultural-plastic disposal (Hemphill 1993). Although machines can aid in lifting the mulch film from the soil, manual labor is used to recover residual mulch fragments. Disposal options include taking them to the landfill or agricultural-plastic recycling facility (Espi et al 2006, Hemphill 1993, Kasirajan and Ngouajio 2012). The soil and vegetable matter stuck to the films significantly increases their weight and, consequently, waste disposal fees. Moreover, most recycling facilities avoid materials with high amounts of dirt because it requires a washing step, making mulch-film recycling less desirable and feasible (Espi et al 2006, Hemphill 1993). Other disposal alternatives are on-site stockpiling or on-site burning (Hemphill 1993, Kasirajan and Ngouajio 2012). The former represents a waste of land, while the latter results in incomplete combustion which produces air pollutants and, potentially, dioxins (Hemphill 1993, Kasirajan and Ngouajio 2012). Although film disposal presents the largest problem for non-degradable plastic films, plastic fragments are of concern. Weathering will make plastic mulch films vulnerable to breakdown (Hayes et al 2017). Even with normal use, there will be tears in the laid mulch films (Rillig 2012). The total recovery of all plastic fragments from soil is unattainable. Residual plastic-mulch pieces in soil are considered a poor farmland management, and detrimentally disturb soil properties (Liu et al 2014). To address these problems, degradable-plastic film mulches were created.

Biodegradable-plastic mulch films

Biodegradable-plastic mulch films (BDMs) are made from a mixture of biobased and synthetic degradable polymers; mixing degradability with durability and stability (Halley et al 2001, Kasirajan and Ngouajio 2012, OMRI 2015). Common biobased polymers used in BDMs include cellulose, starch, polylactic acid (PLA), and polyhydroxyalkanoate (PHA) (Halley et al 2001, Kasirajan and Ngouajio 2012, OMRI 2015). The synthetic biodegradable polyester polymers are commonly poly butylene-co-adipate terephthalate (PBAT) (Kasirajan and Ngouajio 2012, OMRI 2015). Multinational plastic companies, like Novamont and BASF, have made their own biodegradable polyester resins for adding to BDMs. Novamont has Mater-Bi®, self-reportedly composed of starch and polyesters; most likely PBAT (Table 1-1) (Sforzini et al 2016). While BASF produces ecovio®, mainly made of PBAT and PLA (Table 1-1) (BASF 2015). These resins have been certified as compostable and soil degradable; which makes them popular among BDMs-producers.

BDMs were made to be composted or mixed into the soil after harvest. After a season of exposure to heat and sunlight, the weathered films are expected to be degraded and mineralized by the soil biota. Soil microorganisms will oxidize BDMs' carbon (C) molecules into carbon dioxide (CO_2) and biomass (Halley et al 2001, Kasirajan and Ngouajio 2012, OMRI 2015). BDM adoption by farmers have been impeded by up-front cost and lack of familiarity to films (Goldberger et al 2015). Growers are discouraged by the up-front cost even though they avoid disposal fees; BDMs can cost around three-times more than PE-mulch rolls (Goldberger et al 2015). Also, previous plastic films with claimed dubious degradability have made adoption of current BDMs cumbersome (Liu et al 2014). Namely, photodegradable and oxo-degradable plastic mulch films contained compounds to speed up degradation, but remained in soils long after incorporation (Kasirajan and Ngouajio 2012).Performance studies of BDMs suggest shorter durability when compared to PE films, however this should be expected, since they are designed to degrade in the environment (Goldberger et al 2015, Kasirajan and Ngouajio 2012). To date, BDMs have been proven to be as good as PE mulches in specialty crop; however more work needs to be done (Goldberger et al 2015, Kasirajan and Ngouajio 2012).

Table 1-1: Plastic mulch films used on our field experiments in Knoxville, TN, and Mount Vernon, WA, USA. Data from (Hayes et al 2017). Key compounds are as disclosed by manufacturers.

Plastic mulch	Manufacturer	Product name	Key compounds[A]
Biodegradable	BioBag Americas, Inc. Dunedin, FL, USA	BioAgri®	Mater-Bi® (grade EF04P) (PBAT+starch)
	Custom Bioplastics, Burlington, WA, USA	Naturecycle	Starch+polyester
	Organix Solutions, Bloomington, MN, USA	Organix A.G. Film™	BASF ecovio® (grade M2351) (PBAT+PLA)
	Metabolix Inc., Cambridge, MA, USA	Experimental film PLA/PHA	Ingeo® PLA / Mirel™ amorphous PHA
Non-degradable	Filmtech, Allentown, PA, USA	Polyethylene mulch film	Linear low density polyethylene

[A] PLA=poly lactic acid; PHA= poly hydroxyalkanoate; PBAT= poly butylene-co-adipate terephthalate.

Plastic-film degradation

Although all plastic films are manufactured to be highly durable, they do not remain undamaged. Plastic-film degradation encompasses all those processes which affect their physicochemical properties (Shah et al 2008). These deteriorating processes can involve heat, light, moisture, pH, and biological activity. A powerful agent in agricultural film deterioration is sunlight, as ultraviolet radiation induces photolysis (Krueger et al 2015, Lucas et al 2008, Shah et al 2008, Sivan 2011). Additionally, infrared radiation induces thermal oxidation, further contributing to abiotic degradation processes (Shah et al 2008). These pretreatments certainly facilitate microbial colonization, and further depolymerization (Shah et al 2008, Sivan 2011). Heterotrophic microbes must break the plastic polymers into oligomers and/or monomers to access its molecules for biomass and energy. However, plastic-film's solid nature along with its poor nutrient availability presents a challenge for microbial adaptation and selection of this substrate (Krueger et al 2015). Furthermore, measuring microbial degradation of plastics can be complicated as most techniques vary, and depend on the plastic used. Table 1-2 shows a summary of some of the most widely used techniques to measure plastic biodegradation. Contrary to marine and freshwater environments, plastic accumulation in soils remain largely unexplored because of the challenge to identify and quantify them. Hence, impacts caused by plastic residue accumulation to the soil microbial community remain obscure (He et al 2018, Rillig 2012, Rochman 2018).

Impacts of plastic mulch film residues in soils

Weathered plastic mulches will break down and accumulate in agriculture fields or compost piles; adding unquantified amounts of small and microplastic (<1 mm in size) particles to that matrix (He et al 2018, Rillig 2012, Sintim et al 2019b). In an experiment with soils and added LDPE microplastic particles, the sorption capacity of soils for two common herbicides was reduced (Hüffer et al 2019). In other words, the addition of the PE particles increased soil mobility of the agriculturally-relevant organic chemicals. Plasticizer compounds leaching into the soil from weathered plastic mulches has been found, and raises additional concerns, as they can accumulate in plants and animals and are known endocrine-disrupting agents (Erythropel et al 2014, Inman et al 1984, Wang et al 2013). Di (2-ethyl hexyl) phthalate (DEHP)—most leached plasticizer from plastic mulch films—has been previously regarded as recalcitrant in soil because of its

poor bioavailability (Cartwright et al 2000). DEHP, and other plasticizers, have been found in soils and crops grown with plastic mulches (Shi et al 2019).

Table 1-2: Techniques used to assess plastic film degradation. Adapted and modified from (Lucas et al 2008).

Techniques	Function	Limitations
Weight loss	Measuring changes in physical weight of the plastic	(i) Requires high change of film weight to detect
Scanning electron microscopy (SEM)	Plastic-surface visualization; Used to see microbial colonization and physical changes of the plastic-film surface	(i) Costly; (ii) electron beam can disrupt film
Fourier-transform infrared spectroscopy (FTIR)	Qualitative functional group profiling of plastic film; Changes in peak intensities correlate with formation and destruction of functional groups in the film	(i) Artifacts can be introduced by handling of the film; (ii) microbes can appear in spectra
Respirometry	Measures cellular productivity; Quantifies metabolism by increasing CO_2 or decreasing O_2 in headspace	(i) Does not account for C used for microbial biomass; (ii) disregards anaerobic respiration
Thermogravimetric analysis (TGA)	Measures mass loss; changes in mass loss are detected upon heating of a sample	(i) temperature exerted by TGA can differ from that experienced by film

Important physical properties of agricultural soils have also been affected by plastic residue. Residual fragments of PE mulches in the arable layer (0-20cm) were found to decrease gravimetric water content and bulk soil density, and increase porosity (Jiang et al 2017). In our field experiments, 2 years of BDM use and incorporation into the soil did not have a consistent influence on soil properties, health indicators and function (Sintim et al., 2019). Season (Fall vs. Spring) and location (Washington vs Tennessee, USA) had a much stronger influence on the mentioned parameters, than mulch treatments did (Sintim et al., 2019). Plastic fragments in soil are also known to affect soil biota. For example, earthworms (*Lumbricus terrestris)* are known to interact, transport through soils, and even consume plastics (Huerta Lwanga et al 2016, Huerta Lwanga et al 2017, Rillig et al 2017, Zhang et al 2018). Additionally, soil-dwelling collembolans (*Folsomia candida*), commonly known as springtails, have been shown to have a reduction in growth and reproduction, and a diversification in their gut microbiota when exposed to microplastics (Zhu et al 2018). *Actinobacteria* and *Proteobacteria* composed around 52% of the enriched gut community of collembolans exposed to microplastics (Zhu et al 2018).

Microbial interactions with plastics in soil ecosystems

Microbial communities have a fundamental role in soil health by cycling nutrients and adding biomass to the soil. The use and incorporation of plastic mulch films affect the microbial communities in various ways, like changing soil microclimate and adding carbon (Bandopadhyay et al 2018). So far, incorporation of BDMs into soils have not been shown to have an effect on soil microbial communities (Bandopadhyay et al 2019, Kapanen et al 2008, Li et al 2014a, Moore-Kucera et al 2014, Sintim et al 2019a). Our research group found that location and season had a much stronger influence on the microbial community structure than mulch treatment (Bandopadhyay et al 2019, Li et al 2014a, Moore-Kucera et al 2014, Sintim et al 2019a). This was also found for extracellular soil enzyme activities; which are used as proxies for microbial activity (Bandopadhyay et al 2019, Li et al 2014a, Sintim et al 2019a). Colonization and carbon incorporation by microbes on plastic mulch films has been shown, but barely explored (Figure 1-1) (Zumstein et al 2018). Weathered and buried plastic mulches have been shown to carry a different microbial community that in soil (Bandopadhyay et al 2019, Zhang et al 2019b). Namely, *Firmicutes* and *Actinobacteria* have been identified to potentially interact with plastic films (Esan et al 2019, Zhang et al 2019b). Taxa from the *Actinobacteria* phylum —particularly *Streptomyces* spp. — are known to be able to degrade

recalcitrant polymers like PE and PHA (Lee et al 1991, Martínez et al 2015, Pometto et al 1992). To understand how microbial communities compare between soil and used plastic mulches, more studies with longer duration must be done. This might bridge the knowledge gap on microbial response to plastic incorporation in soils, and give insights on potential mechanisms of plastic degradation.

Mechanisms of microbial plastic degradation

Understanding mechanisms of microbial colonization and degradation of plastics is fundamental to better inform our plastic-bound microbial community studies. In nutrient-scarce environmental settings, plastic debris incentivizes the early colonizers who attach to the selective environment, and rapidly create biofilms (Figure 1-2) (Zettler et al 2013). Furthermore, microbial respiration on these surfaces create a phenomenon where nutrients are concentrated, and in turn invites more microbial colonization (Zettler et al 2013, Zobell and anderson 1936). As plastic debris can sorb compounds into its surface, more microorganisms would try and colonize these nutrient-rich materials (Hüffer et al 2019, Zettler et al 2013). Temperature, pH, humidity, presence or absence of oxygen, water availability, and other environmental conditions will influence microbial degradation of plastic (Kale et al 2015). Naturally selected enzymes, used by fungi and bacteria to breakdown recalcitrant organic molecules like lignin, have been applied to breakdown plastics. For example, laccases, secreted by lignin-degrading fungi, have been used to degrade PE; cell-free enzymes reduced PE's molecular weight by 20% when incubated together (Bhardwaj et al 2012). Extracellular enzymes hydrolyze the polymer chains of plastic, which become short chains of oligomers, dimers, and monomers. These are further utilized by the microorganism in their diverse catabolic pathways, like the citric acid (TCA) cycle, provide biomass and/ energy to the cell, and produce water, and carbon dioxide or methane (Bhardwaj et al 2012, Kale et al 2015).

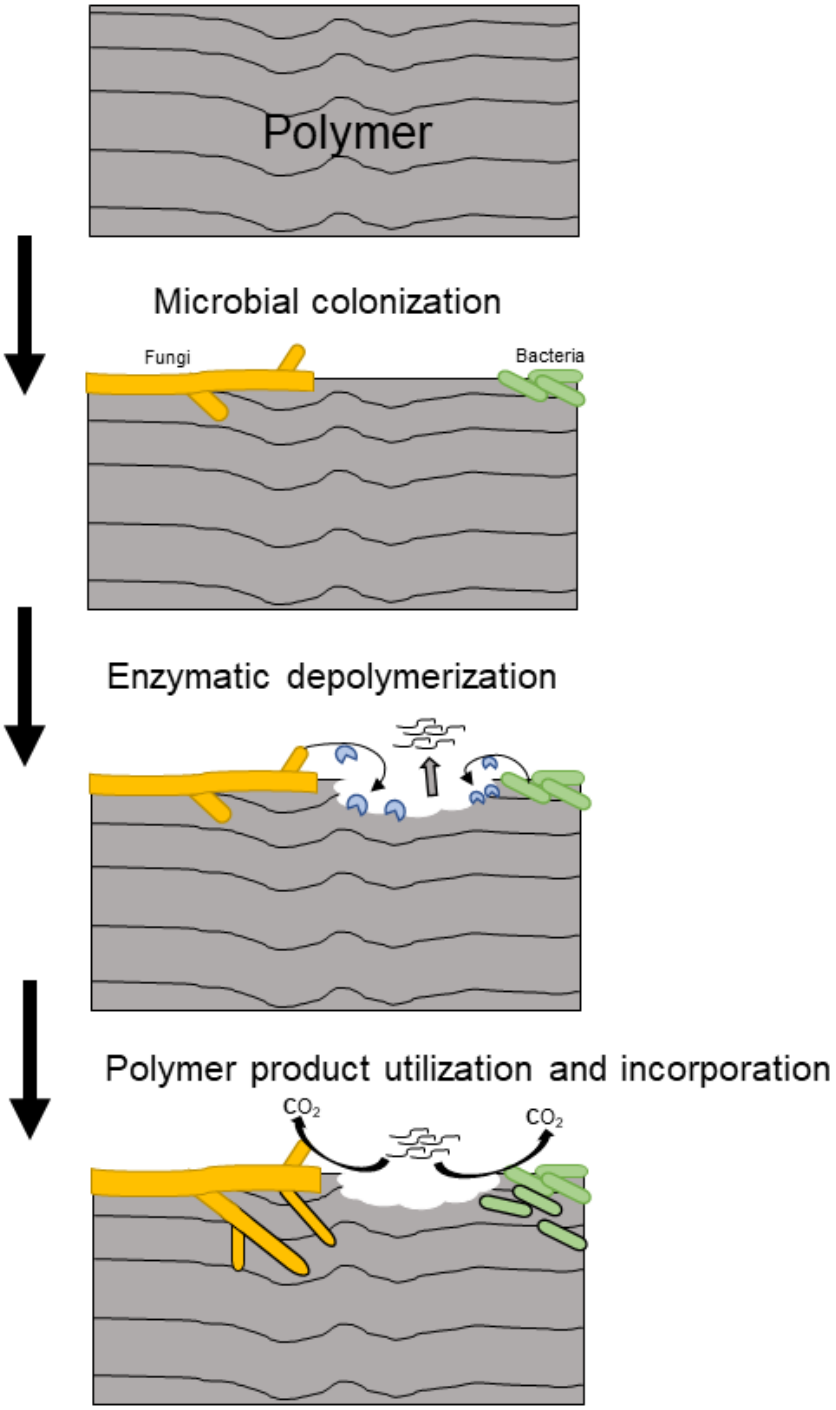

Figure 1-1: Schematic representation of colonization, depolymerization and degradation of a plastic polymer by fungi and bacteria. Note how the enzymes are used to breakdown the plastic polymers, and to incorporate plastic carbon molecules into the microorganism's biomass. The image is adapted from Zumstein et al (2018).

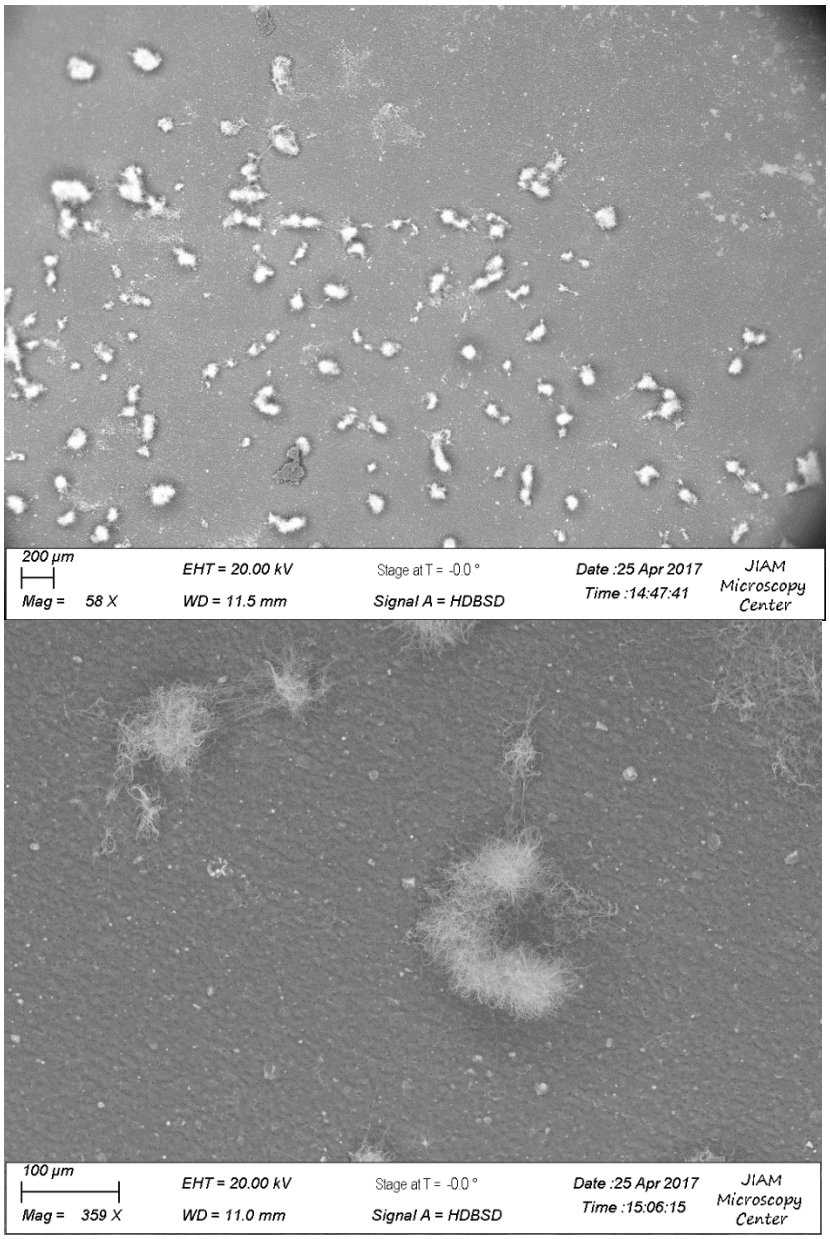

Figure 1-2: Scanning electron microscopy image of a *Streptomyces* sp. colonizing unweathered Naturecycle BDM film surface after a week of incubation on noble agar plate in the lab. Samples and images taken by José E. Liquet y González.

CHAPTER 2
EFFECTS OF LONG-TERM USE AND INCORPORATION OF BIODEGRADABLE PLASTIC MULCH FILMS ON SOIL MICROBIAL COMMUNITY STRUCTURE AND ACTIVITY

Experiment was conceived by Sreejata Bandopadhyay (SB), Jose Enrique Liquet y Gonzalez (JELG), and Jennifer M. DeBruyn (JMD). Sample collection was done by SB, JELG, JMD, Henry Sintim, Mallari Starrett (MS), and Hadaly Serrano Ruiz (HSR). DNA extraction and quantification were performed by JELG, MS, and HSR. qPCR reactions were performed by JELG. Enzyme assays were performed by JELG, HSR, and MS. Whole-community sequencing was prepared by JELG. Data analysis and writing were done by JELG.

Abstract

Plastic mulch films have become widely used in agriculture for altering the soil's microclimate, lowering water-use, suppressing weed growth, and increasing crop production. The most commonly use plastic mulch films are made from low-density polyethylene plastic (PE mulch). However, the disposal of used PE mulch has resulted in an environmental issue as they are not degradable, nor readily recyclable. Using biodegradable-plastic mulch films (BDMs) provides an alternative to PE mulches, as BDMs are made to be soil degradable or compostable. BDMs must pass tests demonstrating that they can degrade 90% by two years. However, *in situ* film breakdown is unpredictable, and information on how BDMs influence soil health in the long-term is missing; both becoming barriers for its wide adoption. Soil microorganisms breakdown BDMs, but knowledge about how buried mulch fragments alter soil microbial community remains limited. Across two diverse locations in USA, we used and incorporated into the soil four BDMs, a PE mulch, and paper mulch treatments for four growing seasons. After the first growing season, we took weathered mulch fragments, placed them in 250 micrometer-size meshbags, and buried them 10 to 20 cm deep in our plot rows. After these four years, we sampled bulk soil and the buried mulch fragments inside meshbags for all the available treatments. To determine alterations in microbial community composition and activity, we analyzed total bacterial and fungal abundance, sequenced the whole bacterial community for both samples, and assayed extracellular enzyme activity on the soil samples. Our analysis indicated that mulch treatment did not alter microbial abundance, or enzyme activity of the bulk soil. However, buried mulches had a higher bacterial abundance, and a more specialized community compared to bulk soil. *Bradyrhizobium* sp. and *Nocardioides* sp. were some of the bacteria found to be more enriched on the buried mulch fragments. Our results indicate that over four years of use and till-down, BDMs do not alter soil microbial community or several extracellular soil enzyme activities. In addition, the buried mulch pieces carry a specialized community with some taxa which

may degrade plastic. This work lays the groundwork for other experiments that further elucidate microbial interactions with buried plastic. Future experiments should be to determine how soil microbes colonize buried mulch, and how environmental factors influence microbial community composition and degradation of plastic mulches.

Introduction

Using plastic mulches has proven to modify soil's microclimate, reduce water use, and increase crop efficiency and production (Feng et al 2019, Seyfi and Rashidi 2007). Consequently, plastic mulch films have become ubiquitous in specialty crop agriculture fields, with Asia leading its consumption (Liu et al 2014, Mormile et al 2017). Their widespread use presents an environmental problem as the most common plastic mulch is low-density polyethylene plastic mulch film (PE mulch), which is not degradable (Kasirajan and Ngouajio 2012, Liu et al 2014). Common disposal options include landfilling, stockpiling, or burning; none are environmentally sustainable (Hemphill 1993, Sintim and Flury 2017, Steinmetz et al 2016). Recycling of PE mulches requires a cleaning step and remains economically unviable (Hemphill 1993, Moore and Wszelaki 2016, MORE-Recycling 2019). The inevitable weathering and disintegration of PE mulches leads to unaccounted incorporation of non-degradable small plastic and microplastic (<1 mm in size) particles into the soil matrix (He et al 2018, Rillig 2012). Currently, the pedologic and edaphic consequences of plastic accumulation remain largely unexplored (He et al 2018, Rillig 2012, Rochman 2018). Although preliminary, PE mulches microparticles have been shown to influence organic contaminant sorption and mobility in soils (Hüffer et al 2019). Residual PE mulch fragments in the arable layer (0 to 20 cm) were found to decrease gravimetric water content and bulk soil density, and increase porosity (Jiang et al 2017).

As an alternative, biodegradable-plastic mulch films (BDMs) are intended to be plowed into the soil, or added into a compost pile, and degraded *in situ* (Bandopadhyay et al 2018, Kasirajan and Ngouajio 2012). BDMs are composed of degradable polyesters (e.g. poly butylene-co-adipate terephthalate (PBAT)) and biobased polymers (e.g., poly lactic acid (PLA), poly hydroxyalkanoate (PHA), and starch). BDMs have been shown to provide similar microclimate influence and yields as PE mulches, across several crops (DeVetter et al 2017, Ghimire et al 2018, Hayes et al 2019, Zhang et al 2019a). However, its adoption, particularly in United States, has been slow due to high upfront-cost and

unpredictable breakdown (Goldberger et al 2015). BDMs are tested for 90% degradability by 2 years in soil or compost in the laboratory. However, *in situ* degradability of BDMs remain inconsistent (i.e., too early in the season, or too late afterwards) with location having a great influence. Location and season influence the BDM-attached microbial community, but we do not understand how this community influence the already obscure *in situ* BDM degradation (Bandopadhyay et al 2019). Furthermore, lack of knowledge on how plowing BDMs into the soil might affect soil health in the long-term is another cause for its low adoption (Goldberger et al 2015). Two of the BDMs used in our study (BioAgri, and an experimental PLA/PHA film) macroscopically degraded >99% and >97%, respectively, after an 18-week incubation in a compost pile (Sintim et al 2019b). But, traces of unknown micro and nanoparticles remained afterwards; leaving questions on how *in situ*-degraded BDMs could influence edaphic factors (Sintim et al 2019b). Potential leaching of plasticizers, like phthalic acid esters, by plastic mulch films raises additional concerns (Wang et al 2013).

Thus far, soil properties, health indicators and function have not been found to be consistently influenced by the use and subsequent incorporation of various BDMs into the soil (Sintim et al 2019a). This field trial was run for two full growing seasons in Tennessee (TN) and Washington (WA), USA. In general, season (i.e. Fall vs. Spring) and location (i.e. TN vs WA) had a much stronger influence on the parameters studied than the mulch treatments (Sintim et al 2019a). Microbial communities, with their nutrient-cycling activity, play a key role in soil health. Studies of BDM influence on soil microbial communities have indicated seasonal and locational effect but not a consistent treatment effect (Bandopadhyay et al 2019, Li et al 2014a, Moore-Kucera et al 2014, Sintim et al 2019a). Extracellular soil enzymes were also found to vary by season and location but not by mulch treatments (Bandopadhyay et al 2019, Li et al 2014a). These studies focused on bulk soil communities but not specifically on communities growing on mulch films. Also, their studies do not sample past two growing seasons, which may not be enough time for BDM incorporation to influence microbial communities and their activity. In TN and WA, we sampled agricultural soils that had four growing seasons of BDM use and subsequent incorporation. Furthermore, we sampled BDM pieces that were buried for 3 growing seasons. We characterized the microbial community abundance and composition of soil and buried mulch samples to determine: (i) if four seasons of BDM use and incorporation have an influence in soil bacterial communities and their extracellular enzyme activities; and (ii) the abundance and composition of the bacterial community associated with buried mulch pieces, to determine taxa

enrichment between buried mulch and soil samples, and between buried BDMs and PE mulch fragments.

Materials and methods

Site description

The experiment was carried out from 2014 to 2018 at the University of Tennessee, East Tennessee AgResearch and Education Center, Plant Sciences Unit in Knoxville, TN (lat. 35°52′52″ N, long. 83°55′27″ W, elevation 270 m) and the Washington State University Northwestern Washington Research and Extension Center at Mount Vernon, WA (48°43′24″N, 122°39′09″W, elevation 6 m), hereafter, sites are referred to as TN and WA, respectively. The TN field site was located in the subtropical southeast United States with an average daily temperature of 23°C, an average relative humidity of 73%, and an average rainfall of 421 mm (30-year average; Arguez et al (2010)). The site has moderately well-drained Shady–Whitwell complex soil characterized as a fine-loamy, thermic Typic Hapludult with a pH of 6.4 and 1.3% organic matter (Moore and Wszelaki 2019). The WA field site was located in the maritime Pacific Northwest, where the summer climate is mild and humid with 15 °C average daily temperature, 82% relative humidity, and 150 mm rainfall (20-year average; AgWeatherNet-Team (2016)). The site has poorly drained Skagit silt loam soil characterized as a fine-silty, mesic Fluvaquentic Endoaquepts with a pH of 6.2 and 2.8% organic matter (Ghimire et al 2018). At TN, a winter wheat cover crop preceded the experiment in 2015 until 2018 (Moore and Wszelaki 2019). At WA, the experiment was preceded by a clover (*Trifolium* sp. L.) winter cover crop in 2015, a winter wheat (*Triticum aestivum* L.) cover crop in 2016 until 2018 (Ghimire et al 2018).

Mulch treatments

The mulch treatments used were four BDMs (BioAgri, Naturecycle, Organix, and an experimental PLA/PHA film), one biodegradable cellulosic-paper mulch (WeedGuardPlus), and one nondegradable PE mulch (Table 2-1). Except for the experimental PLA/PHA mulch, the mulches were commercially-available products. The plastic mulches were black, and paper mulch was brown. The treatment plots were established in a completely randomized block design with four replications. The seven mulch and the bare ground treatments were

randomized within location in 2015, plot assignments did not change across time (2015–2018). This was to avoid cross-treatment mulch and soil contamination, as BDMs were tilled into the soil at the end of every harvesting season.

Field deployment of mulches and sample collection

Mulch was laid by machine (Model 2600 Bed Shaper; Rain-Flo Irrigation, East Pearl, PA) in all plots at the time of row bed shaping in late May; with the only exception of Naturecycle in 2015, which had to be hand laid. Plots were five beds wide and 9 m long, and beds were spaced 2.1-2.4 m center-to-center. Pie pumpkin (*Cucurbita pepo* L.), bell peppers (*Capsicum annuum* L.), and sweet corn (*Zea mays* L.) were grown in the field trials. Pumpkin was grown in 2015 and 2016 in both locations. In 2017 and 2018, peppers were grown in TN and sweet corn was grown in WA. More information about the field experiments and cropping system effects can be found in Ghimire et al (2018), Moore and Wszelaki (2019).

Table 2-1: Mulch treatments information and properties. Table modified from Hayes et al (2017).

Mulch treatments[z]	**Manufacturer**	**Key components[y]**	Thickness (µm)
PLA/PHA[x]	Metabolix Inc., Cambridge, MA, USA	Ingeo® PLA / Mirel™ amorphous PHA	37
BioAgri®	BioBag Americas, Inc. Dunedin, FL, USA	Mater-Bi® grade EF04P (blend of starch and PBAT)	29
Naturecycle	Custom Bioplastics, Burlington, WA, USA	Starch-polyester blend	57
Organix A.G. Film™	Organix Solutions, Bloomington, MN, USA	ecovio® grade M2351 (blend of PLA and PBAT)	20
Polyethylene	Filmtech, Allentown, PA, USA	Linear low density polyethylene	40
WeedGuardPlus®	Sunshine Paper Co., Aurora, CO, USA	Cellulose	562

[z]Green color indicates biodegradable plastic mulch films, orange color indicates biodegradable paper mulch film, and grey indicates non-degradable plastic mulch film.
[y]PLA= polylactic acid; PHA= polyhydroxyalkanoate; PBAT= poly(butylene adipate-co-terephthalate).
[x]Experimental film

At the end of each of the four seasons (2015 to 2018), PE mulch was removed from the field, and BDMs and paper mulch were tilled into the soil. At the end of the first season (2015), pieces of field-weathered mulches (10 cm × 12 cm) were cut and placed into white nylon meshbags (250-µm mesh opening, Industrial Netting, Inc., Minnesota, USA), which were closed with stainless steel staples. Mesh of 250-µm opening size was chosen to optimize the capture of undegraded mulch fragments and minimize loss of fragments falling through a larger mesh opening size. The meshbags were attached to a 4-mm thick nylon string, with each meshbag placed 2 cm apart, with six replicates so that we could sample them destructively six times (twice per year). In October of 2015, the meshbags were buried at about 10-cm depth in the respective plots from where the plastic were sampled (e.g. BioAgri pieces were buried in the plots using BioAgri mulch.) where they remained for three years. Paper mulch was retrieved and buried only in Mount Vernon, but not in Knoxville because it had fully disintegrated on the surface by the end of the 2015 season. From 2016 to 2018, meshbags were removed from the soil when: (i) sampling for analysis at 6-month intervals for 3 years; and (ii) during major field operations each year, such as tillage. When meshbags needed to be temporarily removed for field operations, they were placed in plastic bags, stored in at 4°C, and re-buried within two weeks. The meshbag samples used in this study were collected from the field sites on September 2018 after three years of burial and stored at -20°C. “Meshbag” samples are identified as “buried mulch” from here on.

Soil was collected from all plots 10cm-deep cores using augers, and approximately 12 cores per plot were composited in buckets. Roots and stones were removed by hand. Augers, buckets and gloves were sterilized with 70% ethanol and wiped before use to avoid soil cross-contamination; gloves were discarded after each plot. All soil samples were stored in coolers with ice packs for transport to the laboratory. Samples from WA were shipped to TN on dry ice. Subsequently, soil samples were stored at -80°C.

Bacteria and fungi community assessment

DNA extractions for soils and buried plastic samples were performed using the DNeasy PowerSoil kit and PowerLyzer 24 (Qiagen, Hilden, Germany). Manufacturer’s instructions were followed, with the only exception of using 0.10 g for buried plastic. Extracted DNA was quantified using Quant-iT™ PicoGreen™ dsDNA assay kit (Life Technologies, Oregon, USA; now Invitrogen™, Thermo Fisher Scientific, Massachusetts, USA) per manufacturer’s instructions, and

fluorescence was quantified using Synergy H1 hybrid plate reader (BioTek, Vermont, USA). Extracted DNA was stored at -20^{o}C. Afterwards it was sent to HudsonAlpha (Alabama, USA) for 16SrRNA-based amplicon library preparation and sequencing. In brief, amplicon libraries were done using 515F (GTGCCAAGCAGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) primers, and sequenced by Illumina MiSeq 250 paired-end reads. After sequencing, primers were removed from the fastq files using Cutadapt v2.3 (Martin 2011). Subsequently, contigs were made and aligned to the SILVA non-redundant database using mothur v1.42.0 (Schloss et al 2009).

Quantitative PCR (qPCR) was done in duplicates with Femto Bacterial and Fungal DNA quantification kits (Zymo Research, California, USA) to quantify 16S rRNA and ITS region genes as a proxy for bacterial and fungal abundance in our samples. We followed manufacturer's instructions and used CFX Connect Real-Time PCR Detection System (Bio Rad, California, USA) to perform the reactions. Standard curves had R-squared of ≥98%.

Extracellular soil enzyme activity assays

Extracellular soil enzyme activity assays were done following Steinweg and McMahon protocol described in Bell et al (2013). Only β-glucosidase (BG), Phosphatase (Phos), β-D-cellobiosidase (CB), and β-xylosidase (XYL), and N-Acetyl-glucosaminidase (NAG) were assayed. We used 4-methylumbelliferone (MUB) as standard, and 50 mM Sodium Acetate or 50 mM MES as buffer depending on the soil pH. Our assays were incubated in the dark for 3 hours at 25^{o}C. Fluorescence was quantified using Synergy H1 hybrid plate reader with auto-gain and excitation-emission wavelengths of 365-450, respectively. Extracellular soil enzyme activity was calculated following Bell et al (2013).

Statistics and bioinformatics

Outliers from the gene marker copy number and enzyme activity data were identified using the following criteria: (i) value outside the interquartile range of the group replicates, and (ii) copy numbers which are ±1 log unit from the rest of their replicate group. A maximum of one outlier was removed from each replicate group. All bacterial and fungal gene copy abundance data is presented in appendix Table 4 and 5, with the removed outliers indicated in red. Bacteria and fungi total abundances were determined by normalizing gene starting quantities to dry weight of the material used (in grams), and then log-

transforming the values. Bacteria:Fungi ratio was calculated dividing bacteria and fungi log-transformed gene copy numbers for each respective replicate. A linear fixed-effects (lmer) model was used to understand if there was an effect of treatment and sample on copy number, with location as random effect. Type III analysis of variance (ANOVA) with Satterthwaite's method was used to test the model. To test differences in enzyme activities, models were made with treatment as a fixed effect and location as a random effect: lmer model for NAG, and generalized linear mixed model using penalized quasi-likelihood (glmmPQL) with Gaussian family for BG, XYL, Phos, and CB. Values from soils with negative enzyme activity were transformed by adding a value that made the activity 0.001 nmol activity/g of dry soil/hr. Taxonomy, shared and metadata files from mothur were analyzed using the vegan (v2.5-5) and Phyloseq (v1.24.2) packages in R programing language (v3.5.3) (McMurdie and Holmes 2013, Oksanen et al 2019, R-Core-Team 2018). Operational taxonomic units (OTU) were assigned based on ≥97% sequence similarity cutoff. Briefly, a permutational multivariate analysis of variance (PERMANOVA) test was done using the adonis function (vegan package) with Bray distance matrix and 999 permutations. The multivariate homogeneity of group variance dispersion test was done using betadisper function (vegan package). Richness and inverse Simpson diversity measurements were calculated (Appendix Code 1), and tested for significance with Kruskal-Wallis rank sum test or ANOVA, depending on the data's distribution. Similarity percentage (SIMPER) tests were done with 999 permutations using the sim function in the vegan package. Principal coordinates analysis (PCoA) with Jaccard distance matrix was done with ordinate function from the Phyloseq package. All statistical analyses were done in R (Appendix Code 2).

Results

Abundance of bacteria and fungi among samples and treatments

The total abundance for bacteria and fungi was measured in buried plastic and soil samples across all available treatments for TN and WA. We measured copy numbers of 16S rRNA and ITS gene as a proxy for bacterial and fungal abundance, respectively, in the extracted DNA of our buried mulch and soil samples. There was a significant difference in bacterial abundance between sample types (buried plastic vs. soil): Bacterial abundance was higher on buried plastic than soil across all locations and treatments $F_{(1,81)}$=201.75, p<2e-16 (Fig. 2-1). However, there was no difference between treatments: $F_{(6,81)}$=0.35, p=0.904 (Fig. 2-1). WA had a higher overall bacterial abundance than TN in both soils and

buried mulches. Fungal abundance was not significantly different between sample types or treatments: $F_{(1,81)}$= 0.193, p<2e-16 and $F_{(6,81)}$= 1.92, p=0.661, respectively (Fig. 2-2). Fungal abundance was higher in WA than TN for both sample types. Buried mulch in TN had higher fungal abundance than its soil. Whereas in WA, soil had a higher abundance than buried mulch. There was a significant effect of sample types on bacteria:fungi abundance ratio: $F_{(1,79)}$= 41.70, p<7.95e-09 (Fig. 2-3). Treatments had no effect on the ratio: $F_{(6,79)}$= 0.47, p=0.826. Across both sites, all treatments had a higher bacterial abundance relative to fungi; except BioAgri soil from WA which had a mean bacteria:fungi ratio of 0.9957. Buried mulches in WA had the highest overall bacterial abundance. In TN, both samples had similar bacterial abundance across all treatments.

Extracellular soil enzyme activity

The extracellular soil enzyme activities were quantified for soil samples across all available treatments in TN and WA. B-glucosidase enzyme activity was non-significant throughout all treatments, p>0.05 (Fig. 2-4). B-D-cellubiosidase enzyme activity was non-significant for all treatments, p>0.05 (Fig. 2-5). Nevertheless, phosphatase enzyme activity was significantly different only in soil with Organix mulch, p=0.0349 (Fig. 2-6). This difference is produced from two Organix soil samples from TN that had a higher than average activity (Fig. 2-6). B-xylosidase and N-acetyl-B-glucosaminidase enzyme activities were non-significant for all treatments, p>0.05 (Fig. 2-7 and 2-8).

Bacterial community sequence analysis and diversity measurements

The whole bacterial community was characterized for TN and WA (Fig. S1 and S2, respectively). There were 6,874,451 and 8,605,285 total identified OTUs in TN and WA, respectively. Principal component analysis was performed for the community data from TN and WA (Fig. 2-9 and 2-10). For TN, a PERMANOVA test revealed significant difference of OTUs between soil and buried mulch samples: $F_{(1,44)}$= 11.99, R-squared= 0.21, p<0.001, but no significant difference between treatments of both samples: $F_{(6,39)}$= 1.14, R-squared= 0.14, p=0.196. However, the permutation tests for homogeneity of multivariate dispersions were significant for samples and treatments: $F_{(1,44)}$= 16.64, p<0.001 and $F_{(6,39)}$= 2.47, p=0.051, respectively.

In WA, the PERMANOVA revealed significant difference of OTUs between soil and buried mulch samples: $F_{(1,48)}$= 43.28, R-squared= 0.43, p<0.001, but no significant difference between treatments for both sample types: $F_{(6,43)}$= 1.29, R-

squared= 0.15, p=0.195. Sample types and treatments significantly influenced multivariate homogeneity of group variance dispersion in WA: $F_{(1,48)}$= 130.74, p<0.001 and $F_{(6,43)}$= 3.08, p=0.022, respectively. Significant dispersion between groups in treatments was caused by the comparison of treatments with different sample types: e.g. microbial communities of BioAgri mulch treatment will be significantly different as there are buried plastic and soil samples for BioAgri mulch treatment, which have significantly different communities (Fig. 2-9 and 2-10).

The richness estimate was significantly different between soil and buried plastic samples, with soil having higher overall richness; in TN Chi-square=29.423, p<0.001, df=1 (Fig. 2-11). There was no significant difference between mulch treatments in terms of richness: $F_{(6,21)}$= 0.76, p=0.608 for soil treatments; $F_{(4,13)}$= 1.44, p=0.274 for buried mulch treatments. Soil had a significantly higher overall diversity (inverse Simpson's index) compared to buried plastic ($F_{(1,44)}$= 12.98, p<0.001) (Fig. 2-11). There was no significant difference between mulch treatments on inverse Simpson's diversity indices: $F_{(6,21)}$= 0.94, p=0.484 for soil treatments; $F_{(4,13)}$= 0.692, p=0.610 for buried mulch treatments.

In WA, richness estimates and inverse Simpson's index between soil and buried plastic samples were significantly different as well, with soil having higher overall richness and diversity measures; Chi-square=36.23, p<0.001, df=1; and Chi-square=30.808, p<0.001, df=1, respectively (Fig. 2-12). However, there was no significant difference between mulch treatments on richness: $F_{(6,21)}$= 0.84, p=0.546 for soil treatments; $F_{(4,17)}$= 0.75, p=0.568 for buried mulch treatments. There was a mixed difference between treatments of the samples on inverse Simpson's diversity measurement: $F_{(6,21)}$= 3.62, p=0.012 for soil treatments was significant; $F_{(4,17)}$= 2.17, p=0.115 for buried mulch treatments was not significant.

SIMPER analysis results are summarized in Tables 2-2 and 2-3. Proteobacteria and Actinobacteria were the most overrepresented phyla in buried mulch samples (Table 2-2). The most abundant phyla in buried BDMs fragments compared to PE mulch were Proteobacteria, Chloroflexi, and Planctomycetes in TN, and Proteobacteria, Verrucomicrobia, and Chloroflexi in WA. *Bradyrhizobium* sp., from the Proteobacteria phylum was found in higher mean abundance on buried BDMs samples than on PE samples (Table2-3). *Sphingomonas* sp., from the Proteobacteria phylum, had a higher mean abundance in buried PE mulch samples than in BDMs samples. Overall bacteria from Proteobacteria and

Actinobacteria phyla were the most represented taxa on buried mulch samples for both locations with 10 and 5 individuals, respectively (Table 2-3).

Discussion

After four seasons of use and incorporation of different BDMs to the soil, there was no significant alteration to the soil bacterial community, regardless of treatment (Fig. 2-1:2-3, 2-9:2:10). Our measured gene copy abundance in TN and WA suggested that variability in bacterial communities was explained by the sample type (i.e., buried plastic vs bulk soil), and not treatments. However, we sampled before plowing BDMs into the soil, and we might have missed a potential short term change in soil microbial community due to mulch carbon addition. Li et al (2014b) found that BDM treatments did influence soil microbial communities, but used different methods: they used a shorter sampling timeline and phospholipid fatty acid (PLFA) profiling to assess the microbial community. These difference in methods might explain the differences between our study and theirs. Our results correlate with findings from our previous research which studied the soil microbial community after the initial 2 seasons (Bandopadhyay et al 2019). Indeed Bandopadhyay et al (2019) found that location and season had an influence on the soil microbial community, whereas mulch treatment did not. Kapanen et al (2008) found no alterations in diversity of ammonia-oxidizing bacteria in agricultural soil with added experimental BDMs. Incorporation of BDMs into soil has been controversial with some growers, as BDMs unpredictably breakdown and their long-term effects to soil remain unknown (Goldberger et al 2015). From a microbial community stand-point, our results join others in showing that BDM use and incorporation into soil do not negatively alter soil microbial composition (Bandopadhyay et al 2019, Kapanen et al 2008, Muroi et al 2016).

The carbon input of mulch added to the soil in our studies (6-25 g carbon per m^2) can be considered small relative to other organic amendments like plant residues (<140 g carbon per m^2) (Al-Kaisi and Yin 2005, Hayes et al 2017). We assayed for extracellular enzymes (EEz) that microorganisms use to cycle different carbon molecules, phosphorus, and nitrogen in soils; nutrients which are desired in agriculture fields. Most of our assayed EEz activities in soil were not influenced by mulch treatments (Fig. 2-4:2-8). Again, this might be because of our pre mulch-incorporation sampling time, missing a short term change in activity, or due to the overall small amount of added carbon throughout the years. Also, latent microorganisms will not produce EEz, and some others will not let

their EEz diffuse away from their cells or polysaccharide matrix; these could explain our undetected changes among treatments (Burns 1982). Only phosphatase activity in TN soil with Organix BDM treatments had a significant increase compared to the other mulch treatments ($p = 0.03$). However, this could be explained by location variation, or within-location variability: as two of the four replicates drove the high activity, while the other two replicates remained within the location activity range (Fig. 2-6). EEz have a high turnover rate, which may explain the high variability seen between replicates. Our findings that mulch treatment did not have a significant effect on EEz activities is consistent with other studies which report that EEz activity is better predicted by environmental factors than by certain soil amendments (Allison and Jastrow 2006, Bailey et al 2011, Geisseler and Horwath 2009, Geisseler et al 2011).

Bacterial communities from buried meshbag samples of PE mulch, BDM, and paper mulch (WeedGuardPlus) significantly differed from the communities in bulk soil samples in both TN and WA (Fig. 2-1). Richness and inverse Simpson's diversity index were significantly higher for soil samples than buried mulch samples (Fig. 2-11 and 2-12). Thus, buried mulch samples have a lower number of unique OTUs, and their bacterial communities are less diverse than their soil counterpart. Similar microbial enrichments on plastics have been reported in soil (Bandopadhyay et al 2019, Esan et al 2019, Zhang et al 2019b) and aquatic (Dussud et al 2018, Parrish and Fahrenfeld 2019, Zettler et al 2013) environments. This enrichment on buried mulch fragments can be driven by weathering of plastic polymers, and by sorption of different organic molecules into plastic surface (Hayes et al 2017, Teuten et al 2009, Wang et al 2019, Zumstein et al 2018). For example, field-weathering of polyester-containing BDMs will reduce its polyester bonds, thus making the film more susceptible to colonization and biotic breakdown (Hayes et al 2017). Bacterial communities from buried mulches were significantly different ($p>0.001$) between treatments, regardless of location (Fig. 2-9 and 2-10). As some of our mulch treatments vary significantly in composition (e.g., PE, cellulose, PLA), this selection was expected. However, the possibility that this selection is the result of a "bag effect" remains open, as we did not include an empty meshbag control in this study. Although, similar trends of plastics having a different microbial community than its adjacent matrix has been observed previously; reducing the possibility that our results were simply due to the physical enclosure of the meshbags (Bandopadhyay et al 2019, Dussud et al 2018, Esan et al 2019, Zhang et al 2019b).

SIMPER analysis revealed bacteria that were significantly more abundant ($p<0.05$) in buried mulch samples than in soil (Table 2-2). Members from Proteobacteria, Actinobacteria and Chloroflexi phyla were the most abundant in buried mulch samples. In particular, *Bradyrhizobium* sp. and *Nocardioides* sp. were found on TN and WA. The genus of *Bradyrhizobium* contains soil free-living or plant-associated Nitrogen fixing bacteria (Jaiswal and Dakora 2019). *Nocardioides* bacteria have been widely found in soils, and some possess pollutant-degrading capabilities (Ikunaga et al 2011, Takagi et al 2009). The most abundant phyla in buried BDMs fragments compared to PE mulch were Proteobacteria, Chloroflexi, and Planctomycetes in TN, and Proteobacteria, Verrucomicrobia, and Chloroflexi in WA. *Bradyrhizobium* sp., from the *Proteobacteria* phylum was found in higher mean abundance on buried BDMs samples than on PE samples, on both locations (Table 2-3). *Sphingomonas* sp., from the Proteobacteria phylum, had a higher mean abundance in buried PE mulch samples than in BDMs samples. Previous research done in our lab which characterized the microbial community of field-weathered mulches found *Sphingomonas* sp. to be enriched for BDMs and PE mulches compared to bulk soils (Bandopadhyay et al in prep). *Sphingomonas* sp. has been found associated to plastics in other studies, and some are known to degrade polyethylene glycol (Debroas et al 2017, Pathak and Navneet 2017, Takeuchi et al 1993). *Sphingomonas* sp. have also been found to degrade organic herbicides and contaminants, thus it is possible that *Sphingomonas* sp. consume organic molecules that are sorbed into the plastic films (Leys et al 2004, Li et al 2017). Overall bacteria from Proteobacteria and Actinobacteria phyla were the most represented taxa on buried mulch samples for both locations with 10 and 5 individuals, respectively (Table 2-3); both phyla have been found in macro and microplastic residues in soil (Zhang et al 2019b).

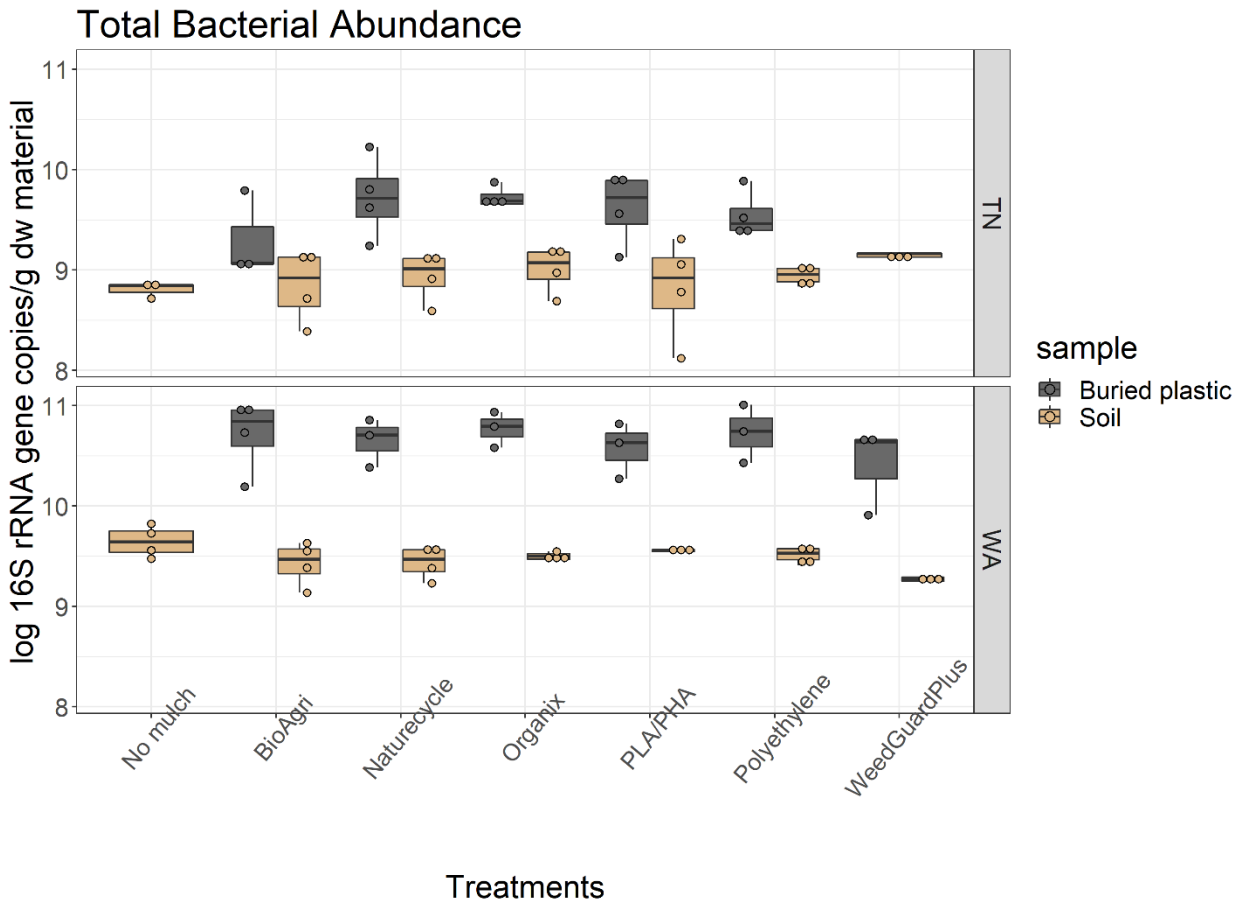

Figure 2-1: Total bacterial abundance across treatments in samples and locations. Bacterial abundance significantly differed between samples ($p<2e-16$), but not between treatments ($p=0.90$). Higher bacterial gene copy numbers were quantified in buried mulch, indicating potential bacterial enrichment on buried mulch pieces. WA had a higher overall abundance of bacteria than TN. The circles represent individual replicate copy number. The dark line in the boxplot represent median, and the whiskers represent values extending no further than 1.5 times the respective hinge (i.e., interquartile range).

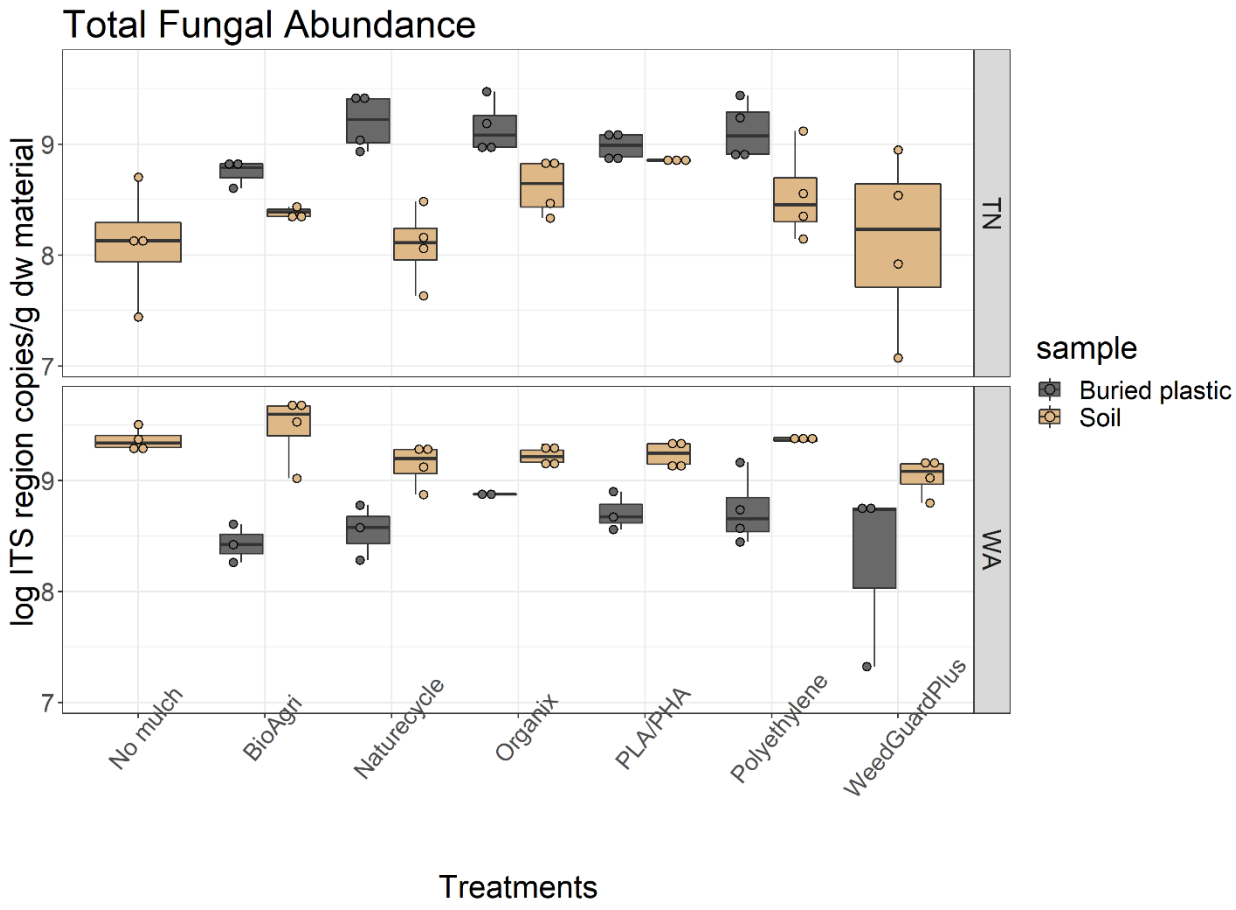

Figure 2-2: Total fungal abundance across treatments in samples and locations. Fungal abundance did not significantly differ between samples (p=0.66) or treatments (p=0.08). Overall, WA had a higher fungal abundance than TN. Contrary to TN, WA soil had a higher fungal abundance than the buried mulch. The circles represent individual replicate copy number. The dark line in the boxplot represent median, and the whiskers represent values extending no further than 1.5 times the respective hinge (i.e., interquartile range).

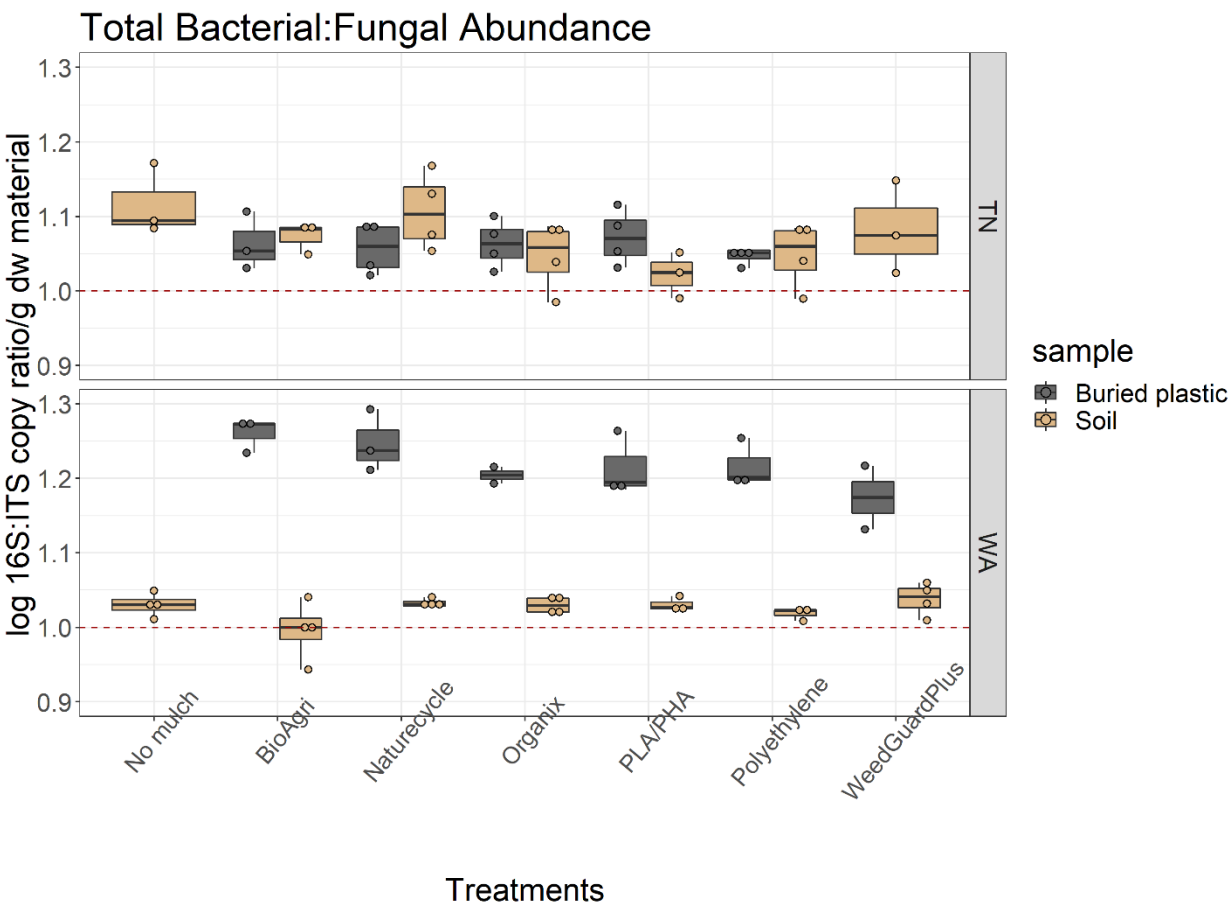

Figure 2-3: Bacteria:Fungi abundance ratio across treatments in samples and locations. The red dash line indicates were bacteria and fungi would be at equal abundance: values above 1.0 represent higher bacterial abundance, and those below 1.0 represent higher fungal abundance. There was a significant effect on bacteria:fungi abundance ratio by sample ($p < 7.95e-09$), but not by treatments ($p = 0.83$). While samples from TN had a similar bacteria:fungi ratio, buried mulches from WA had the highest overall abundance of bacteria. The circles represent individual replicate ratio numbers. The dark line in the boxplot represent median, and the whiskers represent values extending no further than 1.5 times the respective hinge (i.e., interquartile range).

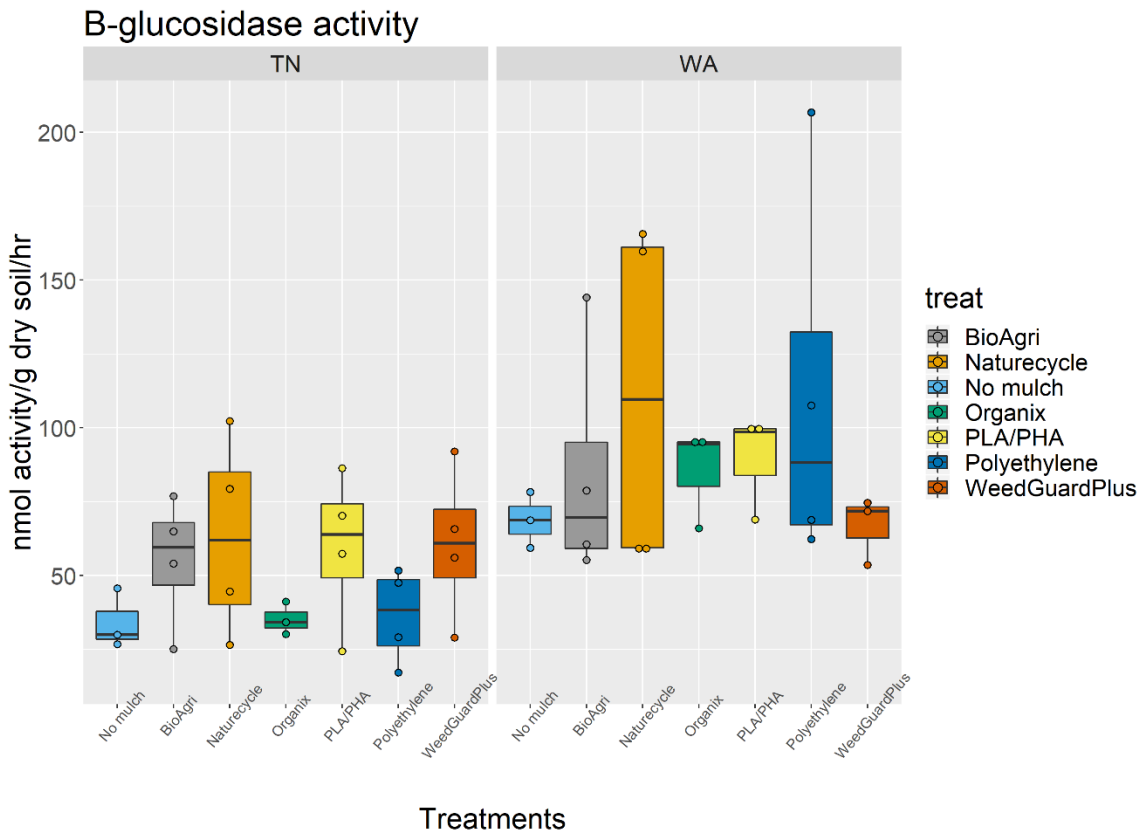

Figure 2-4: B-glucosidase activity across treatments and locations. There was no significant effect of treatments on the enzyme activity ($p>0.05$ for all treatments). The circles represent individual replicate ratio numbers. The dark line in the boxplot represent median, and the whiskers represent values extending no further than 1.5 times the respective hinge (i.e., interquartile range).

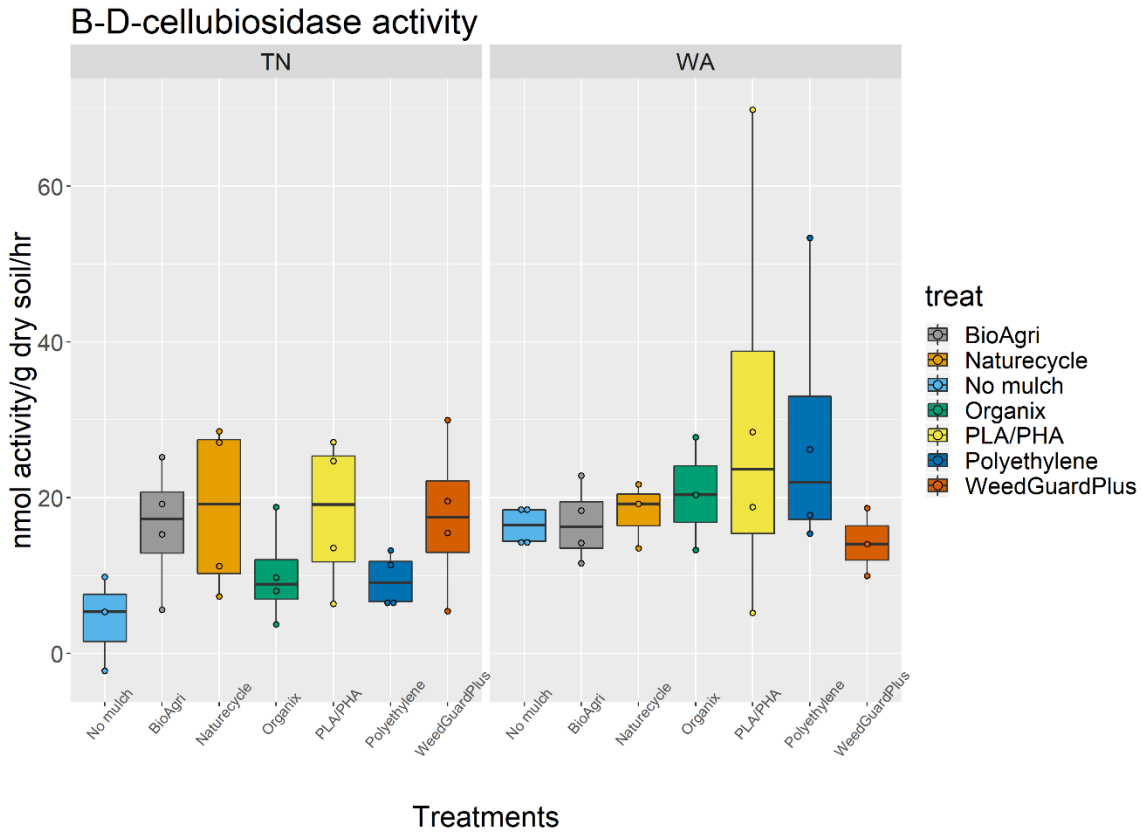

Figure 2-5: B-D-cellubiosidase activity across treatments and locations. There was no significant effect of treatments on the enzyme activity ($p>0.05$ for all treatments). The circles represent individual replicate ratio numbers. The dark line in the boxplot represent median, and the whiskers represent values extending no further than 1.5 times the respective hinge (i.e., interquartile range).

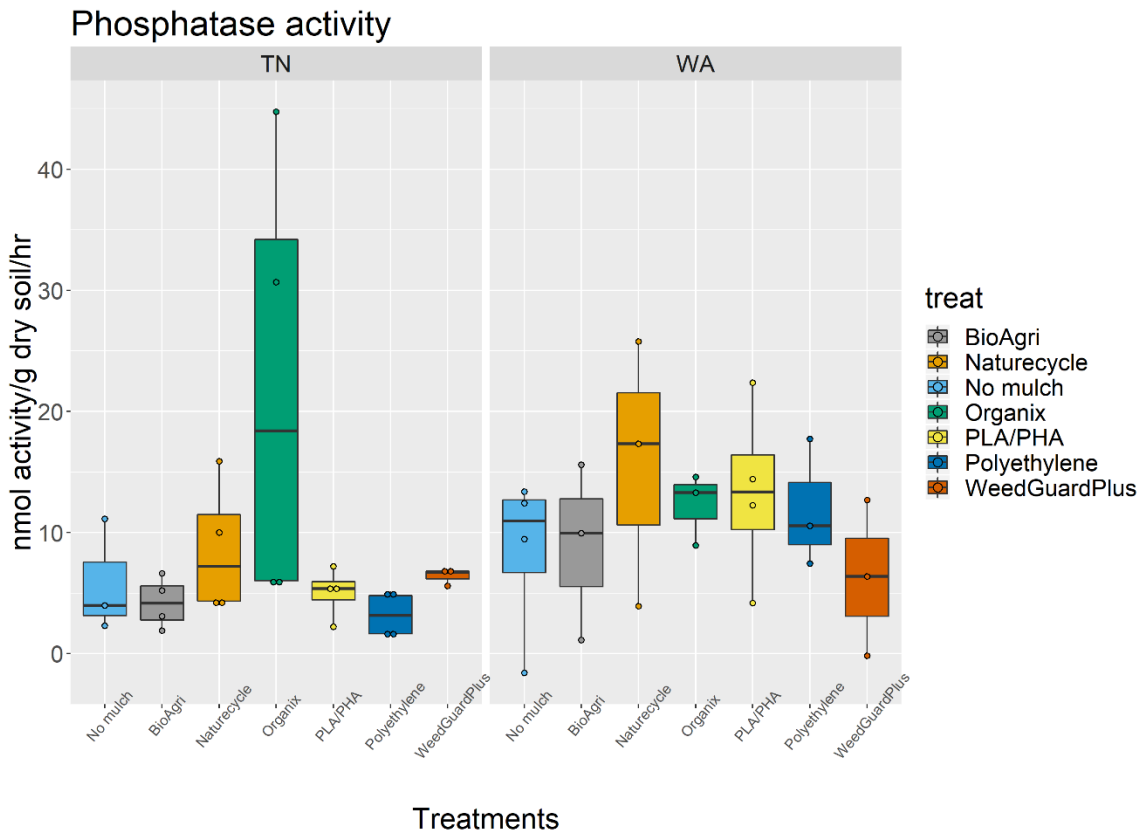

Figure 2-6: Phosphatase activity across treatments and locations. Only Organix had a significant effect on the enzyme activity (p=0.03). The Organix effect on phosphatase activity was particularly high in TN. The circles represent individual replicate ratio numbers. The dark line in the boxplot represent median, and the whiskers represent values extending no further than 1.5 times the respective hinge (i.e., interquartile range).

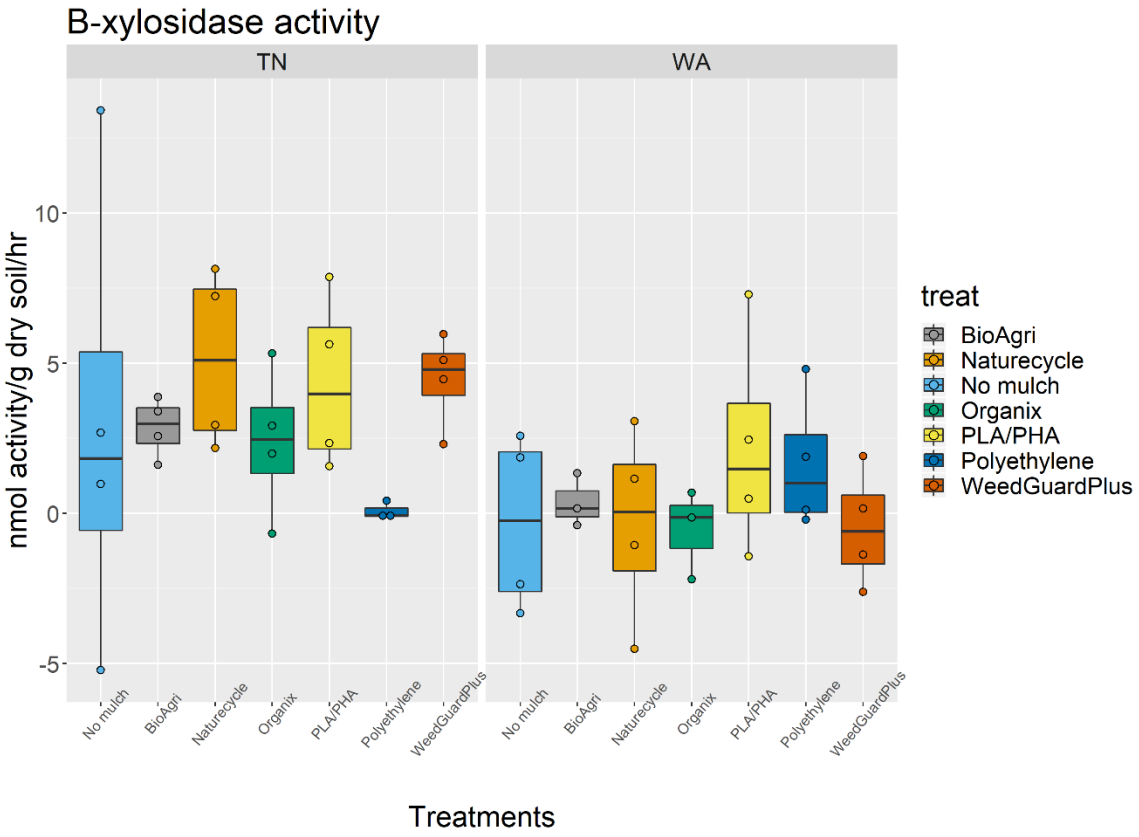

Figure 2-7: B-xylosidase activity across treatments and locations. There was no significant effect of treatments on the enzyme activity ($p>0.05$ for all treatments). The circles represent individual replicate ratio numbers. The dark line in the boxplot represent median, and the whiskers represent values extending no further than 1.5 times the respective hinge (i.e., interquartile range).

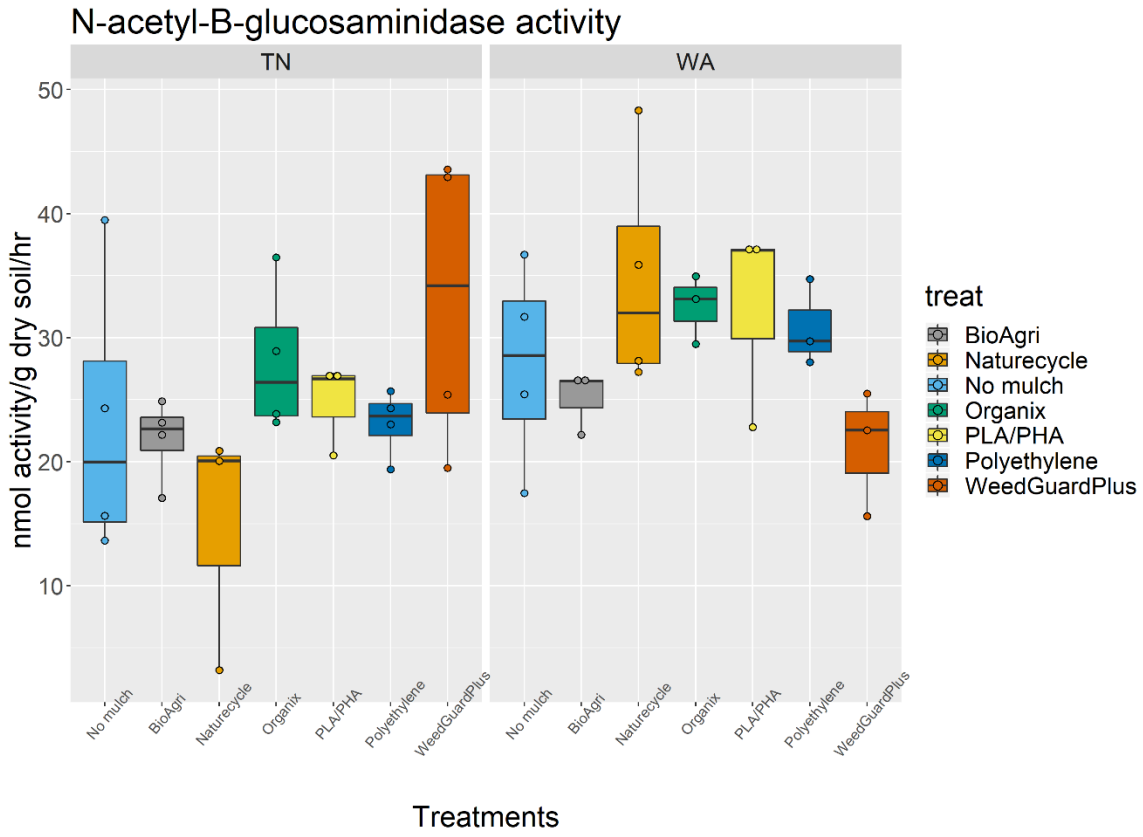

Figure 2-8: N-acetyl-B-glucosaminidase activity across treatments and locations. There was no significant effect of treatments on the enzyme activity ($p>0.05$ for all treatments). The circles represent individual replicate ratio numbers. The dark line in the boxplot represent median, and the whiskers represent values extending no further than 1.5 times the respective hinge (i.e., interquartile range).

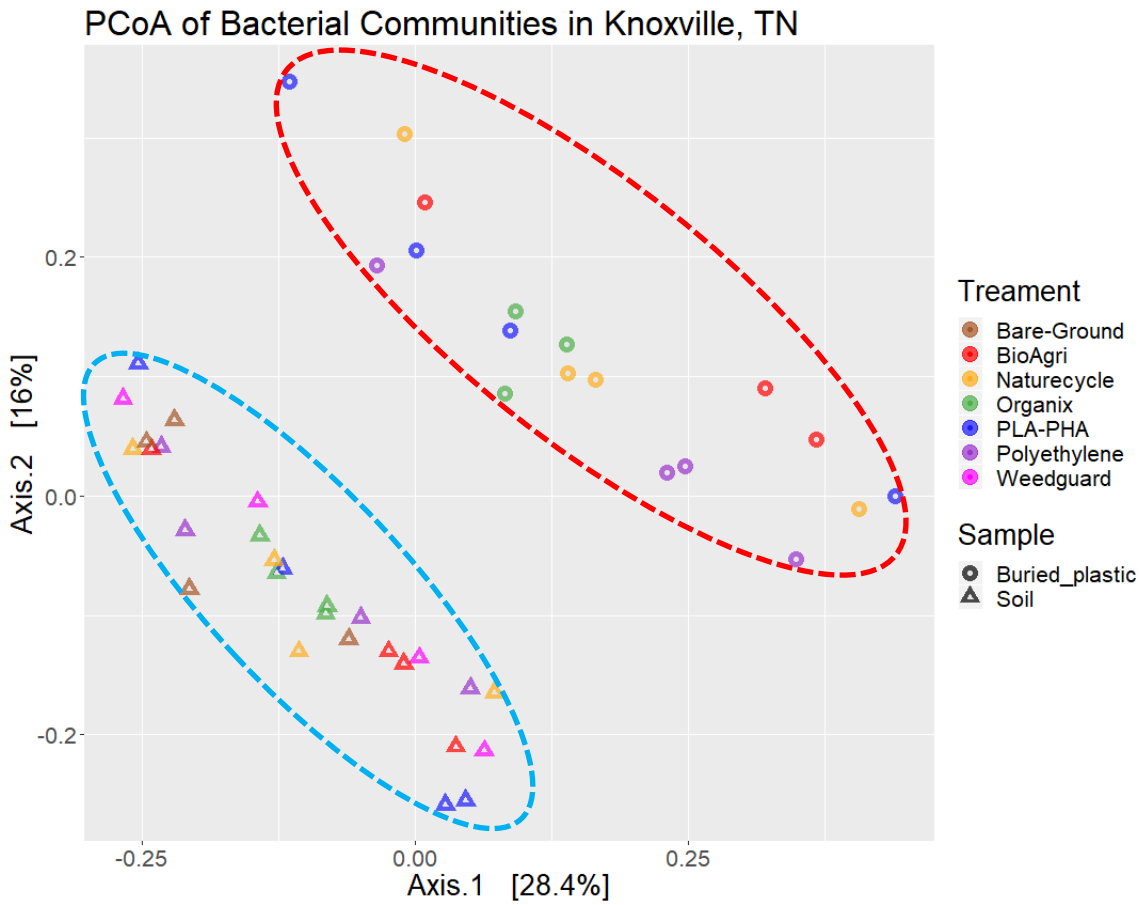

Figure 2-9: Principal coordinates analysis for bacterial community data in TN. Triangles represent soil samples, while circles represent buried mulch. The dashed circles indicate soil and buried mulch samples; colored blue and red, respectively. PERMANOVA and group dispersion estimates for samples were both significantly different between sample types, $p<0.001$.

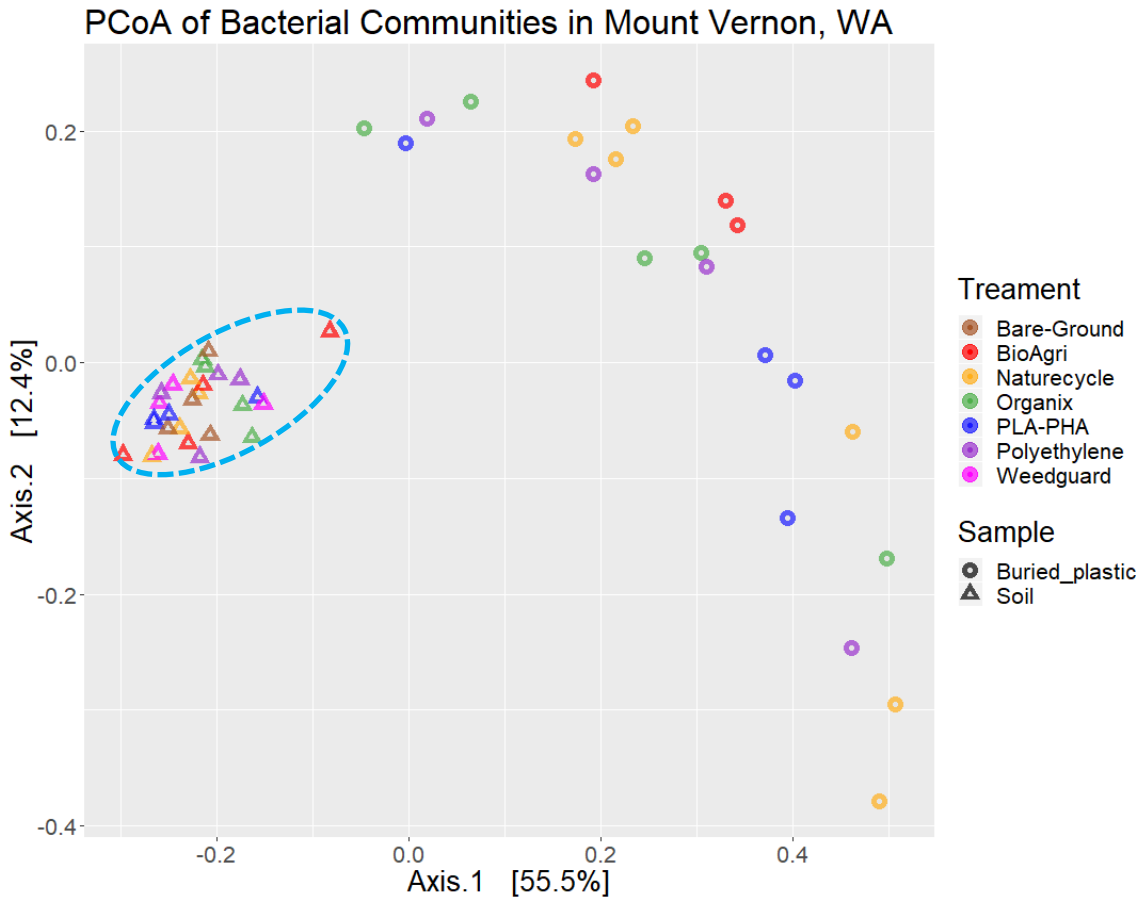

Figure 2-10: Principal coordinates analysis for bacterial community data in WA. Triangles represent soil samples, while circles represent buried mulch. The dashed circles indicate soil and buried mulch samples; colored blue and red, respectively. PERMANOVA and group dispersion estimates for samples were both significantly different between sample types, $p<0.001$.

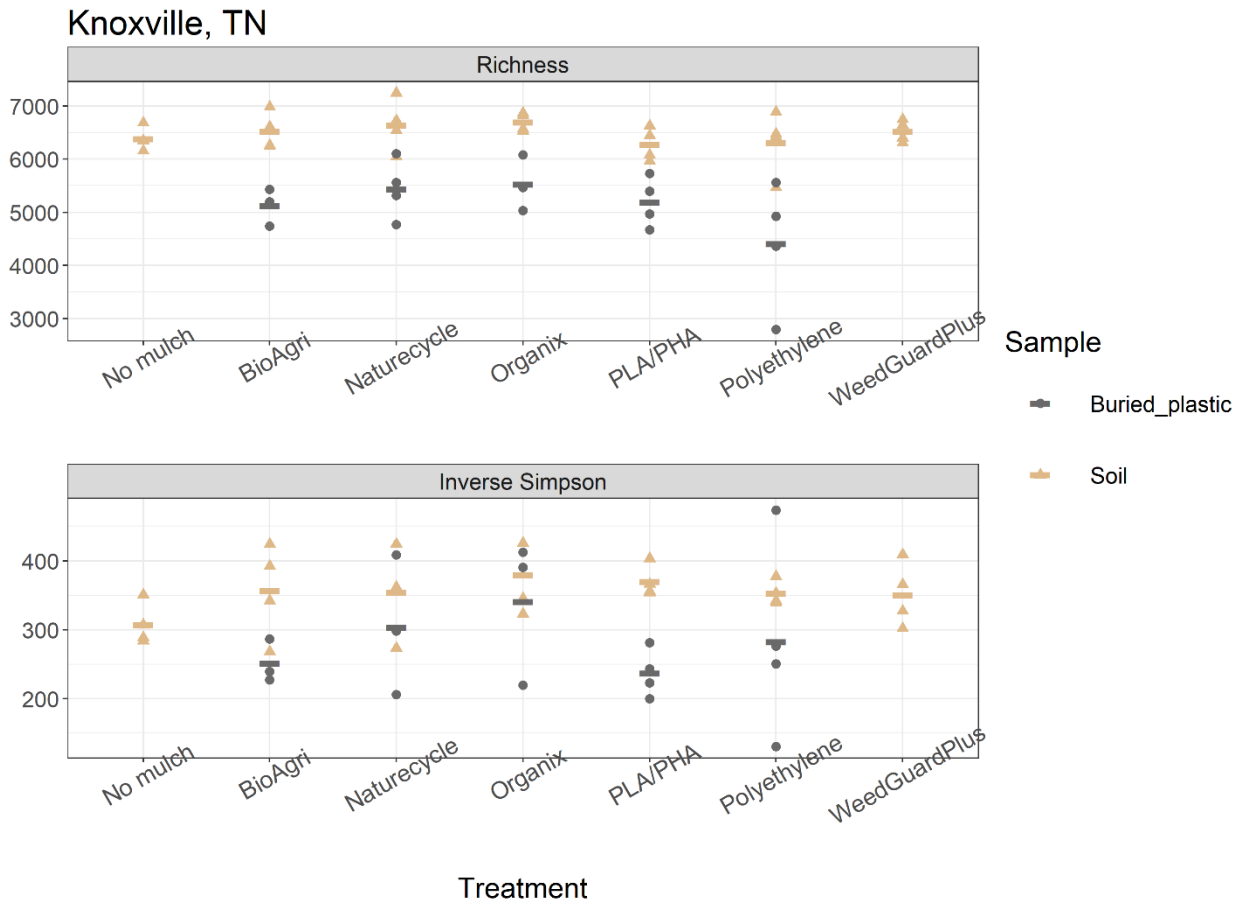

Figure 2-11: Richness and inverse Simpson's diversity measurements for all treatments of buried mulch and soil samples in Knoxville, TN. Filled circles and triangles represent the individual measurements of buried mulch and soil, respectively, while the horizontal line represents the mean. The richness estimate was significantly different between samples, Chi-square=29.42, $p<0.001$, df=1. There was no significant difference between treatments of the samples on richness: $F_{(6,21)}= 0.76$, $p=0.608$ for soil treatments; $F_{(4,13)}= 1.44$, $p=0.274$ for buried mulch treatments. Inverse Simpson's diversity index between samples was also significant, $F_{(1,44)}= 12.98$, $p<0.001$. There was also no significant difference between treatments of the samples on inverse Simpson's diversity measurement: $F_{(6,21)}= 0.94$, $p=0.484$ for soil treatments; $F_{(4,13)}= 0.69$, $p=0.610$ for buried mulch treatments.

Figure 2-12: Richness and Inverse Simpson's diversity measurements for all treatments of buried mulch and soil samples in Mount Vernon, WA. Filled circles and triangles represent the individual measurements of buried mulch and soil, respectively, while the horizontal line represents the mean. Richness estimates and inverse Simpson's index between samples were significantly different as well; Chi-square=36.23, p<0.001, df=1; and Chi-square=30.80, p<0.001, df=1, respectively. However, there was no significant difference between treatments of the samples on richness: $F_{(6,21)}$= 0.84, p=0.546 for soil treatments; $F_{(4,17)}$= 0.75, p=0.568 for buried mulch treatments. There was a mixed difference between treatments of the samples on inverse Simpson's diversity measurement: $F_{(6,21)}$= 3.62, p=0.012 for soil treatments was significant; $F_{(4,17)}$= 2.17, p=0.115 for buried mulch treatments was not significant.

Table 2-2: SIMPER analysis results identifying taxa that were significantly enriched in buried mulch samples compared to bulk soil. Mean relative abundance values in buried mulch (in bold) are significantly different than soil, p<0.001. Permutations=999.

Phyla	Genus	Mean contribution to overall dissimilarity	Mean abundance in soil	Mean abundance in buried mulch
		Knoxville, TN		
Proteobacteria	*Bradyrhizobium*	1.099e-02	4.475e+02	**3.776e+03**
Chloroflexi	*KD4-96*_unclassified	5.103e-03	1.319e+03	**2.665e+03**
Firmicutes	*Bacillus*	3.970e-03	4.767e+02	**1.649e+03**
Actinobacteria	*Nocardioides*	2.849e-03	2.765e+02	**1.096e+03**
Chloroflexi	*KD4-96*_unclassified	2.804e-03	4.084e+02	**1.205e+03**
Chloroflexi	*Roseiflexus*	2.154e-03	2.188e+02	**8.538e+02**
		Mount Vernon, WA		
Actinobacteria	*Nocardioides*	1.305e-02	8.246e+02	**5.248e+03**
Proteobacteria	*Rhodanobacter*	1.295e-02	7.865e+02	**4.840e+03**
Proteobacteria	*Bradyrhizobium*	1.125e-02	1.176e+03	**5.028e+03**
Actinobacteria	*Catenulispora*	8.493e-03	2.732e+02	**3.260e+03**
Proteobacteria	*Xanthomonadaceae*_unclassified	7.216e-03	1.203e+03	**3.193e+03**
Proteobacteria	*Rhodanobacter*	6.970e-03	3.794e+02	**2.704e+03**

Table 2-3: SIMPER analysis results identifying significantly enriched taxa in buried BDM samples compared to buried PE samples. Mean relative abundance values in bold are significantly different, 0.001<p<0.05. Permutations=999. *****No taxa were significantly more abundant on BDMs in WA, p>0.05.

Phyla	Genus	Mean contribution to overall dissimilarity	Mean abundance on BDM	Mean abundance on PE
		Knoxville, TN		
Proteobacteria	*Bradyrhizobium*	1.060e-02	**4.548e+03**	1.073e+03
Proteobacteria	*Comamonadaceae*_unclassified	1.849e-03	**7.572e+02**	1.417e+02
Chloroflexi	*KD4-96*_unclassified	2.831e-04	**1.782e+02**	8.900e+01
Planctomycetes	*Pirellula*_unclassified	7.026e-04	**2.849e+02**	6.225e+01
Proteobacteria	*Rhizobiales_unclassified*	9.273e-05	**5.550e+01**	4.600e+01
Proteobacteria	*Sphingomonas*	2.760e-03	6.947e+02	**1.531e+03**
Acidobacteria	uncultured_unclassified	1.414e-03	6.165e+02	**1.058e+03**
Actinobacteria	*Nocardioidaceae*_unclassified	3.108e-03	4.065e+02	**1.411e+03**
Actinobacteria	*Streptomyces*	3.198e-03	5.643e+02	**1.577e+03**
Proteobacteria	*Rhizobiales*_unclassified	1.230e-03	2.420e+02	**6.327e+02**
		Mount Vernon, WA		
Proteobacteria	*Bradyrhizobium*	1.349e-02	1.898e+03*****	6.000e00
Proteobacteria	*Rhodanobacter*	1.548e-02	2.993e+03*****	2.000e+01
Verrucomicrobia	*DA101*_unclassified	2.763e-04	8.200e+01*****	7.300e+01
Proteobacteria	*Rhodanobacter*	7.636e-03	2.493e+03*****	1.160e+02
Chloroflexi	*JG30-KF-CM45*_unclassified	1.281e-03	4.767e+02*****	2.250e+02
Proteobacteria	uncultured_unclassified	5.525e-05	2.225e+01	**4.300e+03**
Actinobacteria	*Solirubrobacter*	3.986e-04	1.590e+02	**2.190e+03**
Proteobacteria	*Sphingomonas*	7.594e-05	2.825e+01	**4.220e+03**
Actinobacteria	*Streptosporangiaceae*_unclassified	3.227e-04	1.660e+02	**8.010e+03**
Actinobacteria	*Solirubrobacterales*_unclassified	8.281e-05	3.350e+01	**1.400e+03**

Appendix

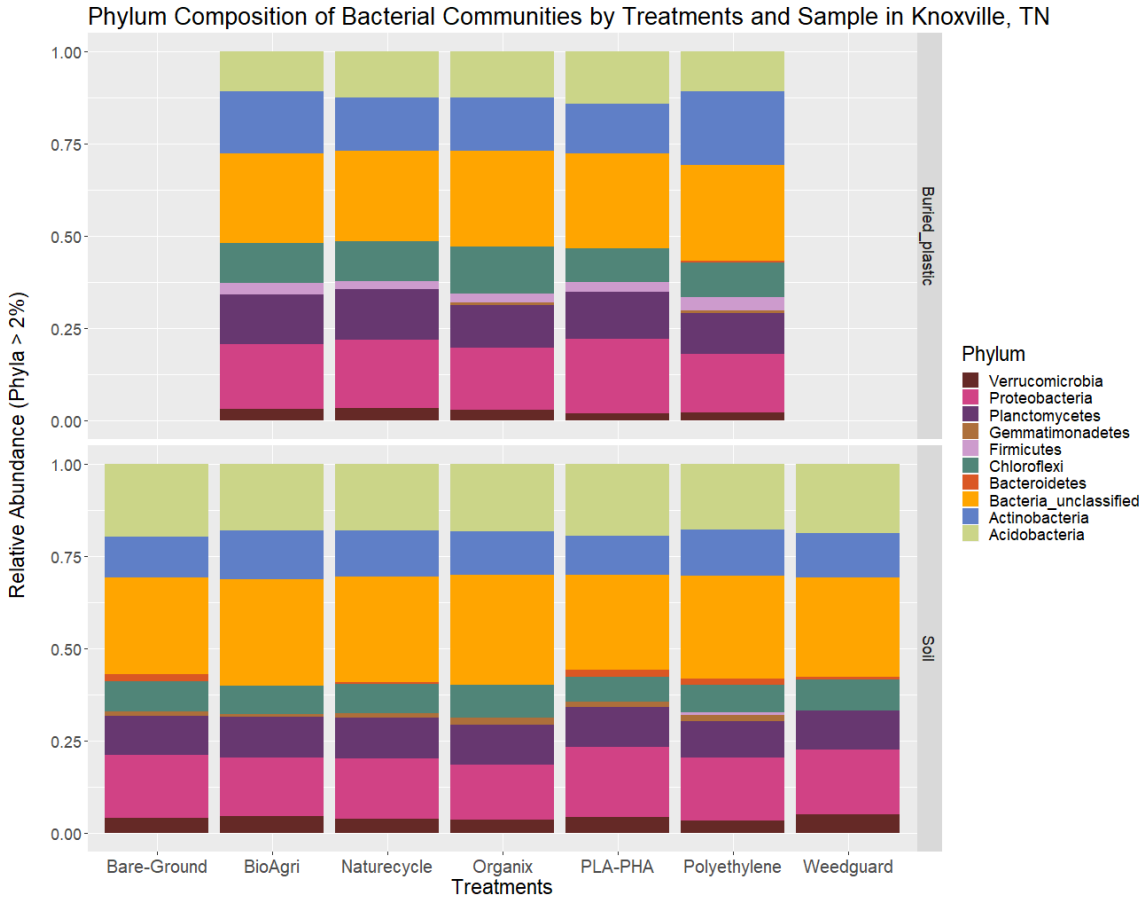

Figure 2-13: Relative abundance of bacterial phyla from buried mulches and soil in Knoxville, TN. Relative abundance phyla represented is over 2%.

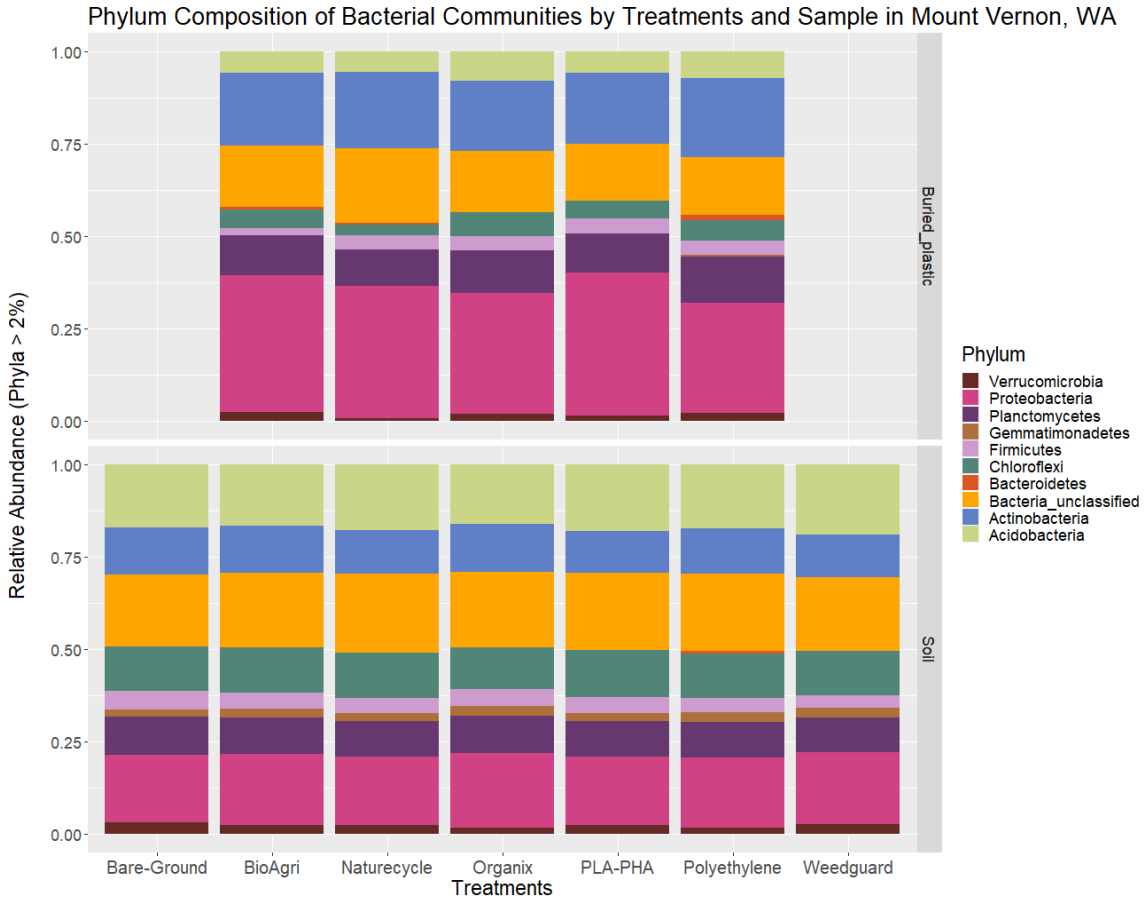

Figure 2-14: Relative abundance of bacterial phyla from buried mulches and soil in Mount Vernon, WA. Relative abundance phyla represented is over 2%.

Table 2-4: Bacterial 16S rRNA gene copy abundances. The cells in red indicate the replicates that were identified as outliers and removed.

Gene target	Location	Sample	Treatment	Rep	Log copies
Bacteria	TN	Buried mulch	BioAgri	2	9.064630629
Bacteria	TN	Buried mulch	BioAgri	5	9.795400531
Bacteria	TN	Buried mulch	BioAgri	1	9.058607724
Bacteria	TN	Buried mulch	Naturecycle	5	9.625587657
Bacteria	TN	Buried mulch	Naturecycle	6	10.22892058
Bacteria	TN	Buried mulch	Naturecycle	1	9.241575809
Bacteria	TN	Buried mulch	Naturecycle	8	9.804497703
Bacteria	TN	Buried mulch	Organix	7	9.71798314
Bacteria	TN	Buried mulch	Organix	5	9.650565209
Bacteria	TN	Buried mulch	Organix	8	9.661733292
Bacteria	TN	Buried mulch	Organix	4	9.87566423
Bacteria	TN	Buried mulch	PLA/PHA	3	9.920992663
Bacteria	TN	Buried mulch	PLA/PHA	1	9.130627023
Bacteria	TN	Buried mulch	PLA/PHA	6	9.565059729
Bacteria	TN	Buried mulch	PLA/PHA	3	9.881429326
Bacteria	TN	Buried mulch	Polyethylene	8	9.888941189
Bacteria	TN	Buried mulch	Polyethylene	7	9.52334905
Bacteria	TN	Buried mulch	Polyethylene	4	9.388725761
Bacteria	TN	Buried mulch	Polyethylene	2	9.397930612
Bacteria	TN	Soil	No mulch	6	8.841936137
Bacteria	TN	Soil	No mulch	2	8.719041565
Bacteria	TN	Soil	No mulch	7	9.240061709
Bacteria	TN	Soil	No mulch	5	8.866209992
Bacteria	TN	Soil	BioAgri	2	8.389703092
Bacteria	TN	Soil	BioAgri	4	9.133235723
Bacteria	TN	Soil	BioAgri	5	9.127518191
Bacteria	TN	Soil	BioAgri	1	8.717668634
Bacteria	TN	Soil	Naturecycle	5	8.916557792
Bacteria	TN	Soil	Naturecycle	6	9.111498361
Bacteria	TN	Soil	Naturecycle	1	8.595359808
Bacteria	TN	Soil	Naturecycle	8	9.125537937
Bacteria	TN	Soil	Organix	7	8.693346289
Bacteria	TN	Soil	Organix	5	8.976518946
Bacteria	TN	Soil	Organix	8	9.198804571
Bacteria	TN	Soil	Organix	4	9.17267882
Bacteria	TN	Soil	PLA/PHA	3	8.783513902
Bacteria	TN	Soil	PLA/PHA	1	8.120492642
Bacteria	TN	Soil	PLA/PHA	6	9.311610695

Table 2-4 (Continued)

Gene target	Location	Sample	Treatment	Rep	Log copies
Bacteria	TN	Soil	PLA/PHA	3	9.058555692
Bacteria	TN	Soil	Polyethylene	8	9.025294449
Bacteria	TN	Soil	Polyethylene	7	9.013667565
Bacteria	TN	Soil	Polyethylene	4	8.835508696
Bacteria	TN	Soil	Polyethylene	2	8.900310918
Bacteria	TN	Soil	WeedGuardPlus	1	8.050590461
Bacteria	TN	Soil	WeedGuardPlus	3	9.094306514
Bacteria	TN	Soil	WeedGuardPlus	2	9.173172959
Bacteria	TN	Soil	WeedGuardPlus	6	9.165127638
Bacteria	WA	Buried mulch	BioAgri	2	10.73405511
Bacteria	WA	Buried mulch	BioAgri	4	10.96826609
Bacteria	WA	Buried mulch	BioAgri	5	10.19351516
Bacteria	WA	Buried mulch	BioAgri	1	10.9526688
Bacteria	WA	Buried mulch	Naturecycle	5	10.85758985
Bacteria	WA	Buried mulch	Naturecycle	6	7.627785401
Bacteria	WA	Buried mulch	Naturecycle	1	10.70895413
Bacteria	WA	Buried mulch	Naturecycle	8	10.3877454
Bacteria	WA	Buried mulch	Organix	7	10.79478923
Bacteria	WA	Buried mulch	Organix	5	10.93621834
Bacteria	WA	Buried mulch	Organix	8	10.58440518
Bacteria	WA	Buried mulch	PLA/PHA	3	10.27498909
Bacteria	WA	Buried mulch	PLA/PHA	1	6.383197873
Bacteria	WA	Buried mulch	PLA/PHA	6	10.81973835
Bacteria	WA	Buried mulch	PLA/PHA	3	10.63215483
Bacteria	WA	Buried mulch	Polyethylene	8	10.43170336
Bacteria	WA	Buried mulch	Polyethylene	7	5.732932518
Bacteria	WA	Buried mulch	Polyethylene	4	10.74654907
Bacteria	WA	Buried mulch	Polyethylene	2	11.00871511
Bacteria	WA	Buried mulch	WeedGuardPlus	1	10.68186165
Bacteria	WA	Buried mulch	WeedGuardPlus	3	5.614410275
Bacteria	WA	Buried mulch	WeedGuardPlus	2	10.63915444
Bacteria	WA	Buried mulch	WeedGuardPlus	6	9.909979472
Bacteria	WA	Soil	No mulch	6	9.558536342
Bacteria	WA	Soil	No mulch	2	9.730424301
Bacteria	WA	Soil	No mulch	7	9.824423418
Bacteria	WA	Soil	No mulch	5	9.476555417
Bacteria	WA	Soil	BioAgri	2	9.387299822
Bacteria	WA	Soil	BioAgri	4	9.552698533

Table 2-4 (Continued)

Gene target	Location	Sample	Treatment	Rep	Log copies
Bacteria	WA	Soil	BioAgri	5	9.63314092
Bacteria	WA	Soil	BioAgri	1	9.139273218
Bacteria	WA	Soil	Naturecycle	5	9.579812239
Bacteria	WA	Soil	Naturecycle	6	9.556797832
Bacteria	WA	Soil	Naturecycle	1	9.384287878
Bacteria	WA	Soil	Naturecycle	8	9.230992632
Bacteria	WA	Soil	Organix	7	9.480568759
Bacteria	WA	Soil	Organix	5	9.521432693
Bacteria	WA	Soil	Organix	8	9.547683126
Bacteria	WA	Soil	Organix	4	9.444642018
Bacteria	WA	Soil	PLA/PHA	3	9.545562717
Bacteria	WA	Soil	PLA/PHA	1	9.249068405
Bacteria	WA	Soil	PLA/PHA	6	9.584449869
Bacteria	WA	Soil	PLA/PHA	3	9.554828883
Bacteria	WA	Soil	Polyethylene	8	9.408960915
Bacteria	WA	Soil	Polyethylene	7	9.574449798
Bacteria	WA	Soil	Polyethylene	4	9.573315735
Bacteria	WA	Soil	Polyethylene	2	9.486647258
Bacteria	WA	Soil	WeedGuardPlus	1	9.271438054
Bacteria	WA	Soil	WeedGuardPlus	3	9.234135883
Bacteria	WA	Soil	WeedGuardPlus	2	9.687915239
Bacteria	WA	Soil	WeedGuardPlus	6	9.311996909

Table 2-5: Fungal ITS gene copy abundances. The cells in red indicate the replicates that were identified as outliers and removed.

Gene target	Location	Sample	Treatment	Rep	Log copies
Fungi	TN	Buried mulch	BioAgri	2	8.6043028
Fungi	TN	Buried mulch	BioAgri	5	8.852161039
Fungi	TN	Buried mulch	BioAgri	1	8.788147273
Fungi	TN	Buried mulch	Naturecycle	5	9.424822671
Fungi	TN	Buried mulch	Naturecycle	6	9.404304583
Fungi	TN	Buried mulch	Naturecycle	1	8.933792655
Fungi	TN	Buried mulch	Naturecycle	8	9.037495268
Fungi	TN	Buried mulch	Organix	7	9.475161642
Fungi	TN	Buried mulch	Organix	5	9.188557497
Fungi	TN	Buried mulch	Organix	8	8.973465799
Fungi	TN	Buried mulch	Organix	4	8.973004929
Fungi	TN	Buried mulch	PLA/PHA	3	8.892934229
Fungi	TN	Buried mulch	PLA/PHA	1	8.855874258
Fungi	TN	Buried mulch	PLA/PHA	6	9.082891708
Fungi	TN	Buried mulch	PLA/PHA	3	9.085662263
Fungi	TN	Buried mulch	Polyethylene	8	9.440403867
Fungi	TN	Buried mulch	Polyethylene	7	9.23771218
Fungi	TN	Buried mulch	Polyethylene	4	8.898826435
Fungi	TN	Buried mulch	Polyethylene	2	8.913217041
Fungi	TN	Soil	No mulch	6	8.155141418
Fungi	TN	Soil	No mulch	2	7.441665966
Fungi	TN	Soil	No mulch	7	8.702078913
Fungi	TN	Soil	No mulch	5	8.101302861
Fungi	TN	Soil	BioAgri	2	7.280528908
Fungi	TN	Soil	BioAgri	4	8.43733062
Fungi	TN	Soil	BioAgri	5	8.388045727
Fungi	TN	Soil	BioAgri	1	8.30915234
Fungi	TN	Soil	Naturecycle	5	7.634509825
Fungi	TN	Soil	Naturecycle	6	8.061299845
Fungi	TN	Soil	Naturecycle	1	8.15978848
Fungi	TN	Soil	Naturecycle	8	8.484996815
Fungi	TN	Soil	Organix	7	8.824127907
Fungi	TN	Soil	Organix	5	8.33204319
Fungi	TN	Soil	Organix	8	8.466877889
Fungi	TN	Soil	Organix	4	8.830274305
Fungi	TN	Soil	PLA/PHA	3	8.871455325
Fungi	TN	Soil	PLA/PHA	1	7.036670229
Fungi	TN	Soil	PLA/PHA	6	8.85299549

Table 2-5 (Continued)

Gene target	Location	Sample	Treatment	Rep	Log copies
Fungi	TN	Soil	PLA/PHA	3	8.841588113
Fungi	TN	Soil	Polyethylene	8	9.119350847
Fungi	TN	Soil	Polyethylene	7	8.350993893
Fungi	TN	Soil	Polyethylene	4	8.145138789
Fungi	TN	Soil	Polyethylene	2	8.554867431
Fungi	TN	Soil	WeedGuardPlus	1	7.072815976
Fungi	TN	Soil	WeedGuardPlus	3	7.921552878
Fungi	TN	Soil	WeedGuardPlus	2	8.53797035
Fungi	TN	Soil	WeedGuardPlus	6	8.948370315
Fungi	WA	Buried mulch	BioAgri	2	8.423241226
Fungi	WA	Buried mulch	BioAgri	4	10.23420497
Fungi	WA	Buried mulch	BioAgri	5	8.260792409
Fungi	WA	Buried mulch	BioAgri	1	8.606588014
Fungi	WA	Buried mulch	Naturecycle	5	8.776953806
Fungi	WA	Buried mulch	Naturecycle	6	7.260365178
Fungi	WA	Buried mulch	Naturecycle	1	8.283700546
Fungi	WA	Buried mulch	Naturecycle	8	8.576612716
Fungi	WA	Buried mulch	Organix	7	8.88216921
Fungi	WA	Buried mulch	Organix	5	10.69424719
Fungi	WA	Buried mulch	Organix	8	8.872385108
Fungi	WA	Buried mulch	PLA/PHA	3	8.672672398
Fungi	WA	Buried mulch	PLA/PHA	1	6.50490037
Fungi	WA	Buried mulch	PLA/PHA	6	8.560843915
Fungi	WA	Buried mulch	PLA/PHA	3	8.899465859
Fungi	WA	Buried mulch	Polyethylene	8	8.737810448
Fungi	WA	Buried mulch	Polyethylene	7	8.447003555
Fungi	WA	Buried mulch	Polyethylene	4	8.57041247
Fungi	WA	Buried mulch	Polyethylene	2	9.164475863
Fungi	WA	Buried mulch	WeedGuardPlus	1	10.29584142
Fungi	WA	Buried mulch	WeedGuardPlus	3	7.321914879
Fungi	WA	Buried mulch	WeedGuardPlus	2	8.741092637
Fungi	WA	Buried mulch	WeedGuardPlus	6	8.759383329
Fungi	WA	Soil	No mulch	6	9.305085788
Fungi	WA	Soil	No mulch	2	9.273918298
Fungi	WA	Soil	No mulch	7	9.504986385
Fungi	WA	Soil	No mulch	5	9.37282886
Fungi	WA	Soil	BioAgri	2	9.021195327
Fungi	WA	Soil	BioAgri	4	9.527579776

Table 2-5 (Continued)

Gene target	Location	Sample	Treatment	Rep	Log copies
Fungi	WA	Soil	BioAgri	5	9.665782499
Fungi	WA	Soil	BioAgri	1	9.689852044
Fungi	WA	Soil	Naturecycle	5	9.276510122
Fungi	WA	Soil	Naturecycle	6	9.29090561
Fungi	WA	Soil	Naturecycle	1	9.121501209
Fungi	WA	Soil	Naturecycle	8	8.872968954
Fungi	WA	Soil	Organix	7	9.132140624
Fungi	WA	Soil	Organix	5	9.332504477
Fungi	WA	Soil	Organix	8	9.173400195
Fungi	WA	Soil	Organix	4	9.254490272
Fungi	WA	Soil	PLA/PHA	3	9.162090462
Fungi	WA	Soil	PLA/PHA	1	9.102073708
Fungi	WA	Soil	PLA/PHA	6	9.341736234
Fungi	WA	Soil	PLA/PHA	3	9.329194726
Fungi	WA	Soil	Polyethylene	8	9.216697541
Fungi	WA	Soil	Polyethylene	7	9.350886068
Fungi	WA	Soil	Polyethylene	4	9.360984146
Fungi	WA	Soil	Polyethylene	2	9.407923479
Fungi	WA	Soil	WeedGuardPlus	1	9.183221492
Fungi	WA	Soil	WeedGuardPlus	3	8.7967685
Fungi	WA	Soil	WeedGuardPlus	2	9.139948895
Fungi	WA	Soil	WeedGuardPlus	6	9.02326474

Appendix code 1

```
# Statistical analysis for my richness and Diversity measurement, adapted from
http://deneflab.github.io/MicrobeMiseq/demos/mothur_2_phyloseq.html


setwd ("C:/Users/JE Liquet y Gonzalez/University of Tennessee/UT_DeBruyn
Laboratory - Documents/BDM.Liquet Project/SoilAndPlastic-community-project-
2018/whole-comm-data/")
library(lmerTest)

tndiv<-read.csv("tn.alphadiv.csv")
wadiv<-read.csv("wa.alphadiv.csv")

#tennessee subsets
tnrichsoil<-subset(tndiv, measure=="Richness" & tndiv$Sample=="Soil", select =
c(mean, Treatment, Sample, measure))
tnrichmesh<-subset(tndiv, measure=="Richness" &
tndiv$Sample=="Buried_plastic", select = c(mean, Treatment, Sample,
measure))

tnsimpsoil<-subset(tndiv, measure=="Inverse Simpson" & tndiv$Sample=="Soil",
select = c(mean, Treatment, Sample, measure))
tnsimpmesh<-subset(tndiv, measure=="Inverse Simpson" &
tndiv$Sample=="Buried_plastic", select = c(mean, Treatment, Sample,
measure))

#Washinton subsets
warichsoil<-subset(wadiv, measure=="Richness" & wadiv$Sample=="Soil",
select = c(mean, Treatment, Sample, measure))
warichmesh<-subset(wadiv, measure=="Richness" &
tndiv$Sample=="Buried_plastic", select = c(mean, Treatment, Sample,
measure))

wasimpsoil<-subset(wadiv, measure=="Inverse Simpson" &
wadiv$Sample=="Soil", select = c(mean, Treatment, Sample, measure))
wasimpmesh<-subset(wadiv, measure=="Inverse Simpson" &
wadiv$Sample=="Buried_plastic", select = c(mean, Treatment, Sample,
measure))

# Anovas for samples
jose<-subset(tndiv,measure=="Richness")
View(jose)
shapiro.test(jose$mean) #not normal
```

```
hist(jose$mean) #right skewed, square root, cubic root and log transformations do not work
tndivmod<-aov(mean~Sample,data=jose)
anova(tndivmod)
kruskal.test(mean~Sample,data=jose) #Kruskal-Wallis chi-squared = 29.423, df = 1, p-value = 5.818e-08
#kruskal.test(mean~Treatment,data=jose), Kruskal-Wallis chi-squared = 6.3957, df = 6, p-value = 0.3804

anova(tndivmod)
#Type III Analysis of Variance Table with Satterthwaite's method
#        Sum Sq Mean Sq NumDF  DenDF  F value Pr(>F)
#treat   0.1838  0.0306     6 81.003   0.3563 0.9043
#sample 17.3508 17.3508     1 81.001 201.7574 <2e-16 ***
#---
#Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
library(fBasics)
normalTest(resid(tndivmod),"da") #residuals are Normal


#Anovas for treatments
library(lmerTest)
shapiro.test(tnrichsoil$mean) #normal
mod<-aov(mean~Treatment,data=tnrichsoil)
anova(mod)
#Analysis of Variance Table

#Response: mean
#         Df  Sum Sq Mean Sq F value Pr(>F)
#Treatment  6  613929  102321  0.7609 0.6085
#Residuals 21 2824071  134480

shapiro.test(tnrichmesh$mean) #not normal
mod<-aov(mean~Treatment,data=tnrichmesh)
library(fBasics)
normalTest(resid(mod),"sw") # residuals are normal!
anova(mod)
#Analysis of Variance Table

#Response: mean
#         Df  Sum Sq Mean Sq F value Pr(>F)
#Treatment  4 2917809  729452   1.447 0.2741
#Residuals 13 6553631  504125

shapiro.test(tnsimpsoil$mean) # normal
```

```
mod<-aov(mean~Treatment,data=tnsimpsoil)
anova(mod)
#Analysis of Variance Table

#Response: mean
#         Df Sum Sq Mean Sq F value Pr(>F)
#Treatment  6  12351  2058.6  0.9447 0.4848
#Residuals 21  45761  2179.1

shapiro.test(tnsimpmesh$mean) # normal
mod<-aov(mean~Treatment,data=tnsimpmesh)
anova(mod)
#Analysis of Variance Table

#Response: mean
#          Df Sum Sq Mean Sq F value Pr(>F)
#Treatment  4  23167  5791.7  0.6921 0.6105
#Residuals 13 108783  8367.9


####################      WA anovas
shapiro.test(warichsoil$mean) #normal
mod<-aov(mean~Treatment,data=warichsoil)
anova(mod)
#Analysis of Variance Table

#Response: mean
#         Df  Sum Sq Mean Sq F value Pr(>F)
#Treatment  6  67756   11293  0.8496 0.5467
#Residuals 21 279133   13292

shapiro.test(warichmesh$mean) #normal
mod<-aov(mean~Treatment,data=warichmesh)
anova(mod)
#Analysis of Variance Table

#Response: mean
#         Df  Sum Sq Mean Sq F value Pr(>F)
#Treatment  4 2917809  729452   1.447 0.2741
#Residuals 13 6553631  504125

shapiro.test(wasimpsoil$mean) # normal
mod<-aov(mean~Treatment,data=wasimpsoil)
anova(mod)
#Analysis of Variance Table
```

```
#Response: mean
#          Df Sum Sq Mean Sq F value Pr(>F)
#Treatment  6 9876.7 1646.11  3.6217 0.01265 *
#Residuals 21 9544.9  454.52
TukeyHSD(mod) # no significant difference between them, p-adjusted>0.05

shapiro.test(wasimpmesh$mean) # not normal
mod<-aov(mean~Treatment,data=wasimpmesh)
normalTest(resid(mod),"da") # residuals are normal!!!!!
anova(mod)
#Analysis of Variance Table

#Response: mean
#          Df Sum Sq Mean Sq F value Pr(>F)
#Treatment  4  42463   10616  2.1772 0.1153
#Residuals 17  82893    4876
```

Appendix code 2

Adapted from
http://deneflab.github.io/MicrobeMiseq/demos/mothur_2_phyloseq.html

```
library(ggplot2)
library(vegan)
library(dplyr)
library(scales)
library(grid)
library(reshape2)
library(phyloseq)
library(wesanderson)

theme_set(theme_bw())

setwd ("C:/Users/JE Liquet y Gonzalez/University of Tennessee/UT_DeBruyn
Laboratory - Documents/BDM.Liquet Project/SoilAndPlastic-community-project-
2018/whole-comm-data/")

list.files()

tax<-
file.choose("bactcomm.trim.contigs.good.unique.good.filter.unique.precluster.pick
.pick.opti_mcc.0.03.cons.taxonomy")
```

```
sharedfile =
"bactcomm.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.opti_
mcc.shared"
taxfile = tax
mapfile = "bactcomm.metadata.csv"

mothur_data  <- import_mothur(mothur_shared_file = sharedfile,
                        mothur_constaxonomy_file = taxfile)

map <- read.csv(mapfile)
head(map)

map <- sample_data(map)
rownames(map) <- map$Sample_name
head(map)

moth_merge <- merge_phyloseq(mothur_data, map)
moth_merge
colnames(tax_table(moth_merge))
colnames(tax_table(moth_merge))<-c("Domain", "Phylum", "Class", "Order",
"Family", "Genus", "Species1","Species2","Species3")

#############DO NOT affect this ERIE/moth_merge, OR DATA WILL BE
SCREWED. THE COLNAMES on erie are the right ones. I do not know why I
cannot change them with aboves command #########
erie<-moth_merge
moth_merge<-erie

####subset data for location!!!!!!!!!
tnsub <- moth_merge %>%
  subset_samples(Location == "TN") %>%
  prune_taxa(taxa_sums(.) > 0, .)

wasub <- moth_merge %>%
  subset_samples(Location == "WA") %>%
  prune_taxa(taxa_sums(.) > 0, .)

tnsoil <- tnsub %>%
  subset_samples(Sample_name == 1:28) %>% #######not working for some
reason !!!!!!!
  prune_taxa(taxa_sums(.) > 0, .)
colnames(sample_data(tnsub))

tnbag <- tnsub %>%
  subset_samples(Sample == "Buried_plastic") %>%
```

```
  prune_taxa(taxa_sums(.) > 0, .)

wasoil <- wasub %>%
  subset_samples(Sample == "Soil") %>%
  prune_taxa(taxa_sums(.) > 0, .)

wabag <- wasub %>%
  subset_samples(Sample == "Buried_plastic") %>%
  prune_taxa(taxa_sums(.) > 0, .)

#####study your data
sample_sum_df <- data.frame(sum = sample_sums(tnsub))

ggplot(sample_sum_df, aes(x = sum)) +
  geom_histogram(color = "black", fill = "indianred", binwidth = 2500) +
  ggtitle("Distribution of sample sequencing depth") +
  xlab("Read counts") +
  theme(axis.title.y = element_blank())
min(sample_sums(tnsub)) #98,819 OTUs counts
mean(sample_sums(tnsub)) #149,444.6 OTUs counts
max(sample_sums(tnsub))# 197,633 OTUs counts
sum(sample_sums(tnsub))#6,874,451 total OTUs

sum(sample_sums(tnbag))#2,986,147 total OTUs
#6874451-2986147= 3,888,304 total OTUs in TN soil

min(sample_sums(wasub)) #25,973 read counts
which.min(sample_sums(wasub))# sample 80 and 34
mean(sample_sums(wasub)) #172,105.7 read counts
max(sample_sums(wasub))# 254,048 read counts
sum(sample_sums(wasub))#8,605,285 total OTUs

sum(sample_sums(wasoil))#5,155,292 total OTUs

sum(sample_sums(wabag)#3,449,993 total OTUs

######Creating stacked barchart
tn_phylum <- tnsub %>%
  tax_glom(taxrank = "Phylum") %>%                    # agglomerate at phylum level
  transform_sample_counts(function(x) {x/sum(x)} ) %>% # Transform to rel.
abundance
  psmelt() %>%                                        # Melt to long format
  filter(Abundance > 0.02) %>%                        # Filter out low abundance taxa
  arrange(Phylum)
phylum_colors <- c(
```

```
  "#CBD588", "#5F7FC7", "orange","#DA5724", "#508578", "#CD9BCD",
  "#AD6F3B", "#673770","#D14285", "#652926", "#C84248",
  "#8569D5", "#5E738F","#D1A33D", "#8A7C64", "#599861")

tn_fam<- tnsub %>%
  tax_glom(taxrank = "Family") %>%                    # agglomerate at phylum level
  transform_sample_counts(function(x) {x/sum(x)} ) %>% # Transform to rel.
abundance
  psmelt() %>%                                        # Melt to long format
  filter(Abundance > 0.025) %>%                       # Filter out low abundance taxa
  arrange(Family)
  my.colors<-c("#89C5DA", "#DA5724", "#74D944", "#CE50CA", "#3F4921",
"#C0717C", "#CBD588", "#5F7FC7",
            "#673770", "#D3D93E", "#38333E", "#508578", "#D7C1B1", "#689030",
"#AD6F3B", "#CD9BCD",
            "#D14285", "#6DDE88", "#652926", "#7FDCC0", "#C84248",
"#8569D5", "#5E738F", "#D1A33D",
            "#8A7C64", "#599861")

#tn_genus0.5 is too low on resolution, i.e., only shows 6 genus

tn_genus0.3<- tnsub %>%
  tax_glom(taxrank = "Genus") %>%                     # agglomerate at phylum level
  transform_sample_counts(function(x) {x/sum(x)} ) %>% # Transform to rel.
abundance
  psmelt() %>%                                        # Melt to long format
  filter(Abundance > 0.03) %>%                        # Filter out low abundance taxa
  arrange(Genus)
my.colors<-c("#89C5DA", "#DA5724", "#74D944", "#CE50CA", "#3F4921",
"#C0717C", "#CBD588", "#5F7FC7",
           "#673770", "#D3D93E", "#38333E", "#508578", "#D7C1B1", "#689030",
"#AD6F3B", "#CD9BCD",
           "#D14285", "#6DDE88", "#652926", "#7FDCC0", "#C84248", "#8569D5",
"#5E738F", "#D1A33D",
           "#8A7C64", "#599861")


wa_phylum <- wasub %>%
  tax_glom(taxrank = "Phylum") %>%                    # agglomerate at phylum level
  transform_sample_counts(function(x) {x/sum(x)} ) %>% # Transform to rel.
abundance
  psmelt() %>%                                        # Melt to long format
  filter(Abundance > 0.02) %>%                        # Filter out low abundance taxa
  arrange(Phylum)
phylum_colors <- c(
```

```
  "#CBD588", "#5F7FC7", "orange","#DA5724", "#508578", "#CD9BCD",
  "#AD6F3B", "#673770","#D14285", "#652926", "#C84248",
  "#8569D5", "#5E738F","#D1A33D", "#8A7C64", "#599861")

wa_fam<- wasub %>%
  tax_glom(taxrank = "Family") %>%                    # agglomerate at phylum level
  transform_sample_counts(function(x) {x/sum(x)} ) %>% # Transform to rel.
abundance
  psmelt() %>%                                        # Melt to long format
  filter(Abundance > 0.025) %>%                       # Filter out low abundance taxa
  arrange(Family)
my.colors<-c("#89C5DA", "#DA5724", "#74D944", "#CE50CA", "#3F4921",
"#C0717C", "#CBD588", "#5F7FC7",
           "#673770", "#D3D93E", "#38333E", "#508578", "#D7C1B1", "#689030",
"#AD6F3B", "#CD9BCD",
           "#D14285", "#6DDE88", "#652926", "#7FDCC0", "#C84248", "#8569D5",
"#5E738F", "#D1A33D",
           "#8A7C64", "#599861")

ggplot(tn_phylum, aes(x = Treament, y = Abundance, fill = Phylum)) +
  facet_grid(sample_Sample~.) +
  geom_bar(stat = "identity", position = "fill") +
  scale_fill_manual(values = phylum_colors) +
  #scale_x_discrete(
   # breaks = c("7/8", "8/4", "9/2", "10/6"),
    #labels = c("Jul", "Aug", "Sep", "Oct"),
    #drop = FALSE
  #) +
  # Remove x axis title
  #theme(axis.title.x = element_blank()) +
  guides(fill = guide_legend(reverse = TRUE, keywidth = 1, keyheight = 1)) +
  ylab("Relative Abundance (Phyla > 2%) \n") +
  ggtitle("Phylum Composition of Bacterial Communities by Treatments and
Sample in Knoxville, TN")+
  theme (axis.text.x = element_text( size=15), text = element_text(size=17))+
  xlab("Treatments")

getPalette = colorRampPalette(brewer.pal(9, "Set1"))
colorCount=length(unique(tn_genus$Genus))

ggplot(tn_genus0.5, aes(x = Treament, y = Abundance, fill = Genus)) +
  facet_grid(sample_Sample~.) +
  geom_bar(colour = "black",stat = "identity", position = "fill") +
  #geom_bar(stat = "identity", position = "fill") +
  #scale_fill_brewer(getPackageName(colorCount)) +
```

```
  guides(fill = guide_legend(reverse = TRUE, keywidth = 1, keyheight = 1)) +
  ylab("Relative Abundance (Genus > 5%) \n") +
  ggtitle("Genus Composition of Bacterial Communities by Treatments and
Sample in Knoxville, TN")+
  theme (axis.text.x = element_text( size=15), text = element_text(size=17))+
  xlab("Treatments")

ggplot(tn_fam, aes(x = Treament, y = Abundance, fill = Family)) +
  facet_grid(sample_Sample~.) +
  geom_bar(colour = "black",stat = "identity", position = "fill") +
  #geom_bar(stat = "identity", position = "fill") +
  #scale_fill_brewer(getPackageName(colorCount)) +
  guides(fill = guide_legend(reverse = TRUE, keywidth = 1, keyheight = 1)) +
  ylab("Relative Abundance (Genus > 2.5%) \n") +
  ggtitle("Family Composition of Bacterial Communities by Treatments and
Sample in Knoxville, TN")+
  theme (axis.text.x = element_text( size=15), text = element_text(size=17))+
  xlab("Treatments")

ggplot(tn_genus0.3, aes(x = Treament, y = Abundance, fill = Family)) +
  facet_grid(sample_Sample~.) +
  geom_bar(colour = "black",stat = "identity", position = "fill") +
  #geom_bar(stat = "identity", position = "fill") +
  #scale_fill_brewer(getPackageName(colorCount)) +
  guides(fill = guide_legend(reverse = TRUE, keywidth = 1, keyheight = 1)) +
  ylab("Relative Abundance (Genus > 3%) \n") +
  ggtitle("Family Composition of Bacterial Communities by Treatments and
Sample in Knoxville, TN")+
  theme (axis.text.x = element_text( size=15), text = element_text(size=17))+
  xlab("Treatments")


ggsave("TN-bact-chart.tiff", width = 15, height = 10, dpi = 300, units = "in",
device='tiff')

ggplot(wa_phylum, aes(x = Treament, y = Abundance, fill = Phylum)) +
  facet_grid(sample_Sample~.) +
  geom_bar(stat = "identity", position = "fill") +
  scale_fill_manual(values = phylum_colors) +
  #scale_x_discrete(
  # breaks = c("7/8", "8/4", "9/2", "10/6"),
  #labels = c("Jul", "Aug", "Sep", "Oct"),
  #drop = FALSE
  #) +
  # Remove x axis title
```

```
  #theme(axis.title.x = element_blank()) +
  guides(fill = guide_legend(reverse = TRUE, keywidth = 1, keyheight = 1)) +
  ylab("Relative Abundance (Phyla > 2%) \n") +
  ggtitle("Phylum Composition of Bacterial Communities by Treatments and
Sample in Mount Vernon, WA")+
  theme (axis.text.x = element_text( size=15), text = element_text(size=17))+
  xlab("Treatments")
ggsave("WA-bact-chart.tiff", width = 15, height = 10, dpi = 300, units = "in",
device='tiff')


############## Creating PCoA #############
scale_reads <- function(physeq, n = min(sample_sums(physeq)), round = "floor")
{

  # transform counts to n
  physeq.scale <- transform_sample_counts(physeq,
                                          function(x) {(n * x/sum(x))}
  )

  # Pick the rounding functions
  if (round == "floor"){
    otu_table(physeq.scale) <- floor(otu_table(physeq.scale))
  } else if (round == "round"){
    otu_table(physeq.scale) <- myround(otu_table(physeq.scale))
  }

  # Prune taxa and return new phyloseq object
  physeq.scale <- prune_taxa(taxa_sums(physeq.scale) > 0, physeq.scale)
  return(physeq.scale)
}

min(sample_sums(tnsub))
    #use the result to substitute n in scale_reads function
tn_scale <-  scale_reads(tnsub, 98819)

min(sample_sums(wasub))
    #use the result to substitute n in scale_reads function
wa_scale <-  scale_reads(wasub, 25973)

sample_data(tn_scale)$Treatments <- factor(
  sample_data(tn_scale)$Treament,
  levels = c("Bare-Groung", "BioAgri", "Naturecycle", "Organix", "Polyethylene",
"PLA-PHA","Weedguard")
)
```

```
sample_data(wa_scale)$Treatments <- factor(
  sample_data(wa_scale)$Treament,
  levels = c("Bare-Groung", "BioAgri", "Naturecycle", "Organix", "Polyethylene",
"PLA-PHA","Weedguard")
)

tn_pcoa <- ordinate(
    physeq = tn_scale,
    method = "PCoA",
    distance = "bray")
wa_pcoa <- ordinate(
  physeq = wa_scale,
  method = "PCoA",
  distance = "bray"
)

tn.pcoa<-plot_ordination(
  physeq = tn_scale,
  ordination = tn_pcoa,
  color = "Treament",
  shape = "Sample",
  title = "PCoA of Bacterial Communities in Knoxville, TN"
) +
  scale_color_manual(values = c("#a65628", "red", "#ffae19",
                                "#4daf4a", "#1919ff", "darkorchid3", "magenta")
  ) +
  geom_point(aes(color = Treament), alpha = 0.7, size = 5) +
  geom_point(colour = "grey90", size = 1.5)

tn.pcoa +
  theme (text = element_text(size=20))

wa.pcoa<-plot_ordination(
  physeq = wa_scale,
  ordination = wa_pcoa,
  color = "Treament",
  shape = "Sample",
  title = "PCoA of Bacterial Communities in Mount Vernon, WA"
) +
  scale_color_manual(values = c("#a65628", "red", "#ffae19",
                                "#4daf4a", "#1919ff", "darkorchid3", "magenta")
  ) +
  geom_point(aes(color = Treament), alpha = 0.7, size = 5) +
  geom_point(colour = "grey90", size = 1.5)
```

```
wa.pcoa +
  theme (text = element_text(size=20))

###############   NMDS plot #######

set.seed(1)
# Ordinate
erie_nmds <- ordinate(
  physeq = erie_scale,
  method = "NMDS",
  distance = "bray"
)

bact.nmds<-plot_ordination(
  physeq = erie_scale,
  ordination = erie_nmds,
  color = "Treament",
  shape = "Sample",
  title = "NMDS of Bacterial Communities"
) +
  scale_color_manual(values = c("#a65628", "red", "#ffae19",
                                "#4daf4a", "#1919ff", "darkorchid3", "magenta")
  ) +
  geom_point(aes(color = Treament), alpha = 0.7, size = 4) +
  geom_point(colour = "grey90", size = 1.5)

bact.nmds +
  facet_wrap(Location~.)+
  theme (text = element_text(size=30))


set.seed(1)

# Calculate bray curtis distance matrix
tn_bray <- phyloseq::distance(tn_scale, method = "bray")
wa_bray <- phyloseq::distance(wa_scale, method = "bray")

# make a data frame from the sample_data
TNsampledf <- data.frame(sample_data(tnsub))
WAsampledf <- data.frame(sample_data(wasub))

# Adonis test
adonis(tn_bray ~ Sample, data = TNsampledf)
#Permutation: free
```

```
#Number of permutations: 999
#Terms added sequentially (first to last)
#          Df SumsOfSqs MeanSqs F.Model      R2  Pr(>F)
#Sample     1    1.1948 1.19478  11.995 0.21421  0.001 ***
#Residuals 44    4.3828 0.09961         0.78579
#Total     45    5.5775                 1.00000

adonis(tn_bray ~ Treament, data = TNsampledf)
#Permutation: free
#Number of permutations: 999
#Terms added sequentially (first to last)
#          Df SumsOfSqs MeanSqs F.Model      R2  Pr(>F)
#Treament   6    0.8362 0.13936  1.1463 0.14992  0.196
#Residuals 39    4.7414 0.12157         0.85008
#Total     45    5.5775                 1.00000

adonis(wa_bray ~ Sample, data = WAsampledf)
#Permutation: free
#Number of permutations: 999
#Terms added sequentially (first to last)
#          Df SumsOfSqs MeanSqs F.Model      R2  Pr(>F)
#Sample     1    3.1711  3.1711  43.287 0.47419  0.001 ***
#Residuals 48    3.5163  0.0733         0.52581
#Total     49    6.6874                 1.00000
adonis(wa_bray ~ Treament, data = WAsampledf)
#Permutation: free
#Number of permutations: 999
#Terms added sequentially (first to last)
#          Df SumsOfSqs MeanSqs F.Model      R2  Pr(>F)
#Treament   6    1.0256 0.17094  1.2982 0.15337  0.195
#Residuals 43    5.6617 0.13167         0.84663
#Total     49    6.6874                 1.00000


#Beta test
TNbeta <- betadisper(tn_bray, TNsampledf$Sample)
permutest(TNbeta)
#Permutation test for homogeneity of multivariate dispersions
#Permutation: free
#Number of permutations: 999
#Response: Distances
#          Df  Sum Sq  Mean Sq     F N.Perm Pr(>F)
#Groups     1 0.10553 0.105531 19.64    999  0.001 ***
#Residuals 44 0.23643 0.005373
```

```
TNbeta2 <- betadisper(tn_bray, TNsampledf$Treament)
permutest(TNbeta2)
#Permutation test for homogeneity of multivariate dispersions
#Permutation: free
#Number of permutations: 999
#Response: Distances
#           Df  Sum Sq  Mean Sq     F N.Perm Pr(>F)
#Groups     6 0.11675 0.0194580 2.4719    999  0.051 .
#Residuals 39 0.30700 0.0078718


WAbeta <- betadisper(wa_bray, WAsampledf$Sample)
permutest(WAbeta)
#Permutation test for homogeneity of multivariate dispersions
#Permutation: free
#Number of permutations: 999
#Response: Distances
#           Df  Sum Sq  Mean Sq     F N.Perm Pr(>F)
#Groups      1 0.49194 0.49194 130.74    999  0.001 ***
#Residuals  48 0.18062 0.00376


WAbeta2 <- betadisper(wa_bray, WAsampledf$Treament)
permutest(WAbeta2)
#Permutation test for homogeneity of multivariate dispersions
#Permutation: free
#Number of permutations: 999
#Response: Distances
#           Df  Sum Sq  Mean Sq     F N.Perm Pr(>F)
#Groups     6 0.34780 0.057967 3.088    999  0.022 *
#Residuals 43 0.80719 0.018772


### alpha diversity

min_lib <- min(sample_sums(tnsub)) ############     replace name


# Initialize matrices to store richness and evenness estimates
nsamp = nsamples(tnsub)
trials = 100

richness <- matrix(nrow = nsamp, ncol = trials)
row.names(richness) <- sample_names(tnsub)
```

```
evenness <- matrix(nrow = nsamp, ncol = trials)
row.names(evenness) <- sample_names(tnsub)

# It is always important to set a seed when you subsample so your result is
replicable
set.seed(3)

for (i in 1:100) {
  # Subsample
  r <- rarefy_even_depth(tnsub, sample.size = min_lib, verbose = FALSE, replace
= TRUE)

  # Calculate richness
  rich <- as.numeric(as.matrix(estimate_richness(r, measures = "Observed")))
  richness[ ,i] <- rich

  # Calculate evenness
  even <- as.numeric(as.matrix(estimate_richness(r, measures = "InvSimpson")))
  evenness[ ,i] <- even
}
# Create a new dataframe to hold the means and standard deviations of richness
estimates
Sample_name <- row.names(richness)
mean <- apply(richness, 1, mean)
sd <- apply(richness, 1, sd)
measure <- rep("Richness", nsamp)
rich_stats <- data.frame(Sample_name, mean, sd, measure)

# Create a new dataframe to hold the means and standard deviations of
evenness estimates
Sample_name <- row.names(evenness)
mean <- apply(evenness, 1, mean)
sd <- apply(evenness, 1, sd)
measure <- rep("Inverse Simpson", nsamp)
even_stats <- data.frame(Sample_name, mean, sd, measure)

alpha <- rbind(rich_stats, even_stats)

s <- data.frame(sample_data(tnsub)) ############     missing WA
colnames(s)[1]<-c("Sample_name")
alphadiv <- merge(alpha, s, by = "Sample_name") #it was SampleID, but I use
Sample_name
#alphadiv <- order_dates(alphadiv) #I dont use this command
colnames(alphadiv)[9]<- c("Treatment") #there was a typo
```

```
write.csv(alphadiv,'tn.alphadiv.csv')

ggplot(alphadiv, aes(x = Treatment, y = mean, color = Sample, group = Sample,
shape = Sample)) +
  geom_point(size = 2.5) +
  stat_summary(fun.y='mean', geom='point', shape='-', size=16, aes(group =
Sample))+
  facet_wrap(~measure, ncol = 1, scales = "free") +
  scale_color_manual(values = c("#696969", "#deb887")) +
  scale_x_discrete(limits = c("No mulch","BioAgri",
"Naturecycle","Organix","PLA/PHA","Polyethylene","WeedGuardPlus"))+
  ggtitle("Knoxville, TN")+
  theme (axis.text.x = element_text(angle=30, size=15), axis.title.y =
element_blank(),text = element_text(size=18))

#Export plot on TIFF with a 1280 width and aspect ratio maintained.
ggsave(filename = "tn-diversity11x8.tiff",width = 11, height = 8, dpi = 300,
device='tiff')
#when "plot" argument is absent, ggsave will defalt to last plot displayed
ggsave(filename = "tn-diversity.eps",width = 11, height = 8, dpi = 300,
device='eps')


################################################ now for Mount, Vernon
WA ############
min_lib <- min(sample_sums(wasub))

# Initialize matrices to store richness and evenness estimates
nsamp = nsamples(wasub)
trials = 100

richness <- matrix(nrow = nsamp, ncol = trials)
row.names(richness) <- sample_names(wasub)

evenness <- matrix(nrow = nsamp, ncol = trials)
row.names(evenness) <- sample_names(wasub)

# It is always important to set a seed when you subsample so your result is
replicable
set.seed(3)

for (i in 1:100) {
  # Subsample
  r <- rarefy_even_depth(wasub, sample.size = min_lib, verbose = FALSE,
replace = TRUE)
```

```
  # Calculate richness
  rich <- as.numeric(as.matrix(estimate_richness(r, measures = "Observed")))
  richness[ ,i] <- rich
  # Calculate evenness
  even <- as.numeric(as.matrix(estimate_richness(r, measures = "InvSimpson")))
  evenness[ ,i] <- even
}
# Create a new dataframe to hold the means and standard deviations of richness
estimates
Sample_name <- row.names(richness)
mean <- apply(richness, 1, mean)
sd <- apply(richness, 1, sd)
measure <- rep("Richness", nsamp)
rich_stats <- data.frame(Sample_name, mean, sd, measure)

# Create a new dataframe to hold the means and standard deviations of
evenness estimatsses
Sample_name <- row.names(evenness)
mean <- apply(evenness, 1, mean)
sd <- apply(evenness, 1, sd)
measure <- rep("Inverse Simpson", nsamp)
even_stats <- data.frame(Sample_name, mean, sd, measure)

alpha <- rbind(rich_stats, even_stats)

s <- data.frame(sample_data(wasub))
alphadiv <- merge(alpha, s, by = "Sample_name") #it was SampleID, but I use
Sample_name
#alphadiv <- order_dates(alphadiv) #I dont use this command
colnames(alphadiv)[9]<- c("Treatment") #there was a typo

ggplot(alphadiv, aes(x = Treatment, y = mean, color = Sample, group = Sample,
shape = Sample)) +
  geom_point(size = 2) +
  stat_summary(fun.y='mean', geom='point', shape='-', size=19, aes(group =
Sample))+
  scale_color_manual(values=c("#696969", "#deb887"))+
  scale_x_discrete(limits = c("No mulch","BioAgri",
"Naturecycle","Organix","PLA/PHA","Polyethylene","WeedGuardPlus"))+
  facet_wrap(~measure, ncol = 1, scales = "free") +
  ggtitle("Mount Vernon, WA")+
  theme(axis.text.x = element_text(angle=30, size=15),axis.title.y =
element_blank(),text = element_text(size=18))
#Export plot on TIFF with a 1280 width and aspect ratio maintained.
```

```
ggsave(filename = "wa-diversity11x8.tiff",width = 11, height = 8, dpi = 300,
device='tiff')
#when "plot" argument is absent, ggsave will defalt to last plot displayed
ggsave(filename = "wa-diversity.eps",width = 11, height = 8, dpi = 300,
device='eps')



#######.....................SIMPER testing


########    function!!!!!!

# convert the sample_data() within a phyloseq object to a vegan compatible data
object
pssd2veg <- function(physeq) {
  sd <- sample_data(physeq)
  return(as(sd,"data.frame"))
}

# convert the otu_table() within a phyloseq object to a vegan compatible data
object
psotu2veg <- function(physeq) {
  OTU <- otu_table(physeq)
  if (taxa_are_rows(OTU)) {
    OTU <- t(OTU)
  }
  return(as(OTU, "matrix"))
}

tnsubENV<-pssd2veg(tnsub)

tnsubotutable<-psotu2veg(tnsub)


library(vegan)
tnsim <- simper(tnsubotutable, tnsubENV$Sample,permutations = 999)
summary(tnsim)
#Contrast: Soil_Buried_plastic

           average       sd  ratio       ava       avb  cumsum     p
Otu00001 1.099e-02 6.494e-03 1.6916 4.475e+02 3.776e+03 0.01963 0.001 ***
  Otu00008 5.322e-03 3.566e-03 1.4925 2.244e+02 1.477e+03 0.02914 0.001
***
  Otu00014 5.313e-03 3.562e-03 1.4919 2.472e+03 1.833e+03 0.03863 0.033 *
```

Otu00002 5.103e-03 4.336e-03 1.1768 1.319e+03 2.665e+03 0.04775 0.001 ***
Otu00029 4.594e-03 2.280e-03 2.0149 1.783e+03 3.789e+02 0.05596 0.001 ***
Otu00012 3.970e-03 1.909e-03 2.0799 4.767e+02 1.649e+03 0.06305 0.001 ***
Otu00022 3.043e-03 1.609e-03 1.8912 1.618e+03 7.035e+02 0.06849 0.001 ***
Otu00018 3.022e-03 2.325e-03 1.2995 1.669e+03 1.536e+03 0.07389 0.859
Otu00003 2.849e-03 2.592e-03 1.0991 2.765e+02 1.096e+03 0.07898 0.001 ***
Otu00016 2.804e-03 1.991e-03 1.4082 4.084e+02 1.205e+03 0.08399 0.001 ***
Otu00004 2.559e-03 2.040e-03 1.2542 1.419e+03 8.806e+02 0.08856 0.225
Otu00007 2.480e-03 1.778e-03 1.3948 2.091e+03 1.763e+03 0.09299 0.676
Otu00035 2.154e-03 2.198e-03 0.9801 2.188e+02 8.538e+02 0.09684 0.001 ***
Otu00011 2.055e-03 1.842e-03 1.1159 1.105e+03 1.165e+03 0.10051 0.002 **
Otu00091 2.043e-03 2.392e-03 0.8541 4.668e+02 6.162e+02 0.10416 0.271
Otu00036 2.030e-03 9.964e-04 2.0378 8.652e+02 2.452e+02 0.10779 0.001 ***
Otu00005 1.978e-03 2.042e-03 0.9691 4.769e+02 9.333e+02 0.11132 0.003 **
Otu00074 1.780e-03 1.414e-03 1.2584 5.842e+02 6.446e+02 0.11450 0.682
Otu00038 1.759e-03 1.216e-03 1.4466 7.599e+02 4.555e+02 0.11765 0.048 *
Otu00113 1.657e-03 2.268e-03 0.7308 1.992e+02 5.508e+02 0.12061 0.006 **
Otu00317 1.623e-03 6.704e-03 0.2421 0.000e+00 4.644e+02 0.12351 0.001 ***
Otu00019 1.551e-03 1.504e-03 1.0308 5.527e+02 8.689e+02 0.12628 0.001 ***
Otu00144 1.539e-03 1.208e-03 1.2734 1.795e+02 6.326e+02 0.12903 0.001 ***
Otu00071 1.435e-03 1.024e-03 1.4011 1.988e+02 6.246e+02 0.13159 0.001 ***
Otu00015 1.432e-03 9.400e-04 1.5238 8.167e+02 6.160e+02 0.13415 0.001 ***
Otu00094 1.393e-03 9.502e-04 1.4656 5.461e+02 1.277e+02 0.13664 0.001 ***
Otu00055 1.390e-03 2.073e-03 0.6703 9.868e+01 4.676e+02 0.13912 0.001 ***
Otu00048 1.383e-03 1.104e-03 1.2522 2.001e+02 5.862e+02 0.14159 0.001 ***
Otu00194 1.371e-03 1.126e-03 1.2173 1.357e+02 5.068e+02 0.14404 0.001 ***

```
tnsimTillage <- simper(tnsubotutable, tnsubENV$Tillage,permutations=999)
summary(tnsimTillage)
#Contrast: Plastic_No_Plastic
```

	average	sd	ratio	ava	avb	cumsum	p	
Otu00001	5.921e-03	6.802e-03	0.8704	2.155e+03	6.026e+02	0.01183	0.924	
Otu00014	4.985e-03	3.441e-03	1.4487	2.261e+03	2.110e+03	0.02178	0.537	
Otu00008	4.583e-03	3.475e-03	1.3185	1.952e+03	1.920e+03	0.03094	0.361	
Otu00002	3.763e-03	3.785e-03	0.9940	1.986e+03	1.447e+03	0.03845	0.634	
Otu00029	3.477e-03	2.450e-03	1.4190	1.222e+03	1.266e+03	0.04540	0.314	
Otu00018	3.180e-03	2.421e-03	1.3136	1.722e+03	1.321e+03	0.05175	0.337	
Otu00004	2.720e-03	2.252e-03	1.2081	1.107e+03	1.494e+03	0.05718	0.122	
Otu00012	2.644e-03	2.195e-03	1.2046	9.154e+02	9.920e+02	0.06246	0.190	
Otu00007	2.445e-03	1.786e-03	1.3689	1.980e+03	1.915e+03	0.06735	0.670	
Otu00003	2.445e-03	2.675e-03	0.9139	5.376e+02	7.652e+02	0.07223	0.103	
Otu00091	2.364e-03	2.644e-03	0.8941	4.726e+02	6.742e+02	0.07695	0.067	.
Otu00317	2.329e-03	7.748e-03	0.3006	2.353e-01	6.960e+02	0.08160	0.266	
Otu00022	2.178e-03	1.519e-03	1.4343	1.240e+03	1.318e+03	0.08596	0.751	
Otu00074	1.861e-03	1.474e-03	1.2629	7.183e+02	2.948e+02	0.08967	0.303	
Otu00016	1.795e-03	1.809e-03	0.9924	7.813e+02	5.468e+02	0.09326	0.729	
Otu00005	1.773e-03	2.079e-03	0.8531	6.251e+02	7.416e+02	0.09680	0.297	
Otu00038	1.740e-03	1.227e-03	1.4181	6.278e+02	6.775e+02	0.10028	0.156	
Otu00011	1.716e-03	1.660e-03	1.0336	1.217e+03	8.770e+02	0.10370	0.588	
Otu00037	1.610e-03	2.198e-03	0.7326	5.173e+02	8.607e+02	0.10692	0.008	**
Otu00030	1.566e-03	1.577e-03	0.9929	4.573e+02	8.014e+02	0.11005	0.021	*
Otu00135	1.517e-03	1.562e-03	0.9711	2.836e+02	4.995e+02	0.11308	0.050	*
Otu00113	1.508e-03	2.248e-03	0.6711	3.180e+02	3.901e+02	0.11609	0.337	
Otu00036	1.499e-03	1.187e-03	1.2627	6.141e+02	6.467e+02	0.11909	0.296	
Otu00059	1.432e-03	1.034e-03	1.3847	7.850e+02	5.494e+02	0.12195	0.437	
Otu00035	1.418e-03	1.817e-03	0.7804	5.170e+02	3.264e+02	0.12478	0.736	
Otu00055	1.349e-03	2.256e-03	0.5980	1.930e+02	3.847e+02	0.12747	0.156	
Otu00132	1.227e-03	1.114e-03	1.1014	2.140e+02	3.870e+02	0.12992	0.023	*
Otu00034	1.226e-03	8.909e-04	1.3757	4.819e+02	4.592e+02	0.13237	0.479	
Otu00052	1.223e-03	8.643e-04	1.4146	3.427e+02	4.618e+02	0.13481	0.294	
Otu00015	1.213e-03	8.917e-04	1.3599	7.786e+02	6.237e+02	0.13724	0.744	
Otu00094	1.209e-03	9.772e-04	1.2375	3.585e+02	4.500e+02	0.13965	0.180	
Otu00023	1.189e-03	1.142e-03	1.0409	6.297e+02	4.500e+02	0.14203	0.621	
Otu00019	1.178e-03	1.241e-03	0.9489	7.258e+02	5.366e+02	0.14438	0.849	
Otu00144	1.130e-03	1.222e-03	0.9247	3.634e+02	3.380e+02	0.14664	0.161	
Otu00260	1.126e-03	1.317e-03	0.8547	1.556e+02	3.151e+02	0.14889	0.056	.
Otu00206	1.118e-03	1.068e-03	1.0477	2.629e+02	2.632e+02	0.15112	0.557	
Otu00048	1.114e-03	1.017e-03	1.0956	3.435e+02	3.731e+02	0.15334	0.224	
Otu00102	1.061e-03	9.093e-04	1.1665	3.675e+02	4.228e+02	0.15546	0.183	
Otu00109	9.993e-04	1.374e-03	0.7271	1.371e+02	3.103e+02	0.15746	0.019	*
Otu00021	9.802e-04	7.774e-04	1.2609	6.453e+02	7.578e+02	0.15942	0.155	

```
library(vegan)
tnsubENVmesh<-subset(tnsubENV, Meshbag=="PE"|Meshbag=="BDM"
,select=Sample_name:Meshbag)
```

```
samplenamestn<-as.character(tnsubENVmesh$Sample_name)
class(samplenamestn)
class(tnsubotutable) #matrix

tnsubotutableMesh<-tnsubotutable[29:46,]
rownames(tnsubotutableMesh)
rownames(tnsubENVmesh)

tnsimMesh <- simper(tnsubotutableMesh, tnsubENVmesh$Meshbag,
permutations = 999)
summary(tnsimMesh)

tnsimMeshDF<-as.data.frame(tnsimMesh$BDM_PE)

tnsimMeshDF[tnsimMeshDF$ava>tnsimMeshDF$avb & tnsimMeshDF$p<0.05,]
#species     average   overall          sd    ratio       ava       avb  ord      cusum
#Otu00001 Otu00001 1.060049e-02 0.5677876 4.728023e-03 2.242056
4548.500000 1073.00     1 0.01866982 0.001
#Otu00033 Otu00033 1.849267e-03 0.5677876 1.053458e-03 1.755426
757.285714  141.75  198 0.16996271 0.037
#Otu00138 Otu00138 2.831414e-04 0.5677876 1.725685e-04 1.640748
178.214286   89.00  785 0.34207604 0.009
#Otu00227 Otu00227 7.026503e-04 0.5677876 4.058775e-04 1.731188
284.928571   62.25 2339 0.42260196 0.004
#Otu00264 Otu00264 9.735996e-05 0.5677876 5.990659e-05 1.625196
38.714286   37.50  321 0.44833162 0.044######## do not use this one
#Otu00570 Otu00570 9.273588e-05 0.5677876 6.142634e-05 1.509709
55.500000   46.00  210 0.58809688 0.039

tnsimMeshDF[tnsimMeshDF$ava<tnsimMeshDF$avb & tnsimMeshDF$p<0.05,]
species     average   overall          sd    ratio       ava       avb  ord      cusum
p
#Otu00004 Otu00004 2.760946e-03 0.5677876 1.838417e-03  1.5018062
694.78571429 1531.00     14 0.04977172 0.005
#Otu00021 Otu00021 1.414402e-03 0.5677876 9.994214e-04  1.4152211
616.50000000 1058.50      7 0.13434743 0.030
#Otu00030 Otu00030 3.108184e-03 0.5677876 1.712426e-03  1.8150764
406.50000000 1411.50     62 0.16192435 0.002
#Otu00037 Otu00037 3.198112e-03 0.5677876 2.791310e-03  1.1457387
564.35714286 1577.50    230 0.17995325 0.004
#Otu00039 Otu00039 1.230083e-03 0.5677876 1.170813e-03  1.0506227
242.00000000  632.75    187 0.18481503 0.009
#Otu00040 Otu00040 1.066318e-03 0.5677876 9.763969e-04  1.0920953
527.57142857  810.25    135 0.18720064 0.008 ##### not use
########################
```

```
wasubENV<-pssd2veg(wasub)

wasubotutable<-psotu2veg(wasub)


library(vegan)
wasim <- simper(wasubotutable, wasubENV$Sample,permutations = 999)
summary(wasim)
#Contrast: Soil_Buried_plastic

average       sd  ratio       ava       avb  cumsum     p
Otu00003 1.305e-02 6.413e-03 2.0353 8.246e+02 5.248e+03 0.02066 0.001 ***
  Otu00006 1.295e-02 1.590e-02 0.8143 7.865e+02 4.840e+03 0.04115 0.001
***
  Otu00001 1.125e-02 9.217e-03 1.2201 1.176e+03 5.028e+03 0.05895 0.001
***
  Otu00010 9.914e-03 3.854e-03 2.5725 3.637e+03 3.397e+02 0.07464 0.001
***
  Otu00017 9.520e-03 1.994e-02 0.4773 8.082e+01 3.491e+03 0.08970 0.030 *
  Otu00020 8.493e-03 8.993e-03 0.9443 2.732e+02 3.260e+03 0.10314 0.001
***
  Otu00013 7.216e-03 6.584e-03 1.0960 1.203e+03 3.193e+03 0.11456 0.001
***
  Otu00002 7.154e-03 3.405e-03 2.1009 3.019e+03 6.669e+02 0.12589 0.001
***
  Otu00025 6.970e-03 6.777e-03 1.0284 3.794e+02 2.704e+03 0.13692 0.001
***
  Otu00032 6.713e-03 9.191e-03 0.7304 1.599e+02 2.426e+03 0.14754 0.001
***
  Otu00028 6.151e-03 2.084e-03 2.9514 2.207e+03 1.752e+02 0.15728 0.001
***
  Otu00005 5.559e-03 3.659e-03 1.5192 3.134e+03 1.380e+03 0.16608 0.001
***
  Otu00011 5.462e-03 2.036e-03 2.6820 2.145e+03 3.350e+02 0.17472 0.001
***
  Otu00026 4.493e-03 3.242e-03 1.3859 6.052e+02 2.102e+03 0.18183 0.001
***
  Otu00027 4.063e-03 1.938e-03 2.0961 1.842e+03 5.109e+02 0.18826 0.001
***
  Otu00024 3.918e-03 1.506e-03 2.6019 1.640e+03 3.465e+02 0.19446 0.001
***
  Otu00008 3.848e-03 1.350e-03 2.8507 1.329e+03 4.086e+01 0.20055 0.001
***
```

Otu00039 3.771e-03 3.051e-03 1.2358 2.089e+02 1.523e+03 0.20652 0.001 ***
Otu00009 3.694e-03 2.933e-03 1.2594 2.513e+03 1.762e+03 0.21237 0.001 ***
Otu00015 3.629e-03 2.112e-03 1.7179 1.761e+03 6.105e+02 0.21811 0.001 ***
Otu00016 3.577e-03 1.977e-03 1.8095 1.563e+03 5.124e+02 0.22377 0.001 ***
Otu00063 3.571e-03 5.440e-03 0.6565 4.814e+01 1.340e+03 0.22942 0.001 ***

```
wasimTillage <- simper(wasubotutable, wasubENV$Tillage,permutations=999)
summary(wasimTillage)
#wasimTillageDF<-as.matrix(summary(wasimTillage))doesnt work

#Contrast: Plastic_No_Plastic
```

	average	sd	ratio	ava	avb	cumsum	p
Otu00006	9.611e-03	1.422e-02	0.6757	2.972e+03	1.296e+03	0.02003	0.721
Otu00003	8.204e-03	7.893e-03	1.0394	2.792e+03	2.704e+03	0.03712	0.756
Otu00013	6.835e-03	8.207e-03	0.8328	1.997e+03	2.337e+03	0.05136	0.152
Otu00001	6.783e-03	8.512e-03	0.7968	3.320e+03	1.449e+03	0.06550	0.986
Otu00010	5.955e-03	4.507e-03	1.3212	2.116e+03	2.408e+03	0.07790	0.875
Otu00017	5.823e-03	1.592e-02	0.3659	2.005e+03	2.392e+02	0.09004	0.906
Otu00020	5.638e-03	7.803e-03	0.7225	1.762e+03	1.034e+03	0.10178	0.824
Otu00032	5.477e-03	9.439e-03	0.5802	1.169e+03	1.118e+03	0.11320	0.462
Otu00025	4.844e-03	6.033e-03	0.8029	1.508e+03	1.068e+03	0.12329	0.743
Otu00002	4.592e-03	3.488e-03	1.3168	1.899e+03	2.252e+03	0.13286	0.777
Otu00005	3.735e-03	3.056e-03	1.2223	2.285e+03	2.605e+03	0.14064	0.896
Otu00028	3.585e-03	2.896e-03	1.2378	1.242e+03	1.538e+03	0.14811	0.779
Otu00026	3.465e-03	3.524e-03	0.9833	1.227e+03	1.381e+03	0.15533	0.314
Otu00009	3.307e-03	2.883e-03	1.1472	2.115e+03	2.396e+03	0.16222	0.414
Otu00011	3.258e-03	2.467e-03	1.3204	1.297e+03	1.514e+03	0.16901	0.890
Otu00004	3.007e-03	2.657e-03	1.1316	2.066e+03	2.445e+03	0.17528	0.601
Otu00042	2.788e-03	2.830e-03	0.9850	9.027e+02	9.751e+02	0.18108	0.389
Otu00027	2.565e-03	1.989e-03	1.2896	1.214e+03	1.390e+03	0.18643	0.731
Otu00015	2.437e-03	1.877e-03	1.2987	1.248e+03	1.278e+03	0.19151	0.782
Otu00024	2.429e-03	1.850e-03	1.3131	9.999e+02	1.295e+03	0.19657	0.564
Otu00016	2.404e-03	1.810e-03	1.3280	1.087e+03	1.143e+03	0.20158	0.957
Otu00008	2.377e-03	1.898e-03	1.2526	7.097e+02	9.284e+02	0.20653	0.446
Otu00039	2.365e-03	2.723e-03	0.8685	8.400e+02	6.200e+02	0.21146	0.801
Otu00023	2.350e-03	1.765e-03	1.3315	1.006e+03	1.091e+03	0.21635	0.905
Otu00063	2.337e-03	4.454e-03	0.5247	7.153e+02	3.048e+02	0.22122	0.814
Otu00031	2.246e-03	3.078e-03	0.7298	8.062e+02	8.122e+02	0.22590	0.494

```
Otu00047 2.099e-03 1.688e-03 1.2434 6.771e+02 7.709e+02 0.23028 0.827
Otu00021 2.080e-03 1.585e-03 1.3123 9.260e+02 1.142e+03 0.23461 0.797
Otu00087 2.079e-03 3.489e-03 0.5959 5.381e+02 3.942e+02 0.23894 0.513
Otu00073 2.001e-03 2.817e-03 0.7104 5.391e+02 6.131e+02 0.24311 0.266
Otu00012 1.982e-03 1.648e-03 1.2030 1.285e+03 1.402e+03 0.24724 0.882

wasubENVmesh<-subset(wasubENV, Meshbag=="PE"|Meshbag=="BDM"
,select=Sample_name:Meshbag)
#select rows named 75:92
rownames(wasubotutable)
#wasubotutableMesh<-wasubotutable[27:45,]
#rownames(wasubotutableMesh) #wrong rows
#"55" "56" "75" "76" "77" "78" "79" "80" "81" "82" "83" "84" "85" "86" "87" "88" "89"
"90" "91"
wasubotutableMesh<-wasubotutable[29:46,]
rownames(wasubotutableMesh) #right rows!

wasimMesh <- simper(wasubotutableMesh,
wasubENVmesh$Meshbag,permutations = 999)
summary(wasimMesh)
wasimMeshDF<-as.data.frame(wasimMesh$BDM_PE)

wasimMeshDF[wasimMeshDF$p<0.05,]
wasimMeshDF[wasimMeshDF$ava>wasimMeshDF$avb,]
wasimMeshDF[wasimMeshDF$ava<wasimMeshDF$avb &
wasimMeshDF$p<0.05,]
#Otu00101 Otu00101 5.525705e-05 0.53354 3.699885e-05 1.4934802
7.21428571  22.25   430 0.4988917 0.003
#Otu00112 Otu00112 3.986688e-04 0.53354 3.226979e-04 1.2354241
51.28571429 159.00   219 0.5174628 0.021
#Otu00135 Otu00135 7.594023e-05 0.53354 6.340546e-05 1.1976923
12.28571429  28.25   422 0.5520678 0.031
#Otu00212 Otu00212 3.227520e-04 0.53354 2.270151e-04 1.4217203
79.78571429 166.00   801 0.6373495 0.030
#Otu00299 Otu00299 8.281599e-05 0.53354 6.264787e-05 1.3219283
15.42857143  33.50   140 0.6994849 0.035
wasimMeshDF[wasimMeshDF$ava>wasimMeshDF$avb,]
#Otu00001 Otu00001 1.349766e-02 0.53354 8.516614e-03 1.5848618
5.817357e+03 1898.25     6 0.02902774 0.630
#Otu00006 Otu00006 1.548746e-02 0.53354 1.263354e-02 1.2259000
3.733286e+03 2993.75    20 0.14409698 0.305
#Otu00018 Otu00018 2.763611e-04 0.53354 2.272859e-04 1.2159183
9.921429e+01   82.00    73 0.25635263 0.964
Otu00025 Otu00025 7.636543e-03 0.53354 6.176957e-03 1.2362953
2.563000e+03 2493.75   116 0.29542522 0.832
```

Otu00027 Otu00027 1.281400e-03 0.53354 9.164433e-04 1.3982321
5.147143e+02 476.75   225 0.30492885 0.973

######identifying the OTUs from SIMPER ######

#convert tax table to DF
mothurTaxTable<-as.data.frame(tax_table(mothur_data))
#access the OTUs I care about
mothurTaxTable["Otu00037",] #rank 2 is phyla, rank 6 is genus

TN plastic enriched, simper
Otu00001, Proteobacteria,Bradyrhizobium
Otu00002, Chloroflexi, KD4-96_unclassified
Otu00012, Firmicutes, Bacillus
Otu00003, Actinobacteria, Nocardioides (this one is rank 8)
Otu00016, Chloroflexi, KD4-96_unclassified
Otu00035, Chloroflexi, Roseiflexus

WA plastic enriched, simper
Otu00003, Actinobacteria, Nocardioides (this one is rank 8)
Otu00006, Proteobacteria, Rhodanobacter
Otu00001, Proteobacteria,Bradyrhizobium
Otu00020, Actinobacteria, Catenulispora (this one is rank 8)
Otu00013, Proteobacteria, Xanthomonadaceae_unclassified
Otu00025, Proteobacteria,Rhodanobacter

mothurTaxTable["Otu00570",]
TN: BDM vs PE enriched, simper
enriched in BDMs, p<0.05
#species
#Otu00001, Proteobacteria,Bradyrhizobium
#Otu00033, Proteobacteria, Comamonadaceae_unclassified
#Otu00138, Chloroflexi, KD4-96_unclassified
#Otu00227 , Planctomycetes, Pirellula_unclassified
Otu00264 , Bacteria_unclassified , Bacteria_unclassified ; do not use
#Otu00570 , Proteobacteria, Rhizobiales_unclassified

mothurTaxTable["Otu00021",]
enriched in PE, p<0.05
#species
#Otu00004 Proteobacteria, Sphingomonas
#Otu00021 Acidobacteria, uncultured_unclassified

#Otu00030 Actinobacteria, Nocardioidaceae_unclassified
#Otu00037 Actinobacteria, Streptomyces
#Otu00039 Proteobacteria, Rhizobiales_unclassified
Otu00040 Actinobacteria, Mycobacterium

WA BDM vs PE enriched, simper
mothurTaxTable["Otu00038",]
enriched in BDMs, p>0.05 no difference is significant!!!!
#species
#Otu00001 , Proteobacteria,Bradyrhizobium
#Otu00006 , Proteobacteria, Rhodanobacter
Otu00014 , Bacteria_unclassified , Bacteria_unclassified
Otu00015 , Bacteria_unclassified , Bacteria_unclassified
Otu00017 , Bacteria_unclassified
#Otu00018 , Verrucomicrobia, DA101_unclassified
#Otu00025, Proteobacteria, Rhodanobacter
#Otu00027, Chloroflexi, JG30-KF-CM45_unclassified
Otu00033, Proteobacteria, Comamonadaceae_unclassified

mothurTaxTable["Otu00299",]
enriched in PE, p<0.05
#species
Otu00072 Bacteria_unclassified
#Otu00101 Proteobacteria, uncultured_unclassified
#Otu00112 Actinobacteria, Solirubrobacter
#Otu00135 Proteobacteria, Sphingomonas
#Otu00212, Actinobacteria, Streptosporangiaceae_unclassified
#Otu00299, Actinobacteria, Solirubrobacterales_unclassified

CONCLUSION AND RECOMMENDATIONS

This research broadly demonstrated that four seasons of use and incorporation of BDMs into the soils did not have a sustained effect in the soil microbial communities compared to PE mulch and no mulch treatments. We also did not find differences between mulch treatments in activity of several carbon, phosphorus and nitrogen cycling extracellular soil enzymes. Nevertheless, we showed that buried mulch fragments have a higher bacterial abundance compared to bulk soil. The mulch-associated bacterial community is less rich and diverse, indicative of a selective enrichment. The buried mulch bacterial community is made of some taxa with potential symbiotic-interactions with plants or diverse compound metabolism. At large, this project is intended to provide information to growers and policy makers on effects of use and incorporation of BDMs into soil. This last decade has been crucial for plastic research: as society, governments and media have realized plastic debris ubiquity, and their potential health and environmental hazards. Strides to curb single-use plastic consumption has been made. However, while most cameras point toward the Great Pacific Garbage Patch, our soils continue to get filled with plastic debris.

With PE mulch films usage expected to raise, we must focus on finding and validating other mulch alternatives. Although BDMs stand out among these alternatives, they remain hindered by their price tag and user's habit to use conventional PE mulch. A change in BDMs adoption will likely be driven by policy change, which is in turn driven by thorough validation of BDMs' safety. This is precisely what our research group did with our USDA-funded four-year field project. Briefly, our group found that soil health was not influenced by BDMs use and incorporation, crop production on BDMs was comparable to PE mulch, most growers would be inclined to use BDMs if they had a competitive incentive (i.e., competitive with growers using inexpensive PE mulches), and that BDMs have specialized microbial communities. Nevertheless, a longer-term field study (5-10 years) would be critical to find if BDMs alter soil health and its microbial community, and to what extent. In our 4-year field research plots, we did not have substantial carry-over of BDMs from previous seasons; accumulation of plastic debris from mulches in field is cited as one of the reasons for its low adoption. A longer-term field project could also be useful to determine this factor. Edaphic factors are location-dependent and take several years to show a sustained alteration. With a long-term field study, and another location, these properties can be further explored, increasing our confidence in BDMs.

Another question that remains is what happens when BDMs are initially tilled in. We think that this time point might have the highest variability among taxa and enzyme activity. A future project could include metagenomic sequencing of soil and buried plastic mulches in a small time-frame subsequently after tillage. This would provide a more exhaustive microbial community analysis to the one done here in a potentially dynamic temporal frame. Furthermore, we would gain an insight into genetic potential of microbial communities, and guide enzyme analysis.

One final recommendation is to continue designing field-like experiment in lab-scale where seasonal inputs of BDMs are added. Lab-scale experiments with varying factors like BDM input concentration, incubation time and changing climate factors are crucial to understand how this factors affect soil health and microbial communities. Furthermore, this experiments are important to understand how climate change will affect BDMs practicality in the future.

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VITA

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