# ASSESSING MICROBIAL COMMUNITY DYNAMICS AND SOIL QUALITY UNDER VARIED SOIL AMENDMENT PRACTICES

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ABSTRACT

This thesis investigates the effects of agricultural soil amendments on the soil microbial community and associated soil nutrients to gain an understanding for the potential implementation of the amendments as practice to promote overall soil health. Study one explored soil fumigation (metam sodium, 1,3-dichloropropenene, and combination) amendments and the potential to recurve impacts fumigation has on the microbial community through cover crop planting (faba bean and winter pea mixture, mustard, radish, and wheat). Results from this study showed that metam sodium+1,3-dichloropropene application resulted in the most shifts in community abundance, with *Planctomycetota*, *Acidobacteriota*, and *Verrucomicrobiota* significantly decreasing (2.39%, 7.48%, and 1.01% to 1.95%, 5.42%, and 0.70%, respectfully). Regarding the eukaryotic population, the dominant phyla *Ascomycota* and *Basidiomycota* showed significant decreases with combination fumigation treatment, decreasing from 11.41% to 6.93% and 0.77% to 0.36%, respectfully. None of the cover crops resulted in changes in the microbial community following fumigation for either prokaryotic or eukaryotic populations.

Study two researched anaerobic digestate as an organic fertilizer alternative and the effect application has on the soil microbial community. Results showed shifts in both prokaryotic and eukaryotic taxa. *Proteobacteria* and *Firmicutes* abundance increased from 18.0% to 19.1% and 4.95% to5.81%, respectfully, and *Chloroflexi* decreased from 6.72% to 5.82%. For the eukaryotic population, the abundance of *Basidiomycota* increased significantly with the application of digestate from 2.4% to 3.9%. The nutrient levels remained stable throughout the duration of the study, apart from total hydrogen which significantly decreased with digestate application from 1.10±0.50 wt% to 0.59±0.22 wt%.

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## 1.1 Introduction

The treatment and management of agricultural land has been a topic of interest for many years. However, more recently, there has been a greater push for the implementation of more sustainable agricultural practices and the creation of a circular economy (Slepetiene et al., 2022; Stockmann et al., 2015). Such practices include the use of feedstock anaerobic digestate as a replacement fertilizer for crops and cover crop planting to promote biological soil health parameters. Both practices have shown several benefits in the maintenance and improvement of agricultural soil including carbon sequestration, reduced nutrient leaching, and increased soil microbial biomass.

When applying treatments to agricultural land, it is always important to consider the implications to soil health. Soil health is critical to the sustainability and functionality of agriculture and the environment (Arias et al., 2005). It considers the soil's "biological, chemical, and physical features" that work in unison to maintain a functional and thriving ecosystem (Abbott & Murphy, 2003). In recent years, microbial communities within soil have become a greater area of interest, acknowledging the role that the biological environment in soil plays in the interconnection of the chemical and physical makeup. The diversity of the microbial communities is so vast, studies have had a hard time quantifying the numbers of different living organisms in soil. This is due impart because each microbial community is very specific to plant type, soil type, global location, and climate, to name a few. It has been reported that billions of microorganisms live in a handful of soil (I. M. Young et al., 2008). Microbial communities' control nutrient cycles within the soil and in turn are an important influence on soil efficiency

and climate change (Balser et al., 2010; Buckley & Schmidt, 2001; Miransari, 2013; Torsvik et al., 1990). Historically, agriculture has namely focused on soil fertility rather than soil health. Soil fertility refers to the "soil chemical fertility and its ability to meet the nutritional needs of plants", ignoring the biological factors, ultimately giving a poor overall understanding of the fertility and health of the soil (Abbott & Murphy, 2003).

Not only does soil health impact the success of agriculture, but it also holds a critical position within the global carbon cycle (Doran & Zeiss, 2000). Soil serves as the largest terrestrial carbon reservoir, accounting for the storage of an estimated 2 to 3 times of that stored in the atmosphere (Arias et al., 2005; Scharlemann et al., 2014; Stockmann et al., 2013; Witzgall et al., 2021). Within current research, there is a lack of knowledge of the amount and spatial distribution of carbon in soil. However, this knowledge is critical to the understanding of the global carbon cycle, carbon management, and climate policy (Scharlemann et al., 2014).

Carbon is stored within soil reservoirs in the form of soil organic carbon (SOC) (Lange et al., 2015; Scharlemann et al., 2014). SOC consists of the "carbon derived from decaying vegetation, fungal, and bacterial growth, and microbial activities of living organisms" (Scharlemann et al., 2014). Carbon storage in terrestrial pools is critical to offsetting the increasing amounts of atmospheric carbon dioxide (Lange et al., 2015). Small changes in SOC can have a significant impact the carbon emissions and therefore contribute to climate change due to the sheer amount of carbon stored in the soil (Arias et al., 2005; Stockmann et al., 2013). Microbial communities highlight the connection between the breakdown of plant-derived carbon into SOC (Witzgall et al., 2021). In a grassland biodiversity experiment (The Jena Experiment), it was shown that higher microbial activity in soil was directly related to an increase in SOC storage (Lange et al.,

2015). This study focused on the application of anaerobic digestate as a soil carbon management method and reviewed its impacts on soil health, microbial community, and land sustainability.

Soil health and fertility is a critical factor in global and environmental health. It also plays an important role within food growth and availability. Ensuring the health of the soil promotes an environment that can help mediate atmospheric carbon as well as promote healthy crops. Anaerobic digestate is widely recognized as an effective fertilizing alternative that helps to return carbon to the soil. However, there exists a knowledge gap in the understanding of anaerobic digestates' impact on microbial communities that exist within the soil to which it is applied. This study wishes to address this knowledge gap by identifying and analyzing the microbial communities that exist within soil that is treated with anaerobic digestate as well as create a general overview of the total soil health by quantifying nutrient and carbon contents.

## 1.2 Soil Health

### 1.2.1 Soil Organic Matter

Soil organic matter (SOM) is made up of three different parts, including the "living organisms, fresh residues, and well-decomposed residues" (Arias et al., 2005; Magdoff & Van Es, 2009). 15% of the organic matter is made up of the living organisms, including microbials, fungi, plant materials, insects, and animals (Magdoff & Van Es, 2009). Others refer to SOM as only the nonliving aspects of organic matter found in soil, depending on the study (Trumbore, 1997). SOM helps determine soil structure and nutrient storage for plant uptake, ultimately impacting plant growth and success (Magdoff & Van Es, 2009; Trumbore, 1997). The residues making up SOM include roots, root exudates, and plant matter such as leaves. These contain carbon dioxide that is fixed by plants. When the microbials and fungi feed on the residues as an energy source, decomposing the organic matter. When this occurs, carbon dioxide is released from the soil

through the process of oxidation, much the same as animals respirating (Lehmann & Kleber, 2015; Magdoff & Van Es, 2009; Trumbore, 1997). SOM contains the most terrestrial carbon, making it an important carbon pool and a major contributor to carbon cycling (Lehmann & Kleber, 2015; Magdoff & Van Es, 2009). Depletion of SOM leads to the release of carbon dioxide into the atmosphere from the soil. This can happen through erosion, deforestation, non-sustainable agricultural practices, and a general decline in the soil health from lack of organic matter input (Magdoff & Van Es, 2009).

Soil organic matter has a positive effect on almost all soil properties, allowing it to establish the basis of a healthy soil (Magdoff & Van Es, 2009). For the large influence SOM has on the productivity and health of soil, it only makes up 1-6% of agricultural topsoil (Arias et al., 2005; Magdoff & Van Es, 2009). However, this small percentage has critical effects on the physical structure and biological aspect of the soil. When organic matter enters the soil, it promotes microbial diversity, which, in turn, results in an increase of available nutrients and aggregation, both promoting the growth of healthy plants (Magdoff & Van Es, 2009). With intensive agricultural practices, SOM can be depleted, resulting in a loss of nutrients and microbial diversity within the soil, impacting the fertility and health of the soil (Arias et al., 2005).

### 1.2.2 Soil Organic Carbon

Soil organic carbon (SOC) is the largest terrestrial carbon pool, housed within soil organic matter (SOM). SOC serves many critical roles within the functioning of the environment. It is crucial to soil health, soil microbial diversity, plant health, crop production, global food security, and the water and carbon cycles (Doran & Zeiss, 2000; Stockmann et al., 2015). In recent years, more attention has been brought to the measurement of global SOC, as concerns for climate change have increased (Stockmann et al., 2015). With change in land use and the climate, soil releases

more carbon dioxide into the atmosphere as degradation of SOM occurs (Doran & Zeiss, 2000; Stockmann et al., 2015). It is important to promote the use of land management practices to prevent increasing land use intensity, soil health degradation, and the release of carbon dioxide into the atmosphere (Grandy & Robertson, 2007). Because SOM can serve as both a source and sink for carbon, it is a new challenge to attempt to find and change agricultural practices to both conserve and absorb carbon stocks (Guo & Gifford, 2002).

Soil organic carbon makes up about 58% of soil organic matter (Stockmann et al., 2013). Carbon enters the soil through organic matter in the SOM. Decomposition of the SOM releases organic carbon compounds into the soil that is then used by microorganisms as an energy source. SOC contains three types of carbon pools, including active, slow, and passive pools (Xu et al., 2016). Decomposition of SOC serves an important role in the carbon cycle by breaking down organic carbon compounds into usable inorganic forms (Xu et al., 2016). There are several conditions that contribute to the regulation of SOC degradation. These include climate, water content, soil structure and properties, and carbon nitrogen ratio (C:N) (Xu et al., 2016). As temperature decreases, SOC stocks increase. It has been found that colder, humid climates have the highest amount of SOC in the soil (Stockmann et al., 2013). In a study by Xu et al., 2016 it was found that the decomposition rates for the active and slow pool types were most impacted by chemical and physical conditions of the soil. These decomposition rates were shown to be the lowest with high clay content, water content, and C:N ratio.

#### 1.2.2.1 Total Organic Carbon Measurement

The total carbon in soil is comprised of both organic and inorganic carbon. To quantify the amount of organic carbon present in soil, or TOC, the organic matter in the soil needs to be destructed. This can be done chemically or by combustion very high temperatures (Schumacher,

2002). The combustion method is known as the catalytic combustion oxidation method. With this method, the soil sample is heated to 720°C or higher. By doing so the carbon in the sample is oxidized and converted to carbon dioxide (Avramidis et al., 2015). It is the carbon dioxide that is measured for carbon quantification. This, however, measures both inorganic and organic carbon, or total carbon (TC). To find TOC, the inorganic carbon (IC) is measured separately (Avramidis et al., 2015). Inorganic carbon in soil is found as carbon derived from carbonates. IC can be measured by adding a small amount of hydrochloric acid. Acidifying the sample converts carbonates to carbon dioxide that is then volatized and measured. TOC is then found by taking the difference of TC and IC (Avramidis et al., 2015; Schumacher, 2002).

### 1.2.3 Soil Nutrients

For the successful production of agricultural crops, 16 to 18 elemental nutrients are required for successful plant growth, including carbon (C), hydrogen (H), nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), iron (Fe), sulfur (S), and calcium (Ca) (Magdoff & Van Es, 2009; Miransari, 2013; Roy et al., 2006). When any one of these nutrients are lacking, the plant cannot complete a successful life cycle, including reproductive success (Roy et al., 2006). Within this list of nutrients, most exist as mineral nutrients while oxygen (O) and carbon (C) are acquired from carbon dioxide and hydrogen (H) is acquired from water (Roy et al., 2006). These nutrients can be broken down into micro- and macronutrients. Those that are needed in large amount are referred to as macronutrients and those required in smaller amounts are micronutrients. Macronutrients include potassium (K), nitrogen (N), phosphorous (P), carbon, hydrogen, oxygen, magnesium, and sulfur (Magdoff & Van Es, 2009; Stockdale et al., 2003).

### 1.2.4 Soil Microbial Community

Microbial populations in the soil play a critical role in the maintenance of elemental nutrients within the soil by "increasing the bioavailability of soil-borne nutrients" for uptake and use by plants (Jacoby et al., 2017). Elemental nutrients are absorbed by plants only in their mineral form (Roy et al., 2006). Soil microbes help to release the nutrients which are bound in organic molecules. Therefore, most nutrients are very minimally bioavailable for plants to use when found in naturally occurring soil ecosystems. By employing metabolic processes, microbes and fungi can mineralize and depolymerize the organic forms of nutrients found in soil. Plants are then able to uptake and use these nutrients via root systems (Jacoby et al., 2017). Because of this relationship, it has been shown that soil microbial communities have a direct impact on plant growth (Jacoby et al., 2017).

C:N ratio refers to the ratio of the amount of carbon to nitrogen contained in organic matter (Magdoff & Van Es, 2009). This ratio has shown to influence the composition of soil microbial communities and is sometimes used as a measurement for such as it varies for plants based on factors such as growth stage (Magdoff & Van Es, 2009; Wan et al., 2015). The C:N ratio is used to help determine the percentage of nitrogen in the SOM (Magdoff & Van Es, 2009). A high ratio means low nitrogen and a low ratio means there is a high percentage of nitrogen. For SOM, the ratio usually falls between 10:1 to 12:1 (Magdoff & Van Es, 2009).

Nitrogen and potassium are the most prevalent mineral nutrients found in plants, making up 80% of the total mineral nutrients (Roy et al., 2006). 19% of mineral nutrients found in plants consists of phosphorus, sulfur, calcium, and magnesium. Nitrogen is the most prevalent mineral and is absorbed by the roots of a plant in the ionic forms of nitrate or ammonium (Magdoff & Van Es, 2009; Roy et al., 2006). Nitrogen within a plant plays a key role in the plant's

development and growth. Deficiency in this nutrient is marked in reduced growth rates and a "spindly appearance" (Roy et al., 2006). It is also an important nutrient to monitor due to its climate implications. Nitrogen is frequently applied as a fertilizer because soil is often lacking the amounts needed for healthy crop production (Magdoff & Van Es, 2009). Excessive fertilization can lead to the release of nitrogen from the soil in runoff and leaching. When not contained in SOM, nitrogen can be converted into nitrous oxide, damaging ozone levels (Magdoff & Van Es, 2009). Microbial communities in soil help to regulate nitrogen levels and make the nutrient available for use. By promoting soil health, microbial communities should theoretically be able to maintain health nitrogen levels from organic matter with less release from the soil.

### 1.2.5 Rhizosphere

Much of the positive interaction between plants, soil microbes, and nutrients occurs in the rhizosphere. The rhizosphere is a term to describe the region of soil with of increased interactions between soil microbes and plant roots, influenced by root secretions (Berendsen et al., 2012; Bonkowski, 2004; Raaijmakers et al., 2009). These interactions control the availability of nutrients found in the rhizosphere (Marschner & Rengel, 2003). This is critical to the processing and signaling of all nutrients a plant uptakes and stimulates mass microbial activity (Bonkowski, 2004). Root exudates contain large amounts of substrates that are easily degradable, most notably, accessible carbon sources (Bonkowski, 2004; Marschner & Rengel, 2003). The carbon in exudates serve as a main food source for microbes. This attracts a diverse, dense microbial population to this soil region, specifically created by the plant itself (Marschner & Rengel, 2003; Raaijmakers et al., 2009). These microbial communities are highly diverse, and much larger genomically than the plant. For this reason, the microbial communities found within

the rhizosphere are often referred to as a “second genome” for the plant (Berendsen et al., 2012). The microbiome found in the rhizosphere can vary from plant species and genotype, as well as location when compared to bulk soil (Berendsen et al., 2012; Qu et al., 2020). There are two aspects of the rhizospheres’ microbial population that promote plant health. One being the influence on nutrient availability to plants, and two being the promotion of disease resistance, creating a form of “feedback regulation” (Qu et al., 2020).

Within the rhizosphere, both harmful and beneficial microorganisms are attracted and exist (Berendsen et al., 2012; Raaijmakers et al., 2009). Because of this, there is a constant battle between the beneficial and pathogenic (Berendsen et al., 2012). These interactions have a direct effect on the pathogenic effects of the pathogen and the outcome of the infection (Raaijmakers et al., 2009). Beneficial microorganisms are able to suppress disease in plants by out-competing pathogens for nutrients through microbial activity, as well as producing antibiotic compounds, known as ‘general disease suppression’(Berendsen et al., 2012). Therefore, the microbial community within the rhizosphere has a direct influence on the growth and survival of the plant.

### 1.2.6 Soil pH

Soil pH has shown a correlation with microbial diversity in soil (Zhalnina et al., 2015). It impacts soils chemically, physically, and biologically (Aciego Pietri & Brookes, 2008). For most crops, a pH ranging from 6 to 7 is best (Magdoff & Van Es, 2009). In soils that are too acidic or too basic, nutrients are not as available, making unideal growth conditions (Magdoff & Van Es, 2009). In a study by Aciego Pietri & Brookes, 2008, correlations found between total and organic carbon, total nitrogen, and pH shows the suggestion of pH impacting the input of SOC. The challenge that comes with the analysis of soil pH is the variation across different soil types, land-use, location, and climate. Although several studies have found that there is a direct

relationship between soil microbial communities and pH, there are conflicting results from others (Kemmitt et al., 2006; Zhalnina et al., 2015). This could be due in part to the variance in soil. Even in soils with a natural gradient of pH there has been difficulty in attributing changes in soil microbial activity and community structure to pH (Kemmitt et al., 2006).

### 1.2.7 Physical Structure of Soil

The physical structure of soil is important in consideration to the overall soil health. Soil structure plays an important role in the functioning of the soil as an environmental mediator, supporting plants and complex microbial communities. It also is a crucial player in carbon sequestration, or carbon storage (Bronick & Lal, 2005). Soil structure can be defined as the arrangement of soil particles, their size and shape, and the presence and location of pores (Bronick & Lal, 2005; Lal, 1991). This arrangement is known as aggregation. Aggregate is a "combination of mineral particles with organic and inorganic substances" (Bronick & Lal, 2005). The process of aggregation is impacted by many different factors, including microbial activity, SOC concentration, land management practices, and plant influences. The formation of aggregates occurs in several stages. One theory, known as the hierarchy theory, suggests that microaggregates are joined with macroaggregates (Bronick & Lal, 2005). Studies have found that good soil structure is favorable for the support of microbial communities and storage of SOC (Bronick & Lal, 2005; Cui & Holden, 2015). Increased aggregation is related to an increase in SOC concentration in soil (Bronick & Lal, 2005). SOC also impacts microbial activity, resulting in microbes having a significant effect on the soil structure through carbon processes that aid in the structure formation (Cui & Holden, 2015). The encouragement of more sustainable farming practices results in better soil structure, health, and crop production.

## 1.3 Agricultural Land Management Practices

### 1.3.1 Soil Fumigation

Soil fumigation was first used in France in 1869 with the application of carbon disulfide to control grape pathogens (Chellemi, 2014; Lembright, 1990). This agricultural treatment is used for pest control and is applied to the soil by injection prior to planting. The liquid chemical, when injected, rapidly volatilizes to form a gas that is able to fill air spaces within the soil and effectively kill fungi, bacteria, nematodes, insects, etc. (Chellemi, 2014; Wilson, 1968). Since the discovery of soil fumigants, numerous compounds have been developed and phased out. For many years, methyl bromide (MeBr) was one of the leading soil fumigants until its phase out in 2005 due to its link to stratospheric ozone damage (Chellemi, 2014; Klose & Ajwa, 2004). This fumigant was a reliable and effective tool for crop protection against pathogens, and after its elimination, researchers worked to find comparable alternatives that would not impact the environment to the extent of methyl bromide (Klose & Ajwa, 2004). Metam sodium (MS) has now become one of the leading fumigants (classified as a nematicide), within the United States, especially within potato production (Cox, 2006). When MS is applied to the soil, it quickly hydrolyzes to form methyl isocyanate (MITC), which is the active pesticidal agent, targeting amino acids within cell walls (Ajwa et al., 2010; Cox, 2006). It is also common practice to combine metam sodium with another nematicide known as 1,3-dichloropropene (1,3-D) (classified as a fungicide) to complement and enhance the pesticidal properties of each compound (Ajwa et al., 2010).

Soil fumigation is most often employed in high value crops with repeated planting in the same location (Chellemi, 2014; Radewald et al., 1987). Fumigation provides a simple, one-time solution to pest management, allowing the user to forego repeated pesticide application (due to

fumigants high toxicity) while treating a range of pathogens without specific fungicide, bactericide, nematicide and herbicide application (due to fumigants broad-spectrum treatment) (Chellemi, 2014). By utilizing fumigants, growers can eliminate crop rotation due to the effective pest control, making the practice financially beneficial. However, the toxicity of the fumigants is documented to be detrimental to beneficial soil microorganisms in addition to pathogens, due to the chemical's broad-spectrum nature. As the soil microbial community is essential to the functioning of a healthily soil, repeated application of fumigants can reduce the overall health of the soil (X. Li et al., 2022; Sederholm et al., 2018). This is where a knowledge gap lies. While soil fumigation remains essential to high-value crop production, depletion of the soil health overtime will result in poor crop yield. To sustain this practice, trends in fumigation research need to address how non-target, beneficial microorganisms can be returned to the soil without impacting the fumigants effects on pathogens (Chellemi, 2014).This study looks at the role cover crops can play when trying to fill this knowledge gap.

### 1.3.2 Cover Crop Planting

Cover crops are used for a variety of reasons and purposes in agricultural soil management and grown most often between cash crop plantings (Dabney et al., 2001; Kaspar & Singer, 2011; Nevins et al., 2018). Some of these purposes include crop yield stability, erosion and nutrient leaching reduction, pest suppression, including weeds, and carbon sequestration (Dabney et al., 2001; Schmidt et al., 2018). Three classifications of cover crops exist, including "catch crops", "green manures", and "living mulch", with each of these groups having a specialized purpose. Catch crops prevent nutrient leaching during fallow periods, green manures are N-fixing to provide nitrogen to soils, and living mulch provides erosion protection, nutrient cycling, and weed suppression (Kaspar & Singer, 2011). Previous studies have shown that cover crop

planting significantly impacts the soil microbial community and structure, many seeing increases in the overall abundance of microbes (Chavarría et al., 2016; Muhammad et al., 2021; Schmidt et al., 2018). Increasingly, cover crops are being explored as sustainable means for promoting, maintaining, or recovering soil health (Collins et al., 2006; Nevins et al., 2018). Because of the crops' ability to increase soil microbial community abundance, cover crops may be a viable option to recurve the impacts fumigation has on the beneficial microbial community. Cover crops have shown to increase microbial populations antagonistic to pathogenic organisms, so their application has the potential to not reverse the fumigants' effects on the pathogenic community (Collins et al., 2006).

### 1.3.3 Sustainable Agricultural Land Management

In recent years, growing attention has been given to soil health as a crucial factor in food security, climate change, water quality, and energy security (Stockmann et al., 2015). With this, the way soil health and fertility are viewed has changed, stemming a push to implement more sustainable agricultural and land management practices that promote more than just the chemical fertility of soil. One major facet of this sustainability effort is on returning carbon to soil pools for storage to help mitigate that released into the atmosphere. Some practices that aid in that respect include the reduction of tillage and the application of organic matter as crop covers to increase residue input (Grandy & Robertson, 2007). Anaerobic digestate can be considered a renewable, organic crop cover that has benefits for overall soil health.

#### 1.3.3.1 Anaerobic Digestate Application

Anaerobic digestate has been identified as a means to return carbon to the soil for storage in an effort to reduce climate change due to greenhouse gases (Chen et al., 2008; Slepetiene et al., 2022). It is a waste treatment process that can be utilized to process agricultural and industrial

waste, resulting in a practice that both minimizes pollution and produces renewable energy (Chen et al., 2008; Lee et al., 2021). Agricultural and industrial waste sources work well for anaerobic digestion because of their high number of biodegradable materials in them (Chen et al., 2008). Anaerobic digestion is a fermentation process that utilizes microorganisms to break down organic matter into biogases and digestate in an anaerobic environment (Bajpai, 2017). The biogases produced mostly consist of methane and carbon dioxide and are utilized as a renewable energy source (Bajpai, 2017; Chen et al., 2008; Lee et al., 2021). This process is also considered "one of the most efficient waste… treatment technologies," and carrying benefits such as its low energy requirement (Chen et al., 2008).

The process of anaerobic digestion can be divided into four different groups, including hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Bajpai, 2017; Broughton, 2009). Within the hydrolysis step, organic polymers, including starches, proteins, and fats, are depolymerized into more basic molecules (sugar, amino acids, glycerol, fatty acids) fermentative microbes aided by enzymes (Bajpai, 2017; Broughton, 2009). Acidogenesis is responsible for converting organic molecules into organic acids by acid-forming bacteria. Acetogenic bacteria then produce acetic acid and hydrogen from the previous organic acids (Broughton, 2009). Finally, methanogenic bacteria produce methane, a component of the produced biogas (Bajpai, 2017; Broughton, 2009; Chen et al., 2008). The entire process is carried out and dictated by the presence of bacteria that work symbiotically to keep advancing the process, with the products of one bacteria type being substrates for another type (Bajpai, 2017). A major factor in the efficiency of anaerobic digestion is particle size. The smaller the particle, the more surface area there is for increased enzymatic activity (Broughton, 2009).

Fertilization is the most practiced way of increasing soil fertility for agricultural land (Tian et al., 2017). Inorganic fertilizers contribute to greenhouse gas emissions as well as water pollution from agricultural runoff. Because of this and its increasing cost, there is a need to turn away from inorganic fertilizers and replace them with effective organic alternatives (Walsh et al., 2012). Anaerobic digestate serves as an organic fertilizer that has been shown to increase or maintain crop yields when compared to inorganic fertilizers (Lee et al., 2021; Walsh et al., 2012). It can be broken down into liquid and dry solids for land application. Anaerobic digestate is ideal for fertilization due to its high nutrient content. Anaerobic digestion is known to preserve critical soil nutrients, specifically nitrogen, phosphorous, and potassium (Barłóg et al., 2020; Lee et al., 2021). In comparison to untreated livestock waste as a fertilizer, there are significant differences in the nutrients and carbon content provided to soil (Barłóg et al., 2020). Anaerobic digestate "contains a more balanced nutrient profile," so the soil doesn't have a need for supplemental nutrient application from inorganic fertilizers (Lee et al., 2021). Overall, there is a lack of knowledge and reliable data concerning crop nutrition due to high variability in environmental study conditions (Lee et al., 2021). Crops with digestate applied showed a greater nitrogen uptake efficiency (Möller & Müller, 2012). This is directly related to an increased ammonia content during digestion. Ammonia is a readily available nitrogen source for plants, and therefore, digestate has a high content of plant-available nitrogen (Möller & Müller, 2012; Walsh et al., 2012).

Regarding the impact of anaerobic digestate on the microbial and biological community of the soil, there is a large knowledge gap. It is known that inorganic fertilizers can result in decreases in soil microbial diversity and respiration (Sabir et al., 2021). More knowledge needs to be found to address the effect of organic fertilizers on the overall health of the soil by

addressing changes in soil biodiversity (Karimi et al., 2022). Finding concurrent data for this topic is difficult due to the wide range of variability in digestate types, the original soil microbial diversity, and other soil parameters such as pH and nutrient content (Karimi et al., 2022). In a study of the current literature available regarding digestate's impact on soil biodiversity, it was found that digestates had a neutral effect on soil biological quality in half of the 222 studies addressed. Of those 222 studies, 7% of the studies showed a negative impact on soil biological diversity and 17% showed that digestate was "less beneficial than other organic fertilizers" (Karimi et al., 2022). Going forward, more studies need to be conducted in the field setting over multiple years to analyze the short- and long-term effects of digestate on soil microbial diversity. With this knowledge, a more conclusive and expansive statement can be made about the relationship between anaerobic digestate and soil health.

## 1.4 Microbial Community Analysis Methods

### 1.4.1 DNA Extraction Methodology

To measure the diversity and activity of soil microbial communities, DNA extraction is used to quantify and analyze the amount of microbial DNA present (Arias et al., 2005; Yeates et al., 1998). The method of extraction of genetic material, specifically found within soils, is highly established and widely used, although there are several different methods that can be utilized (Yeates et al., 1998). In a study performed by Yeates et al., 1998, four different methods of DNA extraction were identified and analyzed for the best method. It was found that the bead-beating method, described in the study, is the most effective at lysis of organisms, and therefore is identified as the method of choice (Yeates et al., 1998). Lysis of cell walls is a major factor in the success and efficiency of DNA extraction as well as the quality of the DNA extracted. When the cell wall is lysed, the genetic material is released and can be accessed by the extraction buffer.

Bead-beating results in higher rates of cell wall lysis, with a lyse efficiency of >90%, and therefore a greater amount of genetic material is released and accessible (De Boer et al., 2010; Robe et al., 2003; Yeates et al., 1998). Bead-beating utilizes mechanical disruptions to enhance extraction (Fujimoto et al., 2004). Beads are added to the tube containing the sample for extraction. The tube is then shaken using a Vortexer to cause collisions between the bead and the sample (Burden et al., 2014; De Lipthay et al., 2004; Fujimoto et al., 2004; Yeates et al., 1998). These collisions cause the lysis of cell walls. This is then followed by DNA purification to isolate the genetic material from soil matter. Chemical lysis is also widely used as a method of extraction of DNA from soil alone or in conjunction with physical methods (i.e., bead-beating) (Robe et al., 2003). The most used chemical lysis agent is a detergent known as sodium dodecyl sulfate (SDS). A detergent is often used with heat treatments and chelating agents (Robe et al., 2003). Between bead-beating and chemical methods, bead-beating shows higher genetic material extraction.

#### 1.4.1.1 16S rRNA Sequencing for Prokaryotic Communities

16s rRNA is used widely for the identification of bacterial species and continues to be the most commonly used genetic marker for taxonomic analysis (Janda & Abbott, 2007). A number of factors surrounding the 16s rRNA gene boost its popularity of use including universal presence in most bacteria, non-changing gene function (highly conserved), and large size, making it ideal for sequencing purposes (Abellan-Schneyder et al., 2021; Janda & Abbott, 2007; Patel, 2001). Studies have found that 16s rRNA sequencing is able to make genus identification most often, special identification in approximately 80% of cases, and may result in no identification for as low as 1% of the isolates, making it a reliable and accurate form of taxonomic identification (Janda & Abbott, 2007; Mignard & Flandrois, 2006).

#### 1.4.1.2 ITS rRNA Sequencing for Fungal Community

ITS rRNA sequencing is used in PCR amplification for fungal genetic material. ITS stands for internal transcribed spacers (Walker et al., 2022). The genes in prokaryotes as well as fungi, specifically the 16s, 23s, and 5s rRNA, are separated by spaces that can vary in size and sequence between species and genus (Couto et al., 2001). The differences in the spacer regions allows for the identification of different species. This type of sequencing has the ability to provide taxonomic information as well as high-resolution amongst fungal communities (O'Brien et al., 2005). The ITS is located between the large and small subunits of the ribosomal RNA gene. It provides a quickly evolving metric for the identification of variation in species as it is the fastest changing genetic region in most organisms (Walker et al., 2022).

## 1.5 Conclusion

Current trends in environmental change and agricultural land management practices are pushing sustainable agriculture to the forefront. By promoting and maintaining soil health, with an emphasis on the biological components, agricultural practices in turn can prevent nutrient leaching, erosion, and soil depletion. Cover crop planting and the application of the digestate byproduct as a fertilizer have shown positive results relating to crop yield and nutrient availability for plants. These practices also hold potential is recurving previous field treatments in fumigation and fertilization. The current knowledge gap exists when it comes to the impact on soil microbial communities, and the interactions these practices may have. Microbial communities within the soil play a key role in many soil processes that maintain soil health and fertility as well as environmental health. There have been emerging studies addressing this gap, however, few results are conclusive due to the large variability associated with digestate, diversity of microbial communities, and physical and chemical soil factors.

The objective of this research is to conduct a long-term field analysis of the soil microbial communities, nutrients, and properties with the application of fumigation, cover crops, and anaerobic digestate and document the resulting shifts in communities due to field treatment.

# CHAPTER 2: EFFECT OF FUMIGATION, COVER CROPS AND POTATO PLANTING ON SHIFTS IN MICROBIAL COMMUNITIES

## 2.1 Summary

Fumigation as a broad-spectrum pesticide can affect both pathogenic and non-target microorganisms in the soil. As microbial communities are critical within the soil ecosystem, depletion of or changes in these communities can result in negative implications for soil health. Because cover crops are used to enhance soil health physically, chemically, and biologically, they might recover the soil health of the fumigated soil. However, little information is available on interactive effect of these practices on soil health especially regarding the soil microbiome. The objective of this study was to investigate microbial community shifts as affected by fumigation and cover crops in different stages of potato field management practices. A year-long field test was conducted in Adkins sandy loam of Eastern Oregon following a split-plot design with fumigation as main plots and cover crops as subplots. Fumigation treatments included non-fumigated control, metam sodium, 1,3-dichloropropenene, and metam sodium+1,3-dichloropropenene. Soil samples were collected around the termination of cover crops and after potato harvesting for soil microbial analysis. 16S and ITS rRNA- gene sequencing analyses were employed to understand the prokaryote and eukaryote populations, respectively. Microbial community analysis indicated no significant interaction between the treatment conditions, with cover crops resulting in no significant shifts in the soil microbial community. Combination fumigation treatments resulted in the largest community shifts for both prokaryotic and eukaryotic communities; however, these changes were not recovered from cover crop planting.

## 2.2 Introduction

Potatoes (*Solanum tuberosum* L.) are one of the leading cash crops in the United States, specifically the Pacific Northwest (PNW) region, with 61% of the U.S. potato production in 2021 coming from the PNW (Hills et al., 2020; United States Department of Agriculture, NASS, 2022). Soil-borne diseases (*Fusarium* dry rot, verticillium wilt, pythium root rot) are major limiting factors in the production of high-yielding, quality potato crops. Thus, soil fumigation has been a widely employed method of soil-borne pest control worldwide, especially within the PNW, targeting a diverse range of organisms, including nematodes, weeds, pathogenic fungi, and insects (Huang et al., 2019; Lembright, 1990). Historically, methyl bromide (MeBr) was a heavily utilized fumigant. However, due to its negative impact on the stratospheric ozone layer, MeBr has been phased out (J. Li et al., 2017). The discontinuation of MeBr prompted the adoption of new or existing chemical soil fumigants. Within the United States, metam sodium (MS) has emerged as the predominant soil fumigant in agriculture, finding widespread use, particularly in potato crops which account for half of its application (Cox, 2006; Sederholm et al., 2018). As a broad-spectrum preplant pesticide, metam sodium effectively controls a range of agricultural pests, including weeds, nematodes, pathogenic fungi, and arthropods (Carlock & Dotson, 2010; J. Li et al., 2017). MS works by hydrolyzing into methyl isocyanate (MITC), a highly toxic and volatile gas, in the presence of water (Ajwa et al., 2010; Sederholm et al., 2018). MITC serves as the pesticidal agent in metam sodium by inactivating specific portions of amino acids (Cox, 2006). Conversion of MS into MITC in soil happens very rapidly, with a conversion efficiency ranging from 87% to 95%; however, this can be impacted by soil characteristics, specifically soil moisture (Zheng et al., 2006). Because MITC possesses a high vapor pressure in relation to MS, it is easily volatilized and dispersed in the gaseous phase, resulting in

atmospheric emissions. Even with the high conversion efficiency, the 10-60% of MITC applied to soil can be lost (van den Berg, 1993; Zheng et al., 2006), resulting in repeated applications and soil exposure creating long-term effects on soil health (Sederholm et al., 2018). Although MS is converted into MITC efficiently, the efficacy of the fumigant is often enhanced with the subsequent use of 1,3-dichloropropene (1,3-D, also known as cis-Telone) (Ajwa et al., 2010). 1,3-D is a nematicide which possesses both fungicidal and insecticidal properties as well, making it a registered fumigant in several countries (Liu et al., 2015). Like MS, 1,3-D undergoes a degradation process within the soil aided by soil bioactivity. Volatilization of 1,3-D is estimated to result in the loss of 20-50% of applied fumigant to the atmosphere, again resulting in repeated applications (Dungan et al., 2001).

In recent years, there has been a growing emphasis on the importance of overall soil health in crop production and global sustainability (Hills et al., 2020). Soil is a significant source of biodiversity for the planet, and in turn, provides numerous services including nutrient cycling, water purification, and carbon storage (Maron et al., 2011). A major indicator of soil health is the microbial structure and diversity of the soil. These microorganisms function within the soil to help maintain the ideal chemical, physical, and biological characteristics that make up soil ecosystems (Abbott & Murphy, 2003; Crecchio et al., 2004; C. Li et al., 2014). However, soil microbial communities are complex ecosystems, and they can be easily influenced by soil properties, such as nutrient availability, pH, and salinity levels (C. Li et al., 2014). This is also true for agricultural management practices, resulting in positive and negative impacts for soil health (Seneviratne, 2009). Previous studies have shown that fumigating soil, namely with MS and 1,3-D, can result in increasing and decreasing shifts in the diversity and functional structure of soil microbial communities, thus disrupting the symbiotic nature of the ecosystem by

impacting nutrient cycling and other functional roles held by microbes (Fang et al., 2020; X. Li et al., 2022; Liu et al., 2015; Sederholm et al., 2018). These shifts in microbial diversity have direct impacts on soil health and resulting plant health and crop production.

Cover crops have been used for years to maintain soil structure by reducing erosion of bare fields during the non-growing season (Muhammad et al., 2021; Poeplau & Don, 2015). They have been increasingly utilized to promote increases in soil organic matter (SOM), decrease nutrient leaching, and reduce pest infection (Daryanto et al., 2018; Muhammad et al., 2021; Poeplau & Don, 2015). Choice of cover crop is important to the crop's successful growth; however, this choice is region-dependent due to differing weather conditions. The weather in Eastern Oregon is semi-arid and can be characterized by dry summers and cold, overcast winters. Therefore, it is crucial to choose a cover crop that will be able to withstand harsh winter conditions. The cover crops chosen for this study were identified due to their root structures, microbial community influence, biomass production, and their ability to survive/thrive in the testing region. Mustard and radish fall under the *Brassicaceae* family. This family of plants can suppress weeds and pathogens due to the isocyanate present in its seeds. This process works similar to that of MS fumigation, allowing *Brassicaceae* to suppress weeds for longer periods of time (Hollister et al., 2013). Radish was also chosen for its rooting ability, as well as protection from erosion and soil compaction (Gruver et al., 2014). A combination of peas and faba beans is favorable due to its ability to rapidly decompose biomass, form symbiotic relationships with nitrogen fixing microbes lessening the need for N fertilization, and its efficient water usage (Mottin et al., 2021; Tribouillois et al., 2015). Winter cereals, such as winter wheat, are excellent and common choices for over-winter crops. Winter wheat is effective at reducing soil erosion, absorbing soil nutrients, suppressing weeds due to its rapid growth, and improving topsoil. All

these functions are enhanced through its fine, yet high-surface area, root system (Clark, 2007; Snapp et al., 2005). In addition, winter wheat is a hearty plant that is able to withstand Oregon winters (Clark, 2007).

It was hypothesized that planting cover crops following fumigation will lead to a measurable increase in beneficial bacteria and fungi in the soil, compared to soils without cover crops, resulting in a diverse microbial population to enhance potato yield and quality. This study addresses two main goals: 1) to examine if the use of cover crops can be a viable alternative to winter fallow, which is generally implemented after fumigation of the soil; and 2) to assess the impacts of cover crop inclusion after fumigation on potato yield and quality.

## 2.3 Materials and Methods

### 2.3.1 Field Experimental Design

A year-long field test was conducted in the Hermiston Agricultural Research and Extension Center (45.81726940164113, -119.2846659758225), Oregon State University (OSU-HAREC) in Hermiston, Oregon from October 2021 through September 2022. Within this region, the soil can be classified as Adkins Series. This taxonomy is defined by deep, well-drained soils which can be described as very fine sandy loam (*Official Series Description - ADKINS Series*, n.d.).

The experimental design utilized a randomized complete block with a split-plot arrangement with three replications, assigning fumigation as the main plot (3.35 m wide by 30.48 m long) and cover crops as subplots (3.35 m wide by 6.10 m long), with a 1.52 m buffer zone between each plot to avoid fumigant crossover. The main plot included no fumigation as control, fumigation with MS, fumigation with 1,3-D (Telone), and the co-fumigation with MS plus 1,3-D. These fumigation treatments were applied on October 1, 2021, using shank injection, with MS being

injected at a depth of 15 cm and 1,3-D injected at a depth of 46 cm. The co-fumigated plots were first treated with MS, followed by 1,3-D 12 hours later.

In mid-October 2021, once the residual fumigants had dissipated completely, the main plots were divided into five subplots. The five cover crop treatments employed were as follows: a control group with no cover crop, a mixture of faba bean and winter pea, mustard, radish, and wheat, with winter pea, faba bean, mustard, radish, and wheat being seeded at rates of 50, 60, 12, 10, and 120 lb./acre, respectively. The cover crops were terminated in early April 2022 via mechanical tilling and incorporated back into the soil at a depth of 20 cm.

In late April 2022, the fields were prepped for potato planting using Russet Burbank. Several fertilizers, including nitrogen, phosphorus, potassium, magnesium, boron, and zinc were applied to the fields prior to planting in accordance with the grower's standards. A controlled-release fertilizer (ESN) was specifically utilized for the nitrogen application. The potato harvest occurred in mid-September 2022, during which parameters including yield, size, specific gravity, and disease incidence were recorded.

### 2.3.2 Soil Sampling

A two-point sampling method was used, where two soil cores were collected per plot at depths of 0-30 cm, with sampling taking place immediately after cover crop termination and after potato harvesting. According to Oregon State University- Soil Health Lab suggested soil storage and preparation procedures, the samples were passed through a 1 mm sieve to remove debris and stored at -20 °C until microbial community analysis could occur.

### 2.3.3 DNA Extraction

Total genetic DNA was extracted using the DNeasy® PowerSoil® Pro kit (Qiagen, Hilden, Germany). DNA concentration was then measured using the NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific Inc., USA).

### 2.3.4 PCR Amplification for Illumina Sequencing

Sequencing of the 16s rRNA and ITS rRNA genes for bacterial and fungal identification were conducted using the universal primers Pro341F (5′-CCTACGGGNBGCASCAG-3′)- Pro805R (5′-GACTACNVGGGTATCTAATCC-3′) (Di Nicolantonio et al., 2023) and ITS1F (5′-CTTGGTCATTTAGGAAGTAA-3′)- ITS2R (5′- GCTGCGTTCTTCATCATGATGC -3′) (Cheng et al., 2021). PCR processes were carried out for both primer sets using the method as follows: initial denaturation at 95 °C for 2 min, 30 denaturation cycles at 95 °C for 20 s, primer annealing at 52 °C for 30 s, elongation at 72 °C for 1 min, and extension of new strands at 72 °C for 10 min. Amplified samples were verified using 2% agarose gel electrophoresis.

### 2.3.5 Sequence Processing

Samples were sequenced at Michigan State University Genomics Core using a MiSeq sequencer (Illumina, Inc., San Diego, CA, USA). The Illumina Fastq files from the high-throughput sequencing were analyzed using Qiime2 (a microbiome bioinformatics platform) to generate taxonomic/phylogenetic data for statistical analysis and comparison of the soil microbial community shift.

### 2.3.6 Community Data Analysis

Alpha diversity was examined using Qiime2 and RStudio, including richness (Shannon-Weiner Species Diversity Index) and evenness (Pielou's evenness) for both bacterial and fungal communities. Changes in these indices were analyzed using two-way analysis of variance

(ANOVA) to determine the relationship between fumigation and cover crops and the treatment interactions. Differences in the indices between fumigation, cover crop, and potato planting were tested using post-hoc Tukey's Honest Significant Difference (HSD) tests. Qiime2 was used to perform principal coordinate analysis (PCoA) to examine beta diversity, based on weighted UniFrac distances. Significant differences in beta diversity were analyzed using permutational multivariate analysis of variance (PERMANOVA, permutations = 9999) in Qiime2. Community composition was analyzed in RStudio (version 2023.09.1). Two-way ANOVA and Tukey's Honest Significant Difference (HSD) tests were used to analyze the shifts in microbial community abundance due to treatment with a significance value of $P \leq 0.05$.

## 2.4 Results

### 2.4.1 Bacterial Alpha and Beta Diversity

Total community shifts were analyzed using alpha and beta diversity indices. Alpha diversity utilized Shannon-Weiner index to quantify the community richness (number) and Pielou's Evenness to measure community evenness (distribution) (Table A 1). Beta diversity used principal coordinate analysis (PCoA) and analysis of similarity (ANOSIM) to measure the similarity or dissimilarity of two communities. No significant difference ($P > 0.05$) was identified using two-way ANOVA to analyze changes in Shannon's index related to fumigation (Figure 1A, Table A 2). Pielou's evenness did show significant differences ($P < 0.05$) from the no fumigation control, with a decrease in the mean index values for the MS+1,3-D treatment (Figure 1D, Table A 2). Cover crop type had no significant effect on community richness and evenness, according to Shannon-Weiner and Pielou's Evenness (Figure 1B/E, Table A 3), possibly due to the limited growth of biomass as cover crops were planted in late October. Potato planting did indicate significant differences ($P < 0.05$) in both alpha diversity indices with

pairwise comparison between samples taken prior to potato planting and those samples taken after potatoes were harvested, with the mean index values decreasing for both community diversity and evenness (Figure 1C/F, Table A 4). Beta diversity was modeled using principal coordinate analysis (PCoA), generated using the weighted UniFrac distances. This analysis indicated no clear shifts in community composition, with no dominant group formation due to fumigation or cover crop type observed (Figure 2A). When using this same analysis to compare samples according to sampling time (before potato planting and after potato harvest), a significant shift in the community was observed (PERMANOVA, $P < 0.05$), with two distinct groupings formed in the PCoA plot, indicating that community composition is most dependent on potato planting (Figure 2B).

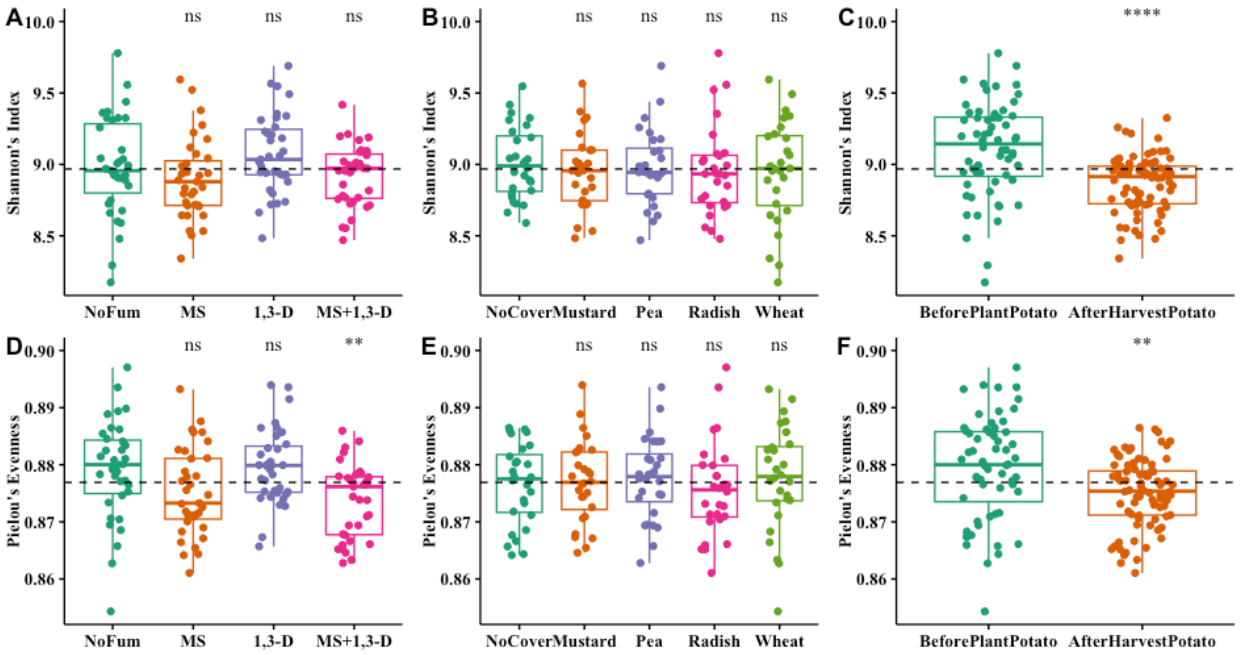


*Figure 1: Alpha diversity indices for the prokaryotic population based on Shannon-Weiner diversity (A,B,C) and Pielou's evenness (D,E,F) for fumigation treatment, cover crop type, and sampling time.*

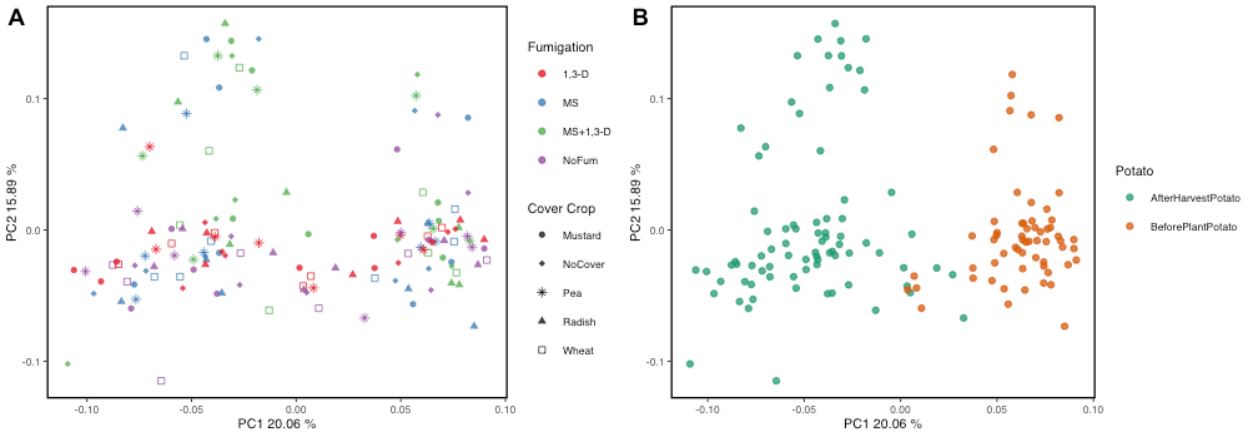


*Figure 2: Beta diversity analysis using principal coordinate analysis (PCoA) based on weighted UniFrac distances for the prokaryotic microbial community for fumigation treatment and cover crop (A) and sampling time (B).*

### 2.4.2 Bacterial taxonomic composition

Two-way analysis of variance (ANOVA) indicated no significant interaction ($P > 0.05$) between fumigation and cover crop application in terms of abundance data (Table A 5, Table A 6) for the identified dominant bacterial phyla and genera. Shifts in the microbial community composition (based on 16s rRNA amplicon sequencing) between fumigation treatments were analyzed using a two-way ANOVA with a 5% significance level (Figure 3A, Table A 7, Table A 8). Application of MS resulted in significant decreases ($P < 0.05$) in abundance for *Acidobacteriota* when compared to no fumigation control, decreasing in abundance from 7.48% to 6.14%. None of the dominant phyla saw a significant shift ($P < 0.05$) in abundance with the application of only 1,3-D. The application of the combined fumigation treatments resulted in significant decreases ($P < 0.05$) for *Planctomycetota*, *Acidobacteriota*, and *Verrucomicrobiota* abundances. The dominant phyla saw no significant difference ($P > 0.05$) due to cover crop type when compared to the no cover control.

Fumigated samples showed no significant shifts in abundance ($P > 0.05$) for the dominant genera *Sphingomonas*, *Bacillus*, *Gaiellales* unclassified and uncultured, *Gemmatimonadaceae*

unclassified and uncultured, *Nocardioides*, and *Micrococcaceae* unclassified when compared to the no fumigation control (Figure 3B). *Micromonosporaceae* unclassified indicated a significant increase ($P < 0.05$) from the no fumigant control when compared with MS+1,3-D fumigation from 1.9% to 2.7% (Table A 8). Similarly, the genus *Actinobacteria* unclassified showed a significant increase ($P < 0.05$) from 4.1% to 5.7% when comparing the non-fumigated control to those samples treated with MS+1,3-D. The dominant genera saw no significant difference ($P > 0.05$) due to cover crop type when compared to the no cover control.

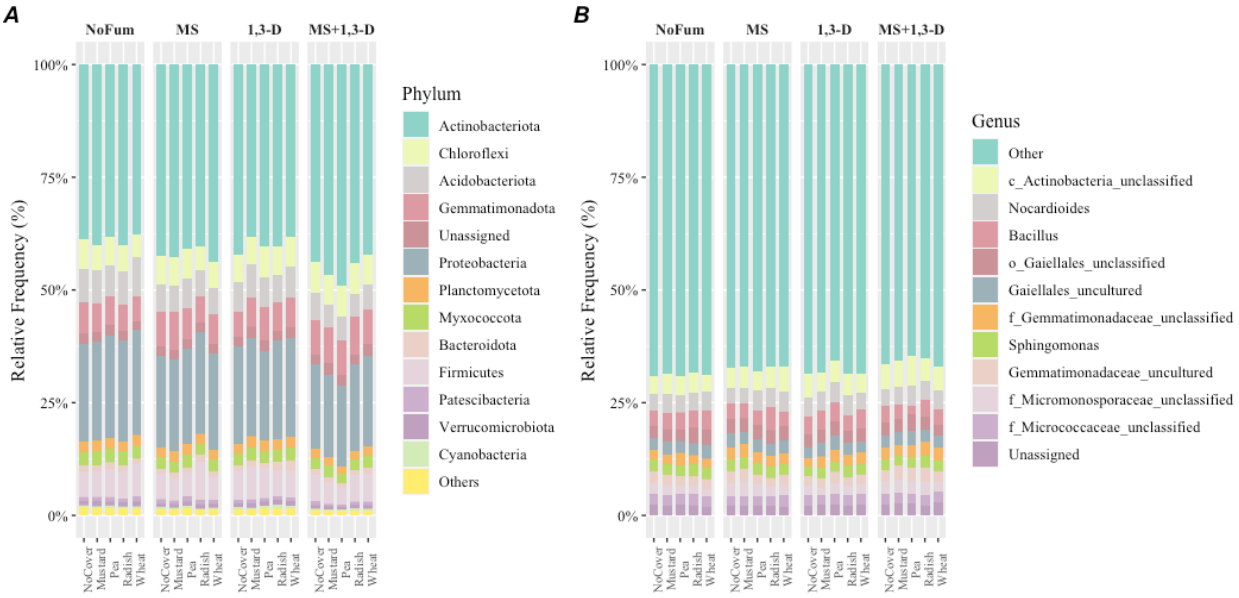


*Figure 3: Relative frequencies of the dominant prokaryotic A) phyla (all phyla accounting for >5% of abundance) and B) genera (all genera accounting for >2% of abundance) according to fumigation and cover crop type based on 16s rRNA amplicon sequencing. Genera outside of the baseline percentages were grouped under "Other".*

### 2.4.3 Eukaryotic Alpha and Beta Diversity

The same alpha and beta diversity analysis performed for the prokaryotic population was utilized for the eukaryotic population (Table A 1). Pairwise comparisons (Tukey's HSD) were used to identify significant differences in Shannon-Weiner diversity index and Pielou's evenness between no fumigation control and fumigation treatments, no cover control and cover crop types, and sampling time (before versus after potato planting) (Table A 2, Table A 3, Table A 4). The

eukaryotic community richness and evenness saw a significant shift ($P < 0.05$) in the form of an overall decrease for samples treated with MS+1,3-D (Figure 4A/D). No other significant shifts were identified for Shannon-Weiner index or Pielou's evenness for fumigation treatments, cover crop types, or sampling time (Figure 4). The PCoA based on weighted UniFrac distances, indicates no significant shifts in community composition due to either fumigation treatment or cover crop type (Figure 5A). Following the same trend seen with the prokaryotic population, the eukaryotic population did experience a shift in community composition due to potato planting, with distinct grouping shown on the PCoA plot for samples taken before potatoes were planted and those taken after potato harvest (PERMANOVA, $P < 0.05$) (Figure 5B).

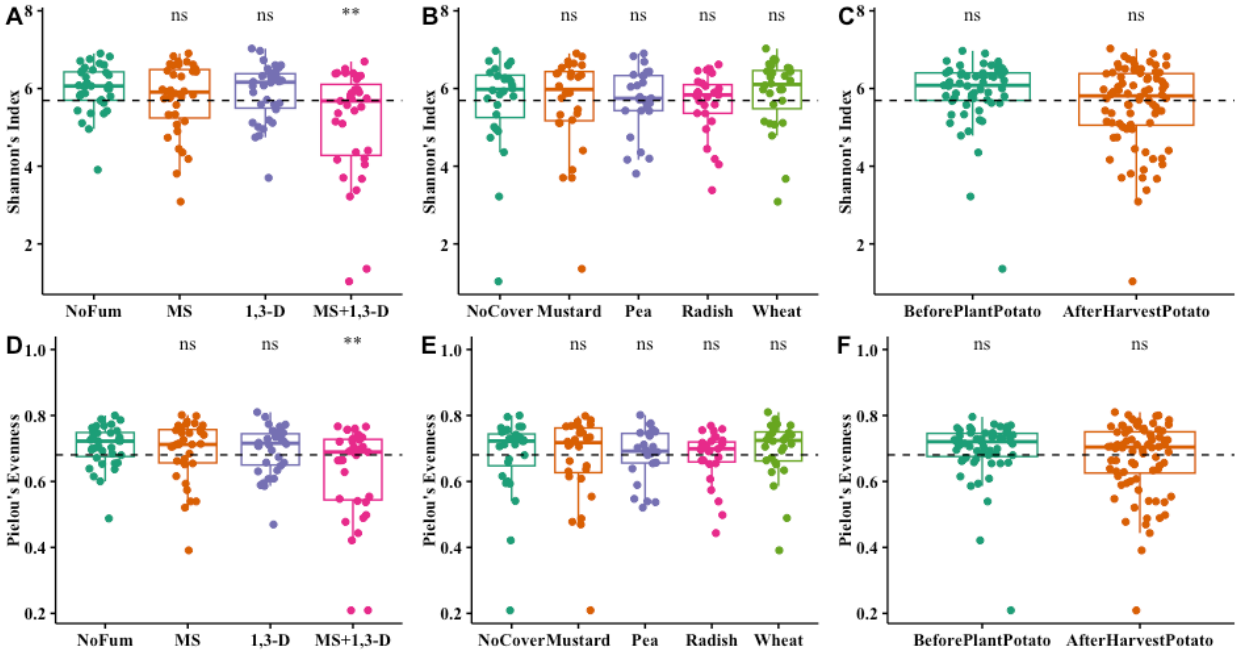


*Figure 4: Alpha diversity indices for the eukaryotic population based on Shannon-Weiner diversity (A, B, C) and Pielou's evenness (D, E, F) for fumigation treatment, cover crop type, and sampling time.*

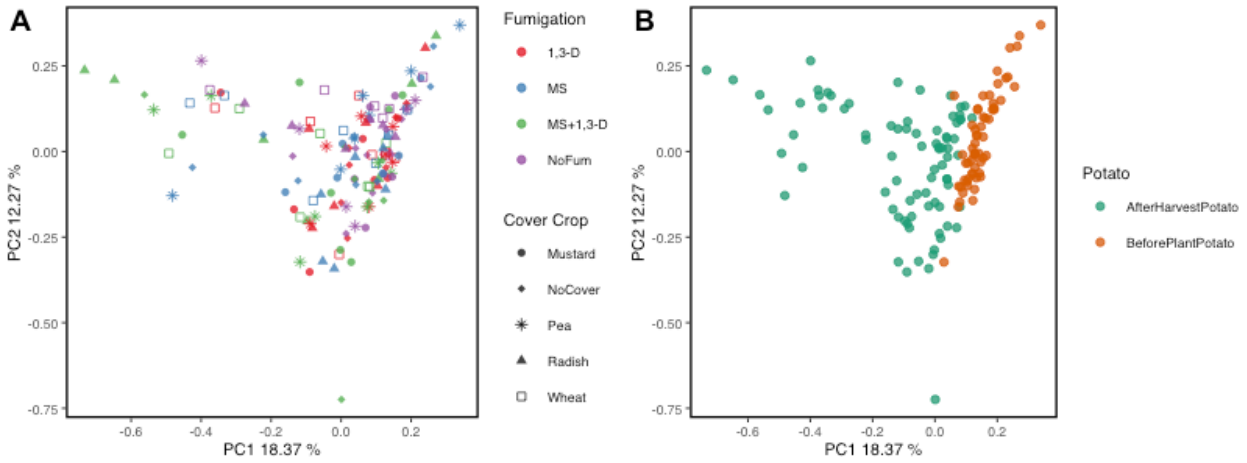


*Figure 5: Beta diversity analysis using principal coordinate analysis (PCoA) based on weighted UniFrac distances for the eukaryotic microbial community for fumigation treatment and cover crop (A) and sampling time (B).*

### 2.4.4 Eukaryotic taxonomic composition

ITS rRNA amplicon sequencing utilizing the United database identified four prominent phyla classifications, including *Eukaryota Incertae sedis* (uncertainty taxonomic group), *Ascomycota*, *Basidiomycota*, and Fungi unassigned (Figure 6A, Table A 5, Table A 6). The abundance of *Basidiomycota* decreased significantly with the application of MS from 0.8% to 0.4% when compared to the no fumigation control (Table A 7). No significant differences ($P > 0.05$) were observed for any of the phyla with the application of 1,3-D. However, the combined fumigation (MS+1,3-D) resulted in significant decreases for *Ascomycota* and *Basidiomycota*. *Eukaryota Incertae sedis* was by far the most abundant from 87.8% to 92.7%. *Eukaryota Incertae sedis* is a taxonomic term used to refer to organisms with an uncertain taxonomic position, therefore, with this classification making up a mean abundance across all conditions of 89.2%, the vast majority of the eukaryotic community composition has uncertain lineage (del Campo et al., 2014).

The dominant eukaryotic genera were identified as *Eukaryota Incertae sedis*, *Alternaria*, *Fusarium*, *Plectosphaerella*, *Inocybe*, *Tetracladium*, *Russula*, *Amphinema*, *Pseudotomentella*, and *Cortinarius* (Figure 6B, Table A 8). *Inocybe* abundance saw significant decreases in

population from the no fumigation control when compared to both MS treatment and the combination treatment, with percentage values decreasing from 0.6% to 0.4% and 0.3% for each treatment, respectfully. The most abundant fungal genus, *Alternaria*, followed a similar trend, decreasing from 6.8% to 4.3% abundance with MS application and 6.8% to 2.7% with MS+1,3-D. Once again *Eukaryota Incertae sedis*, accounted for much of the eukaryotic population, with an average abundance of 78.7%, however no significant differences were observed for fumigation treatments. Results did not indicate any significant shifts in the eukaryotic community due to cover crop type when compared to the no cover control.

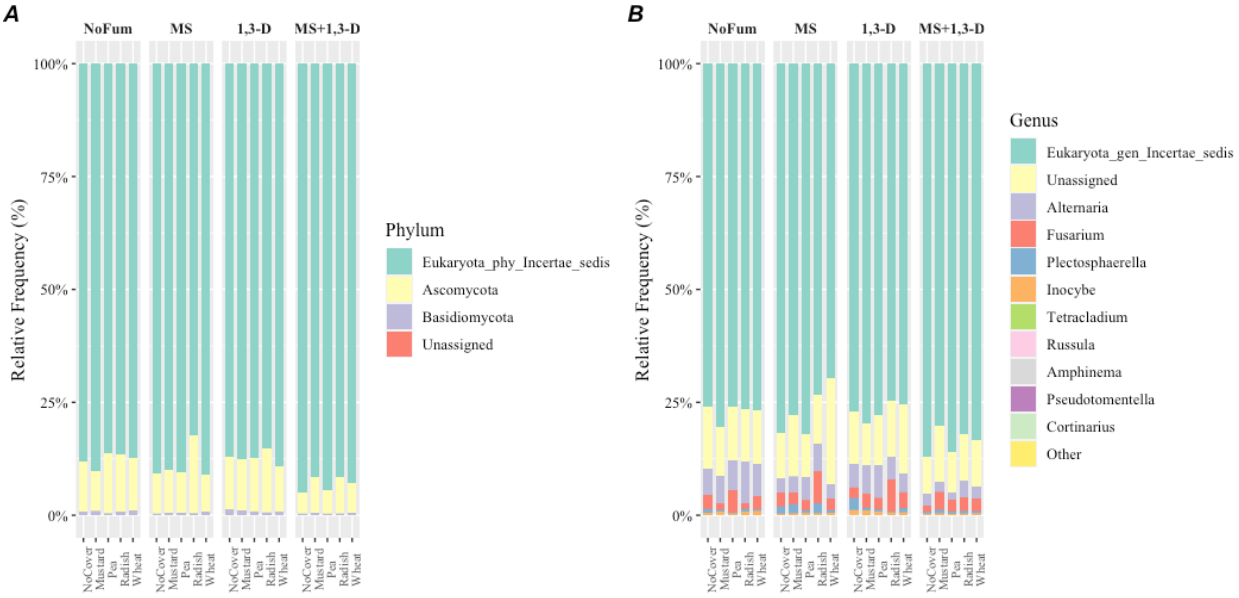


*Figure 6: Relative frequencies of the dominant eukaryotic A) phyla and B) genera (top 10 most abundant) according to fumigation and cover crop type based on ITS rRNA amplicon sequencing. Genera outside of the 10 most abundant "Other".*

### 2.4.5 Potato Planting Impacts on Soil Community Composition

As shown with beta diversity analysis using weighted UniFrac distances, the composition of the soil microbial community (Table A 9) significantly shifted due to potato planting. No significant interaction between potato planting and fumigation or cover crop was observed. Pairwise comparison between soil samples collected prior to potato planting and those collected after

potato harvest resulted in significant shifts in the *Chloroflexi* and *Acidobacteriota* phyla abundances (Figure 7A). A significant decrease was observed for the *Proteobacteria*, *Myxococcota*, *Verrucomicrobiota*, *Cyanobacteria*, and *Bacteroidota* populations. All other dominant prokaryotic phyla saw no significant change in abundance due to potato planting.

Regarding the eukaryotic population, the most dominant phylum, *Eukaryota Incertae sedis*, did not see any significant shift due to potato planting (Figure 7B). However, increases were observed in *Ascomycota* abundance and decreases in *Basidiomycota* abundance.

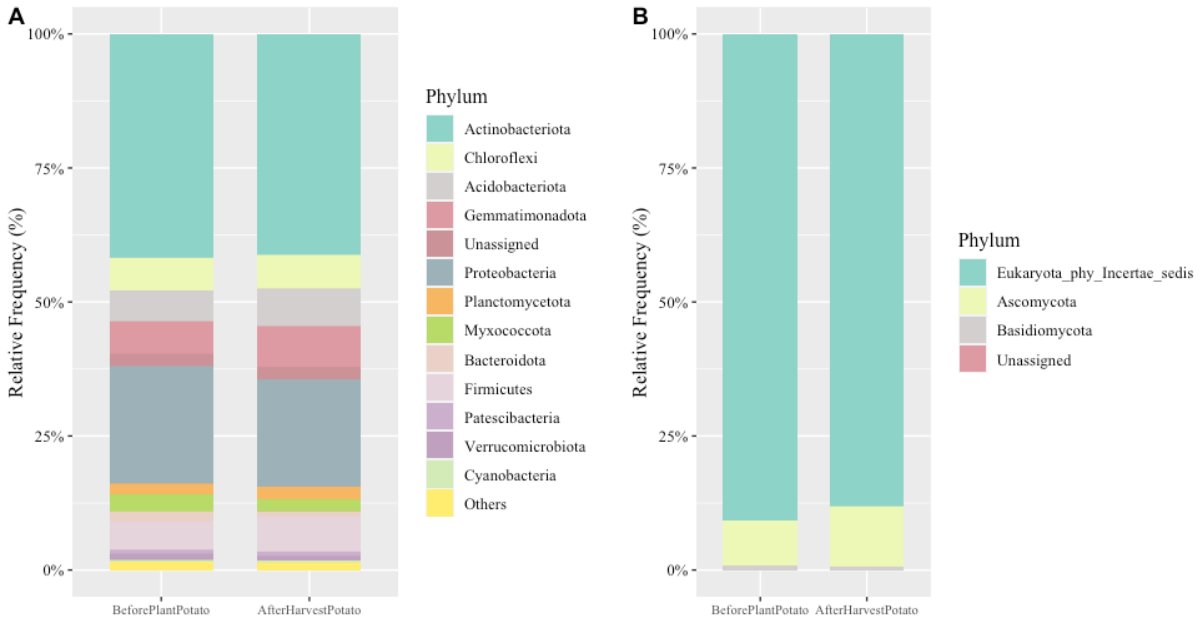


*Figure 7: Relative frequencies of the dominant A) prokaryotic phyla and B) eukaryotic phyla according to sampling time (before planting potatoes v. after harvest) based on 16s and ITS rRNA amplicon sequencing.*

## 2.5 Discussion

This study examined the effects of fumigation and subsequent cover crop planting on soil microbial community shifts and potato crop yield through the analysis of abundance and diversity over a one-year period. Composition and diversity of soil microbial communities was analyzed based on observed number of amplicon sequencing variants (ASVs).

### 2.5.1 Fumigation Effects on Community

It has been documented in many different studies that soil fumigation impacts the soil microbial community. Conversely within this study, soil fumigation did not result in an overall shift in the microbial community structure for the prokaryotic or eukaryotic microorganisms, as shown by the PCoA analyses. However, at the phylum and genus levels, shifts in the abundance of the most dominant groups were observed for fumigation treatments. Significant shifts resulted from the application of MS or the combination of MS+1,3-D. These results were supported by the alpha diversity indices that showed a lower trend for both diversity and evenness for MS and MS+1,3-D treatments. This study agreed with previous studies that observed the least impact on soil communities with the application of 1,3-D alone (Ibekwe et al., 2001; Liu et al., 2015). As with most other studies, the dominant prokaryotic phyla identified included *Actinobacteriota, Acidobacteriota, Chloroflexi, Firmicutes, Bacteroidota,* and *Proteobacteria* (Fang et al., 2020; J. Li et al., 2017; Liu et al., 2015; Sederholm et al., 2018).

*Actinobacteria* was the most abundant prokaryotic phyla throughout the duration of the study, with steady maintenance of its abundance levels despite treatment methods, remaining largely unaffected. *Nocardioides*, one of the main genera identified within the *Actinobacteria* phylum, again, did not see any significant shifts due to treatment. *Actinobacteria* are common soil microbes and are documented to be plentiful and resistant organisms, which may account for the lack of changes in observed abundance (Hazarika & Thakur, 2020; Sederholm et al., 2018). The results are in agreement with those found in previous studies regarding microbial changes due to MS application, where *Actinobacteria* remained unaffected by the fumigant application (Sederholm et al., 2018).

The phylum *Acidobacteriota* experienced the largest decrease in abundance with the application of MS (26% decrease) and MS+1,3-D (18.6% decrease), demonstrating a susceptibility to fumigation treatment. These results were consistent with those observed in other studies for treatment with MS, which also saw a steady decrease in *Acidobacteriota* over time (Sederholm et al., 2018). *Acidobacteriota* is again a common phylum of soil microorganism and is thought to be primarily made up of oligotrophic aerobic heterotrophs (Crits-Christoph et al., 2022).

*Bacillus*, the most abundant genus identified within the *Firmicutes* phylum making up about 3.68% of the sequenced community, did not see any significant shifts due to fumigation application; however, the *Firmicutes* phylum did experience a decrease with both MS and MS+1,3-D application. This decrease is not consistent with previous studies' results that saw a consistent increase in *Firmicutes* throughout the duration of the study (Sederholm et al., 2018). *Firmicutes* in general are resistant bacteria with low GC contents and rigid cell walls (Sederholm et al., 2018). *Bacillus* can be classified as an endospore-forming, plant growth-promoting rhizobacteria (PGPR) (Gurikar et al., 2022). Despite this, the decreasing trend with MS application may be attributed to an increase in *Proteobacteria*, which can serve in an antagonistic role towards *Firmicutes* (Lee et al., 2021). There was no significant increase ($P < 0.05$) in *Proteobacteria* noted, however a 5.8% increase was observed for samples treated with MS and a 3.11% increase for those treated with MS+1,3-D.

The bacterial phylum, *Verrucomicrobiota*, experienced significant decreases with MS+1,3-D application which disagrees with previous findings (Fang et al., 2020). This phylum and its species are often thought to be of low abundance in soil and can be classified as oligotrophic. A decrease in this phylum is interesting because of its oligotrophic properties;

however, due to its lower abundance, it may have been outcompeted by more abundant oligotrophic taxa (Bergmann et al., 2011; Fang et al., 2020).

Within the eukaryotic community, only four phyla classifications were identified: *Eukaryota Incertae sedis*, *Ascomycota*, *Basidiomycota*, and Fungi unassigned. Because 89.2% of the sequenced ASVs were classified as *Eukaryota Incertae sedis*, much of the community identified is of unknown taxonomy. Of the identified phyla, *Basidiomycota* decreased significantly with both MS and MS+1,3-D application (X. Li et al., 2022). *Inocybe* was identified as a dominant genus within the *Basidiomycota* phylum which followed the same decreasing trend. This genus can be classified as a mycorrhizal symbiont of plants and aids in the uptake and utilization of nutrients, establishing themselves as a beneficial soil microorganism, possibly decreasing because of the elimination of weeds and other field vegetation following fumigation (W. Li et al., 2021). *Ascomycota* only saw significant decreases with the combined fumigation treatment. *Ascomycota* is a major phylum within the fungal domain, containing one of the most important crop pathogens, *Fusarium* spp. Species of *Fusarium* are responsible for *Fusarium* dry rot in potatoes, one reason why fumigation is utilized. No significant changes in this genus were observed with fumigation treatments, which may be attributed to the pathogen's ability to live in the soil as chlamydospores for years (R. Li et al., 2016). Additionally, this resistance to change could be due to an increase in fumigant-resistant strains with intensive fumigation (R. Li et al., 2016). None of the eukaryotic community responded significantly to 1,3-D fumigation alone.

### 2.5.2 Cover Crop Effects on Community

Cover crop planting resulted in few changes in the microbial community. The only prokaryotic phyla to show notable shifts in abundance were *Gemmatimonadota* and *Patescibacteria*, although not statistically significant. *Gemmatinoadota* saw decreases in samples planted with

radish cover crops. This bacterial phylum is a commonly occurring microorganism in terrestrial environments where it can be classified as a generalist. Studies have found that this phylum has positive correlations to vegetation restoration, rainfall reductions, and neutral pH (Mujakić et al., 2022), which is opposing to the results found from this study, with population decreasing with vegetation increase. *Patescibacteria* saw increases with both radish and wheat cover crop planting. No prokaryotic genera were impacted by cover crop planting. Additionally, none of the eukaryotic phyla or genera showed significant shifts in abundance due to cover crop planting. Overall, radish cover crops resulted in the largest community shift which may be due to the plants robust rooting ability, despite it producing the least dry root biomass (Gruver et al., 2014; Kim, 2023). Past studies have supported findings that cover crop planting shifts the microbial population; however, the results from this study did not reflect these changes (Leite et al., 2021). This may be due in part to shifts due to fumigation, although no significant interaction was identified between these treatments in relation to microbial abundance, despite a trend of higher mean microbial biomass in no fumigation for all the cover crop types except mustard (data not shown). Additionally, cover crops were planted in late October and the 2021-2022 winter growing season proved to be a drying environment, causing limited growth of cover crops, possibly resulting in the lack of community restoration observed following fumigation.

### 2.5.3 Pathogen Control

The targeted pathogens within this study included *Fusarium* dry rot (*Fusarium* spp.), Verticillium wilt (*Verticillium dahlia*), and Pythium root rot (*Pythium* spp.). *Fusarium oxysporum* was identified as the fourth most dominant fungal species under all fumigation treatments, including the absence of any fumigation treatment, with *F. oxysporum* accounting for 2.8%, 3.5%, 3.8%, and 2.7% of the eukaryotic population treated with no fumigation, MS, 1,3-D,

and MS+1,3-D, respectfully. With all fumigation treatments and cover crop types, including controls from both variables, *F. oxysporum* abundance did not significantly differ; however, small increases in the population did occur with MS and 1,3-D treatment. Within the *Ascomyota* phylum, a second pathogen was identified as *Alternaria tenuissima*, which experienced population declines with MS and MS+1,3-D fumigation, wheat cover crop planting, and potato planting. This species is the cause of *Alternaria* rot and economic losses in the potato industry (Leng et al., 2022). This decreasing trend highlights the ability of fumigation to eliminate pathogenic species. *Verticillium dahlia* and *Pythium* spp. did not return any hits in the sequenced genomes, indicating that the fumigation was effective against these pathogens or that they were not detectable in the soil prior to fumigation.

## 2.6 Conclusion

In this study, the effects of fumigation, cover crops, potato planting, and their interactions were evaluated. None of these treatments showed significant interactions when analyzing microbial abundance levels. Fumigation was the only treatment to show significant shifts in the community individually at the phylum and genus levels; however, the overall composition of the community did not show a prominent shift due to fumigation type. MS and MS+1,3-D caused the most significant shifts, with the combination treatment decreasing alpha diversity indices. Treatment with 1,3-D alone caused virtually no shifts in the community when compared to the non-fumigated control. Similarly, cover crop planting did not result in a significant shift in composition, and only resulted in notable shifts for two dominant bacterial phyla. Radish cover crops seemed to have the largest impact on the community, although limited in its reach. Potato planting resulted in the only significant overall community composition shift; however, the most common prokaryotic and eukaryotic phyla within soil still maintained their dominance. It

suggested a recovery of the community found in non-fumigated controls, with phyla that decreased from fumigation increasing and vice versa.

To draw definitive conclusions on the effect of cover crops on the soil microbial community and their ability to recurve effects of fumigation treatments, further years of study need to be conducted as well as sampling before any treatment is applied, after fumigation only, after cover crop planting, and after potato harvest.

# CHAPTER 3: SOIL CHEMICAL PROPERTIES AND MICROBIAL COMMUNITY COMPOSITION IN A YEAR-ROUND CORN PRODUCTION FIELD TREATED WITH ANAEROBIC DIGESTATE

## 3.1 Summary

Anaerobic digestate is ideal for fertilization due to its high nutrient content and its ability to preserve critical soil nutrients, specifically nitrogen, phosphorous, and potassium. A year-long field test was conducted in Central Michigan from November 2022 through October 2023 to investigate the soil microbial community shifts as affected by anaerobic digestate application in a corn field. Nine replicate samples were collected monthly during the non-growing season (September–April) and biweekly during the growing season (May–August). Each sample underwent analysis for microbial community composition, nutrient levels (P, K, Ca, Mg, Na, Al, S, Zn, Mn, Fe, Cu, B), elemental levels (CHN analysis), pH, and electrical conductivity (EC). Both prokaryotic and eukaryotic communities responded to digestate application with shifts in community composition. The most dominant prokaryotic community was *Actinobacteriota*, making up an average of 43% of the population across factors; however, the phylum did not see any significant changes due to digestate application. Phyla affected include *Proteobacteria* and *Firmicutes*, which increase in abundance from 18.0% to 19.1% and 4.95% to5.81%, respectfully, and *Chloroflexi*, which significantly decreased from 6.72% to 5.82% with digestate application. The eukaryotic population saw the abundance of *Basidiomycota* increase significantly with the application of digestate from 2.4% to 3.9% and *Eukaryota Incertae sedis* abundance decrease from 70.2% to 65.5%. The eukaryotic phylum *Ascomycota* was found to have a strong correlation to soil nitrogen levels; however, neither microbe nor nutrient shifts in the study due to digestate.

## 3.2 Introduction

The promotion of alternative energy production methods and sustainable agriculture practices is a rising topic of interest (Lamolinara et al., 2022). As the world population continues to grow, so does the demand on food production, the environment, and agriculture (Sapp et al., 2015). Efforts to reduce fossil fuel emissions have been and continue to be a challenge when addressing the environment and its sustainability, and a possible solution lies in the form of biogas production. A major facet of the United States economy is animal production. Annually, $143.4 billion dollars is generated from cattle and dairy products (U.S. Department of Agriculture, Economic Research Service, 2024). With that comes the production of an excess of manure. This manure has the potential to serve as a feedstock for bioenergy production.

Biogas production via anaerobic digestion is a major player in sustainability efforts in agriculture practice, as it efficiently reduces greenhouse gas (GHG) emissions and serves as an effective waste management practice (Lamolinara et al., 2022; Möller & Müller, 2012). Anaerobic digestion is a natural process by which fermentation is utilized to breakdown organic matter, producing carbon dioxide, methane, and a digestate slurry as outputs (Bajpai, 2017). The anaerobic digestate (AD) by-product is a nutrient rich slurry that is made up of 90-95% of the feedstock originally fed into the digester (Lamolinara et al., 2022; Möller & Müller, 2012). This high nutrient content makes digestate an ideal organic fertilizer, as it can efficiently return essential nutrients back to the soil and provide plant-available nutrients (Lamolinara et al., 2022). Fertilization is one of the most practiced and simplest ways of increasing the soil fertility in agricultural lands, while also being an accessible and easy method for the mitigation of environmental impacts of agricultural practices and the promotion of the economic possibility of biogas production (Iacovidou et al., 2013; Lamolinara et al., 2022). Chemical fertilization is a

major contributor to GHG emissions and water pollution via groundwater leaching. Many agricultural practices focus on the physical and chemical makeup of the soil, neglecting the biological activity that takes place within the environment as well. Thus, agricultural practices, such as the application of inorganic fertilizers, have been shown to have negative influences on the soil microbial community and associated soil microbial biomass (Kibblewhite et al., 2008; Sapp et al., 2015). Digestate, when applied to the field, has shown to result in higher organic carbon (C) and nitrogen (N) (ammonium), which in-turn is beneficial to the stimulation of soil organic matter (SOM) and the overall biological health of the soil (García-Sánchez et al., 2015). Benefits of digestate application to soil also includes the promotion of nutrient cycling, carbon sequestration, and the maintenance of soil structure (Doyeni et al., 2021). By prioritizing the biological, chemical, and physical properties of the soil, digestate application has shown comparable crop yields to inorganic alternatives, while being a more sustainable and economic approach (Alburquerque et al., 2012; García-Sánchez et al., 2015).

This study addresses the year-round impact liquid digestate application has on the biological, chemical, and physical properties of the soil, and how these properties shift with influences from time of year, treatment, and soil properties. We hypothesize that digestate application will stimulate an increase in essential soil nutrients (carbon (C), nitrogen (N), phosphorous (P), and potassium (K)), as well as a positive increase in soil microbial community diversity, as quantified by diversity metrics.

## 3.3 Materials and Methods

### 3.3.1 Field Experiment Design

A year-long field test was conducted in a 7.3-acre field located on Michigan State University farmland in East Lansing, Michigan from November 2022 through October 2023. Prior to this

study, the field was treated with liquid digestate for eight years and was slated to be sowed with corn in the growing season. The sampling site was divided into nine sampling points (replicates) each located 20 m apart with a depth of 10 m from the adjacent road, covering a total of 0.62 acres of the field (field map shown in Figure 8). Liquid digestate was applied to the field in mid-April 2023 at a rate of 8400 gal/acre and mechanically tilled following application. Following digestate application, corn was planted in mid-May. Fertilizer was applied to the field (35 gal/acre; 28% Urea Ammonium Nitrate (UAN)) in late June 2023, and corn was harvested in late September. A table of sampling dates and field operations can be found in Table B 1. It is important to note that due to normal field operations, the field sampling points were remapped prior to April, May, July, and October sampling events, due to tilling, planting, corn growth, and harvest. However, sampling points were kept as close as possible, utilizing surveying and GPS equipment.

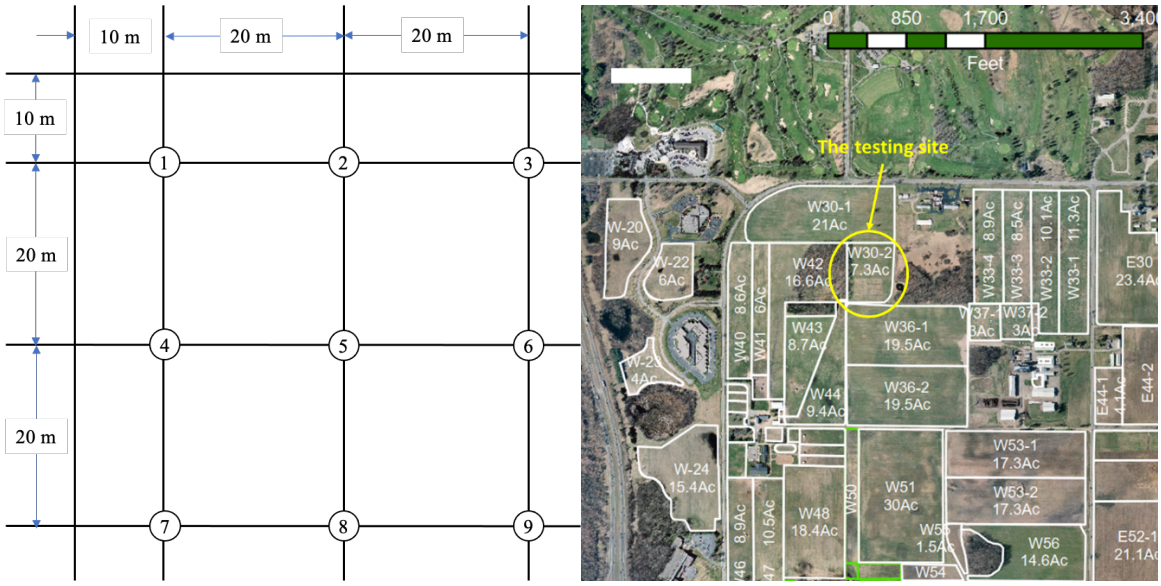

*Figure 8: Sampling area with labeled sampling points (left); the testing site on MSU farmland (W30-2 in yellow circle) (right).*

### 3.3.2 Soil Sampling

Each sampling event consisted of nine soil samples taken monthly during the non-growing season (September-May) and biweekly during the growing season (June-August), starting November 2022. A sampling probe was used, with the depth of the samples being 12 in. Samples were passed through a US standard 5 mesh (4,000 □m) sieve and stored at -20°C between analyses.

### 3.3.3 Soil Property Data

The pH and electrical conductivity (EC) were measured using a standard pH and EC probe (Thermo Scientific™ Orion Star™ A212 pH and Conductivity Benchtop Meter, Pittsburgh, PA). Laboratory pH measurement protocol specifies a slurry containing a 1:2 ratio of soil to deionized water (10 g soil, 20 mL water) be mixed and allowed to equilibrate with atmospheric carbon dioxide for 30 minutes prior to measurements. Prior to each measurement event, the pH and EC probes were calibrated using standard pH solutions of 4, 7, and 10 and EC solutions of Orion 100 µS, 1413 µS and/or 12.9 mS standards. pH was additionally measured by Dairy One (Ithaca, NY) during nutrient analysis, and these values were ultimately used in the report.

### 3.3.4 Soil Nutrients

Elemental levels in the soil were analyzed for total carbon (TC), total hydrogen (TH), total nitrogen (TN), and total sulfur (TS). This analysis for TC and TH was performed by combustion using automatic analyzers. For each sampling round, 3 rotating samples were selected for analysis. Approximately 1g of soil from each of the selected samples was sent for CHS analysis at Atlantic Microlabs (Norcross, GA). TN was measured using TNM-L unit (Shimadzu Corporation, Kyoto, Japan). To prepare the samples for measurement, a 1:2 slurry of soil to

deionized water (10 g soil, 20 mL water) was mixed, allowed to equilibrate for 30 minutes, and then homogenized via centrifugation until all soil particles are separated from the mixture.

Soil nutrient levels were analyzed using the Morgan method of soil nutrient extraction. For each sample, approximately 100 g of dry soil was weighed and sent out for analysis at Dairy One (Ithaca, NY). These analyses returned results describing phosphorous (P), potassium (K), calcium (Ca), magnesium (Mg), sodium (Na), aluminum (Al), sulfur (S), zinc (Zn), manganese (Mn), iron (Fe), copper (Cu), and boron (B) contents within the soil samples. A soil condition test was also performed with Dairy One for sufficiency levels of available nutrients (SLAN), which helps determine soil fertility levels with values below 76 indicating low biological activity, 76-200 moderate activity, and 201-350 high activity.

### 3.3.5 DNA Extraction

DNA was extracted using the Qiagen DNeasy PowerSoil Pro Kit (Qiagen, Germany) and the provided protocol. Extractions were suspended in 100 μL of 10 mM Tris-HCl (pH 8.5) and concentration and purity of the DNA solution was measured using a NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific Inc., USA) to verify the quality of the extraction for downstream purposes. Extractions were then stored in -80°C between analyses.

### 3.3.6 PCR Amplification for Illumina Sequencing

The DNA extractions for all collected sample batches were amplified using standard polymerase chain reaction (PCR) via a thermocycler. Sequencing of the 16s rRNA and ITS genes for bacterial and fungal identification in were conducted using the universal primers Pro341F (5′-CCTACGGGNBGCASCAG-3′)- Pro805R (5′-GACTACNVGGGTATCTAATCC-3′) and ITS1F (5′-CTTGGTCATTTAGGAAGTAA-3′)- ITS2R (5′- GCTGCGTTCTTCATCATGATGC -3′). PCR processes were carried out for both primer sets using the method as follows: initial

denaturation at 95 °C for 2 min, 30 denaturation cycles at 95 °C for 20 s, primer annealing at 52 °C for 30 s, elongation at 72 °C for 1 min, and extension of new strands at 72 °C for 10 min. Agarose gel electrophoresis (2.0% (w/v) agarose concentration and 1× TAE run buffer) was conducted to verify quality and size and to ensure the amplified samples were uncontaminated. Amplified samples were then diluted 7x with 30 μL of 10 mM Tris-HCl and sent for high-throughput sequencing.

### 3.3.7 Sequence Processing

Samples underwent high-throughput sequencing (Illumina MiSeq flow cell) were sequenced at Michigan State University Genomics Core. The Illumina Fastq files from the high-throughput sequencing were then analyzed using QIIME2 (a microbiome bioinformatics platform) to generate taxonomic/phylogenetic data for statistical analysis and comparison of the soil microbial community shift.

### 3.3.8 Statistical Analysis

Microbial analysis was performed in RStudio (Version 2023.09.1+494) using R libraries Qiime2R, Vegan, ggplot2, tidyverse, and DescTools. Alpha diversity was examined for richness (Shannon-Weiner Species Diversity Index) and evenness (Pielou's evenness) for both bacterial and fungal communities. Changes in these indices were analyzed using two-sample Wilcoxon Signed Rank test and Kruskal-Wallis test with Conover's pair-wise rank comparison post-hoc tests at a 5% significance level. Community composition based on taxonomic/phylogenetic data was graphed based on relative abundance and shifts in community were analyzed using the same non-parametric tests at a 5% significance level.

Soil property and nutrient data analysis was performed in RStudio using R libraries vegan, ggplot2, pastecs, and DescTools. Again, changes in soil properties/nutrients were analyzed using

two-sample Wilcoxon Signed Rank test and Kruskal-Wallis test with Conover's pair-wise rank comparison post-hoc tests at a 5% significance level. Redundancy analysis (RDA) was conducted to correlate changes in soil properties/nutrients to shifts in specific taxa abundance.

## 3.4 Results

### 3.4.1 Bacterial Alpha and Beta Diversity

Alpha diversity indices for the prokaryotic population showed significant variation for several factors. Pairwise comparison using two-sample Wilcoxon Signed Rank test showed a significant difference for richness (Shannon's index) (Figure 9A/B) and evenness (Pielou's evenness) (Figure 10A/B) indices, with no anaerobic digestate application (AD) and no crop having higher indices means (Table B 2, Table B 4). Shifts in diversity indices due to sampling date (time of year) were analyzed using the Kruskal-Wallis test and Conover's pair-wise rank comparison post-hoc test if an overall model significance was identified. Soil richness had a decrease in late summer, with late July and both August sampling events being significantly lower than December, February, March, April, and June samplings (Figure 9C). Community evenness was highest during the winter months, decreasing after AD application, and then gradually increasing towards late summer. December showed significantly higher richness when compared to May, late June, and late August samplings (Figure 10C). Weighted UniFrac distances (beta diversity metric) showed no significant group formation, and therefore, no significant shift in overall community diversity at the prokaryotic level (Figure B 1A).

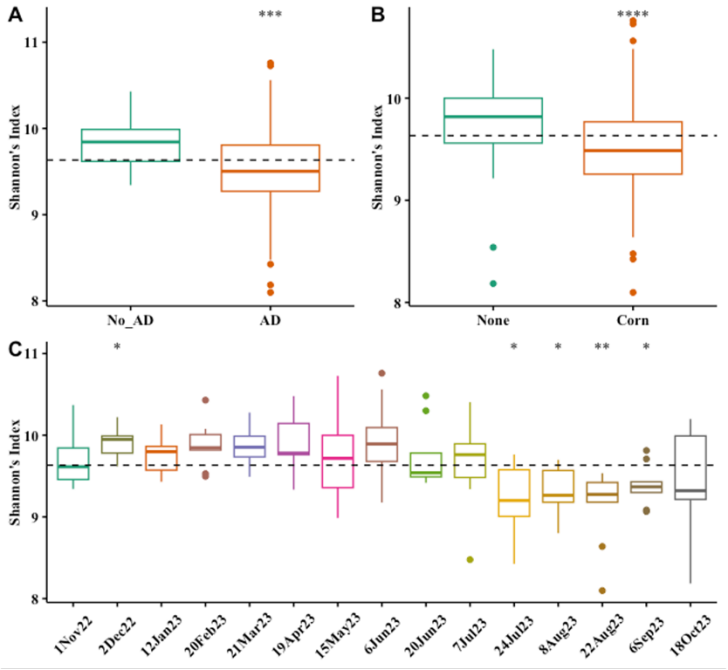


*Figure 9: Alpha diversity indices for the prokaryotic population based on Shannon-Weiner richness (A, B, C) for digestate treatment, planting, and sampling time. The significance codes: 0 '***'0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.*

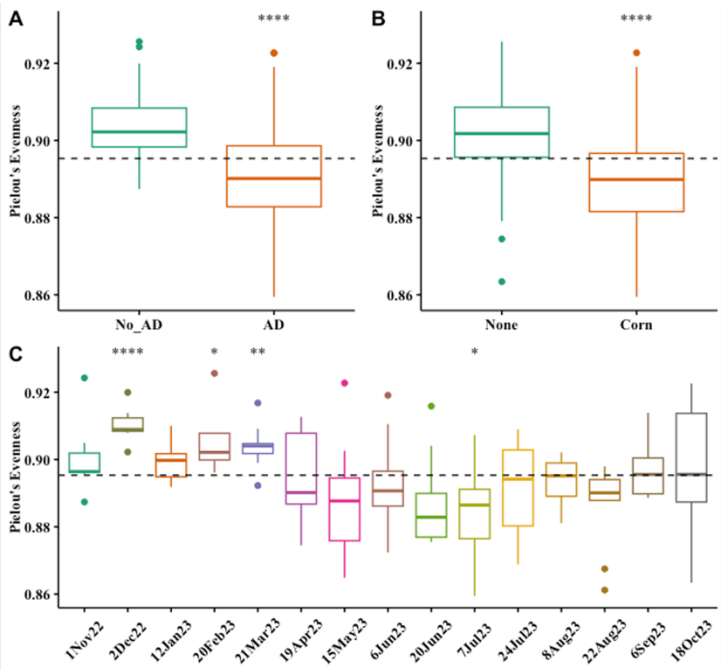


*Figure 10: Alpha diversity indices for the prokaryotic population based on Pielou's Evenness (A, B, C) for digestate treatment, planting, and sampling time. The significance codes: 0 '***'0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.*

### 3.4.2 Bacterial taxonomic composition

16s rRNA sequencing identified 10 dominant phyla, including *Actinobacteriota*, *Proteobacteria*, *Acidobacteriota*, *Chloroflexi*, *Firmicutes*, *Verrucomicrobiota*, *Myxococcota*, *Gemmatimonadota*, *Planctomycetota*, and *Bacteroidota* (Figure 11). Many of these phyla are the same as those identified in Chapter 2. Pairwise comparison between phyla abundances with treatment (No AD, AD) and crop status (None, Corn) were analyzed using Wilcoxon Signed Rank test to identify any significant shifts in population. Treatment with AD resulted in more significant shifts in population abundance than crop planting, with *Proteobacteria* (P = 0.0295) and *Firmicutes* (P = 0.0087) abundance increasing from 18.0% to 19.1% and 4.95% to5.81%, respectfully (Table B 4, Table B 5). *Chloroflexi* (P = 0.0018) significantly decreased from 6.72% to 5.82% with AD application. Crop sowing and growth resulted in significant decreases for *Acidobacteriota* (P = 0.0427), *Myxococcota* (P = 0.0161), and *Planctomycetota* (P = 0.0176). No increases were observed in response to corn planting and growth. Several of the dominant phyla saw significant shifts in abundance throughout the year. *Actinobacteriota* was at peak abundance in May, making up 49.1% of the bacterial population with this number decreasing through the rest of the year to 39.6% in October. *Chloroflexi* was most abundant during December at 8.98% of the population with significant decreases when compared to this number in April, early June, early July, September, and October. Another phylum that showed the most abundance during the colder months was *Myxococcota*. This phylum was the most abundant in October at 3.02% of the population and the least abundant in May at 1.76% of the population. *Planctomycetota* was most abundant in November at 1.84%, dropping to values between 1.30% and 1.6% of the population during the growing season. *Bacteroidota* increased significantly from a low of 0.49% in December to a peak of 1.66% in late July.

Several genera saw significant decreases in abundance with AD application, including *Vicinamibacterales* uncultured, *Chloroflexi*-KD4-96, *Solirubrobacterales bacterium* 67-14, *Vicinamibacteraceae*, and *Xanthobacteraceae* unassigned (Figure 12, Table B 6). *Nocardioides* and *Bacillus* both increased in abundance with AD application, with *Nocardioides* peaking in abundance in May after AD application and *Bacillus* in late July after UAN fertilizer application. Regarding planting, *Vicinamibacterales* uncultured, KD4-96, and *Xanthobacteraceae* unassigned followed the same decreasing trend seen with AD application, with peak abundance during the non-growing season and significantly differing from growing-season abundance. *Vicinamibacterales* uncultured specifically saw its highest abundance in December at 3.41%, decreasing significantly when compared to April, June, and early July samplings. *Actinobacteria* unassigned and *Nocardioides* increased with corn planting, again with abundance peaks in May for both genera.

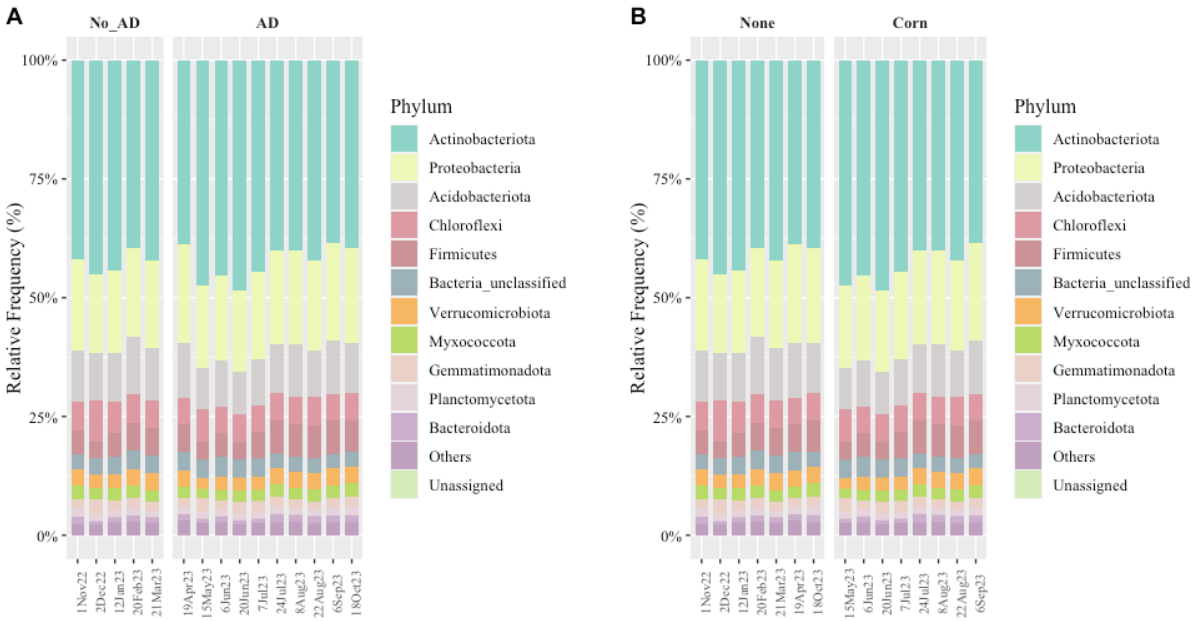


*Figure 11: Relative frequencies of the dominant prokaryotic phyla according to A.) treatment (No AD, AD) and B.) crop (None, Corn) based on 16s rRNA amplicon sequencing. Phyla outside of the ten most abundant were grouped under "Others".*

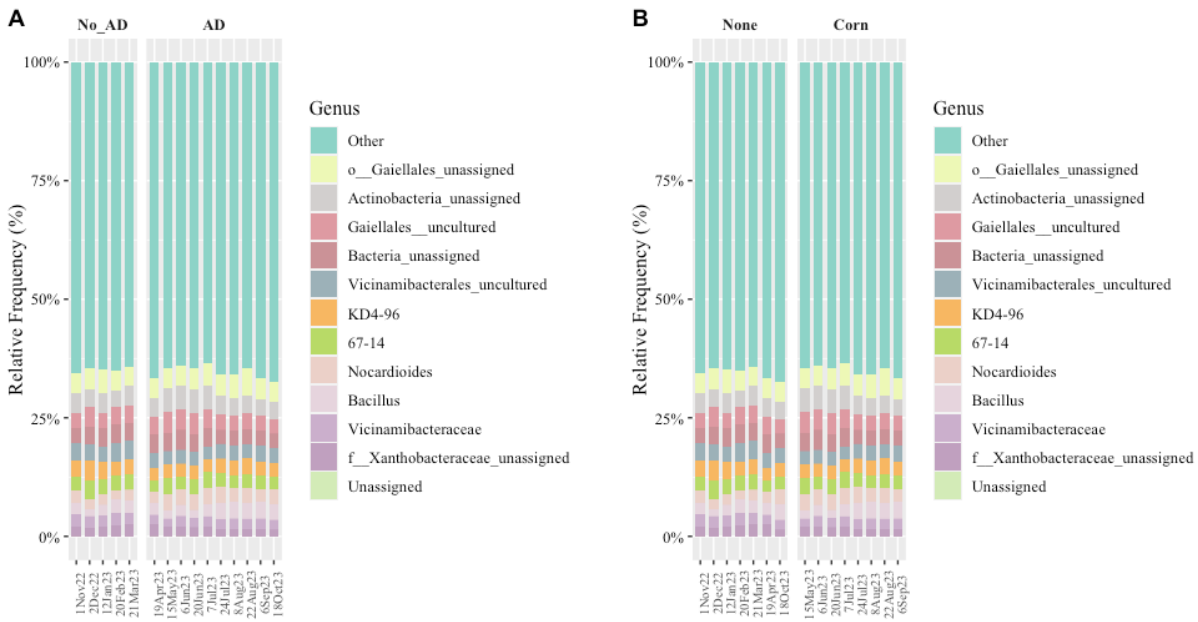


*Figure 12: Relative frequencies of the dominant prokaryotic genera according to A.) treatment (No AD, AD) and B.) crop (None, Corn) based on 16s rRNA amplicon sequencing. Genera outside of the ten most abundant were grouped under "Others".*

### 3.4.3 Eukaryotic Alpha and Beta Diversity

Analysis of the alpha diversity indices for the eukaryotic population revealed a stable community in terms of richness (Shannon's Index) and evenness (Pielou's) (Table B 2). Two-sample Wilcoxon Signed Rank test was used to identify significant differences in Shannon-Weiner diversity index and Pielou's evenness between treatment application (No AD, AD) and crop planting (None, Corn) (Table B 3). The Kruskal-Wallis test was used to identify and model significance for shifts due to sampling time, and Conover's pair-wise rank comparison post-hoc test was employed if an overall model significance was identified. No significant differences ($P > 0.05$) were identified for the eukaryotic richness and evenness with both AD application and corn growth (Figure 13A/B, Figure 14A/B). Community richness did see several significant deviations from the mean, with April, late July, and late August samplings (Figure 13C). Results from Conover's post-hoc indicated that April sampling significantly differed from March, late July, late August, and September samplings. Evenness analysis across sampling times revealed

no significant differences throughout the year (Figure 14C). Weighted UniFrac distances (beta diversity metric) showed no significant group formation, and therefore, no significant shift in overall community diversity at the eukaryotic level (Figure B 1B).

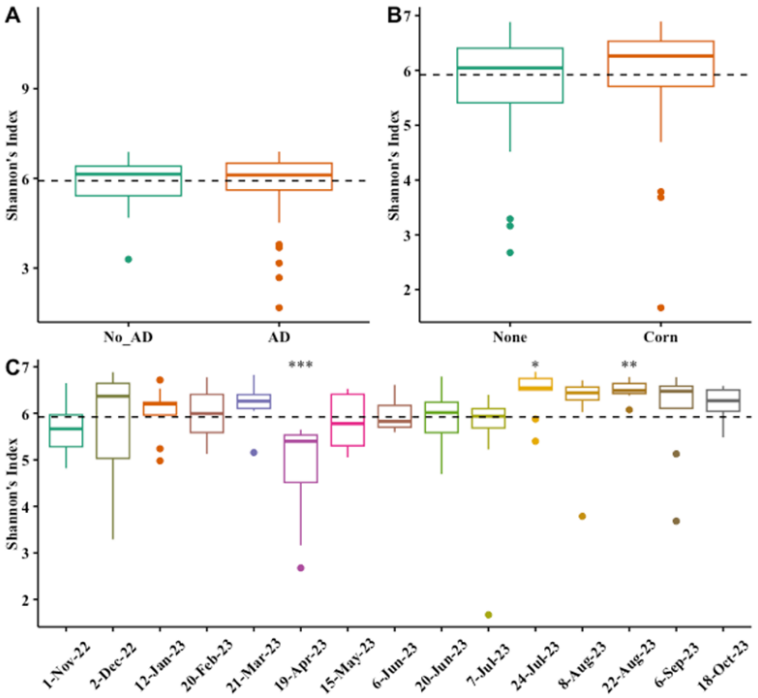


*Figure 13: Alpha diversity indices for the eukaryotic population based on Shannon-Weiner richness (A, B, C) for digestate treatment, planting, and sampling time. The significance codes: 0 '***'0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.*

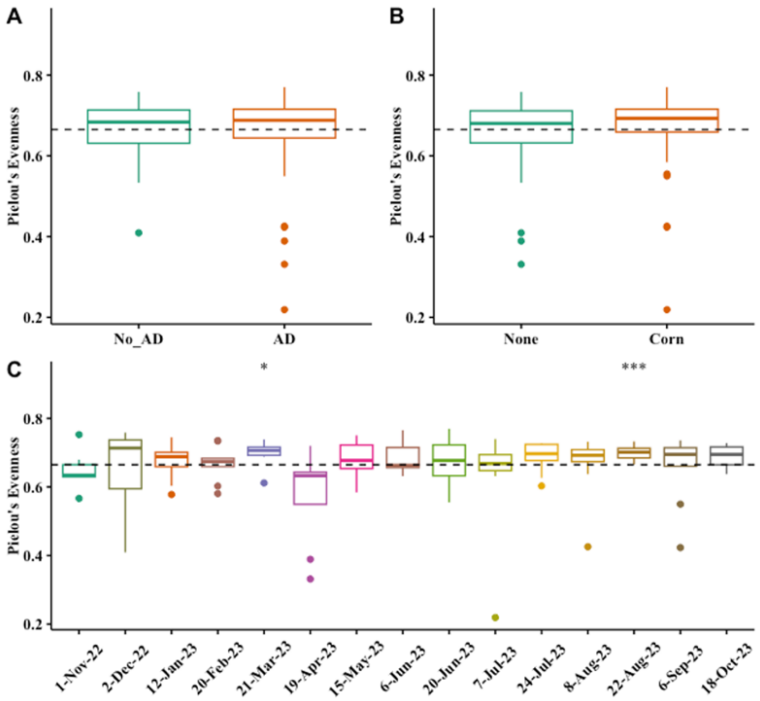


*Figure 14: Alpha diversity indices for the eukaryotic population based on Pielou's Evenness (A, B, C) for digestate treatment, planting, and sampling time. The significance codes: 0 '***'0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.*

### 3.4.4 Eukaryotic taxonomic composition

ITS rRNA amplicon sequencing utilizing the United database revealed nearly identical dominant phyla as those identified in Chapter 2, including *Eukaryota Incertae sedis* (uncertainty taxonomic group), *Ascomycota*, *Basidiomycota*, Fungi unassigned, and *Fungi Incertae sedis* (uncertainty taxonomic group) (Figure 15A/B, Table B 4). The abundance of *Basidiomycota* and the population of unclassified eukaryotes increased significantly with the application of AD from 2.4% to 3.9% (*Basidiomycota*) and 13.1% to 18.6% (Unassigned) when compared to the no AD control (Table B 5). *Eukaryota Incertae sedis* was, again, the most abundant, showing a significant decrease with AD application from 70.2% to 65.5%. All other phyla saw no significant change due to AD application. Corn planting the same trend, with *Basidiomycota* and Unassigned increasing from 2.7% to 3.9% and 15.1% to 18.2%, respectively, and *Eukaryota Incertae sedis* decreasing from 69.1% to 65.2%. All other phyla remained unaffected by crop

planting. The only phyla to display shifts due to time of year (sampling time) was *Basidiomycota*, increasing significantly during May and early June to 8.4% and 4.0% when compared to November sampling, where the phyla made up 1.8% of the population.

The dominant eukaryotic genera were identified as *Eukaryota Incertae sedis*, *Fusarium*, *Inocybe*, *Plectosphaerella*, Fungi unassigned, *Alternaria*, *Amphinema*, *Russula*, *Clavulina*, and *Cortinarius* (Figure 16A/B). *Inocybe* and *Alternaria* abundance saw significant increases in population with AD application, with percentage values decreasing from 2.2% to 3.7% and 0.02% to 0.19%, respectfully (Table B 6). A decrease in abundance was also observed for *Plectosphaerella*, decreasing from 2.2% to 1.1%. Once again, the same trend was observed with crop planting, resulting in significant increases for *Inocybe* and *Cortinarius*, and a decrease in *Eukaryota Incertae sedis*. Sampling time resulted in an increase in *Inocybe* abundance when comparing November and May, while a decrease was observed for *Plectosphaerella* between November and April. No other genera saw significant shifts from AD, corn planting, or sampling time.

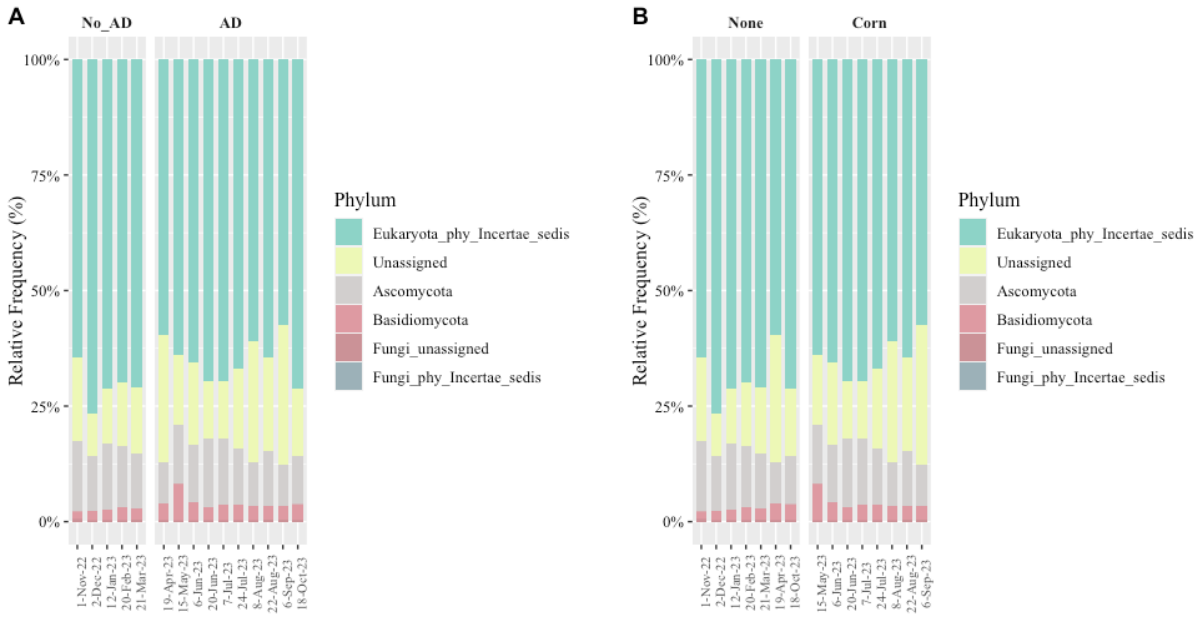


*Figure 15: Relative frequencies of the dominant eukaryotic phyla according to treatment (No AD, AD) and crop (None, Corn) based on 16s rRNA amplicon sequencing. Phyla outside of the ten most abundant were grouped under "Others".*

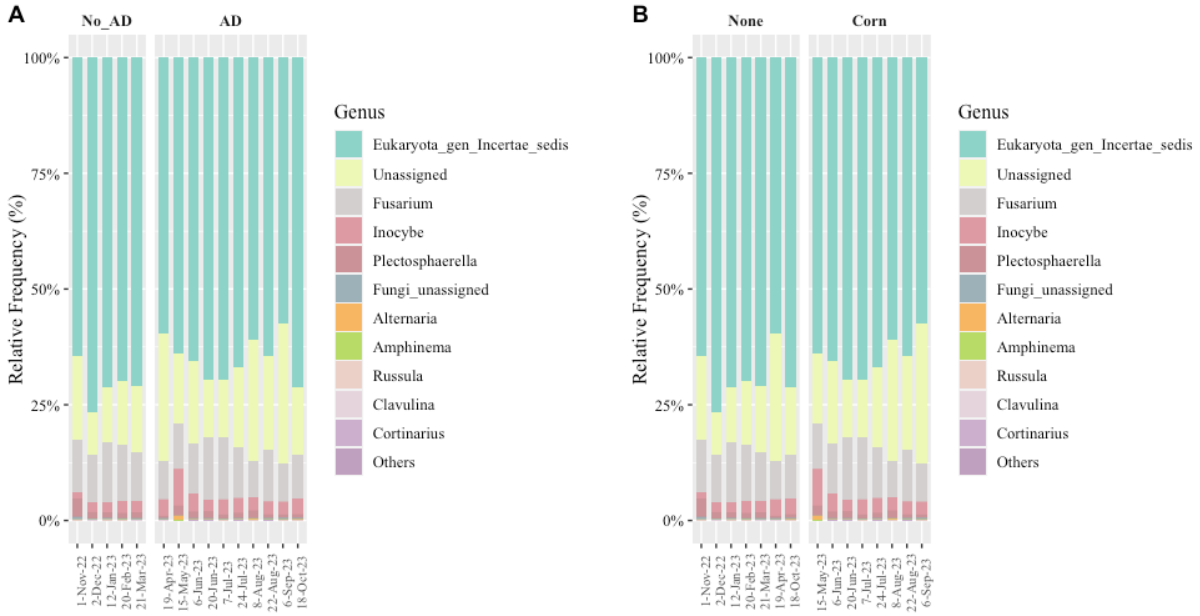


*Figure 16: Relative frequencies of the dominant eukaryotic genera according to treatment (No AD, AD) and crop (None, Corn) based on ITS rRNA amplicon sequencing. Genera outside of the ten most abundant were grouped under "Others".*

### 3.4.5 Soil Properties

All data analysis for soil properties data was performed using RStudio. Data was compared to identify any significant changes in levels due to treatment application, crop planting, or time of year (Table B 7, Table B 8). Within the study, the AD-treated and non-AD-treated soils remained consistent in pH levels, with no significant difference (Wilcoxon, $P = 0.87$) being identified throughout the duration of the field testing (Figure 17A). The same trend was observed for pH with crop planting and sampling time (time of year), with mean pH staying at a value of approximately 6.7 (Figure 17B/C). EC was not influenced by treatment type ($P = 0.\ 10$); however, EC levels did see fluctuations with crop planting ($P < 0.001$), with a mean EC measurement of 114.65 μS with no crop and 191.4 μS with crops. (Figure 18A/B) Time of year also influenced EC ($P < 0.001$), with most observed differences occurring when comparing summer months to February and March (Figure 18C). Sufficiency levels of available nutrients (SLAN) did not significantly fluctuate due to any of the treatment factors (Figure 19).

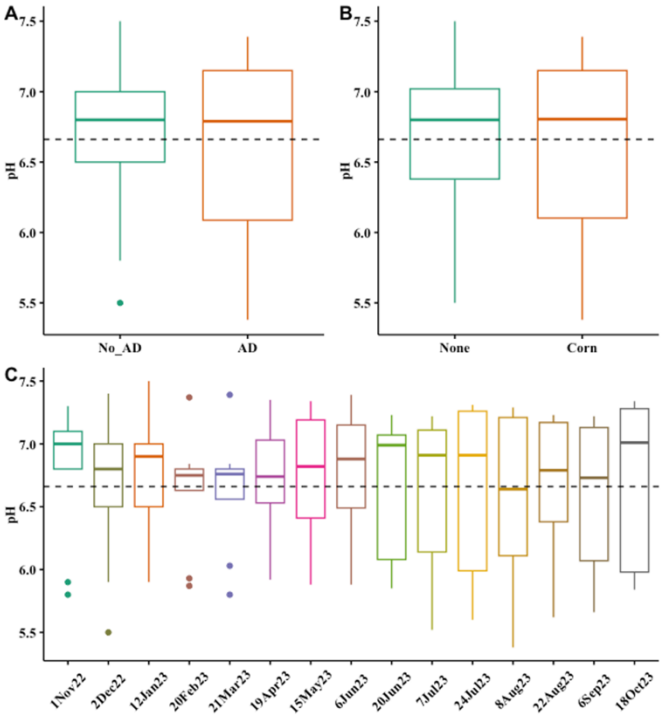


*Figure 17: Shifts in pH levels of soil based on A.) AD treatment, B.) crop planting, and C.) time of year. The significance codes: 0 '***'0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.*

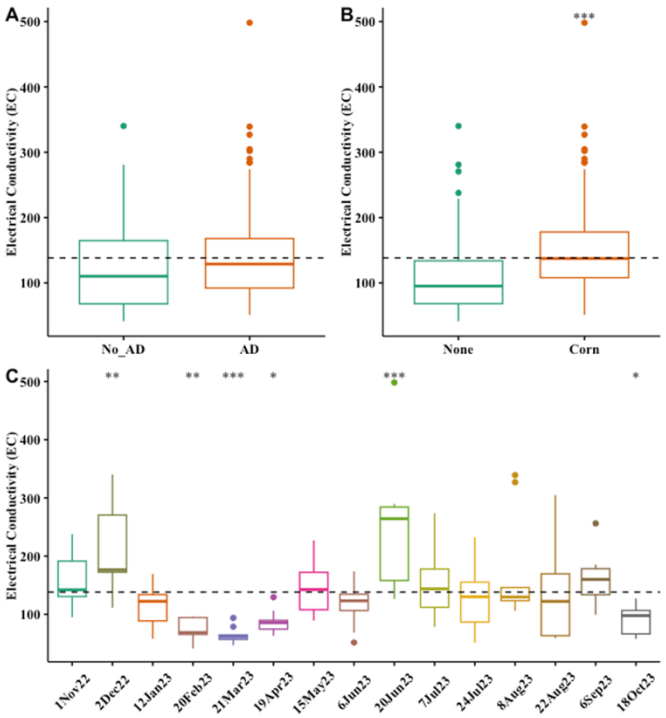


*Figure 18: Shifts in electrical conductivity (EC) levels of soil based on A.) AD treatment, B.) crop planting, and C.) time of year. The significance codes: 0 '***'0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.*

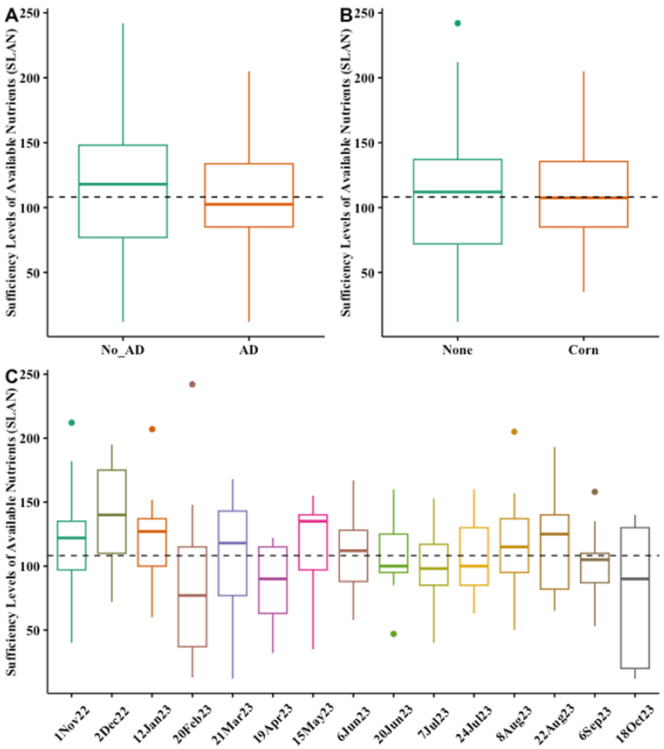


*Figure 19: Shifts in SLAN levels of soil based on A.) AD treatment, B.) crop planting, and C.) time of year. The significance codes: 0 '***'0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.*

### 3.4.6 Soil Nutrients

Changes in soil nutrient levels were analyzed using pairwise comparison for treatment, crop, and sampling time effects. From these results, it was found that the amount of total carbon (TC) in the soil was not impacted by the treatment type as no significant changes (P = 0.72) were identified between No-AD and AD treated samples (Figure 20A, Table B 9). This was true of TC for crop planting and sampling time effects as well, with the measured levels remaining relatively constant throughout the duration of the study, with a mean value of 1.23±0.34 wt% (Figure 20B/C, Table B 10). Both total hydrogen (TH) and total sulfur (TS) experienced shifts with treatment, crops, and sampling time. TH decreased with AD application and crop planting, with the overall mean shifting from 1.10±0.50 wt% to 0.59±0.22 wt% and 0.60±0.21 wt%, respectively (Figure 21A/B, Table B 9,Table B 10). The overall model for the analysis of changes due to time of year showed significance for TH; however, further pairwise comparisons

did not indicate any significant changes between months (Figure 21C). TS followed an opposite trend to that of TH, resulting in increases after AD application and crop planting from 5.48±1.17 ppm to 6.93±1.36 ppm and 5.94±1.23 ppm to 7.07±1.38 ppm (Figure 22A/B, Table B 9, Table B 10). Regarding sampling times, TS differed significantly from March to May, late June, and early July samplings, where it increased from a mean of 5.30 ppm to 7.47-8.06 ppm (Figure 22). After early July sampling, the TS levels steadily decreased for the remainder of the study. Total nitrogen (TN) experienced no significant shifts due to AD application; however, a significant increase in TN was observed with corn growth from a mean of 7.83±4.85 mg-N/L to 12.65±4.84 mg-N/L (Figure 23A/B, Table B 9, Table B 10). TN followed an increasing trend during the growing months, with higher levels being measured in May (17.13±3.13 mg-N/L) following AD application and late July (14.27±1.23) after fertilization with UAN. After August sampling, TN levels in the soil began to decrease (Figure 23C).

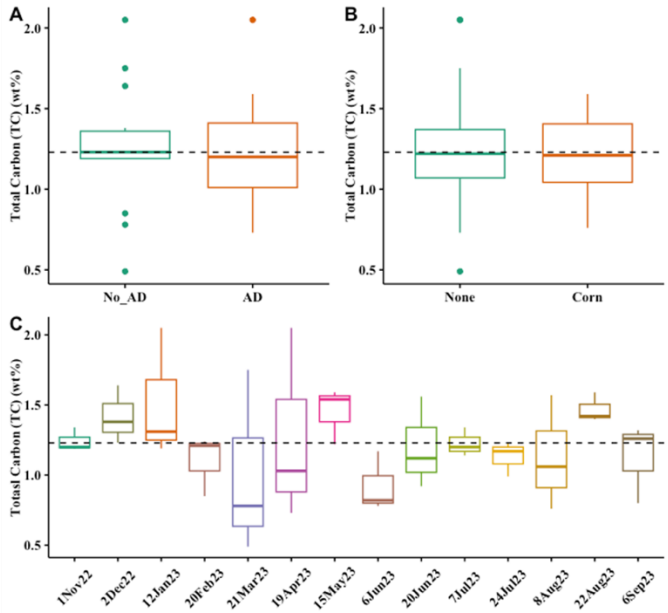


*Figure 20: Shifts in total carbon (TC) levels in soil based on A.) AD treatment, B.) crop planting, and C.) time of year. The significance codes: 0 '***'0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.*

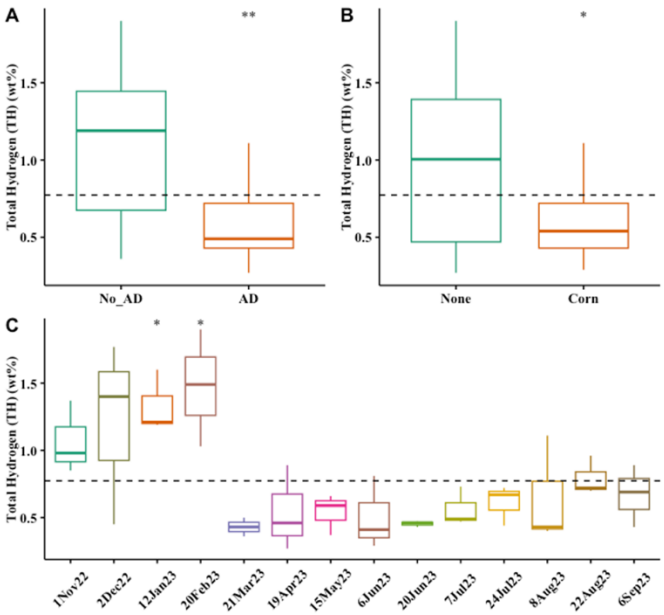


*Figure 21: Shifts in total hydrogen (TH) levels in soil based on A.) AD treatment, B.) crop planting, and C.) time of year. The significance codes: 0 '***'0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.*

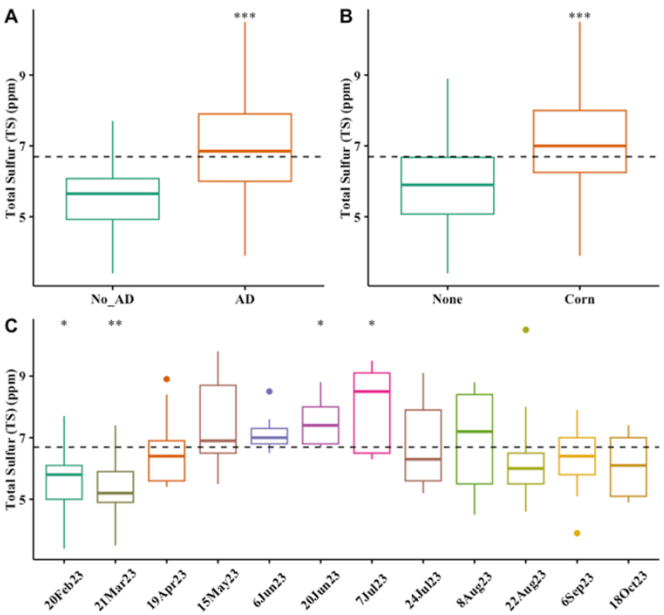


*Figure 22: Shifts in total sulfur (TS) levels in soil based on A.) AD treatment, B.) crop planting, and C.) time of year. The significance codes: 0 '***'0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.*

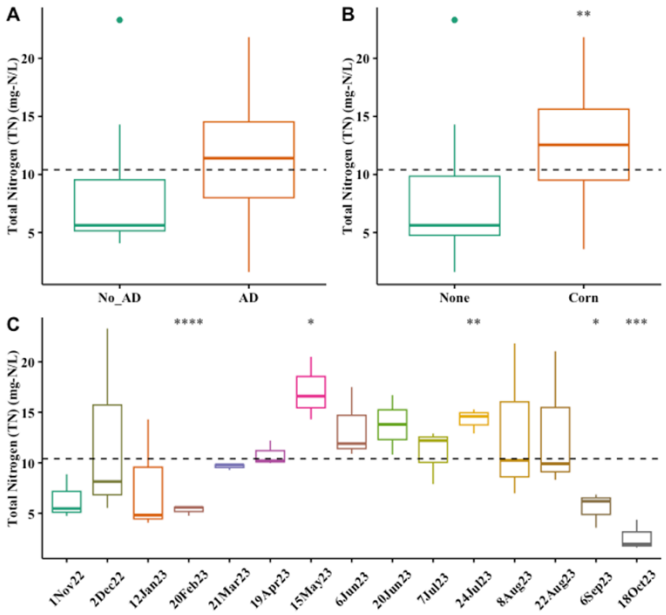


*Figure 23: Shifts in total nitrogen (TN) levels in soil based on A.) AD treatment, B.) crop planting, and C.) time of year. The significance codes: 0 '***'0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.*

The minerals analyzed for shifts in levels due to field treatment and sampling time were phosphorous (P) (Figure 24) and potassium (K) (Figure 25). Both minerals showed no significant changes due to AD application, crop planting, or sampling time, with mean values remaining around 2.27±1.02 ppm and 84.25± 22.09 ppm, respectfully (Table B 9, Table B 10).

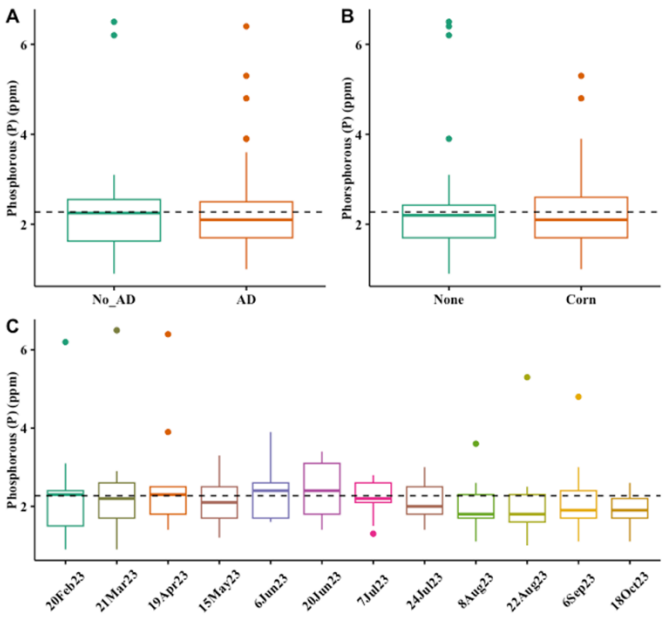


*Figure 24: Shifts in phosphorous (P) levels in soil based on A.) AD treatment, B.) crop planting, and C.) time of year. The significance codes: 0 '***'0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.*

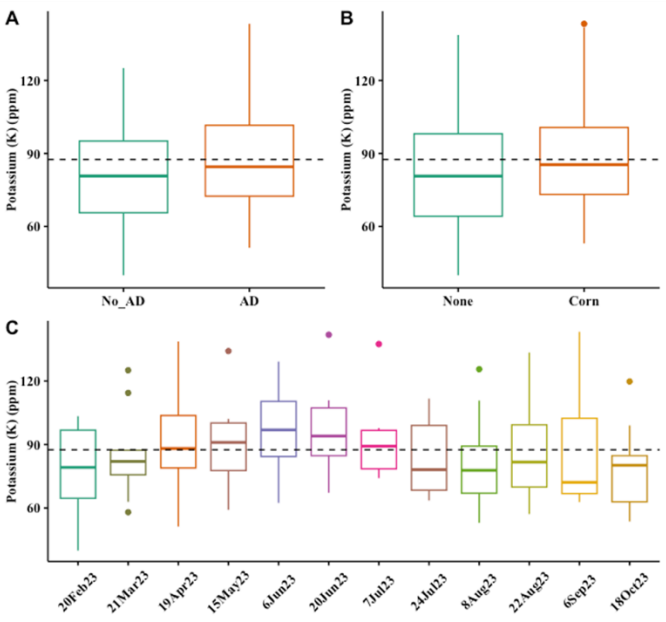


*Figure 25: Shifts potassium (K) levels in soil based on A.) AD treatment, B.) crop planting, and C.) time of year. The significance codes: 0 '***'0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.*

RDA was performed to determine the relationships between the dominant prokaryotic and eukaryotic phyla and the environmental conditions (soil properties and nutrients). RDA for the prokaryotic community found the first two axes explain 19.98% and 7.87% of the total

variation in prokaryotic data. Significance testing (ANOVA) found that TN and K were significant terms in the model; however, no strong relationship between those parameters and dominant phyla could be determined (Figure 26). The first two axes of RDA explain 16.09% and 9.87% of the total variation in the eukaryotic data (Figure 27). Significance testing (ANOVA) revealed no model significance, and therefore no significant parameters contributing to the bacterial community-environment relationship. However, the magnitude of the contributions can be analyzed and related to the dominant phyla. *Ascomycota* shows a strong positive correlation to TN, while other phyla show little correlation to any environmental parameter.

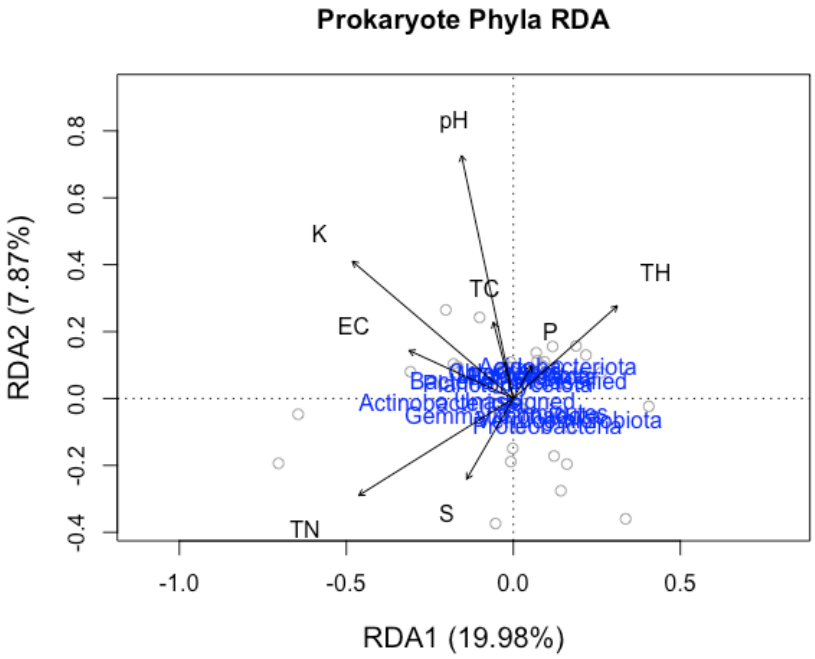


*Figure 26: Redundancy Analysis (RDA) ordinate plot for the relationships between prokaryotic phyla and soil properties. Black arrows indicate the direction and magnitude of environmental parameters.*

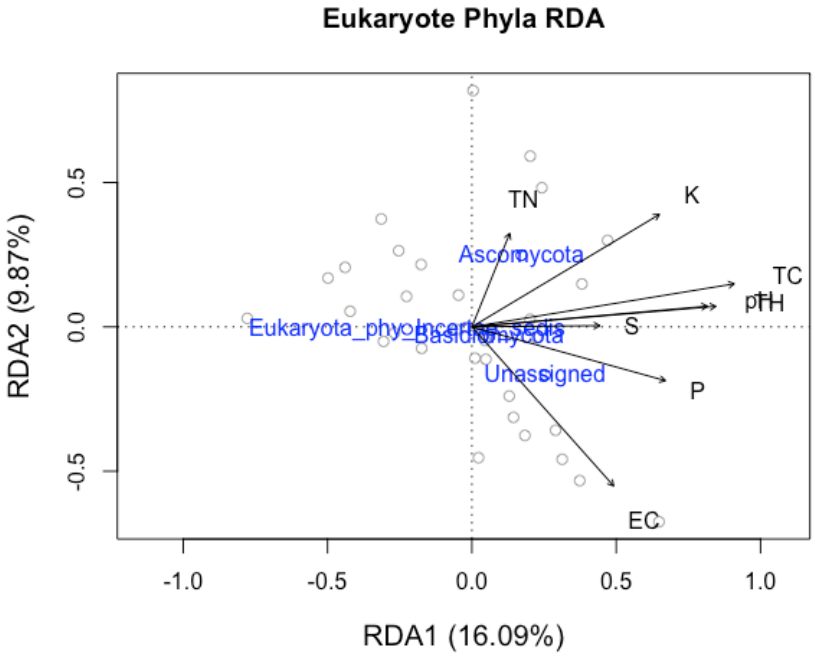


*Figure 27: Redundancy Analysis (RDA) ordinate plots for the relationships between eukaryotic phyla and soil properties. Black arrows indicate the direction and magnitude of environmental parameters.*

At the genus level, RDA analysis uncovered further relationships between dominant prokaryotic and eukaryotic genera and environmental parameters. Parameters considered included pH, electrical conductivity, temperature, carbon, hydrogen, nitrogen, sulfur, potassium, and phosphorous. RDA for the prokaryotic community found the first two axes explain 20.06% and 11.35% of the total variation in prokaryotic data. Significance testing (ANOVA) did not find the model to be significant; however, positive correlations were found for *Vicinamibacterales* uncultured and P, *Chloroflexi*-KD4-96 and TC, and Nocardioides with TN and air temperature (Figure 28). For the eukaryotic community, the first two axes explain 24.60% and 14.00% of the variation in the data. The model was found to be significant, with K being a significant term in the model. K was positively correlated with Plectosphaerella, with TC having a negative correlation (Figure 29). TH was positively correlated to Fusarium, which had a negative relationship with EC, temperature, S, and P.

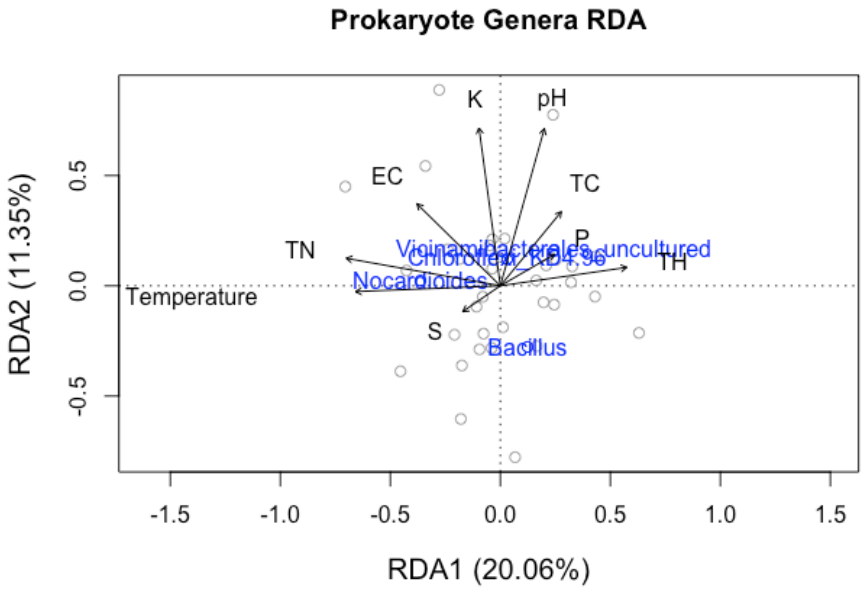


*Figure 28: Redundancy Analysis (RDA) ordinate plots for the relationships between prokaryotic genera and soil properties. Black arrows indicate the direction and magnitude of environmental parameters.*

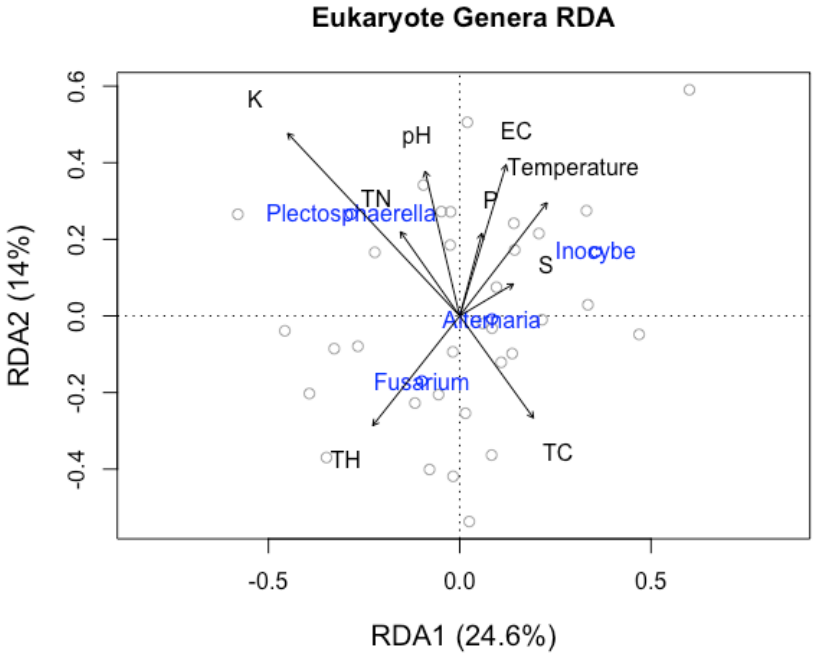


*Figure 29: Redundancy Analysis (RDA) ordinate plots for the relationships between eukaryotic genera and soil properties. Black arrows indicate the direction and magnitude of environmental parameters.*

## 3.5 Discussion

### 3.5.1 Effects of Anaerobic Digestate and Planting Alpha and Beta Diversity

Shifts in community evenness and diversity varied between prokaryotic and eukaryotic communities. Both evenness and richness significantly decreased for the prokaryotic community with AD application and crop planting. The significant shifts in alpha diversity indices in April and late July were consistent with the application of AD and UAN fertilizer, and therefore an influx of specific species that may outcompete others or the influx of nutrients allowed fast-growing bacteria to quickly colonize and outcompete the other bacteria in the area, ultimately lowering the diversity observed. The eukaryotic community, however, did not see these changes and remained stable in evenness and richness throughout the year-long study. It is important to understand that these indices only consider the sheer number of species and overall species diversity. They do not consider the specific species within the group, and so only mark a generic shift in the population density of the community. Just because these indices did or did not significantly differ between treatment, crop, and time of year does not indicate that there were no changes within the soil microbial community. At the species level, the number of species identified may not significantly differ, but the species that make up that number could shift, as evident by the relative abundances calculated for the most prominent phyla. Beta diversity had no significant formation of sampling groups when compared between treatments, indicating that the application of AD did not result in a change in the ecosystem diversity.

### 3.5.2 Effects of Anaerobic Digestate on Microbial Community Composition

The application of anaerobic digestate to the soil did result in changes in microbial community composition for both prokaryotic and eukaryotic populations. As with the results found in Chapter 2, the most dominant phylum in in the prokaryotic community throughout the duration

of the study was *Actinobacteriota*, making up ~43% of the prokaryotic population. *Actinobacteria* is a diverse bacterial phylum that proliferates in a wide range of habitats, including the rhizosphere and soil (Ul-Hassan & Wellington, 2009). A majority of the *Actinobacteriota* phylum consists of saprophytic species, able to decompose plant and animal organic matter, so increases in this phylum could be expected with the application of AD. However, within this study no significant change in the phylum was documented. Previous studies have found results that both support and conflict with these findings. One study documented shifts in *Actinobacteria* phylum after AD application to soil, while another found *Actinobacteria* to be unaffected (Pathan et al., 2021; Ren et al., 2020). Several *Actinobacteria* genera were identified as dominant within the soil community, including *Gaiellales* unassigned, *Actinobacteria* unassigned, *Gaiellales* uncultured, *Solirubrobacterales bacterium* 67-14, *Nocardioides*. Of these genera, *Solirubrobacterales bacterium* 67-14 and *Nocardioides* significantly decreased and increased, respectively, with AD application. Shifts in genera but not the overall phyla suggests that changes in abundance occurred at lower-taxonomic levels.

*Proteobacteria* followed an increasing trend in abundance with the application of AD, making up ~18-19% of the community composition. Similar studies that observed the impact of manure fertilization on microbial communities found the same increasing trend with application (W. Li et al., 2020). This same study was able to correlate increases in *Proteobacteria* to carbon metabolism, and, with the influx of organic matter into the environment with AD application, these bacteria were able to proliferate with the available carbon input. The *Xanthobacteraceae* family was the most abundant of the *Proteobacteria* identified, which interestingly declined in abundance with AD application. This decline is unpredicted, as *Xanthobacteraceae* is a versatile family of aerobic chemoheterotrophs and facultative chemolithoautotrophy, as well as nitrogen

fixation, so should theoretically be able to adjust to environmental changes relatively quickly (A. Oren, 2014).

*Chloroflexi* is a common resident of the soil microbial community, making up about 6.1% of the prokaryotic community in this study. This phylum experienced the largest shift with AD application when compared to the dominant phyla, experiencing a 13.3% decrease in population. Its most dominant genus was identified as *Chloroflexi*-KD4-96, which remained unaffected by digestate application, indicating other, less dominant taxonomies shifted. The large decrease in phylum population does not align with previous studies; however, the lack of change in the dominant genera does (Ren et al., 2020). This study found that *Chloroflexi* was largely unaffected by AD application. The genus *Chloroflexi*-KD4-96 is commonly found in soil; however its exact role is still unknown (Kujala et al., 2018).

*Firmicutes* significantly increased with digestate application, making up 5.81% of the population. This phylum is considered oligotrophic, and thus is fast growing and able to adjust quickly to nutrient influxes. This is consistent with results from previous studies that observed increases in the abundance of *Firmicutes* with AD application (Ren et al., 2020). The dominant genus identified within this phylum was *Bacillus* which followed the same trend with an increase in abundance after AD application. This change could also be related to UAN fertilization, as the abundance of the genus peaked in late July following application. This is also consistent with results from (Ren et al., 2020).

The eukaryotic community was largely unidentified, so conclusions as to how the community was impacted by treatment, crop, and sampling time are limited. The eukaryotic population had a large increase in the abundance of the *Basidiomycota* phylum with AD application. As elaborated on in Chapter 2, this phylum makes up approximately one third of all

fungi, and contains five of the dominant eukaryotic genera identified in this study, including *Inocybe*, *Amphinema*, *Russula*, *Clavulina*, and *Cortinarius* (Taylor et al., 2015b). *Inocybe* and *Cortinarius* were the only genera to experience changes in abundance due to field treatment. Both genera are classified as mycorrhizal fungi, meaning they form symbiotic associations with host plants. *Basidiomycota* taxa are often referred to as wood-decaying fungi, serving in breaking-down the lignin in plant cell walls (Bentil, 2021). Observed increases in this phylum and associated genera could be related to the influx of organic matter via AD application as well as root establishment by corn (Li et al., 2021). Additionally, studies into the dominant fungal species found within digesters found populations of *Basidiomycota* (D. Young et al., 2018). Taking this into consideration, the significant increase in *Basidiomycota* fungi after the application of AD could be attributed to a population found within the digestate. *Ascomycota* is the most diverse fungal phylum, with the most abundant genus in this study being *Fusarium* (Taylor et al., 2015a). *Fusarium* is a pathogenic fungus that poses a major concern for food and feed globally (Thrane, 2014). It is the most commonly reported disease infecting corn, where it can result in severe stalk rot and wilting (L. Oren et al., 2003). Although no significant shifts in population abundance was observed for this phylum, its persistence is important to note due to its pathogenic background. It was also found from RDA, that *Ascomycota* has a strong correlation to nitrogen levels, which is substantiated by its role in nitrogen cycling (Nelson et al., 2015).

### 3.5.3 Effects of Corn Growth on Microbial Community Composition

*Actinobacteria* followed the same trend with crop planting and growth as seen with digestate application; there was no change in the phylum population abundance. However, again similarly to the AD treatment scenario, *Actinobacteria* unassigned and *Nocardioides* significantly increased with corn planting. Past studies have established positive correlations between plant

growth and *Actinobacteria* abundance, with this phylum being described as plant growth promoting and disease suppressing (Sapp et al., 2015).

*Acidobacteriota* is an extremely abundant and common phylum in soil environments, primarily classified by heterotrophic metabolism with a large role in carbon cycling (Sapp et al., 2015). Within this study, a significant decrease in the phylum was observed with corn growth. This may be attributed to the mid-July UAN fertilization event. A previous study found that application of liquid UAN resulted in decreases in abundance for *Acidobacteriota* (Ren et al., 2020). This is explained by the copiotrophic hypothesis. With nitrogen influx, the copiotrophic groups (fast growth rates) are able to quickly adjust to the nutrient-rich environment, while the oligotrophic groups (slow growth rate, i.e. *Acidobacteriota*) are unable to adjust to the change as quickly, and as a result, decline (Ren et al., 2020).

*Myxococcota* significantly decreased with crop planting, showing its lowest abundance in May immediately following planting. *Myxococcota* is a very unique prokaryotic phylum due to its predation capabilities and fruiting body formation (L. Li et al., 2023). The decline in population with crop planting is interesting for a predatorial phylum, as this capability should theoretically make the bacterial resistant. However, this type of bacteria is known to have pack-like predatory behavior, so an adverse event that causes a sharp decline may be hard for the phylum to recover with less opportunity for socialization (L. Li et al., 2023). No previous studies were able to be found documenting shifts due to planting and crop growth associated with *Myxococcota*.

*Planctomycetota* experienced a significant decrease in response to crop planting and growth. This phylum is often associated with a diverse set of role in the nitrogen cycle, so a

decrease in the population during the growing season is interesting with the influx of nitrogen from fertilization (Vourlitis et al., 2024).

Eukaryotic community shifts following planting and crop growth followed the same trend in community shifts seen with AD application.

### 3.5.4 Effects of Anaerobic Digestate and Planting on Soil Properties

The soil pH and SLAN levels were not impacted by AD application, corn growth, or time of year. The pH levels remained within the optimum range (6.0-7.0) for corn growth and nutrient availability (*Assessing Nutrient Availability for Corn*, 2021). SLAN values were acceptable, with mean values depending on treatment and growth staying around 100-117. This indicates that the soil falls into the "moderate activity" range (76-200) for soil biological activity related to the levels of available nutrients in soil. Electrical conductivity did see significant increases with both AD application and corn growth. Studies have shown positive correlations with this parameter between crop yield, soil water content, and soil texture (Johnson et al., 2005; Stadler et al., 2015). Other studies show varying results on changes in soil property levels when treated with digestate, with one study seeing significant differences in EC levels (Gómez-Brandón et al., 2016).

### 3.5.5 Effects of Anaerobic Digestate and Planting on Soil Nutrient Levels

The amount of total carbon was not impacted by the treatment type as no significant changes ($P = 0.72$) were identified between No-AD and AD treated samples. Total nitrogen (TN), again saw no significant change ($P= 0.79$) after AD application, suggesting that AD application will not contribute to over-nitrification of the ecosystem; however, it did see higher levels in months directly after AD application and UAN fertilizer application. Total hydrogen (TH) indicated a significant shift ($P= 0.002$) in levels, seeing a decrease after AD was applied. This could be

caused by an increase in microbes that utilize $H_2$ as an electron donor in respiration, including Acetogens and Methanogens, resulting in a decrease in hydrogen faster than it can be replaced (Piché-Choquette & Constant, 2019). Overall, these results indicate that the application of digestate had little significant impact on the associated soil properties. This could be due to soil stabilization over time, as the study field had been treated with digestate each growing season for 8 years prior. One study found that digestate application resulted in few significant differences in soil nutrient levels, with changes in the microbial community occurring at a faster rate than nutrient levels (Odlare et al., 2008). This could be attributed to shifts in clade diversity while guild diversity remains intact. Further analysis into the metabolisms of the dominant species could reveal more thoroughly the reasoning for the lack of soil property shifts.

## 3.6 Conclusion

In this study, the effects of anaerobic digestate application and corn planting in a year-round field test were evaluated through microbial analysis and soil property and nutrient measurements. The application of digestate did result in shifts in both prokaryotic and eukaryotic populations; however, the overall community richness and evenness only shifted for the prokaryotic community. These results were very similar to those observed with corn planting and growth. The treatment effects on communities and nutrient levels were most prominent immediately following AD application and UAN fertilizer application, most likely due to organic matter and nutrient influxes. Nutrient levels within the soil remained stable throughout the study with exception to TH and TS levels. Soil microbial communities can be very well-established and resilient, meaning that short-term changes in soil treatment may not result in significant changes within the soil, so the continuation of this research will be beneficial in establishing a long-term trend in microbial community composition and structure. Establishing a true control within the

field design may also be beneficial to monitor the isolated effect of year-round microbial cycles related to weather and life-cycle behaviors.

# CHAPTER 4: CONCLUSIONS AND FUTURE WORK

## 4.1 Conclusions

Soil is a fundamental aspect in ensuring global access to food, economic growth, and environmental sustainability. By implementing sustainable and nourishing agricultural practices, soil and its associated micro-environment can be preserved for continued use. Understanding and complementing beneficial soil dynamics biologically, chemically, and physically is essential in this preservation. This thesis aimed to address the knowledge gaps that exist in the understanding of the relationship between soil microbial communities and more sustainable agricultural practices.

This research demonstrates that fumigation and fertilization, both AD and chemical fertilizers, effect the soil microbial community by triggering significant shifts for the bacterial and fungal communities. Cover crop planting to recurve fumigation effects did not show anticipated results, with very few significant shifts in the community observed for all cover crop types. Within these results, it is important to note that pathogenic taxa, including *Fusarium*, *Verticillium*, and *Pythium*, did not see significant changes with any of the treatments. This has both positive and negative implications, as with the fumigation treatments, it is hoped that pathogens will be eliminated or severely reduced in their abundance. However, no significant change in pathogen abundance with AD application is positive, as it indicates that application does not have the potential of stimulating pathogen growth. Soil property and nutrient analysis showed very few changes of note. However, small fluctuations were observed with TN levels peaking directly following AD application and UAN fertilizer application.

Overall, the results of this work demonstrate a proof of concept for continued research into these areas. Soil is an extremely resilient ecosystem, so establishing long-term research is

essential in understanding and uncovering the dynamics of the soil and responses to environmental change and stressors.

## 4.2 Future Work

Work into cover crops and their ability to recurve fumigation effects as well as community changes due to digestate application should be continued to establish the long-term trends in microbial shifts. With any field study there are many uncontrolled variables due to environmental variance. Because of this, field tests benefit from long-term studies. The longer a study is performed, the higher the quality of data collected. This is especially true when looking at the cover crop aspect of Chapter 2. During the 2021-2022 winter growing-season, environmental conditions were not ideal for optimized cover crop growth, impacting the rooting of the plants and therefore the change, or lack thereof, in community composition and structure. By continuing this research for multiple years, an overall trend can be established, especially as it pertains to the environmental factors, and one can more confidently draw conclusions about the potential of cover crops as a sustainable means of soil health recovery. Future work should also explore further the metabolic classification of the microbial community and how the dominant metabolic pathways shift with treatment and time. By understanding the metabolisms involved and how they relate to soil health and crop growth and yield, one can optimize soil conditions to promote the establishment of certain microbes for the benefit of the soil and economic profitability of the field.

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# APPENDIX A: CHAPTER 2 SUPPLEMENTARY DATA

*Table A 1: Diversity metrics of microbial communities based on fumigation, cover crop, and potato treatment**

| ***Treatment*** | ***Sequencing*** | ***Sample ID*** | ***Shannon [a]*** | ***Pielou's [b]*** |
|---|---|---|---|---|
| Fumigation | 16S rRNA | No Fum | 9.002 | 0.879 |
| | | MS | 8.914 | 0.876 |
| | | 1,3-D | 9.065 | 0.880 |
| | | MS+1,3-D | 8.916 | 0.874 |
| | ITS rRNA | No Fum | 5.990 | 0.706 |
| | | MS | 5.738 | 0.691 |
| | | 1,3-D | 5.901 | 0.696 |
| | | MS+1,3-D | 5.140 | 0.629 |
| Cover Crop | 16S rRNA | No Cover | 9.008 | 0.877 |
| | | Mustard | 8.949 | 0.877 |
| | | Pea | 8.978 | 0.878 |
| | | Radish | 8.953 | 0.876 |
| | | Wheat | 8.978 | 0.878 |
| | ITS rRNA | No Cover | 5.652 | 0.677 |
| | | Mustard | 5.613 | 0.669 |
| | | Pea | 5.714 | 0.685 |
| | | Radish | 5.613 | 0.675 |
| | | Wheat | 5.863 | 0.697 |
| Potato | 16S rRNA | Before Potato | 9.121 | 0.880 |
| | | After Potato | 8.865 | 0.875 |
| | ITS rRNA | Before Potato | 5.898 | 0.696 |
| | | After Potato | 5.539 | 0.669 |

a. Shannon's index (richness of the microbial community).

b. Pielou's evenness (evenness of the microbial community)

*. Data in this table are the average of four replicates.

*Table A 2: P-values for the comparison of the alpha diversity index for microbial communities between fumigation treatments**

| ***Microbial*** | ***Alpha diversity index*** | ***Model Significance*** | ***NoFum vs MS*** | ***NoFum vs 1,3-D*** | ***NoFum vs MS+1,3-D*** |
|---|---|---|---|---|---|
| Bacteria | Shannon | . | *0.6018* | *0.5556* | *0.81* |
| | Pielou's | ** | *0.1365* | *0.9897* | *0.0182* |
| Eukaryote | Shannon | ** | *0.708* | *0.98* | *0.0029* |
| | Pielou's | ** | *0.926* | *0.9749* | *0.0103* |
| Comparison Methods | | ANOVA [a] | Pairwise Comparison (Tukey's HSD) | | |

*. Data in this table was calculated by R software.

a. The significance. codes: 0 '***'0.001 '**' 0.01 '*' 0.05 '.' 0.1 '' 1.

*Table A 3: P-values for the comparison of the alpha diversity index for microbial communities between cover crop treatments**

| ***Microbial*** | ***Alpha diversity index*** | ***Model Significance*** | ***NoCover vs Mustard*** | ***NoCover vs Pea*** | ***NoCover vs Radish*** | ***NoCover vs Wheat*** |
|---|---|---|---|---|---|---|
| Bacteria | Shannon | | *0.9402* | *0.9949* | *0.9568* | *0.9388* |
| | Pielou's | | *0.9999* | *0.9898* | *0.9881* | *0.9873* |
| Eukaryote | Shannon | | *0.9999* | *0.9993* | *1.0000* | 0.933 |
| | Pielou's | | *0.9987* | *0.9985* | *1.0000* | *0.9496* |
| Comparison Methods | | ANOVA [a] | Pairwise Comparison (Tukey's HSD) | | | |

*. Data in this table was calculated by R software.

a. The significance. codes: 0 '***'0.001 '**' 0.01 '*' 0.05 '.' 0.1 '' 1.

*Table A 4: P-values for the comparison of the alpha diversity index for microbial communities between sampling time (before/after potato planting)**

| ***Microbial*** | ***Alpha diversity index*** | ***ANOVA [a] (P value)*** |
|---|---|---|
| Bacteria | Shannon | *3.5e-07 **** |
| | Pielou's | *0.00094 **** |
| Eukaryote | Shannon | *0.039 ** |
| | Pielou's | *0.14* |

*. Data in this table was calculated by R software.

*Table A 5: The abundance of the microbial community at the phylum level for fumigant treated soil**

| ***Gene Sequencing*** | ***Microbial*** | ***Phylum*** | ***Fumigation (Relative abundance %)*** | | | |
|---|---|---|---|---|---|---|
| | | | ***NoFum*** | ***MS*** | ***1,3-D*** | ***MS+1,3-D*** |
| 16 S rRNA | Bacteria | *Actinobacteriota* | 39.03 | 41.96 | 39.95 | 45.31 |
| | | *Chloroflexi* | 5.93 | 6.10 | 6.35 | 6.64 |
| | | *Acidobacteriota* | 7.48 | 6.14 | 6.80 | 5.42 |
| | | *Gemmatimonadota* | 6.24 | 7.31 | 6.54 | 7.93 |
| | | *Unassigned* | 2.14 | 2.11 | 2.28 | 2.48 |
| | | *Proteobacteria* | 22.55 | 21.13 | 21.44 | 18.74 |
| | | *Planctomycetota* | 2.39 | 2.28 | 2.41 | 1.95 |
| | | *Myxococcota* | 3.06 | 2.57 | 2.75 | 2.35 |
| | | *Bacteroidota* | 1.32 | 1.26 | 1.54 | 1.12 |
| | | *Firmicutes* | 6.31 | 5.99 | 6.60 | 5.56 |
| | | *Patescibacteria* | 0.93 | 0.97 | 0.83 | 0.72 |
| | | *Verrucomicrobiota* | 1.01 | 0.81 | 0.98 | 0.70 |
| | | *Cyanobacteria* | 0.41 | 0.38 | 0.47 | 0.31 |
| | | *Others* | 1.20 | 0.98 | 1.06 | 0.77 |
| | | **Total** | **100.0** | **100.0** | **100.0** | **100.0** |
| ITS rRNA | Eukaryote | *Eukaryota Incertae sedis* | 87.83 | 88.90 | 87.29 | 92.71 |
| | | *Ascomycota* | 11.41 | 10.65 | 11.86 | 6.93 |
| | | *Basidiomycota* | 0.77 | 0.44 | 0.84 | 0.36 |
| | | *Unassigned* | 0.00 | 0.01 | 0.00 | 0.00 |
| | | **Total** | **100.0** | **100.0** | **100.0** | **100.0** |

*. Data in this table are the average of four replicates.

*Table A 6: The abundance of the microbial community at the phylum level for cover crop treated soil**

| ***Gene Sequencing*** | ***Microbial*** | ***Phylum*** | ***Cover Crop (Relative abundance %)*** | | | | |
|---|---|---|---|---|---|---|---|
| | | | ***No Cover*** | ***Mustard*** | ***Pea*** | ***Radish*** | ***Wheat*** |
| 16 S rRNA | Bacteria | *Actinobacteriota* | 41.8 | 42.3 | 42.5 | 41.1 | 40.4 |
| | | *Chloroflexi* | 6.49 | 6.13 | 6.58 | 6.03 | 6.06 |
| | | *Acidobacteriota* | 6.60 | 6.26 | 6.39 | 6.13 | 6.86 |
| | | *Gemmatimonadota* | 7.00 | 7.53 | 7.19 | 6.75 | 6.65 |
| | | *Unassigned* | 2.25 | 2.28 | 2.31 | 2.17 | 2.27 |
| | | *Proteobacteria* | 20.7 | 20.4 | 20.3 | 21.7 | 21.7 |
| | | *Planctomycetota* | 2.22 | 2.32 | 2.28 | 2.18 | 2.28 |
| | | *Myxococcota* | 2.61 | 2.74 | 2.65 | 2.67 | 2.75 |
| | | *Bacteroidota* | 1.27 | 1.35 | 1.08 | 1.39 | 1.48 |
| | | *Firmicutes* | 6.00 | 5.74 | 5.63 | 6.76 | 6.35 |
| | | *Patescibacteria* | 0.88 | 0.79 | 0.88 | 0.85 | 0.90 |
| | | *Verrucomicrobiota* | 0.85 | 0.89 | 0.83 | 0.92 | 0.90 |
| | | *Cyanobacteria* | 0.38 | 0.36 | 0.41 | 0.42 | 0.39 |
| | | *Others* | 1.04 | 0.94 | 1.03 | 0.98 | 1.03 |
| | | **Total** | **100.0** | **100.0** | **100.0** | **100.0** | **100.0** |
| ITS rRNA | Eukaryote | *Eukaryota Incertae sedis* | 90.02 | 90.03 | 89.77 | 86.36 | 89.86 |
| | | *Ascomycota* | 9.36 | 9.26 | 9.78 | 13.15 | 9.37 |
| | | *Basidiomycota* | 0.61 | 0.71 | 0.45 | 0.48 | 0.77 |
| | | *Unassigned* | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 |
| | | **Total** | **100.0** | **100.0** | **100.0** | **100.0** | **100.0** |

*. Data in this table are the average of four replicates.

*Table A 7: P values for the comparison of the dominant phyla abundances for microbial communities between fumigation treatments**

| ***Microbial*** | ***Phylum*** | ***Model Significance*** | ***NoFum vs MS*** | ***NoFum vs 1,3-D*** | ***NoFum vs MS+1,3-D*** |
|---|---|---|---|---|---|
| Bacteria | *Actinobacteriota* | | *0.7773* | *0.7116* | *0.1000* |
| | *Chloroflexi* | | *0.9993* | *0.3446* | *0.3057* |
| | *Acidobacteriota* | *** | *0.0387* | *0.9391* | *0.0024* |
| | *Gemmatimonadota* | | *0.6541* | *0.8717* | *0.0847* |
| | *Unassigned* | * | *0.9479* | *0.4436* | *0.2122* |
| | *Proteobacteria* | . | *0.8161* | *0.9982* | *0.1027* |
| | *Planctomycetota* | ** | *0.7518* | *0.7673* | *0.0304* |
| | *Myxococcota* | | *0.3079* | *0.9450* | *0.0904* |
| | *Bacteroidota* | | *0.9993* | *0.3917* | *0.8920* |
| | *Firmicutes* | | 0.9589 | *0.7407* | *0.5771* |
| | *Patescibacteria* | | *0.9497* | *0.9536* | *0.2560* |
| | *Verrucomicrobiota* | ** | *0.1474* | *0.9994* | *0.0107* |
| | *Cyanobacteria* | | *0.9795* | *0.6690* | *0.5835* |
| | *Others* | *** | *0.2083* | *1.0000* | *0.0012* |
| Eukaryotes | *Eukaryota Incertae sedis* | *** | *0.2600* | *0.4927* | *0.5155* |
| | *Ascomycota* | ** | *0.5585* | *0.9048* | *0.0159* |
| | *Basidiomycota* | *** | *0.0175* | *0.2632* | *0.0071* |
| | *Unassigned* | | *0.8153* | *0.9826* | *0.9680* |
| Comparison Methods | | ANOVA[a] | | Tukey's HSD | |

*. Data in this table was calculated by R software.

a. The significance. codes: 0 '***'0.001 '**' 0.01 '*' 0.05 '.' 0.1 '' 1.

*Table A 8: P values for the comparison of the dominant genera abundances for microbial communities between fumigation treatments**

| ***Microbial*** | ***Phylum*** | ***Model Significance*** | ***NoFum vs MS*** | ***NoFum vs 1,3-D*** | ***NoFum vs MS+1,3-D*** |
|---|---|---|---|---|---|
| Bacteria | *Other* | | *0.9588* | *0.8900* | *0.9309* |
| | *c_Actinobacteria_unclassified* | * | *0.6680* | *0.5188* | *0.0056* |
| | *Nocardioides* | | *0.9986* | *0.7247* | *0.9940* |
| | *Bacillus* | . | *0.9963* | *0.6094* | *0.5621* |
| | *o_Gaiellales_unclassified* | | *0.9850* | *0.9524* | *0.9999* |
| | *Gaiellales_uncultured* | | *0.9949* | *0.9998* | *0.9969* |
| | *f_Gemmatimonadaceae_unclassified* | | *0.3295* | *0.6165* | *0.1459* |
| | *Sphingomonas* | | *0.9751* | *0.9883* | *0.8337* |
| | *Gemmatimonadaceae_uncultured* | . | *0.9873* | *0.9231* | *0.0818* |
| | *f_Micromonosporaceae_unclassified* | * | *0.2182* | *0.9678* | *0.0610* |
| | *f_Micrococcaceae_unclassified* | | *0.7695* | *0.9863* | *0.9988* |
| | *Unassigned* | * | *0.9479* | *0.4436* | *0.2122* |
| Eukaryotes | *Eukaryota_gen_Incertae_sedis* | ** | *0.2600* | *0.4927* | *0.5155* |
| | *Unassigned* | | *0.9993* | *0.9537* | *0.6658* |
| | *Alternaria* | *** | *0.0042* | *0.7509* | *0.0000* |
| | *Fusarium* | | *0.9526* | *0.6596* | *1.0000* |
| | *Plectosphaerella* | | *0.3290* | *0.4379* | *0.9869* |
| | *Inocybe* | *** | *0.0186* | *0.1935* | *0.0076* |
| | *Tetracladium* | | *1.0000* | *0.9969* | *0.3806* |
| | *Russula* | | *1.0000* | *0.2384* | *1.0000* |
| | *Amphinema* | | *0.5002* | *0.5680* | *0.4508* |
| | *Pseudotomentella* | | *0.4301* | *0.9422* | *0.4301* |
| | *Cortinarius* | | *0.6560* | *0.2556* | *0.3773* |
| | *Other* | | *0.2801* | *0.9964* | *1.0000* |
| Compariso n Methods | | ANOVA[a] | | Tukey's HSD | |

**. Data in this table was calculated by R software.

a. The significance. codes: 0 '***'0.001 '**' 0.01 '*' 0.05 '.' 0.1 '' 1.

*Table A 9: The abundance of the microbial community at the phylum level for soil before and after potato planting**

| ***Gene Sequencing*** | ***Microbial*** | ***Phylum*** | ***Potato (Relative abundance %)*** | |
|---|---|---|---|---|
| | | | ***No Potato*** | ***Potato*** |
| 16 S rRNA | Bacteria | *Actinobacteriota* | 41.87 | 41.33 |
| | | *Chloroflexi* | 6.11 | 6.36 |
| | | *Acidobacteriota* | 5.73 | 7.01 |
| | | *Gemmatimonadota* | 6.06 | 7.72 |
| | | *Unassigned* | 2.25 | 2.26 |
| | | *Proteobacteria* | 22.0 | 20.2 |
| | | *Planctomycetota* | 2.10 | 2.38 |
| | | *Myxococcota* | 3.22 | 2.28 |
| | | *Bacteroidota* | 1.86 | 0.90 |
| | | *Firmicutes* | 5.46 | 6.61 |
| | | *Patescibacteria* | 0.82 | 0.89 |
| | | *Verrucomicrobiota* | 1.04 | 0.76 |
| | | *Cyanobacteria* | 0.45 | 0.35 |
| | | *Others* | 1.02 | 0.99 |
| | | **Total** | **100.0** | **100.0** |
| ITS rRNA | Eukaryote | *Eukaryota Incertae sedis* | 90.85 | 87.93 |
| | | *Ascomycota* | 8.42 | 11.56 |
| | | *Basidiomycota* | 0.73 | 0.51 |
| | | *Unassigned* | 0.01 | 0.00 |
| | | **Total** | **100.0** | **100.0** |

*. Data in this table are the average of four replicates.

# APPENDIX B: CHAPTER 3 SUPPLEMENTARY DATA

*Table B 1: Field sampling and treatment schedule*

| ***Batch*** | ***Samples*** | ***Treatment*** | ***Crop*** | ***Date*** | ***Field Management*** |
|---|---|---|---|---|---|
| 1 | B1-1, B1-2, B1-3, B1-4, B1-5, B1-6, B1-7, B1-8, B1-9 | No AD | None | 11/1/22 | |
| 2 | B2-1, B2-2, B2-3, B2-4, B2-5, B2-6, B2-7, B2-8, B2-9 | No AD | None | 12/2/22 | |
| 3 | B3-1, B3-2, B3-3, B3-4, B3-5, B3-6, B3-7, B3-8, B3-9 | No AD | None | 1/12/23 | |
| 4 | B4-1, B4-2, B4-3, B4-4, B4-5, B4-6, B4-7, B4-8, B4-9 | No AD | None | 2/20/23 | |
| 5 | B5-1, B5-2, B5-3, B5-4, B5-5, B5-6, B5-7, B5-8, B5-9 | No AD | None | 3/21/23 | |
| 6 | B6-1, B6-2, B6-3, B6-4, B6-5, B6-6, B6-7, B6-8, B6-9 | AD | None | 4/19/23 | AD applied; 4/11/23 |
| 7 | B7-1, B7-2, B7-3, B7-4, B7-5, B7-6, B7-7, B7-8, B7-9 | AD | Corn | 5/15/23 | Corn planted; 5/15/23 |
| 8 | B8-1, B8-2, B8-3, B8-4, B8-5, B8-6, B8-7, B8-8, B8-9 | AD | Corn | 6/6/23 | |
| 9 | B9-1, B9-2, B9-3, B9-4, B9-5, B9-6, B9-7, B9-8, B9-9 | AD | Corn | 6/20/23 | Chemical fertilizer; 6/28/23 |
| 10 | B10-1, B10-2, B10-3, B10-4, B10-5, B10-6, B10-7, B10-8, B10-9 | AD | Corn | 7/7/23 | |
| 11 | B11-1, B11-2, B11-3, B11-4, B11-5, B11-6, B11-7, B11-8, B11-9 | AD | Corn | 7/24/23 | |
| 12 | B12-1, B12-2, B12-3, B12-4, B12-5, B12-6, B12-7, B12-8, B12-9 | AD | Corn | 8/8/23 | |

Table B1 (cont'd)

| | | | | | |
|---|---|---|---|---|---|
| 13 | B13-1, B13-2, B13-3, B13-4, B13-5, B13-6, B13-7, B13-8, B13-9 | AD | Corn | 8/22/23 | |
| 14 | B14-1, B14-2, B14-3, B14-4, B14-5, B14-6, B14-7, B14-8, B14-9 | AD | Corn | 9/6/23 | Corn harvested; 9/26/23 |
| 15 | B15-1, B15-2, B15-3, B15-4, B15-4, B15-6, B15-7, B15-8, B15-9 | AD | None | 10/18/23 | |

*Table B 2: Diversity metrics of microbial communities based on digestate application and crop planting**

| ***Treatment*** | ***Sequencing*** | ***Sample ID*** | ***Shannon [a]*** | ***Pielou's [b]*** |
|---|---|---|---|---|
| Anaerobic Digestate | 16S rRNA | No AD | 9.820 | 0.902 |
| | | AD | 9.540 | 0.891 |
| | ITS rRNA | No AD | 5.933 | 0.669 |
| | | AD | 5.916 | 0.663 |
| Crop | 16S rRNA | No Crop | 9.771 | 0.901 |
| | | Corn | 9.513 | 0.890 |
| | ITS rRNA | No Crop | 5.809 | 0.658 |
| | | Corn | 6.020 | 0.671 |

*. Data in this table was calculated by R software.
a. Shannon's index (richness of the microbial community).
b. Pielou's evenness (evenness of the microbial community)

*Table B 3: Comparison of the alpha diversity index for microbial communities between sampling time**

| ***Microbial*** | ***Alpha diversity index*** | ***No AD vs. AD*** | ***No Crop vs. Corn*** |
|---|---|---|---|
| Bacteria | Shannon | *>0.001* | *>0.001* |
| | Pielou's | *>0.001* | *>0.001* |
| Eukaryote | Shannon | *0.6558* | *0.06429* |
| | Pielou's | *0.9907* | *0.2908* |
| Comparison Method | | *Wilcoxon Signed Rank Test* | |

*. Data in this table was calculated by R software.

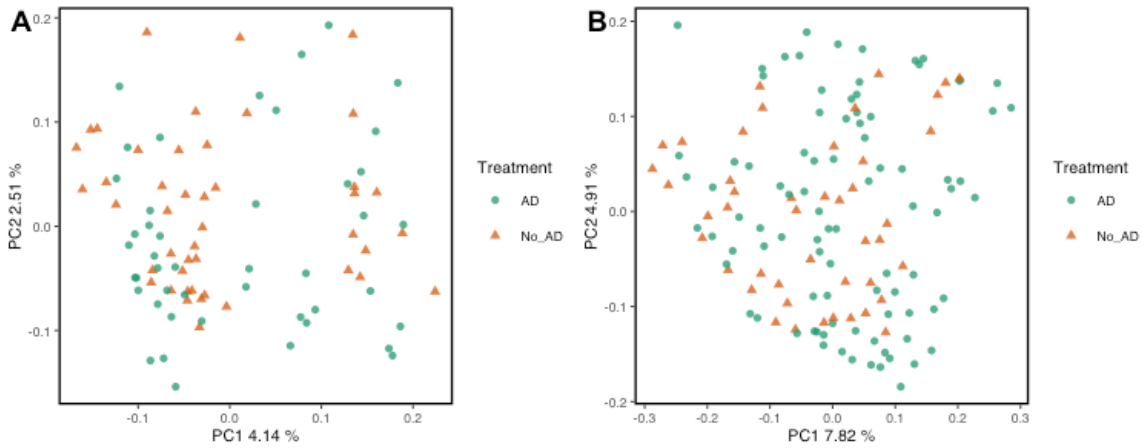


*Figure B 1: Beta diversity analysis using principal coordinate analysis (PCoA) based on weighted UniFrac distances for the prokaryotic (A) and eukaryotic (B) microbial communities for digestate treatment.*

*Table B 4: The abundance of the microbial community at the phylum level for digestate treated soil and crop planting*

| ***Gene Sequencing*** | ***Microbial*** | ***Phylum*** | ***Fumigation (Relative abundance %)*** | | | |
|---|---|---|---|---|---|---|
| | | | ***No AD*** | ***AD*** | ***No Crop*** | ***Corn*** |
| 16 S rRNA | Bacteria | *Actinobacteriota* | 42.65 | 42.89 | 41.87 | 43.63 |
| | | *Proteobacteria* | 18.04 | 19.06 | 18.71 | 18.73 |
| | | *Acidobacteriota* | 10.77 | 10.00 | 10.74 | 9.83 |
| | | *Chloroflexi* | 6.72 | 5.82 | 6.38 | 5.90 |
| | | *Firmicutes* | 4.95 | 5.81 | 5.30 | 5.72 |
| | | *Bacteria_unclassified* | 3.67 | 3.45 | 3.61 | 3.45 |
| | | *Verrucomicrobiota* | 3.22 | 3.05 | 3.24 | 2.99 |
| | | *Myxococcota* | 2.59 | 2.44 | 2.61 | 2.38 |
| | | *Gemmatimonadota* | 2.25 | 2.17 | 2.20 | 2.19 |
| | | *Planctomycetota* | 1.55 | 1.47 | 1.57 | 1.44 |
| | | *Bacteroidota* | 1.06 | 1.29 | 1.17 | 1.24 |
| | | *Others* | 2.53 | 2.53 | 2.58 | 2.48 |
| | | *Unassigned* | 0.01 | 0.02 | 0.02 | 0.02 |
| | | **Total** | **100.0** | **100.0** | **100.0** | **100.0** |
| ITS rRNA | Eukaryote | *Eukaryota Incertae sedis* | 70.23 | 65.46 | 69.12 | 65.24 |
| | | *Unassigned* | 13.12 | 18.58 | 15.09 | 18.22 |
| | | *Ascomycota* | 13.92 | 11.84 | 12.72 | 12.38 |
| | | *Basidiomycota* | 2.37 | 3.88 | 2.74 | 3.93 |
| | | *Fungi_unassigned* | 0.36 | 0.25 | 0.34 | 0.24 |
| | | *Fungi Incertae sedis* | 0.00 | 0.00 | 0.00 | 0.00 |
| | | **Total** | **100.0** | **100.0** | **100.0** | **100.0** |

*Table B 5: P values for the comparison of the dominant phyla abundances for microbial communities between digestate treatment and crop planting**

| ***Microbial*** | ***Phylum*** | ***No AD vs AD*** | ***No Crop vs Corn*** |
|---|---|---|---|
| Bacteria | *Actinobacteriota* | *0.9907* | *0.1332* |
| | *Proteobacteria* | *0.0295* | *0.9701* |
| | *Acidobacteriota* | *0.0560* | *0.0427* |
| | *Chloroflexi* | *0.0018* | *0.1821* |
| | *Firmicutes* | *0.0087* | *0.2001* |
| | *Bacteria_unclassified* | *0.0757* | *0.1601* |
| | *Verrucomicrobiota* | *0.1796* | *0.1413* |
| | *Myxococcota* | *0.1347* | *0.0161* |
| | *Gemmatimonadota* | *0.9164* | *0.9595* |
| | *Planctomycetota* | 0.2330 | *0.0176* |
| | *Bacteroidota* | *0.1130* | *0.6640* |
| | *Others* | *0.8064* | *0.4494* |
| | *Unassigned* | *0.2308* | *0.2333* |
| Eukaryotes | *Eukaryota Incertae sedis* | *0.0229* | *0.0069* |
| | *Unassigned* | *0.0081* | *0.0053* |
| | *Ascomycota* | *0.1371* | *0.9525* |
| | *Basidiomycota* | *>0.001* | *0.0017* |
| | *Fungi_unassigned* | *0.7456* | *0.8375* |
| | *Fungi Incertae sedis* | *0.2207* | *0.1044* |
| Comparison Method | | Wilcoxon Signed Rank Test | |

***.** Data in this table was calculated by R software.

*Table B 6: P values for the comparison of the dominant genera abundances for microbial communities between digestate treatment and crop planting **

| ***Microbial*** | ***Phylum*** | ***No AD vs AD*** | ***No Crop vs Corn*** |
|---|---|---|---|
| Bacteria | *o__Gaiellales_unassigned* | *0.3640* | *0.1190* |
| | *Actinobacteria_unassigned* | *0.1904* | *0.0292* |
| | *Gaiellales__uncultured* | *0.8794* | *0.3532* |
| | *Bacteria_unassigned* | *>0.001* | *>0.001* |
| | *Vicinamibacterales_uncultured* | *>0.001* | *0.0352* |
| | *KD4-96* | *0.0757* | *0.1601* |
| | *67-14* | *0.0065* | *0.5769* |
| | *Nocardioides* | *>0.001* | *>0.001* |
| | *Bacillus* | *0.0038* | *0.0788* |
| | *Vicinamibacteraceae* | *0.0476* | *0.1413* |
| | *f__Xanthobacteraceae_unassigned* | *0.0402* | *0.0479* |
| | *Other* | *0.3375* | *0.3671* |
| | *Unassigned* | *0.2308* | *0.2333* |
| Eukaryotes | *Eukaryota Incertae sedis* | *0.0229* | *0.0069* |
| | *Unassigned* | *0.0082* | *0.0053* |
| | *Fusarium* | *0.2442* | *0.9420* |
| | *Inocybe* | *>0.001* | *0.0013* |
| | *Plectosphaerella* | *0.0021* | *0.3813* |
| | *Fungi_unassigned* | *0.7456* | *0.8375* |
| | *Alternaria* | *>0.001* | *>0.001* |
| | *Amphinema* | *0.9051* | *0.6508* |
| | *Russula* | *0.1104* | *0.6100* |
| | *Clavulina* | *0.4903* | *0.8143* |
| | *Cortinarius* | *0.1613* | *0.0025* |
| | *Others* | *0.4377* | *0.0736* |
| Comparison Method | | Wilcoxon Signed Rank Test | |

***.** Data in this table was calculated by R software.

*Table B 7: Soil properties for non-AD treated vs. AD treated soil*

| ***Variable*** | ***No AD Mean (±SD)*** | ***Range*** | ***AD Mean (±SD)*** | ***Range*** |
|---|---|---|---|---|
| pH[a] | 6.68±0.50 | 5.50-7.50 | 6.65±0.58 | 5.38-7.39 |
| Electrical Conductivity (µS/cm) [b] | 124.42±69.24 | 41.30-340.20 | 145.16±78.24 | 51.09-498.30 |
| SLAN[a] | 116.91±52.04 | 12.00-242.00 | 103.81±39.24 | 12.00-205.00 |

a: Value obtained from Daity One Inc.
b: Value obtained using Thermo Scientific™ Orion Star™ A212 Conductivity Benchtop Meter

*Table B 8:Soil properties for no crop samples vs. crop samples*

| ***Variable*** | ***No Crop Mean (±SD)*** | ***Range*** | ***Corn Mean (±SD)*** | ***Range*** |
|---|---|---|---|---|
| pH[a] | 6.68±0.52 | 5.50-7.50 | 6.64±0.59 | 5.38-7.39 |
| Electrical Conductivity (µS/cm) [b] | 114.64±61.45 | 41.30-340.20 | 158.90±81.25 | 51.09-498.30 |
| SLAN[a] | 106.94±51.89 | 12.00-242.00 | 109.26±36.42 | 35.00-205.00 |

a: Value obtained from Daity One Inc.
b: Value obtained using Thermo Scientific™ Orion Star™ A212 Conductivity Benchtop Meter

*Table B 9: Soil nutrient composition for non-AD treated vs. AD treated*

| ***Variable*** | ***No AD Mean (±SD)*** | ***Range*** | ***AD Mean (±SD)*** | ***Range*** |
|---|---|---|---|---|
| Phosphorous (ppm)[a] | 2.51±1.53 | 0.90-6.50 | 2.23±0.89 | 1.00-6.40 |
| Potassium (ppm)[a] | 81.62±21.31 | 39.90-125.10 | 88.69±22.18 | 51.20-143.30 |
| Carbon (wt%)[b] | 1.26±0.38 | 0.49-2.05 | 1.21±0.32 | 0.73-2.05 |
| Hydrogen (wt%)[b] | 1.10±0.50 | 0.36-1.90 | 0.59±0.22 | 0.27-1.11 |
| Nitrogen (mg-N/L)[c] | 8.27±5.02 | 4.07-23.30 | 11.47±5.30 | 1.60-21.82 |
| Sulfur (ppm)[a] | 5.48±1.17 | 3.40-7.70 | 6.93±1.36 | 3.90-10.50 |

a: Value obtained from Dairy One Inc.

b: Value obtained from Atlantic Microlabs (Norcross, GA)

c: Value obtained from TNM-L unit (Shimadzu Corporation, Kyoto, Japan)

*Table B 10:Soil nutrient composition for no crop samples vs. crop samples*

| ***Variable*** | ***No Crop Mean (±SD)*** | ***Range*** | ***Corn Mean (±SD)*** | ***Range*** |
|---|---|---|---|---|
| Phosphorous (ppm)[a] | 2.41±1.35 | 0.90-6.50 | 2.20±0.80 | 1.00-5.30 |
| Potassium (ppm)[a] | 83.03±22.42 | 39.90-138.70 | 89.75±21.74 | 53.00-143.30 |
| Carbon (wt%)[b] | 1.26±0.42 | 0.49-2.05 | 1.21±0.28 | 0.76-1.59 |
| Hydrogen (wt%)[b] | 1.01±0.52 | 0.27-1.90 | 0.60±0.21 | 0.29-1.11 |
| Nitrogen (mg-N/L)[c] | 7.83±4.85 | 1.60-23.30 | 12.65±4.84 | 3.57-21.82 |
| Sulfur (ppm)[a] | 5.94±1.23 | 3.40-8.90 | 7.07±1.38 | 3.90-10.50 |

a: Value obtained from Dairy One Inc.

b: Value obtained from Atlantic Microlabs (Norcross, GA)

c: Value obtained from TNM-L unit (Shimadzu Corporation, Kyoto, Japan)

APPENDIX C: RSTUDIO CODE FOR MODELING AND ANALYSIS

***Calculation of Alpha Diversity Metrics***

*Analysis repeated for both 16s and ITS rRNA data

```
# Load packages
library(tidyverse)
library(qiime2R)
library(ggplot2)
library(RColorBrewer)
library(DescTools)

# Read in metadata
metadata<-read_csv(" file_name",
                   col_types = cols(.default = col_character()))

# Read in diversity metric vectors from Qiime2
shannon<-read_qza("shannon_vector.qza")
pielou<-read_qza("evenness_vector.qza")

# Move the sample names to a new column that matches metadata to all of them to be merged
shannon<-shannon$data %>% rownames_to_column("sample_ID")
pielou<-pielou$data %>% rownames_to_column("sample_ID")

# Merge dataframes
metadata<-
  metadata %>%
  left_join(shannon)
metadata<-
  metadata %>%
  left_join(pielou)
head(metadata)

# Print descriptive statistics for metrics based on factors
by(metadata, metadata$Factor, stat.desc)

# Check normality assumption
shapiro.test(metadata$shannon_entropy)
shapiro.test(metadata$pielou_evenness)

# Pairwise comparison between factor conditions for diversity indices
wilcox.test(diversity_metric~Factor, data=metadata)

kruskal.test(diversity_metric~Factor, data=metadata)
ConoverTest(diversity_metric~Factor, method="bonferroni", data = metadata)


## Boxplots for Alpha Diversity ------------------------------------------------------------------------------------

# Shannon Entropy
```

```
# New data frame for Shannon entropy
dat_S <- metadata[, c("Factor", "Factor", "Factor", "Factor", "shannon_entropy")]
dat_S

# Reorder data for plotting
sample_order <- c("Order in which samples were collected
")
treat_order <- c("Order you wish to see factors appear on plot")

dat_S <- dat_S %>%
  mutate(Sample_Order_Factor = factor(Sample_Order_Factor, levels=sample_order))
dat_S <- dat_S %>%
  mutate(Treatment_Factor= factor(Treatment_Factor, levels=treat_order))


# Shannon Boxplot
p1<-ggboxplot(dat_S, x = "Factor", y = "shannon_entropy", color="Factor",
            legend = "none", palette=brewer.pal(n = 8, name = 'Dark2')) +
  theme(
      axis.text.x=element_text(family="Times New Roman",face="bold",color="black",size=10,vjust =
0.5),
      axis.text.y=element_text(family="Times New Roman",face="bold",color="black",size=10),
      axis.title.x=element_blank(),
      axis.title.y=element_text(family="Times New Roman",face="bold",size=10))+labs(y="Shannon's
Index")+
  geom_hline(yintercept = mean(dat_S$shannon_entropy), linetype = 2)+
  stat_compare_means(label = "p.signif", method = "wilcox.test",ref.group = "Factor_Control",hide.ns =
TRUE, label.y=11.25,family="Times New Roman")
p1

-----------------------------------------------------------------------------------------------------------------------

# Pielou's Evenness
# New data frame for Pielou's Evenness
dat_P <- metadata[, c("Factor", "Factor", "Factor", "Factor", "pielou_evenness")]

# Reorder data for plotting
sample_order <- c("Order in which samples were collected
")
treat_order <- c("Order you wish to see factors appear on plot")

dat_P <- dat_P %>%
  mutate(Sample_Order_Factor = factor(Sample_Order_Factor, levels=sample_order))
dat_P <- dat_P %>%
  mutate(Treatment_Factor= factor(Treatment_Factor, levels=treat_order))

## Pielou's Boxplot
P1<-ggboxplot(dat_P, x = "Factor", y = "pielou_evenness", color = "Factor",
            legend = "none", palette=brewer.pal(n = 8, name = 'Dark2')) +
  theme(
```

```
      axis.text.x=element_text(family="Times New Roman",face="bold",color="black",size=10,vjust =
0.5),
      axis.text.y=element_text(family="Times New Roman",face="bold",color="black",size=10),
      axis.title.x=element_blank(),
      axis.title.y=element_text(family="Times New Roman",face="bold",size=10))+labs(y="Pielou's
Evenness")+
  geom_hline(yintercept = mean(dat_P$pielou_evenness), linetype = 2
  stat_compare_means(label = "p.signif", method = "t.test",ref.group = "Factor_Control",hide.ns=TRUE,
label.y=0.93,family="Times New Roman")
p1
```

### *Plotting of Beta Diversity Distance Matrix*

*Analysis repeated for both 16s and ITS rRNA data

```
# Load packages
library(tidyverse)
library(qiime2R)
library(ggplot2)
library(RColorBrewer)
library(DescTools)

# Read in metadata
metadata<-read_csv("metadata.csv")

# Read in distance matrix from Qiime2
wunifrac<-read_qza("weighted_unifrac_pcoa_results.qza")

# Plot PCoA
p1<- wunifrac$data$Vectors %>%
  dplyr::select(SampleID, PC1, PC2) %>%
  left_join(metadata) %>%
  ggplot(aes(x=PC1, y=PC2, color=`Factor1`, shape=`Factor2`)) +
  geom_point(alpha=0.8) +
  theme_q2r() +
  scale_shape_manual(values=c(16,18,8,17,0), name="Factor2") + #see
  scale_color_brewer(palette = "Set1")+
  xlab(paste("PC1",round(100*uwunifrac$data$ProportionExplained[1],2),"%")) +
  ylab(paste("PC2",round(100*uwunifrac$data$ProportionExplained[2],2),"%"))
p1
```

### *Microbial Abundance Plotting and Analysis*

*Analysis repeated for both 16s and ITS rRNA data

```
# Load packages
library(paletteer)
library(ggplot2)
library(tidyverse)
library(egg)
library(RColorBrewer)
```

```
library(DescTools)

## Phylum Level
# Read in abundance data
abundance_table <- read_csv("OTU_Abundance _Table_Phylum.csv",
            col_types = cols(otu_id = col_character(),
                        .default = col_number()))

# Pivot table
dat <- abundance_table %>%
  pivot_longer(-otu_id, names_to = "sample_ID", values_to = "count")

# Read in OTU key
OTU_Taxonomy <- read_csv("OTU_Taxonomy_Table.csv",
            col_types = cols(.default = "character"))

# Merge abundance and OTU key
dat <- dat %>%
  left_join(OTU_Taxonomy, by = "otu_id")
dat

# Read in metadata
metadata <- read_csv("metadata.csv",
             col_types = cols(.default = col_character()))

# Merge metadata, abundance, and OTU key
dat <- dat %>%
  left_join(Meta, by = "sample_ID")
dat

# Reorder data for plotting
phyla_order <- c("dominant_phyla_names")
sample_order <- c("Order in which samples were collected")
treat_order <- c("Order you wish to see factors appear on plot")

dat <- dat %>%
  mutate(Phylum = factor(Phylum, levels = phyla_order))
dat <- dat  %>%
  mutate(Sample_Order_Factor = factor(Sample_Order_Factor, levels=sample_order))
dat <- dat %>%
  mutate(Treatment_Factor= factor(Treatment_Factor, levels=treat_order))

# Plot abundance barchart
# Create color palette to fit number of phyla
getPalette = colorRampPalette(brewer.pal(12, "Set3"))(14)

# Plot
p1<-dat %>%
  ggplot(aes(x = Factor2, y = count)) +
  facet_grid(~ Factor1, scales = "free_x", space = "free_x") +
  geom_bar(aes(fill = Phylum), stat = "identity", position = "fill", width = 0.7) +
```

```
  scale_fill_manual(values = getPalette)+
  scale_y_continuous(name = "Relative Frequency (%)", labels = scales::percent) +
  theme(axis.text.x = element_text(family="Times New Roman",angle = 90, size = 8),
        axis.text.y = element_text(family="Times New Roman",color = "black"),
        axis.title.x=element_blank(),
        strip.text = element_text(family="Times New Roman",face = "bold"),
        strip.background = element_blank(),text=element_text(family="Times New Roman", size=12))
p1
```

---

```
## Genus Level
# Read in abundance data
abundance_table <- read_csv("OTU_Abundance _Table_Genus.csv",
                col_types = cols(otu_id = col_character(),
                                 .default = col_number()))

# Pivot table
dat <- abundance_table %>%
  pivot_longer(-otu_id, names_to = "sample_ID", values_to = "count")

# Read in OTU key
OTU_Taxonomy <- read_csv("OTU_Taxonomy_Table_Genus.csv",
                col_types = cols(.default = "character"))

# Merge abundance and OTU key
dat <- dat %>%
  left_join(OTU_Taxonomy, by = "otu_id")
dat

# Read in metadata
metadata <- read_csv("metadata.csv",
                 col_types = cols(.default = col_character()))

# Merge metadata, abundance, and OTU key
dat <- dat %>%
  left_join(Meta, by = "sample_ID")
dat

# Reorder data for plotting
genus_order <- c("dominant_genera_names")
sample_order <- c("Order in which samples were collected")
treat_order <- c("Order you wish to see factors appear on plot")

dat <- dat %>%
  mutate(Genus = factor(Genus, levels = genus_order))
dat <- dat  %>%
  mutate(Sample_Order_Factor = factor(Sample_Order_Factor, levels=sample_order))
dat <- dat %>%
  mutate(Treatment_Factor= factor(Treatment_Factor, levels=treat_order))
```

```
# Plot abundance barchart
# Create color palette to fit number of genera
getPalette = colorRampPalette(brewer.pal(12, "Set3"))(14)

# Plot
p1<-dat %>%
  ggplot(aes(x = Factor2, y = count)) +
  facet_grid(~ Factor1, scales = "free_x", space = "free_x") +
  geom_bar(aes(fill = Genus), stat = "identity", position = "fill", width = 0.7) +
  scale_fill_manual(values = getPalette)+
  scale_y_continuous(name = "Relative Frequency (%)", labels = scales::percent) +
  theme(axis.text.x = element_text(family="Times New Roman",angle = 90, size = 8),
        axis.text.y = element_text(family="Times New Roman",color = "black"),
        axis.title.x=element_blank(),
        strip.text = element_text(family="Times New Roman",face = "bold"),
        strip.background = element_blank(),text=element_text(family="Times New Roman", size=12))
p1
```

---

```
## Statistical Shifts for Phyla and Genera
# Read in relative abundance data
RelAb <- read_csv("RelAbundance_Phylum.csv",
                  col_types = cols(otu_id = col_character(),
                                   .default = col_number()))

# Pivot table
dat <- RelAb %>%
  pivot_longer(-otu_id, names_to = "sample_ID", values_to = "count")
dat

# Read in OTU key
OTU_Taxonomy <- read_csv("OTU_Taxonomy_Table_Phylum.csv",
                  col_types = cols(.default = "character"))

# Merge relative abundance and OTU key
dat <- dat %>%
  left_join(OTU_Taxonomy, by = "otu_id")
dat

# Read in metadata
metadata <- read_csv("metadata.csv",
                   col_types = cols(.default = col_character()))

# Merge metadata, abundance, and OTU key
dat <- dat %>%
  left_join(Meta, by = "sample_ID")
dat
```

---

```
# Filter data by dominant phylum
Phylum1<- dat %>%
  filter(Phylum=='Phylum_Name')

Phylum2<- dat %>%
  filter(Phylum=='Phylum_Name')

Phylum3<- dat %>%
  filter(Phylum=='Phylum_Name')

Phylum4<- dat %>%
  filter(Phylum=='Phylum_Name')

Phylum5<- dat %>%
  filter(Phylum=='Phylum_Name')

Phylum6<- dat %>%
  filter(Phylum=='Phylum_Name')

Phylum7<- dat %>%
  filter(Phylum=='Phylum_Name')

Phylum8<- dat %>%
  filter(Phylum=='Phylum_Name')

Phylum9<- dat %>%
  filter(Phylum=='Phylum_Name')

Phylum10<- dat %>%
  filter(Phylum=='Phylum_Name')

Phylum11<- dat %>%
  filter(Phylum=='Phylum_Name')

Phylum12<- dat %>%
  filter(Phylum=='Phylum_Name')

-----------------------------------------------------------------------------------------------------------------------

# Test assumptions
#Normality
shapiro.test(Phylum1$count)
shapiro.test(Phylum2$count)
shapiro.test(Phylum3$count)
shapiro.test(Phylum4$count)
shapiro.test(Phylum5$count)
shapiro.test(Phylum6$count)
shapiro.test(Phylum7$count)
shapiro.test(Phylum8$count)
shapiro.test(Phylum9$count)
shapiro.test(Phylum10$count)
```

```
shapiro.test(Phylum11$count)
shapiro.test(Phylum12$count)

---------------------------------------------------------------------------------------------------------------------------

# If assumptions are satisfied, parametric test method
t.Phylum1<-t.test(count ~ Factor1, data = Phylum1, var.equal=FALSE)
t.Phylum1

t.Phylum2<-t.test(count ~ Factor1, data = Phylum2, var.equal=FALSE)
t.Phylum2

t.Phylum3<-t.test(count ~ Factor1, data = Phylum3, var.equal=FALSE)
t.Phylum3

t.Phylum4<-t.test(count ~ Factor1, data = Phylum4, var.equal=FALSE)
t.Phylum4

t.Phylum5<-t.test(count ~ Factor1, data = Phylum5, var.equal=FALSE)
t.Phylum5

t.Phylum6<-t.test(count ~ Factor1, data = Phylum6, var.equal=FALSE)
t.Phylum6

t.Phylum7<-t.test(count ~ Factor1, data = Phylum7, var.equal=FALSE)
t.Phylum7

t.Phylum8<-t.test(count ~ Factor1, data = Phylum8, var.equal=FALSE)
t.Phylum8

t.Phylum9<-t.test(count ~ Factor1, data = Phylum9, var.equal=FALSE)
t.Phylum9

t.Phylum10<-t.test(count ~ Factor1, data = Phylum10, var.equal=FALSE)
t.Phylum10

t.Phylum11<-t.test(count ~ Factor1, data = Phylum11, var.equal=FALSE)
t.Phylum11

t.Phylum12<-t.test(count ~ Factor1, data = Phylum12, var.equal=FALSE)
t.Phylum12

# If factor has more than 2 levels use ANOVA for parametric
aov.Phylum<-aov(count~Factor1*Factor2, data = Phylum)
summary(aov.Phylum) #sig difference for fumigation

# If ANOVA found to be significant for one or more factors
hsd_test <- TukeyHSD(aov.Phylum,which="Factor1")
hsd_test

---------------------------------------------------------------------------------------------------------------------------
```

```
# If assumptions not satisfied, non-parametric test method
# Wilcoxon Signed Rank test for 2 level
wilcox.Phylum1<-wilcox.test(count ~ Factor1, data = Phylum1)
wilcox.Phylum1

wilcox.Phylum2<-wilcox.test(count ~ Factor1, data = Phylum2)
wilcox.Phylum2

wilcox.Phylum3<-wilcox.test(count ~ Factor1, data = Phylum3)
wilcox.Phylum3

wilcox.Phylum4<-wilcox.test(count ~ Factor1, data = Phylum4)
wilcox.Phylum4

wilcox.Phylum5<-wilcox.test(count ~ Factor1, data = Phylum5)
wilcox.Phylum5

wilcox.Phylum6<-wilcox.test(count ~ Factor1, data = Phylum6)
wilcox.Phylum6

wilcox.Phylum7<-wilcox.test(count ~ Factor1, data = Phylum7)
wilcox.Phylum7

wilcox.Phylum8<-wilcox.test(count ~ Factor1, data = Phylum8)
wilcox.Phylum8

wilcox.Phylum9<-wilcox.test(count ~ Factor1, data = Phylum9)
wilcox.Phylum9

wilcox.Phylum10<-wilcox.test(count ~ Factor1, data = Phylum10)
wilcox.Phylum10

wilcox.Phylum11<-wilcox.test(count ~ Factor1, data = Phylum11)
wilcox.Phylum11

wilcox.Phylum12<-wilcox.test(count ~ Factor1, data = Phylum12)
wilcox.Phylum12

# If factor has more than 2 levels use Kruskal for non-parametric
kruskal.Phylum<-kruskal.test(count~Factor2, data = Phylum)
kruskal.Phylum

# If Kruskal test found to be significant
ConoverTest(count~Factor2, method="bonferroni", data = Phylum)
```

***Soil Properties Descriptive Statistics and Analysis***

```
# Load packages
```

```
library(pastecs)
library(DescTools)
library(ggplot2)

## Soil Properties Data
## Read in meta data containing soil properties
metadata<-read_csv("Soil_Properties_Metadata.csv")

# Compute descriptive statistics by factor
by(metadata, metadata$Factor1, stat.desc)
by(metadata, metadata$Factor2, stat.desc)
```

---

```
## Test assumptions
# Normality
shapiro.test(metadata$pH) #not normal
shapiro.test(metadata$EC) #not normal
shapiro.test(metadata$SLAN) # normal
```

---

```
## Statistical Shifts for Soil Properties
# If non-normal, Wilcoxon test; If normal, t-test with unequal variance
wilcox.test(pH~Factor, data=metadata)
wilcox.test(EC~Factor, data=metadata)
t.test(SLAN~Factor, data=metadata,var.eqaul=FALSE)
```

---

```
## Plotting Soil Properties
# Reorder data for plotting
sample_order <- c("Order in which samples were collected")
treat_order <- c("Order you wish to see factors appear on plot")

metadata <- metadata %>%
  mutate(Sample_Order_Factor = factor(Sample_Order_Factor, levels=sample_order))
metadata <- metadata %>%
  mutate(Treatment_Factor = factor(Treatment_Factor, levels=treat_order))

# Boxplots for Soil Properties
p1<-ggboxplot(metadata, x = "Factor", y = "pH", color = "Factor", legend = "none",
palette=brewer.pal(n= 8, name = 'Dark2')) +
  theme(
        axis.text.x=element_text(family="Times New Roman",face="bold",color="black",size=10,vjust =
0.5),
        axis.text.y=element_text(family="Times New Roman",face="bold",color="black",size=10),
        axis.title.x=element_blank(),
        axis.title.y=element_text(family="Times New Roman",face="bold",size=10))+labs(y="pH")+
  geom_hline(yintercept = mean(metadata$pH), linetype = 2)+
```

```
  stat_compare_means(label = "p.signif", method = "wilcox.test",ref.group =
"Factor_Control",hide.ns=TRUE, family="Times New Roman")
p1

p2<-ggboxplot(metadata, x = "Factor", y = "EC", color = "Factor", legend = "none",
palette=brewer.pal(n= 8, name = 'Dark2')) +
  theme(
       axis.text.x=element_text(family="Times New Roman",face="bold",color="black",size=10,vjust =
0.5),
       axis.text.y=element_text(family="Times New Roman",face="bold",color="black",size=10),
       axis.title.x=element_blank(),
       axis.title.y=element_text(family="Times New Roman",face="bold",size=10))+labs(y=" Electrical
Conductivity (EC)")+
  geom_hline(yintercept = mean(metadata$EC), linetype = 2)+
  stat_compare_means(label = "p.signif", method = "wilcox.test",ref.group =
"Factor_Control",hide.ns=TRUE, family="Times New Roman")
p2

p3<-ggboxplot(metadata, x = "Factor", y = "SLAN", color = "Factor", legend = "none",
palette=brewer.pal(n = 8, name = 'Dark2')) +
  theme(
       axis.text.x=element_text(family="Times New Roman",face="bold",color="black",size=10,vjust =
0.5),
       axis.text.y=element_text(family="Times New Roman",face="bold",color="black",size=10),
       axis.title.x=element_blank(),
       axis.title.y=element_text(family="Times New Roman",face="bold",size=10))+labs(y=" Sufficiency
Levels of Available Nutrients (SLAN)")+
  geom_hline(yintercept = mean(metadata$SLAN), linetype = 2)+
  stat_compare_means(label = "p.signif", method = "wilcox.test",ref.group =
"Factor_Control",hide.ns=TRUE, family="Times New Roman")
p3
```

***Soil Nutrient Descriptive Statistics and Analysis***

```
# Load packages
library(pastecs)
library(DescTools)
librar(ggplot2)

#Read in metadata for nutrient data
metadata<-read.csv("Soil_Nurtient_Metadata.csv")

# Compute descriptive statistics by factor
by(metadata, metadata$Factor1, stat.desc)
by(metadata, metadata$Factor2, stat.desc)

--------------------------------------------------------------------------------------------------------------------------

## Test assumptions
# Normality
shapiro.test(metadata$TC) #normal
shapiro.test(metadata$TH) #not normal
```

```
shapiro.test(metadata$TN) # normal
shapiro.test(metadata$TS) # normal
shapiro.test(metadata$P) #not normal
shapiro.test(metadata$K) #not normal
```

---

```
## Statistical Shifts for Soil Nutrients
# If non-normal, Wilcoxon test; If normal, t-test with unequal variance
t.test(TC~Factor, data=metadata, var.equal=FALSE)
wilcox.test(TH~Factor, data=metadata)
t.test(TN~Factor, data=metadata, var.equal=FALSE)
t.test(TS~Factor, data=metadata, var.equal=FALSE)
wilcox.test(P~Factor, data=metadata)
wilcox.test(K~Factor, data=metadata)
```

---

```
## Plotting Soil Nutrients
# Reorder data for plotting
sample_order <- c("Order in which samples were collected")
treat_order <- c("Order you wish to see factors appear on plot")

metadata <- metadata %>%
  mutate(Sample_Order_Factor = factor(Sample_Order_Factor, levels=sample_order))
metadata <- metadata %>%
  mutate(Treatment_Factor = factor(Treatment_Factor, levels=treat_order))

# Boxplots
p1<-ggboxplot(metadata, x = "Factor", y = "TC", color = "Factor", legend = "none",
palette=brewer.pal(n= 8, name = 'Dark2')) +
  theme(
      axis.text.x=element_text(family="Times New Roman",face="bold",color="black",size=10,vjust =
0.5),
      axis.text.y=element_text(family="Times New Roman",face="bold",color="black",size=10),
      axis.title.x=element_blank(),
      axis.title.y=element_text(family="Times New Roman",face="bold",size=10))+labs(y="Total Carbon
(TC) (wt%)")+
  geom_hline(yintercept = mean(metadata$TC), linetype = 2)+
  stat_compare_means(label = "p.signif", method = "wilcox.test",ref.group =
"Factor_Control",hide.ns=TRUE, family="Times New Roman")
p1

p2<-ggboxplot(metadata, x = "Factor", y = "TH", color = "Factor", legend = "none",
palette=brewer.pal(n= 8, name = 'Dark2')) +
  theme(
      axis.text.x=element_text(family="Times New Roman",face="bold",color="black",size=10,vjust =
0.5),
      axis.text.y=element_text(family="Times New Roman",face="bold",color="black",size=10),
      axis.title.x=element_blank(),
```

```
      axis.title.y=element_text(family="Times New Roman",face="bold",size=10))+labs(y="Total
Hydrogen (TH) (wt%)")+
  geom_hline(yintercept = mean(metadata $TH), linetype = 2)+
  stat_compare_means(label = "p.signif", method = "wilcox.test",ref.group =
"Factor_Control",hide.ns=TRUE, family="Times New Roman")
p2

p3<-ggboxplot(metadata, x = "Factor", y = "TN", color = "Factor", legend = "none",
palette=brewer.pal(n= 8, name = 'Dark2')) +
  theme(
      axis.text.x=element_text(family="Times New Roman",face="bold",color="black",size=10,vjust =
0.5),
      axis.text.y=element_text(family="Times New Roman",face="bold",color="black",size=10),
      axis.title.x=element_blank(),
      axis.title.y=element_text(family="Times New Roman",face="bold",size=10))+labs(y="Total
Nitrogen (TN) (mg-N/L)")+
  geom_hline(yintercept = mean(metadata $TN), linetype = 2)+
  stat_compare_means(label = "p.signif", method = "wilcox.test",ref.group =
"Factor_Control",hide.ns=TRUE, family="Times New Roman")
p3

p4<-ggboxplot(metadata, x = "Factor", y = "TS", color = "Factor", legend = "none",
palette=brewer.pal(n= 8, name = 'Dark2')) +
  theme(
      axis.text.x=element_text(family="Times New Roman",face="bold",color="black",size=10,vjust =
0.5),
      axis.text.y=element_text(family="Times New Roman",face="bold",color="black",size=10),
      axis.title.x=element_blank(),
      axis.title.y=element_text(family="Times New Roman",face="bold",size=10))+labs(y="Total Sulfur
(TS) (ppm)")+
  geom_hline(yintercept = mean(metadata $TS), linetype = 2)+
  stat_compare_means(label = "p.signif", method = "wilcox.test",ref.group =
"Factor_Control",hide.ns=TRUE, family="Times New Roman")
p4

p5<-ggboxplot(metadata, x = "Factor", y = "P", color = "Factor", legend = "none", palette=brewer.pal(n =
8, name = 'Dark2')) +
  theme(
      axis.text.x=element_text(family="Times New Roman",face="bold",color="black",size=10,vjust =
0.5),
      axis.text.y=element_text(family="Times New Roman",face="bold",color="black",size=10),
      axis.title.x=element_blank(),
      axis.title.y=element_text(family="Times New Roman",face="bold",size=10))+labs(y="Phosphorous
(P) (ppm)")+
  geom_hline(yintercept = mean(metadata$P), linetype = 2)+
  stat_compare_means(label = "p.signif", method = "wilcox.test",ref.group =
"Factor_Control",hide.ns=TRUE, family="Times New Roman")
p5

p6<-ggboxplot(metadata, x = "Factor", y = "K", color = "Factor", legend = "none", palette=brewer.pal(n=
8, name = 'Dark2')) +
```

```
  theme(
      axis.text.x=element_text(family="Times New Roman",face="bold",color="black",size=10,vjust =
0.5),
      axis.text.y=element_text(family="Times New Roman",face="bold",color="black",size=10),
      axis.title.x=element_blank(),
      axis.title.y=element_text(family="Times New Roman",face="bold",size=10))+labs(y="Potassium
(K) (ppm)")+
  geom_hline(yintercept = mean(metadata$K), linetype = 2)+
  stat_compare_means(label = "p.signif", method = "wilcox.test",ref.group =
"Factor_Control",hide.ns=TRUE, family="Times New Roman")
p6
```

***RDA Analysis***

*Analysis repeated for both 16s and ITS rRNA data

```
## Load packages
library(vegan)
library(ggplot2)

--------------------------------------------------------------------------------------------------------------------------

## Data prep
# Read in abundance data and with metadata and soil property/nutrient data
df<-read.csv("RDA_metadata.csv",header=TRUE)
# Ensure there are no N/A values in data frame
df<-na.omit(df)

# Convert abundance data to relative abundance
spec.h<-decostand(df[c(2:5)],method="hellinger")

# Standardize soil property/nutrient data
t.env<-decostand(df[,c(10:17)], method="standardize")
env.stand<- t.env

--------------------------------------------------------------------------------------------------------------------------

# Conduct RDA
spec.rda <- rda(spec.h ~ ., env.stand)
summary(spec.rda)
perc <- round(100*(summary(spec.rda)$cont$importance[2, 1:2]), 2)

# Plot RDA
model <- ordiplot(spec.rda, type = "none", scaling=2, cex=10, main = "Eukaryote Phyla RDA", xlab =
paste0("RDA1 (", perc[1], "%)"), , ylab = paste0("RDA1 (", perc[2], "%)"), cex.lab=1.25)
points(spec.rda, col="darkgrey", cex=1)
points(spec.rda, dis="sp", col="blue")
text(spec.rda, dis="sp", col="blue")
text(spec.rda, dis="bp",col="black")

# Verify parameters are significant in model
spec.rda1 <- step(spec.rda, scope=formula(spec.rda), test="perm")
```

```
summary(spec.rda1)

vif.cca(spec.rda1)

# Run signficicance testing for parameters
anova(spec.rda, perm.max=1000) #model significance
anova(spec.rda, by="axis", perm.max=1000) #axes significance
anova(spec.rda, by="terms", perm.max=1000) # term significance
anova(spec.rda, by="margin", perm.max=1000) #term order significance
```