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# Investigating potential indicators of soil health through microbiome response to environmental and anthropogenic stressors

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# Investigating potential indicators of soil health through microbiome response to environmental and anthropogenic stressors

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Traditionally, the analysis of soil health has overlooked the biological component of soil due to poor understanding of connections between the microbiome and empirically measured soil health indicators. The purpose of this study was to assess the effects of environmental and anthropogenic stressors on the soil microbiome, with the aim of identifying measurable soil biological indicators. Chosen soils were examined under distinct conditions to evaluate the effect of selected environmental and anthropogenic stressors on the microbiome. Soil biological responses were analyzed via enzymatic response, microbial functional genes, and microbial community. Environmental factors such as soil moisture and organic matter showed significant influence on the microbiome with each selected biological indicator showing importance. Anthropogenic factors provided various responses dependent largely on the nature of the soil amendment. This study demonstrates that in addition to traditional soil health indicators, soil biological indicators should be included in the process of determining healthy soils.

# DEDICATION

This thesis is dedicated to those who have supported and encouraged me throughout my education experience, especially my dad, Gerald. Thank you for guiding me through the highs and the lows.

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# CHAPTER I

# AN INTRODUCTION TO SOIL HEALTH AND ENVIRONMENTAL AND ANTHROPOGENIC STRESS

Since the dawn of agriculture, soil and its management have been an ongoing subject of interest and research. Early agronomists noted the influence of plant interactions with soil and spread agricultural practices throughout early civilizations and cultures. The formation of healthy, productive soil became a necessity for civilization growth. Through centuries of crop production and more recent innovations in agriculture and ecology, scientists understand that soil formation is an intricate process subject to the interplay of multiple physical, chemical, and biological parameters; as such, soil is the most complex biomaterial on Earth.

Soil health was initially described using the term "soil quality" by Mausel in 1971. It was subsequently defined by Doran and Parkin (1994) as "the ability of soils to contribute to environmental quality, and to promote animal and human health, as well as crop productivity." At this time, disagreements between the usage of soil "quality" and "health" arose. Though the use of the terms continued to be debated, one point of agreement was that soil health was a reflection of the living component of soil. This concept was reiterated by Doran and Zeiss (2000), whose definition of soil health included a reference to a "vital living system." The recognition that soil microbiota comprise a substantial aspect of soil health has only become increasingly emphasized during recent years.

The soil microbial community, which consists of metabolically active and potentially metabolically active microbes, has a principal influence on soil health and plays an intricate part in many different aspects of maintaining a healthy soil. Microbes play a large role in organic matter break down and the fate of carbon (Schimel and Schaeffer, 2012). Similarly, microbes play parts in the nitrogen (Jetten, 2008; Li, 2018), phosphorous (Richardson and Simpson, 2011), and potassium cycles (Das, 2016). While modern agriculture systems operate using high nutrient inputs as Pimentel et al. (1995) described, scientists are just now beginning to understand the critical role of microorganisms in the soil-plant-water system. Likewise, studies have shown that adding beneficial microorganisms to the soil can maximize nutrient uptake (Kirankumar et al. 2008) and enhance resistance to abiotic stress (Selvakumar et al. 2012). These studies illustrate how optimizing the microbial population to achieve peak biogeochemical cycling is key to soil health and agriculture sustainability.

Traditionally, the health of a soil has been measured using a set of numerically measurable indicators. Some of these are relatively recalcitrant characteristics such as soil texture, pH, organic matter, and cation exchange capacity (CEC). While pH can vary across soils, organic matter is built up over longer periods of time and will sway little in the span of a growing season (Magdoff & Weil, 2004). This high carbon material acts as a source of nutrients while providing more surface area within the soil for greater water retention and cation exchange capacity. Soil carbon and nitrogen levels, as well as other plant macronutrients, are also included in many soil health analyses. Essentially, these assessments provide a snapshot of the nutrient availability of the soil system. This availability is variable throughout a growing season and the analysis of these nutrients is often used to determine fertilization needs. However, these indications do little to account for the microbial health of the system.

In recent years, the recognition of the soil microbiome has led to the exploration of the addition of biological analyses to supplement the traditional soil health indicators. Conventional analyses of soil microbes have included plating of culturable bacterial and fungal communities, enzyme analyses, and direct counts of bacteria and fungi. Recently, DNA (e.g., quantitative polymerase chain reaction) and genomic approaches are available for in-depth quantitative and qualitative analyses of the microbiome (Brooks et al., 2018, 2019). These approaches use targeted sequencing and metagenomics to indicate community composition and abundance, and to evaluate potential functionality.

No soil characteristic (e.g., organic matter content, pH, soil texture, or bacterial community) can be used as the sole indicator of soil health. The health of a soil system is influenced by all of these characteristics and therefore must be estimated from a complete evaluation and summary of all indicators. Holistic analyses offer the ability to navigate the relationships between abiotic and biotic soil indicators and their environmental stressors.

Ecological “stressors” are often described as disturbances or threats; however, they may also positively influence the growth of the soil microbial community (Meisner et al., 2017). Soil microorganisms are subject to different environmental stressors throughout the seasons depending on the type of soil they inhabit and the geographic and climactic conditions of that soil. These environmental stressors include (but are not limited to) changes in soil moisture, water activity, temperature, and pH (Manzoni et al., 2012; Rocca et al. 2019).

Soil type, as an inherent soil property, has direct and indirect influence on many potential environmental stressors. Research demonstrates that soil type plays a large role in determining microbial communities (Lamarche et al., 2007; Carletti et al., 2009; Wakelin et al., 2008). For instance, soils with clay particles will exhibit a high CEC, which increases negative charges

within the soil that allows for increased absorption of positively charged elemental nutrients. Clay particles will also, because of their small size and higher CEC, be more poorly drained and have an increased soil moisture capacity than soils with larger grain sizes, allowing clay soils to resist drought conditions. Sandy or silty soils are not able to hold onto elemental nutrients as tightly and are slightly more susceptible to the effects of drought; however, these soils drain water more effectively (Rawls et al., 2003).

Low soil moisture, brought on by periods of drought, is perhaps the most common environmental stress on soil microorganisms, which reduces respiration and activity (Guntinas et al. 2013, Yuste et al. 2007). Soil microorganisms can resist drought through production of exopolysaccharides, making up the structural skeleton of biofilm, which "can provide a microenvironment that holds water and dries more slowly than its surroundings" (Roberson and Firestone, 1992). Due to drought concerns, soil moisture is highly correlated with the functional potential of the microbiome (Brockett et al. 2011).

Soil organic matter contributes to soil water holding capacity (Libohova et al., 2018), nutrient availability and reduced leaching (Overstreet and DeJong-Hughes, 2009), infiltration, and aeration. Organic matter has generally high water availability and therefore holds water much longer than the inorganic, mineral soil faction. Soils that are low in organic matter will experience drought effects earlier and more severely than those that have high organic matter content, because organic matter acts as a "sponge" (Bhadha et al., 2017). Under prime circumstances, an influx of organic matter will also raise the soils CEC over time (Murphy, 2015), providing more nutrient availability along with greater water holding capacity, due to high surface area and pore space.

Soil pH has also been found to be a predictor of bacterial community composition and nutrient status (Lauber et al. 2008). Rousk et al. (2009) found that soil pH had a direct correlation with soil microbial community structure. Changes in pH would put acclimated communities under stress and force a community change. In natural field conditions, changing the pH of the environment may lower the metabolic activity of the microbial community by up to 50% (Fernandez-Calviño and Bååth, 2010). Soil pH also influences the CEC of a soil. This influence on CEC can then have effects that have been mentioned above such as water holding capacity and nutrient availability.

Soil microorganisms are also highly influenced by anthropogenic activities. Rodriguez-Eugenio et al (2018) extensively describes both anthropogenic and natural soil degradation processes along with the effects of anthropogenic pollution. Among these is the continual use of fertilizer which can induce a stressing effect on the soil microbiome. Any fertilizer additive promotes a chemical change within the soil, as the main goal of fertilization is to artificially and rapidly boost plant nutrient availability. The addition of these chemicals also often stimulates a pH change immediately surrounding the fertilized soil area, which leads to effects on the immediate surrounding soil microbes (Belay et al., 2002; Zheng et al., 2017). Long term soil fertilization, particularly with mineral nutrients, has been shown to alter microbial interactions, biogeochemical processes, and the bacterial community structure at multiple soil depths (Li et al., 2014; Dai et al., 2018).

Another form of fertilizer addition is via “organic” waste products such as manure or municipal sewage biosolids. Addition of microplastics by way of sewage sludge can also have a stressing effect on the soil microbiome. Recent estimates are that 63,000-430,000 and 44,000-300,000 tons of microplastics are added annually to agricultural farmlands in Europe and North

America, respectively (Nizzetto et al. 2016). Sewage is known to contain large amounts of microplastics including synthetic fibers sourced from washing machines (Ziajahromi et al., 2017; Henry et al., 2019). Personal care products also contribute to the microplastic load in sewage, with inputs from toothpaste, soaps, and facial scrubs (Napper et al., 2015). During wastewater treatment, the majority of microplastics partition into the solid phase, and subsequently become part of the biosolids fraction (Gatidou et al., 2019). The introduction of these microplastics can change the structure of the soil (by expanding pore space and providing rigid structure), alter the soil chemistry, and ultimately reshape the bacterial community (Kim and Rillig, 2022).

Soil microorganisms are critical to the resiliency and sustainability of agricultural production systems. Many production systems heavily rely on soil bacteria to store and cycle nutrients to produce fertile conditions (Schimel and Schaeffer, 2012; Li et al., 2019; Richardson and Simpson, 2011). Legumes, which include the principal crop soybeans, are especially dependent on nitrogen fixing bacteria, providing a great example of how critical the microbiome is for agricultural systems.

Though the literature provides examples of how soil health is connected to the soil microbiome, there are fewer studies demonstrating the use of molecular or ‘omics techniques to define soil biological health. Many attempts have been made to quantify soil health using multiple physical, chemical, and biological indicators. However, these approaches require quantification and scoring of each indicator, and arbitrarily assigning a “weight” to each indicator. Other attempts have been made to scale down the number of used indicators, such as the Soil Health Institute’s recommendation of three measurements of soil health that are based on nutrient cycling and soil structure (organic carbon concentration, carbon mineralization potential,

aggregate stability) (Soil Health Institute, 2023). My approach focuses on actual measurement of soil microbial community activity coupled with the breadth and depth of DNA based methods.

To address this gap in the research, I propose the use of molecular techniques to accomplish these objectives:

i.) **Objective 1:** To evaluate the influence of environmental stressors such as soil moisture, organic matter, and biological pest pressure on the soil microbiome.

ii.) **Objective 2:** To evaluate the influence of anthropogenic stressors such as synthetic soil amendments and microplastic pollution on the soil microbiome.

These objectives were accomplished through the use of controlled benchtop experiments complimented by real world field experiments. An analysis of selected response variables were conducted to determine their potential fitness as indicators of soil biological health. To establish a list of criteria for indicating soil biological health, modifications can be made to the criteria applied to fecal indicator bacteria described by Cabelli (1977) and the criteria for an ideal indicator organism described by Gerba (2009). Thus, a suitable biological health indicator should have the following criteria: 1) the indicator should consistently respond to the soil stressor; 2) it must be detectable in sufficient numbers; 3) the methodology should be feasible for widespread use; and 4) it should be broadly applicable to a variety of soil types.

Soil microorganisms are critical for the resiliency and sustainability of agricultural production systems, and their evaluation with regard to community structure, function, and metabolic activity will lend new meaning to the term “precision agriculture”.

# CHAPTER II

# EVALUATING THE INFLUENCE OF ENVIRONMENTAL STRESSORS SUCH AS SOIL MOISTURE, ORGANIC MATTER, AND BIOLOGICAL PEST PRESSURE ON THE MICROBIOME

## Introduction

The soil microbiome is an important aspect of soil-plant interactions. Soils found in each ecoregion are under the influence of the climactic and weather conditions of that region. These may include but are not limited to temperature variations, inputs from native flora and fauna, susceptibility to drought, precipitation patterns, etc.

Soil moisture is a primary environmental stressor that is highly correlated to the functional potential of soil microorganisms (Brockett et al., 2012). Microorganisms endure fluctuating moisture levels year-round and may experience multiple desiccation and rewetting events during a growing season. To prevail in these conditions, microbes must adapt to the water potential of their surroundings, for example, microbes accumulate solutes to retain water within their cells (Schimel, 2018).

In 1983, Orchard and Cook discovered that soil respiration rate (soil respiration was used as an indicator of microbial activity) is proportional to soil water content; thus, bacterial respiration decreases in low moisture soil environments. Brockett et al. (2012) found that soil moisture was positively correlated with total microbial biomass, while Conant et al. (2004) found that on average soil respiration was 2.5 times greater under wetter conditions. While an optimal

moisture availability is the ideal goal for any soil, literature suggests that a lack of soil water content decreases bacterial activity (Schimel, 2018; Brockett et al., 2012).

Organic matter acts like a sponge within the soil, increasing the water holding capacity of soil. Libohova et al. (2018) found that soil organic matter can hold up to 100% of its weight in available water (water that is immediately available for plant or microbial uptake). The high carbon material holds onto water and acts as a desiccation buffer in dry soils. A lack of organic matter will result in a lower water holding capacity and therefore often result in soil moisture stress (Libohova et al., 2018); however, microbial life has found ways to overcome such stressors. Exopolysaccharides are residues that are secreted by bacteria into their immediate environment to provide a barrier against desiccation. These natural polymers are known to hold multiple times their weight in water and "can provide a microenvironment that holds water and dries more slowly than its surroundings" (Roberson and Firestone, 1992). The production of these residues allows for bacterial resistance to desiccation and may be one way that bacterial communities in low organic matter soils can overcome soil moisture stressors.

While not typically measured in environmental samples, water activity is a direct measure of the water available to the microbiome as well as to plants. According to the U.S. Food and Drug Administration (FDA 1984), water activity is the ratio between the vapor pressure within the substance (usually food) and the vapor pressure of distilled water under completely identical conditions. In short, water activity is the measure of water that is available for use by life forms. By this definition, there is a measurable situation where the amount of water cannot support life. Therefore, below a certain water activity ($A_w$) threshold, no microbial life can exist (Stevenson et al. 2015). Through experimentation, water activity has been shown to be the ultimate determinant for biotic activity amongst extremophiles (bacteria that inhabit

extreme environments) and a common value of about 0.61 $A_w$ is the limit of activity for all three domains of life (Stevenson et al. 2015). However, literature suggests that most bacteria cannot multiply below 0.90 $A_w$ (Moyano et al., 2013; Manzoni et al., 2012). This presents a situation in which most soil bacteria would not actively multiply in drier locations or under drought stress. These situations are likely to produce unique community structures with moisture stress tolerant microbes.

While there are 12 general soil types, or orders, in the United States, there are tens of thousands of local soil series that each exhibit their own microbial community. A field may comprise multiple soil series, leading to variable community structures within a field setting. Each of these soil series are made up of a mixture of clay, sand, and silt particles making each series chemically unique. Soils with a high percentage of sand particles will have a low cation exchange capacity (CEC) and low water holding capacity. Clay soils experience the highest CEC (>30 meq/100 g soil) while sandy soils have low CEC values (~5 meq/100 g soil) (Saha, 2022). Loamy soils (generally a decent mixture of all three components) will fall between clay and sand on the CEC scale, depending on the soil texture. Organic material will also raise soil CEC and can influence a soil's microbial community structure and functioning (Bending, Turner, and Jones, 2002).

Leaching, or the draining away of nutrients due to the movement and percolation of water input, can be experienced by all soil types. This effect will move available soil nutrients further down into the soil profile and away from topsoil horizons (eventually reaching the groundwater system). Courser soils, such as sandy soils, will likely experience both greater quantities of leaching effects and at a faster pace than finer grained soils, such as clay (Vinten et al., 1994; Shepherd and Bennet, 1998). This is due to more pore space leading to lower CEC and faster

water movement. Inversely, finer soils such as clay will experience leaching at a slower pace, however it will still occur. Higher levels of organic material will also raise the CEC and water holding capacity of a soil and can therefore lessen the effects of leaching (Bigelow, Bowman, and Cassel, 2001).

It is well documented that soil pH is a strong predictor of bacterial community composition (Wan et al. 2019; Rousk et al. 2010; Bartram et al. 2014). While pH can be affected by many influences, most natural determinants are unwavering, such as a soil's parent material. The parent material, or the geologic material that gives rise to a soil, can affect the pH and the amount of nutrients that are naturally in the soil (Vestin et al. 2006). Much of the pH dependent nutrient availability is determined by the pH influence on soil CEC. By increasing the pH of a soil, soil CEC will also increase. This brings greater nutrient availability and therefore a possible change in microbial community structure. At neutral pH, conditions are suitable for most bacteria. In cases of acidic or alkaline soils, these low and high pH values will present a stressful environment for bacteria to grow. While soil pH usually stays consistent, soils with lower buffer holding capacity (sandy soils) may experience greater pH swings. These relatively quick pH changes can stress the microbiome.

Assessment of the soil microbiome's response to environmental stressors will provide critical information about the resiliency of the microbiome found in agricultural soil. Ultimately, a measurement of the microbiome's response could lead to identifying potential soil microbiological indicators sensitive to environmental change. Using the metrics directly related to microbiome function (pH, $A_w$, gene quantification, enzyme activity), the effects of soil moisture, organic matter, and biological pest pressure (i.e. nematode presence) on the microbiome was evaluated. This study was conducted through a series of three controlled

experiments at the microcosm and field level (Table 2.1) to test the following research hypothesis: (1) low moisture will have a negative influence on the soil microbiome, while high moisture will positively influence the soil microbiome, and (2) soil organic matter and biological pest pressure will result in different community structures and metabolic activities within the microbial community.

## Materials and Methods

To accomplish the objective of this study, soil samples were collected from a combination of ongoing small-plot field experiments and controlled microcosm-based experiments whereby environmental stressors include soil moisture, organic matter, and biological pests.

### Soil Moisture and Organic Matter– Microcosm-scale

Controlled microcosm-based experiments were conducted in Tucson, AZ using six jars each filled with 300 grams of field-moist soils. The experiment comprised a 2x3 design with two soil types replicated three times, subjected to a wetting and drying cycle. The soil types comprised a low organic matter soil (Brazito sandy loam; 1.10% organic matter) and a high organic matter soil (Gila fine sandy loam; 8.84 % organic matter). The six microcosms began at field-moist conditions (timepoint, $T_1$) were then allowed to dry out in a 35°C incubator to create moisture stress. Once the soil reached <1%-3% moisture content ($T_2$), the microcosms remained incubated for an additional seven days of moisture stress ($T_3$). Following $T_2$ and $T_3$, the microcosms were re-wetted to their respective original moisture ($T_4$). Samples were collected at $T_1$ through $T_4$ from each microcosm, packed and refrigerated. Fifty-gram samples were sent via

overnight shipping in a ~4℃ container from Tucson, AZ to Starkville, MS. Laboratory analyses and assays were carried out upon receiving samples.

**Soil Moisture – Small plot-scale**

Soil moisture stress was also evaluated under field conditions by using previously established field small-plot experiments in Mississippi. These experiments were conducted to provide "real world" systems whereby moisture varies based on natural rainfall or irrigation. Three experimental field sites were chosen on Mississippi State University research stations. The first field (site 1) was located at the Pontotoc Ridge – Flatwoods Branch Experiment Station located in Pontotoc, MS (Atwood silt loam) and is part of a five-year study; samples were collected in the third-year growing season. The study site was cropped in corn (*Zea mays*) and consisted of a randomized complete block design (3x5) where fertilizer type (control, urea ammonia-nitrate, broiler litter) was the main treatment and was replicated five times. Fertilizers were applied at agronomic rates recommended for corn and the site uses no till soil conservation. To investigate the impact of weather conditions on microbiome function in the experimental plots, the plots were sampled twice during the growing season under contrasting moisture conditions. At the perceived driest part of the season (field dry conditions at ~20 days without substantial rainfall) and one to two days following a substantial rain event (field-moist condition) soil samples were collected from all field plots. Due to the climate in Mississippi, these were the closest moisture designations approximating a dry and high moisture condition, respectively. To compare microbiome function under different moisture stress, samples from all plots under dry conditions were compared to samples from all plots under wet conditions.

The second field (site 2) was located at the R. R. Foil Plant Science Research Center located in Starkville, MS (Leeper silty clay loam) and is part of a five-year study; samples were

collected in the third-year growing season. The study site was cropped in corn (*Zea mays*) and consisted of a randomized complete block design (3x8) where fertilizer type (control, urea ammonia-nitrate, and broiler litter) was the main treatment and was replicated eight times. Fertilizers were applied at agronomic rates recommended for corn and the site used minimal tillage. To investigate the impact of weather conditions on microbiome function in the experimental plots, the plots were sampled twice during the growing season under contrasting moisture conditions. At the perceived driest part of the season (field dry conditions at ~20 days without substantial rainfall) and one to two days following a substantial rain event (field-moist condition) soil samples were collected from all field plots. Due to the climate in Mississippi, these were the closest moisture designations approximating a dry and high moisture condition, respectively. To compare microbiome function under different moisture stress, samples from all plots under dry conditions were compared to samples from all plots under wet conditions.

The third field (site 3) was located at the R. R. Foil Plant Science Research Center located in Starkville, MS (Leeper silty clay loam, Marietta fine sandy loam) and was a controlled field irrigation study conducted for one year. The study site was cropped in cotton (*Gossypium hirsutum*) and consisted of a randomized complete block design (3x3) consisting of irrigation volume (high, low, control) as the main effect and replicated three times; however, samples were collected only during the irrigation event whereby the high and low treatments were considered field-moist, and the control treatment was considered dry. This site used minimal tillage.

Samples from all fields comprise two randomized cores (a cylindrical sub-surface soil sample) (0-15 cm) taken within the furrow. Furrows were sampled to negate the effect of the plant influence. Samples were stored on ice in the field and in transport. Laboratory analyses and assays were carried out upon samples return to the lab in Starkville, MS.

**Biological Stressor – Small plot-scale**

Analysis of biological pest (i.e., nematode infestation) stress on the soil microbiome was conducted by collecting soil from a known nematode, *Meloidogyne incognita*, infested field in Clarkton, MO at a University of Missouri experimental station (Bosket fine sandy loam). Samples were collected from a field planted with both cotton (*Gossypium hirsutum*) resistant to nematodes and a susceptible variety. All experimental units (four row plots) were exposed to nematodes, with only the susceptibility of the plants (resistant and susceptible) to nematode infestation being a treatment factor and were replicated six times. Single soil cores were collected from near the root zone of selected cotton plants (0-15cm). Six cores were collected from the susceptible and six cores collected from the resistant plots during a one-time sampling event. Samples were transported back to the Starkville, MS lab stored on ice where laboratory analyses and assays were carried out upon receiving samples.

## Laboratory Assays and Analyses

Samples from each experiment were subject to the same soil physical, chemical, and biological analyses described below.

i) pH Measurement

Soil pH was measured using a 1:1 ratio with dH2O. The slurry was then measured using an Oakton pH/CON 510 series pH meter.

ii) Soil Moisture Content

Gravimetric moisture content was assessed by weighing 10g (moist weight) of soil, drying at 104°C for 24h and reweighing the dried sample. Moisture content was measured using the following equation:

$$\text{moisture content} = [\text{soil wet weight} - (\text{dry weight of soil and weigh boat} - \text{weight of weigh boat})] / \text{soil wet weight} * 100 \quad (2.1)$$

iii) Soil Water Activity

Soil water activity was assessed via the AquaLab Water Potential Meter using the chilled mirror technique. Water potential was measured in MPa and converted to water activity using

$$A_w = P/P0 \quad (2.2)$$

where $A_w$ is water activity of a solid or liquid material, P is the vapor pressure of a solid or liquid material, and P0 is the vapor pressure of pure water.

iv) Enzyme Analysis

For soil enzyme analysis, n-acetylglucosaminidase, phosphomonoesterase, and B-glucosidase was measured using microplate fluorometric methods as described by Deng et al., (2011). Briefly, a 1 g aliquot of soil was added to 120 ml of sterile dH2O, homogenized via stomacher, and transferred to horizontal shaker for 30 min set at 65 rpm. A 100 µl aliquot (quadruplicate) was then subjected to the microplate assay whereby pH for each individual soil enzyme was adjusted to either 5.5 or 6.0 via buffered solution, and methylumbelliferyl (MUF) based substrates are added to the microplates to assess enzyme activity. MUF substrates comprise MUF- -D glucoside, -n-acetyl- -D glucosaminide, and -phosphate. Appropriate sample matrix, MUF standard curve, and experimental controls were added to each plate and group of plates. Plates are incubated for 1 h at 37°C. Plate fluorescence was read on a Biotek plate reader with excitation at 365 nm and emission at 450 nm. Soil enzymatic activity is expressed as millimoles MUF $kg^{-1}$ $h^{-1}$.

v) Nucleic Acid Extraction

DNA was extracted from each soil sample using standard DNA extraction kits. Briefly, total microbial DNA was extracted (0.25 g soil) using MP Biomedical FastDNA Spin Kits (MP Biomedical; Santa Ana, CA) and a FastPrep-24 homogenizer (MP Biomedical) following the recommended manufacturer's protocol. Extracted DNA was checked for quantity and quality prior to subjecting it to polymerase chain reaction (PCR). Extracted DNA was frozen at -80°C until processed for high throughput sequencing and PCR-based assays.

vi) Quantitative PCR (qPCR)

DNA was subjected to quantitative polymerase chain reaction to assess levels of 16S rRNA (16S), 18S rRNA, urease (*ure*C), rubulose-1,5-biphosphate carboxylase (*cbb*LR) and phosphatase (*pho*A) enzyme genes present in the soil samples. 16S rRNA (Nadkarni et al., 2002), *ure*C (Koper et al., 2004), and *pho*A (Han et al., 2012) primers and assay conditions were selected or modified from the literature. Quantitative PCR assays were conducted as stated in Brooks et al. (2019) using sybrgreen chemistry and duplicated. A standard curve comprised of serial 10-fold dilutions of IDT g-block designed DNA controls or known positive controls was subjected to melt curve analysis confirmation. All values were reported as GU dry $g^{-1}$ (genomic units per dry g of soil).

vii) 16S rRNA Targeted Sequencing

Soil microbial genomic DNA was submitted for 16S rRNA library preparation and sequencing through Microbiome Insights (Vancouver, BC, Canada) using the Illumina MiSeq DNA sequencing platform. 16S rRNA targeted sequencing was followed as stated in Brooks et al. (2019). Soil microbial DNA was collected for all soil samples regardless of endpoint assay and was archived. Selected time points were then subjected to MiSeq sequencing and analyses.

Briefly, the MiSeq SOP proposed by Kozich et al. (2013) was followed using 2x250 base pair V2 chemistry. Library preparation and quality-assurance and control measures was conducted prior to sequencing. The V4 region of the 16S rRNA gene was amplified and sequenced.

The Mothur platform (v. 1.47.0) was used for bioinformatic 16S rRNA sequence analyses and carried out according to Kozich et al (2013). Briefly, libraries were contiged and curated to reduce errors and low-quality sequences. Sequences were then aligned relative to Silva alignment reference files (release 128), and taxonomically classified using the most current Ribosomal Database Project reference files (RDP version 16). Sequences which did not align or classify as Eubacteria were removed from the libraries, and chimera sequences were screened within Mothur. Operational Taxonomic Unit (OTU) and phylotype analyses were conducted using Mothur (Kozich et al., 2013). Operational Taxonomic Units was assigned at a 3% dissimilarity and taxonomy-based analyses were conducted at the genus taxon. OTU-based analysis consisted of alpha- and beta-diversity analyses comprised of rarefaction, inverse Simpson, AMOVA, and HOMOVA commands run in Mothur. Alpha-diversity analysis function rarefaction generate sample richness curves while inverse Simpson is the inverse of the Simpson diversity estimator, estimating richness in a community with uniform evenness. Beta-diversity analysis function AMOVA (analysis of molecular variance) was used to test the genetic diversity within two populations while HOMOVA (homogeneity of molecular variance) was used to test homogeneity within two or more populations.

viii) Statistical Analysis

Data were analyzed by first $\log_{10}$ transforming raw data collected from each assay (except 16S rRNA sequencing, pH, $A_w$) to achieve normal distribution and homogeneity of variance. Each experiment (soil moisture and organic matter, soil moisture, biological pest) was

treated as an independent experiment and analyzed separately. Dependent variables for each experiment were: pH, $A_{w,}$ soil moisture content, enzyme analysis, quantitative PCR (qPCR), and 16S rRNA targeted sequencing. Data were analyzed in SAS using a two-way ANOVA for the soil moisture and organic matter microcosm experiment whereby soil moisture (field-moist, dry, dry +, rewet) and organic matter (high, low) are the independent variables, and the interactions between the two were estimated (soil moisture x organic matter). A one-way ANOVA whereby soil moisture (field-moist, dry) was considered the independent class variable was run for the small plot soil moisture experiments. All fields had fertilizer applications; however, the effect of fertilizer was ignored to focus on the soil moisture. A one-way ANOVA whereby nematode susceptibility (nematode resistant cotton and susceptible cotton) was considered the independent class variable. Data from 16S rRNA high throughput sequencing were analyzed using Mothur described above. A one-way ANOVA was conducted on inverse Simpson values according to the treatments of the specific experiment from which the data originated. Figures were created in program R (4.1.3) using Rstudio (2022.02.2+485) and the following packages ggplot2, ggfortify, ggpubr, tidyverse, patchwork.

## Results and Discussion

To investigate the effects of environmental stressors on the soil microbiome, a series of experiments were conducted at the microcosm and small plot scale.

### Soil Moisture and Organic Matter – Microcosm-scale

The controlled microcosm experiment was conducted in conjunction with the University of Arizona (Tucson, AZ). The experiment used high organic matter and low organic matter field

soils collected at field moist condition and established a timepoint experiment consisting of four timepoints.

Overall, the effect of organic matter and soil moisture was evident on numerous physical and biological metrics. For example, the overall effect of soil organic matter was shown to be significant ($F(1,4) = 19.88$, $p = 0.0112$) on water activity levels. When the microcosms were introduced to drought stress ($T_2$), the sandy soil was not capable of holding a biologically relevant $A_w$ (<0.85) (Stevenson et al., 2015) (Figure 2.1) due largely to the low surface area and low cation exchange capacity of the soil (Gaines & Gaines, 1994; Huang & Hartemink, 2020). These attributes will not allow sand particles to hold tightly to water molecules, allowing more complete drying than clay or loamy soils or those with a higher organic matter content (Table 2.2). However, the highly porous sandy soil did exhibit a fast resurgence of water availability when rewetting occurred, resulting in no significant differences between the different organic matter soils at $T_4$ ($t(12) = -1.62$, $p = 0.7347$). This demonstrates that in an Arizona sandy loam soil, an abiotic pressure, such as extreme drought, would manifest into a measurable metric of soil health, such as water activity, but may be dependent on the organic matter content of that soil.

Soil pH levels were shown to be significantly higher amongst the high organic matter soil samples ($\bar{x} = 7.69$) than levels in the low organic matter soil samples ($\bar{x} = 7.46$) ($F(1,4) = 8.35$, $p = 0.0446$). However, soil pH only seemed to be affected by moisture amongst the low organic matter soils as drying stress significantly increased the soil pH at $T_2$ ($t(12) = -4.79$, $p = 0.0073$). This is understandable as low organic matter soils do not have the cation holding capacity of other soils, likely causing an increase in pH during ammonification when the soils were dried (Haynes & Swift, 1989). Ultimately, high organic matter buffers the soil and prevents any

deviation from optimal soil pH. While the shift in pH was statistically significant, due to most microbial life thriving in pH levels between 6 and 8, the shift recorded among low organic matter soils likely would not negatively affect the soil microbiome but could cause minor shifts in microbial abundance and diversity (Pepper, Gerba, & Gentry, 2015).

Organic matter content was shown to be a significant factor in the microbiome's response to moisture levels as measured by soil gene abundance levels. The abundance of the 16S rRNA gene was significantly higher amongst the high organic matter soil ($F(1,4) = 360.82$, $p < 0.0001$), regardless of moisture condition (Figure 2.2). Likewise, 18S rRNA gene abundance showed statistically higher levels within the high organic matter soil ($F(1,4) = 192.8$, $p = 0.0002$) (Figure 2.3). These abundance differences are likely because high organic matter soils provide more access to nutrients for microbial life, support higher moisture and water availability levels, and keep pH levels more stable than low organic matter soils (Khosro et al., 2011). Organic matter also provides more surface area to soil particles, adding to the inhabitable volume of space microbes can occupy and therefore a greater potential for microbial activity (Tuson & Weibel, 2013). Drought stress was also shown to significantly affect the level of 16S rRNA gene abundance regardless of organic matter content ($t(12) = 5.82$, $p = 0.0015$ for low organic matter soil; $t(12) = 5.09$, $p = 0.0045$ for high organic matter soil) (Figure 2.2). This is likely tied to the biological availability of water to the soil bacteria as mentioned earlier.

Soil *ure*C gene abundance showed a significantly higher level within the high organic matter soils than the low organic matter soils ($F(1,4) = 75.47$, $p = 0.0010$) (Table 2). This is logically expected given the results above. Similarly, *ure*C gene abundance was shown to be affected by drought stress amongst the low organic matter soil ($t(12) = 4.25$, $p = 0.0177$) as saturated soils provide for a less stressful environment than dry soils, thus promoting increased

gene abundance. The abundance of the *cbbL*R gene levels were also found to be significantly greater amongst the high organic matter soils ($F(1,4) = 18.06$, $p = 0.0132$). This was the only significant difference found with no differences associated with the effects of soil moisture. The *pho*A gene abundance levels were also significantly elevated amongst the high organic matter soil ($F(1,4) = 37.29$, $p = 0.0036$). The importance of organic matter to *pho*A is most evident in the rewetting time point ($T_4$) where the higher organic matter soil exhibited a higher abundance of *pho*A genes ($t(12) = 4.24$, $p = 0.0134$) (Figure 2.4). This result again points to the importance of organic matter in the soil as it is able to hold conditions at a more optimal state for bacterial gene abundance through a period of drought, which allowed for a more significant rebound after periods of drought stress.

Enzyme levels amongst this experiment were shown to be influenced by both soil organic matter and soil moisture level. β-glucosidase was shown to be affected significantly by organic matter as higher levels were found in the high organic matter soil ($F(1,4) = 66.80$, $p = 0.0012$). Interestingly though, as the soils came under drought stress, the high organic matter soils did not hold significantly higher β-glucosidase levels over the low organic matter soils ($t(12) = 1.05$, $p = 0.9565$). In fact, while the low organic matter soils show no significant change from soil moisture fluctuations, the high organic matter soils show a large drop in β-glucosidase levels after drying (drop from $\bar{x} = 179$ pmol $h^{-1}$ to $\bar{x} = 62$ pmol $h^{-1}$) ($t(12) = 4.19$, $p = 0.0194$). This is supported in the literature as Sardans and Peñuelas (2005) have shown a decrease in β-glucosidase and other enzymes during drought conditions. The levels of n-acetylglucosaminidase showed similar direction with high organic matter being significantly higher than low organic matter soils ($F(1,4) = 282.28$, $p < 0.0001$). Unlike β-glucosidase, the high organic matter soils were found to have significantly higher n-acetylglucosaminidase levels throughout the moisture

stress time points, with the exception of $T_2$ (high organic matter $\bar{x} = 63$ pmol $h^{-1}$; low organic matter $\bar{x} = 41$ pmol $h^{-1}$). Interestingly, the soil moisture stress did affect the high organic matter soils as a large drop in n-acetylglucosaminidase levels happened after drying (drop from $\bar{x} = 120$ pmol $h^{-1}$ to $\bar{x} = 63$ pmol $h^{-1}$) ($t(12) = 5.18$, $p = 0.0039$) ($T_2$). Phospohomonoesterase levels did not show a significant difference between high and low organic matter ($F(1,4) = 4.50$, $p = 0.1012$), however, soil moisture stress did show an effect. The low organic matter soil was shown to lose much of its phospohomonoesterase potential after being dried ($t(12) = -6.72$, $p = 0.0004$) ($T_2$). This was also evident at $T_3$ as the low organic matter soil showed low levels of phosphomonoesterase in comparison to the levels found in the high organic matter soil at the same moisture condition (high organic matter $\bar{x} = 118$ pmol $h^{-1}$; low organic matter $\bar{x} = 73$ pmol $h^{-1}$) ($t(12) = 6.30$, $p = 0.0007$). Findings by Hueso, Hernandez, and Garcia (2011) also found similar trends amongst enzyme levels in a drought scenario. A caveat associated with the measure of soil enzyme activity is the *ex-situ* nature of the assay. A potential source of error associated with soil enzyme analysis is the addition of sterile dH20 to the aliquoted soil, which may limit any negative effect of drought or moisture. Thus, the microbial population can respond quickly, and free or membrane bound enzymes can respond quickly (Meisner, Baath & Rousk, 2013). While this does have the potential to skew the data from the “dry” timepoints, there are no other methods of measuring enzyme activity.

Alpha and beta diversity were also estimated by use of 16S rRNA targeted sequencing. A two-way ANOVA of the inverse Simpson estimation found no significance for the effects of organic matter on the 16S rRNA diversity ($F(1,15) = 0.01$, $p = 0.908$) and likewise no significance under any of the moisture levels. Beta diversity was separately analyzed for the effects of soil moisture and organic matter and showed that AMOVA ($F(1,21) = 104.72$, $p <$

0.001) and HOMOVA (B = 3.43, $p < 0.001$) community metrics were significantly different between organic matter groups. However, soil moisture did not affect the bacterial community AMOVA ($F(1,21) = 1.11$, $p = 0.271$) or HOMOVA (B = 0.33, $p = 0.071$) which were statistically similar under dry and field-moist conditions. This indicates that organic matter had a greater influence on bacterial community diversity because the organic matter, hence available nutrients will select for specific bacterial populations, while it is possible that a more long-term drought stress would be needed to facilitate change in the bacterial diversity.

**Soil Moisture – Small plot-scale**

Three previously established (3+ years) field small-plot experiments were used in Mississippi to determine the effect of moisture levels experienced through a growing season on the soil biology *in-situ*.

Water activity levels at all three sites were significantly greater under field-moist conditions when compared to dry ($p < 0.05$). However, the dry field conditions remained very high ($A_w > 0.97$) above the $A_w$ 0.85 biological threshold because clay dominated soils like those found in Mississippi will naturally hold water much longer in times of drought (Rawls, Gimenez, & Grossman, 1998) due to high surface area, thus high CEC. While water activity was statistically significant in differences between dry and field-moist, it may not be biologically relevant due to the $A_w$ remaining above the critical threshold.

Soil pH at site 1 showed statistically significant higher levels under saturated conditions ($F(1,28) = 5.48$, $p = 0.0266$). This pH change was not expected since addition of water creates redox reactions within the soil, dropping pH. However, this change may be biologically important as a pH increase (5.4 in the dry soil to 5.8 in the saturated soil) would likely promote a shift in the microbial population. Saturated soils at site 2 showed an expected decrease in pH

level ($F(1,46) = 14.56$, $p = 0.0004$) (7.1 in the dry soil to 6.9 in the saturated soil). This reduction may also be biologically important as a pH decrease could have a similar effect on the soil microbial population. Site 3 showed no significant difference in pH between saturated and dry soils ($F(1,10) = 2.22$, $p = 0.1669$). Soil pH has been suggested by a number of studies to be a quality indicator of overall soil health (He et al., 2021; Raghavendra et al., 2020); however, despite these moisture stressors changing the soil pH, the effect may not be biologically relevant.

Soils from site 1 did not show a significant change in 16S rRNA gene abundance between the dry and saturated conditions ($F(1,28) = 0.34$, $p = 0.5662$) (Figure 2.5). This likely is due to the sites high-water activity and the undisturbed nature of the field (no-till management). Site 2 showed a significantly higher 16S rRNA gene abundance in the saturated soil ($F(1,46) = 19.38$, $p < 0.0001$); however, this was not replicated by the site 3 field ($F(1,10) = 1.33$, $p = 0.2751$) (Figure 2.5). Each field's 18S rRNA gene abundance trends were very similar to their 16S rRNA gene abundance (Table 2.3). The soil from site 1 did not show a significant difference in 18S gene abundance ($F(1,28) = 0.66$, $p = 0.4235$); however, the saturated samples from site 2 produced a higher abundance than the dry samples ($F(1,46) = 25.15$, $p < 0.0001$). The site 3 samples did not show a significant difference amongst 18S rRNA gene abundance ($F(1,10) = 0.33$, $p = 0.5788$). The rRNA gene results do not corroborate with results observed in the microcosm experiment, likely due in large part to different soil types and the uncontrolled environmental conditions found within the field experiments.

Within site 1, the *ure*C gene abundance acted as expected, with saturated soils showing about a $\log_{10}$ increase in abundance over dry soils ($F(1,28) = 24.87$, $p < 0.0001$) (Table 2.3). At site 2, dry soil samples had a higher abundance than saturated samples ($F(1,46) = 38.56$, $p < 0.0001$), which was the opposite of expected and opposed results from the other field sites. The

site 3 samples did not show a significant difference between the two conditions ($F(1,10) = 3.13$, $p = 0.1075$). These opposing results may suggest other soil conditions which may affect *ure*C levels, such as presence of plant material and debris or use of cover crops (Adeli et al., 2020), which may select for specific *ure*C enriching microbial population.

Contrary to expectation, the site 1 soil indicated a significant decrease of *cbbL*R gene levels when the soil was saturated ($F(1,28) = 131.09$, $p = 0.0001$) (Figure 2.6). As this gene is involved in $CO_2$ fixation, saturated conditions that inhibit fixation might be selecting against the *cbbL*R gene, lowering abundance. The difference shown was large enough to suggest that moisture stress may have an opposing biologically significant influence in the site 1 soil (Atwood silt loam). The *cbbL*R gene levels did not show significant differences in the site 2 field ($F(1,46) = 0.02$, $p = 0.8995$) indicating that moisture had very little effect on the gene's abundance in that system. The site 3 field did not show a statistical difference between moisture levels ($F(1,10) = 3.95$, $p = 0.0748$), but the saturated soils did show high variation amongst *cbbL*R gene levels suggesting some influence on the soil microbiome.

The *pho*A gene abundance did not show a significant statistical difference between moisture treatment at any of the three sites ($F(1,28) = 0.28$, $p = 0.5978$, $F(1,46) = 2.46$, $p = 0.1233$; and $F(1,10) = 1.90$, $p = 0.1984$ respectively). This is a glaring difference to the results given from the microcosm experiment, highlighting the effect of a real world field experiment over that of a controlled benchtop study.

Overall enzyme levels were not consistently affected by soil moisture levels. For example, β-glucosidase (dry $\bar{x} = 128$ pmol $h^{-1}$; saturated $\bar{x} = 205$ pmol $h^{-1}$) and phosphomonoesterase (dry $\bar{x} = 930$ pmol $h^{-1}$; saturated $\bar{x} = 1122$ pmol $h^{-1}$) enzyme levels from site 1 were significantly affected by moisture levels, while only site 2 showed significant

differences in n-acetylglucosominidase levels (dry $\bar{x} = 106$ pmol $h^{-1}$; saturated $\bar{x} = 217$ pmol $h^{-1}$) ($p < 0.05$). One reason for the inconsistent effect of moisture on enzyme levels may have to do with the agronomic practices at each site. For example, site 1 uses a no tillage practice, greatly reducing the ability for water to penetrate the soil. In that soil environment, field saturation could make a difference for the soil biological life. Conversely, site 2 and 3 both use a tillage practice, allowing for moisture to be taken up more readily by the soil, therefore reducing the difference between field dry and field saturated and lowering the stress put upon the soil microbiome. Like the microcosm experiment, the results of the enzyme analysis expressed here could report a potential error in that 120 ml of sterile dH20 is added to the aliquoted soil. As discussed above, the added moisture limits any negative effect of drought or moisture potentially causing a misrepresentation in the data.

Targeted 16S rRNA sequence results of *in-situ* soil moisture treatments from site 1 showed no significant differences from an inverse Simpson ANOVA ($F(1,16) = 0.06$, $p = 0.804$) and no significant differences in both AMOVA ($F(1,16) = 1.05$, $p = 0.403$) and HOMOVA ($B = 0.26$, $p = 0.158$). Likewise, the field samples from site 2 found no significant differences under the saturated versus dry field conditions when analyzed by an ANOVA of the inverse Simpson index ($F(1,22) = 0.23$, $p = 0.640$) and no significance in both AMOVA ($F(1,22) = 1.91$, $p = 0.093$) and HOMOVA ($B = 0.001$, $p = 0.975$). The site 3 field also produced no significance from the inverse Simpson ANOVA ($F(1,10) = 0.09$, $p = 0.767$) and no significance in both AMOVA ($F(1,10) = 0.69$, $p = 0.796$) and HOMOVA ($B = 8.55 \times 10^{-5}$, $p = 0.987$). These results are likely due to the short periods of time between rainfall events where the measured microbial populations may go dormant in the dry soil but would remain detectable.

**Biological Stressor – Small plot-scale**

Nematode susceptibility among cotton plants in Missouri did not significantly affect selected microbial response metrics in bulk soil. Nematodes (*Meloidogyne incognita*, cause of root knot, in this instance) are parasitic to the cotton plant in their spatial vicinity and form cysts on the roots of the plant (Hewezi and Baum, 2013), which ultimately reduces plant viability and growth. The lack of treatment effect in this experiment indicates that the microbial community associated with each plot was not impacted by the pest infestation or plant molecular response or that the effect was not measurable. However, it is well known that the stress on the plant imparts a change in the soil microbiome (Abdul Rahman et al., 2021; Santos-Medellin et al., 2021). This effect may be more associated, hence measurable, in rhizobiome populations. Since the stress is more closely associated with the plant susceptibility phenotype, any sort of mensurate stress placed on the bulk soil microbiome by the nematodes would not likely have been measurable among every sample, eliminating or obscuring any significance that plant susceptibility may have on the microbiome. While some change in available nutrients would occur due to the presence of nematodes (Gebremikael et al., 2016), the change was not large enough to significantly affect the microbial community and be recognizable through the used methods (Table 2.4).

Similarly, 16S targeted sequencing results indicated that the presence of a nematode pest produced no significance from an inverse Simpson ANOVA ($F(1,10) = 2.11$, $p = 0.177$), AMOVA ($F(1,10) = 0.73$, $p = 0.721$), or HOMOVA ($B = 0.66$, $p = 0.600$).

**Indicator Selection**

While the effect of the selected environmental and biological stressors on the soil microbiome were often inconsistent under these experimental conditions, some metrics may have

potential as suggested biological indicators of soil health as suggested by the series of field and microcosm-based experiments. Water activity (water directly available to biological life) was shown to be an important indicator of the microbiome's health under extreme conditions related to low moisture circumstances. However, this may be connected strongly to soil type and geographical region. High clay soils, like those found in Mississippi, showed limited $A_w$ significance as the clay particles held water for biological use under low moisture stress. Sandy soils, such as those found in Arizona, were not able to secure biologically available water under drought conditions and therefore are much more likely to use the water activity assay. This dependency on soil type limits water activity's potential to be added to general soil health tests.

The 16S and 18S gene abundance assay using quantitative PCR showed support as an effective and potentially reliable indicator of soil health. In general, analysis of 16S and 18S gene abundance was significantly different amongst both soil moisture and organic matter stressors that would allow for an overall idea of the microbiome structure and abundance within a tested soil. Addition of this assay would be economically feasible and relatively quick to perform and interpret, providing a valuable tool for microbial soil health analysis. While some significance was also found amongst the functional genes, the consistency in response of these selected functional genes may not be high enough to suggest use as a soil health indication strategy. However, further investigation on other functional genes not tested here is suggested as there are multiple genetic biochemical pathways found within the microbiome (Jansson and Hofmockel, 2018). Analysis of other genes might provide important results in the field of soil health indicators.

Finally, enzyme analysis did provide some important evidence of biological soil health especially amongst β-glucosidase and n-acetylglucosaminidase associated with organic matter.

However, these experiments have shown that assessing enzyme activity is most useful for very specific cases, such as high organic matter soils, while the effect of soil moisture may be masked by the ex-situ nature and the addition of moisture during the assay. This limits the assay's ability to measure direct stressor effects and potentially causes misrepresentation in the data. Because of this, and due to the amount of experience and work needed for the assay, this form of enzyme activity measurement may not be the best fit for determining potential biological indicators of soil health.

## Conclusions

Traditional indicators of soil health have previously not taken into consideration the biological aspect of soil. Using the selected biological indicators to analyze the described environmental stressors, organic matter and soil moisture levels provided a mensurate response from the microbiome. While it is well known that organic matter is a key indicator of soil health and that saturated soils will provide better environmental conditions for biological life than dry soils, support can be raised from these results for the inclusion of biological soil health measurements into the soil health discussion. Furthermore, while this study focused on a wide variety of measurements, there is a substantial depth of unexplored genes, enzymes, and targeted and shotgun sequencing which may elucidate many of these interactions. Overall, the methods used in this experiment show that the influence of environmental stressors on the microbiome, such as organic matter and soil moisture, do influence soil biological health.

## Tables

Table 2.1 Environmental Stressor Experiments

| Stressor (Independent Variable) | Design & Location | Treatment Levels | # of Observations (n) | Statistical Analysis |
|---|---|---|---|---|
| Soil Moisture & Organic Matter | Microcosm- Tucson, AZ (Timepoint) | High OM x Field Moist ($T1$) | 3 | Two way ANOVA |
| | | High OM x Dry ($T_2$) | 3 | |
| | | High OM x Dry + ($T_3$) | 3 | |
| | | High OM x Rewet ($T_4$) | 3 | |
| | | Low OM x Field Moist ($T_1$) | 3 | |
| | | Low OM x Dry ($T_2$) | 3 | |
| | | Low OM x Dry + ($T_3$) | 3 | |
| | | Low OM x Rewet ($T_4$) | 3 | |
| Soil Moisture | Small plot- Site 1 Pontotoc, MS | Saturated | 15 | One way ANOVA |
| | | Dry | 15 | |
| | Small plot- Site 2 Starkville, MS | Saturated | 24 | One way ANOVA |
| | | Dry | 24 | |
| | Small plot- Site 3 Starkville, MS | Saturated (Irrigation) | 8 | One way ANOVA |
| | | Dry (No Irrigation) | 4 | |
| Biological Pest Pressure | Small plot- Clarkton, MO | Plant Susceptible | 6 | One way ANOVA |
| | | Plant Not Susceptible | 6 | |

Brief description of the nature of each environmental stressor experiment. Stressors were analyzed by microcosm benchtop experiment and small plot field experiment. Number of observations relate to samples within each treatment. OM refers to organic matter. $T_x$ refers to timepoint. Plant (Not) Susceptible refers to susceptibility of cotton plant in field to nematode infection.

Table 2.2 Microcosm Soil Moisture and Organic Matter Stressor Results

| Time-point | Moisture | Organic Matter Level | 16S rRNA | 18S rRNA | *ure*C | $A_w$ | pH |
|---|---|---|---|---|---|---|---|
| | | | ---$log_{10}$ GU dry $g^{-1}$--- | | | | |
| $T_1$ | Field wet | High | $12.27_{\pm 0.07}^{**}$ | $12.80_{\pm 0.20}$ | $9.99_{\pm 0.22}$ | $0.98_{\pm 0.00}$ | $7.63_{\pm 0.10}^{*}$ |
| | | Low | $11.33_{\pm 0.12}$ | $12.30_{\pm 0.06}$ | $9.37_{\pm 0.18}$ | $0.99_{\pm 0.00}$ | $7.09_{\pm 0.05}$ |
| $T_2$ | Dry | High | $11.57_{\pm 0.09}^{***}$ | $12.50_{\pm 0.21}^{**}$ | $9.77_{\pm 0.06}^{**}$ | $\underline{0.55_{\pm 0.00}}$ | $7.69_{\pm 0.03}$ |
| | | Low | $10.53_{\pm 0.12}$ | $11.53_{\pm 0.07}$ | $8.55_{\pm 0.22}$ | $\underline{0.57_{\pm 0.01}^{*}}$ | $7.66_{\pm 0.01}$ |
| $T_3$ | Dry + | High | $11.57_{\pm 0.09}^{***}$ | $12.90_{\pm 0.15}^{***}$ | $9.69_{\pm 0.12}^{**}$ | $\underline{0.50_{\pm 0.00}}$ | $7.73_{\pm 0.05}$ |
| | | Low | $10.23_{\pm 0.07}$ | $11.33_{\pm 0.12}$ | $8.42_{\pm 0.06}$ | $\underline{0.49_{\pm 0.01}}$ | $7.54_{\pm 0.22}$ |
| $T_4$ | Re-wet | High | $12.17_{\pm 0.09}^{**}$ | $13.03_{\pm 0.09}^{*}$ | $10.33_{\pm 0.09}^{**}$ | $0.99_{\pm 0.00}$ | $7.70_{\pm 0.01}$ |
| | | Low | $11.27_{\pm 0.09}$ | $12.17_{\pm 0.13}$ | $9.37_{\pm 0.08}$ | $1.00_{\pm 0.00}$ | $7.54_{\pm 0.06}$ |

Averages for the Soil Moisture and Organic Matter Stressor experiment. Data presented after log transformation. $T_x$ refers to timepoint. ureC = urease gene. $A_w$ = water activity. $A_w$ = 0.85 is considered threshold for biologically available water; below this water is not freely available for bacteria (_). * indicates significant difference below the 0.05 level. ** indicates significant difference below the 0.01 level. *** indicates significant difference below the 0.0001 level. Pairwise comparisons made via post hoc Tukey corrected t-tests within timepoint.

Table 2.3 Field Soil Moisture Stressor Results

| Site | Moisture Level | 16S rRNA | 18S rRNA | *ure*C | pH |
|---|---|---|---|---|---|
| | | ---$log_{10}$ GU dry $g^{-1}$--- | | | |
| Site 1 | Saturated | $11.87_{\pm 0.09}$ | $13.17_{\pm 0.10}$ | $11.76_{\pm 0.15}^{***}$ | $5.81_{\pm 0.13}^{*}$ |
| | Dry | $11.93_{\pm 0.05}$ | $13.08_{\pm 0.06}$ | $10.97_{\pm 0.06}$ | $5.38_{\pm 0.12}$ |
| Site 2 | Saturated | $12.06_{\pm 0.05}^{***}$ | $13.33_{\pm 0.04}^{***}$ | $10.0_{\pm 0.05}$ | $6.91_{\pm 0.05}$ |
| | Dry | $11.75_{\pm 0.05}$ | $12.98_{\pm 0.05}$ | $10.52_{\pm 0.07}^{***}$ | $7.13_{\pm 0.04}^{**}$ |
| Site 3 | Saturated (Irrigation) | $12.30_{\pm 0.08}$ | $13.28_{\pm 0.06}$ | $10.83_{\pm 0.10}$ | $7.38_{\pm 0.06}$ |
| | Dry (No Irrigation) | $12.15_{\pm 0.06}$ | $13.33_{\pm 0.02}$ | $10.55_{\pm 0.06}$ | $7.22_{\pm 0.10}$ |

Averages for the Soil Moisture small-plot field experiment. Data presented after log transformation. ureC = urease gene. * indicates significant difference below the 0.05 level. ** indicates significant difference below the 0.01 level. *** indicates significant difference below the 0.0001 level. Pairwise comparisons made via post hoc Tukey corrected t-tests.

Table 2.4 Biological Pressure Results

| Plant Susceptibility | 16S rRNA | 18S rRNA |
|---|---|---|
| | ---$log_{10}$ GU dry $g^{-1}$--- | |
| Susceptible | $11.80_{\pm 0.07}$ | $12.58_{\pm 0.07}$ |
| Control | $11.82_{\pm 0.08}$ | $12.43_{\pm 0.05}$ |

Averages for the Biological Pest Pressure experiment. Data presented after log transformation. Plant Susceptibility refers to the cotton plant's susceptibility to nematode damage.

## Figures

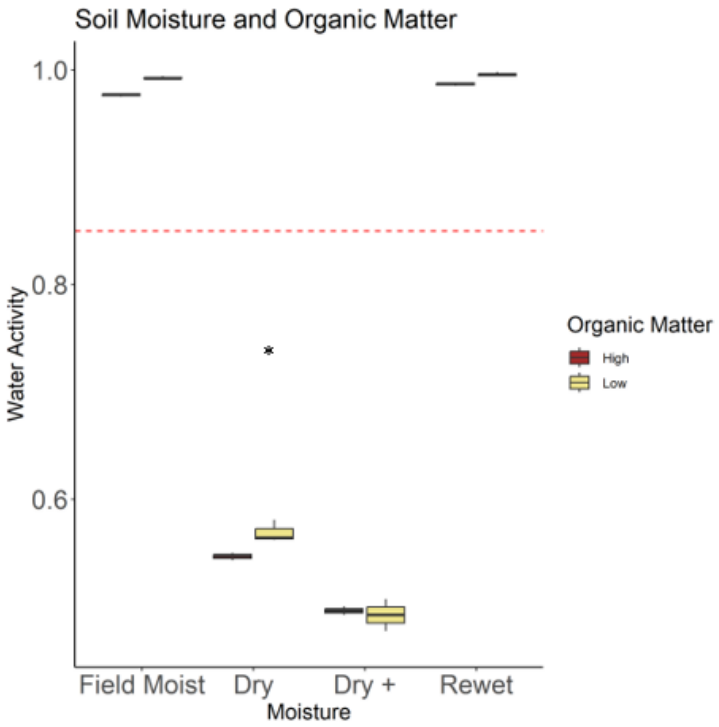


Figure 2.1 Microcosm Soil Moisture and Organic Matter- $A_w$

Figure shows $A_w$ from microcosm-scale soil moisture and organic matter experiment. Microcosm experiment took place in Tucson, AZ. Results below 0.85 are not biologically relevant due to water being unavailable for biological life. Organic matter levels described in legend are as follows: High = ~8% organic matter, Low = ~1% organic matter. * indicates significant difference at the 0.05 level within pairwise ANOVA analysis.

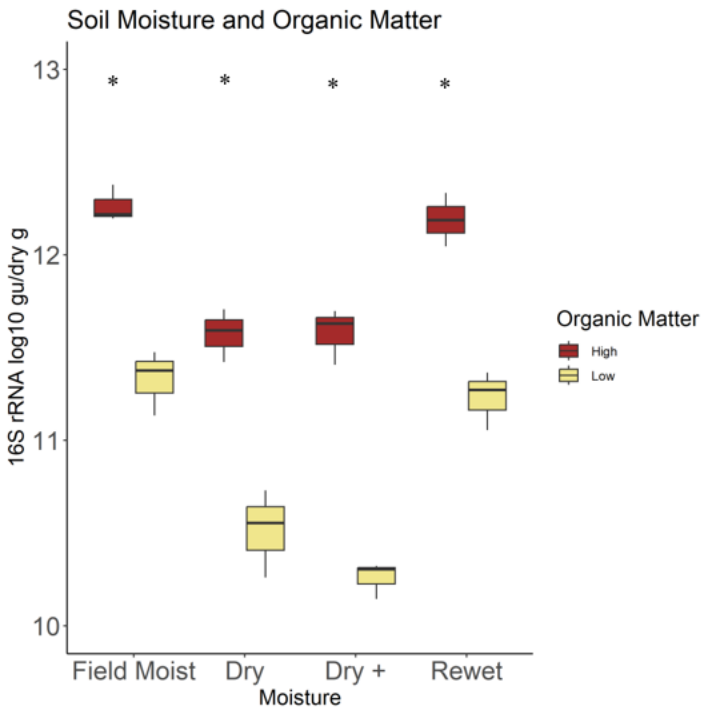


Figure 2.2 Microcosm Soil Moisture and Organic Matter- 16S rRNA Gene Abundance

Figure shows 16S rRNA gene abundance from microcosm-scale soil moisture and organic matter experiment. Microcosm experiment took place in Tucson, AZ. Data presented after $\log_{10}$ transformation. Organic matter levels described in legend are as follows: High = ~8% organic matter, Low = ~1% organic matter. * indicates significant difference at the 0.05 level within pairwise ANOVA analysis.

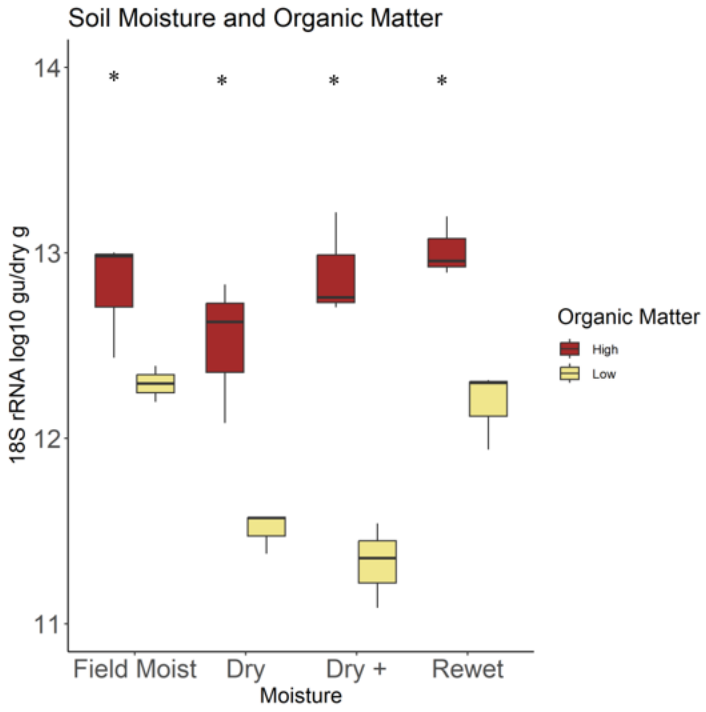


Figure 2.3 Microcosm Soil Moisture and Organic Matter- 18S rRNA Gene Abundance

Figure shows 18S rRNA gene abundance from microcosm-scale soil moisture and organic matter experiment. Microcosm experiment took place in Tucson, AZ. Data presented after log10 transformation. Organic matter levels described in legend are as follows: High = ~8% organic matter, Low = ~1% organic matter. * indicates significant difference at the 0.05 level within pairwise ANOVA analysis.

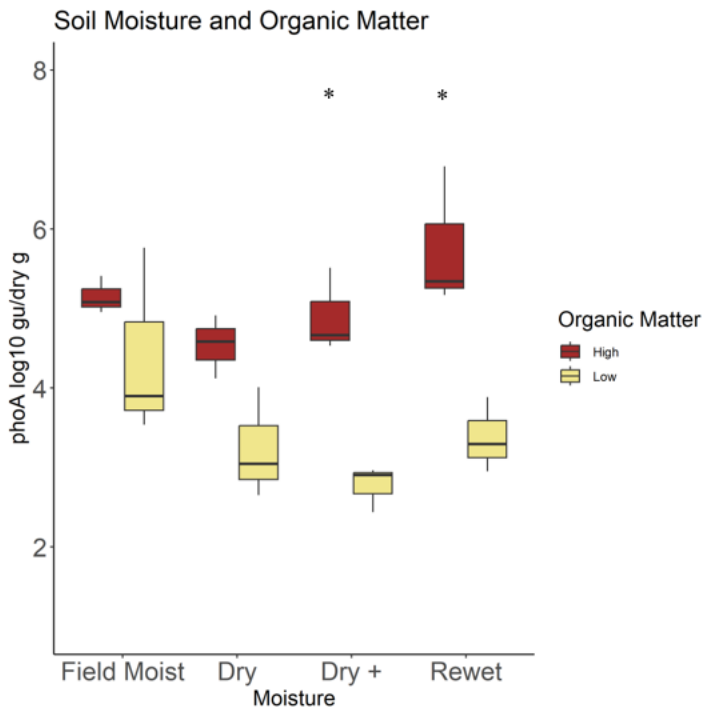


Figure 2.4 Microcosm Soil Moisture and Organic Matter- phoA Gene Abundance

Figure shows phoA gene abundance from microcosm-scale soil moisture and organic matter experiment. Microcosm experiment took place in Tucson, AZ. Data presented after log10 transformation. Organic matter levels described in legend are as follows: High = ~8% organic matter, Low = ~1% organic matter. * indicates significant difference at the 0.05 level within pairwise ANOVA analysis.

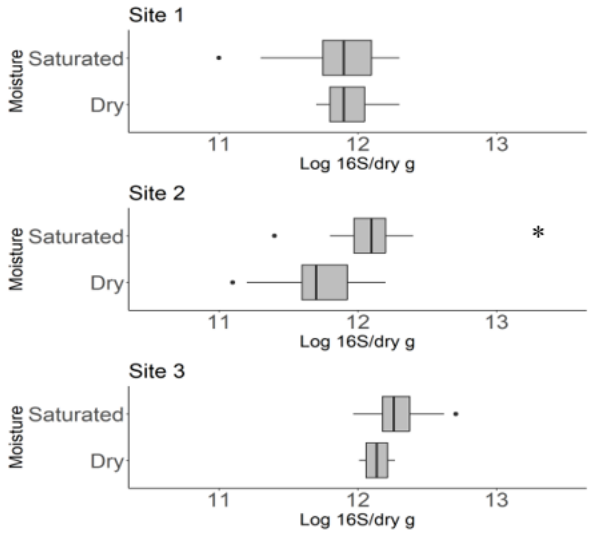


Figure 2.5 Field Soil Moisture- 16S rRNA Gene Abundance

Figure shows 16S rRNA gene abundance from Mississippi field soil moisture experiment. Data presented after $\log_{10}$ transformation. Dots on graph indicate statistical outliers. Horizontal axis in $\log_{10}$ GU units per dry g of soil. Vertical axis represents moisture condition. * indicates significant difference below the 0.05 level within ANOVA analysis.

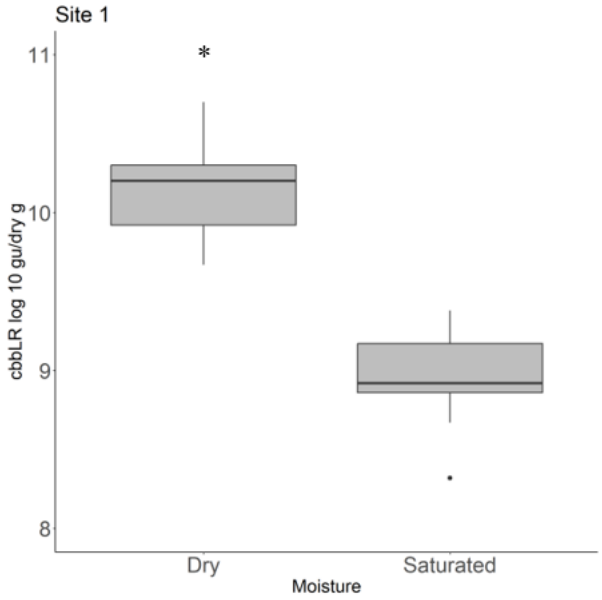


Figure 2.6 Field Soil Moisture- Site 1 cbbLR Gene Abundance

Figure shows cbbLR gene abundance from site 1 of the Mississippi field soil moisture experiment. Data presented after log10 transformation. Dots on graph indicate statistical outliers. Vertical axis in log10 GU units per dry g of soil. * indicates significant difference at the 0.05 level within ANOVA analysis.

# CHAPTER III

# EVALUATING THE INFLUENCE OF ANTHROPOGENIC STRESSORS SUCH AS SYNTHETIC SOIL AMENDMENTS AND MICROPLASTIC POLLUTION ON THE SOIL MICROBIOME

## Introduction

Most soil microbiomes are anthropogenically influenced to some degree. Humans have influenced soil microbiomes through agriculture and other activities, particularly land leveling, plowing, landfills, intensive agricultural practices, and pollution. Even the most remote soils are now experiencing the effects of change due in part to human activity and pollution (Feng et al., 2020).

The application of fertilizer, more specifically the long-term use of fertilizer, is a widespread practice in row-crop production systems with influence on the soil microbiome. Fertilizer has been known to alter the community structure of microorganisms and does so in several ways (Li et al., 2014; Wang et al., 2017; Zhang et al., 2012). Most notably, the addition of any type of fertilizer enacts a chemical change within the soil environment. When this chemical change occurs, changes in pH also occur in the immediate vicinity of the fertilizer application (Belay et al. 2002). This pH swing will introduce a pH stress on the microorganisms in the immediate area.

Dai et al. (2018) explains the effect of long-term nitrogen fertilization which ultimately decreases the bacterial community's diversity. The influx of elemental nutrients alters the biogeochemical processes that take place within the soil and therefore reshapes the interactions

that the bacteria in the community share with each other. Soil microorganisms are the engine that drives soil nitrogen mineralization (Li et al. 2019). Ultimately, the addition of nitrogen fertilizer will influence the abundance of nitrogen-cycling microorganisms (Du et al. 2019).

Another fertilizer option available is “organic” fertilizers such as manures and biosolids. These additives are high carbon materials that provide large amounts of organic matter for microbial use. Organic fertilizers act as a nutrient source of nitrogen, potassium, and phosphorous for both crops and microbes. However, these organic fertilizers may also input a certain community bias into the soil (Schlatter et al., 2019). This bias comes from the microbiome that may already be present within the fertilizer source due to the high carbon makeup of the material.

The addition of microplastics via agricultural application of biosolids is shown to also have a stressing effect on the soil microbiome (Fei et al., 2020; Lin et al., 2020). Nizetto et al. (2016) found in recent estimates that 63,000-430,000 tons of microplastics are added annually to agricultural farmlands in North America and 44,000-300,000 tons in Europe. These microplastics find their way into the biosolids fraction during wastewater treatment where the majority of microplastics partition into the solid phase (Gatidou et al., 2019). Sources of microplastics include synthetic fibers from washing machines (Ziajahromi et al., 2017; Henry et al., 2019), toothpaste, soaps, and facial scrubs (Napper et al., 2015).

Within the soil, microplastics produce a change in the structure of the soil (by expanding pore space and providing rigid structure) and often alter the soil chemistry through the addition of the synthetic chemicals used in production of microplastics. This change in soil chemistry is a major stressor for microbial communities that ultimately alters the enzymatic activities of soil microorganisms (Fei et al., 2020). The result is often a microbial community shift to microplastic

tolerant populations and potential plastic degrading organisms (Huang et al., 2019; Huang et al., 2021; Pinnell and Turner, 2019). Some studies have also shown that nitrogen fertilizers accelerate decomposition of microplastics (Zhang et al., 2020; Huang et al., 2019).

Harrison et al. (2014) and Zhang et al. (2019) state that microplastics provide habitat for microbes as the rigid structure of the plastic provides a large surface area potentially for biofilm production (McCormick et al., 2014; Miao et al., 2019). In the case of biofilm spread, Yuan et al. (2020) and Shabbir et al. (2020) have shown that the time it takes for degradation of the microplastic particles lessens. There are even some aspects of microplastics that have been found to benefit some aspect of the soil. For instance, de Souza Machado et al. (2018) found that additions of microplastics could increase the water holding capacity and maintain soil moisture for a longer time. However, most current research, such as Lin et al. (2020) considers that even though microbial activity might increase with microplastic introduction, there are indirect ecological effects that are not beneficial to the microbiome.

Glucose is a simple sugar that is used as a key energy source by living organisms of all sizes. Microbial life is no exception to the use of glucose breakdown for energy production. Within the cell, glucose is broken down through the process of glycolysis, gaining energy from the sugar compound. An addition of glucose into the microbial environment would therefore logically increase the availability of energy for the cell. Since organic matter is often the provider of carbon-based sugars and metabolites, a low amount of organic matter will indicate a metabolite low ecosystem for microbial life. The practice of glucose addition into the environment is therefore mostly performed under low organic matter situations where the primary source of organic carbon for uptake is root turnover and exudates (Hoyle, Murphy, & Brookes, 2008). This sugar addition provides for more immediately available carbon sources

than what little crop residues are left behind in the soil (Blagodatskaya et al., 2009). Chenu, Hassink, and Bloem (2001) also found that glucose addition into soil increased the number of bacteria and fungi within both clay and sandy soils. This makes sense as the extra metabolites would allow for an increase in metabolism and growth for the duration that the glucose stayed available (Reischke, Kumar, & Baath, 2015).

In this experiment, the effect of anthropogenic stressors, such as synthetic soil amendments, and microplastic pollution, on the microbiome were evaluated through the use of microcosm and field-based experiments (Table 3.1) to test the following research hypotheses: (1) soils high in microplastics will increase microbial activity, however a bacterial community shift will occur when compared to low microplastic fields, and; (2) the use of inorganic fertilizer will produce microbial communities with lower abundance while the use of organic fertilizer will result in greater microbial community abundance, and; (3) the addition of glucose into the environment will allow for an increase in microbial activity and abundance.

## Materials and Methods

To accomplish the objective of this study, soil samples were collected from a combination of ongoing small-plot field experiments, as well as field soil subjected to controlled microcosm-based experiments whereby manipulation of anthropogenic stressors included fertilizer use, glucose addition, and microplastic addition.

### Fertilizer – Microcosm-scale

Controlled microcosm-based experiments were conducted in Tucson, AZ using six jars filled with field-moist soil. The experiment comprised a 2x3 design where a field-collected soil (Gila fine sandy loam) was subjected to an addition of urea solution, including a control, each

subject to three sampling timepoints and replicated three times. Timepoint 1 ($T_1$) was the initial sample collection at time of urea or control addition (3.31 ml of 2% urea solution or sterile $dH_2O$). All microcosms were then incubated at 35℃ for three days before sampling $T_2$. An additional three-day incubation period followed ($T_3$). Fifty-gram timepoint samples were sent via overnight shipping in a ~4℃ container from Tucson, AZ to the lab in Starkville, MS. Laboratory analyses and assays were carried out upon receiving samples.

**Fertilizer – Small plot-scale**

Fertilizer experiments were also evaluated under field conditions by using previously established small-plot field experiments in Mississippi. These experiments were conducted to provide "real world" systems whereby fertilizer addition was based on an agronomic rate of both inorganic and organic nitrogen sources for the cash crop. Two experimental field sites were chosen on Mississippi State University research stations.

The first field (site 1) was located at the Pontotoc Ridge – Flatwoods Branch Experiment Station located in Pontotoc, MS (Atwood silt loam) and was part of a five-year study; samples were collected in the third year growing season. The study site was cropped in corn (*Zea mays*), used no till soil conservation, and consisted of a randomized complete block design (3x10) where fertilizer type (control, urea ammonia-nitrate, broiler litter) was the main treatment and was replicated ten times. Fertilizer application rates were as follows: broiler litter application (10,394 kg/ha), urea-ammonium nitrate application (3,757 kg/ha at planting and 8,766 kg/ha at squaring stage), and control plots with no fertilizer applications.

The second field (site 2) was located at the R. R. Foil Plant Science Research Center located in Starkville, MS (Leeper silty clay loam) and is part of a five-year study; samples were collected in the third year growing season. The study site was cropped in corn (*Zea mays*), used

minimal tillage, and consisted of a randomized complete block design (3x16) where fertilizer type (control, urea ammonia-nitrate, broiler litter) was the main treatment and was replicated sixteen times. Fertilizer application rates were as follows: broiler litter application (10,394 kg/ha), urea-ammonium nitrate application (3,757 kg/ha at planting and 8,766 kg/ha at squaring stage), and control plots with no fertilizer applications.

Samples from all fields comprise two randomized cores (0-15 cm) taken within the furrow. Furrows were sampled to negate the effect of the plant influence. Samples were stored on ice in the field and in transport. Laboratory analyses and assays were carried out upon samples return to the lab in Starkville, MS.

**Glucose – Microcosm-scale**

Controlled microcosm-based experiments were conducted in Tucson, AZ using six jars filled with field-moist soil. The experiment comprised a 2x3 design where field collected soil (Gila fine sandy loam) was subjected to an addition of 0.01% glucose solution and included a control ($dH_2O$ received at the same volume), each subject to six timepoint sampling events and replicated three times. After an initial timepoint ($T_1$), where soil microcosms were initially sampled directly after glucose addition, microcosm experiments were sampled at five additional timepoints (three, six, nine, fourteen and twenty-one days) to measure biological response variables over time. Fifty-gram timepoint samples were sent via overnight shipping in a ~4°C container from Tucson, AZ to the lab in Starkville, MS. Laboratory analyses and assays were carried out upon receiving samples.

**Microplastic – Microcosm-scale**

Controlled microcosm-based experiments were conducted in Tucson, AZ using six jars filled with 600 grams of field-moist soil. The experimental design comprised a 2x3 where two different soil types (Brazito sandy loam and Gila fine sandy loam) were subjected to an addition of microplastic solution (solution containing 1-4 um clear microspheres) and included a control, each subject to six timepoint sampling events and replicated three times. Samples from both soils were analyzed together to limit influence of organic matter. The entire experiment was repeated with each soil type. Timepoint 1 ($T_1$) was the initial sampling directly after microplastic solution was added (~$10^8$ microspheres per gram of soil). Microcosm experiments were then sampled at five additional timepoints (forty-eight hours, one week, one month, three months, and six months post exposure) to measure biological response variables over time. Fifty-gram timepoint samples were sent via overnight shipping in a ~4°C container from Tucson, AZ to the lab in Starkville, MS. Laboratory analyses and assays were carried out upon receiving samples.

**Microplastic – Small plot-scale**

Soils indirectly exposed to microplastics were also evaluated under field conditions by using fields from Tucson, AZ that had been previously applied with municipal wastewater treatment plant Class B biosolids. Class B biosolids are known to contain microplastics and are one of the main anthropogenic sources of microplastics (Crossman et al., 2020). These experiments were conducted to provide “real world” systems whereby biosolid addition has occurred for the previous twenty-five years and should represent a “naturally” high exposure to microplastics. The study comprised of a 3x6 design whereby biosolid application rate (high, low, control) was replicated six times. Treatments comprised three biosolid application rates as follows: high biosolid rate (89,668 dry kg/ha), low biosolid rate (29,142 dry kg/ha), and control

field with no biosolid application. Sampling occurred during a one-time sampling event, collected from the middle of treatment plots and samples were sent via overnight shipping in a ~4°C container from Tucson, AZ to the lab in Starkville, MS. Laboratory analyses and assays were carried out upon receiving samples.

## Laboratory Assays and Analyses

All samples collected from each experiment were subject to the following physical, chemical, and biological analyses.

i) pH Measurement

Soil pH was measured using a 1:1 ratio with dH2O. The slurry was then measured using an Oakton pH/CON 510 series pH meter.

ii) Soil Moisture Content

Gravimetric moisture content was assessed by weighing 10g (moist weight) of soil, drying at 104°C for 24h and reweighing the dried sample. Moisture content was measured using the following equation:

$$\text{moisture content} = [\text{soil wet weight} - (\text{dry weight of soil and weigh boat} - \text{weight of weigh boat})] / \text{soil wet weight} * 100 \quad (3.1)$$

iii) Soil Water Activity

Soil water activity was assessed via the AquaLab Water Potential Meter using the chilled mirror technique. Water potential was measured in MPa and converted to water activity using

$$A_w = P/P0 \quad (3.2)$$

where $A_w$ is water activity of a solid or liquid material, P is the vapor pressure of a solid or liquid material, and P0 is the vapor pressure of pure water.

iv) Enzyme Analysis

For soil enzyme analysis, n-acetylglucosaminidase, phosphomonoesterase, and B-glucosidase were measured using microplate fluorometric methods as described by Deng et al., (2011). Briefly, a 1 g aliquot of soil was added to 120 ml of sterile dH2O, homogenized via stomacher, and transferred to horizontal shaker for 30 min set at 65 rpm. A 100 µl aliquot (quadruplicate) was then subjected to the microplate assay whereby pH for each individual soil enzyme is adjusted to either 5.5 or 6.0 via buffered solution, and methylumbelliferyl (MUF) based substrates are added to the microplates to assess enzyme activity. MUF substrates comprise MUF- -D glucoside, -n-acetyl- -D glucosaminide, and -phosphate. Appropriate sample matrix, MUF standard curve, and experimental controls were added to each plate and group of plates. Plates are incubated for 1 h at 37°C. Plate fluorescence was read on a Biotek plate reader with excitation at 365 nm and emission at 450 nm. Soil enzymatic activity was expressed as millimoles MUF $kg^{-1}$ $h^{-1}$.

v) Nucleic Acid Extraction

DNA was extracted from each soil sample using standard DNA extraction kits. Briefly, total microbial DNA was extracted (0.25 g soil) using MP Biomedical FastDNA Spin Kits (MP Biomedical; Santa Ana, CA) and a FastPrep-24 homogenizer (MP Biomedical) following the recommended manufacturer's protocol. Extracted DNA was checked for quantity and quality prior to subjecting it to polymerase chain reaction (PCR). Extracted DNA was frozen at -80°C until processed for high throughput sequencing and PCR-based assays.

vi) Quantitative PCR (qPCR)

DNA was subjected to quantitative polymerase chain reaction to assess levels of 16S rRNA (16S), 18S rRNA, urease (*ure*C), rubulose-1,5-biphosphate carboxylase (*cbb*LR) and

phosphatase (*pho*A) enzyme genes present in the soil samples. 16S rRNA (Nadkarni et al., 2002), *ure*C (Koper et al., 2004), and *pho*A (Han et al., 2012) primers and assay conditions were selected or modified from the literature. Quantitative PCR assays was conducted as stated in Brooks et al. (2019) using sybrgreen chemistry and duplicated. A standard curve comprised of serial 10-fold dilutions of IDT g-block designed DNA controls or known positive controls was subjected to melt curve analysis confirmation. All values were reported as GU dry $g^{-1}$ (genomic units per dry g of soil).

vii) 16S rRNA Targeted Sequencing

Soil microbial genomic DNA was submitted for 16S rRNA library preparation and sequencing through Microbiome Insights (Vancouver, BC, Canada) using the Illumina MiSeq DNA sequencing platform. 16S rRNA targeted sequencing was followed as stated in Brooks et al. (2019). Soil genomic DNA was collected for all soil samples regardless of endpoint assay and was archived. Selected time points were then subjected to MiSeq sequencing and analyses. Briefly, the MiSeq SOP proposed by Kozich et al. (2013) was followed using 2x250 base pair V2 chemistry. Library preparation and quality-assurance and control measures were conducted prior to sequencing. The V4 region of the 16S rRNA gene was amplified and sequenced.

The Mothur platform (v. 1.40.3) was used for bioinformatic 16S rRNA sequence analyses and carried out according to Kozich et al (2013). Briefly, libraries were contiged and curated to reduce errors and low-quality sequences. Sequences were then aligned relative to Silva alignment reference files (release 128), and taxonomically classified using the most current Ribosomal Database Project reference files (RDP version 16). Sequences which did not align or classify as Eubacteria were removed from the libraries, and chimera sequences were screened within Mothur. Operational Taxonomic Unit (OTU) and phylotype analyses were conducted

using Mothur (Kozich et al., 2013). Operational Taxonomic Units were assigned at a 3% dissimilarity and taxonomy-based analyses were conducted at the genus taxon. OTU-based analysis consisted of alpha- and beta-diversity analyses comprised of rarefaction, invsimpson, AMOVA, and HOMOVA commands. Alpha-diversity analysis function rarefaction generate sample richness curves while inverse Simpson is the inverse of the Simpson diversity estimator, estimating richness in a community with uniform evenness. Beta-diversity analysis function AMOVA (analysis of molecular variance) was used to test the genetic diversity within two populations while HOMOVA (homogeneity of molecular variance) was used to test homogeneity within two or more populations. Post hoc pairwise comparisons were used when evaluating fertilizer amendments.

viii) Statistical Analysis

Data were analyzed by first $\log_{10}$ transforming raw data collected from each assay (except 16S rRNA sequencing, pH, and $A_w$) to achieve normal distribution and homogeneity of variance. Each experiment was treated as an independent experiment and analyzed separately. Dependent variables for each experiment were: pH, $A_w$, soil moisture content, enzyme analysis, quantitative PCR (qPCR), and 16S rRNA targeted sequencing (fertilizer experiments only). Data were analyzed in SAS using a one-way ANOVA whereby fertilizer (i.e., urea application, broiler litter, and control), glucose addition, or microplastic contamination (i.e., microplastic solution or biosolid addition and control) was considered independent class variables for each respective experiment. Pairwise comparisons were made via post hoc Tukey corrected t-tests within timepoints. Due to low detection and a non-normal distribution, a nonparametric test was used when analyzing *pho*A abundance levels within both the field and microcosm microplastic experiments. Data from 16S rRNA high throughput sequencing were analyzed using MOTHUR

described above. A one-way ANOVA was conducted on inverse Simpson values according to the treatments of the specific experiment from which the data originated. Figures were created in program R (4.1.3) using Rstudio (2022.02.2+485) and the following packages ggplot2, ggfortify, ggpubr, tidyverse, and patchwork.

## Results and Discussion

To investigate the effects of anthropogenic stressors on the soil microbiome response variables, a series of experiments were conducted at the microcosm and small plot scale.

### Fertilizer – Microcosm-scale

The effect of added urea fertilizer did not result in significant differences in water activity levels between control and urea treated soils ($p > 0.05$). However, a significant statistical difference was found at $T_3$ (day six) between the urea and control microcosms ($F(1,4) = 56.58$, $p = 0.0017$). At this time point, the water activity of the urea amended soil was higher ($A_w = 0.936$) than that of the control soil ($A_w = 0.870$). While this measurement is statistically significant, the importance may be minimal as the water activity of the control soil does not drop below the 0.85 threshold described by Stevenson et al. (2015). The increase in $A_w$ due to urea application into the soil may be of importance in certain scenarios, for example this may be due to urea's preference to absorb moisture and begin to dissolve through chemical reactions, the process of hydrolysis that ultimately creates ammonium (Jones et al., 2013). Through moisture absorption (particularly in humid settings), urea applications may be able to supply a subtle amount of moisture to the immediate surrounding soil particles and therefore slightly increase water availability for the immediate microbiome.

Similar to water activity, addition of urea solution into the microcosm did not have an overall effect on the pH of the soil. However, at $T_2$, pH was significantly lower amongst the urea added soil, relative to the control ($F(1,4) = 72$, $p = 0.0011$) (Table 3.2). This drop makes sense as urea fertilizer is known to reduce soil pH (Chien, Gearhart, & Collamer, 2008; Hao et al., 2020). However, the significance of this drop may be mitigated as microcosms at the next time point were similar regardless of treatment. The overall pH change was minor enough that effects on the microbiome would be minimal with the exception of short-term influence.

Soil 16s rRNA gene abundance showed no statistical significance between sample treatments overall, ($F(1,16) = 0.57$,$p = 0.4624$); however, 16S rRNA levels initially decreased in urea treated soils at $T_1$ ($F(1,4) = 16$, $p = 0.016$) (Figure 3.1), before increasing to that of the controls in subsequent time points. This is likely because of a disruption in the soil chemistry and nitrifying bacteria inhibition under a sudden excess of ammonium concentration (Cheng et al., 2023). Abundance levels of 18S rRNA showed no statistical differences amongst timepoints ($F(1,16) = 0.40$, $p = 0.5384$).

Functional gene abundance levels were also found to be similar between treatments. Neither *ure*C nor *cbbL*R ($F(1,16) = 0.16$, $p = 0.6987$ & $F(1,16) = 0.4$, $p = 0.5380$ respectively) showed any patterns when analyzed by time point. While the levels of *pho*A revealed what could be a weak trend towards abundance rising with time, the importance of this is disputable over such a short time period (six days) ($F(1,16) = 1.23$, $p = 0.2833$).

Enzyme levels were also not significantly influenced by addition of urea solution. β-glucosidase levels ($F(1,16) = 0.31$, $p = 0.5857$) varied between time points with no recognizable pattern (control $\bar{x} = 90$ pmol $h^{-1}$; urea $\bar{x} = 99$ pmol $h^{-1}$). N-acetylglucosaminidase showed no statistical difference between urea amended and control samples (control $\bar{x} = 98$ pmol $h^{-1}$; urea

$\bar{x}$ = 100 pmol $h^{-1}$) ($F(1,16) = 0.01$, $p = 0.9197$) and phosphomonoesterase soil levels were also not statistically significant (control $\bar{x}$ = 249 pmol $h^{-1}$; urea $\bar{x}$ = 230 pmol $h^{-1}$) ($F(1,16) = 1.23$, $p = 0.2832$). A caveat associated with the measure of soil enzyme activity is the *ex-situ* nature of the assay and the timing of which enzymatic activity measurements were taken. While each timepoint was measured independently, the measurements did not take place at the same time as the sample extraction, which may skew data as enzyme activity could have changed in the time between sampling and enzyme assay. However, all timepoints were treated the same.

Alpha and beta diversity were also calculated for the fertilizer stressor using 16S rRNA targeted sequencing. The microcosm scale Arizona soils showed no significant differences from an ANOVA based on the inverse Simpson index ($F(1,13) = 2.31$, $p = 0.152$) and likewise no significant differences in AMOVA ($F(1,13) = 1.03$, $p = 0.360$) or HOMOVA ($B = 0.31$, $p = 0.163$). Lack of statistical significance found amongst diversity data were likely due to the short time frame of the experiment and the use of high organic matter soil.

**Fertilizer – Small plot-scale**

As a complement to the microcosm experiment above, two field sites were selected whereby broiler litter and urea-ammonium nitrate were used as nitrogen fertilizer sources. However, soil from both sites demonstrated a lack of statistical difference in microbial responses based on fertilizer treatments (Table 3.3). This is somewhat surprising as the addition of organic fertilizer (broiler litter) was expected to result in greater microbial community abundance over inorganic fertilizer (urea-ammonium nitrate) (Figure 3.2). Soil measurements such as water activity (site 1 $F(2,27) = 0.15$, $p = 0.8592$; site 2 $F(2,45) = 0.89$, $p = 0.4186$) and pH (site 1 $F(2,27) = 0.77$, $p = 0.4742$; site 2 $F(2,45) = 0.25$, $p = 0.7797$) were also not significantly affected by fertilizer treatments at these locations. These results may be due to field histories where both

fertilizer types have been used (long standing history of fertilizer use), or because established nutrient conditions and abundant access to crop root metabolites and crop residues at each field allows for thriving microbial populations regardless of added nutrient fertilizer (Chavez-Romero et al., 2016). A different target (i.e., selected genes of a different pathway or focus) may illuminate change in this scenario.

The selected field sites also underwent alpha and beta diversity analysis using 16S rRNA targeted sequencing. While the field samples from site 1 showed no significance in the inverse Simpson ANOVA ($F(2,15) = 0.21$, $p = 0.814$), beta diversity (AMOVA) for the site showed differences based on fertilizer treatments ($F(2,15) = 1.93$, $p = 0.024$), specifically post hoc pairwise comparisons of control and litter along with litter and urea ($F(1,10) = 2.18$, $p = 0.029$ and $F(1,10) = 2.76$, $p = 0.030$ respectively). HOMOVA results for site 1 were also significant ($B = 0.85$, $p = 0.020$). The field samples from site 2 showed no significance in the inverse Simpson ANOVA ($F(2,21) = 0.38$, $p = 0.689$) but beta diversity (AMOVA) was again significantly different between fertilizer treatments ($F(2,21) = 2.35$, $p = 0.013$), specifically post hoc pairwise comparisons of litter and urea ($F(1,14) = 3.08$, $p = 0.022$). HOMOVA results for the site 2 field were not significant ($B = 0.01$, $p = 0.991$). This indicates that while abundance via qPCR metrics and alpha diversity analysis yielded no significant differences, beta diversity analyses indicated there were differences in bacterial community profiles, specifically the makeup and diversity of urea and litter treated soils.

**Glucose – Microcosm-scale**

The effect of added glucose (0.01%) did not significantly affect $A_w$ levels between control and glucose treated soils, and a drop in $A_w$ levels below the 0.85 threshold described by Stevenson et al. (2015) was not observed. Similar to water activity, addition of glucose solution

into the microcosm did not have an overall effect on the pH of the soil. However, at $T_5$ a significant drop in pH ($F(1,4) = 8.88$, $p = 0.0407$) is shown amongst the added glucose samples. This drop is likely due to the rising fungal population as described below. As the fungal metabolism of the glucose increases and growth occurs, a small local pH change from release of organic acids caused by fungal metabolism is likely (Rosling et al., 2004). Overtime, this slight change can be noticeable, leading to the significance found at $T_5$. This is not important biologically, however, as the slight pH change from 7.4 to 7.2 would have minimal effects on the microbiome.

The addition of glucose into the microcosms also significantly affected fungal abundance. 18S rRNA gene abundance ($F(1,34) = 4.75$, $p = 0.0363$) was shown to react to the glucose addition with higher abundance among glucose samples, while 16S rRNA did not show any significance ($F(1,34) = 0.75$, $p = 0.3939$) (Table 3.4). From the observed measurements, it appears that 18S rRNA levels may be more indicative of carbon stress (Figure 3.3). This makes sense as many fungi are saprophytic and thrive in high carbon environments. Likewise, fungal growth has been shown to be more influenced than bacteria at high glucose concentrations (Reischke, Rouske, & Baath, 2014).

Functional gene abundance levels were also found to be without significance. Neither *ure*C nor *cbbL*R ($F(1,34) = 0.04$, $p = 0.8508$ and $F(1,34) = 0.21$, 0.6462 respectively) showed any patterns when analyzed by time point. While *pho*A abundance revealed no overall significance ($F(1,34) = 0.01$, $p = 0.9101$), $T_4$ shows that glucose treated soils showed a higher abundance of *pho*A than the control samples ($F(1,4) = 15.08$, $p = 0.0178$). Huang et al. (2021) found that an increase in labile carbon (glucose in this case) increased soil available phosphorous and increased phosphorous metabolizing genes. This is likely because when glucose metabolism

increases and growth occurs, internal phosphorous demands would increase as phosphorous is a key component of the DNA structure, which may explain these results.

Enzyme levels were not significantly influenced by addition of glucose solution into the microcosm soil. β-glucosidase levels ($F(1,34) = 0.34$, $p = 0.5660$) showed very little difference between control and glucose amendments. N-acetylglucosaminidase also showed no statistical difference ($F(1,34) = 1$, $p = 0.3245$), however, analysis of each time point indicated that at $T_5$ there was a significant difference between the glucose amended soils which showed lower enzyme activity than the control samples (control $\bar{x} = 100$ pmol $h^{-1}$; glucose $\bar{x} = 82$ pmol $h^{-1}$) ($F(1,4) = 113.78$, $p = 0.0004$). This may be due to the microbial population's ability to rely on added glucose for nutrition, negating the need for secreted enzymes used in organic matter breakdown. Phosphomonoesterase soil levels were also not statistically significant ($F(1,34) = 0.24$, $p = 0.6273$).

**Microplastic – Microcosm-scale**

An introduction of microplastic solution into soil microcosms of two sandy Arizona soils (high organic matter and low organic matter) resulted in no significant differences in microbial responses between treatment groups. Data were analyzed via one-way ANOVA whereby organic matter treatments were combined and separate, with no difference in interpretation. A nonparametric test for *pho*A abundance levels was used because levels were often below detection, thus a non-normal distribution. Overall, each assay resulted in biological responses that were not significantly different ($p > 0.05$) based on microplastic exposure (Table 3.5).

The microplastic amended samples did show a slightly higher pH than control at $T_1$ (forty-eight hour post spike) showing slight significance ($F(1,10) = 5.38$, $p = 0.0428$). This is likely due to the alkaline nature of microplastics (Zhao, Lozano, & Rillig, 2021; Li & Liu, 2022).

However, this effect was most likely not biologically important as the pH change was minimal. Additionally, at $T_1$ the *pho*A gene abundance levels were significantly different between treatments ($F(1,10) = 5.34$, $p = 0.0434$) as the microplastic amended soils showed lower levels. However, statistical significance was slight and the biological importance of this may be minimal as the *pho*A gene abundance showed no overall significance amongst microplastic amended soils compared to control samples ($p = >0.05$).

The results of this experiment do not align with the presented hypothesis, in which a acute biological response to microplastics was expected. Microplastics are known to break down very slowly in nature, so the timeframe of this experiment may not have allowed for sufficient decomposition (Zhang et al., 2021), or possibly for ample biological interactions. Also, the size, color, and shape of the microplastics used in this experiment may have influenced the result, as the mechanically sheered plastics (1-4 um clear microspheres) in this study would not represent the variety of those found within a plastic polluted natural soil (Xu et al., 2022; Henry et al., 2019). The soil microbiome may have also not been influenced by the microplastic presence or may have treated the plastics as a surface to colonize, no different than sand or soil particles (Harrison et al., 2014). Many bacterial communities may even be using microplastics as a distinct habitat, creating a situation where the microplastics are not a stressor but rather sought out and used by biological life (Zhang et al., 2019). If this is the case, the properties and presence of the microplastics within the soil may not be as detrimental to the soil microbiome health as previously thought.

**Microplastic – Small plot-scale**

Microbial responses to long term biosolid amendments (microplastic particles are common within biosolids (Nizetto et al., 2016; Gatidou et al., 2019)) in Arizona field

experiments did not show statistical significance within any of the measured biological metrics. It's important to state that no "real world" field samples contaminated with microplastics will be without further confounding factors which also bear influence on the observed metrics. In this case, the effect of microplastics in these samples cannot be divorced from the effect of biosolids. However, although it was not statistically significant, the observed $A_w$ of the control plots and high rate biosolid application plots both fell below the 0.85 threshold (Stevenson et al., 2015) in over half of the measured samples. Likewise, the low rate biosolid application also resulted in two sample measurements falling below this threshold. With both application rates ultimately measuring higher than the control plots in $A_w$ (Table 3.6), it seems that microplastic presence, or more likely addition of biosolid amendment (biosolids provide greater surface area and water retention), may improve biological water availability (Wang et al., 2022; de Souza Machado et al., 2019). Because the effects of microplastics cannot be separated from biosolids, it is possible that negative effects caused by microplastic presence could be overshadowed by the positive effects on the microbiome that are experienced from 25 years of biosolid application. However, study of biosolid application is one of the only reliable ways to analyze effects of microplastics in an agricultural field setting.

While the results of this experiment contradict the presented hypothesis, the significance of this experiment may point towards microplastics having minimal chemical influence on the soil microbiome, acting as an inert substrate. Under real world conditions such as those found in this experiment, the presence of microplastics may not be the most pressing stress or the top priority for the microbiome. In this case, the soil microbiome may not have been influenced by microplastic presence in order to respond to pressing environmental issues such as drought or temperature induced stress. The microbiome may be indifferent to microplastic presence if these

materials are not shown to affect their immediate environmental conditions. Alternatively, the microbiome may also react to microplastic stress in a way that is not observed by the chosen biological metrics. One potential avenue of research will be identifying potential genes associated with microplastic or complex carbon breakdown through the use of metagenomics for example.

**Indicator selection**

While the effect of the selected environmental and biological stressors on the soil microbiome were often inconsistent under these experimental conditions, some metrics may have potential as biological indicators of soil health as suggested by the series of field and microcosm-based experiments. Of the selected potential indicators tested in this study, none have been shown to be effective in determining biological soil health under the described anthropogenic stress situations. While beta diversity did show evidence of change within long term fertilizer conditions, only a general evaluation was given leaving the positive or negative effects of treatment unknown. Because of this, the importance of using 16S rRNA beta diversity metrics from a soil health perspective is undetermined. Water activity was shown to be an important indicator of the microbiome's health under extreme conditions related to low moisture circumstances, especially in the microplastic field situation. However, this has been shown to be connected strongly to soil type, organic matter content, and geographical region, limiting its potential to be added to soil health tests.

Quantitative PCR and targeted genes were also not useful in determining effects on the microbiome based on anthropogenic stressors. While some significance was identified between treatment and control groups amongst timepoint data, the general lack of significant differences imparted by the stressors would make suggesting functional gene abundance as a preferred

measure or indicator of anthropogenic stress difficult based on these results. However, even though a positive response was found only once (18S rRNA amongst glucose samples), analysis of 16S rRNA and 18S rRNA gene abundance would be the most useful indicator of those selected here, given the previous chapter's results. This would allow for a general idea of the structure and abundance of microorganisms within a tested soil, giving a "snapshot" of how current practices are influencing the soil microbiome. Addition of this assay would also be economically feasible and relatively quick to perform and interpret.

Finally, enzyme analysis did not provide important evidence of biological health within the study. These results, along with the expertise needed in performing the assay and analyzing the results, support the position that the used enzyme analysis would not be suggested as a biological soil health indicator.

## Conclusion

Traditional indicators of soil health have previously not taken into consideration the biological aspect of soil. Using the selected biological indicators to analyze the described anthropogenic stressors, only fertilizer inputs and glucose amendments (both at the microcosm scale) resulted in a mensurate response from the microbiome. While the microplastic experiments (small plot-scale and microcosm scale) had in minimal statistical significance, this lack of significance is important as microplastic interactions in the soil are not yet fully understood and the time scale of this microcosm experiment was most likely too short. Furthermore, while this study focused on a wide variety of measurements, there is a substantial depth of unexplored genes, enzymes, and targeted and metagenomic sequencing which may elucidate many of these interactions. Overall, the methods used in this experiment show that the

influence of anthropogenic stressors on the microbiome, such as synthetic soil amendments and microplastic pollution, are more complex and warrant additional research.

## Tables

Table 3.1 Anthropogenic Stressor Experiments

| Stressor | Design & Location | Treatment | # of Observations (n) | Statistical Analysis |
|---|---|---|---|---|
| Fertilizer | Microcosm- Tucson, AZ (Timepoint) | Control | 9 | One way ANOVA + ANOVA @ each tp |
| | | Urea | 9 | |
| | Small plot- Site 1 Pontotoc, MS | Control | 10 | One way ANOVA |
| | | Broiler Litter | 10 | |
| | | Urea | 10 | |
| | Small plot- Site 2 Starkville, MS | Control | 16 | One way ANOVA |
| | | Broiler Litter | 16 | |
| | | Urea | 16 | |
| Glucose | Microcosm- Tucson, AZ (Timepoint) | Control | 18 | One way ANOVA + ANOVA @ each tp |
| | | 0.01% Glucose | 18 | |
| Microplastic | Microcosm- Tucson, AZ (Timepoint) | Control | 36 | One way ANOVA + ANOVA @ each tp + nonparametric test for analysis of *pho*A |
| | | MP Addition | 36 | |
| | Small plot- Tucson, AZ | High Biosolid | 6 | |
| | | Low Biosolid | 6 | |
| | | Control | 6 | |

Brief description of the nature of each anthropogenic stressor experiment. Stressors were analyzed by microcosm benchtop experiment and small plot field experiment. Timepoint experiments were subject to one way ANOVA at each timepoint. Number of observations relate to samples within each treatment. Control refers to no added treatment. Microplastic microcosm study included a high organic matter and a low organic matter soil that were analyzed together, providing for higher n.

Table 3.2 Microcosm Fertilizer Stressor Results

| Timepoints | Fertilizer | 16S rRNA | 18S rRNA | pH |
|---|---|---|---|---|
| | | ---$log_{10}$ GU dry $g^{-1}$--- | | |
| $T_1$ (0 days) | Control | $11.80_{\pm 0.00}$* | $12.60_{\pm 0.20}$ | $7.40_{\pm 0.08}$ |
| | Urea | $11.67_{\pm 0.03}$ | $12.53_{\pm 0.07}$ | $7.30_{\pm 0.08}$ |
| $T_2$ (3 days) | Control | $11.80_{\pm 0.06}$ | $12.47_{\pm 0.03}$ | $7.33_{\pm 0.02}$** |
| | Urea | $11.73_{\pm 0.12}$ | $12.40_{\pm 0.17}$ | $7.13_{\pm 0.00}$ |
| $T_3$ (6 days) | Control | $11.43_{\pm 0.07}$ | $12.60_{\pm 0.06}$ | $7.01_{\pm 0.06}$ |
| | Urea | $11.43_{\pm 0.03}$ | $12.57_{\pm 0.03}$ | $7.03_{\pm 0.02}$ |

Averages for the Fertilizer Stressor experiment. Data presented after log transformation. $T_x$ refers to timepoint. * indicates significant difference below the 0.05 level. ** indicates significant difference below the 0.01 level. Pairwise comparisons made via post hoc Tukey corrected t-tests within timepoint.

Table 3.3 Small Plot Fertilizer Stressor Results

| Site/Design | Fertilizer | 16S rRNA | 18S rRNA | pH |
|---|---|---|---|---|
| | | ---$log_{10}$ GU dry $g^{-1}$--- | | |
| Field Site 1 | Control | $11.86_{\pm 0.11}$ | $13.00_{\pm 0.09}$ | $5.68_{\pm 0.18}$ |
| | Broiler Litter | $12.02_{\pm 0.08}$ | $13.24_{\pm 0.10}$ | $5.68_{\pm 0.13}$ |
| | Urea | $11.83_{\pm 0.07}$ | $13.14_{\pm 0.10}$ | $5.42_{\pm 0.19}$ |
| Field Site 2 | Control | $11.87_{\pm 0.07}$ | $13.14_{\pm 0.08}$ | $7.03_{\pm 0.06}$ |
| | Broiler Litter | $11.94_{\pm 0.07}$ | $13.24_{\pm 0.06}$ | $7.04_{\pm 0.04}$ |
| | Urea | $11.91_{\pm 0.08}$ | $13.08_{\pm 0.08}$ | $6.99_{\pm 0.07}$ |

Averages for the Fertilizer Stressor experiment. Data presented after log transformation.

Table 3.4 Glucose Stressor Results

| Timepoints | Glucose | 16S rRNA | 18S rRNA | pH |
|---|---|---|---|---|
| | | ---$log_{10}$ GU dry $g^{-1}$--- | | |
| $T_1$ (0 days) | 0.01% Glucose | $11.33_{\pm 0.13}$ | $11.93_{\pm 0.19}$ | $7.02_{\pm 0.08}$ |
| | Control | $11.43_{\pm 0.11}^{*}$ | $12.17_{\pm 0.06}^{*}$ | $7.28_{\pm 0.14}$ |
| $T_2$ (3 days) | 0.01% Glucose | $12.03_{\pm 0.32}$ | $12.40_{\pm 0.12}^{*}$ | $7.04_{\pm 0.03}$ |
| | Control | $11.50_{\pm 0.10}$ | $12.17_{\pm 0.06}$ | $7.38_{\pm 0.17}$ |
| $T_3$ (6 days) | 0.01% Glucose | $11.57_{\pm 0.18}$ | $12.50_{\pm 0.10}^{**}$ | $7.00_{\pm 0.06}$ |
| | Control | $11.48_{\pm 0.16}$ | $12.17_{\pm 0.06}$ | $7.11_{\pm 0.16}$ |
| $T_4$ (9 days) | 0.01% Glucose | $10.93_{\pm 0.15}$ | $12.20_{\pm 0.06}$ | $7.25_{\pm 0.13}$ |
| | Control | $11.27_{\pm 0.26}$ | $12.03_{\pm 0.14}$ | $7.39_{\pm 0.08}$ |
| $T_5$ (14 days) | 0.01% Glucose | $11.83_{\pm 0.07}$ | $12.20_{\pm 0.06}$ | $7.24_{\pm 0.02}$ |
| | Control | $11.70_{\pm 0.06}$ | $12.17_{\pm 0.15}$ | $7.39_{\pm 0.05}^{*}$ |
| $T_6$ (21 days) | 0.01% Glucose | $11.63_{\pm 0.09}$ | $12.37_{\pm 0.09}$ | $7.47_{\pm 0.12}$ |
| | Control | $11.83_{\pm 0.07}$ | $12.33_{\pm 0.20}$ | $7.34_{\pm 0.13}$ |

Averages for the Glucose Stressor experiment. Data presented after log transformation. $T_x$ refers to timepoint. * indicates significant difference below the 0.05 level. ** indicates significant difference below the 0.01 level. Pairwise comparisons made via post hoc Tukey corrected t-tests within timepoint.

Table 3.5 Microcosm Microplastic Stressor Results

| Timepoints | Microplastic Treatment | 16S rRNA ---$log_{10}$ GU dry $g^{-1}$--- | 18S rRNA ---$log_{10}$ GU dry $g^{-1}$--- | $A_w$ |
|---|---|---|---|---|
| $T_1$ (0 days) | Control | $10.96_{\pm 0.59}$ | $10.70_{\pm 0.37}$ | $0.99_{\pm 0.00}$ |
| | Microplastic Addition | $10.77_{\pm 0.61}$ | $10.55_{\pm 0.35}$ | $0.99_{\pm 0.00}$ |
| $T_2$ (2 days) | Control | $10.78_{\pm 0.57}$ | $10.64_{\pm 0.35}$ | $0.99_{\pm 0.00}$ |
| | Microplastic Addition | $11.07_{\pm 0.61}$ | $11.00_{\pm 0.34}$ | $0.99_{\pm 0.00}$ |
| $T_3$ (1 week) | Control | $11.17_{\pm 0.29}$ | $11.15_{\pm 0.22}$ | $1.00_{\pm 0.00}$ |
| | Microplastic Addition | $11.20_{\pm 0.29}$ | $11.18_{\pm 0.17}$ | $0.99_{\pm 0.00}$ |
| $T_4$ (1 month) | Control | $10.46_{\pm 0.23}$ | $9.91_{\pm 0.35}$ | $1.00_{\pm 0.00}$ |
| | Microplastic Addition | $8.97_{\pm 1.80}$ | $9.30_{\pm 1.87}$ | $0.99_{\pm 0.00}$ |
| $T_5$ (3 months) | Control | $10.87_{\pm 0.08}$ | $10.47_{\pm 0.44}$ | $1.00_{\pm 0.00}$ |
| | Microplastic Addition | $10.90_{\pm 0.07}$ | $10.24_{\pm 0.40}$ | $1.00_{\pm 0.00}$ |
| $T_6$ (6 months) | Control | $10.43_{\pm 0.19}$ | $7.52_{\pm 0.80}$ | $0.99_{\pm 0.00}$ |
| | Microplastic Addition | $9.91_{\pm 0.43}$ | $4.20_{\pm 1.91}$ | $0.99_{\pm 0.00}$ |

Averages for the Microplastic Stressor experiment. Data presented after log transformation. $T_x$ refers to timepoint. Aw = water activity.

Table 3.6 Field Microplastic Stressor Results

| Microplastic Treatment | 16S rRNA ---$log_{10}$ GU dry $g^{-1}$--- | 18S rRNA ---$log_{10}$ GU dry $g^{-1}$--- | $A_w$ |
|---|---|---|---|
| High Biosolid Rate | $10.67_{\pm 0.13}$ | $11.20_{\pm 0.40}$ | $\underline{0.80_{\pm 0.07}}$ |
| Low Biosolid Rate | $10.55_{\pm 0.20}$ | $10.38_{\pm 0.18}$ | $0.86_{\pm 0.05}$ |
| Control | $10.50_{\pm 0.40}$ | $10.41_{\pm 0.21}$ | $\underline{0.71_{\pm 0.07}}$ |

Averages for the Microplastic Stressor experiment. Data presented after log transformation. Aw = water activity. Aw = 0.85 is considered threshold for biologically available water; below this water is not freely available for bacteria (_).

## Figures

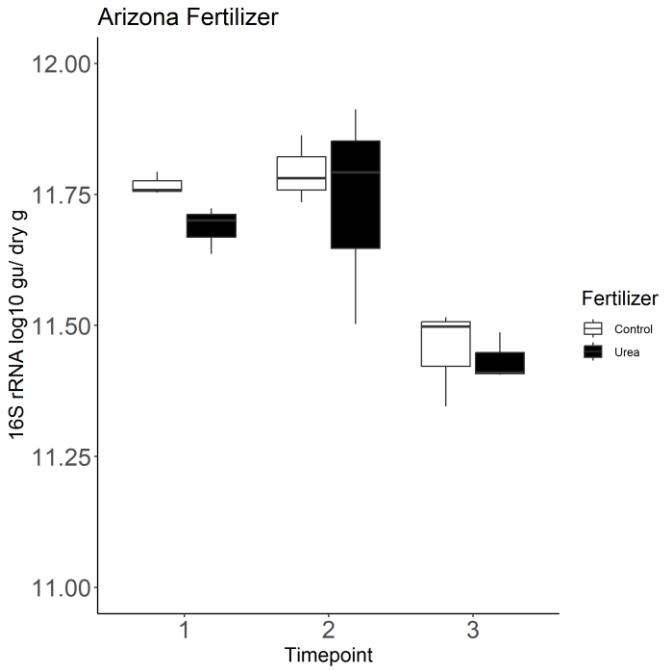


Figure 3.1 Fertilizer- Microcosm 16S rRNA Gene Abundance

Figure shows 16S rRNA gene abundance from microcosm scale fertilizer stressor experiment. Microcosm experiment took place in Tucson, AZ. Data presented after $\log_{10}$ transformation. Vertical axis in $\log_{10}$ GU units per dry g of soil. Legend describes control vs urea addition.

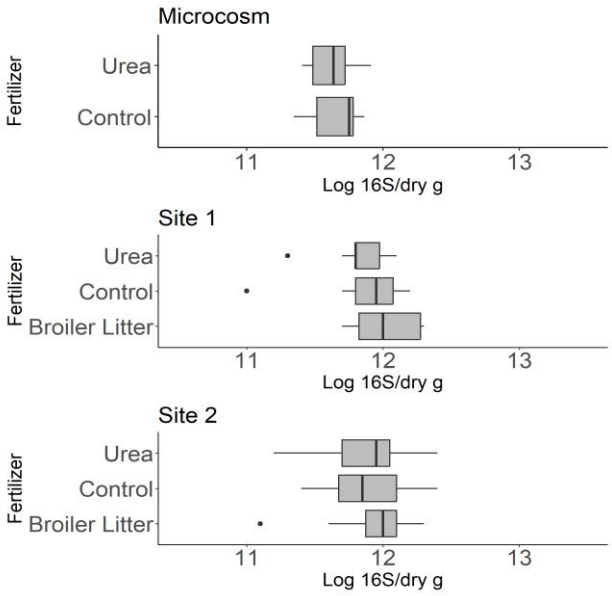


Figure 3.2 Fertilizer- 16S rRNA Gene Abundance

Figure shows 16S rRNA gene abundance from fertilizer stressor experiment. Microcosm experiment took place in Tucson, AZ while Site 1 and Site 2 fields were located in Mississippi. Data presented after $\log_{10}$ transformation. Dots on graph indicate statistical outliers. Horizontal axis in $\log_{10}$ GU units per dry g of soil.

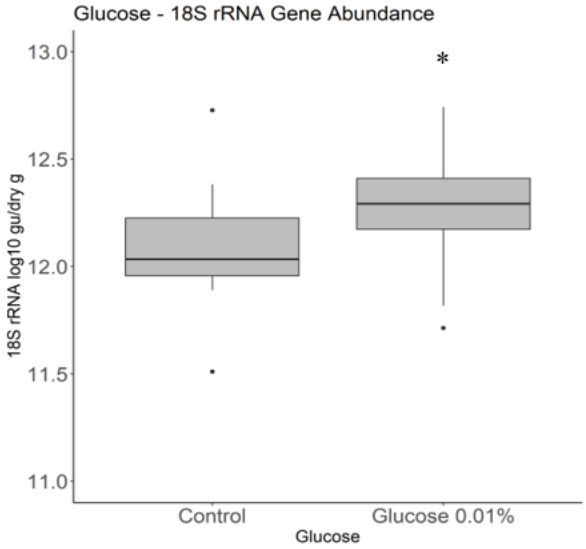


Figure 3.3 Glucose- 18S rRNA Gene Abundance

Figure shows 18S rRNA gene abundance from microcosm scale glucose stressor experiment performed in Tucson, AZ. Glucose addition was a 0.01% glucose solution. Data presented after $\log_{10}$ transformation. Dots on graph indicate statistical outliers. Vertical axis in $\log_{10}$ GU units per dry g of soil. * indicates significant difference below the 0.05 level within ANOVA analysis.

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