RHIZOBIOME DYNAMICS IN PLANT GROWTH PROMOTION AND ABIOTIC STRESS RESPONSE

by

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DEDICATION

This thesis is dedicated to the next generation of women in STEM. Together may we build a better, brighter future for ourselves and those we are paving the way for.

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ABSTRACT

Soil microorganisms play vital roles in global nutrient cycling. Understanding the complex relationships between plants and soil microbes and their implications is one of the greatest challenges facing microbial ecology today. Soil microbes can play beneficial roles in supporting plant growth by increasing access to nutrients, water, and decreasing plant stress signaling under abiotic stresses such as drought and heat. With increasing climate variability due to climate change, it is imperative to make scientifically informed management decisions to best support global biodiversity and plant productivity in natural and agroecosystsms. In this dissertation I summarize four separate investigations of plant-microbe interactions. The first is using nitrogen-fixing cyanobacterial biofertilizers to promote plant growth of perennial second generation bioenergy crops switchgrass (*Panicum virgatum*) and tall wheatgrass (*Agropyrun elongatum*). The second and third studies seek to better understand plant-microbe carbon exchange under drought stress in the native North American prairie grass blue grama (*Bouteloua gracilis*). The final study explores the potential microbial contribution to heat tolerance of panic grass (*Dichanthelium lanuginosum*) across a natural soil temperature gradient in Yellowstone National Park. Next-generation amplicon sequencing using the Illumina Miseq platform is the primary technique utilized across the three studies to investigate microbial community dynamics.

The main results of the biofertilizer study were that tall wheatgrass is better suited to the SW Montana growing season than switchgrass, and similar plant yields were achieved with the cyanobacterial biofertilizer as with urea chemical fertilizer without negatively impacting the microbial community diversity. The first blue grama study found that severe and mild drought had distinct, phylogenetically linked responses within the blue grama rhizobiome with *Planctomycetes*, *Thermoproteota* (ammonia-oxidizing archaea), and *Glomeromycetes* (arbuscular mycorrhizal fungi) exhibiting notably altered relative abundances. The second blue grama study found that climate legacy plays an important role in shaping blue grama drought response. Finally, from the *D. lanuginosum* study in Yellowstone National Park we learned that pH and temperature both strongly influence community composition, and that *D. lanuginosum* selects for unique community members in its rhizosphere at higher temperatures. Collectively, these studies contribute to furthering our understanding of the dynamics of plant-associated microbiomes.

CHAPTER ONE

PLANT RHIZOBIOMES IN NATURAL AND AGROECOSYSTEMS, AND THE IMPACTS OF CLIMATE CHANGE

Plants rely on microbes to provide the oxygen, food, shelter, medicine, fuel, and innumerous bioproducts that humans and animals alike depend upon for life on Earth. Every one of the hundreds of thousands of known plant species is colonized by millions of bacteria, archaea, and fungi, many of which are essential to healthy plant functioning (van der Heijden et al. 2008). The interactions of plants and microbes are influenced by a variety of abiotic factors including water availability, temperature, light, nutrient availability, and soil chemistry but are ultimately regulated by the climate environment in which they live as temperature and precipitation patterns largely dictate global plant distribution (Nottingham et al. 2018, Woodward and Williams 1987).

Increased variability in temperature and precipitation patterns due to climate change is impacting global plant productivity and distribution in both natural and agroecosystems. The latest report of the Intergovernmental Panel on Climate Change (IPCC 2023) describes significant globally distributed increases of extreme climate events (heat waves, drought, heavy precipitation) in the past 50 years with expectations for increases frequency and intensity in the future. These changes in climate patterns have severely decreased agricultural productivity in many parts of the world and exacerbated the degradation of natural terrestrial ecosystems such as grasslands (IPCC 2023, Gornall et al. 2010).

The need to understand plant-microbe relationships has been widely recognized in agricultural ecosystems through efforts to improve the sustainability of crop production (Bender

et al. 2016), particularly in light of climate change. To address the above concerns, understanding and potentially manipulating the interplay between plants and their belowground microbiomes will be key to developing sustainable plant management solutions. In addition, understanding how plant-microbe interactions are impacted by changing climate conditions will help predict future changes in plant distribution and global nutrient cycling. However, the high levels of diversity among plants and within their microbiomes make it extremely difficult to untangle the web of interactions between organisms and their activities in different soils and across climate conditions. Despite the rapid advances in the fields of plant and soil microbiomes in recent years, there remain significant knowledge gaps which hinder our ability to predictably manage plant microbiomes to improve plant tolerance to abiotic stress. In the following chapter I review the central literature surrounding soil microbiomes and specifically *Poaceae* rhizobiomes, as well as the current state of knowledge regarding soil microbiome modulation of plant responses to climate change.

The Soil Ecosystem

Soil is a vital natural resource that plays a crucial role in supporting life on Earth. All living organisms depend on cyclical transformations of essential elements including hydrogen (H), oxygen (O), carbon (C), nitrogen (N), sulfur (S) and phosphorus (P), much of which occurs belowground in terrestrial ecosystems, namely soils. The definition of soil has evolved considerably since the formal development of soil science in the 1800s; a current definition developed by van Es (2017) is: “The layer(s) of generally loose mineral and/or organic material that are affected by physical, chemical, and/or biological processes at or near the planetary surface and usually hold liquids, gases, and biota and support plants,” see also Dazzi and Lo

Papa (2022). Soil is the repository for more than 75% of the earth's terrestrial C (Lal 2004) and is responsible for providing humanity with 98.8% of its food while also contributing to greenhouse gas regulation, flood mitigation and support for both natural resources and urban development (Kopittke et al. 2019).

Among the most diverse ecosystems, soil is home to organisms ranging from micron-scale bacteria to macrofauna including insects, worms, and small mammals (Bardgett and van der Putten 2014). At the microbial level, every teaspoon of soil is home to over 100 million microorganisms and global soils contain roughly 5% of all prokaryotic life forms (Schloss and Handelsman 2006). It is estimated that less than 1% of soil microbes have been cultured, and while we have identified many thousands of species of soil bacteria, archaea, fungi, and viruses there are likely many thousands more that we have yet to discover (Schloss et al. 2016).

Major Microbial Taxa in Soil

Bacteria. Bacteria are the most abundant and diverse group of soil microorganisms. Some of the major soil phyla include: *Proteobacteria* (particularly classes *Alphaproteobacteria* and *Betaprotobacteria*), *Actinobacteria*, *Acidobacteria*, *Planctomycetes, Chloroflexi*, *Verrucomicrobia, Bacteroidetes, Gemmatimonadetes,* and *Firmicutes* (Delgado-Baquerizo et al. 2018). The diversity of bacteria in soil is frequently in the range of several hundred to several thousand unique species (Goemann et al. 2021; Schloss and Handelsman 2006). While some species of bacteria have well-known functions in soil such as nitrogen-fixers among the *Burkholderia* and *Cyanobacteria*, some phyla including the *Planctomycetes, Verrucomicrobia* and *Gemmatimonadetes* have very few cultured representatives and little is known regarding their ecological functions despite their high abundance and ubiquity across many soils.

In a recent meta-analysis, globally dominant soil bacteria were found to fall into five unique ecological clusters that describe habitat preferences as: high pH, low pH, drylands, low plant productivity, and dry-forest environments (Delgado-Baquerizo et al. 2018). In the following chapters, the environments studied largely fall under "drylands" cluster in which *Actinobacteria* and *Proteobacteria* tend to dominate.

Fungi. Soil fungi are responsible for breaking down complex organic compounds, such as lignin and cellulose, therefore contributing greatly to nutrient cycling. The fungal phyla include *Ascomycota*, *Basidiomycota*, *Blastocladiomycota*, *Chytridiomycota*, and *Mucoromycotina*. Fungal phylogenetics and classification have improved dramatically with the advent of next generation sequencing technology, however due to the complexity of their eukaryotic genomes, taxonomic resolution at the species level is still generally unreliable in datasets from highly diverse ecosystems such as soil. Most fruiting fungi (ie. macroscopic mushroom-forming) are found among the *Basidiomycota* with notable *Ascomycete* exceptions including truffles (*Tuber sp.*) and morel mushrooms (*Morchella sp.*). *Saccharomyces cerevisiae* (baker's yeast) is also a notable *Ascomycete*.

In soils, mycorrhizal fungi are of particular importance as they often form mutualistic associations with plant roots. In exchange for plant-derived carbon (C) from photosynthate, mycorrhizae can provide access to limiting nutrients such as water, nitrogen, and phosphorus. Arbuscular mycorrhizal fungi (AMF, class *Glomeromycetes* within phylum *Mucoromycotina*) are the most common mycorrhizal organisms. AMF are associated with greater than 200,000 plant species representing more than 80% of all terrestrial plant species (van der Heijden et al. 2015). These fungi penetrate plant roots and form small microstructures (arbuscules) within plant

root cells which are the site for nutrient transfer to and from the fungus. One of the hallmarks of AMF is mycelial growth throughout the soil which allows the fungi to transport nutrients from distal mycelial filaments back to the arbuscules where the exchange of these nutrients for plant carbon occurs.

Archaea. Archaea are a group of microorganisms characterized by unique membranes, cells walls, and informational processing machineries distinct from bacteria (Garrett and Klenk 2007). The importance of archaea in soil ecosystems has become dramatically more apparent with the advent of next-generation sequencing. Until the early 1990s, archaea were thought to thrive primarily in extreme environments such as hot springs or hydrothermal vents and contribute little to global nutrient cycling (Offre, Spang, and Schleper 2013). However, we now know that many species of mesophilic and heterotrophic archaea are commonly abundant in soil ecosystems (Kletzin 2007). Archaea belonging to the *Thermoproteota* (formerly *Tharumaracheaota*) and *Euryarchaeota* are commonly identified in terrestrial environments. Many members of these phyla are involved in nutrient cycling processes, including nitrification, denitrification, methanogenesis or methane oxidation, sulfate reduction, iron oxidation and organotrophy (Offre, Spang, and Schleper 2013; Kletzin 2007).

Viruses. The contribution of viruses to soil ecosystem functioning is one of the largest gaps in soil ecology. With advances in metagenomic and metatranscriptomic analysis capabilities we are only beginning to understand the complex interactions viruses and phage have within soil microbial ecosystems and the consequences for ecosystem functioning. Emerson (2019) and Jansson (2023) recently reviewed the current knowledge in viral soil ecology and concluded that the diversity of soil viruses is unknown but that they are largely distinct from currently

characterized viruses. In addition, soil viruses appear to be extremely responsive to changes in their environment which likely has important implications for downstream ecosystem functioning that has yet to be determined. In Chapter 4 I will discuss *Dichanthelium lanuginosim*, a grass species unique to thermal features in Yellowstone National Park which is able to survive in soil temperatures up to 65 °C (Márquez et al. 2007) largely due to a tripartite relationship with a fungal endophyte *Curvularia protuberata* and a fungal virus CThTV.

Classifying Microbial Taxa

Soil samples typically contain many thousands of individual taxa, commonly described as "operational taxonomic units" (OTUs). OTUs differentiate genetic sequences into individuals with a 97% identity similarity cutoff. However, in recent years a shift has been made to "zero radius OTUs; ZOTUs" and/or "amplicon sequence variants; ASVs." ZOTUs and ASVs attempt to improve the resolution between individuals based on 100% sequence similarity cutoff (Edgar 2016b; Callahan, McMurdie, and Holmes 2017). While this approach improves differentiation between individuals, in some cases a single "species" can be split across multiple individuals.

Given that the vast majority of soil microbes are uncultivated and have only been identified *in vitro* via sequencing, taxonomic identification is typically dependent on sequence similarity to previously deposited data in public repositories such as the National Center for Biotechnology Information (NCBI, Sayers et al., 2021), the Ribosomal Database Project (RDP, Cole et al., 2014), Silva (Quast et al. 2013), and the Genome Taxonomy Database (GTDB, Chaumeil et al., 2022) to name a few. Importantly, taxonomic identification of microbial species is dependent on the taxa present in a given database which tends to be biased towards well-studied, culturable organisms. However, one method to overcome database taxonomy limitations

is to construct phylogenetic trees thereby placing lesser known or previously undocumented taxa amongst closely related taxa.

The Rhizobiome

Determining the influence of plants on soil microbial communities and vice versa is of particular interest for developing plant management strategies and understanding soil nutrient cycling. Differences between soil collected from the rhizosphere, the area of soil influenced by plant roots, compared to bulk soil have been well-documented. In a recent global meta-analysis, Ling et al. (2022) found that across plants, environments, and land-use types, the rhizosphere microbiome (hereafter the rhizobiome) typically has lower (0.9-5.3%) diversity than bulk soils and rhizobiome community composition is typically a subset of bulk soil members. The rhizosphere is a hotspot of nutrient exchange with rapid turnover of plant rhizodeposits (material deposited by plant roots) and both plant and microbial biomass turnover. As such, with higher resource availability there is more competition between microbial community members and therefore more fast-growing, copiotrophic organisms such as many members within the *Proteobacteria* and *Bacteroides* phyla tend to dominate the rhizobiome. The rhizobiome also tends to have greater concentrations of plant pathogens. In contrast, bulk soils tend to have greater relative proportions of *Gemmatimonadetes*, *Nitrospirae*, *Acidobacteria*, and *Chloroflexi* which are mostly slower-growing organisms (Ling, Wang, and Kuzyakov 2022).

Microorganisms within the rhizobiome can be beneficial, harmful, or have no effect on plant health and fitness depending on the microbes and plant involved. Beneficial microbes can increase plant pathogen resistance, retain more water, improve nutrient uptake and accessibility, and generally improve plant growth (Olanrewaju et al. 2019). Some of the most common

organisms identified as plant growth promoting rhizobacteria (PGPR) include: *Agrobacterium, Arthrobacter, Azotobacter, Azospirillum, Allorhizobium, Bacillus, Bradyrhizobium, Burkholderia, Caulobacter, Chromobacterium, Erwinia, Flavobacterium, Frankia, Mesorhizobium, Micrococcous, Pseudomonas, Rhizobium* and *Serratia* species (Gupta et al. 2015). There are several mechanisms through which PGPR influence plant growth which can be separated into direct and indirect categories. As reviewed by Gupta et al. (2015), direct PGPR influenced processes include: nitrogen fixation, phosphate solubilization, potassium solubilization, siderophore production, and phytohormone production (i.e. indole acetic acid, cytokinins, and gibberellins). Indirect mechanisms include the production of antibiotics and lytic enzymes which serve as biocontrol against other microorganisms for the benefit of the plant host, and biofilm formation which can improve nutrient solubility and protection from desiccation and other stresses.

Plants may regulate the rhizobiome composition and activity through the secretion of a wide variety of compounds collectively termed “root exudates.” Root exudates consist of both low-molecular weight compounds such as amino acids, sugars, organic acids and other secondary metabolites, and high-molecular weight compounds such as polysaccharides and proteins (Badri and Vivanco 2009). While some of these compounds are hypothesized to be simply waste products (Bais et al. 2006), there is considerable evidence that root exudates can attract or repel certain rhizobiome microbes with significant benefits to plant growth (Badri and Vivanco 2009; Haichar et al. 2014; Rolfe, Griffiths, and Ton 2019). Zhou et al. (2021) and Zhalnina et al. (2018) demonstrated plants may adjust root exudation patterns to tailor microbial recruitment for plant nutritional requirements at different life stages. Plant photosynthesis mode

is also known to influence root exudation patterns due to differences in dominant sugar compositions between C_3 and C_4 plants (Gupta et al. 2015). Although carbon is not typically the limiting nutrient in soils, root exudation constitutes a significant carbon cost for plants and must be tightly regulated (Lynch & Ho, 2005), although the mechanisms behind root exudation regulation are largely not yet understood.

Poaceae and their Rhizobiomes

Poaceae (formerly *Gramineae*) is one of the largest plant families containing the world's approximately 10,000 species of grasses. Among these species are the major cereal crops: maize, wheat, barley, oats, rice, sorghum, rye, and millet which supply 51% of global dietary calories and 47% of protein (Lobell and Gourdji 2012). In addition to food, various grasses including maize, switchgrass, and miscanthus are increasingly being cultivated as renewable sources of bioenergy (Goemann et al. 2021; Varnero et al. 2018). Others such as bamboo are important sources of textiles (Nayak and Mishra 2016), and lawn grasses typically make up 50-70% of urban green spaces (Hedblom et al. 2017). Natural grassland ecosystems play critical roles in global nutrient cycling and are biodiversity hotspots. Some grassland ecosystems can be home to nearly 100 plant species per square meter (Petermann and Buzhdygan 2021) and belowground, grasslands are among the most microbially diverse terrestrial ecosystems (Bastida et al. 2021a).

Given the global economic and ecological importance of many *Poaceae* species, continuing to cultivate or maintain these plants is critical to supporting global needs for food, fiber, fuel, and natural resource conservation. This is a daunting challenge in light of a growing global population facing the intensifying effects of global climate change. Increasing frequency and intensity of droughts and heat stress are already having devastating effects on global crop

production (Lesk, Rowhani, and Ramankutty 2016), and grasslands are currently among the most threatened global ecosystems due to land use change and degradation (Epstein et al. 2021; Habel et al. 2013).

Due to their economic importance, a number of recent studies have investigated the core rhizobiomes of key *Poaceae* species such as maize (Walters et al. 2018), rice (Edwards et al. 2015), wheat (Mahoney, Yin, and Hulbert 2017), and switchgrass (Singer et al. 2019). Core microbiome members are shared features across host-associated microbial communities and are hypothesized to reflect underlying functional relationships (Shade and Stopnisek 2019). These core microbiomes are largely species specific – in wheat the most abundant bacterial families are *Sphingobacteriaceae* and *Gemmatimondaceae* (Corneo et al. 2016; Mahoney, Yin, and Hulbert 2017), while core members that were shared among 27 maize lines in three separate fields across a five-year span were all *Proteobacterial* families including *Agrobacterium*, *Bradyrhizobiaceae*, *Devosia*, *Comamonadaceae*, *Pseudomonas*, and *Sinobacteraceae* (Walters et al. 2018). The switchgrass core microbiome is also distinct from these, dominated by *Streptomyces*, *Sphingobium, Bacillaceae, Ralstonia, and Olivibacter* (Singer et al. 2019). Importantly, plant genotypes and environmental conditions were found to have non-negligible influences in rhizobiome composition in each of the above studies. In addition, within a single plant species developmental stage influences the rhizobiome composition. Xiong et al. (2021) studied the rhizobiome of maize and observed an increase in bacterial and fungal richness from the seedling to tasseling stage, which likely was due to the increase in rhizodeposits and plant organic matter present in the soil later in development. This high level of variability confounds the ability to develop widely adoptable plant management strategies. However, due to high levels functional

redundancy among microbial communities (Louca et al. 2018), it may be more important for plant microbiome management strategies to focus on core functionality rather than taxonomic composition within the rhizobiome (Ling, Wang, and Kuzyakov 2022, Matthews et al. 2021).

Among grasses, the border between rhizosphere and bulk soils can be blurred due to the dense, fibrous structure of grass roots (Ravenek et al. 2016). In an extensive meta-analysis, Ling et al. (2022) observed similar alpha diversity between bulk and rhizosphere soils in uncultivated grasslands, although *Poaceae* croplands tended to have higher alpha diversity in bulk soils compared to rhizosphere soils. This difference between grasslands and *Poaceae* croplands is likely due to the greater plant density and species diversity typical of grasslands.

Climate Change

Climate change is having and will continue to have profound effects on soil ecosystem functioning and therefore global agriculture and natural resource management. Increased temperatures and variability in precipitation patterns are posing significant challenges to food production, crop yields, and overall agricultural sustainability (Lesk, Rowhani, and Ramankutty 2016). Understanding the impacts of climate change on plant-microbe interactions is vital to not only adapting agricultural practices to future climate scenarios, but also to improve our ability to model and predict changes in global nutrient cycling.

Drought Stress

Climate change can also disrupt rainfall patterns, leading to changes in precipitation frequency and intensity. Across the globe, some regions are expected to experience more frequent and intense droughts, while others may face increased rainfall and flooding (IPCC

2018). These changes can negatively impact crop water availability, soil moisture levels, and nutrient leaching, affecting crop growth and productivity (Kim and Jehanzaib 2020). Changes in precipitation can also exacerbate soil degradation and erosion processes. Intense rainfall events can lead to increased runoff, soil erosion, and nutrient loss, degrading soil quality and reducing agricultural productivity (Kaur et al. 2020; Wantzen and Mol 2013). Soil erosion in turn impacts water quality in rivers and lakes due to sediment and nutrient runoff (Issaka and Ashraf 2017).

Drought is more damaging to global crop productivity than any other environmental factor (Berdugo et al. 2020; Cattivelli et al. 2008; F. T. de Vries et al. 2016). In a large-scale epoch analysis, Lesk et al. (2016) found that national cereal production was reduced by 10.1% during droughts from 1964-2007 which typically equated to six years of production growth. Reduced photosynthetic assimilation and thereby decreased yield is a hallmark of plant drought stress as stomatal closure to limit water loss is one of the earliest responses (Lawlor and Tezara 2009). Plants can also synthesize a wide variety of osmoprotectants such as prolines and soluble sugars to maintain cell turgor pressure during extended drought stress (Ali et al. 2022).

Transfer of plant carbon to root exudates under drought stress appears to be dependent on plant growth strategy among other factors (Kaštovská et al. 2015). Williams and de Vries (2020) hypothesized that although fast-growing plants may shut down more quickly in response to drought, greater investment in root exudation upon the release from drought may help them out-compete slower growing plants which are not as affected by drought due to a greater investment in fungal associations.

The response of the rhizobiome to drought stress exhibits strong phylogenetic signal with many clades of bacteria responding roughly in unison (Naylor et al. 2017; Xu et al. 2018). Gram

positive bacteria are generally enriched due to their thicker cell walls and the more widespread ability to sporulate which increases their drought stress tolerance over Gram negative bacteria (Nocker et al. 2012). Accordingly, *Actinobacteria, Chloroflexi*, and *Firmicutes* are Gram positive bacteria frequently enriched under drought conditions (Lewin et al. 2016; Xu and Coleman-Derr 2019). There is evidence that these shifts in community composition are plant-mediated as studies such as Castro et al. (2019) have demonstrated strong correlations between the abundance of these lineages and stress-induced plant metabolite production. However, the interacting influences of plant species, soil types, and the conditioning of historic climate conditions complicate our understanding of these plant-microbe interactions, and more research is needed to explore these relationships across species and systems. In Chapters 3 and 4 I explore the effects of increasing drought severity and the influence of climate legacy on the above- and belowground drought responses of blue grama (*Bouteloua gracilis*), a C4 grass native to North American shortgrass prairies.

Heat Stress

Due to increased concentrations of greenhouse gases in the atmosphere, global mean temperature is predicted to increase by 0.3-4.8 C by 2100 (IPCC, 2018). Increased temperatures can lead to heat stress in plants, resulting in reduced photosynthesis and increased water requirements (Jagadish, Way, and Sharkey 2021; X. Li et al. 2018). Temperature variability is also expected to increase with more severe high-temperature spikes (Redden et al. 2014). Extreme heat events can cause crop damage, reduce yields, and result in crop failure (Bakhshandeh et al. 2019; Ortiz-Bobea et al. 2019). At the molecular level, high temperatures can increase the concentration of free radicals and reactive oxygen species in plant tissues which

can result in enzyme denaturation (Ihsan et al. 2019). Heat stress can also alter grain quality; several studies have shown that heat stress tends to decrease starch content in cereals (Yang et al. 2018) and increase protein content (Beckles and Thitisaksakul 2014; Wang and Frei 2011). The timing of heat stress is also important as extreme heat can be particularly damaging at sensitive developmental periods such as early establishment, flowering, and gametogenesis resulting in decreased yields (Jagadish, Way, and Sharkey 2021). In addition, Somayanda et al. (2019) identified 23 °C as the critical threshold for nighttime heat stress beyond which significant decreases in productivity were observed across ten genotypes of winter wheat.

The vast majority of microbes are mesophilic in nature with optimum growth temperatures between 20-45 °C (Maitra et al. 2021). Riah-Anglet et al. (2015) observed that extreme heat stress (50 °C) significantly decreased enzymatic activity and fungal but not bacterial total biomass in both cropland and grassland soil, and the grassland soil community was found to be more resistant to heat stress. However, interpreting the impact of heat stress on soil microbial communities is complicated by the high levels of diversity typical of rhizobiomes. While increases in alpha diversity have been observed after heat stress, this may be due to detection of more rare species previously below sequencing detection limits which are then able to fill niches left by the loss of more dominant organisms under heat stress (van der Voort et al. 2016).

Compounding Effects of Multiple Stressors

Changes in temperature and precipitation patterns can also alter the timing and duration of growing seasons. This can affect crop phenology, disrupt pollination cycles, and impact the synchronization between crops and their natural pollinators. Farmers may need to adjust planting

and harvesting schedules to adapt to shifting growing seasons. Seasonal changes in microbial communities have been documented in many systems (Guo et al. 2022; Waldrop and Firestone 2006a). Changes in timing and duration of growing seasons also have cascading effects on natural ecosystems and their multi-trophic food webs. As climate conditions shift, the suitability and productivity of certain crops in specific regions may change. Some regions may become less favorable for current crops due to increasing temperatures or water scarcity, while other areas may become more suitable. This can require farmers to adapt by shifting their crop choices and cropping systems to match the changing climatic conditions. Changes in forage availability and quality can also impact grazing systems and feed availability for livestock.

Climate change can also influence the geographic range, population dynamics, and behavior of pests and diseases that affect crops. Warmer temperatures can lead to the expansion of pests' ranges, increased pest reproductive rates, and altered patterns of disease transmission. This can result in higher pest and disease pressure, requiring additional pest management strategies and increased pesticide use. The global economic burden of fungal diseases was estimated at $60 billion in 2019 (Fisher et al. 2012). The spread of fungal pathogens is especially concerning as they are notoriously difficult to control. Because fungi are eukaryotes, it is difficult to limit fungal control applications from having deleterious off-target effects on crops or other microbes (Papadopoulou et al. 2022).

Adaptation and mitigation strategies are crucial to minimize the impacts of climate change on agriculture. These may include developing climate-resilient crop varieties, improving water management practices, implementing sustainable soil and land management techniques, adopting precision agriculture technologies, promoting agroforestry and conservation farming

practices, and supporting farmers with climate information and knowledge to make informed decisions.

Managing the Soil Microbiome

Beneficial microbes in agricultural practices can enhance soil fertility, reduce the dependence on chemical inputs, improve crop yields, and promote sustainable farming methods (Bender, Wagg, and van der Heijden 2016). Adapting crops and their microbiomes to current and future climate scenarios is a current priority in agriculture.

Biofertilizers and Bioinoculants

Microbial-based biofertilizers and inoculants (hereafter biofertilizers and bioinoculants) are typically bacteria and fungi cultivated and applied to plants to promote plant growth and/or health (plant growth promoting microbes, PGPM). The biofertilizer market is rapidly growing as interest in sustainable fertilizers and climate adaptation increases. Valued at $3.0B in 2023, the biofertilizer market is expected to increase 12.1% between 2023 and 2029 (Mordor Intelligence 2023). Inoculating crops with PGPM can help mitigate the effects of climate stress and promote plant adaptation. This can improve nutrient availability and uptake efficiency, reducing the need for synthetic fertilizers and promoting sustainable nutrient management. Some of the most common PGPM include *Streptomyces sp., Pseudomonas sp., Burkholderia sp*. *Cyanobacteria,* and AM fungi. Many of these organisms have been developed for use as inoculants to assist in plant growth promotion.

In Chapter 2 I discuss the use of a nitrogen-fixing cyanobacteria as a biofertilizer to support the growth of second-generation bioenergy crops with the goal of decreasing the overall

carbon footprint of producing energy, while also benefiting the soil microbiome and biogeochemistry. Theoretically, if a nitrogen-fixing PGPM could be established on the soil surface it could provide soil stability through the formation of a biocrust and an inexhaustible source of plant-available N (and C from microbial turnover and stimulation of decomposers to process plant litter). The combination of the biofertilizer with perennial crops such as switchgrass or tall wheatgrass is especially promising for soil carbon sequestration due to the limited need for tillage (Gay et al. 2022). However, most non-legume crop plants cannot rely on biologically fixed N alone and will still need additional fertilization to achieve production goals.

Some microbes can also help alleviate drought stress. These microbes can help mitigate the effects of drought and support plant water availability, reducing irrigation requirements and enhancing water-use efficiency. AMF are particularly well-suited as PGPM to alleviate drought stress as the small size of mycelial hyphae in relation to fine roots (2-20 uM vs. < 2 mm) allows the hyphae to penetrate smaller soil pores to access water and transport it back to the larger plant roots (Aufrecht et al. 2017; Fricker et al. 2017). *Streptomyces* (*Actinobacteria*) utilized as bioinoculants have also been shown to contribute to increased water use efficiency (Ardakani et al. 2011), synthesis of plant growth hormones (Solans et al. 2011), and generation of reactive oxygen species scavengers and osmoprotectants (Hasanuzzaman et al. 2013).

Due to the threat of increased temperatures as discussed above, there is also interest in integrating microbial heat stress tolerance traits into agriculture. In Chapter 5 I explore the rhizosphere of a highly heat-tolerant grass, *Dichanthlium lanuginosum*, using both amplicon and metagenomic sequencing to identify possible beneficial microbes and functional genetics that

contribute to the plant's ability to grow in soils up to 65 °C near hot springs in Yellowstone National Park.

Importantly, there is much debate about the use of bacterial and fungal inoculants to benefit agricultural production, especially regarding the release of genetically modified species. It is impossible to predict the long-term consequences of introducing non-native and/or bioengineering organisms into new environments. One possibility is that they will simply die and not have any effect, positive or negative, other than providing some amount of C into the system. Another possibility is the over-establishment of these organisms to the detriment of the overall biodiversity of the ecosystem. Once again, the incredible complexity of the plant-microbe-soil system makes determining which of these outcomes are more likely nearly impossible. In addition, while PGPM often perform well in greenhouse and pilot studies, due to the complexity of microbe-microbe, plant-microbe, plant-soil-microbe, and plant-soil-microbe-climate interactions, many commercial inoculants have limited success outside of the climate and crop systems where they are developed. It is unlikely that a "silver bullet" PGPM exists due to the plasticity and multifunctionality of most all microorganisms. Rather, the development of crop-specific, or perhaps even field-specific microbial consortia has been proposed. However, developing individualized consortia scalable to commercial farming operations poses a significant logistical and economical challenge.

In summary, microbial communities associated with plants can influence their resilience to climate stress. Harnessing knowledge about plant-microbe interactions can inform crop breeding programs to develop climate-resilient varieties that can better withstand extreme temperatures, water stress, and other environmental challenges. Additionally, research and

innovation in microbial ecology, microbiome analysis, and biotechnological applications can further advance the utilization of microbes for climate-smart agriculture.

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CHAPTER TWO

ABOVEGROUND AND BELOWGROUND RESPONSES TO CYANOBACTERIAL BIOFERTILIZER SUPPLEMENT IN A SEMI-ARID, PERENNIAL BIOENERGY CROPPING SYSTEM

Contribution of Authors and Co-Authors

Manuscript(s) in Chapter(s) 2

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ORIGINAL RESEARCH

Aboveground and belowground responses to cyanobacterial biofertilizer supplement in a semi-arid, perennial bioenergy cropping system

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Abstract

The need for sustainable agricultural practices to meet the food, feed, and fuel demands of a growing global population while reducing detrimental environmental impacts has driven research in multi-faceted approaches to agricultural sustainability. Perennial cropping systems and microbial biofertilizer supplements are two emerging strategies to increase agricultural sustainability that are studied in tandem for the first time in this study. During the establishment phase of a perennial switchgrass stand in SW Montana, USA, we supplemented synthetic fertilization with a nitrogen-fixing cyanobacterial biofertilizer (CBF) and were able to maintain aboveground crop productivity in comparison to a synthetic only (urea) fertilizer treatment. Soil chemical analysis conducted at the end of the growing season revealed that late-season nitrogen availability in CBF-supplemented field plots increased relative to urea-only plots. High-throughput sequencing of bacterial/archaeal and fungal communities suggested fine-scale responses of the microbial community and sensitivity to fertilization among arbuscular mycorrhizal fungi, Planctomycetes, Proteobacteria, and Actinobacteria. Given their critical role in plant productivity and soil nutrient cycling, soil microbiome monitoring is vital to understand the impacts of implementation of alternative agricultural practices on soil health.

KEYWORDS

barley, biodiversity, cyanobacteria, microbiome, nitrogen, soil, switchgrass

1 | INTRODUCTION

Over the last century, conventional agricultural practices, including synthetic fertilization of nitrogen (N), tillage, and the use of chemical herbicides, have benefited humanity by drastically improving agricultural productivity, but at a significant cost to soil and ecosystem health (Chen et al., 2014; Rockström et al., 2009). For example, agricultural intensification and mismanagement of fertilizer application have led to soil acidification, decreased

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soil organic carbon (SOC), nutrient loss through leaching and gaseous emission, and declines in microbial diversity and functioning (Barak et al., 1997; Tian et al., 2020; Tsiafouli et al., 2015). The degradation of soil microbial communities in particular has become an increasing concern as microbes including bacteria, archaea, and fungi are critical regulators of soil organic matter dynamics, nutrient availability, and subsequent crop productivity (Saleem et al., 2019; Singh et al., 2020; van der Heijden et al., 2008; Wagg et al., 2014). To mitigate or reverse these effects, the expansion in both research and practice of sustainable cropping systems has become a focus to address negative environmental impacts while meeting, or surpassing, conventional cropping system yields (Hobbs et al., 2008). The use of perennial cropping systems and the use of microbial biofertilizers as supplements to synthetic fertilizers are two emerging strategies to address the negative impacts of agriculture on the environment, and specifically the mismanagement of nitrogen (N) fertilizer (Robertson & Vitousek, 2009). These approaches provide multiple key ecosystem services (improved C cycling and microbial diversity, reduced nutrient loss) in comparison to conventional agricultural practices (Jesus et al., 2016; Robertson & Vitousek, 2009; Werling et al., 2014), and in combination could result in dramatically improved sustainability.

Perennial crop cultivation has increased significantly in the United States in recent years, particularly in the use of switchgrass (*Panicum virgatum*) for cellulosic bioenergy production (Mitchell et al., 2008; Robertson et al., 2017; Wright & Turhollow, 2010). Switchgrass cropping systems may also play an integral role with emerging climate-smart technologies such as bioenergy with carbon capture and storage (BECCS) because deep-rooting perennial plants transfer large amounts of carbon belowground (Jarchow et al., 2015; Stoy et al., 2018). While it remains unlikely that any single species will become a universal feedstock for bioenergy production (Jessup, 2009), perennial grasses have many environmental advantages (enhanced biodiversity, pest suppression, and pollination) compared to annual species (de Oliveira et al., 2018; Fazio & Monti, 2011; Lai et al., 2018; Pugesgaard et al., 2015; Werling et al., 2014). Furthermore, there is high economic potential for some perennial bioenergy crops (Adler et al., 2007; Wright & Turhollow, 2010), and although spatial and temporal variations in yield can be high (Schmer et al., 2009), consideration of regional biophysical factors during species selection can help optimize production. However, the net benefits of conversion to perennial bioenergy systems depend largely on the retention of ecosystem carbon (C) and N (Robertson et al., 2011; Robertson & Vitousek, 2009). While perennial grass systems tend to accumulate soil organic carbon (SOC) when converted from annual-based systems (Lemus & Lal, 2005; Liebig et al., 2008), variability in SOC accrual has been documented across agroecosystems (Ye & Hall, 2019). In modeling scenarios developed by Dolan et al. (2020), future climate projections indicated that much of the Upper Missouri River Basin (UMRB) of North America has high potential for second-generation bioenergy production. However, the productivity of switchgrass, a C4 species adapted to warm climate regions (Sanderson et al., 2006), has not been rigorously documented in field experiments across agricultural regions of the semi-arid UMRB. Given the extent of marginal land and relatively low quality of soils across the UMRB, the ecosystem services provided by perennial cropping systems could be highly beneficial (Emery et al., 2017; Gelfand et al., 2013). As in all bioenergy cropping systems, nutrient, and specifically N, loss in annually harvested biomass must be matched with appropriate annual inputs. Thus, combining a perennial cropping system with a biofertilizer might offer a sustainable approach to replacing annual N loss (from harvest) through the input of an initially less reactive form of N that is not as likely to be lost quickly from volatilization or leaching.

Biofertilizers are inoculants of living microorganisms applied to plants or soil to support plant growth by increasing nutrient availability (Bhattacharjee & Dey, 2014; Pellegrino et al., 2015). N-fixing cyanobacteria are gram-negative bacteria that often exhibit filamentous growth and are widespread in soil and aquatic habitats, and that have been used as biofertilizers for many years in rice paddy operations (Alvarez et al., 2021; De, 1939; Prasanna et al., 2015). They also can promote the growth of a wide variety of terrestrial crops with additional benefits to soil quality (Nain et al., 2010; Swarnalakshmi et al., 2013; Watanabe et al., 1979). Cyanobacterial-based biofertilizers (CBF) have been shown to (1) maintain plant productivity when used as a supplement to replace 25%–50% synthetic NPK fertilizer (Chittapun et al., 2018; El-Beltagy et al., 2016), (2) increase SOC through photosynthesis and biomass turnover (Yilmaz & Sonmez, 2017), (3) decrease soil erosion through the production of exopolysaccharides (Falchini et al., 1996; Malam Issa et al., 2006), and (4) increase a variety of soil enzymatic activities (Nisha et al., 2018). In addition, when compared to synthetic fertilizers, CBF provides a more gradual release of nutrients to the soil which can benefit crop growth and quality (Coppens et al., 2016; Mulbry et al., 2007). In contrast, there is ample evidence that urea application, which accounts for >55% of global N production and is the most dominant crop N fertilizer (FAO, 2019), is susceptible to large losses of ammonia (Pan et al., 2016) and nitrous oxide (Bouwman et al., 2002) to the

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atmosphere and nitrate to groundwater (Di & Cameron, 2002). In regards to soil N dynamics in bioenergy cropping systems, if fertilization of N is managed to match crop needs (Ruan et al., 2016), perennial systems have a greater potential for N retention in comparison to annual crops via reduced loss of nutrients through leaching, runoff, and greenhouse gas flux coupled with increased N use efficiency (Culman et al., 2013; Liebig et al., 2008). While the reported benefits of CBF are numerous, the effects of CBF on the soil microbial community and nutrient cycling in field studies remain a large knowledge gap. The vital roles soil microbes play in decomposition and nutrient cycling make understanding the impact of biofertilizers on native soil microbial communities critical to their successful implementation in sustainable agricultural systems.

Together, perennial bioenergy crops and biofertilization could significantly promote agricultural sustainability by improving nutrient retention and microbially mediated ecosystem processes. To our knowledge, this is the first study to investigate these strategies in tandem. The goal of this study was to analyze aboveground and belowground treatment effects of CBF on perennial crop establishment, soil microbial community diversity, N availability, and SOC in a field-based, randomized plot experiment. We hypothesized that (1) supplementing synthetic fertilizer with CBF would maintain aboveground biomass production compared to synthetic fertilization alone and that the slower mineralization rate of CBF would benefit late-season crop productivity, (2) CBF supplementation would prolong N availability, and (3) CBF supplementation would benefit microbial diversity compared to synthetic fertilization alone and response to fertilization would be phylogenetically conserved in bacterial/archaeal and fungal communities.

To test these hypotheses, we compared aboveground and belowground responses of switchgrass field plots treated with synthetic urea fertilizer, a mixture of urea and CBF, or untreated (controls). As perennial grasses often take a year to establish and can suffer from weed competition in the early establishment phases, barley was intercropped to outcompete weeds and served as an early indicator of fertilizer effects, as barley has a higher N demand than switchgrass (Ball & O'Sullivan, 1987; Lemus et al., 2008). To preserve the switchgrass stand integrity during establishment, we measured aboveground productivity non-destructively using the Normalized Difference Vegetation Index (NDVI) as a proxy for aboveground biomass (Garroutte et al., 2016). Soil chemistry and microbial diversity using high-throughput sequencing were measured throughout the growing season to determine the chemical and biological effects of CBF implementation.

2 | MATERIALS AND METHODS

2.1 | Site description and experimental design

Field plots are located at the Montana State University (MSU) Arthur H. Post Research Farm (45.66 N, −111.15 W) located 10 km west of Bozeman at 1450 m elevation where mean annual temperature and precipitation are 6.5°C and 408 mm year^{-1} (Figure S1). The soil at the Post Farm is broadly classified as an Amsterdam silt loam which is a fine-silty, mixed, superactive, frigid Typic Haplustoll (100 g kg^{-1} clay, 810 g kg^{-1} silt, 90 g kg^{-1} sand; Engel et al., 2017). The preceding crop was Proso millet (*Panicum milaceum*) which was fertilized with 67 kg N ha^{-1} urea in spring 2017. Barley (*Hordeum vulgare*, Hayse forage variety) and switchgrass (*Panicum virgatum*, Dakota variety) were seeded on April 16, 2018 at 16 kg ha^{-1} with a no-till disk seeder. Three treatments were established across replicated (n = 5) 3.34 m^2 plots: (1) 100 kg N ha^{-1} urea (urea), (2) 100 kg N ha^{-1} 50:50 CBF/urea mix (CBF:Urea), and (3) control plots that received no experimental N addition (control). To minimize edge effects, the plots were arranged in a randomized block design with buffer alleyways (1.2 m) between replication rows that were seeded but left untreated (Figure S2). We elected to supplement the synthetic fertilizer with a half-dose of CBF as recent literature found replacing 50% of the synthetic fertilizer application with CBF resulted in the greatest yields of various crops compared to synthetic fertilizer alone (Chittapun et al., 2018; Naher, 2018; Nain et al., 2010). Notably, we were limited by production capacity to expand the field plot treatments to include a CBF-only treatment to separate CBF effects from urea effects. However, this design allowed us to build off of the existing literature and study CBF in a practical manner similar to what producers would typically utilize (Naher, 2018). Fertilizer application timing was based off common regional application practice for urea and was applied on April 22nd 2018 (Jacobsen et al., 2005). Urea plots were hand-spread and received all 100 kg ha^{-1} in April, whereas CBF:Urea plots only received 50 kg ha^{-1}. The remaining 50 kg ha^{-1} of CBF were split into five application periods (June 2, June 15, June 28, July 12, and August 09) applied in 10 kg ha^{-1} increments explained in detail below. Supplemental sprinkler irrigation (n = 4 events), totaling ~112 mm of precipitation equivalent, was applied after fertilization applications to all plots. This was done to help the biofertilizer infiltrate below the soil surface when natural precipitation events were lacking. Due to cost constraints, soil chemistry and microbial community results are reported from a subset of 3 out of the 5 treatment replicates.

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2.2 | CBF cultivation

From previous screening of several cultured representatives of N-fixing cyanobacterial species, we selected *Anabaena cylindrica* (UTEX 1611) for use as CBF as we verified that it grows robustly in liquid media, has a relatively high N fixation rate in liquid culture, and does not produce any known cyanotoxins (Weeks, 2013). Elemental analysis was conducted on an EA Flash 2000 and indicated that CBF biomass contained 9% N, 1% P, 1% K, and 50% C. For each application period, we cultivated a total of 800 L liquid culture using 3 × 200 L raceway ponds and 4 × 50 L bag reactors (Figure 1). At the time of harvest, bags and raceways were pumped into conical tanks to gravity settle overnight before harvesting the concentrated biomass. Dry biomass weights (in mg/L) were measured in triplicate by vacuuming the concentrated culture onto 0.2 μM filters and drying overnight at 60°C. Dry weights were used to calculate the volume of culture to be applied to each plot. CBF was cultured for 2 weeks prior to harvesting, resulting in five CBF applications over the growing season. Due to limited production capacity, living CBF biomass was supplemented with commercially available, dried *Spirulina* (*Arthrospira platensis*) biomass (10% N, 1% P, 1% K) at a final biomass ratio of 20:80 *A. cylindrica*:*Spirulina* to meet the goal of 50% CBF-derived N for the CBF:Urea supplement treatment. The multiple smaller applications and mixture with *Spirulina* were necessary due to limitations in culture volume of *A. cylindrica*.

2.3 | Sampling and analyses

Crop productivity and growth were monitored in situ using a novel remote sensing device (Arable, USA) designed to measure a suite of crop management indices including hourly spectrometer data. A wide variety of vegetation indices are used to assess vegetation/crop status via remote sensing devices metrics. Here, we use the Normalized Difference Vegetation Index (NDVI), one of the most widely used vegetation index, that has been correlated to aboveground biomass, canopy area, and leaf N content (Cabrera-Bosquet et al., 2011; Teal et al., 2006; Tucker, 1979), where NDVI = (NIR − red)/(NIR + red) and NIR is the fraction of near-infrared radiation that is emitted back to the sensor and red is the fraction of red radiation emitted back to the sensor. The index reports values from −1 to 1 and generally represent the vegetative greenness within the spectrometer's footprint. Arable Mark devices were installed directly following seeding on April 26, 2018. Arable Marks were mounted on 2 m tall steel posts (7.5 cm diameter) in the center of each plot, installed at 1 m above maximum crop height, and stayed in place for the duration of the growing season (Figure S3). In all, six Arable Marks were deployed for the study across two randomly selected plots from each treatment. The raw mean daily NDVI values from each Arable sensor were aggregated by month to test for statistical differences between treatments across the growing season. Barley biomass was harvested July 23, 2018 at peak forage biomass by shearing 10 cm above ground surface in 1 m^2 blocks at the center of each plot. The biomass was air-dried for 1 week at 40°C and subsequently weighed as dry biomass.

Field plots were sampled prior to seeding in April 2018 to establish background soil conditions and then monthly to capture temporal and compositional variance in the soil microbial community and shifts in N availability. Due to the small area of individual plots, one fixed-depth soil core (Oakfield Apparatus) was taken from each plot and broken up into 0–15 and 15–30 cm soil depth increments. Three plot replicates were haphazardly selected for soil chemical and microbial community analyses. The soil was then homogenized in separate pools by depth and each pool was subsampled for microbial and chemical analyses. The biological subsamples were immediately flash frozen in an ethanol-dry ice bath and temporarily stored on dry ice before transfer to long-term storage at −80°C. Before soil processing, a small subsample was separated and massed before being dried at 105°C for 48 h and massed again for volumetric soil moisture content (Figure S4). To determine bulk soil total nitrogen (TN) and soil organic carbon (SOC), samples were immediately transported back to the lab and oven-dried at 65°C for at least 24 h, passed through a 2 mm sieve, and milled for 12 h in stainless steel jars on a



FIGURE 1 Cyanobacterial culturing systems in the Montana State University Plant Growth Center greenhouse: 200 L raceway ponds (left) and 50 L photobioreactors (right)

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slow rolling tumbler. Samples were prepared in duplicate as SOC quantification required acidification of the soil using 1 and 2 M HCl to remove the inorganic C fraction. After drying for 48 h, %C and %N were determined using a Costech ESC 4010 elemental analyzer. Duplicates were run for ~10% of all samples to ensure analytical precision. Inorganic nitrogen (NO_3^--N and NH_4^+-N) was processed within 12 h of soil collection by using ~10 g of the homogenized field moist soil core, with coarse/fine root material removed, and then extracted in 100 ml of 2 M KCl by shaking at 200 RPM for 30 min. Following shaking, the samples were filtered through Whatman grade-2 filter paper and frozen until analysis. We determined extractant concentrations of NO_3^--N and NH_4^+-N colorimetrically using an automated flow injection system (Lachat QuickChem 8500, Hach Company). NH_4^+-N was determined using the indophenol method while NO_3^--N was determined using a cadmium column reduction and coupled colorimetry. Soil pH was measured in 2:1 deionized water to soil solution using a glass electrode (Mettler Toledo).

2.4 | DNA extraction, amplification, and sequencing

To obtain high-resolution representation of the soil microbial community and its response to fertilization, we performed rRNA gene amplicon sequencing across three of the treatment replicates at three time points ($n = 54$). The selected cores were specifically paired with those used for inorganic-N extraction to directly link measures of the microbiome and N availability. Genomic DNA was extracted from 0.5 g frozen soil samples using the Fast DNA SPIN Kit for soil (MP Biomedicals) according to the manufacturer's instructions. We targeted the V4 region of the 16S rRNA gene using f515a and r806b (GTGYCAGCMGCCGCGGTAA and GGACTACVSGGGTATCTAAT, respectively) from the Earth Microbiome Project (Thompson et al., 2017), and the D2 hypervariable region of the fungal LSU using LR22r and LR3 (Mueller et al., 2016). PCRs were carried out in 20 μl volumes with each standard reaction mix containing 1 U Phusion high-fidelity polymerase (ThermoFisher Scientific), a final concentration of 1x Phusion HF reaction buffer, 200 μM dNTPs, 0.5 μM each primer, 1 μM BSA, 1 mM MgCl, sterile molecular grade water, and 5 μl (10 ng) genomic DNA as a template. PCR conditions consisted of initial denaturation at 98°C for 30 s, followed by 22 cycles of 98°C for 30 s, 55°C for 30 s, 72°C for 45 s and a final extension at 72°C for 5 min. Barcoding was conducted using the Illumina Nextera Indexing Kit D, with eight cycles of indexing PCR in 20 μl volumes using the same concentrations as PCR1 with 10 μl template DNA. PCR products were checked for quality and length in a 1% agarose gel and quantitated using the Quant-iT™ dsDNA Kit (Invitrogen) with a BioTek H2 plate reader. The two libraries were pooled at 4 nM and loaded onto an in-house Illumina MiSeq (Illumina). Reads were merged, trimmed, and dereplicated with USEARCH, and zero-radius operational taxonomic units (ZOTUs) were identified with UNOISE3 (v.11.0.667; Edgar, 2010). Similar to amplicon sequence variants, UNOISE3 identifies ZOTUs by correcting point errors and filtering out chimeric amplicons to obtain accurate predictions of true biological sequences that are superior to utilizing a 97% identity cutoff (Callahan et al., 2017). Representative 16S and LSU ZOTUs were classified against their respective databases using the online Bayesian classifier of the Ribosomal Database Project (Set 18 for bacteria, Set 11 for fungi; Wang et al., 2007). All reads classified to chloroplast (16S), or animalia, protozoa, or viridiplantae (LSU) were removed prior to analysis, and datasets were randomly subsampled according to the sample with the lowest read number for statistical analysis using the R package *vegan* (Oksanen et al., 2019). The data are accessible at MG-RAST under accession numbers: mgm4916997.3 (LSU) and mgm4916998.3 (16S).

2.5 | Phylogenetic trees

Reference phylogenies for the 16S and fungal LSU were constructed using full-length and near full-length sequences downloaded from GenBank. For fungi, we focused on sequences associated with the Assembling the Fungal Tree of Life project (Kauff et al., 2007). For bacteria and archaea, we used sequences from both isolates and metagenome assemblies to better cover the tree of life, including a subset of the Candidate Phylum Radiation. Reference sequences were aligned using mafft (Katoh & Standley, 2013; Stamatakis, 2006) and maximum likelihood trees were constructed using RAxML with the GTR + gamma model. ZOTUs were aligned and placed onto their respective reference phylogenies using pplacer (Matsen et al., 2010). Trees were visualized and annotated using the interactive tree of life (iTOL; Letunic & Bork, 2019).

2.6 | Data analysis

To test the effect of fertilizer treatment on soil chemistry by month, we used linear mixed models using fertilizer treatment and depth as fixed effects and plot replicates as a random effect (to account for the non-independence of replicate soil samples taken within a replicate) using the lme4 package (Bates et al., 2015). To assess whether there

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were differences in soil chemistry based on fertilizer treatment, we performed type III ANOVA; if fixed effects accounted for variation, we compared post-hoc differences using pairwise Tukey comparisons using the emmeans package (Lenth et al., 2020). For NDVI analysis, we fit linear models with a fixed effect of treatment and fit one-way ANOVA's. If treatment accounted for variation, we used Tukey's comparison to examine pairwise comparisons for fertilizer treatment effects. We report all soil chemistry and NDVI results as model mean values (±1 SE) and C:N as molar ratios. For soil dissolved inorganic nitrogen (DIN; NO_3^--N and NH_4^+-N) analysis, we compared concentrations at the individual soil depths (0–15 and 15–30 cm) as well as the mean aggregated total depth (0–30 cm). pH is reported as the mean of hydrogen ion concentrations followed by a log10 back transformation with uncertainty reported as a 95% confidence interval (CI). For NO_3^--N and NH_4^+-N samples that were under the detection limit, we substituted a value that was ½ the lower detection limit. All data were assessed for influential points, homogeneity of variance, and normality. Log transformations were performed on data that did not fit the assumption of normality. Treatment differences were declared significant at $p = .05$ for NDVI and microbial community analyses and $p = .10$ for NO_3^- and NH_4^+ because of the inherent temporal and spatial variability of soil N transformations and the limited number of cores taken per plot (Moebius-Clune et al., 2008).

We calculated alpha diversity and beta diversity of the bacterial/archaeal and fungal communities using the phyloseq package (McMurdie & Holmes, 2013) with three separate methods for alpha diversity: ZOTU richness, Shannon's diversity index (Shannon, 1949), and Faith's Phylogenetic Diversity (PD; Faith, 1993), and unweighted UniFrac for phylogenetic-based beta diversity (Lozupone & Knight, 2005). We used PERMANOVA to test for treatment, soil depth, and seasonal effects on community beta diversity (Anderson, 2017). To assess individual ZOTU responses to fertilizer treatment, response ratios were calculated by taking the difference between the read count for each ZOTU in a fertilized treatment and that of the control, non-fertilized field plots divided by the total number of reads for that ZOTU in both the treatment and control:

$$\frac{\text{ZOTU sum Treatment} - \text{ZOTU sum Control}}{\text{ZOTU sum Treatment} + \text{ZOTU sum Control}}. \quad (1)$$

The response ratio calculation was permuted 999 times for each ZOTU and averaged to acquire a final response ratio. The output of Equation (1) is the responses on the scale from −1 to 1. Response classifications were determined by splitting the responses into quartiles. To investigate differences in response ratios on the scale of individual ZOTUs, the vegan package was utilized to conduct SIMPER analysis of ZOTUs driving the differences between the response ratios in treatment groups (Oksanen et al., 2019). The phylosig package was utilized to test Pagel's lambda and Abouheif's C_{mean} measures of phylogenetic signal (Keck et al., 2016). Prior to calculation, ZOTU tables were trimmed to include only ZOTUs with 10 or greater total reads to reduce erroneous classifications due to sampling error. We performed all analyses in R version 3.6.1 (R Core Team, 2019).

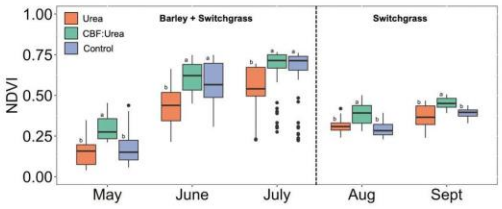


FIGURE 2 Box plots (median, 5th and 95th percentiles) of daily mean NDVI values ($n = 980$) aggregated by month across the three fertilizer treatments. The dashed line represents when the barley intercrop was harvested. Differences ($p < .05$) between aggregated daily means (Table S1) are denoted by letter signifiers

3 | RESULTS

3.1 | Barley and switchgrass growth

Fertilizer treatment influenced NDVI throughout the growing season ($F_{10,952} = 5.37$, $p < .0001$). Compared to the control and urea treatments, mean daily NDVI values were higher in the CBF:Urea treatments during the second half of the 2018 growing season (Figure 2; Table S1). Barley plant biomass was similar between the urea and CBF:Urea treatments ($p = .189$) with 8806 ± 1362 and 7288 ± 774 kg ha^{-1}, respectively (Figure S5). However, both urea and CBF:Urea treatments significantly increased aboveground plant biomass compared to the unfertilized control at 4170 ± 1143 kg ha^{-1} ($p = .001$ and $p = .02$, respectively).

3.2 | Soil chemistry

Prior to experimental treatments, soils at the 0–15 cm soil depth had a pH of 8.0 (95% CI: 7.8–8.2) and low background SOC (1.00% ± 0.03) and TN (0.11% ± 0.002), and a C:N of (10.42 ± 0.45). For the 15–30 cm soil depth, we report a pH of 8.0 (95% CI: 7.8–8.4), SOC of (0.50% ± 0.03), and TN of (0.07% ± 0.002) and a C:N of (7.37 ± 0.45). SOC and C:N

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ratios at the 0–15 cm soil depth declined by 16% and 10% across all treatments ($F_{1,14}$ = 22.88, p = .0029, $F_{1,14}$ = 6.02, p = .025) from pretreatment (April) to the end of the season (September). Total soil N and soil pH did not vary across fertilizer treatments or through time (Tables S2 and S3).

Experimental treatments had two major effects on soil DIN across the cumulative growing season (May–September). First, the CBF:Urea treatment resulted in overall higher mean concentrations of NH_4^+-N compared to unfertilized controls (0–30 cm, p = .01), whereas the urea treatment concentrations were not different than the unfertilized controls (0–30 cm, p = .36). There was suggestive evidence that this effect was stronger at the 0–15 cm depth (p = .09). Second, the urea treatments had higher mean concentrations of NO_3^--N at the 15–30 cm depth compared to the unfertilized controls (p = .05) while there was no difference between the CBF:Urea and control treatments (p = .43).

Additionally, we found evidence for seasonal influence over fertilizer treatment effect on DIN ($F_{4,139}$ = 13.95, $p \leq .0001$; Figure 3). During the spring (May and June), fertilizer treatment affected the NO_3^--N concentrations at both soil depths ($F_{2,54}$ = 12.3, $p \leq .001$, $F_{2,54}$ = 3.37, p = .071; Figure 3). Urea concentrations were greater than the unfertilized control (p = .006, Figure 3) at the 15–30 cm depth while there was no difference between the CBF:Urea and control treatments (p = .16, Figure 3). In contrast, when just analyzing the spring months (May and June) NH_4^+-N concentrations, we found no differences among fertilizer treatment or depths. During the late season (August and September), we report evidence that fertilizer treatment affected DIN concentrations ($F_{2,53}$ = 5.11, p = .009). The CBF:Urea treatment had higher concentrations of NH_4^+-N compared to the unfertilized controls (p = .007; Figure 3) with marginal evidence of a difference between CBF:Urea and urea treatments (p = .12). Furthermore, CBF:Urea was the only treatment with a difference in NH_4^+-N concentrations between the two soil depths (p = .02), with higher concentrations at 0–15 cm. In contrast, when analyzing just the late season, we did not find any differences in NO_3^--N concentrations among fertilizer treatment or soil depths.

3.3 | Microbial community characterization

In total, we sequenced the 16S and LSU communities from 54 soil samples throughout the growing season in 2018. We obtained 17,449 ZOTUs with 18,073–175,266 (mean 67,329) reads per sample for the 16S rRNA gene and 6,574 OTUs with 16,699–127,884 (mean 54,498) reads per sample of the LSU rRNA gene fungal subset. The most abundant bacterial phyla observed in this collective community included Proteobacteria (22%), Acidobacteria (17%), Actinobacteria (16%), and Bacteroidetes (12%; Figure 4a). Composition of the bacterial/archaeal community at the phylum level was stable across time, depths, and treatments (Figure 4a). The fungal community comprised Ascomycota (38%), Basidiomycota (21%), Glomeromycota (21%), Chytridiomycota (9.7%), Blastocladiomycota (2.9%), and the remaining were unclassified fungi incertae sedis (6.21%; Figure 4b). The Glomeromycota was the most dynamic phylum which increased in relative abundance over time from 13% in April to 32% in September

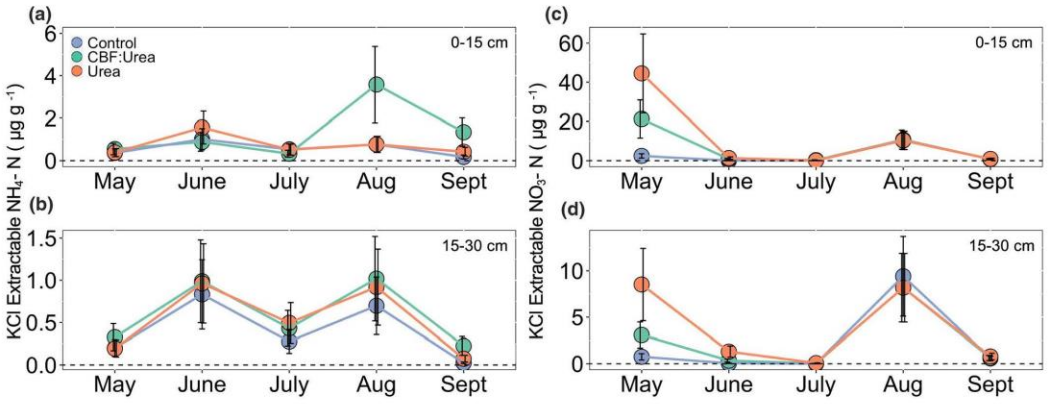


FIGURE 3 Time series of extractable KCl NH_4^+-N and NO_3^--N across the growing season at two soil depths, 0–15 cm (a, c), and 15–30 cm (b, d) (Tables S4 and S5). Note differences in y-axis scales with lower concentrations of both NH_4^+-N and NO_3^--N at the 15–30 cm depth

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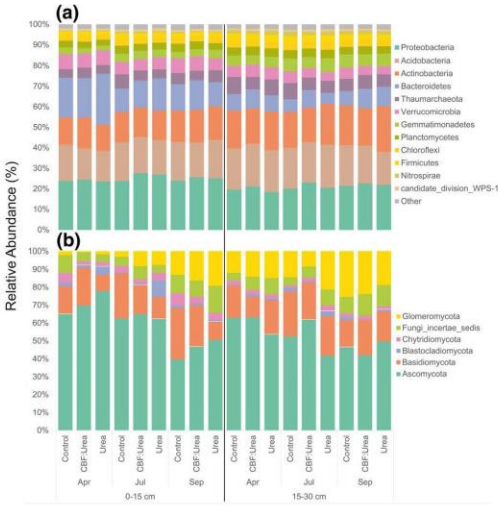


FIGURE 4 Relative abundance at phylum level of (a) bacterial/archaeal and (b) fungal community phyla by treatment, soil depth, and time

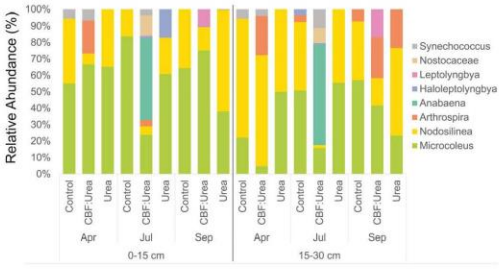


FIGURE 5 Relative abundance of cyanobacterial genera across time, treatments, and soil depth

while the Ascomycota decreased from 44% to 30% in this timeframe. In addition, Glomeromycota had a greater relative abundance in the deeper soil in general (30% vs. 15%) and was also slightly greater in the urea-treated plots than in the CBF:Urea and control plots (25% vs. 18% and 22%, respectively).

3.4 | Cyanobacterial response to fertilizer treatment

Cyanobacteria were rare, averaging only 0.07% of the total community over the entire season. At the genus level, the cyanobacteria were dominated by *Microcoleus* sp. and *Nodosilinea* sp., and BLASTn of these ZOTUs indicated that *Microcoleus vaginatus* was the primary species present (Figure 5). The CBF species *Anabaena cylindrica* was not detected in any field plots prior to application and was only detected in CBF:Urea plots in July.

3.5 | Bacterial/archaeal response to fertilizer treatment

As shown in Figure 6, alpha diversity of the bacterial/archaeal community had similar trends among diversity metrics with no strong evidence for differences by treatment, time, or soil depth except for phylogenetic diversity (Figure 6a,b). There was strong evidence of a seasonal shift in phylogenetic alpha diversity with an increase from April to July with community mean branch lengths of 802 ± 18 and 875 ± 16 (SE), respectively ($F_{(2,48)} = 5.41$, $p = .008$; Figure 6b). September PD was similar July PD with a mean branch length of 853 ± 14. CBF:Urea plots trended toward greater alpha diversity although there was high variability between plots. The beta diversity of the bacterial/archaeal community indicated a strong separation by soil depth ($F_{(2,48)} = 3.49$, $p = .003$) but not by treatment ($p = .939$) or time ($p = .088$; Figure S6a, Table S6), suggesting that distinct communities were present at each soil depth, but the respective levels of diversity were similar.

Response ratios of the changes in relative abundance of ZOTUs across the phylogenetic tree indicated that bacteria generally had neutral responses across the phylogenetic tree (Figure 7a). However, Pagel's λ indicated a significant phylogenetic signal in the bacterial/archaeal ($\lambda = 0.23$, $p = 2.60e^{-101}$) and confirmed with Abouheif's C_{mean} (Table S7) meaning that response ratios of closely related ZOTUs were more similar to each other than would be expected under random trait distribution. Density plots of response ratios confirmed that the bacterial/archaeal community had primarily neutral to weak responses (Figure 7a insert) and that the differences in responses between the two fertilizer treatments were slight. However, the CBF:Urea treatment had slightly more strong negative and weak-to-moderately strong positive responses than the urea treatment while the urea treatment had more neutral responses. Through our SIMPER analysis, we identified 12 ZOTUs driving the differences in response ratios between the urea and CBF:Urea bacterial/archaeal community (Table S8; $p < .001$). The four most influential ZOTUs contributing to were classified to Planctomycetaceae and two Actinobacterial families, Micromonosporaceae and Acidimicobiaceae while the others also included Proteobacteria, Candidatus Uhrbacteria, Acidobacteria, Tenericutes, Abditibacteriota, and Bacteroidetes.

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3.6 | Fungal response to fertilizer treatment

Similar to the findings for the archaea/bacteria, we also observed also high variability in the alpha diversity of the fungal community with weak evidence for a difference between treatments regardless of the diversity metric. However, there was evidence for a difference in taxonomic richness over time ($F_{(2,48)} = 5.59$, $p = .007$; Figure 6c). The number of fungal ZOTUs was highest in April (274 ± 11), followed by September (250 ± 9) and July (225 ± 13). Like the bacteria, beta diversity of the fungal community indicated a strong separation by depth but not by treatment or time (Figure S6b). Through SIMPER analysis, we identified 15 ZOTUs driving the difference between the urea and CBF:Urea fungal communities, which appeared to be primarily driven by arbuscular mycorrhizal fungi (AMF). The four most influential ZOTUs driving 76% of the difference between the two communities all belonged to the Glomeromycota, with the urea treatment having greater abundance than the CBF:Urea treatment (Table S9). The remaining ZOTUs were classified as Eurotiomycetes, Monoblepharidomycetes, Agaricomycetes, Pezizomycetes, Sordariomycetes, and Zygomycota incertae sedis. However, the two communities were found to be 74% different overall, with individual ZOTUs making up at most 0.07% of this difference.

In contrast to the bacterial/archaeal community, fungi exhibited polarized responses to fertilization with primarily strong positive or negative responses from either urea or CBF:Urea treatments compared to the non-fertilized control (Figure 7b). Phylogenetic signal was also detected in the fungal community ($\lambda = 0.621$, $p = 9.9e^{-37}$), which was confirmed with Abouheif's C_{mean} (Table S7) suggesting that, albeit weakly, response ratios of closely related ZOTUs were more similar to each other than would be expected under random trait distribution. The fungal community was polarized with primarily strong positive or strong negative responses and confirmed the similarity between the two treatments (Figure 7b insert). In contrast to the bacterial/archaeal community, the urea treatment had slightly more strong negative and strong positive responses in the fungal community than the CBF:Urea treatment and the CBF:Urea treatment had a greater frequency of moderate-to-neutral responses.

4 | DISCUSSION

Maintaining crop productivity while reducing negative environmental impacts is critical to increasing the sustainability of agricultural practices. Perennial cropping systems may play an important role in future sustainability initiatives, but their implementation in dryland agriculture is limited and as a result the impacts on soil C and N dynamics are unclear (Wang et al., 2015). In addition, understanding the complexities of how the soil microbiome effects ecosystem function and contributes to soil health is currently a top research priority in the field of microbial ecology (Bender et al., 2016; Fierer et al., 2007; Mendes et al., 2013). Furthermore, field experiments and data demonstrating the effects of algal biofertilizers on the structure and functioning of microbial communities in agricultural systems are scarce. Therefore, this study aimed to contribute to these knowledge gaps by evaluating the effects of CBF supplementation on switchgrass stand establishment and to characterize the initial soil microbial community and N availability.

4.1 | Fertilizer effects on plant productivity

Supplementing 50% of the synthetic fertilizer application with CBF compared to 100% urea application resulted in similar barley biomass and provided field evidence for sustained increases in late season NDVI for an establishing perennial switchgrass crop (Figure 2). Similarly, in a study with potted rice, a combination of *Nostoc* sp. CBF with a half-dose of synthetic fertilizer resulted in the highest yield of rice grain compared to CBF or synthetic fertilizer alone (Chittapun et al., 2018). It is likely that by combining synthetic and biofertilizers, plants can take advantage of readily available synthetic N then utilize the

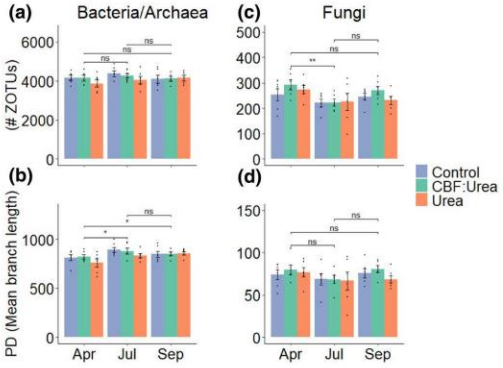


FIGURE 6 Alpha diversity of bacterial/archeal (a, b) and fungal (c, d) communities by treatment and time. Two measures of alpha diversity, #OTUs (A, C), and phylogenetic diversity (mean branch length), (b, d). Error bars represent standard error (SE) from the mean ($n = 6$). Statistics compare diversity between time points based on Tukey–Kramer multiple comparison tests. *$p < .05$ and **$p < .01$; ns, non-significant

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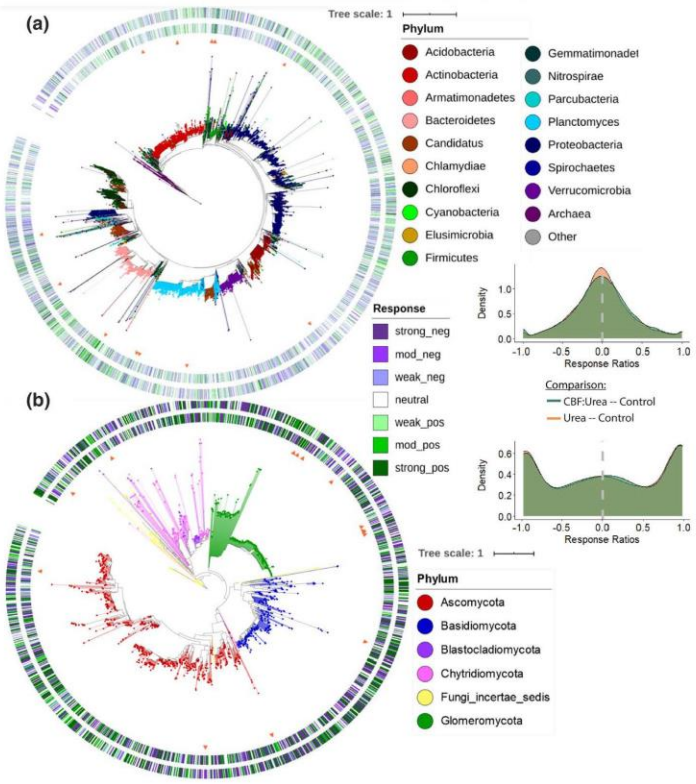


FIGURE 7 Phylogenetic trees of (a) bacteria and archaea (16S) and (b) fungal (LSU) communities colored by phylum. Response ratios are represented by colored circles around the trees. The inner circle represents the Urea–Control comparison while the outer circle represents the CBF:Urea–Control comparison. Darker colors indicate stronger responses with shades of green indicating positive responses (i.e., present in fertilized plots but not present in non-fertilized plots), likewise negative responses are in shades of purple. Neutral responses are in white. Triangles indicate ZOTUs identified by SIMPER analysis. Insets are histograms of the response ratio comparisons for each community

more slowly mineralized biofertilizer biomass throughout the growing season (Coppens et al., 2016; Kubheka et al., 2020).

4.2 | N and C responses to fertilization

Studies carried out with various CBF species have often shown that CBF can maintain or improve upon crop growth compared to synthetic fertilizers with additional benefits to soil quality including increased nutrient availability longer than synthetic fertilizers (Manjunath et al., 2016; Prasanna et al., 2012). We observed significantly higher late-season NH_4^+-N concentrations in shallow soil across the CBF:Urea plots compared to the urea fertilized plots or the non-fertilized controls in August (Figure 3). Furthermore, *Anabaena* CBF was detectable in July but not in September (Figure 5), which when considered with the increased soil NH_4^+ in the CBF:Urea plots, suggesting that the algal biomass was mineralized into available N over that time period, as observed by Coppens et al. (2016). This highlights the potential for biofertilizer supplements to extend N availability well into the late growing season and support a more closed N cycle in comparison to the conventional N application via urea pellets. We also observed higher levels of NO_3^--N in the urea-only treatment at the 15–30 cm soil depth across the entire growing season, and especially in the early season (Figure 3). This is noteworthy as NO_3^--N is a highly soluble and mobile form of N that, at this soil depth and early stage in stand development, is highly susceptible to eventual groundwater loss. We did not expect to see shifts in bulk soil chemistry after one growing season (Schrumpf et al., 2011); however, the ubiquitous decline in soil SOC across all treatments (Table S2) is an important observation in regards to potential ecosystem consequences (e.g., decrease in SOC) during the establishment of switchgrass as a bioenergy crop in the UMRB. However, this observation was made over a single growing season with a limited sample size of soil cores. Detecting SOC is notoriously difficult because of spatial heterogeneity (Conant & Paustian, 2002), and will require further investigation with a specific sampling design before conclusions can be drawn about a potential crop-induced mechanism for SOC loss.

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4.3 | Microbial response to fertilization

While overall diversity was minimally affected by treatment in either the bacterial/archaeal or fungal communities, the response ratio analysis across the phylogenetic tree revealed finer-scale responses within each community. Specifically, as shown in Figure 7, the fungal community was polarized with the majority of the identified ZOTUs falling under strong positive or strong negative response classifications while bacterial/archaeal responses were centered around neutral. The lack of response in the bacterial community contradicts several long-term studies that reported opposite findings (Ai et al., 2018; Marschner et al., 2003), but this is potentially due to the lack of soil chemical changes since bacteria tend to be more sensitive to changes in abiotic factors such as pH (Strickland & Rousk, 2010). We identified several ZOTUs driving small differences between the urea and CBF:Urea treatments which primarily belonged to the Planctomycetes, Actinobacteria, and Proteobacteria phyla. While different Planctomycetes ZOTUs were enriched in either the urea or CBF:Urea treatments, the Actinobacteria and Proteobacteria ZOTUs were enriched only in the urea treatments. Long-term N fertilization has been linked to increases in the relative abundances of Actinobacteria and Proteobacteria, which has been explained by their generally copiotrophic life strategies (Dai et al., 2018; Horton et al., 2017). In addition, members of the Actinobacteria, Proteobacteria, and Planctomycetes have all been identified as potential indicators of changes in pH and C:N ratios (Hermans et al., 2017) which may suggest more microscale differences in soil chemical conditions than we were unable to detect. Furthermore, the high level of heterogeneity within soil and the legacy effects of synthetic fertilization applied to these experimental field plots may have contributed to a lack of broad community changes (Fan et al., 2019). Alternatively, since strong effects of synthetic fertilization on the microbial community tend to occur at high levels of synthetic fertilizer application (>200 kg N ha^{-1}; Fierer et al., 2012; Treseder, 2008), the moderate levels used in this study (100 kg N ha^{-1}) may not be sufficient to lead to significant shifts.

In the fungal community, the greater abundance of arbuscular mycorrhizal fungi (AMF) species in urea plots driving the difference between the urea and CBF:Urea treatments was surprising as the negative impact of fertilization on AMF has been reported across multiple studies and systems (Chen et al., 2016; Ding et al., 2017; Egerton-Warburton et al., 2007; Williams et al., 2017; Zhu et al., 2018). *Glomus* sp. in particular have been reported to be sensitive to increasingly competitive environments such as those created by increased fertilization (Liu et al., 2015). However, the legacy of synthetic fertilization in these experimental plots may have conditioned these members of the community so that they were less able to utilize the organic source of N from the CBF. The hyphae of AMF are known to preferentially uptake NH_4^+ and also have a limited enzymatic repertoire which results in a dependence on other saprobic members of the microbial community to mineralize complex organic matter (Jansa et al., 2019). We hypothesize that a decreased ability of the fungal community to utilize the N derived from CBF biomass may have contributed to the differences in late-season NH_4^+ we observed. Long-term studies comparing organic versus synthetic fertilization have reported maintenance of or increases in fungal diversity in organic systems (Kamaa et al., 2011; Kazeeroni & Al-Sadi, 2016; Liu et al., 2020), with some exceptions attributed to indirect effects of plant biomass (Piazza et al., 2019). It is thus possible that there may be a lag period for some community members during the transition from previously synthetic to organic fertilization.

In both bacterial/archaeal and fungal communities, a weak phylogenetic signal was detected, indicating that closely related species tended to respond to fertilization more similarly to each other than to randomly selected species across the phylogenetic tree. For example, although we were unable to statistically detect any specific clades that responded strongly to either fertilization treatment, it appeared that certain clades within the Firmicutes, Bacteroidetes, and Candidatus phyla responded coherently in a positive manner, while a number of archaea were negatively affected by fertilization of either type, warranting further investigation into the ecosystem functions performed by these groups. The relatively short length of this study (one growing season) and the possible effects of "relic" DNA in the soil environment on diversity measures emphasize the need for longer-term investigation of the effects of CBF on the soil microbial community (Carini et al., 2016). In addition, the contribution of the differences in abundances of individual ZOTUs to the overall community dissimilarities between treatments was exceedingly small. This suggests that even when supplementing CBF at a half-dose of synthetic fertilizer, the overriding effects of N addition on the soil microbial community are likely driven by the urea fertilization.

5 | CONCLUSIONS

Supplementing 50% of urea fertilizer with CBF in an outdoor field study maintained statistically similar barley biomass and increased late-season NDVI of switchgrass, indicating potential for CBF efficacy in the

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UMRB region. An increase in late-season N availability and evidence for mineralization of the CBF from July to September supported the potential benefits of the slower mineralization rate of CBF compared to urea. Supplementing urea fertilizer with CBF did not significantly alter bacterial or fungal composition or diversity over the growing season; however, in-depth analysis of responses across the phylogenetic tree revealed the communities had fine-scale dynamic responses. Regardless of fertilizer type, fungi had stronger responses to fertilization than bacteria with polarized strong positive and negative responses, although phylogenetic signal was detected in both kingdoms suggesting cohesive responsiveness within specific clades. In addition, we identified specific ZOTUs that had strong responses to either fertilization treatment which suggests possible avenues of further ecosystem function investigations. Importantly, the neutral response of the microbial community to biofertilizer supplementation may be construed as a lack of negative impact of the biofertilizer supplement on the soil microbial community, although longer-term studies need to be undertaken to determine if this is the case. Taken together, the results from this study are promising for the future implementation of perennial cropping with biofertilization in dryland agricultural systems.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTIONS

BP, PM, HG, JG, JB, and BMP collaborated on study design. HG, JG, and PM were responsible for field sampling and HG, JG, and RCM performed the sample processing, and bioinformatic and statistical analyses. BP, RCM, JB, JG, and HG contributed to data interpretation. All authors contributed to writing the manuscript.

CONSENT FOR PUBLICATION

All coauthors consent to publication, and no human subjects were used in this study.

DATA AVAILABILITY STATEMENT

Sequence data are available at MG-RAST under accession numbers mgm4916997.3 (LSU) and mgm4916998.3 (16S). The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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CHAPTER THREE

SEVERE AND MILD DROUGHT CAUSE DISTINCT PHYLOGENETICALLY LINKED SHIFTS IN THE BLUE GRAMA (*BOUTELOUA GRACILIS*) RHIZOBIOME

Contribution of Authors and Co-Authors

Manuscript(s) in Chapter(s) 3

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Contributions: Sample processing, bioinformatic and statistical analyses, data interpretation, primary manuscript author

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Abstract

Plants rely on a diverse rhizobiome to regulate nutrient acquisition and plant health. With increasing severity and frequency of droughts worldwide due to climate change, untangling the relationships between plants and their rhizobiomes is vital to maintaining agricultural productivity and protecting ecosystem diversity. While some plant physiological responses to drought are generally conserved, patterns of root exudation (release of small metabolites shown to influence microbes) and the consequential effects on the plant rhizobiome can differ widely across plant species under drought. To address this knowledge gap, we conducted a greenhouse study using blue grama (*Bouteloua gracilis*), a drought-tolerant C4 grass native to shortgrass prairie across North American plains, as a model organism to study the effect of increasing drought severity (ambient, mild drought, severe drought) on root exudation and the rhizobiome. Our previous results demonstrated physiological effects of increasing drought severity including an increase in belowground carbon allocation through root exudation and shifts in root exudate composition concurrent with the gradient of drought severity. This work is focused on the rhizobiome community structure using targeted sequencing and found that mild and severe drought resulted in unique shifts in the bacterial + archaeal and fungal communities relative to ambient, non-droughted controls. Specifically, using the change in relative abundance between ambient and drought conditions for each ZOTU as a surrogate for population-scale drought tolerance (e.g., as a response trait), we found that rhizobiome response to drought was non-randomly distributed across the phylogenies of both communities, suggesting that *Planctomycetota*, *Thermoproteota* (formerly *Thaumarchaeota*), and the *Glomeromycota* were the primary clades driving these changes. Correlation analyses indicated weak correlations between

droughted community composition and a select few root exudate compounds previously implicated in plant drought responses including pyruvic acid, D-glucose, and myoinositol. This study demonstrates the variable impacts of drought severity on the composition of the blue grama rhizobiome and provides a platform for hypothesis generation for targeted functional studies of specific taxa involved in plant-microbe drought responses.

Introduction

Drought is one of the most important abiotic factors limiting plant growth and development (Berdugo et al., 2020; de Vries et al., 2016). Increasing precipitation variability and rising temperatures due to climate change are predicted to increase drought intensity and the rate at which droughts develop (Mukherjee & Mishra, 2021). These changes will likely have dramatic consequences on the productivity and distribution of plant life in both agricultural and natural ecosystems. Understanding plant-microbe interactions in a wide variety of contexts is critical to making informed management decisions in agriculture and natural resource conservation in the face of global climate change.

The plant rhizosphere, the region of soil adjacent to plant roots that is directly influenced by plant growth, respiration, and nutrient exchange, is the epicenter of a complex web of plant-microbe interactions. The rhizosphere hosts a wide diversity of microorganisms including bacteria, archaea, and fungi collectively referred to as the rhizobiome that can improve plant access to nutrients and aid in plant survival under abiotic stress. Mutualistic fungi such as many arbuscular mycorrhizal fungi (AMF) can increase water exchange and nutrient mining to plants under drought stress in exchange for plant C inputs (S. Mathur et al., 2019). Bacteria including *Streptomyces sp*. (Abbasi et al., 2020; Li et al., 2020) and *Burkholderia sp.* (Huang et al., 2017;

Naveed et al., 2014) have been utilized as plant inoculants to protect against drought stress. These and other drought-tolerant bacteria offer benefits to plants including the solubilization of mineral phosphates, nitrogen fixation, and production of plant growth-promoting hormones such as indole acetic acid (Dahal et al., 2017; Passari et al., 2016).

However, it remains difficult to discern broad patterns in rhizobiome community responses to drought stress due to variation in plant species, soil type, and climate. This is partly because we do not fully understand the influence of plant carbon inputs into the soil, namely as root exudates, on the rhizosphere microbiome. Root exudates are small molecules released by plant roots that frequently include sugars, amino acids, and organic acids (Bais et al., 2006). Many of these compounds have been found to have osmoprotectant or chemical signaling properties in various plant systems (Vries et al., 2019; Williams & de Vries, 2020). With recent advances in metabolomics technology, we are only beginning to understand the effects of changes in the quantity and composition of root exudates on rhizobiome community composition and function under increasing drought severity.

The goal of the present study was to examine how increasing drought severity influences the rhizobiome community diversity and composition of *B. gracilis*. Blue grama is a widespread, drought-tolerant C4 grass native to North America that accounts for 75-90% of net primary productivity of grasslands it inhabits and is a major contributor to belowground C storage (Costello, 1944). Blue grama is utilized for conservation, rangeland seeding, landscaping, reclamation, and erosion control and is also valuable for livestock and wildlife because it provides highly palatable forage year-round (Costello, 1944). The level of precipitation in the driest month of the year has historically been a strong influence on the distribution of blue grama

across the central and western Great Plains from Canada to northern Mexico (Avendaño-González & Siqueiros-Delgado, 2021). The importance of the blue grama rhizobiome in mediating its drought tolerance is not well understood, although Ulrich et al. (2019) demonstrated that growing blue grama without its microbiome (grown in sterilized soil) resulted in less germination, less biomass, and lower drought tolerance compared to rhizobiome-intact plants. Prior results from this study indicated that increasing drought severity on blue grama grass (*Bouteloua gracilis*) resulted in strong shifts in root exudate composition with compounds such as sucrose, myo-inositol, pyruvic acid, and L-threonine primarily driving differences between drought severity treatments (Ulrich et al., 2022).

Here we further explored the above greenhouse experiment that subjected blue grama seedlings to three levels of drought severity by measuring the diversity and composition of the rhizosphere bacterial + archaeal and fungal communities using next generation amplicon sequencing. We tested two hypotheses: 1. Severe drought will have a stronger effect on the rhizobiome diversity and composition than mild drought and 2. drought-induced changes in root exudate composition would correlate to changes in rhizobiome composition. Understanding how increasing drought severity alters plant-soil feedbacks in drought-tolerant plants will provide important insights into plant-microbe mechanisms that may be applied to less drought-tolerant species (Mathur & Roy, 2021). Disentangling these relationships will also improve our abilities to model global nutrient dynamics and develop microbially enhanced food production and natural resource management solutions in the face of climate change.

Methods

Study Site and Experimental Design

Blue grama was grown from commercial seed (Wind River Seed Co., Manderson, WY, USA) in a greenhouse (Bozeman, MT, USA) in small trays in a 50:50 mix of sand:soil collected from the top soil layer (0-10 cm) from a pinyon-juniper woodland near Bozeman, MT, which is the general ecosystem where blue grama naturally occurs (*Bouteloua gracilis*, n.d.; Coffin & Lauenroth, 1992). This ensured microbes relevant to blue grama would be available (Naylor & Coleman-Derr, 2018). Seeds were planted on October 1, 2019. After 30 days of initial growth, 120 seedlings of similar size were selected and transplanted with the soil from the trays to larger pots (~0.75 L, 6.4 cm diameter, 25.4 cm height) with sterilized playground sand (Ulrich et al., 2019). Greenhouse conditions during the study (Oct-Jan 2019) consisted of a 16-hour photoperiod, daytime temperature of 23.8 °C, nighttime temperature of 21.1 °C, and average daytime PAR of 500 μmol m^2 s^{-1}. Plants were watered to field capacity and allowed to acclimate to greenhouse conditions for 30 days. Then, established plants were divided into three treatment groups: ambient, mild drought, and severe drought (n = 40 plants per treatment). The ambient treatment group received normal watering to field capacity. The mild drought treatment group was watered half as frequently to maintain 50% of field capacity. The severe drought treatment group was watered 25% as frequently to maintain 25% of field capacity. Treatments lasted 30 days. These drought severity levels were selected based on previous work where blue grama required 33 days of drought to desiccate when water was completely withheld (i.e. 100% reduction in water) (Ulrich et al., 2019). In this study, less severe drought treatment levels (50% and 75% vs. 100% reduction in water) were selected to induce plant stress response without

killing the plants, allowing measurement of shifts in physiology and root exudate concentration and composition as a function of drought severity.

Plant and Soil Measurements and Collection

Plant physiology analysis is described in full in (Ulrich et al., 2022). Briefly, to determine how drought severity treatment influenced plant physiology, predawn leaf water potential, leaf gas exchange (photosynthesis, stomatal conductance) and root and shoot biomass were measured to determine root to shoot biomass ratios (root:shoot) in each treatment group before (T1) and after (T2) 30 days of treatment (December 19-January 18). Predawn leaf water potential was measured using a Scholander type pressure chamber (model 1505D, PMS Instruments, Corvallis, OR, USA). Gas exchange was measured using a portable photosynthesis instrument equipped with an infrared gas analyzer (LI-6800, Licor, Lincoln, NE USA) on 5 plants randomly selected from each treatment group. Plant physiology results demonstrating the effectiveness the drought treatments are listed in Figure S1.

At time of harvest 50 mL each of rhizosphere and bulk soil fractions were collected separately, flash-frozen in an ethanol-dry ice bath and transferred to long-term storage at -80 °C for microbial analysis. For rhizosphere collection a soil core was taken as close to the plant as possible directly adjacent to the root crown while the bulk soil core was collected close to the edge of the pot (~3 cm away). Root exudates were collected via vacuum filtration at the time of harvest and transferred to long-term storage at -80 °C. Root exudate collection procedures followed the method of Phillips et al. (2008). Briefly, plants were removed from pots and roots were rinsed of soil in DI water and dipped in Gibco Antibiotic-Antimyotic solution (ThermoFisher Scientific CA, USA) to halt the microbial production of C compounds. This

ensured that we collected exudates from the plant and not microbes. Plants were subsequently transplanted to filter flasks of 60-120 mesh glass beads (100 mL) that do not provide a C source but still provide mechanical pressure on the root system, resembling natural soil conditions. Plant roots were flushed with 100 mL of sterile water using a vacuum pump connected to the filter flasks, another 100 mL was added, and plants were allowed to release exudates over a 41-hour period. The remaining solution was filtered (0.22 μm) and collected for root exudate concentration and composition analyses. Root exudates were frozen and stored at −80 °C until analysis. Exudate samples were submitted to the Environmental Molecular Science Laboratory (EMSL) for gas chromatography-mass spectrometry (GCMS) analysis and data preprocessing. GCMS methods are described in Ulrich et al. (2022).

DNA Extraction, Amplification, and Sequencing

To obtain high-resolution representation of the soil microbial community and its response to drought, we performed rRNA gene amplicon sequencing on all replicates of bulk and rhizosphere soil for all treatment and pretreatment (T1) samples (n=15 each for T1 rhizosphere and bulk soils, n=5 for post-drought (T2) rhizosphere and bulk samples across three treatments, total n=60). Genomic DNA was extracted from 0.5 g frozen soil samples using the Fast DNA SPIN Kit for soil (MP Biomedicals, Santa Ana, CA) according to the manufacturer's instructions. We targeted the V4 region of the 16S rRNA gene using 515F (Parada et al., 2016) and r806b (GTGYCAGCMGCCGCGGTAA and GGACTACVSGGGTATCTAAT, respectively) from the Earth Microbiome Project (Thompson et al., 2017), and the D2 hypervariable region of the fungal large subunit (LSU) using LR22R (Mueller et al., 2016) and LR3. PCR reactions were carried out in 20 μL volumes containing 1 U Phusion high-fidelity

polymerase (ThermoFisher Scientific, MA, USA), a final concentration of 1x Phusion HF reaction buffer, 200 μM dNTPs, 0.5 μM each primer, 1 μM BSA, 1 mM MgCl, sterile molecular grade water, and 5 μL (10 ng) genomic DNA as a template. PCR conditions consisted of initial denaturation at 98 ˚C for 30s, followed by 22 cycles of 98 ˚C for 30 s, 55 ˚C for 30 s, 72 ˚C for 45 s and a final extension at 72 ˚C for five minutes. Samples were purified using AMPure XP (Beckman-Coulter, Brea CA, USA) magnetic beads and barcoding was conducted using the Illumina Nextera Indexing Kit D, with 10 cycles of indexing PCR in 20 μL volumes using the same concentrations as PCR1 with 10 μL template DNA. Samples were purified again with magnetic beads and PCR products were checked for quality and length in a 1% agarose gel and quantitated using the Quant-iT™ dsDNA Kit (Invitrogen, Carlsbad CA, USA) with a BioTek H2 plate reader. Individual samples for each community were combined at equimolar concentrations and the two libraries were pooled at 4 nM and loaded onto an in-house Illumina MiSeq (Illumina, San Diego CA, USA) and sequencing using the V3 600 cycle kit.

Bioinformatics

Reads were merged, trimmed, and dereplicated with USEARCH, quality filtered with an ee-value of 1.0 and zero-radius operational taxonomic units (ZOTUs) were identified with UNOISE3 (v.11.0.667) (Edgar, 2010). A total of 4,668,982 16S and 4,872,815 LSU high quality reads were clustered into 15,541 and 6,893 ZOTUs respectively. Representative 16S and LSU ZOTUs were classified against their respective databases using a subset of the Genome Taxonomy Database (GTDB, Chaumeil et al., 2022) for 16S and the Ribosomal Database Project (RDP, Cole et al., 2014) v11 database for LSU using SINTAX (Edgar, 2016). All reads classified to chloroplast (16S), or animalia, protozoa, or viridiplantae (LSU) were removed prior to

downstream analysis. For statistical analyses, datasets were randomly subsampled according to the sample with the lowest read number. To construct phylogenetic trees for environmental ZOTUs, we used reference phylogenies constructed using full length and near full length sequences of isolates and metagenome assemblies downloaded from GenBank, focusing on GTDB sequences for 16S and Assembling the Fungal Tree of Life sequences for LSU (Lutzoni et al., 2004). Reference sequences were aligned using mafft (v.7.250, Katoh & Standley, 2013), and a maximum likelihood tree was constructed with the general time reversible (GTR) + gamma model using RAxML (v7.2.7 Stamatakis, 2014). Environmental sequences were aligned to the reference using mafft with the –add flag and then mapped onto the reference tree using pplacer (v1.1.alpha19-0-g807fbf3, Matsen et al., 2010) with reference-aligned sequences. Trees were visualized and annotated using the interactive Tree of Life software (v6.8.1, Letunic & Bork, 2019).

Statistics and Data Analysis

Community analyses were conducted using R software (v4.3.0, R, 2023) in RStudio (RStudio Team, 2020). Data management was handled primarily with the tidyverse package (Wickham et al., 2019). Community ordination and alpha diversity analyses were performed using the packages vegan (Oksanen et al., 2019), picante (Kembel et al., 2010) and phyloseq (McMurdie & Holmes, 2013). We used PERMANOVA (Anderson, 2017) to test for treatment, timepoint, and bulk vs. rhizosphere effects on community beta diversity computed using unweighted UniFrac (Lozupone & Knight, 2005). To explore the effect of time on community composition we conducted Wilcoxen Signed Rank Tests to test differences in relative abundances between T1 (pretreatment) and ambient samples at T2 (post-drought).

To examine whether ZOTU responses to drought were non-randomly distributed across the bacterial + archaeal and fungal phylogenies in the blue grama rhizosphere, we performed phylosignal analysis using response ratios as a response trait on T2 (post-drought) rhizosphere samples. Response ratios were computed by dividing the difference between the read counts in the treatment (severe or mild drought) and ambient conditions by the total read count for a ZOTU. Response ratios were permuted 999 times for each ZOTU and averaged across 5 replicate samples in each treatment. Phylosignal analyses were performed using phytools (Revell, 2012) and geiger (Pennell et al., 2014) for Pagel's lambda and Blomberg's K (Diniz-Filho et al., 2012). Packages phylobase (R Hackathon et al., 2020) and adephylo (Jombart et al., 2010) were utilized for Abouheif's C_{mean}. One-way t-tests were then conducted across taxonomic levels to determine which clades had response ratios significantly different from 0. All t-tests were adjusted with Benjamini-Hochberg (BH) multiple comparisons corrections, and we report the adjusted p-values. To aid in visualization of the phylogenetic signal analysis, the response ratio for each ZOTU and for clades with significantly positive or negative clade-wide response ratios were mapped back to the phylogenetic trees using the color strip tree annotation template provided by the interactive Tree of Life software (Letunic & Bork, 2019).

Having identified several clades of bacteria, archaea, and fungi which had significant clade-wide response to severe drought, we performed network analysis to determine whether there were any detectable inter-kingdom (i.e. bacterial-fungal) associations in relative abundances between drought and ambient conditions. Sparcc networks were constructed and analyzed using the NetCoMi package (Peschel et al., 2021) in RStudio with specific parameters listed in the Supplementary Information. We utilized the differential network analysis module in

the NetCoMi package to determine the effects of mild and severe drought on the top 50 of these potential associations. Differential network analysis uses permutation tests to assess whether associations between taxa are different between two user-defined treatment groups, in our case defined as mild drought vs. ambient and severe drought vs. ambient conditions (Peschel et al., 2021). All figures were edited for publishing quality in Adobe Illustrator 2023 27.5.0.

To calculate correlations between root exudation and community composition we selected root exudate compounds identified as driving the differences between treatment groups (ambient, mild drought, and severe drought) reported in Figure 3 of Ulrich et al. (2022). Correlation analysis was performed using the envfit function in the vegan package. Envfit correlation p-values were also BH-corrected for multiple comparisons.

Results

Drought Effects on Rhizobiome Composition

We found severe drought resulted in significantly altered beta diversity of the bacterial + archaeal and fungal communities compared to the ambient treatment in T2 (Bacteria + archaea: $F_2 = 1.41$, $R^2 = 0.0466$, $p = 0.01$, Fungi: $F_2 = 1.34$, $R^2 = 0.183$, $p = 0.025$, Fig 1). In comparison, mild drought conditions had minimal effects on the rhizobiome beta diversity. We also detected decreased dispersion in the bacterial + archaeal community ($F_2 = 4.81$, $p = 0.029$) in the severe drought condition compared to ambient. We did not find evidence for differences in alpha diversity between drought conditions (mild or severe) and ambient conditions or between mild and severe drought, in either the bacterial + archaeal or fungal communities across any of the three metrics tested: species richness, Shannon's diversity, or Faith's phylogenetic diversity (Fig.

1, Table S1), although we did detect a positive effect of time (T2 > T1) across all three metrics in the fungal community (Table S1).

Beta diversity also differed between T1 and ambient conditions at T2 in both the bacterial + archaeal (F_2 = 1.64, R^2 = 0.0271, p = 0.007, Table 1) and fungal communities, although the difference between T1 and T2 ambient conditions in the fungal community was marginal (F_2 = 1.59, R^2 = 0.0264, p = 0.059, Table 1). The differences between T1 and ambient-T2 in the bacteria + archaea were likely driven by the detection of *Armatimonadota* and *Nitrospirota* above 1% abundance only at T1, and detection of *Fibrobacterota* only in ambient-T2 samples (Figure S2a). We did not detect any significant differences in relative abundances of fungal classes between T1 and ambient-T2 (Figure S2b). We also did not detect differences between T1 and T2 in beta diversity between bulk and rhizosphere samples, or effects of any interactions between the combinations of soil compartment, time, and treatment (Table 1).

Distribution of Drought Response Across the Rhizobiome Phylogeny

Phylosignal analysis using response ratios as a response trait on the bacterial + archaeal and fungal trees indicated that changes in relative abundance between the mild drought and ambient treatments as well as the severe drought and ambient treatments were not randomly distributed across either tree (Table 2). In the bacterial + archaeal community, all three phylosignal tests (Pagel's Lambda, Blomberg's K, and Abouheif's C_{mean}) rejected the null hypothesis of random distribution of the response ratio trait. In the fungal community for both the mild drought-ambient and severe drought-ambient comparisons, Blomberg's K test did not reject the null hypothesis but the other two tests were found to be significant.

In the mild drought–ambient comparison, global analysis of response ratios detected one archaeal phylum, four bacterial phyla, and one fungal class which had clade-wide response ratios significantly different from zero (i.e., increased or decreased relative abundance in drought vs. ambient conditions). *Thermoproteota* (archaea also known as *Thaumarchaeota*), and bacterial *Chloroflexota* and *Actinobacteriota* had positive phylum-wide response ratios (greater relative abundance in mild drought vs. ambient conditions) while bacterial *Omnitrophota* and *Planctomycetota* had negative phylum-wide response ratios (Table S2). The fungal class *Eurotiomycetes* within the *Ascomycota* was the only fungal clade detected and had a negative class-wide response ratio in the mild drought-ambient conditions comparison. At the family level for bacteria + archaea, we identified 15 families across the phylogeny with positive response ratios, and six with negative response ratios (Fig. 3A inner circles, Table S2). Most of the families with positive response ratios belonged to the *Actinobacteriota* phylum, including: *Rubrobacteraceae, Solirubrobacteraceae, Gaiellaceae*, *Illumatobacteraceae*, *Geodermatophilacaeae*, *Microbacteriaceae*, *Propionibacteriaceae*, and *UB11606* (order *Acidimicrobiales*). The other clades with positive response ratios included three families within the *Alphaproteobacteria* (*Devosiaceae, Xanthobacteraceae*, and *Beijerinckiacaeae*), *Roseiflexiaceae* (*Chloroflexota*), one *Acidobacteriota* family, *UBA5704 (Thermoanaerobaculia)*, and the only family present within the *Thermoproteota*, *Nitrososphaeraceae*. Negative family-wide responses included: *Bryobacteraceae* (*Acidobacteriota*), *Nevskiaceae* and *Burkholderiaceae* (*Gammaproteobacteria*), *SM1A02* and *PALSA_1355* (*Planctomycetota*) and *J027* (*Chloroflexota*).

In the severe drought-ambient comparison, we detected many more bacterial + archaeal clades with significantly positive or negative response ratios (11 phyla and 45 classes/families vs. 5 phyla and 22 classes/families in the mild drought-ambient comparison), with only a few overlapping between the two comparisons (Fig. 3A outer circles, Table S2). At the phylum level, the *Thermoproteota* and *Chloroflexota* maintained positive response ratios, while the *Planctomycetota* flipped from negative to positive. We also detected positive response ratios in the *Verrucomicrobiota* and *Acidobacteriota* which were not significant in the mild drought-response ratios. Likewise, the *Firmicutes*, *Gemmatimonadota*, and *Bdellovibrionota* were found to have negative response ratios only in the severe drought-ambient comparison. While the *Gemmatimonadota* have been associated with drought-resistance in some studies (Guo et al., 2023; Wang et al., 2022) there is evidence that members of this phylum are sensitive to dry-wet cycling (Qi et al., 2022) which the droughted samples experienced in this study. Although the *Actinobacteriota* did not have a significant phylum-wide response ratio in the severe drought-ambient comparison, we did detect positive response ratios in the *Rubrobacteraceae*, *Gaiellaceae,* and *IMCC26256* (order *Acidimicrobiia*) families within the *Actinobacteriota* as in the mild drought-ambient comparison. Unique to the severe drought-ambient comparison, actinobacteriotal family *Steptomycetaceae* had a negative response ratio. Within the *Proteobacteria* we observed positive response ratios in both drought treatments in the *Devosiaceae* and *Xanthobacteraceae,* and negative response ratios in both treatments in the *Burkholderiaceae* and *Nevskiaceae*. *Alphaproteobacteria* families *Sphingomonadaceae* and *Rhodobacteraceae* had negative response ratios unique to the severe drought-ambient comparison, and we observed a positive response ratio in the *Steroidobacteraceae* family in the

severe drought-ambient comparison and a positive response in the *Beijerinckiaceae* family only in the mild drought-ambient comparison.

In the fungal community, severe drought also had a greater impact on response ratios than mild drought compared to ambient conditions. With the severe drought-ambient comparison we detected positive response ratios in the *Mucoromycotina* and *Ascomycota* phyla, and negative response ratio in the *Chytridiomycota.* We detected 4 classes which had positive response ratios including: *Glomeromycetes*, *Sordariomycetes, Dothideomycetes*, and the *Eurotiomycetes* (Fig. 3B). The *Chytridiomycetes* were the only class with a clade-wide negative response ratio. Within the *Glomeromycetes* class there was a notable sub-clade, BLAST-identified as *Funneliformis sp*., which had negative response ratios although these did not influence the clade-wide positive response.

Impact of Drought Severity on Rhizobiome Associations

To determine the effects of drought severity on community associations, we performed network analyses using differential networks of mild drought-ambient and severe drought-ambient communities. In the mild drought-ambient differential network, we identified a number of bacterial-fungal associations affected by drought (Fig. 4a). *Acidobacterial* class *Vicinamibacteria, Actinobacteriota* class *Thermoleophilia*, and *Chloroflexota* classes *Ktdedonobacteria* and *Ellin6529* were the most connected classes in the network. The fungal class *Leotiomycetes* appeared to have several associations with bacterial classes that were negative under ambient conditions but positive under mild drought conditions (pink lines) including associations with *Ktedonobacteria, Thermoleophilia, Actinomycetia, Rubrobacteria*, and *Phycisphaerae.* In contrast, bacterial class *Actinomycetia* had several associations that were

positive under ambient conditions but negative under mild drought (blue lines) including associations with *Acidimicrobia*, *Alphaproteobacteria*, *Gammaproteobacteria*, and *Saccharimonadia*. Some classes such as *Binatia*, *Planctomycetes*, and *Blastocatellia* had only positive associations with other taxa in both ambient conditions (green lines). Several fungal classes had primarily negative associations regardless of treatment group (black lines) including the *Glomeromycetes*, *Pezizomycetes*, *Dothideomycetes*, and *Sordariomycetes*.

In the severe drought-ambient differential network, *Planctomycetes* was the most connected class and appeared to have strong tradeoffs of positive associations in the ambient vs. severe drought communities. *Planctomycetes* associated positively with 11 classes in the ambient condition that were negative associations in the severe drought condition (purple lines) including *Actinomycetia*, *Blastocatellia*, *Ellin* 6529, *Gemmatimonadetes*, *Ktedonobacteria*, *Rubrobacteria*, Thermoleophilia, and fungal classes *Ascomycota incertae sedis*, *Eurotiomycetes*, and *Leotiomycetes*. In contrast, *Planctomycetes* were positively associated with a different set of 9 classes in the severe drought condition that were negative associations in ambient condition. These included *Alphaproteobacteria*, *Bacilli*, *Bdellovibrionata*, *Chlamydiia*, *Gammaproteobacteria*, *Thermoanaerobaculia*, and *Zygomycota incertae sedis* *Nitrososphaeraceae* was always positively associated with *Planctomycetes* while the majority of the fungal classes including the *Tremellomycetes*, *Agaricomycetes*, *Microbotryomcetes*, *Chytridiomycetes*, *Exobasidiomycetes*, and *Dothidiomycetes* were always negatively associated with *Planctomycetes* regardless of treatment.

Connections to Plant Physiology & Root Exudation

Previous results focusing on changes in blue grama root exudation in response to increasing drought severity found significantly increased total organic carbon in root exudate extracts under severe drought compared to ambient conditions (Ulrich et al., 2022). There were ten root exudate compounds driving the greatest differences in exudate composition across the three treatments (ambient, mild drought and severe drought). These compounds were sucrose, D-arabinose, pyruvic acid, D-mannose, sedoheptulose anhydride monohydrate, tagatose, D-glucose, L-threonine, and 4-guanidinobutyric acid (Ulrich et al., 2022). Here we detected weak evidence for a correlation between the composition of these root exudates across samples with the rhizosphere bacterial + archaeal (Mantel $R^2 = 0.299$, $p = 0.084$) and fungal (Mantel $R^2 = 0.289$, $p = 0.087$) community compositions (beta diversity). In the bacterial + archaeal community, the best possible model correlating root exudates to community composition included only myoinositol and D-arabinose (Mantel $R^2 = 0.269$, $p=0.081$). For the fungal community, the best possible model included pyruvic acid, D-mannose, tagatose, and D-glucose (Mantel $R^2 = 0.296$, $p = 0.083$). Pearson correlations between taxa with significant clade-wide response ratios (i.e., those indicated by Figure 3) and root exudates were also conducted but no significant correlations were detected.

Discussion

Severe Drought had Stronger Effects on the Blue Grama Rhizobiome than Mild Drought

In support of our first hypothesis, severe drought had a greater impact on the blue grama rhizobiome composition than mild drought compared to ambient, non-droughted controls. Shifts

in overall composition of the bacterial + archaeal and fungal communities were detected, and a decrease in dispersion (e.g., reduced beta diversity) among bacterial + archaeal community members was observed during severe drought conditions compared to the ambient treatment (Fig. 1). In contrast, mild drought did not induce a significant shift in beta diversity among either the bacteria + archaea or fungi (Fig. 1). The greater impact of severe drought on the soil rhizobiome compared to mild (or moderate) drought has been hypothesized in modeling efforts such as demonstrated by Wang and Allison (2021). Simmons et al. (2020) also demonstrated that severe drought had a greater impact than moderate drought on rhizosphere bacterial community composition across multiple species of millet.

The lack of drought treatment effects (either mild or severe) on alpha diversity in either the bacterial + archaeal or fungal communities (Fig. 2) indicated that the changes in community composition were primarily due to shifts in relative abundances of existing members. Importantly, greenhouse studies limit dispersal which can have significant effects on community diversity under abiotic stress (Evans et al., 2020). However, the controlled conditions of greenhouse studies allow for treatment effect isolation and deliberately limiting dispersal effects improves the ability to detect shifts more directly in existing community membership. Phylogenetic signal is considered a useful metric for studying the effects of drought on the soil microbiome as many clades at different taxonomic levels tend to respond roughly in unison (Koyama et al., 2018; Naylor et al., 2017; Xu et al., 2018). Accordingly, phylogenetic signal in comparisons of both mild drought-ambient and severe drought-ambient rhizobiomes were detected using response ratios of the differences in relative abundances of taxa between treatment groups as a response trait (Fig. 3).

Several clades of microorganisms have been found to be resilient to drought, including the *Actinobacteriota, Firmicutes, Mucoromycotina* (which includes the *Glomeromycetes*), and various subclades among the *Ascomycota* (Santos-Medellín et al., 2021; Xu et al., 2018). Consistently, we observed positive clade-wide responses (i.e. increased relative abundance in severe drought vs. ambient conditions) in all of these phyla with the exception of the *Firmicutes,* which in our study exhibited a clade-wide negative response in severe drought (i.e. decreased relative abundance vs. ambient conditions) but not mild drought. Overall, severe drought resulted in a greater number of significant response ratio changes (either positive or negative) across the bacterial + archaeal and fungal phylogenies than mild drought. For the fungal community in particular, the lack of significant response under mild drought conditions may reflect the greater water stress tolerance amongst mycelial community members (Santos-Medellín et al., 2021). Plant-microbe interactions are often the most beneficial when they help mediate access to the most limiting nutrient in an environment, such as water during drought stress (Ruth et al., 2011). Due to the lack of strong compositional effects in mild drought conditions, we hypothesize that blue grama may be able to self-regulate and tolerate mild drought. However, the greater investment in root exudation observed under severe drought stress (Ulrich et al. 2022) may be a mechanism to stimulate microbial mutualisms (i.e. the positive response ratios across the mycorrhizal *Glomeromycetes*) to help alleviate severe drought stress (Bécard & Piché, 1989; Vries et al., 2019).

Within the *Actinobacteriota,* more positive response ratios were detected in the mild drought than severe drought compared to ambient conditions, although some families such as *Rubrobacter,* which are known to be highly tolerant to desiccation (Naylor et al., 2017; Tóth et

al., 2017), had positive response ratios in both drought severity treatments. Interestingly, while the actinobacteriotal genus *Streptomyces* has been found to alleviate drought stress in multiple agricultural plant species including tomatoes (Abbasi et al., 2020), maize (Warrad et al., 2020), and wheat (Li et al., 2020), here we observed a decrease in relative abundance of *Streptomycetaceae* in response to severe drought compared to ambient. Likewise, while we detected decreased relative abundances of *Burkholderiaceae (Alphaproteobacteria)* in both mild and severe drought compared to ambient, *Burkholderia* sp. have been used as plant inoculants to successfully protect against drought stress in *Arabidopsis*, maize, wheat, tomato, and bell peppers (Huang et al., 2017; Naveed et al., 2014; Tallapragada et al., 2016). Importantly, inoculants are most commonly applied to annual, domesticated crops with the exception of some trees (Bizos et al., 2020; Umashankar et al., 2012). Wild, perennial plant species such as blue grama typically have greater genetic diversity and often greater natural drought tolerance than cultivated species (Budak et al., 2013) which likely results in unique drought response patterns in the rhizobiome. The results presented here may also serve to broaden the known range of drought response patterns across plant species.

While the majority of the *Mucoromycotina* had positive response ratios in the severe drought-ambient comparison, we observed a distinct sub-group of *Funneliformis* sp. within the *Glomeromycetes* which had negative response ratios. This negative response is surprising as *Funneliformis sp.* are arbuscular mycorrhizal fungi which have been utilized as inoculants for several plants including thyme, soybeans and groundcherries to improve plant growth during water stress (Amani Machiani et al., 2021; Reyes et al., 2019). However, recent studies of bacterial colonization of mycorrhizal spores (Lasudee et al., 2018; Schrey et al., 2012) have

identified *Streptomyces sp.* as among the most common endophytic mycorrhizal colonizers, and of *Funneliformis sp.* specifically. A tight association between *Funneliformis sp*. and *Streptomyces sp.* may explain the negative response ratio we observed in the *Streptomycetaceae* in the severe drought-ambient comparison. These results are also further evidence that drought-tolerant blue grama may not rely on the same drought response mechanisms that have been observed in other plant species.

Insights on Inter-kingdom Associations via Network Analysis

Network analysis enables the identification of co-occurrence patterns between microbial taxa which may provide clues to shared niche spaces, nutrient acquisition strategies, or even direct symbioses (Barberán et al., 2012). These analyses provide an important foundation for hypothesis generation through which future experiments can be designed to determine the extent and conditionality of associations between taxa. Here we observed several distinct network patterns between mild drought-ambient and severe drought-ambient networks (Fig. 4).

In the severe drought-ambient differential network the *Planctomycetes* was the bacterial class most highly connected to other bacterial and fungal classes. There were 11 positive associations with *Planctomycetes* in ambient conditions which were negative associations in severe drought conditions, nine associations which were negative in ambient conditions but positive in severe drought conditions, three that were always positive regardless of treatment, and seven which were always negative regardless of treatment conditions. *Planctomycetota* are among the most commonly detected clades in aquatic and terrestrial ecosystems (Delgado-Baquerizo et al., 2018; Wiegand et al., 2018), but due to their low cultivability, little is known about the extent of their metabolic capabilities and influence on nutrient cycling. Wang et al.

(2015) reported the ability of *Planctomycetota* species to degrade complex heteropolysaccharides in soil. Elevated or maintained relative abundance of *Planctomycetota* in response to drought has also been observed in several studies (Dai et al., 2019; Sheik et al., 2011). In addition, some *Planctomycetota* have been found to display antifungal properties. Graca et al. (2016) explored 40 *Planctomycetota* isolates to identify bioactive molecules and found that 43% of their study group contained genetic markers for antifungal activity which aligns with our finding that many fungal classes were negatively associated with *Planctomycetes* regardless of treatment condition. Although this apparent relationship was not influenced by drought and was not detected at all in mild drought conditions, it is additional evidence of possible functional attributes of a largely uncharacterized clade of microorganisms that warrants further investigation.

The mild drought-ambient differential network was largely unique from the severe drought-ambient network. Here, *Planctomycetes* only exhibited positive associations with the most connected taxa. These most connected taxa included *Thermoleophilia* (*Actinobacteriota*), *Ktedonobacteria* (*Chloroflexota*), and *Vicinamibacteria* (*Acidobacteriota*). *Ktedonobacteria* have been identified as a keystone specialist group in tundra ecosystems with positive correlations to lower moisture content (Wong et al., 2023) and while much of their metabolic capability remains undetermined, Zheng et al. (2019) observed strong functionality related to plant decomposition through cellulolytic activity. Although *Vicinamibacteria* were not detected in the mild drought-ambient comparison of our response ratio analysis, closely related *Bryobacteraceae* exhibited a negative response, and family *SCN_69_37* of the *Vicinamibacteria* did have a positive response ratio in the severe drought-ambient condition. *Vicinamibacteria*

have also previously been reported as highly drought tolerant (Huber et al., 2022) although their mechanism of survival is not yet understood.

The differential networks were further utilized to investigate possible inter-kingdom associations of interest. We hypothesized that the observed clade-wide positive response observed in the response ratios of the *Thermoproteota* (ammonia-oxidizing archaea) under severe drought may be linked to the concurrent increase in *Glomeromycetes* (arbuscular mycorrhizal fungi) relative abundance via turnover of organic N-rich root exudates such as urea, uracil and various amino acids that were detected under the severe drought conditions (Ulrich et al., 2022). Arbuscular mycorrhizal fungi (AMF) are well known plant growth promoters and can help alleviate drought stress through hyphal mining for water and nutrients in the bulk soil adjacent to plant roots (S. Mathur et al., 2019; Wu et al., 2013). However, due to low saprotrophic ability, AMF proliferation is highly reliant on other soil microbes to degrade organic compounds and release available nitrogen (Bukovská et al., 2016). Despite exhibiting strong phyla-wide positive response ratios in severe drought conditions, network analysis did not detect any direct associations between *Thermoproteota* and *Mucoromycotina*. Importantly, although sparcc network statistics are compositionally aware, users are required to manually set thresholds for taxa inclusion that may bias networks towards more highly abundant taxa (Matchado et al., 2021). Members of *Thermoproteota* were among the most abundant ZOTUs so associations with the relatively rarer *Mucoromycotina* may exist but were not detected in the sparcc analysis. Future work would also benefit from the inclusion of non-plant controls to disentangle plant-specific microbiome responses distinct from responses of the soil microbiome alone to drought (Bandopadhyay et al., 2023).

Lack of Difference in Rhizosphere vs. Bulk Samples Suggests a Wider Scope of the Rhizosphere Zone of Influence

Differentiating rhizosphere from bulk soil is common practice in soil microbial ecology studies to determine the influence of plants on the soil microbiome, and differences in community composition between rhizosphere and bulk soils have been well-documented (Castellano-Hinojosa et al., 2021; Ling et al., 2022). However, here we did not detect any significant differences between rhizosphere and bulk soil samples. We hypothesize that the small diameter of our pots (6.4 cm) did not allow for sufficient coring distance from plant roots to make this distinction as the roots largely filled the available space. This finding challenges a common working definition of rhizosphere soil as that which is specifically attached to roots (Chaparro et al., 2014; Shami et al., 2022; Zhu et al., 2020). Kuzyakov and Razavi (2019) reviewed the temporal dynamics and spatial stationarity of plant rhizospheres and found that the rhizosphere generally extends 0.5-4 mm from plant roots. Due to the 4-dimensional (3D-space + 1D-time), fibrous, high-density and high turnover rate of root structure of most grasses including *B. gracilis* (Ravenek et al., 2016), it is very possible that the vast majority of the soil within our pots were within this zone of influence. In a recent global meta-analysis of rhizosphere vs. bulk soil microbiomes, Ling et al. (2022) also found minimal differences in alpha diversity between bulk and rhizosphere soils in grasslands. Since plant species, soil type and texture, nutrient availability and microbiome activity all influence plant-microbe interactions (Jiang et al., 2017; Ling et al., 2022), it follows that the size of the zone of influence of the rhizosphere would be similarly variable.

Root Exudate and Microbiome Composition Correlations

In partial support of our second hypothesis, potential exudate compounds of interest which were primarily sugars and organic acids correlated marginally with the microbiome composition metrics. One possible compound of interest is pyruvic acid which is typically in high abundance for normal functioning photosynthesis, glycolysis, and the TCA cycle. Maurer et al. (2021) found high levels of pyruvate and amino acid exudation that had stimulatory effects on denitrification enzyme activity. Interestingly, while some compounds such as urea, arbutin, L-proline, and D-gluconic acid were only detected in the mild and severe drought treatment root exudates (Ulrich et al., 2022), no correlations between these compounds and shifts in the microbial community composition were detected here. As suggested by Bandopadhyay et al. (2023), perennial grass species may not be as reliant on stress signaling to microbes via root exudation due to their relatively greater investment in belowground root architecture compared to many annual plant species. Another possibility, as suggested by Karlowsky et al. (2018), is that root exudation during drought may be more important for the reinitiation of soil microbial activity following rewetting of the system. The authors suggest that further studies incorporating recovery from drought are needed to test this hypothesis across different soils, plants, and environments. In addition, utilizing metatranscriptomics to measure differential expression of metabolic pathways in the rhizobiome under drought will also be an important step in linking plant-microbe responses to drought. This would bypass the limitations of DNA-based analyses which cannot distinguish the contribution of active microbes to ecosystem processes (Carini et al., 2016). Metatranscriptomics would also provide evidence for potential utilization of root exudates. Experiments could then be designed to explore specific relationships between active microbial taxa and plant physiology.

Conclusion

Here we show that the blue grama rhizobiome underwent significant community shifts as a result of severe drought and was only marginally affected by mild drought. We detected phylogenetically linked tradeoffs in community composition as a result of severe drought conditions including increased relative abundances of arbuscular mycorrhizae (*Glomeromycetes*), ammonia oxidizing archaea (*Thermoproteota*), and *Planctomycetota*. Although we detected weak evidence for correlations between root exudate composition and community beta diversity, these analyses provide a platform for future exploration of similar multi-omic datasets. Altogether this study provides an important step towards understanding plant-microbe feedbacks under drought conditions and provides a foundation for future targeted investigations of the potential protective effects of root exudates and the rhizobiome against drought stress.

Data Availability

All sequencing data are accessible on the NCBI SRA database under bioproject PRJNA1006788. Other data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author Contributions

D.E.M.U. conceived the ideas, designed the methodology with the help of H.M.G., R.C.M., and L.V.G.G.; D.E.M.U. established and maintained the greenhouse experiment with the help of H.M.G., R.C.M., and B.M.P. H.M.G. performed DNA extraction, sequencing, and bioinformatics; H.M.G., R.C.M., D.E.M.U. and B.M.P. contributed to data analysis. H.M.G. was the primary author of the manuscript. All authors contributed critically to the written manuscript and gave final approval for publication.

Figures and Tables

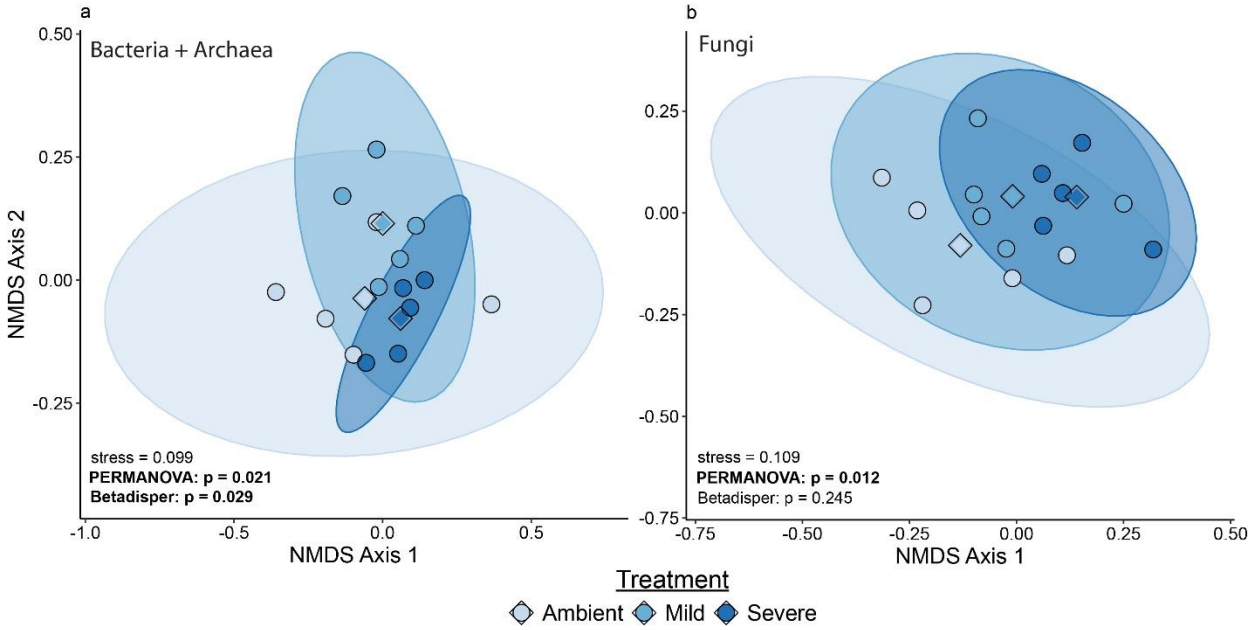


Figure 1. Beta diversity measured with unweighted Unifrac of bacterial + archaeal community (a) and fungal community (b) at T2. Points represent individual samples with a larger diamond representing the treatment centroid. Ellipses indicate the 95th percentile area of community distribution. Legend shows symbol and ellipsis color.

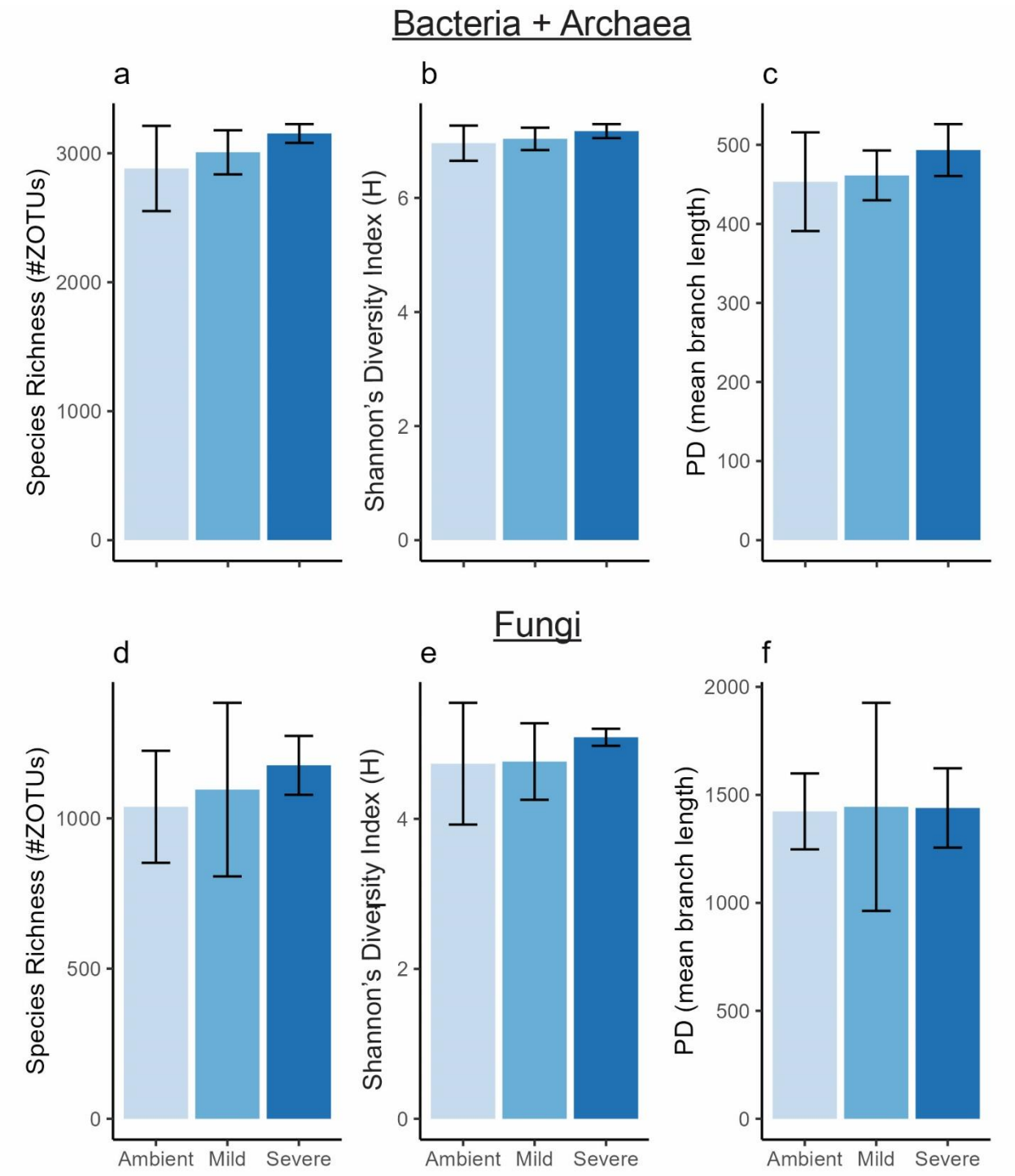


Figure 2. Alpha diversity of bacterial/archaeal community (a-c) and fungal community (d-f) measured by species richness, Shannon's diversity index (H), and Faith's Phylogenetic Diversity (PD) at T2. Error bars are ± SE.

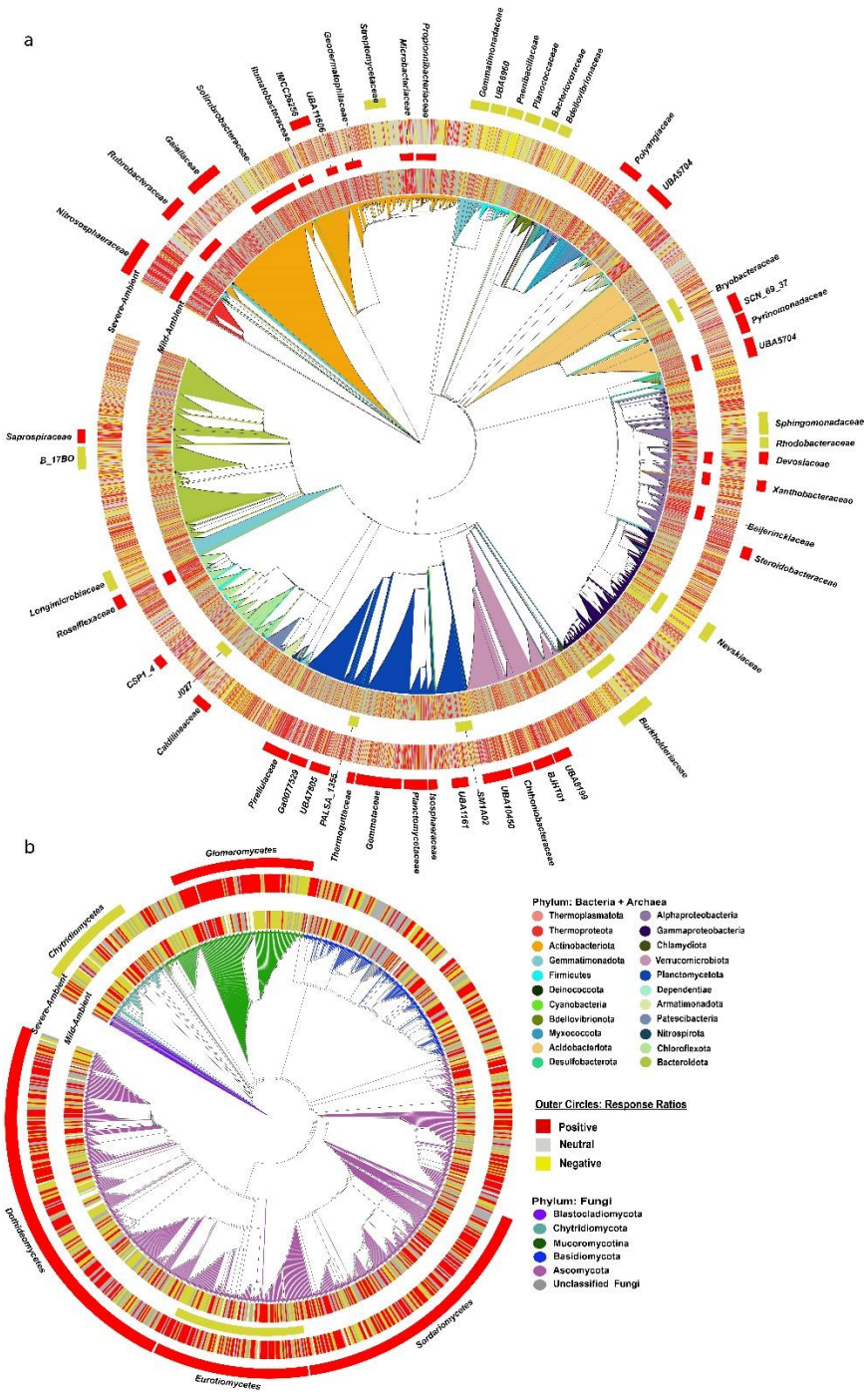


Figure 3. Phylogenetic trees of bacterial + archaeal (a) and fungal (b) communities, colored by phyla. The 1st ring for each the Mild Drought-Ambient comparison (inner circles) and Severe Drought-Ambient comparison (outer circles) indicates positive (red), neutral (gray) or negative (yellow) response ratios at the ZOTU level, then families for the bacteria + archaea and classes for fungi with significantly positive (red) or negative (yellow) response ratios are indicated by red (positive) or yellow (negative) colored strips for both drought-ambient comparisons.

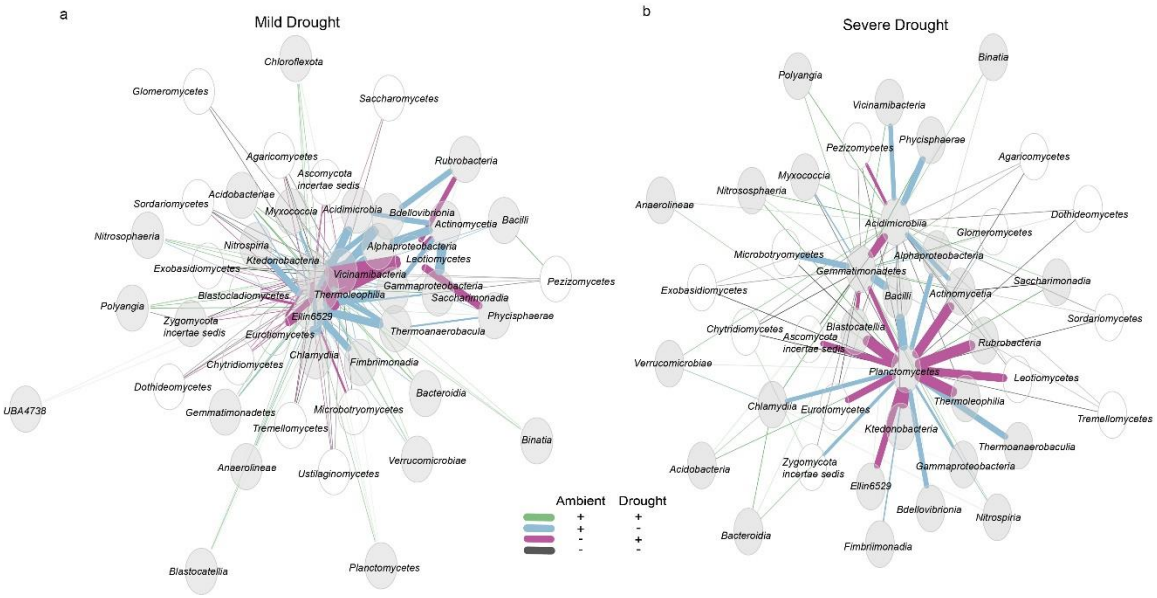


Figure 4. Sparcc differential network visualizing the top 50 associations of the total microbiome community at the class level under (a) mild drought vs. ambient conditions, and (b) severe drought vs. ambient conditions. Bacterial and archaeal nodes are gray while fungal nodes are unfilled. Line thickness indicates strength of association. All associations are significant with BH-adjusted $p < 0.05$.

Table 1. Summary of Beta Diversity Test Statistics (PERMANOVA)

	Variable	**Df**	**SumofSqs**	**R^2**	**F**	**P-value**
Bacteria + Archaea	Treatment (Drought)	2	0.471	0.0466	1.41	**0.01**
	Time	1	0.274	0.0271	1.64	**0.007**
	Microhabitat	1	0.173	0.0161	1.04	0.289
	Treatment:Time	2	0.383	0.0379	1.15	0.114

Table 1 Continued.

	Variable	**Df**	**SumofSqs**	**R2**	**F**	**p-value**
Bacteria + Archaea	Treatment:Microhabitat	2	0.333	0.0330	1.00	0.405
	Time:Microhabitat	1	0.160	0.0156	0.958	0.482
	Treatment:Time:Microhabitat	2	0.321	0.0318	0.965	0.553
	Beta dispersion	2	0.00336	NA	4.81	**0.029**
Fungi	Treatment (Drought)	2	0.370	0.1832	1.34	**0.025**
	Time	1	0.1706	0.02635	1.59	*0.059*
	Microhabitat	1	0.0803	0.01239	0.746	0.772
	Treatment:Time	2	0.2077	0.0321	0.965	0.477
	Treatment: Microhabitat	2	0.2412	0.0373	1.12	0.258
	Time: Microhabitat	1	0.078	0.0120	0.725	0.815
	Treatment:Time: Microhabitat	2	0.2633	0.0406	1.22	0.151
	Beta dispersion	2	0.001022	0.000511	0.766	0.486

Table 2. Summary of Phylosignal Test Results

	Mild Drought		**Severe Drought**	
Test	**Stat**	**P-value**	**Stat**	**P-value**
	Bacteria + Archaea			
Pagel's Lambda	0.702	**2.21e-144**	0.701	**1.26e-144**
Blomberg's K	0.0270	**0.001**	0.0179	**0.011**
Abouheif's C_{mean}	0.107	**0.001**	0.103	**0.001**
	Fungi			
Pagel's Lambda	0.793	**1.51e-35**	0.808	**2.12e-51**
Blomberg's K	0.0267	0.157	0.0287	0.134
Abouheif's C_{mean}	0.132	**0.001**	0.198	**0.001**

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CHAPTER FOUR

PRECIPITATION LEGACY INFLUENCES BLUE GRAMA PHYSIOLOGY AND MICROBIOME RESPONSE TO DROUGHT STRESS

Contribution of Authors and Co-Authors

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Abstract

Climate legacy is an important factor dictating the level of current-day resiliency of grasslands to extreme climate change-driven changes in precipitation patterns. The collective activity of plants and microbes within grassland ecosystems drive many globally important ecosystem services. While climate legacy has been shown to influence the response of both plants and microbes, the effects on their interactions remains relatively unexplored. Here we sought to fill this knowledge gap by exploring the physiological response of blue grama (*Bouteloua gracilis*) to drought stress and subsequent recovery when grown in native *B. gracilis* rhizosphere soils with differing precipitation legacies along a 200 mm mean annual precipitation (MAP) gradient. Plants grown with rhizobiomes collected from either end of our precipitation gradient exhibited the least drought stress and significantly increased root exudate TOC and TN. The rhizosphere bacterial + archaeal community of plants grown in *B. gracilis* rhizosphere soil from our lowest MAP site had positive responses in relative abundance amongst the *Acidobacteriota*, *Actinobacteriota*, *Armatimonadota*, *Chloroflexota*, and *Planctomycetota* phyla at peak drought conditions that were not observed in other soils. While all plants grown in all soils recovered physiologically post-drought, the rhizosphere community of our highest MAP site had a unique signature of largely positive responses in relative abundance of bacterial + archaeal phyla upon recovery post-drought compared to the other soils that exhibited more negative or mixed responses. Collectively, these results provide valuable insights into the influence of climate legacy on the rhizosphere diversity of an important grassland species, the variability of root exudation patterns under drought stress within a single species, and the potential links between blue grama and its microbiome in response to drought stress.

Introduction

Grasslands are among the most species-rich habitats on Earth, both above ground with nearly 100 plant species per square meter in some cases (Petermann and Buzhdygan 2021), and belowground with extremely high soil microbial diversity (Delgado-Baquerizo et al. 2018; Bastida et al. 2021b). Together, plants and microbes provide numerous valuable ecosystem services of grasslands including forage for livestock and wildlife, carbon sequestration, pollination, erosion control, and climate cooling (Petermann and Buzhdygan 2021; Dass et al. 2018). Plant and microbial activities are tightly regulated by soil moisture and can be largely constrained by historical precipitation patterns in a given ecosystem (Averill, Waring, and Hawkes 2016). Many microbial functions including carbon cycling and storage are similarly regulated by water availability (Manzoni, Schimel, and Porporato 2012).

Variation in precipitation patterns can strongly impact grasslands (Knapp and Smith 2001) and the legacy of prior years' precipitation can be an important predictor of future above- and belowground productivity (Waring and Hawkes 2018). Hawkes et al. (2017) showed that precipitation history strongly dictated soil respiration responses to elevated soil moisture, indicating that the composition and functioning of the native soil microbiome across climate and geographic gradients will affect subsequent ecosystem responses to perturbation. Other recent studies have investigated plant-microbe interactions under drought in soils with either high (F. T. de Vries et al. 2019; Fuchslueger et al. 2014) or low (F. T. de Vries et al. 2019) historical precipitation and found inconsistent responses between plant species, highlighting the need to study a single plant species across a historical precipitation gradient.

Plants may select for and attract beneficial microbes (bacteria, archaea, fungi, etc.) which can increase plant access to nutrients and water (Ling, Wang, and Kuzyakov 2022). Chemical signaling via root exudates is one of the mechanisms plants employ to modulate their microbiomes. Root exudates can include a variety of metabolites including sugars, amino acids, and organic acids (Zhao et al. 2021; Prescott et al. 2020; Pausch and Kuzyakov 2018). Evidence is mounting that plants modulate the release of root exudates in response to environmental conditions (Zhalnina et al. 2018; Lu et al. 2018). However, the external dynamics driving the magnitude of root exudation responses are still relatively unexplored due to the vast diversity of plant species and the interacting effects of plant species, microbiome compositions, and abiotic conditions.

The objective of this study was to determine the influence of precipitation legacy on C allocation and microbiome restructuring of *Boutelous gracilis* under drought stress. *B. gracilis* (blue grama) is a drought-tolerant C4 grass native to short grass prairies across the western and southwestern regions of the U.S (Bushey et al. 2023). This species has great ecological and economical importance as it is a major contributor to belowground carbon storage, is quality forage for livestock and wildlife, and is often utilized in conservation and restoration efforts (Costello 1944; Bushey et al. 2023). Prior experiments with blue grama have shown that *B. gracilis* increases root exudate TOC under drought driven by increased exudation of many osmoprotectants and microbial signaling compounds (Ulrich et al. 2022). The level of drought severity has a significant effect on the blue grama rhizobiome composition (Goemann et al. 2023, in publication). However, it is unknown whether these effects are shared across blue grama rhizospheres with differing climate legacies.

We collected rhizosphere soil from four native populations of blue grama that spanned a 200 mm annual precipitation gradient and conducted a greenhouse experiment to implement a drought with a subsequent recovery period using blue grama plants from a common seed source grown in the four soils with differing precipitation histories. The approach was designed to examine the role of the soil microbiome in driving blue grama responses to drought, rather than genotypic responses of the plant. We hypothesized that: i) precipitation history will influence the native blue grama rhizobiome ii) blue grama plants will experience the least drought stress in soils with rhizobiomes originating from sites with lower historic MAP, iii) root exudation will increase with drought stress, and iv) blue grama rhizosphere community will have unique precipitation legacy signatures among the four soils. This experimental plan also lent itself toward characterizing the core blue grama rhizosphere across environments which contributes to current goals in the plant-microbiome field to better understand the dynamics of plant-microbe holobionts (Vandenkoornhuyse et al. 2015). This work is an important contribution to understanding how historic climate conditions shape the blue grama soil microbiome and how climate legacy affects plant-microbe interactions.

Methods

Site Selection, Soil Collection and Preparation

To determine soil collection locations along a mean annual precipitation (MAP) gradient within native populations of blue grama (*Bouteloua gracilis*), we compared blue grama distribution data from the SEInet database (SEINet 2023) with PRISM historical climate data (PRISM Climate Group 2010). Four sites were chosen based on the past 10-year averages of MAP and current presence of blue grama: Dillon (250 mm), Ruby Reservoir (330 mm), Madison

River (400 mm) and Paradise Valley (450 mm). Approximately 20 L of topsoil (0-15 cm soil depth) were collected from established stands of blue grama at each of these sites managed by the U.S. Bureau of Land Management (BLM) in Southwestern Montana, USA on 6/02/22 (Figure 1). Site descriptions are provided in Table 1 including soil characteristics accessed from the National Resource Conservation Service Web Soil Survey (Soil Survey Staff 2023). Written permission to collect soil at each site was acquired from the respective regional BLM offices. To examine the variation in the blue grama rhizobiome across the sites, an additional five replicates of background blue grama rhizosphere soil (directly adjacent to the root balls of living blue grama plants) were collected in 15 mL Falcon tubes at each site within the same soil collection area, transferred to ice for transport and stored at -20 °C until DNA extraction.

Soil was transported to the Montana State University (MSU) Plant Growth Center and prepared for experimental conditions within 48 hrs. of field collection. The soils were first sieved to remove rocks and large debris (> 1 cm). Then, to retain the soil microbiomes while minimizing nutrient and texture differences between the soils, experimental pots (D40H Deepots, Stuewe & Sons, Inc., OR, USA) were prepared using a mixture of 30% field soil, 69% 30 mesh graded, washed and kiln dried sand (Sakrete, GA, USA), and 1% autoclaved coconut fiber (Thrive, CA, USA) to minimize pot compaction. All tools were ethanol-sanitized between soils. Pots were washed, bleach-sterilized (1%), and dried prior to use. Pots prepared with each of the four sand:soil mixtures were then seeded with ~10-20 blue grama seeds (Wind River Seed Co., Manderson, WY, USA, n = 440 total), covered with plastic wrap and watered daily to maintain moisture levels for one week until all pots had produced several 0.5” seedlings at which time the plastic wrap was removed and all pots were thinned to one plant. Pots were numbered and

randomized by soil origin, and locations on the greenhouse bench were re-randomized weekly to minimize greenhouse microsite effects (Fig S1). Greenhouse conditions during the study (Jun-Sept) consisted of a 16-hour photoperiod, daytime temperature of 23.8 °C, nighttime temperature of 21.1 °C, and average daytime PAR of 500 μmol m^2 s^{-1}. Plants were watered daily to maintain field water capacity and allowed 60 days of pre-growth prior to the drought experiment.

Drought Experiment

After the pre-growth period (06/08/22-08/07/22), n = 16 plants for each site were randomly assigned to either drought (no watering) or ambient treatments (continued daily watering to maintain field water capacity). To track water stress, five additional replicate plants for each treatment group were utilized for destructive harvesting to measure weekly predawn and midday leaf water potentials. Leaf water potentials were measured using a Scholander type pressure chamber (model 1505D, PMS Instruments, Corvallis, OR, USA). A previous study of blue grama determined the turgor loss point to be -2 MPa predawn water potentials (Ulrich et al. 2019b). We chose -2 MPa for this experiment as a goal for peak drought so as to stress the plants without killing them. In this experiment -2 MPa (or peak drought) was reached by more than one plant in at least one soil after two weeks (Fig. S2), after which the drought treatment ended. One half of each treatment group (n=8) was then destructively harvested for plant physiology measurements and microbiome analyses. The remaining droughted plants were then returned to ambient watering conditions for a recovery period until predawn and midday leaf water potentials returned to ambient levels (which took two weeks) at which time the remainder of the plants were harvested.

Plant Physiology and Root Exudates

To determine how precipitation legacy and drought treatment influenced plant physiology, we measured predawn and midday leaf water potential, leaf gas exchange (photosynthesis, stomatal conductance), root and shoot biomass (root, shoot), and root exudate carbon and nitrogen in each treatment group at peak drought (Day 14) and post recovery (Day 28). Gas exchange was measured using a portable photosynthesis instrument equipped with an infrared gas analyzer (LI-6800, Licor, Lincoln, NE USA) on 5 plants randomly selected from each treatment group. At time of each harvest, soil for microbial analysis was collected by removing plants from the experimental pots into small, ethanol-sterilized containers, thoroughly homogenizing the soil, then harvesting 50 mL of the soil into sterile collection tubes. Soil samples were stored briefly on ice, then transferred to long-term storage at -80 °C. Root exudates were collected via the Phillips et al. (2008) method at the time of each harvest and transferred to long-term storage at -80 °C. Briefly, plants were removed from pots and roots were rinsed of soil in DI water and dipped in Gibco antimicrobial-antimyotic solution (ThermoFisher Scientific, CA USA) to halt the microbial production of C compounds. This ensured that we collected exudates from the plant and not microbes. Plants were subsequently transplanted to filter flasks of 60-120 mesh glass beads (100 mL) that do not provide a C source but still provide mechanical pressure on the root system, resembling natural soil conditions. Plant roots were flushed with 100 mL of sterile water using a vacuum pump connected to the filter flasks, another 100 mL was added, and plants were allowed to release exudates over a 48-hour period. The remaining solution was filtered (0.22 µm) and collected for root exudate concentration and composition analyses. Root exudates were frozen and stored at − 80 °C until analysis. Exudate samples were analyzed for total carbon, total inorganic carbon, non-purgeable organic carbon, and total nitrogen at the

Montana State University Environmental Analytical Laboratory on a Shimadzu TOC-V-TN (Shimadzu, Columbia MD, USA).

Soil DNA Extraction, Amplification and Sequencing

To obtain high-resolution representation of the soil microbial communities and their response to drought, we performed rRNA gene amplicon sequencing on all replicates of soil for each soil, treatment, and timepoint. As the plant roots largely filled the available space in the pots, we considered all collected soil as "rhizosphere" soil. Genomic DNA was extracted from 0.5 g frozen soil samples using the Fast DNA SPIN Kit for soil (MP Biomedicals, Santa Ana, CA) according to the manufacturer's instructions. We targeted the V4 region of the 16S rRNA gene using 515F (Parada, Needham, and Fuhrman 2016) and 806R-b (GTGYCAGCMGCCGCGGTAA and GGACTACVSGGGTATCTAAT, respectively) from the Earth Microbiome Project (Thompson et al., 2017). Reactions were carried out in 20 μL volumes containing 1 U Phusion high-fidelity polymerase (ThermoFisher Scientific, MA, USA), a final concentration of 1x Phusion HF reaction buffer, 200 μM dNTPs, 0.5 μM each primer, 1 μM BSA, 1 mM MgCl, sterile molecular grade water, and 5 μL (10 ng) genomic DNA as a template. PCR conditions consisted of initial denaturation at 98 ˚C for 30s, followed by 22 cycles of 98 ˚C for 30 s, 55 ˚C for 30 s, 72 ˚C for 45 s and a final extension at 72 ˚C for five minutes. Samples were purified using AMPure XP (Beckman-Coulter, Brea CA, USA) magnetic beads and barcoding was conducted using the Illumina Nextera Indexing Kit D, with 10 cycles of indexing PCR in 20 μL volumes using the same concentrations as PCR1 with 10 μL template DNA. Samples were purified again with magnetic beads and PCR products were checked for quality and length in a 1% agarose gel and quantitated using the Quant-iT™ dsDNA Kit (Invitrogen,

Carlsbad CA, USA) with a BioTek H2 plate reader. Individual samples for each community were combined at equimolar concentrations and the two libraries were pooled at 4 nM and loaded onto an in-house Illumina MiSeq (Illumina, San Diego CA, USA) and sequenced using the V3 600 cycle kit.

Analysis of the fungal rhizosphere community was also attempted but due to technical difficulties achieving high quality sequencing data with similar replication to the 16S community, fungal results are reported as anecdotal in the Supplemental Information. Fungal sequencing samples were prepared as described above. We targeted the D2 hypervariable region of the fungal LSU using primers LR22R (Mueller, Gallegos-Graves, and Kuske 2016) and LR3.

Bioinformatics

Reads were merged, trimmed, and dereplicated with USEARCH, quality filtered with an ee-value of 1.0 and zero-radius operational taxonomic units (ZOTUs) were identified with UNOISE3 (v.11.0.667) (Edgar, 2010). A total of 10,854,982 reads were clustered into 31,205 ZOTUs. Representative 16S ZOTUs were classified using a subset of the Genome Taxonomy Database (GTDB, Chaumeil et al., 2022). All reads classified to chloroplast were removed prior to downstream analysis. Fungal ZOTUs were likewise curated, using the Ribosomal Database Project for classification (Cole et al. 2014). For statistical analyses, the datasets were randomly subsampled according to the sample with the lowest read number for statistical analysis (18,840). To construct a 16S phylogenetic tree for environmental ZOTUs, we used reference phylogenies constructed with full length and near full length sequences of isolates and metagenome assemblies downloaded from GenBank, focusing on GTDB sequences. Reference sequences were aligned using mafft (Katoh and Standley 2013), and a maximum likelihood tree was

constructed with the GTR + gamma model using RAxML (Stamatakis 2014). Environmental sequences were aligned to the reference using mafft with the –add flag and then mapped onto the reference tree using pplacer (Matsen, Kodner, and Armbrust 2010) with reference-aligned sequences.

Statistical Analysis

Plant physiology, root exudate chemistry and microbial community analyses were conducted using R software (v. 4.3.0, R 2023) in RStudio (RStudio Team 2020). Data management was handled primarily with the tidyverse package (Wickham et al. 2019). We utilized a two-way ANOVA framework with site, treatment, and timepoint effect variables. Tukey post-hoc tests using the multcomp package (Hothorn, Bretz, and Westfall 2008) were utilized to determine significant group differences the between treatments, sites, and timepoints. Plant physiology measurements were tested for normality using Shapiro-Wilk tests. No data transformations were found to be necessary.

Community ordination and alpha diversity analyses were performed using the packages vegan (Oksanen et al., 2019), picante (Kembel et al. 2010) and phyloseq (McMurdie and Holmes 2013). We used PERMANOVA (Anderson 2017) to test for treatment, site, and timepoint effects on community beta diversity computed using unweighted UniFrac (Lozupone and Knight 2005). Background blue grama rhizobiome samples from each sampling site were analyzed separately from the experimental data to determine site differences. The indicspecies package (Cáceres and Legendre 2009) was used to identify ZOTUs driving the differences between the four sites.

To examine how ZOTU responses to drought were distributed in the bacterial + archaeal communities across the four soils we compared response ratios from droughted and ambient

samples at each timepoint. Response ratios were computed by dividing the difference between the read counts in the treatment (drought) and ambient conditions by the total read count for a ZOTU. Response ratios for each ZOTU were permuted 999 times for each ZOTU and averaged across 6 replicate samples in each treatment. One-way student's t-tests were then conducted across taxonomic levels to determine which clades had response ratios significantly different from 0. All t-tests were adjusted with Benjamini-Hochberg (BH) multiple comparisons corrections. All figures were edited for publishing quality in Adobe Illustrator 2023 27.5.0.

Results

Original Site Differences in Blue Grama Rhizobiome

The alpha and beta diversity of the blue grama rhizobiome were significantly different across the four collection sites (Fig. 2a, 2b-d). The bacterial + archaeal communities significantly separated by site based on unweighted Unifrac distance ($R^2 = 0.345$, $F_3 = 2.649$, $p = 0.001$, Table S1). The Dillon site (lowest historical MAP) generally had the lowest alpha diversity (Faith's Phylogenetic Diversity (PD): 406 ± 30 and species richness: 2175 ± 233, 95% CI) while Madison River had the highest (Faith's PD: 484 ± 48, species richness: 2562 ± 247, 95% CI, $p_{Tukey} = 0.021$). Shannon's Diversity Index (H) increased slightly along the precipitation gradient with Madison River and Paradise Valley having marginally greater evenness than Dillon ($p_{Tukey\ MR\text{-}DL} = 0.0835$, and $p_{Tukey\ PV\text{-}DL} = 0.0651$). These compositional differences were driven primarily by the relative abundances of *Chloroflexota*, *Gemmatimonadota*, and *Verrucomicrobiota* phyla across the four sites (Fig. 2e). At the ZOTU level, we utilized indicator species analysis to determine the ZOTUs driving the differences between the four soils. We identified 724 indicator ZOTUs in Dillon soil, 194 in Ruby Reservoir soil, 784 in Madison River soil, and 545 in

Paradise Valley soil. Most of the ZOTUs identified by the indicator species analysis belonged to the phyla *Acidobacteriota, Actinobacteriota, Alphaproteobacteria, Chloroflexota, Planctomycetota,* and *Verrucomicrobiota* (Fig. 2f).

Despite these differences in community composition across the four collection sites, we identified eleven bacterial + archaeal families comprising the "core microbiome" of the blue grama rhizosphere across the sites (Fig. 3) based on prevalence in least 50% of samples (10/20) and a minimum relative abundance of 1% in the community. These families consisted of: one archaeal family, *Nitrosphaereaceae*, known to be primarily ammonia oxidizers, and bacterial families *Rubrobacteraceae* and *Solirubrobacteraceae (Actinobacteria*), *Gemmatimonadaceae (Gemmatimonadota), Pyrinomonodaceae* and *UBA2999 (Acidobacteria), Chitinophagaceae (Bacteroidota), Xanthobacteraceae* and *Beijinckeraceae (Alphaproteobacteria), Burkholderiaceae (Betaproteobacteria),* and *UBA1050 (Candidatus Saccharibacteria). Nitrososphaeraceae* had the greatest prevalence and abundance with a > 5% abundance in 100% of the samples, while *Burkholderiaceae* was only present at close to 1% abundance in 50% of the samples.

Plant Physiology in Response to Drought and Recovery

Drought treatment had significant effects on plant physiology. Leaf water potentials at both predawn and midday were significantly lower in droughted plants after two weeks (peak drought. Fig. 4a,b). Leaf predawn water potential measurements indicated that plants experienced increasing water stress in soils with increasing historical MAP across the four sites at peak drought. Plants grown in the Dillon soil were least drought stressed (-1.229 ± 0.324 95% CI MPa) with predawn leaf potentials beign significantly higher than that of plants grown in the

other three soils. In contrast, plants grown in Paradise Valley soil where historic MAP is the greatest were the most drought stressed (-1.854 ± 0.219 95% CI MPa). Plants grown in Ruby Reservoir and Madison River site soils had predawn leaf water potentials in the middle of the two extremes although we did not detect significant differences between Ruby Reservoir, Madison River, and Paradise Valley. Similarly, midday leaf water potentials had significantly greater magnitude at peak drought compared to ambient plants, averaging -2.13 ± 0.132 across all droughted plants vs. -0.712 ± 0.105 MPa across all ambient plants. In contrast to predawn leaf water potentials, midday leaf water potentials did not significantly differ among the four soils.

Total plant biomass did not significantly differ among the four soils, nor between drought and ambient treatments at either timepoint (Fig. 4c,d). However, gas exchange (photosynthesis and stomatal conductance) significantly decreased in all four soils in response to drought (Fig. 5e,f). Photosynthesis averaged 0.146 ± 1.21 μmol CO_2 m^{-2} s^{-1} in droughted samples vs. 11.4 ± 1.49 (95% CI) in ambient plants. Stomatal conductance (to water vapor) averaged 0.00527 ± 0.00548 mol H_2O m^{-2} s^{-1} in the droughted treatment for all four soils vs. 0.0472 ± 0.00894 (95% CI) in the control treatment for all four soils. Paradise Valley plants in the drought treatment did not have detectable differences from the control treatment for both photosynthesis and stomatal conductance, or from the other sites among the drought treatment. All other sites had significantly decreased photosynthesis and stomatal conductance from their respective control treatments.

After two weeks of recovery, predawn leaf water potential, photosynthesis, and stomatal conductance of plants in the drought treatment returned to the same levels as the control treatment across the four soils. Predawn and midday leaf water potentials were also measured in

the week between peak drought and the final recovery endpoint, at which point the water potentials had already recovered to pre-drought levels and we did not detect any evidence for site differences in the rate of recovery. We also did not detect any significant soil site effect differences, nor differences between control treatments from the peak drought to recovery timepoint. Total biomass and root:shoot ratios did not significantly differ between peak drought and recovery for any treatment groups.

Root Exudate Chemistry Response to Drought and Recovery

Root exudate chemistry significantly differed among the four soils in response to drought. At peak drought, total carbon (TC) was significantly greater in the drought treatment than the control treatment in all soils except for Madison River (Fig. 5a). Within the drought treatment TC was significantly higher in Dillon samples compared to Ruby Reservoir and Madison River (Dilon: 0.185 ± 0.0359, Ruby Reservoir: 0.106 ± 0.0183, Madison River: 0.0838 ± 0.0274 95% CI mg C/L/mg root mass), but not compared to Paradise Valley (0.136 ± 0.0653 95% CI mg C/L/mg root mass) samples. The significant differences among soils and between treatments in TC were primarily driven by changes in total organic carbon (TOC) rather than inorganic carbon (TIC) as only Paradise Valley had significantly greater TIC in the drought treatment than in the control treatment. Dillon and Paradise Valley soil samples had significantly greater TOC in droughted vs. ambient samples (Dillon: 0.0730 ± 0.0216 vs. 0.0345 ± 0.00768 95% CI; Paradise Valley: 0.0652 ± 0.0371 vs. 0.0238 ± 0.00614 95% CI, Fig. 4b), while no soil site differences were detected for TIC (Fig. 5c). Treatment differences in root exudate total nitrogen (TN) between ambient and droughted samples at peak drought were only detected in Dillon and Paradise Valley soils (Fig. 5d). Drought doubled Dillon soil root exudate TN to

0.00781 ± 0.00308 from 0.00350 ± 0.00145 (95% CI) mg N/L/mg root mass in the control treatment.

Upon recovery from drought, we did not detect any differences in root exudate chemistry between the drought and control treatments in any of the four soils. There were also no significant differences in root exudate TC, TOC, TIC, or TN between the soils in the control treatment at the peak drought vs. the recovery timepoint.

Microbial Community Response to Drought and Recovery

Soil origin site influenced the bacterial + archaeal community of the blue grama rhizosphere in response to drought. Although we did not detect treatment effects on beta diversity (unweighted Unifrac) at either the peak drought or recovery timepoints, the microbial communities retained soil site differences (Fig. 6, Table S2). The Ruby Reservoir group was also observed to have marginally decreased dispersion compared to Madison River (p.adj = 0.070) and Paradise Valley (p.adj = 0.065) communities at peak drought regardless of treatment, which was significant compared to Madison River (p.adj = 0.027) at the recovery timepoint.

To identify significant changes in relative abundances among sites, treatments and timepoints, we compared response ratios between the control treatment and drought treatment communities for each soil at each timepoint. At the phylum level we observed several distinct responses among the four soils (Fig 7). At the peak drought timepoint, Dillon samples had the most phyla with significantly positive response ratios which included the *Acidobacteriota*, *Actinobacteriota*, *Armatimonadota*, and *Planctomycetota.* All the phyla with at least ten representative members in the Ruby Reservoir soil exhibited negative or neutral response ratios. In samples with Madison River soil, we detected positive response ratios in *Bdellovibrionata* and

Chlamydiota phyla while most other phyla had negative response ratios. *Tectomicrobia* was the only phyla with a positive response ratio in Paradise Valley soils. Several phyla responded in unison across the four soils including negative response ratios in *Bacteroidota, Elusimicrobiota, Gemmatimonadota, Gammaproteobacteria* (class), *Myxococcota, Omnitrophota,* and *Patescibacteria.*

In contrast, after the two weeks of recovery we did not detect any phyla with significant response ratios that were uniformly positive or negative among all four soils. Dillon and Ruby Reservoir soil samples retained essentially the same response ratios as in the peak drought comparison. Madison River response ratios were also largely similar with the exception of a positive response ratio in the *Firmicutes* phylum. However, many of the Paradise Valley phyla had shifted so that all had positive or neutral response ratios except for the *Dormibacteria*.

We further explored the response of the core microbiome of blue grama identified in the background soils to the drought and subsequent recovery. Among the families identified as comprising the core microbiome (50% prevalence across the four soils, with at least 1% relative abundance), response ratio trends varied across the four soils (Fig. 8). At the peak drought timepoint in the Dillon soil, all core members had neutral responses except *Gemmatimonadetes* (negative) and *Solirubrobacteraceae* (positive) which was the only significantly positive response ratio across the four soils at peak drought. *Solirubrobacteraceae* was also positive at the recovery timepoint for Dillon. Ruby Reservoir had mostly negative response ratios across the core families (7/11 in peak drought, 5/11 in recovery) with the rest being neutral. Madison River response ratios were similar to Ruby Reservoir with (8/11 in both peak drought and recovery), although in recovery the Madison River *Beijinckeraceae* had a positive response ratio. For

Paradise Valley soils most responses were neutral with the exceptions of *Burkholderiaceae*, *Chitinophagaceae*, and *Gemmatimonadaceae* (all negative) in Peak Drought, and *Chitinophagaceae*, *Rubrobacteraceae*, and *Pyrinomonadaceae* (all positive) after the recovery period. Few core families responded in unison across either of the two timepoints. At peak drought, *Gemmatimonadaceae* (negative), *Beijerinckaceae*, *Nitrososphaeraceae*, and *Rubrobacteraceae* (neutral) were the only families that responded in unison. *Xanthanobacteraceae* was the only family that responded in unison in the recovery timepoint (neutral).

Discussion

Precipitation and temperature patterns are the major determinants of the global distribution of grasslands which account for 40% of the earth's terrestrial area (Loveland et al. 2010; Petermann and Buzhdygan 2021). The effects of increased frequency and severity of droughts predicted by many climate change models (Trenberth et al. 2014; A. Dai 2011) are major concerns for future grassland productivity and species distribution. Soil climate history has been shown to structure the soil microbiome in other systems (Hawkes et al. 2017; Waldrop and Firestone 2006b), but how that history primes plant-microbe interactions in response to future climate conditions has yet to be extensively investigated. Therefore, we sought to better understand how blue grama microbiomes collected along a 200 mm historical precipitation gradient influence the physiological response of *B. gracilis*, a widespread grassland species dominant across many North American short grass prairies, to drought and subsequent recovery.

Soil Precipitation Legacy Influenced Blue Grama Rhizosphere Composition

In support of our hypothesis, soil site origin had a significant effect on the blue grama bacterial + archaeal rhizosphere community alpha and beta diversity. The differences in beta diversity were driven primarily by different relative abundances of *Chloroflexota*, *Gemmatimonadota*, and *Verrucomicrobiota*. Interestingly, the relative abundances of these phyla somewhat followed the precipitation gradient across the sites; we observed greater *Gemmatimonadota* and *Verrucomicrobiota* within increasing MAP and the opposite trend was observed for *Chloroflexota* with the exception of the Paradise Valley soil. While *Verrucomicrobiota* are known to be sensitive to moisture and decrease in relative abundance with decreasing moisture (Moreno-Espíndola et al. 2018; Martins et al. 2023), the response of *Gemmatimonadota* to soil moisture is unclear as different studies have found contrasting patterns (Yao et al. 2017; Fawaz 2013). *Chloroflexota* have a wide variety of mixotrophic metabolisms and are often highly abundant in arid soils (Sterling et al. 2022). Yao et al. (2017) also observed a decrease in *Chloroflexota* across a much narrower precipitation gradient (215-261 mm MAP) in the Mongolian steppe. Indicator analysis also found that most of the indicator ZOTUs driving the differences between sites belonged to the highly abundant, highly diverse *Actinobacteriota*, *Acidobacteriota*, *Planctomycetota*, *Chloroflexota*, and *Verrucomicrobiota*.

A prior effort to define the core blue grama root microbiome found little variation among blue grama bacterial root endophytes across a strong elevation and precipitation gradient in SW Arizona, USA (Rubin et al. 2020). However, the external rhizosphere experiences much greater influence from other plants and micro-climate conditions than internal endophytes and the diversity of the rhizosphere is generally much greater than that of the endosphere (Moronta-

Barrios et al. 2018; Y. Guo et al. 2021). In addition, fewer species are able to colonize roots due to the highly specific plant-microbe signaling required to initiate endosphere colonization (Knights et al. 2021; Lundberg et al. 2012). Despite the differences across sites, members of our identified core microbiome such as the *Chitinophagaceae* family were also previously identified as core members of the blue grama endophytic microbiome by Rubin et al. (2020). As suggested by their name, *Chitinophagaceae* produce chitinolytic enzymes which are thought to be important for biological control of fungal pathogens (Singh et al. 2013).

Several of the core community members we observed are involved in soil nitrogen and phosphorous cycling. The *Nitrososphaeraceae,* the most prominent family in the core, are ammonium oxidizing archaea which control the rate limiting step of nitrification, the conversion of ammonium to nitrite. Some members of *Nitrososphaeraceae* also contain genes typically involved in phosphorus solubilization suggesting *Nitrososphaeraceae* associated with plants may also contribute to increased phosphorous availability (Wu et al. 2021). Other members of the core microbiome included commonly plant-associated, nitrogen-fixing families such as the *Burkholderiaceae*, *Beijinckeraceae*, and *Xanthobacteraceae* (Ling, Wang, and Kuzyakov 2022). Although the *Actinobacteriotal* families *Rubrobacteraceae* and *Solirubrobacteraceae* are not well studied, phosphorous solubilization is a widespread trait amongst the *Actinobacteriota* (Boubekri et al. 2022). *Burkholderiaceae* and *Chitinophagaeceae* were also identified as core members of the wheat microbiome across African and European soils (Simonin et al. 2020), which suggests these clades may be broadly important amongst *Poaceae* microbiomes.

Soil Precipitation Legacy Marginally Influenced Plant Physiological Drought Response

In partial support of our hypothesis, plants grown in soil originating from the site with historically the lowest MAP (Dillon) exhibited the lowest water stress at peak drought as measured by leaf predawn water potentials. However, there was no site effect among the midday leaf water potentials at peak drought. Both predawn and midday leaf water potentials are commonly used to assess plant water status (Charrier 2020; Tosin et al. 2021). While predawn leaf water potential is assumed to be in equilibrium with the soil water potential and less affected by daily temperature and sunlight variability, midday leaf water potential is more representative of the maximum water stress plants are currently experiencing (Martínez-Vilalta et al. 2014).

Contrary to our hypothesis, plants grown in Paradise Valley treatment group soils were the only group for which we found no evidence of a significant decrease in gas exchange (photosynthesis or stomatal conductance) from the respective control treatment group at peak drought. Photosynthesis in blue grama is strongly dependent on soil moisture (Thomey et al. 2014) so our particular drought may have quickly caused our plants to close stomata and halt gas exchange. In addition, we did not observe any changes in biomass or root:shoot ratios while in prior experiments we observed a significant increase in root:shoot ratio in response to 30 days of severe drought (Ulrich et al. 2022). This may be due to the short duration of our study (14 days of drought) which limited our ability to distinguish physiological effects between plants in our experimental soils. However the peak drought for this study was intentionally defined as when water potentials reached the turgor loss point of blue grama (~ -2 MPa, Ulrich et al. 2019) as exploring the recovery response was also an important objective.

Regardless of soil climate history, all plants in all soils were able to recover to ambient levels of water potential, photosynthesis, and stomatal conductance within two weeks. This high level of plasticity is indicative of the resilient nature of blue grama to drought stress. In a regional synthesis of long-term vegetation monitoring data in the Chihuahuan Desert, Munson et al. (2013) reported that *B. gracilis* can tolerate down to 163 mm summer precipitation before decreasing in stand productivity. Interestingly, at our Dillon site, average summer precipitation for the past 10 years was less than 100 mm, which suggests that the range of local adaptation to water availability may be wider than expected.

Soil Precipitation Legacy Influenced Root Exudation Patterns Under Drought and Recovery

The two soils on the extremes of the historic precipitation gradient (Dillon, low; Paradise Valley, high) showed the most variability in root exudation patterns in response to drought. Both Dillon and Paradise Valley samples had increased TC and TOC, and TN at peak drought compared to ambient samples, and Paradise Valley also had increased TIC. Samples in Ruby Reservoir soil only had increased root exudate TC, and we did not detect any significant change in Madison River root exudates. Increased root exudate TOC in blue grama has previously been reported in response to severe but not mild drought (Ulrich et al. 2022). We hypothesize that the differences in root exudation observed here despite the minimal differences in aboveground plant stress may be suggestive of different microbiome management strategies to regulate drought stress, especially as it also possible that the root exudate composition may have shifted regardless of changes (or lack thereof) in total exudation (Tiziani et al. 2022).

In addition, the recovery of root exudate C and N to ambient levels as drought conditions were released is further evidence that altering root exudation is an integral part of the plant

drought response. The return to “normal” root exudation patterns has been described in several species and the timing appears to depend on species and level of drought severity (Gargallo-Garriga et al. 2018; Canarini, Merchant, and Dijkstra 2016).

Collectively, the plants grown in soils collected on either end of our MAP gradient (Dillon: 250 mm MAP, Paradise Valley: 450 mm MAP) appeared to have tolerated the drought slightly better than plants in Ruby Reservoir or Madison River soil, and had the greatest changes in root exudation. Importantly, if these plants were indeed slightly less stressed at peak drought, it is possible they could have tolerated a longer period of drought prior to recovery compared to plants grown in either Ruby Reservoir or Madison River soil microbiomes. In addition, the lack of difference in total plant biomass despite increased root exudate TOC in Dillon and Paradise Valley plants is further evidence of better plant functioning compared to the Ruby Reservoir or Madison River samples.

Bushey et al. (2023) suggested that among blue grama plant populations there may be a plasticity-sensitivity tradeoff when comparing plant drought responses. They found that blue grama plants adapted to arid sites were more sensitive and showed less osmotic adjustment plasticity in response to changes in moisture levels while plants from more mesic sites exhibited greater plasticity and less sensitivity. Here we purposefully utilized a single genotype of *B. gracilis* to explore the relative contribution of the rhizosphere microbiome to plant physiological response to drought. We speculate that the marginally less drought stress observed in Dillon may be indicative of plant genotype-soil matching, as the MAP of the seed origin location (Manderson WY) is most similar to that of Dillon. However, this does not explain the similar response observed in the Paradise Valley soils. Anecdotally, the soil texture and nutrient

availability in the background soils may have played non-inconsequential role in the experiment, despite the greenhouse pot mixtures being mostly sand. The Paradise Valley pots in particular became extremely cement-like which may have impacted root functioning. We expect this was likely due to unique edaphic characteristics of the Paradise Valley soil, but these differences were not characterized for this study.

Distinct Rhizosphere Community Responses to Drought

Mirrored Precipitation Legacy Effects on Plant Response

Despite experiencing drought and subsequent recovery, the four soils maintained their significant differences in beta diversity observed in the background soils. Although drought did not have a detectable effect on the overarching beta diversity of the four soils individually, in contrast to our hypothesis response ratio analysis indicated a variety of soil-specific changes in the rhizosphere communities that may be indicative of legacy effects of precipitation history in these microbiomes. While legacy effects are not uncommon in microbial responses to drought such as respiration (Hawkes, Shinada, and Kivlin 2020; Glassman et al. 2018), they do not always occur (Preece et al. 2019) for reasons that have yet to be determined. Across the precipitation gradient, shifts in the bacterial + archaeal communities reflected the trends we observed in the root exudates and aboveground plant physiology. We observed the most positive response ratios among bacterial + archaeal phyla in the Dillon at peak drought, while the majority of the Ruby Reservoir, Madison River and Paradise Valley community members exhibited primarily negative or neutral response ratios. In contrast, after the recovery period, the Paradise Valley community exhibited the most positive response ratios compared to the other soils. Interestingly, we observed the same general response pattern among core microbiome members.

Several trends among the response ratios of the observed rhizosphere were similar to those observed in other drought stress studies. Many Gram-positive bacteria such as the *Actinobacteriota* and *Firmicutes* are frequently enriched in drought conditions due to their thick peptidoglycan layers and common ability to sporulate offering protection from desiccation (Xu et al. 2018; Tocheva, Ortega, and Jensen 2016). Here, *Actinobacteriota* only had positive response ratios in Dillon soil, while there were no significant response ratio shifts detected for the *Firmicutes.* Goemann et al. (unpublished 2023) also observed positive response ratios across the *Actinobacteria* in the blue grama rhizobiome under both mild and severe drought. Interestingly, at peak drought the only two phyla with positive response ratios in the Madison River soil (2nd highest historical MAP) were *Bdellovibrionata* and *Chlamydiota*. Both of these phyla had negative response ratios in Dillon and Ruby Reservoir soils (Paradise Valley neutral). *Bdellovibrionata* contains predominantly predatory bacteria (Kamada, Wakabayashi, and Naganuma 2023) which commonly prey upon many Gram-negative bacteria (Johnke et al. 2020). The increased relative abundance of *Bdellovibrionata* in Madison River soils may therefore partly explain the negative response ratios observed across many of the Gram-negative phyla including *Aquificota, Armatimonadota, Bacteroidota, Desulfobacterota, Gemmatimonadota, Methylomirabilota, Planctomycetes,* and *Verrucomicrobiota.*

We hypothesize that the distinct response patterns between soil microbiomes may be evidence of several putative plant-soil feedback mechanisms. First, the positive responses among the Dillon soil community members (lowest historic MAP of our precipitation gradient) in conjunction with greater root exudate TOC and TN and marginally less aboveground drought stress may be further evidence of positive plant-soil feedbacks strengthened by microbial

adaptation to prior drought stress (de Vries et al. 2023; White et al. 2013). Secondly the positive responses of the Paradise Valley soil community members upon recovery from drought may be indicative of the importance of root exudation in resuscitation of community activity post-drought stress (Karlowsky et al. 2018). However, the DNA-based analyses used here do not capture changes in the activity within the rhizosphere. It is very likely that changes in the activity of core members is more dramatic in response to drought than relative abundance, however, exploring changes in relative abundance across our four sites with differing climate legacies provides some indication of the impact of climate legacy on community resistance to drought stress. Lastly, as all plants in all four soils of different climate legacies were able to recover physiologically after drought despite differences in exudation patterns and community responses, it is possible that: (i) blue grama responsivity to moisture outweighed reliance on microbial partners, (ii) differences in community response time due to their respective climate legacies may have had a greater impact in a longer-term drought (Malik and Bouskill 2022), or (iii) that blue grama is able to tailor its response pattern to its environment and available microbial community to achieve the same outcome of plant persistence. Additional experimental evidence is needed to further disentangle these plant-soil feedback mechanisms; the authors suggest broadening the climate legacy gradient, analyzing microbial activity via metatranscriptomics, and utilizing a full common-garden factorial experimental design with both plant genotypes and soil microbiomes.

Conclusion

Here we show that climate history strongly shapes the blue grama rhizosphere bacterial + archaeal community, and that those differences likely contribute to plant physiology responses to drought. We observed distinct root exudation patterns to drought stress across soils with differing

climate legacies and return of altered root exudation to ambient levels upon plant recovery from drought. Changes in root exudation were mirrored in response ratio patterns of the bacterial + archaeal community, providing evidence for positive plant-soil feedbacks in previously drought-adapted soils. This study also improves our understanding of the blue grama core microbiome and its response to drought which will aid in understanding and predicting future distribution patterns of this dominant grassland plant.

Data Availability

All sequencing data will published on the NCBI SRA database upon submission of the manuscript for publication. Plant physiology and root exudation data is available upon reasonable request to the authors.

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Figures and Tables

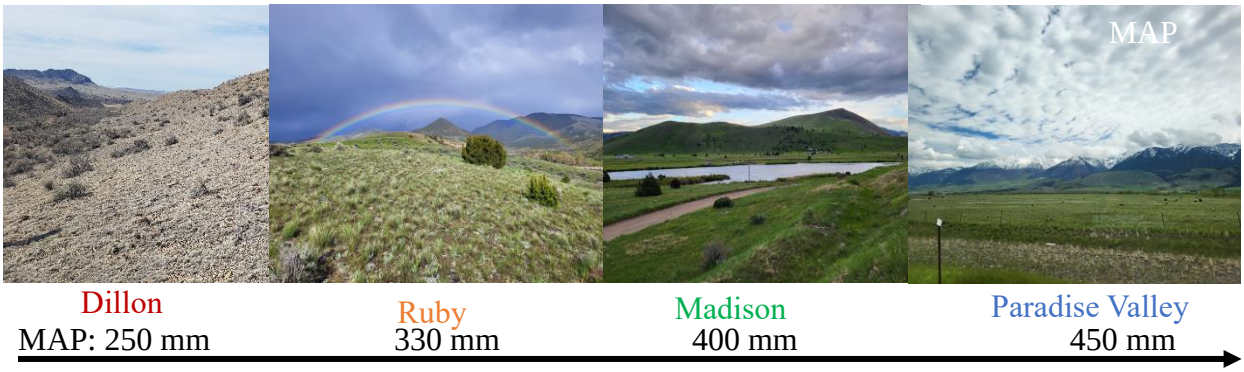


Figure 1. Sampling locations along a 200 mm historic precipitation gradient in SW Montana from left to right in order of increasing MAP. All soil samples were collected on 6/02/22.

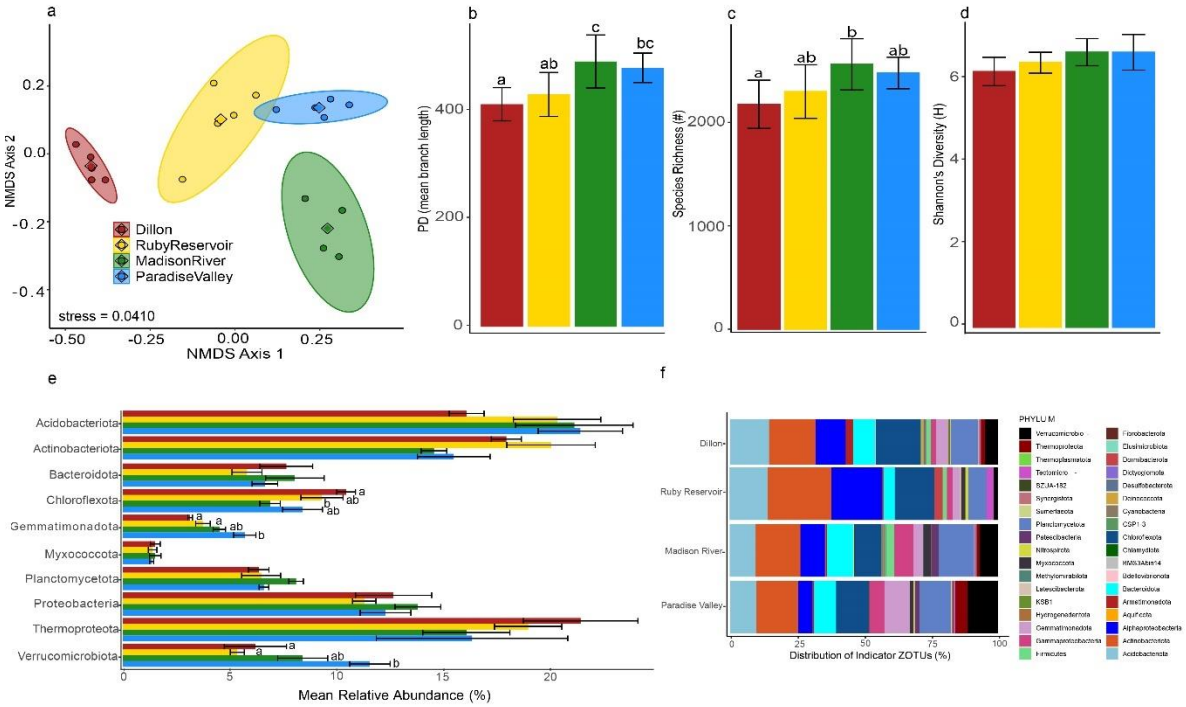


Figure 2. Summary of blue grama rhizosphere microbiomes from each of the four original soil sites. A) unweighted Unifrac distance, b-d) alpha diversity including phylogenetic diversity (Faith's PD), Shannon's diversity index and observed species richness where error bars are 95% CI, e) mean relative abundances where error bars are ± SE and f) distribution of indicator ZOTUs across bacterial + archaeal phyla among the four sites. Letters indicate $p < 0.05$ based on post-hoc Tukey tests for multiple comparisons. Absence of letters indicates $p > 0.05$ between sites.

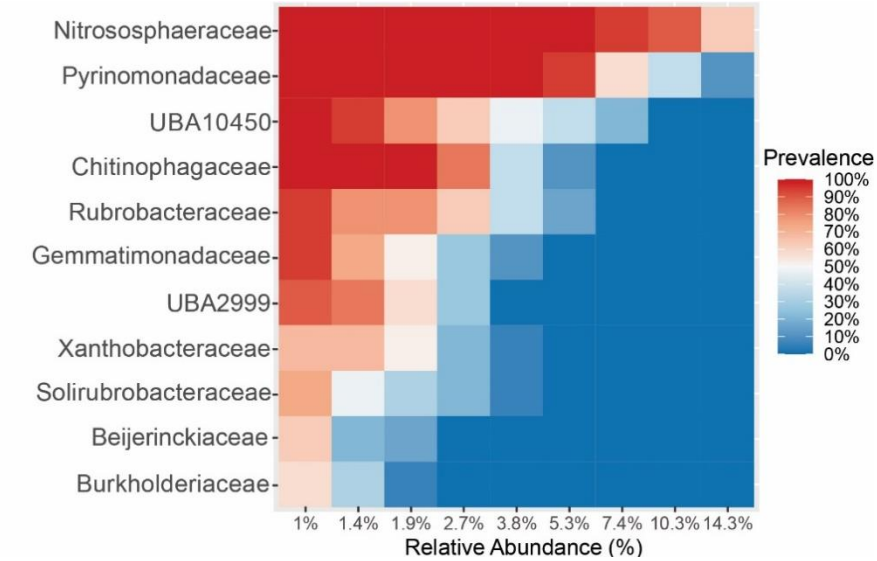


Figure 3. Core bacterial + archaeal families shared between the four sites. Core members are present at least 1% abundance in at least 50% of samples.

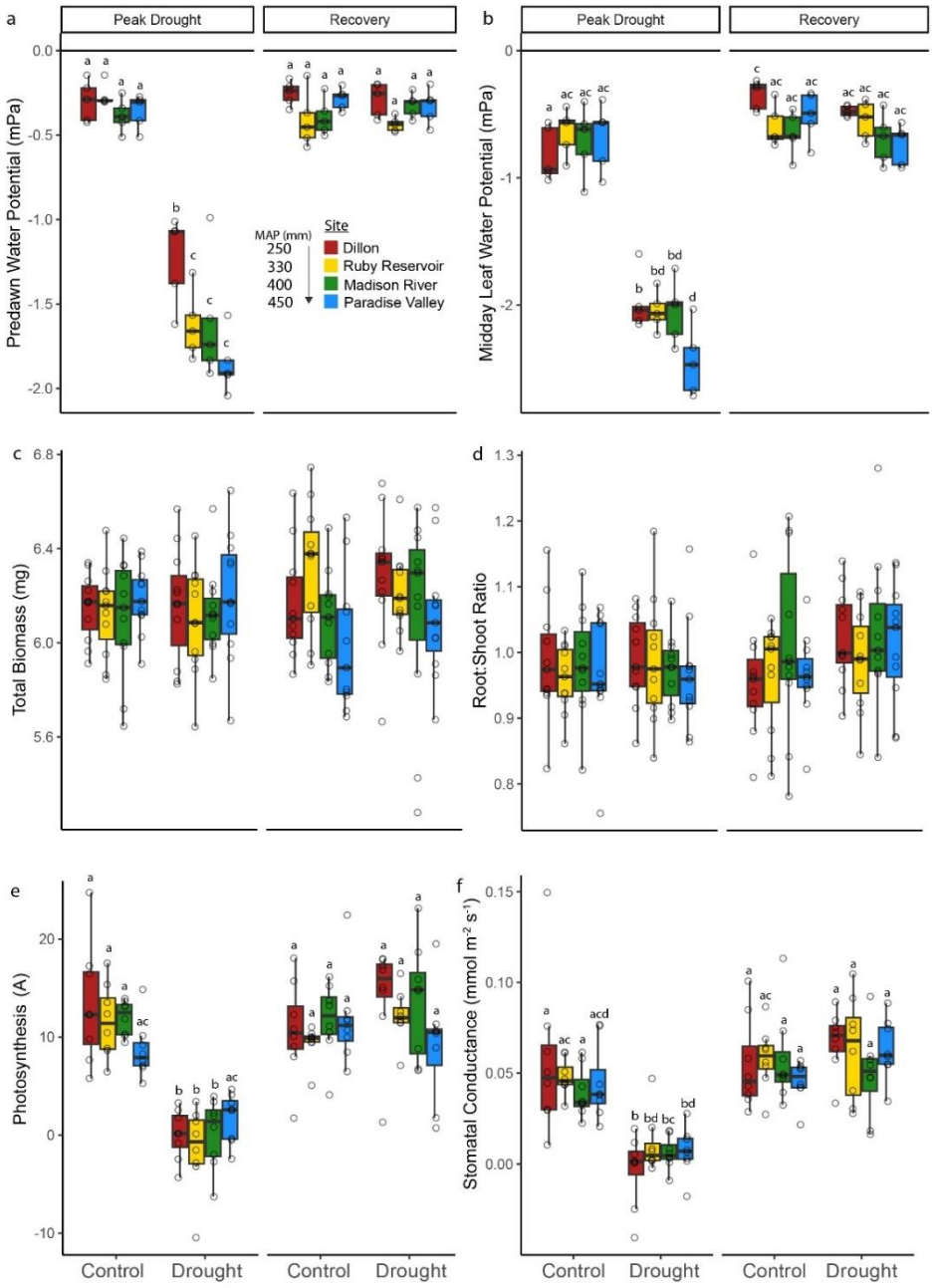


Figure 4. Plant physiology in response to drought treatments and recovery across the four sites; a) predawn water potential, b) midday water potential, c) total biomass, d) root:shoot ratio, e) photosynthesis, and f) stomatal conductance. Boxes indicate first and third quartiles, whiskers are at most 1.5*IQR. Points indicate individual samples. Letters denoting alpha < 0.05 differences within timepoints were calculated via post-hoc Tukey tests. Absence of letters indicates no significant differences were detected between treatment groups.

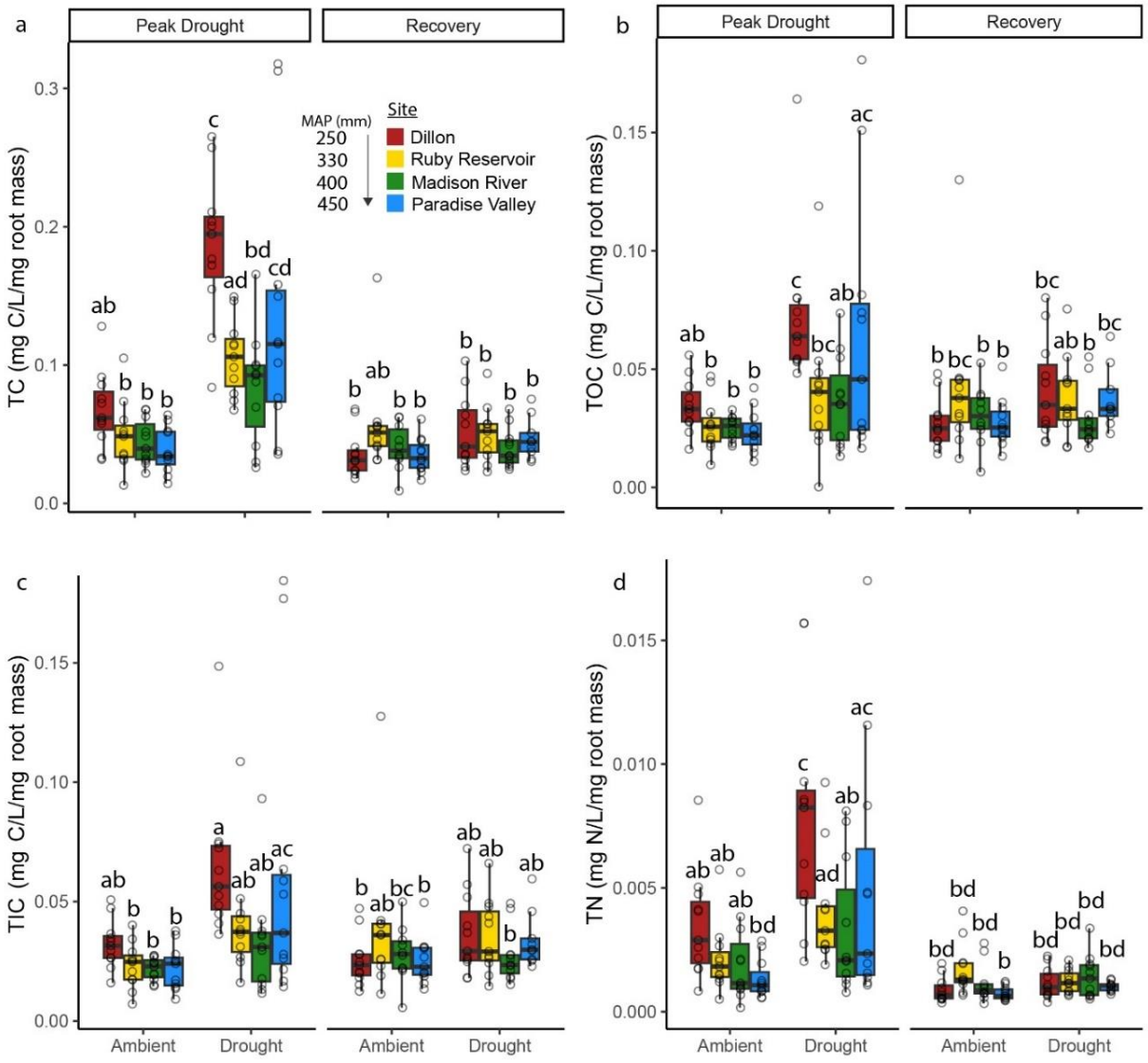


Figure 5. Root exudate chemistry including a) TOC, b) TIC, and c) TN. Boxes indicate first and third quartiles, whiskers are at most 1.5*IQR. Points indicate individual samples. Letters denoting alpha < 0.05 differences within timepoints were calculated via post-hoc Tukey tests.

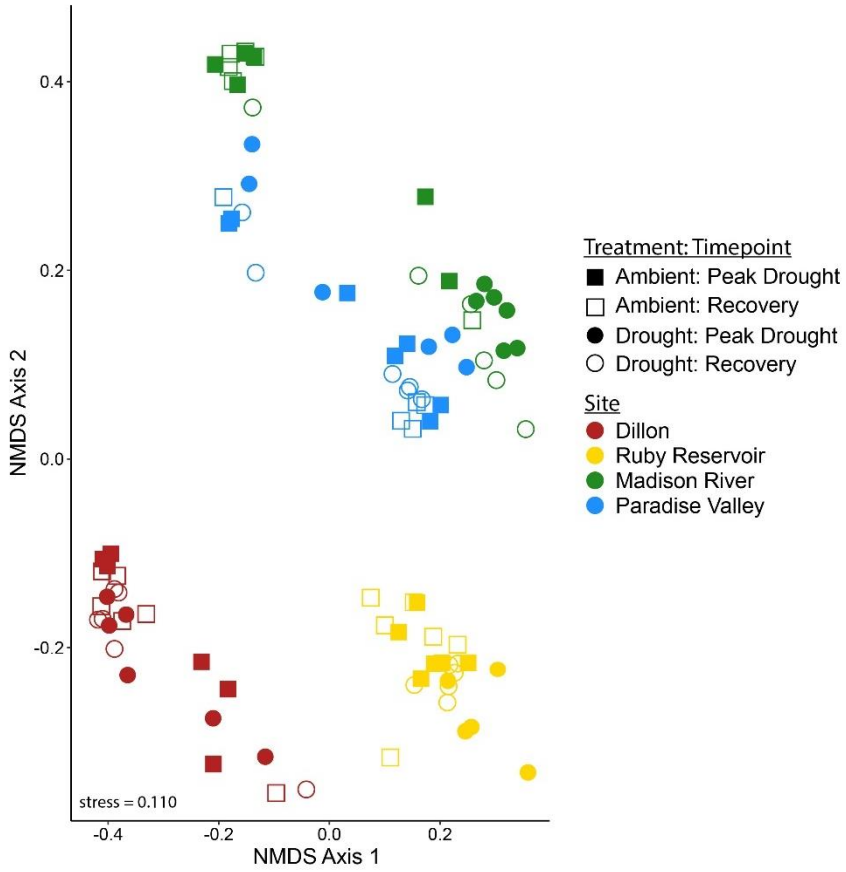


Figure 6. Unweighted Unifrac ordination of the bacterial + archaeal community in the four soils after harvesting at Peak Drought and Recovery timepoints (n=6). Individual samples are colored by site and shapes indicate ambient vs. drought treatment conditions, filled shapes indicate Peak Drought while open shapes indicate the Recovery timepoint.

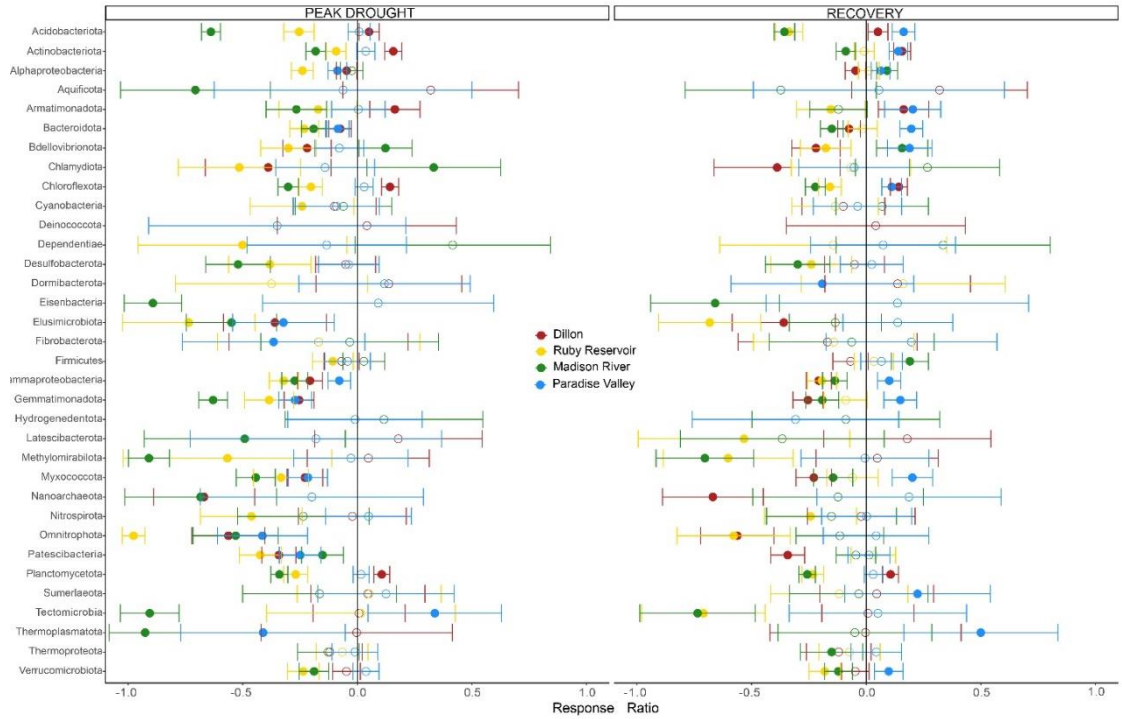


Figure 7. Mean response ratios of bacterial + archaeal phyla in ambient vs. drought comparisons across the four soils and two timepoints (n=6). Positive response ratios indicate greater relative abundance in drought vs. ambient treatment. Error bars are 95% confidence intervals. Filled dots indicate ratios with BH-adjusted p-values significantly different from 0 (p.adj < 0.05).

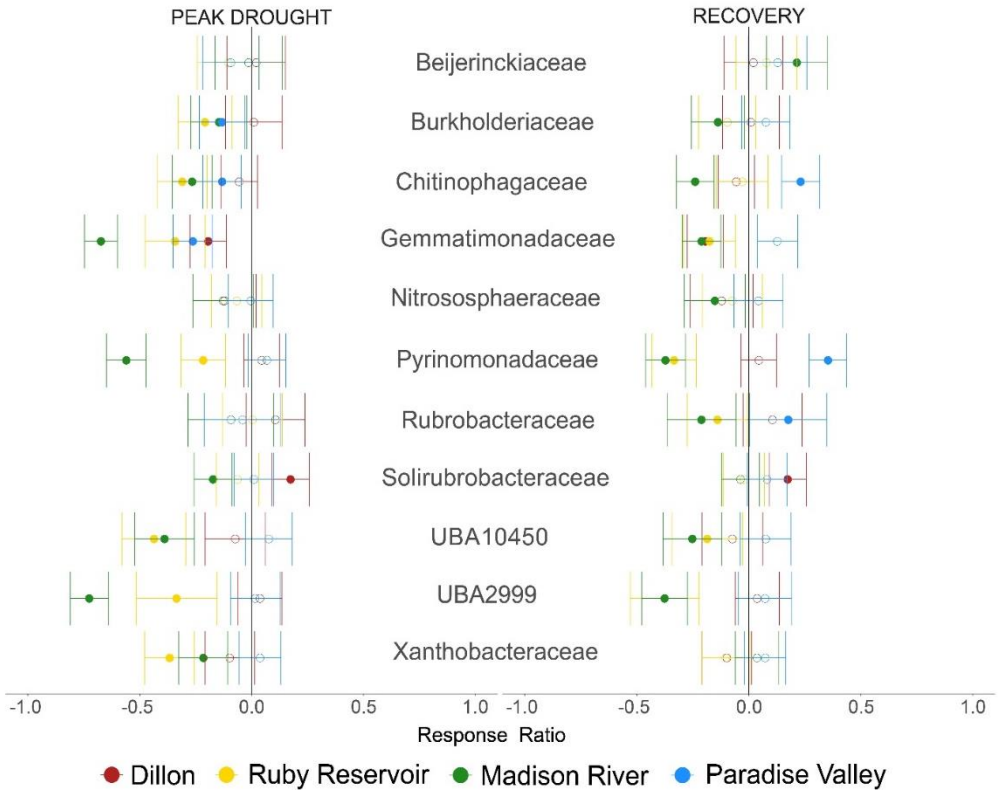


Figure 8. Response ratios of core bacterial + archaeal families. Error bars are 95% CI. Filled circles represent response ratio means significantly different from 0 (p.adj < 0.05).

Table 1. Summary of Soil Collection Sites

Collection Site	GPS Coordinates	Soil Description	MAP (mm)	pH	Organic matter (%)	Water Storage (cm)
Dillon	45.456886, -112.563360	Pensore-Crago, cool-Rock outcrop complex	250	8.0	2	2.74
Ruby Reservoir	45.224214, -112.117752	Musselshell-Amesha, bedrock substratum, complex, cool	330	7.9	2	4.89
Madison River	45.617925, -111.561111	Kalsted sandy loam	400	7.2	1.5	3.58
Paradise Valley	45.442490, -110.650840	Beaverell, stony-Attewan complex	450	7.2	2	2.96

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CHAPTER FIVE

RHIZOBIOME BIOGEOGRAPHY OF THE THERMAL TOLERANT DICHANTHELIUM LANUGINOSUM IN YELLOWSTONE NATIONAL PARK

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Abstract

The plant rhizobiome provides a wide variety of benefits to the plant including adaptation to abiotic stresses such as high temperatures. Although temperature is one of the strongest drivers of global plant distribution, the effect of temperature on rhizobiome assembly is poorly studied and has rarely been studied in natural ecosystems. Yellowstone National Park offers a unique environment in which to better understand plant-microbe interactions in relation to soil temperature due to its high distribution of geothermal features. We investigated the bacterial + archaeal rhizosphere assembly of the highly thermotolerant panic grass (*Dichanthelium lanuginosum*) across a natural temperature gradient from 24 to 61 °C which also encompassed a soil pH range of 3.1-9.5 across six distinct geothermal sites? in Yellowstone National Park. As the first characterization of the *D. lanuginsum* rhizosphere bacterial + archaeal community, we observed that site-specific characteristics, temperature, and pH all influence the *D. lanuginosum* rhizobiome assembly. Phylogenetic diversity significantly decreased with increasing soil temperature. We also observed several rhizosphere-specific trends in community composition with increasing temperatures which suggested that thermotolerant microbes may benefit from plant colonization at high temperatures.

Introduction

The upper temperature limit of plant life on Earth is generally assumed to be 60 °C (Fitter and Hay 2012). Most plants thrive closer to 25 °C and can experience severe damage to growth and productivity if exposed to temperatures above 40 °C for extended periods of time (Iloh et al. 2014, Jagadish, Way and Sharkey 2021). However, heat-tolerant plants have evolved

mechanisms to mitigate the effects of high temperatures, such as increased synthesis of heat shock proteins, antioxidants and other protective compounds, and altered root morphology (Stout and Al-Niemi 2002, Germino and Wraith 2003). The rhizosphere microbiome can also putatively contribute to plant heat tolerance by maintaining nutrient turnover and water access under stressful conditions (De Zelicourt, Al-Yousif, and Hirt 2013), although the mechanisms of heat tolerance provided by the rhizosphere are not yet well understood.

One notable example of microbial contribution to plant heat tolerance is panic grass (*Dichanthelium lanuginosum*). *D. lanuginosum* is a C4 grass of particular interest as previous studies have discovered a unique, tripartite relationship between the plant, a fungal endophyte *Curvularia proterberata,* and a fungal virus (CThTV) which results in extremely high plant heat-tolerance up to soil temperatures of 65 °C (Márquez et al. 2007; Redman et al. 2002). Thermophilic bacteria and archaea are abundant in geothermal springs and sediments (Inskeep et al. 2013, Lynes et al. 2023, Peach et al. 2022), but it is unknown to what extent these extremophiles also naturally colonize plant rhizospheres. It is possible that other organisms are also contributing to *D. lanuginosum* fitness in high temperature soils but have yet to be discovered. In support of this theory, Appoloni et al. (2008) observed unique compositions of arbuscular mycorrhizal fungi in thermal tolerant plants including *D. lanuginosum* across YNP, but their contribution to plant fitness remains unclear.

The goal of this study was to investigate the *D. lanuginosum* microbiome across a natural thermal gradient within Yellowstone National Park (YNP). YNP offers a unique ecosystem favorable for testing hypotheses related to plant thermal tolerance due to the widespread thermal

features across the Park and limited diversity of plant life growing near and around geothermal features of various temperatures (Stout and Al-Niemi 2002).

During two sampling seasons, we collected *D. lanuginosum* rhizosphere and bulk soil samples from six distinct thermal sites in YNP representing a temperature range from 24 to 61 °C which coincidentally also included a soil pH range of 3.1-9.5 (Fig. 1a,b). We hypothesized that i) site, temperature, and pH would affect the alpha and beta diversity of the *D. lanuginosum* rhizosphere differently from that of bulk soils, ii) the compositional differences between rhizosphere and bulk communities would increase with increasing temperature, and iii) we will be able to detect a distinct group of plant-associated community members at high temperatures.

Methods

Sample Locations and Collection

Soil samples were collected from six thermal sites in Yellowstone National Park in September of 2020 and 2021 (Figure 1). At each sampling location paired, 5 cm deep soil cores were taken to capture rhizosphere (immediately adjacent to a *D. lanuginosum* plant), and bulk soil (at least 1 ft. from the nearest plant) using a handheld soil corer. Digital temperature probes were used to record temperature to 5 cm soil depth at both rhizosphere and bulk soil locations to ensure that soil of the same temperature was collected for each. Soils were collected in 15 mL Falcon tubes and flash frozen in an ethanol-dry ice bath prior to storage on dry ice for transport back to the lab (apprx. 4 days due to nature of the backcountry sampling trips) and long-term storage at -80 °C. One representative sample was used to measure pH for each collection location. pH was measured using a 1:10 soil:Nanopure water solution, mixed on a rotary mixer for 30 min. and the sediment was allowed to settle for 1 hr. prior to pH measurement.

Soil DNA Extraction, Amplification, and Sequencing

To obtain high-resolution representation of the soil microbial communities and their response to drought, we performed rRNA gene amplicon sequencing on all rhizosphere and bulk soil samples from each of the six sites. Genomic DNA was extracted from 0.5 g frozen soil samples using the Fast DNA SPIN Kit for soil (MP Biomedicals, Santa Ana, CA) according to the manufacturer's instructions. We targeted the V4 region of the 16S rRNA gene using 515F (Parada, Needham, and Fuhrman 2016) and 806R-b (GTGYCAGCMGCCGCGGTAA and GGACTACVSGGGTATCTAAT, respectively) from the Earth Microbiome Project (Thompson et al., 2017). Reactions were carried out in 20 μL volumes containing 1 U Phusion high-fidelity polymerase (ThermoFisher Scientific, MA, USA), a final concentration of 1x Phusion HF reaction buffer, 200 μM dNTPs, 0.5 μM each primer, 1 μM BSA, 1 mM MgCl, sterile molecular grade water, and 5 μL (10 ng) genomic DNA as a template. PCR conditions consisted of initial denaturation at 98 ˚C for 30s, followed by 22 cycles of 98 ˚C for 30 s, 55 ˚C for 30 s, 72 ˚C for 45 s and a final extension at 72 ˚C for five minutes. Samples were purified using AMPure XP (Beckman-Coulter, Brea CA, USA) magnetic beads and barcoding was conducted using the Illumina Nextera Indexing Kit D, with 10 cycles of indexing PCR in 20 μL volumes using the same concentrations as PCR1 with 10 μL template DNA. Samples were purified again with magnetic beads and PCR products were checked for quality and length in a 1% agarose gel and quantitated using the Quant-iT™ dsDNA Kit (Invitrogen, Carlsbad CA, USA) with a BioTek H2 plate reader. Individual samples for each community were combined at equimolar concentrations and the two libraries were pooled at 4 nM and loaded onto an in-house Illumina MiSeq (Illumina, San Diego CA, USA) and sequenced using the V3 600 cycle kit.

Bioinformatics

Reads were merged, trimmed, and dereplicated with USEARCH, quality filtered with an ee-value of 1.0 and zero-radius operational taxonomic units (ZOTUs) were identified with UNOISE3 (v.11.0.667) (Edgar, 2010). A total of 4,668,982 high quality reads were clustered into 15,541 ZOTUs. ZOTUs were classified using a subset of the Genome Taxonomy Database (GTDB, Chaumeil et al., 2022) using SINTAX (Edgar 2016a). All reads classified to chloroplast were removed prior to downstream analysis. For statistical analyses, the dataset was randomly subsampled according to the sample with the lowest read number for statistical analysis (5,466). To construct phylogentic trees for environmental ZOTUs, we first constructed reference phylogenies using full length and near full length sequences of isolates and metagenome assemblies downloaded from GenBank, focusing on GTDB sequences. Reference sequences were aligned using mafft (Katoh and Standley 2013), and a maximum likelihood tree was constructed using RAxML with the GTR + gamma model (Stamatakis 2014). Environmental sequences were aligned to the reference using mafft with the –add flag and then mapped onto the reference tree using pplacer (Matsen, Kodner, and Armbrust 2010) with reference-aligned sequences. The tree was then visualized and annotated using the interactive Tree of Life software (Letunic and Bork 2019).

Statistics and Data Analysis

To explore the effects of site, temperature, microhabitat and pH we performed PERMANOVA analysis using a mixed effects model. Linear regressions using the multcomp package in R (Hothorn, Bretz, and Westfall 2008) were utilized to explore relationships between relative abundances of community diversity and specific phyla with temperature and pH.

Indicator species analysis was conducted using the indicspec package in R (v4.3.0, Cáceres and Legendre 2009).

Results

Beta Diversity of the *D. lanuginosum* Rhizobiome

Site, temperature, and pH all played significant roles in structuring the *D. lanuginosum* bacterial + archaeal rhizosphere community with both individual effects ($P_{site} = 0.001$, $P_{temp} = 0.002$, $P_{pH} = 0.004$) and their interactions ($P_{site:temp:pH} = 0.003$) measured by PERMANOVA (Fig. 2a, Table S1). Microhabitat (bulk soil vs. rhizosphere) was not a significant predictor of community beta diversity ($P_{comp} = 0.993$). We also detected significant differences in beta dispersion between sites which were primarily driven by Mud Volcano samples having significantly lower dispersion than all sites except West Thumb (Fig. 2b).

To determine the influence of temperature and pH on beta diversity across the six sites, we performed PCoA and regression analyses (Fig. 2c,d). PCoA indicated that the first two axes of the ordination explained 9.4% and 5.8% of community variability. While temperature appeared to be the most divergent across the y-axis, pH was a strong driver of separation along the x-axis although the majority of the samples collected were below pH 5 (Table S1). Regression analysis indicated that the weighted Unifrac distance between bulk and rhizosphere samples across the six sites increased significantly with temperature (Fig. 2e, $R^2 = 0.273$, $P = 0.001$). In contrast, we did not find a significant relationship between pH and bulk vs. rhizosphere Unifrac distance (Fig. 2f, $R^2 = 0.0648$, $P = 0.0826$), although a quadratic regression suggested that the distances were greatest at the two extremes of pH and lowest at circum-neutral soil pH.

Alpha Diversity Across the Temperature Gradient

Alpha diversity for all six sites was largely similar and we detected only a few significant site differences in species richness, Faith's phylogenetic diversity, or Shannon's Diversity Index and between bulk and rhizosphere soils (Fig 3). Species richness was influenced by site (F_3 = 3.06, p = 0.0164) but not by microhabitat (F_1 = 0.759, p = 0.387), and Mud Volcano had greatest species richness than West Thumb and White Creek sites (SR Fig. 3a). Faith's phylogenetic diversity (PD) was similar between sites but we did detect significantly greater PD overall in the rhizosphere vs. bulk soil (Fig. 3b, F_1 = 5.864, p = 0.02). We did not detect any significant differences between sites or microhabitats for Shannon's Diversity Index (H) indicates that evenness was similar across the *D. lanuginosum* rhizosphere and adjacent bulk soil communities.

To understand the influence of temperature and pH on the alpha diversity of the *D. lanuginosum* rhizosphere bacteria + archaea, we performed regression analysis on PD vs. temperature and pH across the six sites. We found that PD negatively correlated with temperature (Fig. 4a, R^2 = 0.0804, p = 0.01), while pH was positively correlated with PD (Fig. 4b, R^2 = 0.158, p = 0.002). We did not find interacting effects of site or microhabitat on these correlations.

Community Composition Across Sites, Temperatures, and pH

Across the six sites, the dominant phyla were bacterial *Acidobacteria*, *Actinobacteria*, *Chloroflexota, Planctomycetota,* and one archaeal phylum, *Thermoproteota,* together averaging 60% of the total community membership (Fig. 5). *Chloroflexota* was the most dominant across all sites and microhabitats (bulk and rhizosphere) averaging 33% of the community. The bulk soil in the Mud Volcano site had a uniquely large relative abundance of *Thermotogota,* a primarily hypothermophilic phylum of bacteria, that was not observed in the rhizosphere or in

any of the other sites. The other most dominant bacterial + archaeal phyla across sites were *Acidobacteria*, *Actinobacteria*, *Chloroflexota*, *Alphaproteobacteria*, and *Thermoproteota* (Fig. 6a).

To explore the effect of temperature on the community composition in the *D. lanuginosum* rhizosphere vs. bulk soils, we performed linear regressions with the major phyla across the temperature gradient. We observed different trends in rhizosphere vs. bulk soils in several phyla (Fig. 6). *Thermoproteota* was observed to increase by 1% for every degree increase in temperature and only in rhizosphere samples ($R^2 = 0.333$, $p < 0.001$), increasing from 2-44% across the 24-61 °C temperature gradient. We also observed a marginally positive relationship in the relative abundance of rhizosphere *Chloroflexota* with temperature ($R^2 = 0.061$, $p = 0.088$). The rhizosphere and *Alphaproteobacteria* had a marginally negative relationship with temperature that was not observed in bulk soils ($R^2_{Rhiz} = 0.167$, $p_{Rhiz} = 0.022$; $R^2_{Bulk} = 0.014$, $p_{Bulk} = 0.259$) while the relative abundance of *Actinobacteria* decreased with increasing temperature in both rhizosphere and bulk soils ($R^2_{Rhiz} = 0.086$, $p_{Rhiz} = 0.063$; $R^2_{Bulk} = 0.151$, $p_{Bulk} = 0.023$). In contrast, several phyla had stronger responses in bulk soils than rhizosphere soils. The bulk soil Cyanobacteria had a strong positive relationship with temperature that was not observed in the rhizosphere ($R^2_{Rhiz} = -0.031$, $p_{Rhiz} = 0.602$; $R^2_{Bulk} = 0.852$, $p_{Bulk} < 0.001$). Bulk soil *Desulfobacterota* also increased marginally with temperature ($R^2 = 0.105$, $p = 0.055$). Lastly, the bulk soil *Acidobacteria* had a marginally negative relationship with increasing temperature ($R^2 = 0.076$, $p = 0.073$). We also explored these relationships across the pH gradient and did not detect any significant associations between the relative abundance of major phyla and pH.

Indicator Species Analysis Across the Temperature Gradient

To identify ZOTUs specifically colonizing the *D. lanuginosum* rhizosphere at high temperatures, we conducted indicator species analysis on bulk vs. rhizosphere samples across the temperature gradient (Fig. 7a). We identified 126 ZOTUs that were indicator species for bulk soils, and 123 for the rhizosphere (Appendix 1). The indicator ZOTUs for both groups were widespread across the phylogeny but primarily consisted of *Acidobacteriota, Actinobacteriota, Armatimonadota, Chloroflexota, Desulfobacterota, Firmicutes, Planctomycetota, Thermoproteota* and *Verrucomicrobiota* phyla and *Alpha-* and *Gammaproteobacterial* classes. We further investigated whether any ZOTUs were associated with temperature by partitioning the temperature gradient across sites into low (24-40 °C) and high (40 – 61 °C) groups as 40 °C is generally the upper limit of optimal temperature for mesophilic organisms. In the low-temperature group, the bulk soil we detected 83 indicator ZOTUs which were primarily *Alphaproteobacteria*, *Acidobacteriota*, *Chloroflexota*, *Actinobacteriota,* and *Planctomycetota* decreasing order of relative distribution (Fig. 7b). The 34 rhizosphere soil indicator ZOTUs in the same temperature range were dominated by *Actinobacteria, Acidobacteria, Planctomycetes* and *Thermoproteota*. In the higher temperature group we detected 43 indicator ZOTUs in bulk soils which were comprised of primarily *Actinobacteria*, *Firmicutes, Planctomycetota*, *Alphaproteobacteria*, *Gammaproteobacteria,* and *Aquificota*. In contrast, the 89 rhizosphere ZOTUs in the same temperature range were dominated by *Chloroflexota, Firmicutes*, *Gemmatimonadota,* and *Acidobacteriota.* We further explored the highest end of the temperature gradient (50 – 61 °C) and identified 26 indicator rhizosphere ZOTUs dominated by *Chloroflexota* and *Firmicutes* which together made up 60% of the ZOTUs. The bulk soils

indicator ZOTUs in this highest temperature group were primarily *Actinobacteriota, Firmicutes, Planctomycetota, Alphaproteobacteria,* and *Aquificota*.

Discussion

Yellowstone National Park has been and continues to be a wellspring of discovery for the scientific community seeking to understand the limitations of biological life. Much of the microbial ecology research in the Park has been focused on characterizing the extremophilic microbes living in hot springs and their sediments (Lynes et al. 2023; Peach et al. 2022; Reichart et al. 2022; Vishnivetskaya et al. 2015; X. Jiang and Takacs-Vesbach 2017; De León et al. 2013; Kozubal et al. 2008; Inskeep et al. 2013) which have greatly broadened our understanding of microbial evolution and biodiversity, and led to many biotechnological applications (Ishino and Ishino 2014; Yaver 2023). In comparison, the soil microbiomes and particularly the plant-microbe holobionts living in the geothermal areas of Yellowstone have been much less explored, and *D. lanuginosum* remains the only thermotolerant plant in which microbial symbionts have been extensively studied. Although the tripartite relationship between *D. lanuginosum*, fungal endophyte *Curvularia protuberata,* and fungal virus CThTV has been well-established (Redman et al. 2002; Márquez et al. 2007), little else is known about the *D. lanuginosum* microbiome and how other microbes including bacteria and archaea in the *D. lanuginosum* rhizosphere may contribute to plant fitness at high temperatures. Here we provide the first biogeographical analysis of the *D. lanuginosum* bacterial + archaeal rhizosphere community across six distinct geothermal areas, a 37 °C temperature gradient, and across soil pHs from 3.1-9.5 in Yellowstone National Park.

Site, Temperature, and pH Outweighed
Microhabitat Effects on Beta Diversity

Contrary to our hypothesis, we did not detect differences in the beta diversity between the two microhabitats (bulk soil vs. *D. lanuginosum* rhizosphere) at any site or for all sites?, or temperature and pH gradients. However, we did observe a positive relationship of paired, unweighted unifrac distances between bulk and rhizosphere samples with increasing temperature. Unifrac uses phylogenetic information to compare the amount of unique evolutionary history (i.e. the fraction of phylogenetic branch length not shared) between collections of sequences (Lozupone and Knight 2005). This indicated that as temperatures increased, bulk and rhizosphere samples did become more phylogenetically distinct. The lack of this relationship using weighted Unifrac distance indicated that rather than shifts in relative abundances between shared members, the collective membership of the rhizosphere and bulk soils became more dissimilar at high temperatures (Lozupone et al. 2007). In contrast, we detected a marginal negative relationship of pH with weighted paired Unifrac distance metric suggesting that as pH increased, the bulk and rhizosphere communities may have become more similar driven by shifts in relative abundances. Although this effect was marginal, the trend is logical as lower pH has been associated with lower microbial diversity (Fierer and Jackson 2006).

Across the six sites, Mud Volcano had lower dispersion than every site except West Thumb, indicating that the Mud Volcano community was more homogeneous compared to the other sites. Notably, samples from Mud Volcano also spanned the most narrow temperature (26-32 °C) and pH (3-4) ranges of the six sites which may explain the increased homogeneity. However, collectively site, temperature, pH, and the interactions between the three explained less

than half of the variation in beta diversity between all samples indicating that other biogeochemical factors not measured here also play important roles in structuring the microbial communities of these soils.

Alpha Diversity was Influenced by Temperature and pH more than Site or Microhabitat

Similar to the beta diversity, we found few differences between bulk and rhizosphere soils across the sites based on either richness (# of species), Faith's phylogenetic diversity (PD, mean branch length), or Shannon's Diversity Index (H, evenness), although across the sites we did observe greater PD in rhizosphere vs. bulk samples. We also observed greater species richness in Mud Volcano sites compared to West Thumb and White Creek.

Faith's phylogenetic diversity was found to have opposite trends with temperature and pH. Aggregated across site and microhabitat, PD decreased with increasing temperature, indicating that higher temperatures have a filtering effect across the phylogeny. In contrast, PD increased with increasing pH, similar to the observation of decreased Unifrac distance between rhizosphere and bulk soils. Importantly, temperature and pH did not follow a linear relationship among samples across or within sites. Nottingham et al. (2018) observed that mean annual temperature (MAT) was the strongest driver of soil community diversity across a 3.5 km elevation gradient spanning 18 °C, and that bacterial alpha diversity increased along the temperature gradient from 6 °C to 24 °C. As 24 °C was the lowest temperature recorded among our samples, the negative trend in PD we observed is further indicative of environmental filtering at temperature extremes. The lack of observed difference between bulk and rhizosphere compartments in this relationship also suggests that the strength of this relationship surpasses

plant selection effects as has been suggested previously (Nottingham et al. 2018; Yao et al. 2017).

Community Composition Revealed Taxa Driving Site Effects

Across the sites and microhabitats, the bacterial + archaeal community was dominated by *Chloroflexota.* The *Chloroflexota* have a wide range of mixotrophic metabolisms and are found in greater abundance in low moisture, and low diversity soils (Yao et al. 2017; Sterling et al. 2022). Hernandez et al. (2020) found *Chloroflexota* to be among the primary successors colonizing new soils post- volcanic eruptions in Chile. *Acidobacteria* and *Actinobacteria* were also dominant across sites and microhabitats which was expected as they are frequently amongst the most abundant soil phyla (Delgado-Baquerizo et al. 2018). The *Thermoproteota* (formerly *Thaumarcheaota*) which are primarily ammonia oxidizing archaea were also among the most dominant phyla across sites and microhabitats.

The uniqueness of the Mud Volcano site was also further demonstrated by the composition of major phyla. In the Mud Volcano bulk soils we detected a large proportion of *Thermotogota* which was not observed in the rhizosphere soils, or in any other site. This observation is especially curious as the *Thermotogota* are known to be highly thermophilic (AlSaade et al. 2022) but the highest soil temperature we observed in Mud Volcano soils was only 32 °C. This contradiction could be partially explained by the transient and heterogeneous nature of heat in geothermal areas (Hurwitz and Lowenstern 2014). The soil temperatures recorded for this study are snapshots for a particular day which may not be fully representative of the historical temperature of that soil. These discrepancies at the Mud Volcano site are

particularly interesting as some of the highest fumarole temperatures in the Park have been recorded in the Mud Volcano area (Chiodini et al. 2012).

Differential Bulk and Rhizosphere Community Composition Along the Temperature Gradient

Temperature influenced some community members more than others and we observed several differences in bulk vs. rhizosphere trends. We observed a significant positive relationship of *Thermoproteota* relative abundance with temperature, and this trend was specific to the rhizosphere. *Thermoproteota* often dominate the archaeal community in mesic soils (Nelkner et al. 2019; JunJie et al. 2019; Nelkner et al. 2023), and also occupy hot springs (Power et al. 2023). Metagenomic analysis of *Thermoproteota* MAGs across European agricultural soils found evidence for plant growth promotion based on genetic potential for nitrogen transformation (i.e. ammonium oxidation), plant signaling, carbon fixation, and exopolysaccharide production (soil stabilization) (Nelkner et al. 2023). We hypothesize that the ability of *Thermoproteota* to survive at high temperatures *and* potentially provide plant beneficial ecosystem services leads to increased selection of *Thermoproteota* by *D. lanuginosum*.

Further evidence for possible plant-associated microbial nutrient cycling was the observation of a rhizosphere-specific marginally positive relationship with *Chloroflexota.* West-Roberts et al. (2021) reported widespread genetic potential for nitrate-nitrite reduction amongst *Chloroflexota* genomes which may link the increased rhizosphere abundance of *Chloroflexota* observed here to that of the ammonia-oxidizing *Thermoproteota*. We also observed an increase in relative abundance of cyanobacteria with increasing temperature which included several nitrogen-fixing genera including *Nostoc* and *Leptolyngbya* but this was only in bulk soils. The cyanobacterial community also included several thermotolerant families including

Thermosynechoccoceae and *Elainacceae* which may have had a competitive niche advantage in the open bulk soils (Alcorta et al. 2020).

Indicator Species Analysis Revealed Distinct Phylogenetic Trends between Rhizosphere and Bulk Soil Communities with Increasing Temperature

Indicator ZOTUs between rhizosphere and bulk soils were largely spread across the phylogeny at lower temperatures but the rhizosphere in particular became more phylogenetically constrained with increasing temperature. In agreement with our hypothesis, we detected 26 ZOTUs that were indicator species of rhizosphere soils at high temperatures. Of these ZOTUs, eight belonged to the *Ktedonobacteraceae* family of the *Chloroflexota.* High levels of cellulolytic potential have been recorded in multiple studies of *Chloroflexota* genomes (West-Roberts et al. 2021; Hug et al. 2013) which may explain this tight association with *D. lanuginosum* in sites where there is little other plant material available. *Ktedonobacteraceae* have also been of recent interest in the discovery of novel secondary metabolites as their ability to survive in a wide range of habitats is putatively linked to high metabolic diveristy (Yabe et al. 2017).

Members of the *Firmicutes* phylum were also predominant among the high-temperature rhizosphere indicator ZOTUs. *Firmicutes* are notorious for the ability to survive in extreme environments due to their widespread ability to form endospores (Filippidou et al. 2016). *Firmicutes* are also commonly found in plant rhizospheres (Breidenbach, Pump, and Dumont 2016; Chaparro, Badri, and Vivanco 2014; Ling, Wang, and Kuzyakov 2022).

Notably, the bootstrap values provided by the SINTAX program indicating the confidence in taxonomic identification were very low (often below 50%) across the indicator

ZOTUs and BLASTn analysis did not improve the identification. This suggests that many of these ZOTUs are quite distinct from those previously deposited in either database and have very likely never been cultivated.

Study Limitations

While this study provides the first characterization of the bacterial + archaeal rhizosphere community of *D. lanuginosum*, there are several technical and biological limitations to consider. The first is that we were unable to find *D. lanuginosum* at the same temperatures at each site, despite the existence of equally hot thermal features. This suggests that there are likely edaphic or biological controls on the distribution of *D. lanuginosum* other than temperature and pH that were not examined in this study. In addition, while amplicon sequencing provides an extremely detailed database of the diversity within the rhizosphere and allows for detection of rare species, it does not provide concrete information regarding the functional potential of these community members that would be provided with a metagenomic analysis may be more important for determining microbial contributions to plant fitness (Compant et al. 2019). Lastly, an important aspect of biogeography is time. Each of the samples collected here represent a single snapshot of environmental conditions. As microbial communities can exhibit seasonal dynamics, a more comprehensive understanding of the *D. lanuginosum* rhizobiome will require additional data to explore whether seasonal shifts in rhizobiome community, encompassing the perennial lifecycle of *D. lanuginosum*, are a defining feature of this system.

Conclusion

Here we show that the rhizosphere bacterial + archaeal community of *D. lanuginosum* is variable across local communities and is influenced by soil temperature and pH. We found evidence for rhizosphere selection particularly at high temperatures where life in the rhizosphere may provide a "safe haven" of microbial flourishment for thermotolerant organisms that would otherwise be largely metabolically inactive in bulk soils (i.e. sporulated). While it is yet unclear whether these organisms provide any plant benefit, this study is the first to characterize the bacterial + archaea in the *D. lanuginosum* rhizosphere, setting a foundation for future studies to build upon. This study also provides rare insights into soil microbiome assembly across a wide natural temperature gradient, leading to hypotheses regarding the abiotic limits for several previously uncharacterized soil microbiota.

Data Availability

Sequencing data will be made available on the NCBI SRA database upon submission of manuscript.

Acknowledgements

This work was supported by the U.S. Department of Agriculture National Institute of Food and Agriculture under award number 2020-07194. The authors also thank Drs. Muneeb Rathore and Margaux Mesle for field assistance in sample collection.

Figures and Tables

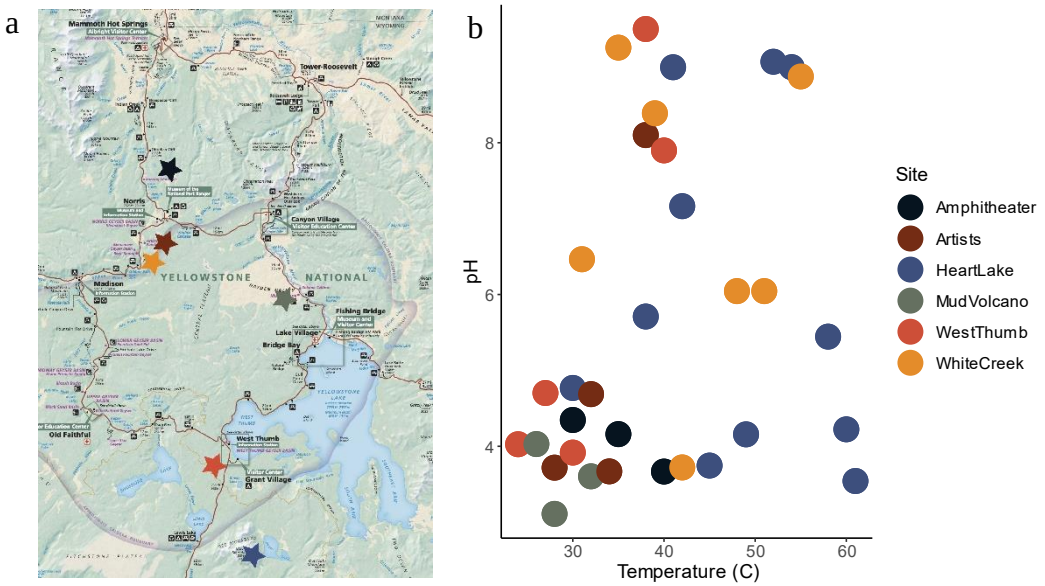


Figure 1. Map of sampling locations in Yellowstone National Park indicated with stars (a), adapted from the United States Geological Survey. (b) Temperature and pH of collected soil samples across sites.

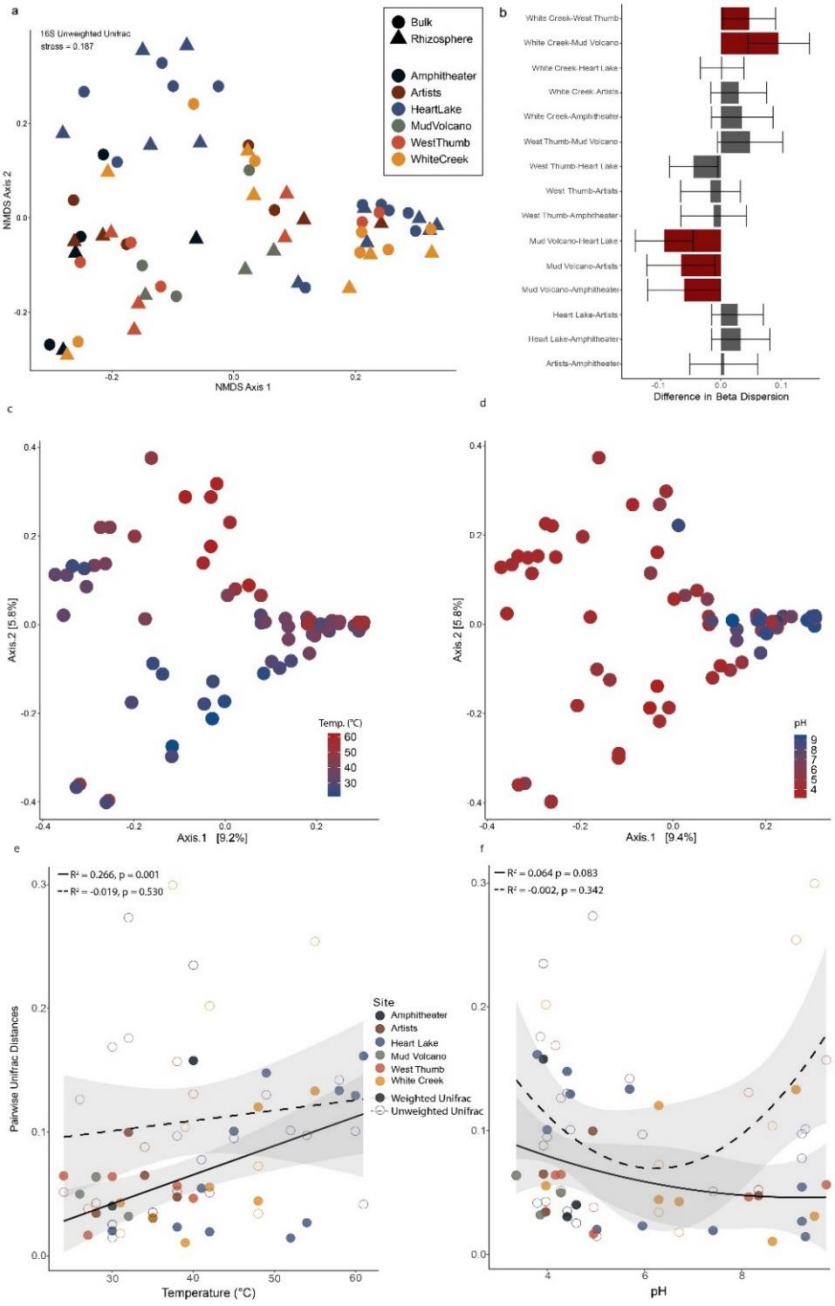


Figure 2. Beta diversity in the bacterial + archaeal community (a) where individual samples are colored by site and shapes indicate bulk (circles) vs. rhizosphere (triangles) samples. Differences in beta dispersion (b) with significantly greater or lower values indicated in red. Error bars are 95% CI. Influences of temperature and pH on beta diversity via PCoA analysis of temperature (c) and pH (d), and regression of pairwise, Unifrac distances between bulk and rhizosphere samples across the six sampling sites vs. temperature (e) and pH (f). Weighted Unifrac distances are filled circles with a solid regression lines, unweighted distances are empty circles with dashed regression lines.

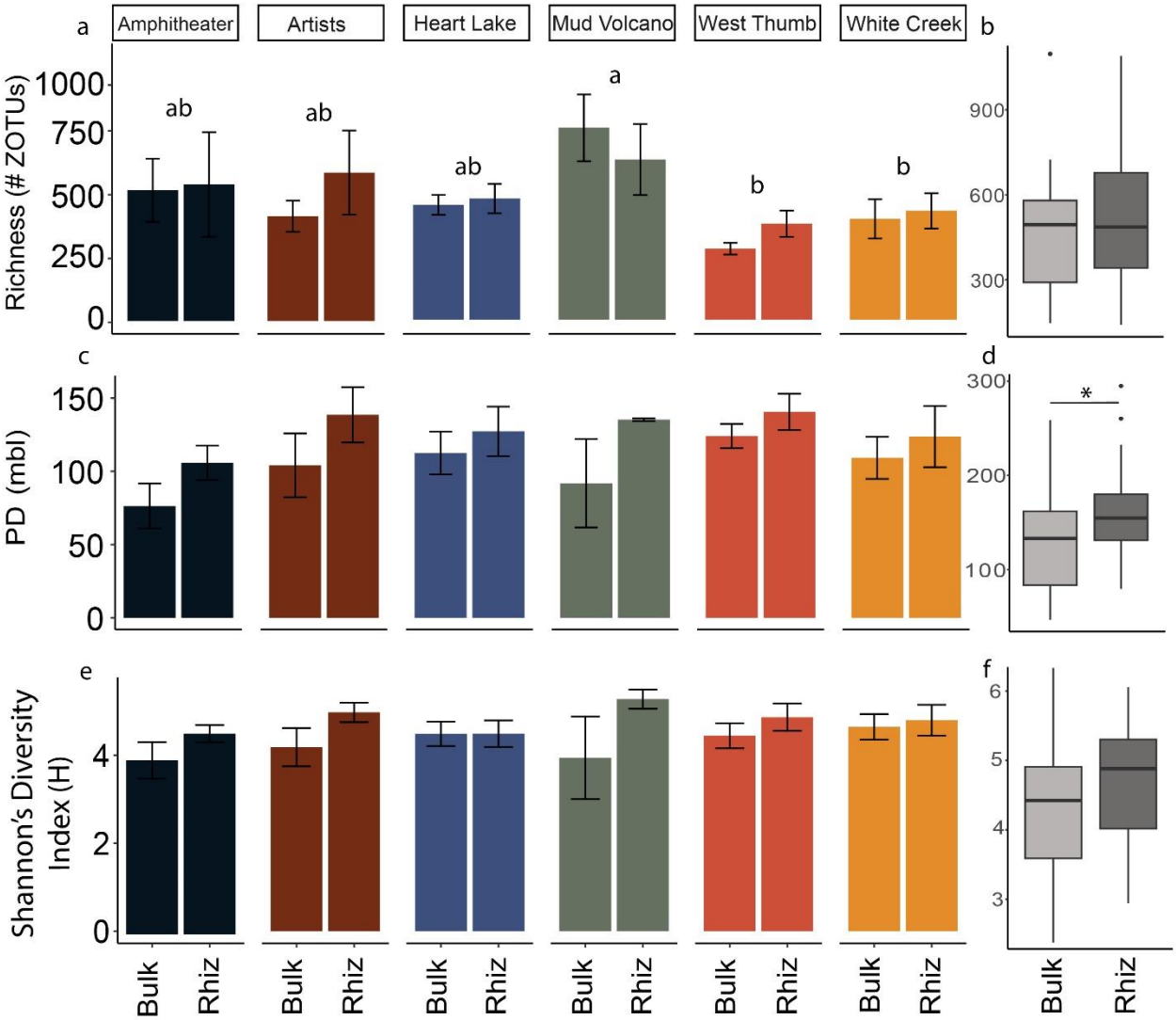


Figure 3. Alpha diversity and richness in bacterial + archaeal community measured by species richness of each site (a) and between bulk soil and rhizosphere for all sites (b), Faith's phylogenetic diversity of each site (c) and between bulk soil and rhizosphere for all sites (d), and Shannon's Diversity Index of each site (e) and between bulk and rhizosphere for all sites (f). Error bars on bar charts are ± SE. Box charts indicate mean values with whiskers of .25 and .75 IQR. * indicates $p < 0.05$, and letters indicate significant differences detected via post-hoc Tukey tests across sites.

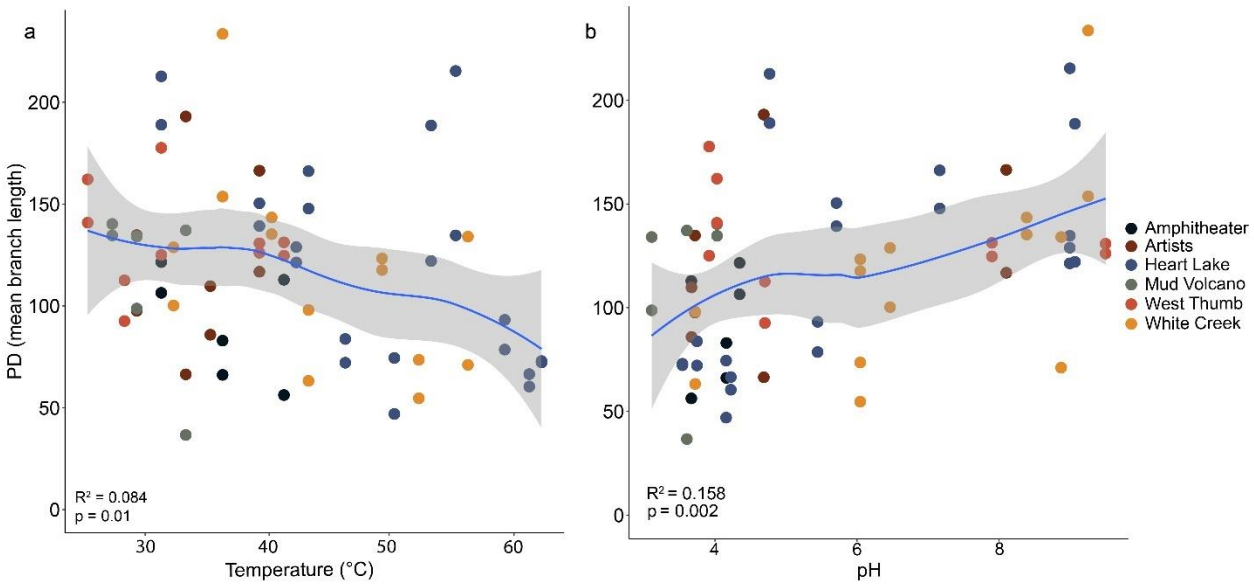


Figure 4. Effects of temperature and pH on phylogenetic diversity across sites. All samples including bulk and rhizosphere are included as no effects of microhabitat were detected.

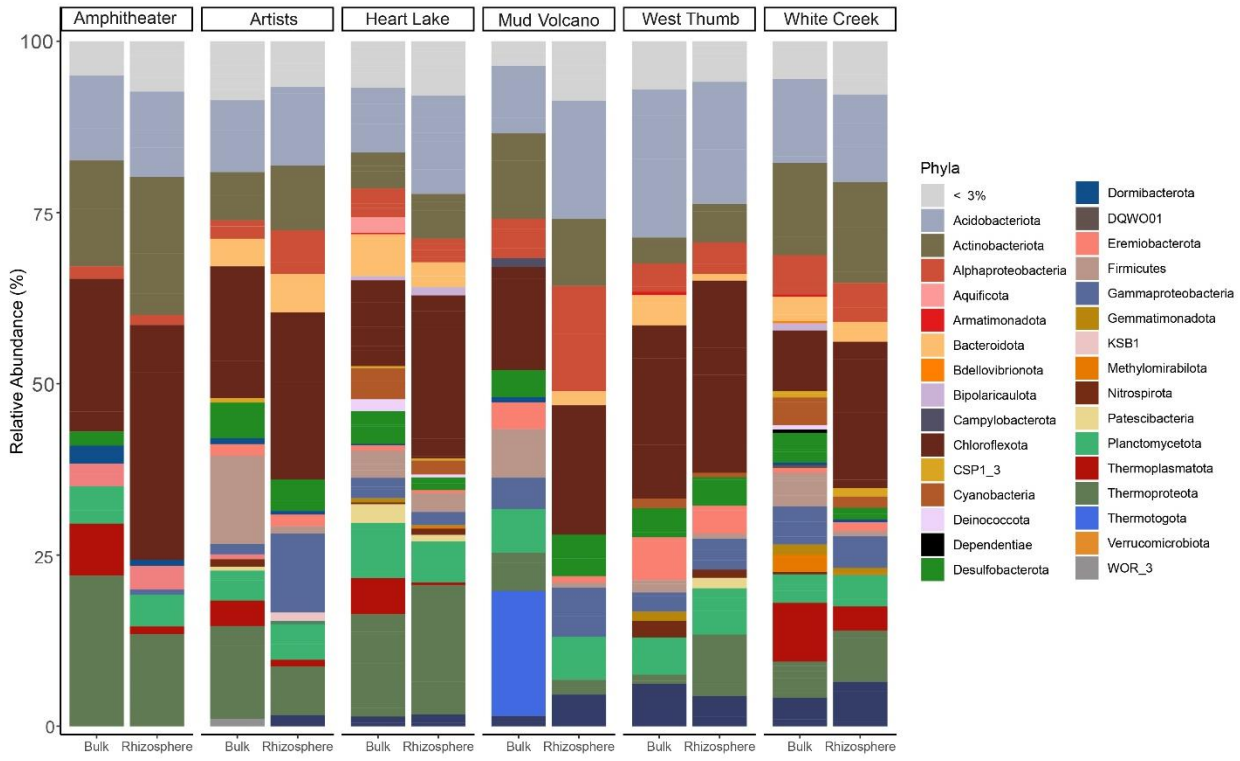


Figure 5. Relative abundances of major phyla across sites and microhabitat.

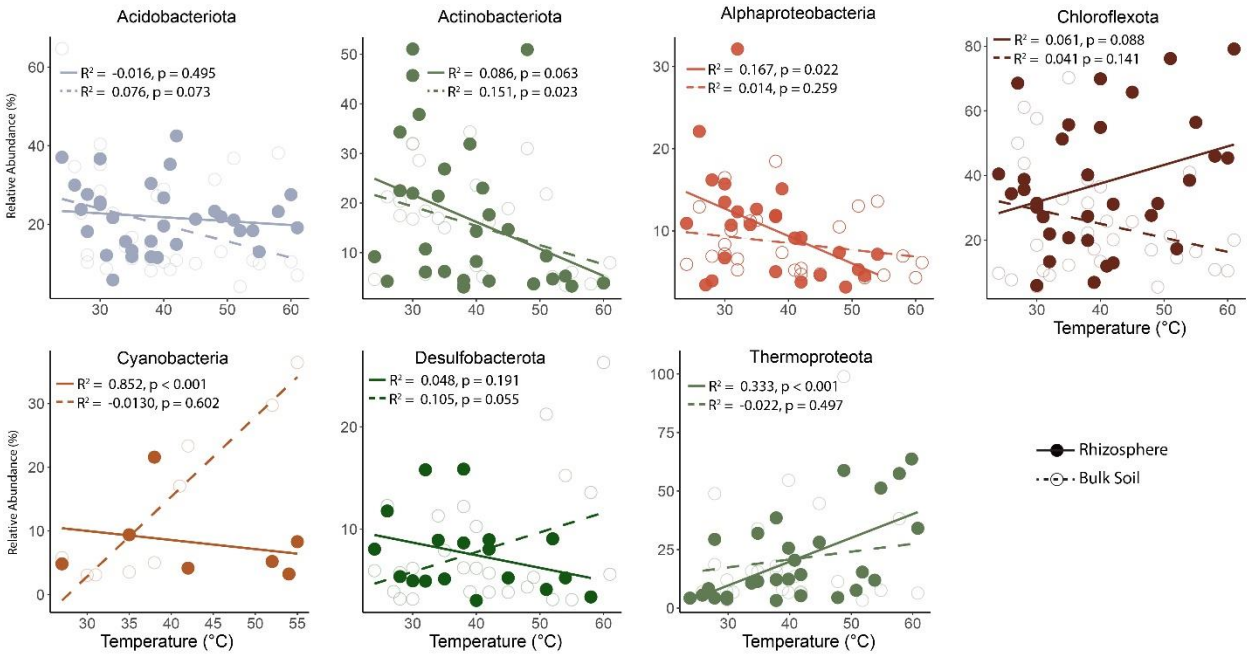


Figure 6. Linear regressions of bacterial + archaeal phyla with significant or marginal relationships with temperature. Rhizosphere samples are indicated by filled circles and straight lines while bulk soil samples are empty circles and dashed lines. Colors correspond with Fig. 5.

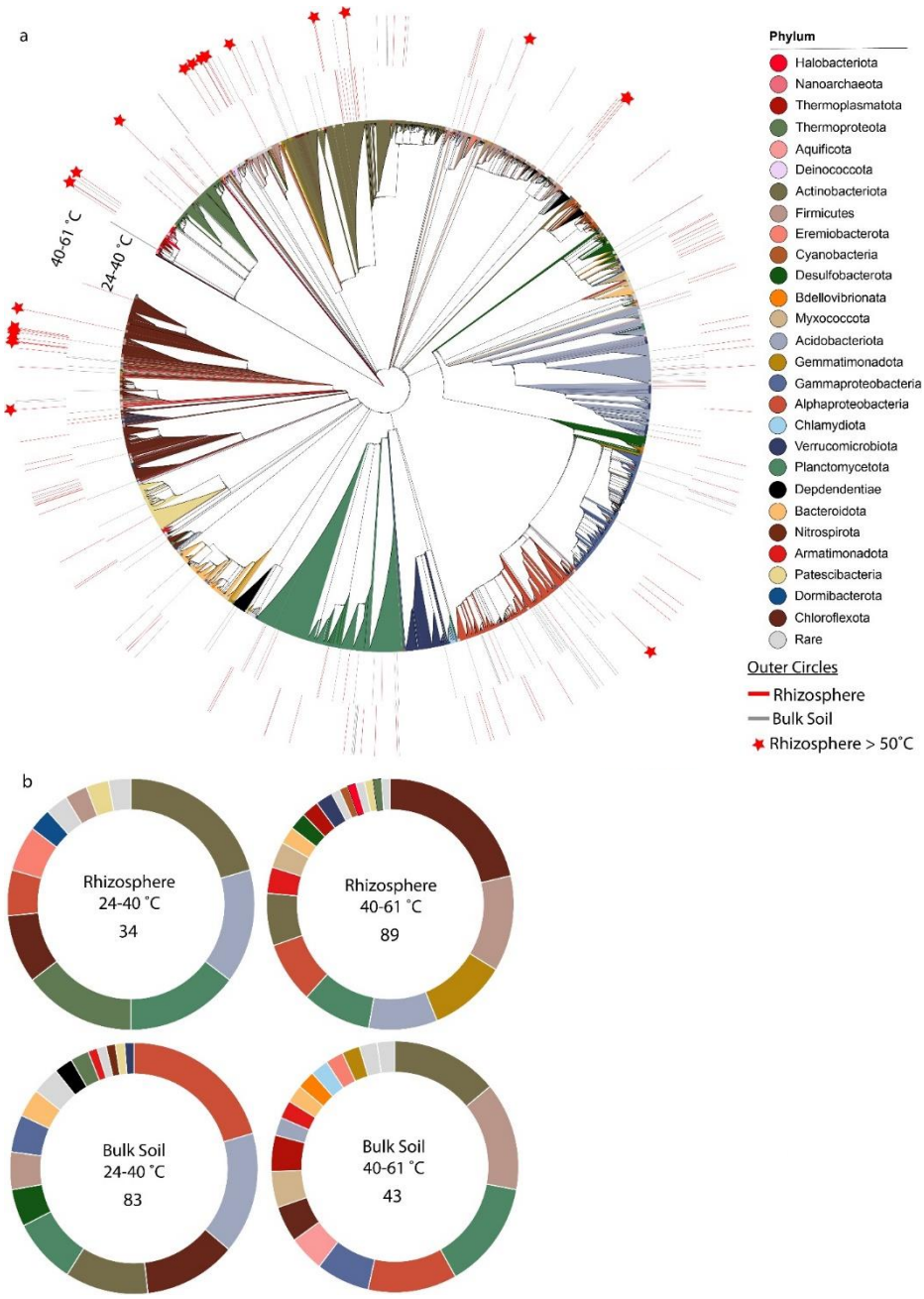


Figure 7. 16S phylogenetic tree of full sequencing dataset (a). Branches within the tree are colored by phyla and legend follows appearance in the tree starting at the *Halobacteriota* (archaea) root. Outer rings describe indicator species analysis of ZOTUs associated with bulks soils (gray), and with the *D. lanuginosum* rhizosphere (red) across temperatures and sites from 24-40 °C (inner circle), and 40-61 °C (outer circle). The subset of high temperature indicator ZOTUs above 50°C in the rhizosphere are indicated with stars. (b) Donut rings indicate the distribution of phyla within the groups of indicator ZOTUs and give the number of ZOTUs identified as indicator species for each.

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CHAPTER SIX

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

The discovery of microbes by Hooke and van Leeuwenhoek in the mid-1600s launched the pursuit of answers to what have become the enduring central questions of microbial ecology: "Who is there?" and "What are they doing?" Pursuing these questions over the past several centuries has led to countless life-saving medicinal and agricultural advancements and has brought us ever closer to understanding the origins of life on earth and the fundamental mechanisms that allow life to exist. Despite this rich history, these two questions remain at the heart of present-day soil microbial ecology. With the increasing concerns for the impacts of climate change on both natural and agroecosystems, understanding how microbes contribute to plant survival under different abiotic stressors is imperative to predicting and adapting future plant fitness and productivity. However, the sheer diversity of plants, microbes, and their innumerable interactions with each other and their surrounding environment presents a daunting challenge which will likely require many more years of global scientific effort to complete.

The work presented in this thesis contributes to furthering our understanding of soil microbial ecology in relation with several economically and ecologically valuable plant species among the *Poaceae.* This thesis presents the first successful field implementation of a cyanobacterial biofertilizer to support the production of tall wheatgrass (*Agropyron elongatum*) and switchgrass (*Panicum virgatum*) as 2nd generation bioenergy crops, an in-depth characterization of the effects of drought on plant-microbe interactions in blue grama (*Bouteloua*

gracilis), and the first insights into the bacterial + archaeal rhizobiome community of the highly heat-tolerant *Dichanthelium lanuginosum* across a wide natural temperature gradient in Yellowstone National Park. Together these studies demonstrate the potential for soil microbiota to be utilized for sustainable agriculture and provide an important foundation for future studies to untangle the complex relationships between plants and their microbiomes.

Chapter two of this dissertation describes the use of nitrogen-fixing cyanobacteria as biofertilizer to supplement the growth of two perennial bioenergy crops, tall wheatgrass (*Agropyron elongatum*) and switchgrass (*Panicum virgatum*) in a semi-arid cropping system and its impact on the soil microbial community and soil chemistry. This study (Goemann et al. 2021) is among the first field-plot scale biofertilizer experiments and demonstrated that cyanobacterial biofertilizer supplement can maintain aboveground productivity in comparison to synthetic fertilizer. We also observed greater late-season nitrogen availability in biofertilized plots compared to synthetically fertilized plots. Next-generation amplicon sequencing of the bacterial + archaeal and fungal communities indicated that biofertilization did not strongly affect the alpha or beta diversity of the soil microbiome after one year of the field study although there were some indications of increased relative abundances of arbuscular mycorrhizal fungi, and members of the *Planctomycetes, Actinobacteriota,* and *Proteobacterial* phyla.

After three years of biofertilization (Gay et al. 2022, Appendix X) the greatest differences observed in the microbial community were more associated with the establishment of the deep-rooting perennial crops rather than the different fertilization practices. Increases in relative abundances common carbon-cycling community members including fungi (*Agaricomycetes*, *Glomeromycetes*, and *Sordariomycetes*) and bacteria (*Actinobacteriota* and *Planctomycetota*)

were found for the tall wheatgrass along with indications of increased soil organic carbon. The cyanobacterial biofertilizer continued to maintain the same level of growth promotion as synthetic fertilizer throughout the three-year field study and the cool-season tall wheatgrass productivity matched similar levels to those recorded in other field studies, achieving 98% greater productivity than switchgrass. The lack of effect of fertilization type on the community diversity within the field plots suggested that: (i) the many years of chemical fertilization prior to the start of the experiment likely has a long-lasting legacy and/or (ii) that low levels of cyanobacterial biofertilization can adequately promote plant growth without negatively affecting the soil microbial community.

Chapter 3 of this dissertation explored the effect of increasing drought severity on the rhizobiome of blue grama (*Bouteloua gracilis*). Prior experimentation with blue grama had shown that (i) the presence of rhizosphere microbes benefited plant growth and photosynthesis prior to extreme drought stress but also increased the susceptibility of blue grama to severe drought (Ulrich et al. 2019b), and (ii) that under severe drought stress blue grama increases root exudation of osmoprotectants and signaling molecules (Ulrich et al. 2022). Chapter 3 was built upon the Ulrich et al. 2022 experiment and shifts in community diversity and composition were investigated across ambient, mild drought, and severe drought conditions. Response ratio analysis indicated that unique phylogenetic clades were differentially affected by mild and severe drought. These results contribute to understanding the variability of plant-microbe drought responses depending on the plant species and the severity of drought.

Chapter 4 of this dissertation further investigated the blue grama drought response by exploring the influence of climate legacy on the plant and microbiome response to drought and

subsequent recovery. Precipitation legacy strongly influenced the blue grama rhizosphere microbiome as indicated by amplicon sequencing of the bacterial + archaeal and fungal communities. When a single blue grama genotype was grown in the native blue grama microbiome soil collected from four sites along a 200 mm mean annual precipitation gradient, distinct responses linked to the precipitation legacy were recorded in plant physiology, root exudation and the microbial community.

We observed indications that plants grown in soil with the lowest MAP (Dillon site, 250 mm) and highest MAP (Paradise Valley, 450 mm) experienced the least drought stress on aboveground physiology. These two sites also displayed the greatest root exudation response with increased TOC and TN at peak drought. Further, the rhizosphere microbiome originating from Dillon soil had the greatest number of positive response ratios among major phyla at peak drought, while the Paradise Valley-originating microbiome had the most positive responses at the recovery timepoint. These results indicate that climate legacy shapes the resistance and resilience of blue grama to drought stress, and plant microbiomes with different climate legacies may have different strategies of plant-microbe feedback depending on the community composition.

Chapter 5 of this dissertation provides the first exploration of the bacterial + archaeal rhizosphere community of the extremely heat-tolerant *Dichanthelium lanunigosum* across a wide temperature range in Yellowstone National Park. Community beta diversity was strongly influenced by site, temperature and pH. Several clades involved in nitrogen cycling appeared to have particularly strong relationships with temperature. Rhizosphere *Thermoproteota*, *Chloroflexota* both increased in relative abundance with increasing temperature, and bulk

Cyanobacteria followed a similar trend. Above 50 °C ZOTUs belonging to *Ktedonobacteraceae* (*Chloroflexota*) and thermophilic *Firmictutes* were indicator species of the *D. lanuginosum* rhizobiome. These trends and indicator species suggest that at higher temperatures *D. lanuginosum* selects for a distinct rhizobiome that may or may not benefit plant fitness.

Future Work

Blue Grama Drought and Heat Stress

Heat and drought stress often happen concurrently and several recent studies have suggested that heat and drought stress in combination have distinct effects on plant-microbe interactions from that of either stress alone (Tiziani et al. 2022). To further understand the blue grama holobiont, investigating the individual and combinatory effects of drought and heat stress will aid in the completion of our understanding of the plant-microbe interactions of this important grassland species. In addition, to better understand the mechanisms of the blue grama stress responses, we recognize the need to investigate the response of the active portion of the microbial community. Therefore, we conducted a multifactorial stress experiment and subjected blue grama to drought, heat, and the combination of both stresses (Appendix B). We then extracted both DNA and RNA from the blue grama rhizobiome. In conjunction with plant physiology and root exudation in response to drought, heat, and drought*heat we will investigate the active and total microbial community response using both the 16S ratios method (Bowsher et al. 2021) and metatranscriptomics.

Preliminary data are provided in Appendix F. Plant physiology results suggest the drought and heat treatments were successful in stressing the experimental blue grama plants. Root exudation chemistry indicate that the active community is notably distinct from the total

community. *Alphaproteobacteria* were a major proponent of the active bacterial + archaeal community under drought stress, *Actinobacteria* were the largest active phyla under heat stress, and the combinatory effect of drought and heat stress resulted in a unique active community composition from either of the effects individually. In addition, when drought stress was ceased, all active microbial communities became more similar to ambient treatments suggesting that in this case drought had a much stronger effect on the community than heat stress, and that the active community is much faster response time that the total community. Future work will further explore the changes in microbial community composition between active and total membership and explore correlations between shifts in the active community and root exudate metabolites.

Blue Grama Climate Legacy Active Metagenomes

As part of the blue grama climate legacy experiment, we also set an objective to track the partitioning of newly fixed carbon through the plant to the active microbiome under drought stress. To do this we conducted $^{13}CO_2$ pulse-labeling just prior to harvesting the experimental plants at the peak drought and recovery timepoints. Plant samples were sent to Cornell University to determine the atom% of ^{13}C in plant tissues. Using the DNA extracted from the rhizosphere of destructively harvested plants as described in Chapter 4, we plan to create heavy- and light-C fractionated metagenomes from each of the experimental groups (four sites, two treatments, two timepoints, three experimental replicates, n=48). We will also conduct heavy- and light-C fractionation on the root exudate samples that were also collected at peak drought and recovery timepoints as described in Chapter 4. This analysis will allow us to precisely determine how root exudate composition shifts during drought stress, which microorganisms

likely utilize those exuded compounds, and what the likely feedbacks are for plant response to drought.

Blue Grama MONet Results

We recently participated in the Environmental Molecular Science Laboratory (EMSL) Molecular Observation Network program to conduct extensive analysis of the soil biogeochemistry and community composition (via metagenomics) at three of our four climate legacy field sites (Dillon, Ruby Reservoir, and Madison River). Soil cores were collected on Jun 23, 2023 and sent to EMSL for analysis. Soil biogeochemistry data will include Fourier-Transform Ion Cyclotron Resonance (FTICR) and Lipidomics Liquid Chromatography-Mass Spectrometry (LC-MS), total organic carbona and total nitrogen, soil hydraulic properties, and X-ray computed tomography. These data in combination with our previous sequencing results will provide an extremely detailed summary of the influence of soil edaphic characteristics as well as climate legacy on the assembly of the blue grama rhizobiome.

Blue Grama Seed Microbiomes

Seed microbiota play important roles in plant fitness as they are the primary inoculum for a plant's microbiome and can be heritable or transmitted horizontally in the environment (Simonin et al. 2020). Seed microbiomes are also gaining interest in agricultural fields as sources for biotechnological innovation and as carriers for biostimulants and bioinocula (Berg and Raaijmakers 2018). Despite their importance, relatively little is known about the dynamics of seed microbiomes across plant species and environments. Expanding our blue grama research to include the effects of distinct seed microbiomes is a logical next step in understanding the blue grama microbiome. Bushey et al. (2023) observed local adaptation of blue grama across an

elevation and precipitation gradient in SW. Arizona, suggesting that some ecotypes were more resilient to drought and temperature stresses than others. I hypothesize that this adaptation likely extends to the seed microbiome, and that diversity of the seed microbiome mirrors the effect of climate legacy observed here in Chapter 4 in structuring the blue grama rhizobiome.

D. lanuginosum Metagenomes and Soil Chemistry

To further understand the D. lanuginosum rhizobiome, we have generated metagenomic data matching that of the amplicon data presented in Chapter 5. We offer two alternate hypotheses: (i) that the functional potential of the rhizobiome will indicate plant-microbe mutualistic pathways that contribute to plant fitness at high temperatures (ii) that the functional potential of the rhizobiome will indicate the rhizosphere offers a “safe haven” for thermotolerant microbes to outcompete typical rhizosphere microorganisms and they do not offer any specific benefits to the plant. We also plant to submit soil samples for soil chemical analysis to better understand what other soil properties are important for the *D. lanuginosum* rhizosphere community assembly.

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APPENDICES

APPENDIX A

SUPPLEMENTAL INFORMATION FOR CHAPTER 2

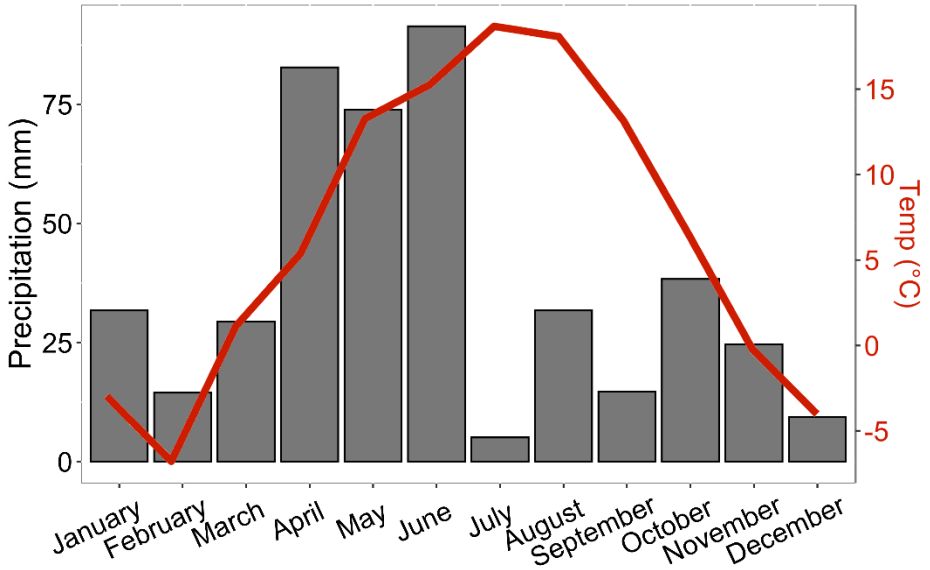


Figure S1. Monthly precipitation (mm) and mean daily temperature (°C) at the Arthur H. Post Research Farm meteorological station during 2018. In total 447.8 mm of precipitation fell in 2018, with 55% falling between April-June.

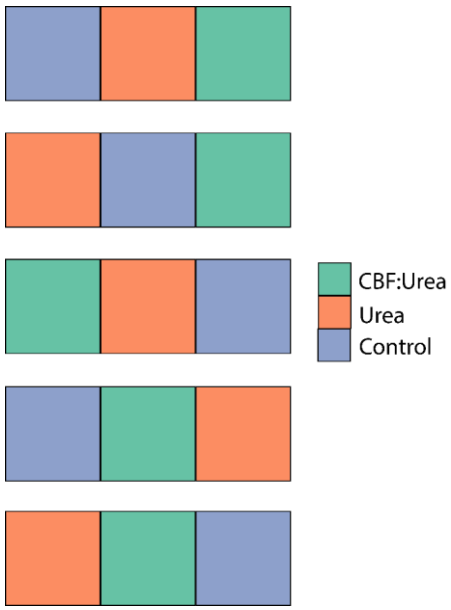


Figure S2. Experimental plot layout, alleys between treatments are not to scale as they were equal width (1.8 m^2 x 1.8 m^2) to experimental plots.

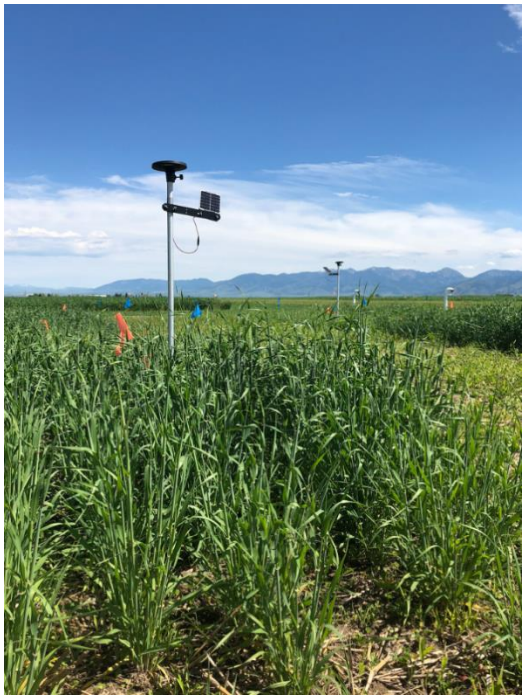


Figure S3. Arable mark NDVI sensor installed in the center of an example plot at 1 m above the max crop height.

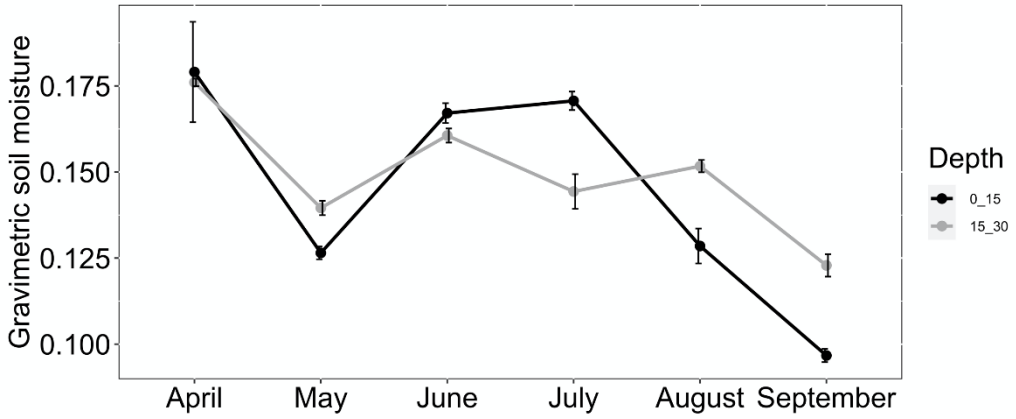


Figure S4. Gravimetric soil moisture collected and analyzed from the same soil cores used for chemical and microbial analysis. Data is averaged (±SE) across all treatment reps at 0-15 cm and 15-30 cm.

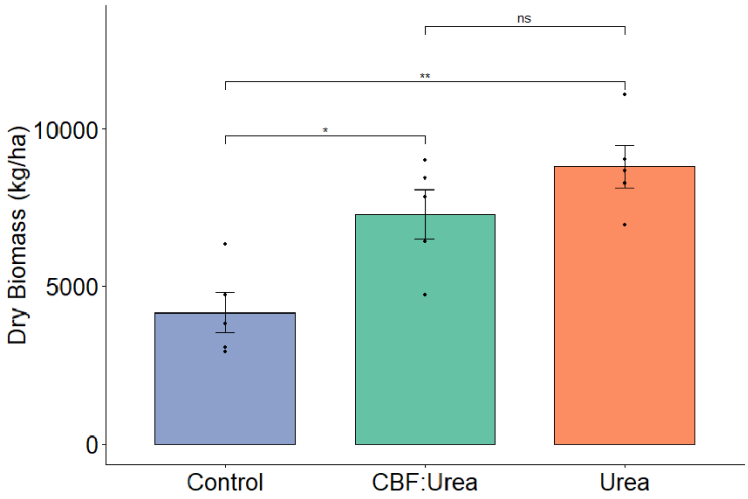


Figure S5. Barley dry biomass harvest under different fertilizer treatments. Bars represent ± SE, n=5. ** $p < 0.01$, * $p < 0.05$ based on Tukey-Kramer multiple comparison test.

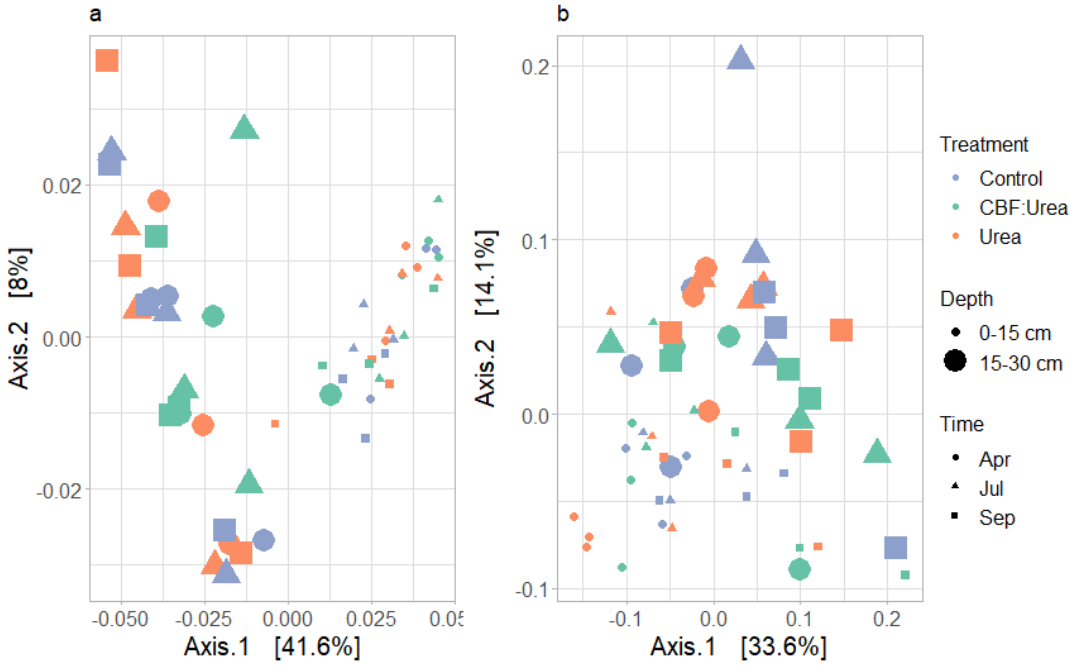


Figure S6. PCoA of weighted Unifrac community dissimilarity for a) bacteria/archaea and b) fungi. Points indicate individual samples, n=3 for each treatment.

Table S1. Summary of mean daily (± SE) NDVI values aggregated by month. Letters denote pairwise fertilizer treatment differences by month where significance is set at $p = 0.05$.

	May	**June**	**July**	**Aug**	**Sept**
Control	0.15(±0.01)[b]	0.43(±0.01)[b]	0.54(±0.01)[b]	0.31(±0.01)[b]	0.37(±0.01)[b]
Urea	0.18(±0.01)[b]	0.57(±0.02)[a]	0.64(±0.01)[a]	0.29(±0.01)[b]	0.39(±0.01)[b]
CBF:Urea	0.30(±0.02)[a]	0.61(±0.01)[a]	0.65(±0.01)[a]	0.39(±0.01)[a]	0.45(±0.01)[a]

Table S2. Total soil N and SOC across fertilizer treatments at two soil depths from pre-treatment to end of the 2018 growing season. Values are means (± SE)

			%N	***%SOC***	***C:N***
April 2018	**0-15 cm**		0.11(±0.002)	1.00(±0.03)	10.42(±0.45)
	15-30 cm		0.07(±0.002)	0.50(±0.03)	7.37(±0.45)
September 2018	**0-15 cm**	Control	0.1(±0.005)	0.85(±0.08)	10.3(±1.26)
		Urea	0.11(±0.005)	0.83(±0.06)	8.5(±1.26)
		CBF:Urea	0.11(±0.005)	0.85(±0.07)	9.5(±1.26)
	15-30 cm	Control	0.08(±0.005)	0.50(±0.12)	7.9(±1.21)
		Urea	0.07(±0.007)	0.51(±0.15)	8.2(±1.26)
		CBF:Urea	0.08(±0.004)	0.51(±0.07)	8.1(±1.27)

Table S3. pH means (95% CI) in April and July 2018. pH was calculated by averaging the hydrogen ion concentrations followed by a log10 back transformation.

April 2018	**pH**
0-15 cm	
	8.0(7.8-8.2)
15-30 cm	
	8.0(7.8-8.4)
July 2018	
0-15 cm	
Control	8.0(7.8-8.2)
Urea	7.9 (7.7-8.1)
CBF:Urea	8.0 (7.7-8.2)
15-30 cm	
Control	8.1(8.0-8.4)
Urea	8.0(7.9-8.1)
CBF:Urea	8.1(7.9-8.2)

Table S4. KCl extractable NO_3-N (μg g^{-1}) model means (±SE) across the 2018 growing season at two soil depths. Letters denote pairwise treatment differences at the respective soil depths by month where significance is set at $p = 0.1$.

Depth	Treatment	May	June	July	August	Sept
0 – 15 cm	Control	2.43(±1.1)[a]	0.048(±0.02)[a]	0.07(±0.03)[a]	10.3(±4.7)[a]	0.80(±0.36)[a]
	Urea	44.51(±20.2)[b]	1.34(±0.61)[b]	0.06(±0.03)[a]	10.4(±4.7)[a]	0.89(±0.41)[a]
	CBF:Urea	21.25(± 9.7)[b]	0.82(±0.37)[b]	0.27(±0.12)[b]	10.8(±4.9)[a]	0.75(±0.34)[a]
15 – 30 cm	Control	0.75(±0.34)[a]	0.05(±0.02)[a]	0.01(±0.01)[a]	9.41(±4.3)[a]	0.58(±0.26)[a]
	Urea	8.52(±3.9)[b]	1.31(±0.60)[b]	0.07(±0.03)[b]	8.17(±3.7)[a]	0.79(±0.36)[a]
	CBF:Urea	3.08(±1.40)[b]	0.34(±0.16)[b]	0.03(±0.01)[a]	8.18(±3.4)[a]	0.62(±0.28)[a]

Table S5. KCl extractable NH_4-N (μg g^{-1}) model means (±SE) across the 2018 growing season at two soil depths. Letters denote pairwise treatment differences at the respective soil depths by month where significance is set at $p = 0.1$.

Depth	Treatment	May	June	July	Aug	Sept
0 – 15 cm	Control	0.37(±0.18)[a]	1.01(±0.5)[a]	0.53(±0.26)[a]	0.77(±0.38)[a]	0.17(±0.09)[a]
	Urea	0.38(±0.19)[a]	1.6(±0.77)[a]	0.52(±0.26)[a]	0.78(±0.38)[a]	0.42(±0.21)[ab]
	CBF:Urea	0.55(±0.27)[a]	0.89(±0.44)[a]	0.33(±0.16)[a]	3.6(±1.8)[b]	1.3(±0.66)[b]
15 – 30 cm	Control	0.20(±0.1)[a]	0.83(±0.41)[a]	0.28(±0.14)[a]	0.70(±0.34)[a]	0.03(±0.01)[a]
	Urea	0.19(±0.1)[a]	0.96(±0.47)[a]	0.50(±0.24)[a]	0.92(±0.45)[a]	0.07(±0.04)[ab]
	CBF:Urea	0.33(±0.16)[a]	0.99(±0.49)[a]	0.43(±0.21)[a]	1.02(±0.50)[a]	0.22(±0.11)[b]

Table S6. PERMANOVA test results for fertilizer, time, and soil depth.

Predictor	**df**	**SumsofSqs**	**MeanSqs**	**F Model**	**R2**	**Pr(>F)**
fertilizer	2	0.00092	0.00046	0.63	0.023	0.939
time	2	0.0022	0.0011	1.49	0.054	*0.088*
depth	1	0.0025	0.0025	3.49	0.063	**0.003**
Residuals	48	0.035	0.00073		0.86	
Total	53	0.041			1.0	

Table S7. Phylosig results for 16S and LSU

	16S		**LSU**	
Phylosig Measure	Value	p	Value	p
Pagel's Lambda	0.234	**$2.60e^{-101}$**	0.621	**$9.9e^{-37}$**
Abouheif's C_{mean}	0.076	**0.001**	0.076	**0.001**

Table S8. SIMPER results for the bacterial/archaeal community at the phylum and family level based on RDP and BlastN classification. % Contrib refers to contribution of each ZOTU to the community difference between CBF:Urea and Urea fertilizer treatments. % Cusum indicates the cumulative sum of the differences between the communities explained by a given ZOTU. The p-value refers to the null hypothesis that the ZOTU is present equally in both communities.

ZOTU						**CBF:Urea**	**Urea**	
	Phylum	**Family**	**% Contrib**	**%Cusum**	**Ratio**	**Mean Abund**	**Mean Abund**	**p**
10978	Planctomycetes	unclassified Planctomycetia	0.07	0.15	0.50	0.28	0	<0.001
12684	Actinobacteria	Micromonosporaceae	0.07	0.35	0.53	0	0.22	<0.001
15193	Planctomycetes	Planctomycetaceae	0.07	0.58	0.50	0	0.28	<0.001
	Actinobacteria	Acidimicrobiaceae	0.07	0.76	0.50	0	0.8	<0.001

Table S8. Continued

	Phylum	Family	% Contrib	%Cusum	Ratio	CBF:Urea Mean Abund	Urea Mean Abund	p
3458	**Proteobacteria**	**Rhodospirillales incertae sedis**	**0.07**	**0.83**	**0.53**	**0.22**	**0**	**<0.001**
5119	Candidatus Uhrbacteria	Candidatus Uhrbacteria	0.07	0.90	0.53	0	0.22	<0.001
5640	Acidobacteria	Solibacteraceae	0.07	0.92	0.53	0	0.22	<0.001
7191	Abditibacteriota	Abitibacteriaceae	0.07	0.97	0.62	0.28	0	<0.001
7474	Planctomycetes	Pirellulaceae	0.07	0.98	0.58	0	0.33	<0.001
8403	Bacteroidetes	Porphyromonadaceae	0.07	1	0.62	0.28	0	<0.001
8488	Tenericutes	Mycoplasmataceae	0.07	1	0.66	0.28	0.056	<0.001
8573	Bacteroidetes	Cytophagaceae	0.07	1	0.55	0.056	0.33	<0.001

Table S9. SIMPER results for the fungal community at class and species level based on RDP and BlastN classification. % Contrib refers to contribution of each ZOTU to the community difference between CBF:Urea and Urea fertilizer treatments. % Cumul indicates the cumulative sum of the differences between the communities explained by a given ZOTU. The p-value refers to the null hypothesis that the ZOTU is present equally in both communities.

ZOTU	Class	Species	% Contrib	%Cusum	Ratio	CBF:Urea Mean Abund	Urea Mean Abund	p
1084	Glomeromycetes	*Glomus sp.*	0.02	0.52	0.65	1.28	20.3	0.003
1158	Glomeromycetes	*Glomus sp.*	0.02	0.65	0.42	0.61	17.9	0.025
1192	Glomeromycetes	*Glomus sp.*	0.02	0.70	0.55	0.00	18.2	0.004
1275	Glomeromycetes	*Rhizophagus sp.*	0.02	0.76	0.42	0.39	17.1	0.050
2456	Eurotiomycetes	*Cladophialophora modesta*	0.00	0.96	0.39	0.00	5.00	0.037
2805	Monoblepharidomycetes	*Hyaloraphidium curvatum*	0.00	0.97	0.75	1.28	2.94	0.045
355	Agaricomycetes	*Thanatephorus sp.*	0.07	0.99	0.57	85.1	6.94	0.037
466	Glomeromycetes	*Glomus sp.*	0.07	1.00	0.57	3.17	66.4	0.026

Table S9. Continued

ZOTU	Class	Species	% Contrib	%Cusum	Ratio	CBF:Urea Mean Abund	Urea Mean Abund	p
578	**Glomeromycetes**	***Glomus sp.***	**0.05**	**1.00**	**0.57**	**1.00**	**49.7**	**0.006**
604	Glomeromycetes	*Glomus sp.*	0.05	1.00	0.53	2.22	45.9	0.018
788	Glomeromycetes	*Glomus sp.*	0.04	1.00	0.41	1.78	30.3	0.038
873	Glomeromycetes	*Glomus sp.*	0.04	1.00	0.40	0.00	31.7	0.032
892	Pezizomycetes	*Iodophanus carneus*	0.03	1.00	0.46	5.50	23.0	0.041
5852	Sordariomycetes	*Hirsutella rhossiliensis*	0.00	1.00	0.38	0.00	0.670	0.050
888	Zygomycota incertae sedis	*Mortierella elongata*	0.03	1.00	0.50	1.17	29.5	0.029

APPENDIX B

SUPPLEMENTAL INFORMATION FOR CHAPTER 3

Table S1. Summary of P-values from linear mixed effects models describing the effects of treatment, day, microhabitat (bulk vs. rhizosphere) and their interactions on microbiome alpha diversity.

	Predictor	Richness (#Zotus)	Shannon's Diversity (H)	Faith's Phylogenetic Diversity (mbl)
Bacteria + Archaea	Treatment	0.284	0.186	0.410
	Microhabitation	0.884	0.583	0.393
	Time	0.158	0.583	0.123
	Treatment*Time	0.608	0.490	0.699
	Treatment*Microhabitat	0.197	0.377	0.120
	Time*Microhabitat	0.326	0.244	0.273
	Treatment*Time*Microhabitat	0.232	0.356	0.175
Fungi	Treatment	0.503	0.613	0.642
	Microhabitation	0.761	0.983	0.421
	Time	**< 0.001**	**0.002**	**< 0.001**
	Treatment*Time	0.187	0.364	0.403
	Treatment*Microhabitat	0.191	*0.0597*	**0.0356**
	Time*Microhabitat	0.299	*0.0856*	0.157
	Treatment*Time*Microhabitat	0.769	0.145	0.674

Table S2. Summary of T-Test results for clade-wide response ratios

	Comparison	Taxonomy	Stat	p.adj
Bacteria + Archaea	Mild-Ambient	**Phylum**		
		Omnitrophota	-0.485	0.0434
		Planctomycetota	-0.0371	0.0485
		Chloroflexota	0.0581	0.0173
		Actinobacteriota	0.12	< 0.001
		Thermoproteota	0.246	< 0.001
		Family		
		J027	-0.359	0.0162
		Burkholderiaceae	-0.106	0.0405
		SM1A02	-0.29	0.0461
		PALSA_1355	-0.318	0.0167
		Bryobacteraceae	-0.176	0.00451
		Nevskiaceae	-0.667	< 0.001
		Beijerinckiaceae	0.281	< 0.001
		Devosiaceae	0.237	0.0196
		UBA5704	0.392	0.0161
		Propionibacteriaceae	0.282	< 0.001
		Geodermatophilaceae	0.268	< 0.001
		Microbacteriaceae	0.143	0.0196
		UBA11606	0.234	0.0461
		Ilumatobacteraceae	0.158	0.0178
		Gaiellaceae	0.221	< 0.001
		Solirubrobacteraceae	0.112	< 0.001
		Rubrobacteraeceae	0.189	< 0.001
		Nitrososphaeraceae	0.246	< 0.001
		Xanthobacteraceae	0.109	< 0.001
		Roseiflexaceae	0.294	0.0001
		Ktedonobacteraceae	0.145	0.027
	Severe-Ambient	**Phylum**		
		Firmicutes	-0.33	< 0.001
		Gemmatimonadota	-0.153	< 0.001
		Bdellovirionota	-0.144	0.00503
		Acidobacteriota	0.054	0.00115
		Chloroflexota	0.0971	< 0.001
		Verrucomicrobiota	0.144	< 0.001
		Planctomycetota	0.153	< 0.001
		Thermoproteota	0.299	< 0.001

Table S2. Continued

	Comparison	Taxonomy	Stat	p.adj
		Family		
Bacteria + Arch	Severe-Ambient	*Nevskiaceae*	-0.435	< 0.001
		Rhodobacteraceae	-0.359	0.0378
		Paenibacillaceae	-0.34	< 0.001
		UBA 6960	-0.337	0.0163
		Bedellovibrionaceae	-0.331	0.00727
		Longimicrobiaceae	-0.315	0.00669
		B_17BO	-0.22	0.0101
		Streptomycetaceae	-0.189	0.00458
		Bacteriovoraceae	-0.166	0.0456
		Planococcaceae	-0.155	0.0199
		Gemmatimonadaceae	-0.151	< 0.001
		Sphingomonadaceae	-0.127	0.0198
		Burkholderiaceae	-0.127	0.0031
		Pyrinomonadaceae	0.0838	0.00919
		Gemmataceae	0.0934	0.00458
		Rubrobacteraceae	0.0999	0.00715
		UBA1161	0.106	0.0137
		UBA10450	0.11	0.0378
		Gaiellaceae	0.127	< 0.001
		Polyangiaceae	0.146	0.0163
		Chthoniobacteraceae	0.149	0.0163
		SCN_69_37	0.156	0.0455
		Caldilineaceae	0.171	0.0125
		Steroidobacteraceae	0.173	0.0121
		Saprospiraceae	0.173	0.0455
		UBA7805	0.174	0.0353
		CSP1_4	0.186	0.00155
		Planctomycetaceae	0.191	0.0125
		Xanthobacteraceae	0.191	< 0.001
		Pirellulaceae	0.205	< 0.001
		IMCC26256	0.211	0.00727
		BJHT01	0.211	0.0112
		UBA8199	0.211	0.0188
		Devosiaceae	0.217	0.0455
		Ga0077529	0.228	< 0.001
		Roseiflexaceae	0.247	0.00386
		Isosphaeraceae	0.305	0.00423

Table S2. Continued

		Taxonomy	Stat	p.adj
Bacteria + Archaea	Severe-Ambient	*Nitrososphaeraceae*	0.312	< 0.001
		Thermoguttaceae	0.361	< 0.001
		UBA5704	0.411	0.000472
Fungi	Mild-Ambient	**Class**		
		Eurotiomycetes	-0.17	0.009
	Severe Ambient	**Phylum**		
		Chytridiomycetes	-0.314	0.00571
		Ascomycota	0.191	< 0.001
		Mucoromycotina	0.512	< 0.001
		Class		
		Chytridiomycetes	-0.33	0.00655
		Dothideomycetes	0.179	0.0019
		Eurotiomycetes	0.188	0.000151
		Sordariomycetes	0.222	< 0.001
		Glomeromycets	0.512	< 0.001

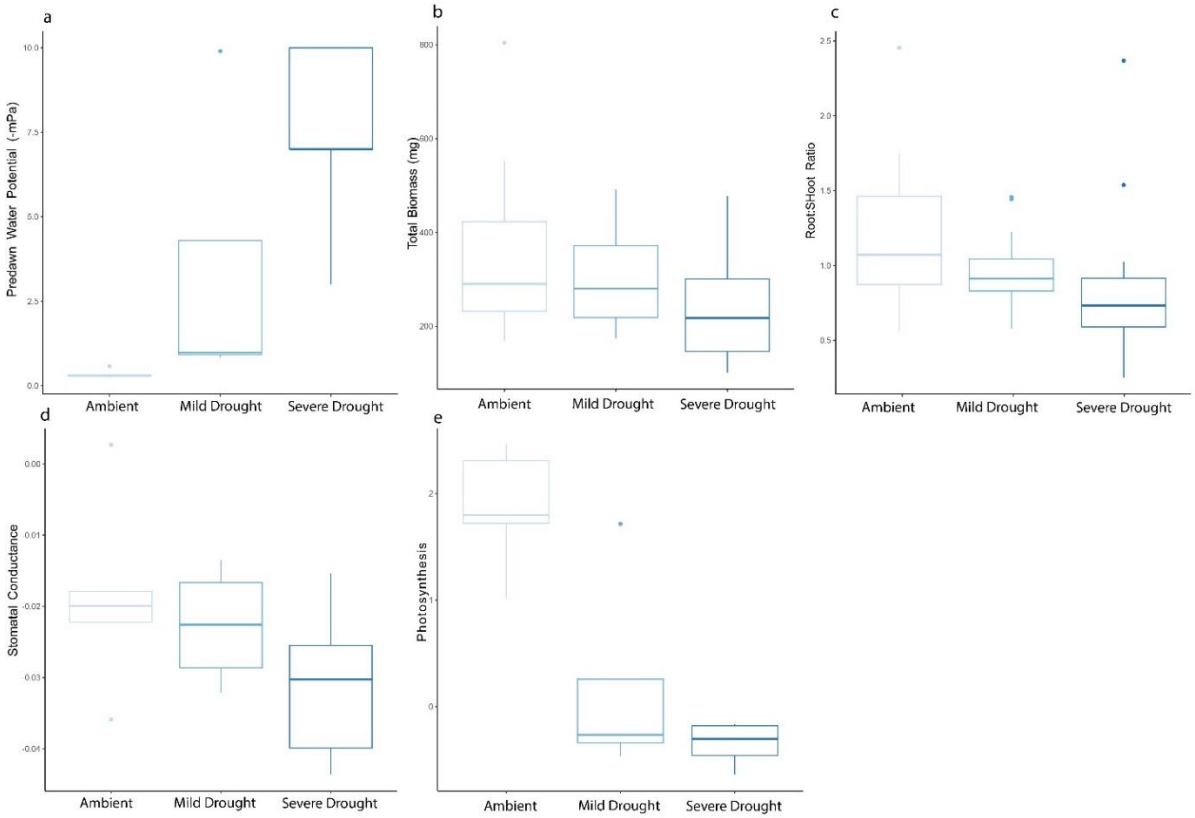


Figure S1. Plant physiology at T2 in response to increasing drought severity.

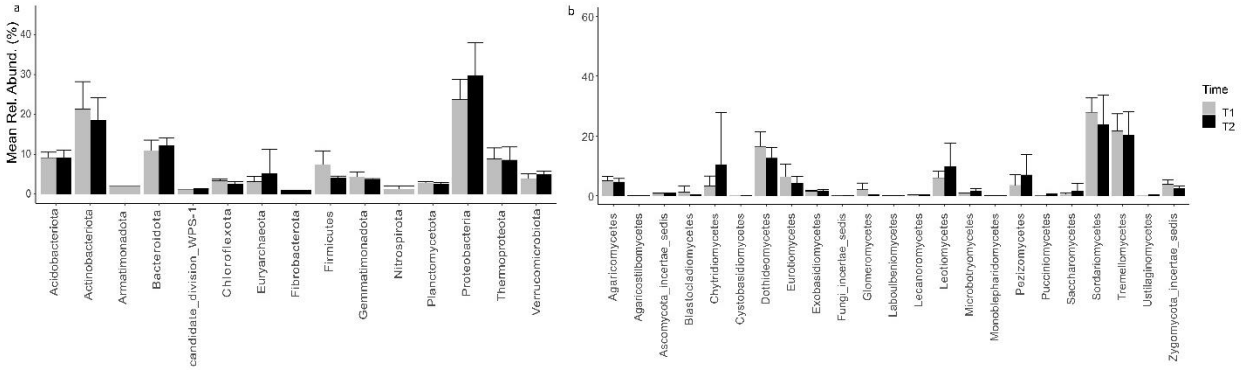


Figure S2. Relative abundances of (a) bacteria + archaeal phyla, and (b) fungal classes at T1 vs. ambient T2. * indicates $p < 0.05$ based on BH-corrected two-way Wilcoxen tests. Error bars are 95% CI.

NetCoMi Parameters:

```
combinedgen<-netConstruct(data=ambcomb_class,
                          data2=sevcomb_class,
                      measure="sparcc",
                      dataType="counts",
                      taxRank = "class",
                      measurePar = list(rep.num=100),
                      filtTax="highestVar",
                      filtTaxPar = list(highestVar=50),
                      filtSamp="totalReads",
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                      normMethod="none",
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```

APPENDIX C

SUPPLEMENTAL INFORMATION FOR CHAPTER 4

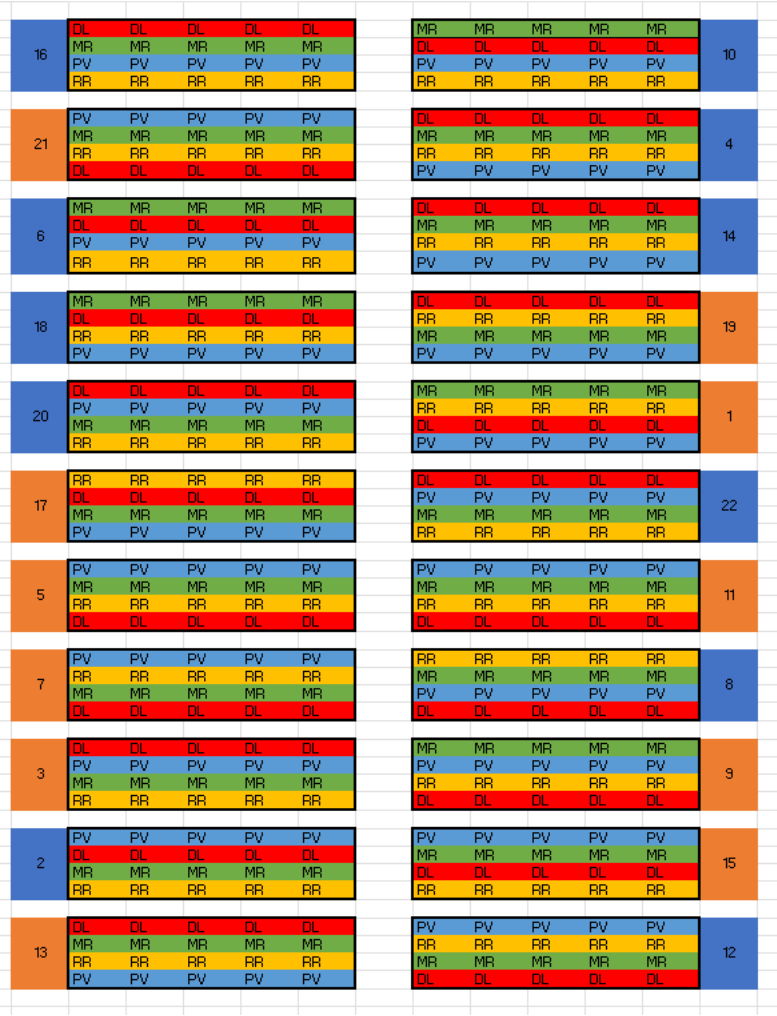


Figure S1. Greenhouse experimental layout. Each block represents a rack of 20 pots, each with one plant. Samples are colored by soil origin site: DL = Dillon, RR = Ruby Reservoir, MR = Madison River, PV = Paradise Valley. Each block (rack) was randomly assigned a number, and each number was randomly assigned to either the drought (orange) or ambient (blue) treatment conditions at the beginning of the drought treatment period. Rack positions were reshuffled weekly within the greenhouse to minimize microclimate influences.

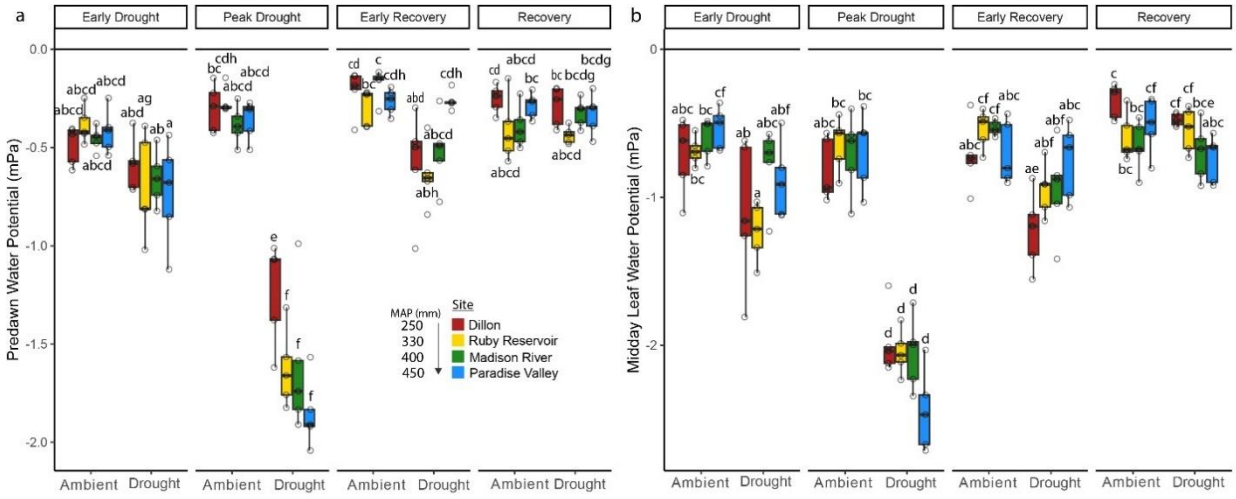


Figure S2. Predawn (a) and Midday (b) leaf water potentials including early drought and early recovery timepoints. Boxes indicate first and third quartiles, whiskers are at most 1.5*IQR. Points indicate individual samples. Letters denoting alpha < 0.05 differences within timepoints were calculated via post-hoc Tukey-Kramer multiple comparison tests.

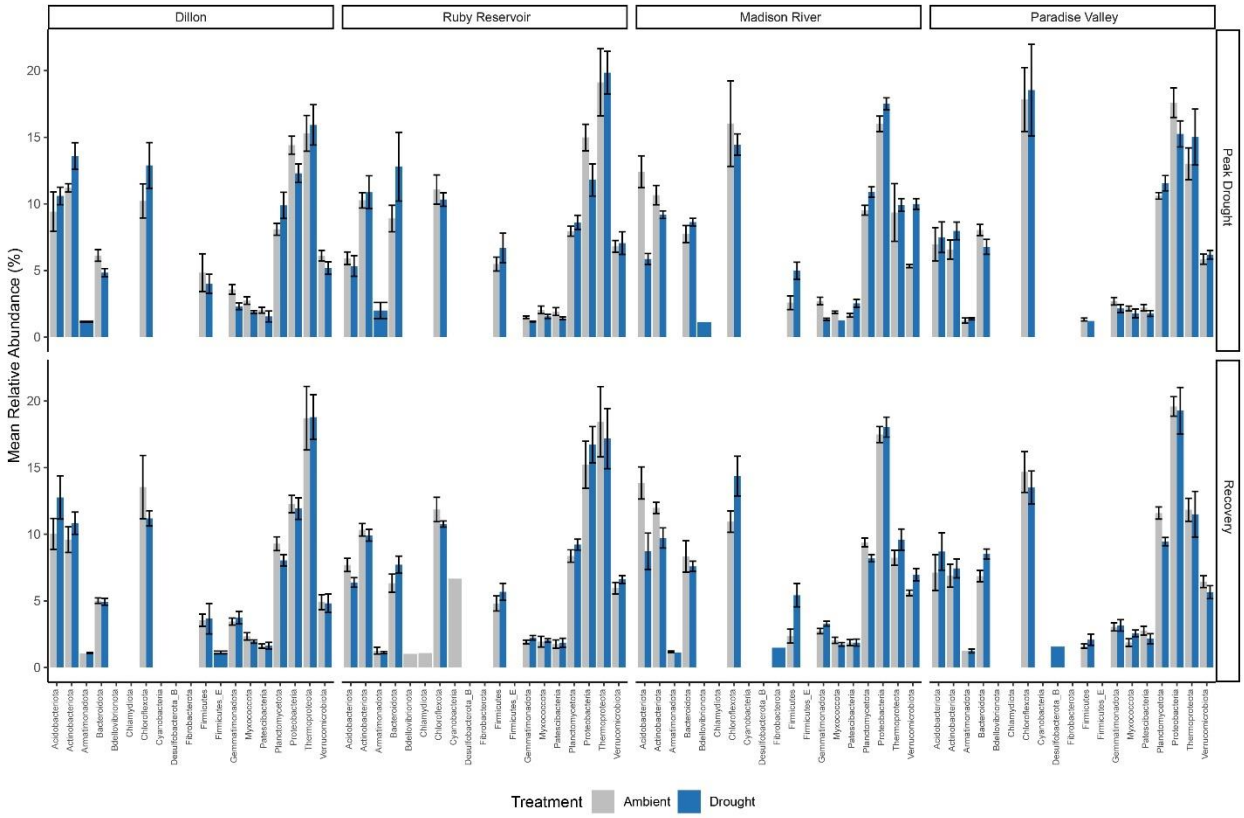


Figure S3. Mean relative abundances of bacterial + archaeal phyla across the four soils, two treatments and two timepoints. Error bars are ± SE.

Table S1. Summary of Background Soil Community Beta Diversity Test Results

PERMANOVA	Df	SumofSqs	R^2	F	Pr(>F)
site	3	2.299	0.34634	2.6493	**0.001**
Residual	15	4.3389	0.65366		
Total	18	6.6379	1.00000		
PermDist	Df	Sum Sq	Mean Sq	F	Pr(>F)
Groups	3	0.051611	0.00172	3.3606	0.04704

Table S1. Continued

PermDist-Tukey		Diff	Lwr	Upr	p adj
	RR_DL	0.04123	5.0976e-05	0.0825	**0.04967**
	MR_DL	0.0175	-0.263e-02	0.0612	0.665
	PV_DL	0.00437	-3.687e-02	0.0456	0.989
	MR_RR	-0.0238	-6.758e-02	0.0199	0.423
	PV_RR	-0.0369	-7.816e-02	0.00433	*0.0872*
	PV_MR	-0.0131	-5.682e-02	0.0307	0.824

Table S2. Summary of Experimental Beta Diversity Test Results

PERMANOVA	Df	SumofSqs	R^2	F	Pr(>F)
Site	3	7.191	0.20995	7.9884	**0.001**
Treatment	1	0.262	0.00766	0.8745	0.715
Timepoint	1	0.274	0.00799	0.9116	0.635
Site:Treatment	3	0.822	0.02400	0.9130	0.790
Site:Timepoint	3	0.881	0.02572	0.9785	0.509
Treatment:Timepoint	1	0.286	0.00835	0.9532	0.493
Site:Treatment:Timepoint	3	0.831	0.02425	0.9227	0.752
Residual	79	23.703	0.69209		
Total	94	34.249	1.00000		

PermDist	Df	Sum Sq	Mean Sq	F	Pr(>F)
Peak Drought	3	0.0055937	0.001864	2.8396	**0.0487**
Recovery	3	0.009536	0.0031785	3.065	**0.03796**

Table S2. Continued

PermDist_Tukey					
Timepoint	Group	Diff	Lwr	Upr	P adj
Peak Drought	RR_DL	-0.0213	-0.0498	0.00725	0.206
	MR_DL	0.0056	-0.0222	0.0336	0.948
	PV_DL	0.00552	-0.0219	0.0329	0.949
	MR_RR	0.0268	-0.00157	0.0555	*0.0700*
	PV_RR	0.0268	-0.00119	0.0549	*0.0651*
	PV_MR	-0.000156	-0.0275	0.0272	0.999
Recovery	RR_DL	-0.0108	-0.0459	0.0243	0.843
	MR_DL	0.0277	-0.00749	0.0628	0.168
	PV_DL	0.00233	-0.0356	0.0382	0.998
	MR_RR	0.0385	0.00334	0.0736	**0.0269**
	PV_RR	0.0132	-0.0228	0.0481	0.762
	PV_MR	-0.0253	-0.0612	0.0106	0.250

Fungal Community Data

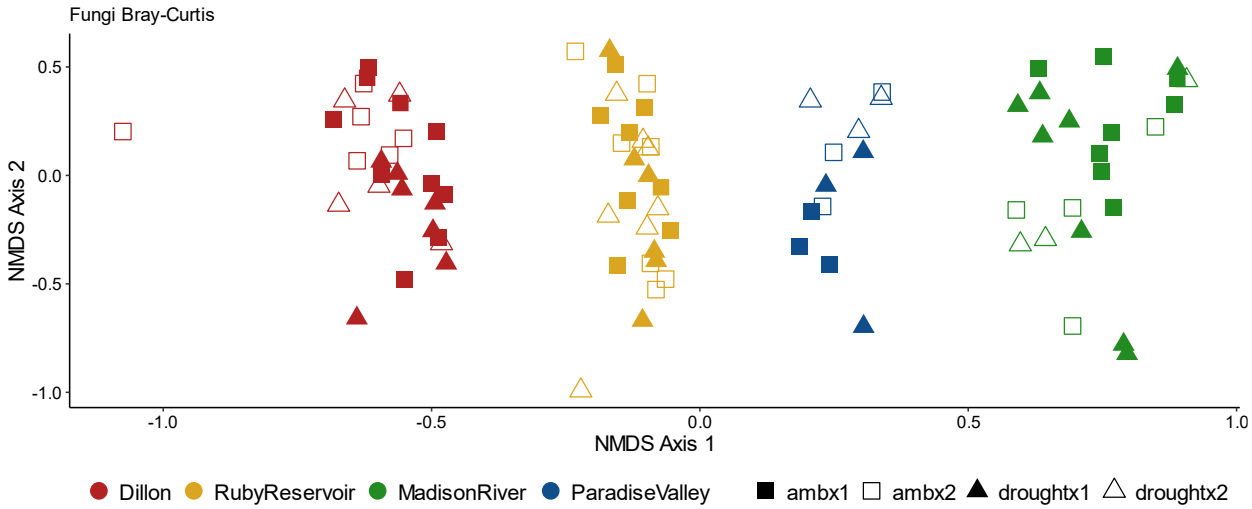


Figure S4. Bray-Curtis ordination of fungal community colored by site. Shapes indiate treatment and timepoint. Filled shapes indicate timepoint 1 (Peak Drought), and empty shapes indicate timepoint 2 (Recovery).

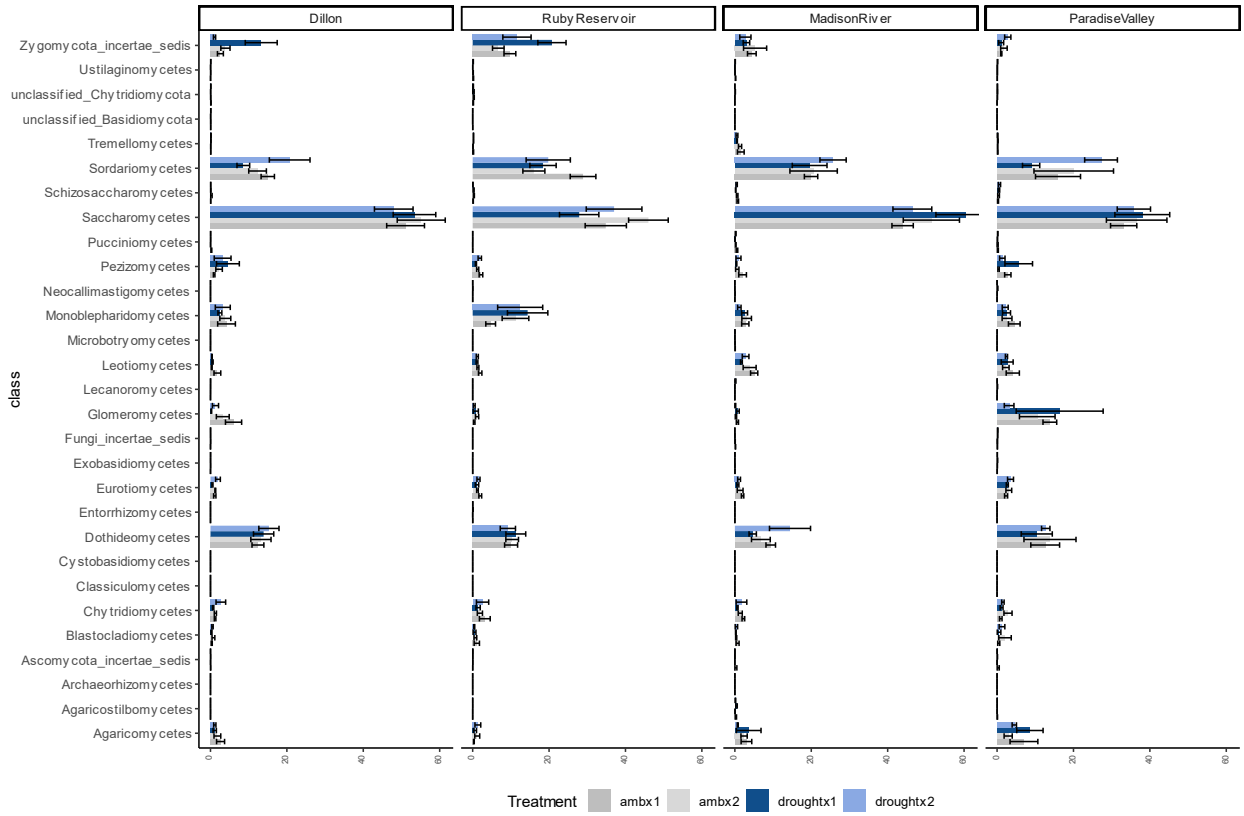


Figure S4. Relative abundances of fungal classes across sites, treatments and timepoints. Error bars are ± SE.

APPENDIX D

SUPPLEMENTAL INFORMATION FOR CHAPTER 5

Table S1. Summary of sample temperatures and pH. Note that each sample had bulk and rhizosphere components.

Sample_ID	Site	Temperature C	pH
AmphitheaterB	Amphitheater	30	4.346
Amphitheater2	Amphitheater	35	4.16
Amphitheater1	Amphitheater	40	3.667
Ob2	Artists	28	3.718
Ob1	Artists	32	4.687
APP34	Artists	34	3.669
Ob3	Artists	38	8.101
HLGB1	HeartLake	30	4.769
PKF	HeartLake	38	5.71
HLGB2	HeartLake	41	8.995
WCxLLx1	HeartLake	42	7.164
HLGB6	HeartLake	45	3.746
HLGB3	HeartLake	49	4.157
WCxLLx2	HeartLake	52	9.068
WCxM	HeartLake	54	8.996
WCxU	HeartLake	58	5.445
HLGB4	HeartLake	60	4.223
HLGB5	HeartLake	61	3.542
MV2	MudVolcano	26	4.029
MV1	MudVolcano	28	3.109
MV3	MudVolcano	32	3.604
WT24	WestThumb	24	4.027
WT27	WestThumb	27	4.702
WT30	WestThumb	30	3.917
WT38	WestThumb	38	9.499
WT40	WestThumb	40	7.899
WhiteCreek5	WhiteCreek	31	6.465
WhiteCreek3	WhiteCreek	35	9.254
WhiteCreek5b	WhiteCreek	39	8.388
Whc1	WhiteCreek	42	3.721
WhiteCreek4b	WhiteCreek	48	6.045
WhiteCreek4	WhiteCreek	51	6.045
WhiteCreek2	WhiteCreek	55	8.874

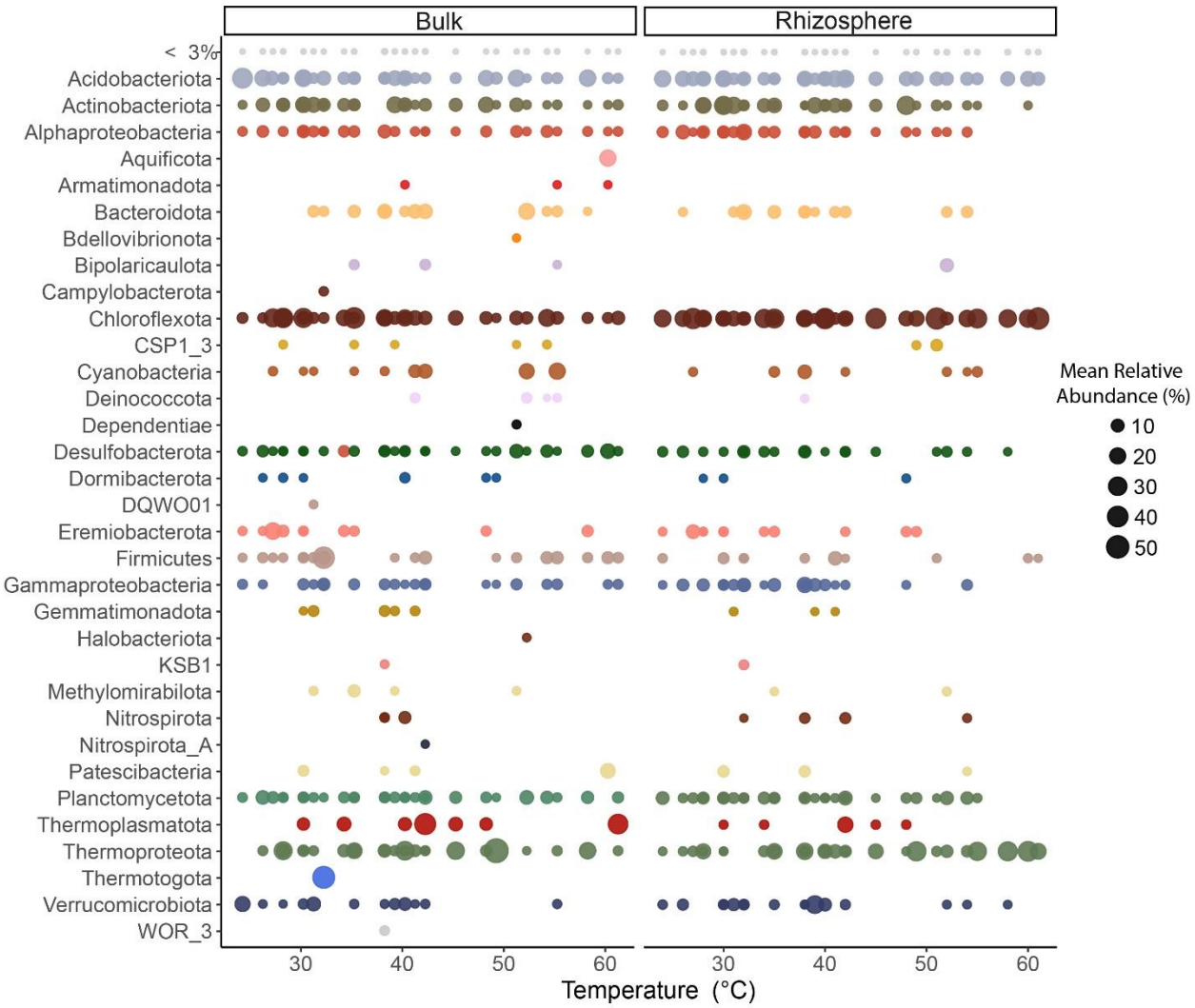


Figure S1. Temperature effects on the relative abundances of bacterial + archaeal phyla across in the *D. lanuginosum* rhizosphere and bulk soils.

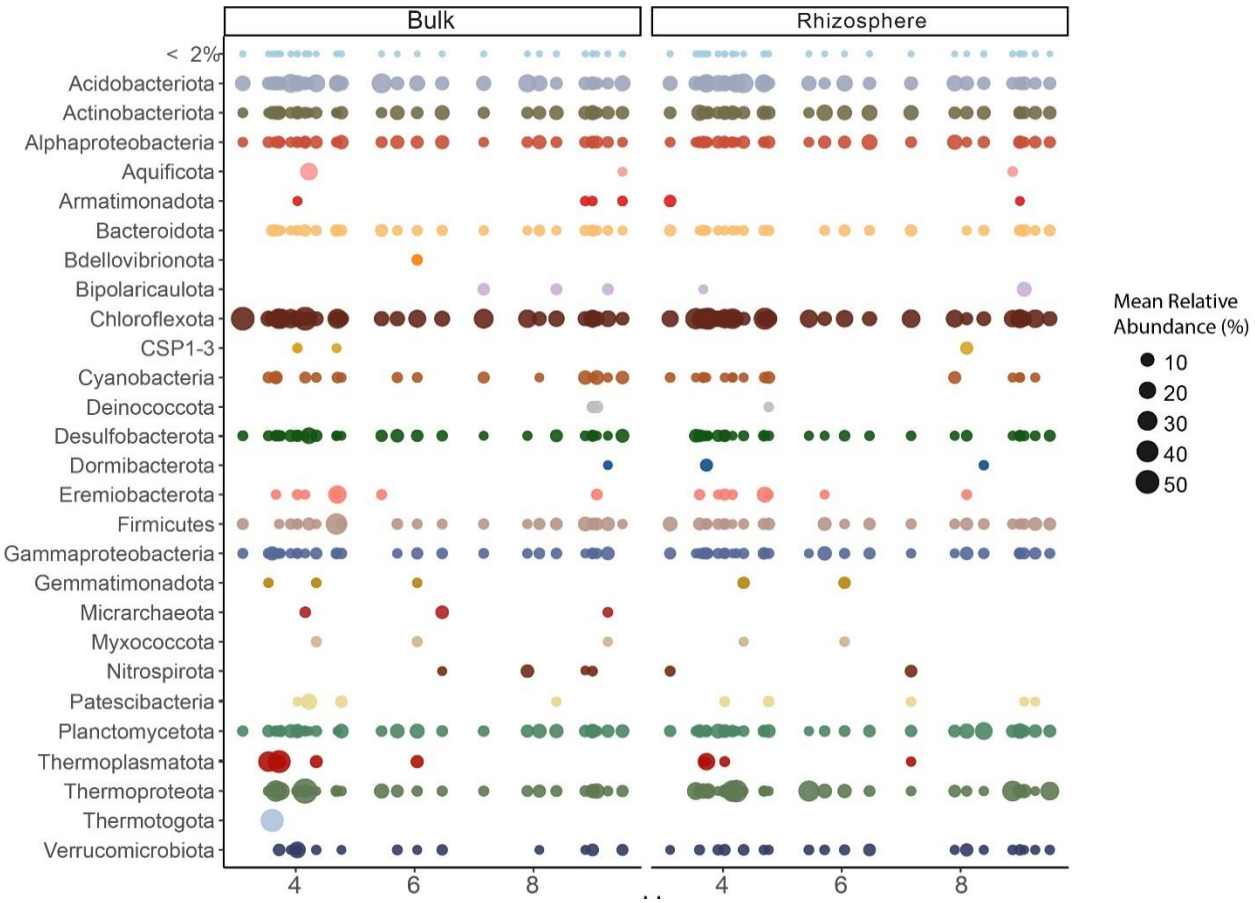


Figure S2. Effect of pH on the relative abundance of bacterial + archaeal phyla in bulk and rhizosphere soil.

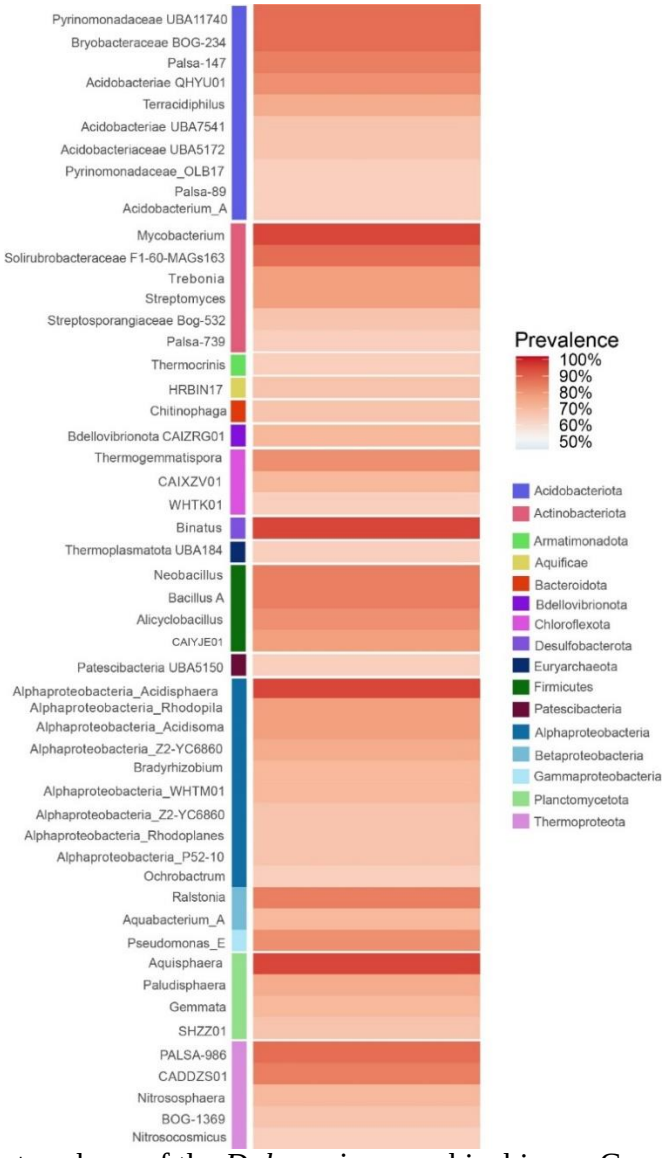


Figure S3. Core bacteria + archaea of the *D. lanuginosum* rhizobiome. Genera are grouped by bacterial + archaeal phylum. The prevalence threshold was set as > 60% of all samples across the six sites in YNP.

APPENDIX E

INFLUENCE OF CYANOBACTERIAL BIOFERTILIZER ON THE SOIL SURFACE CRUST

Overview

As a continuation of the field study described in Chapter 2, we continued to apply biofertilizer and monitor the soil chemistry and microbial community at the Post Farm tall wheatgrass and switchgrass field plots through 2020. In addition to the paired samples collected for soil chemistry and microbial analysis, a 0-1 cm sample was also collected explore the effects of the cyanobacterial biofertilizer specifically on the top soil crust where we expected the impacts of the biofertilizer to be the greatest on the soil microbial community.

Methods

See Goemann et al. (2021) and Gay et al. (2022) for site description and experimental setup. In addition to the soil collected at 0-15 and 15-30cm, surface crust samples (0-1 cm) were collected from tall wheatgrass plots in April, July and September in 2019 and 2020, and in April of 2021 after the project was finished and no more biofertilizer was applied. Samples were flash-frozen in an ethanol-dry ice bath immediately after collection and stored on dry ice until transport to lab and long-term storage at -80 °C. DNA extraction, sequencing and bioinformatics were conducted for the soil bacterial + archaeal community using 16S amplicon sequencing as described in Chapters 2-5. Below are preliminary figures summarizing the results of the soil crust dataset.

Results

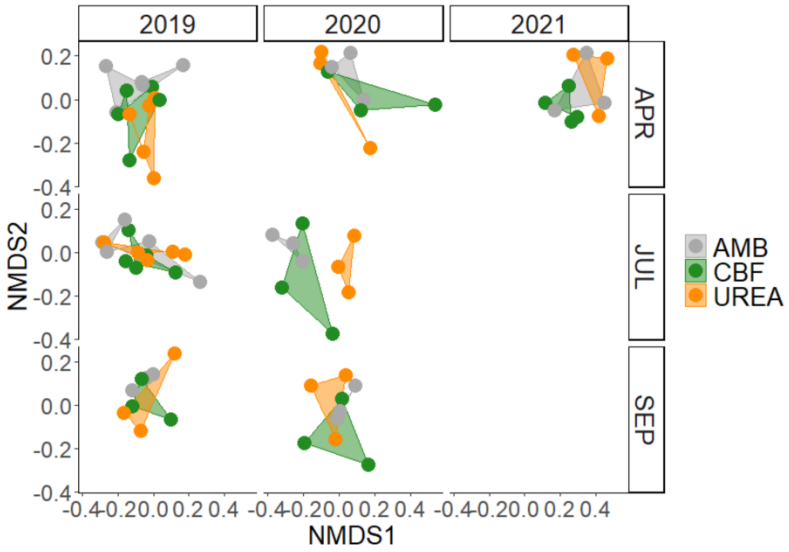


Figure 1. Bray-Curtis ordination of 16S crust community by treatment and sampling timepoint.

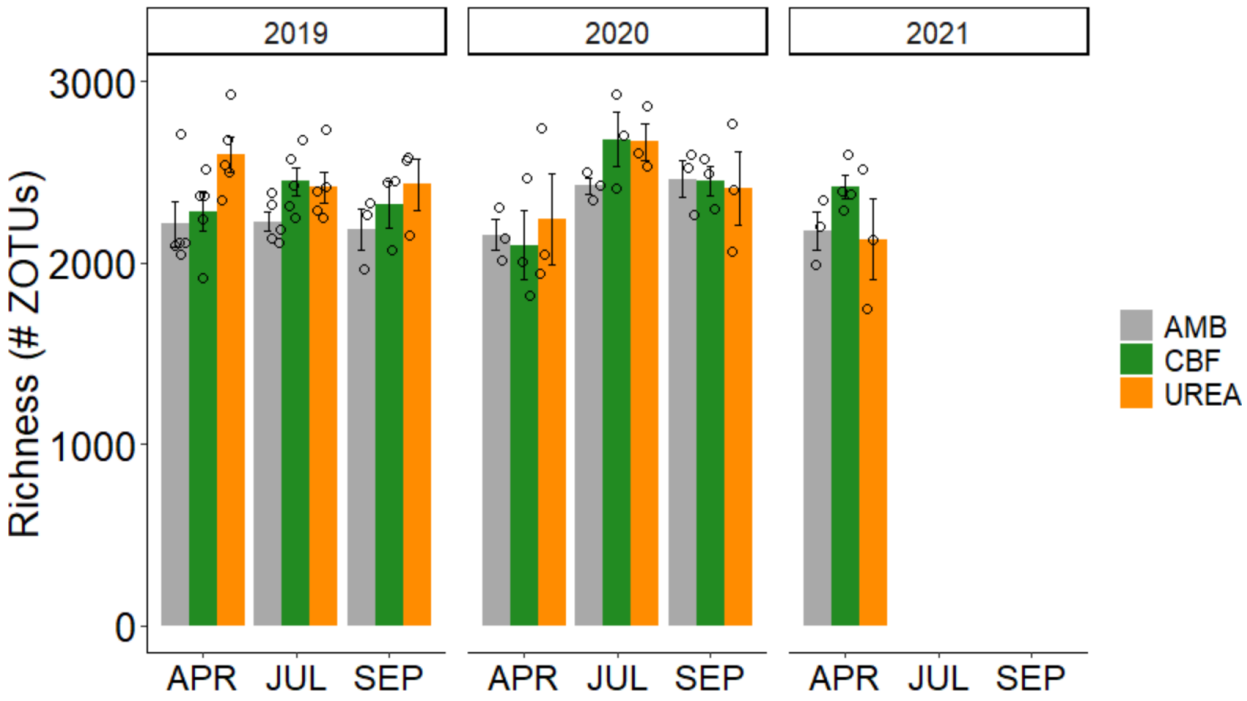


Figure 2. Alpha diversity of 16S crust community by treatment and time.

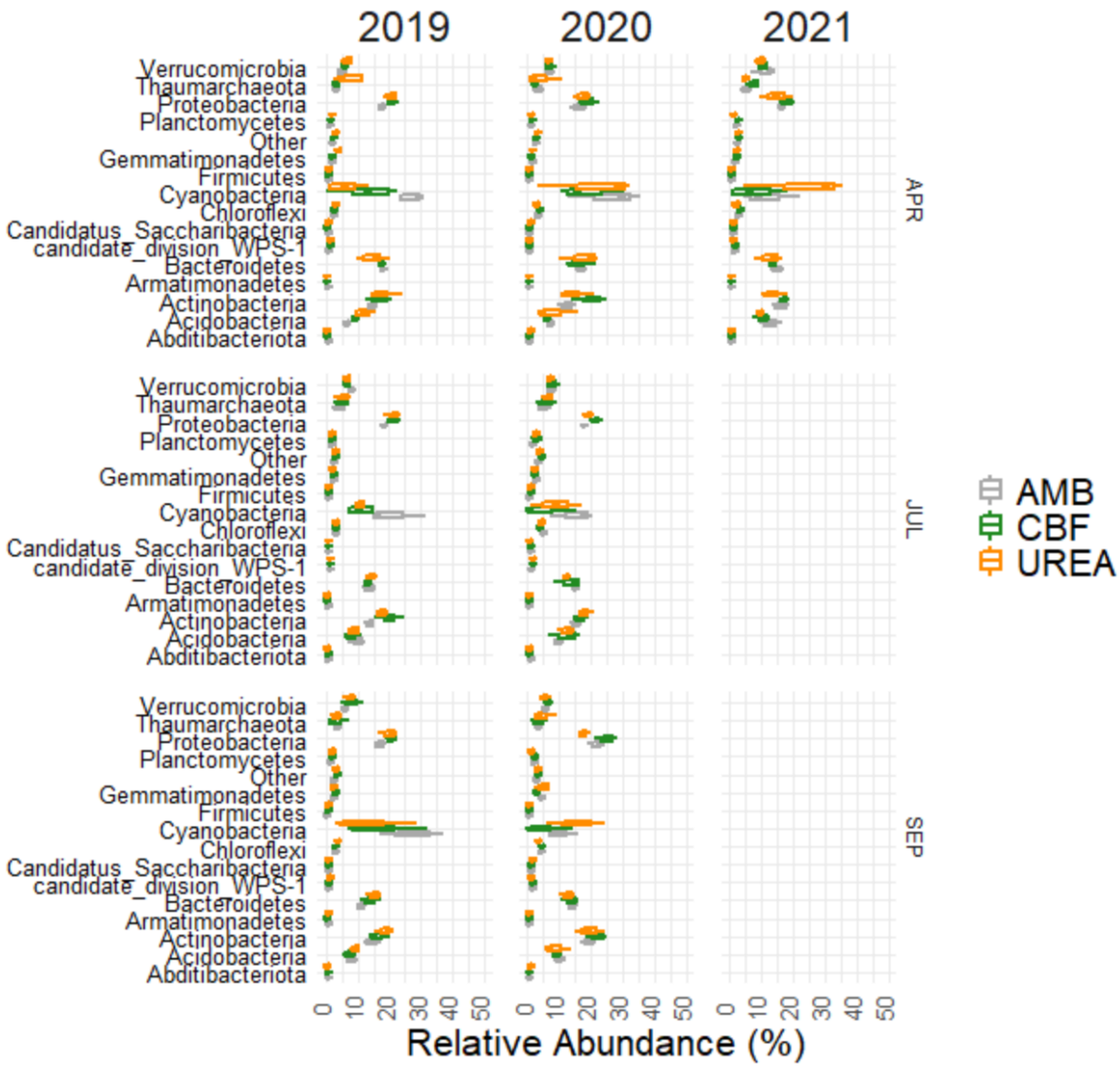


Figure 3. Relative abundance of bacterial + archaeal phlya across treatments and timepoints.

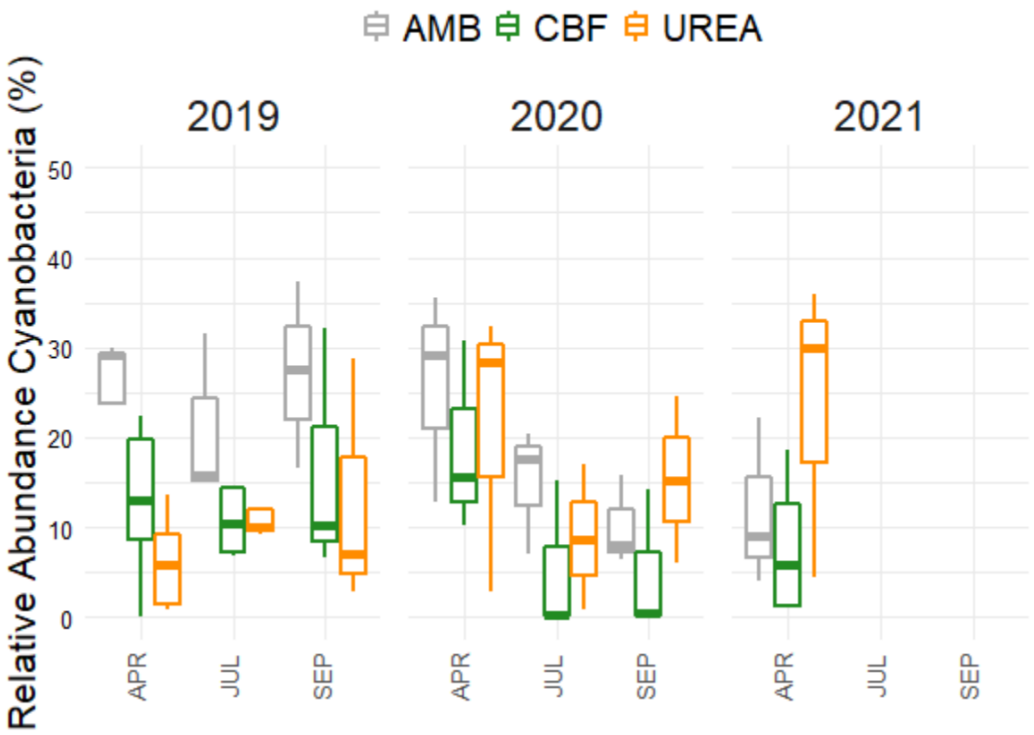


Figure 4. Relative abundance of the Cyanobacterial phylum across treatments and time.

References

Gay J.D., Goemann H.M., Currey B., Stoy P.C., Miller P., Poulter B., Peyton B.M., Brookshire E.N.J. 2022. Climate mitigation potential and soil microbial response of cyanobacteria-fertilized bioenergy crops in a cool semi-arid cropland. *GCB Bioenergy*. 14(12). https://doi.org/10.1111/gcbb.13001

APPENDIX F

BLUE GRAMA DROUGHT*HEAT

Overview

To explore the combinatory effects of drought and heat on the blue grama physiology as well as the importance of the blue grama microbiome to its stress response, we conducted a greenhouse experiment designed to test the effects of each stress alone and in combination. To investigate the influence of microbes on the blue grama stress response we compared live and autoclaved soil.

Methods

The greenhouse experiment was conducted from May 2021-September 2021. Experimental pots (D40H Deepots, Stuewe & Sons, Inc., OR, USA) were prepared using a standard greenhouse soil mixture curated in bulk at the Montana State University Plant Growth Center. To test the importance of the microbiome in the plant stress response, a batch of sterilized greenhouse soil mixture was also prepared by triple-autoclaving for 70 min. over the course of 3 days to allow the soil to cool between autoclaving events. All tools were ethanol-sanitized. Pots were washed, bleach-sterilized (1%), and dried prior to use. Prepared pots were then seeded with ~10 blue grama seeds (Wind River Seed Co., Manderson, WY, USA, n = 220 total), every other day to maintain moisture levels for one week until all pots had produced several 0.5” seedlings at which time all pots were thinned to one plant. Greenhouse conditions during the study (Jun-Sept) consisted of a 16-hour photoperiod, daytime temperature of 23.8 °C, nighttime temperature of 21.1 °C, and average daytime PAR of 500 μmol m^2 s^{-1}. Plants were allowed 30 days of pre-growth prior to the experiment.

After 30 days, the plants were moved to two controlled growth chambers and plants grown in either live or autoclaved soil were randomly assigned to ambient, drought, heat, and drought*heat treatment groups (n=20). One growth chamber was set to the ambient temperature of 25 °C, while the other was constantly heated at 28 °C. The drought treatment was a 50% reduction in water compared to ambient plants kept at field watering capacity.

At 20, 40, and 60 days of drought a subset of plants (n=5) were destructively harvested. All harvest procedures including measuring photosynthesis and stomatal conductance, plant biomass, root exudate collection, and soil collection for DNA and RNA extraction were conducted as described in Chapter 4 of this thesis. Root exudates were sent to the Environmental Molecular Science Laboratory for metabolomic analysis, and to the Montana State University Environmental Analytical Laboratory for total carbon and nitrogen analysis. At Day 60 droughted plants were returned to ambient watering levels for a recovery period of 20 days. Note that due to concerns that the plants were becomingly overly droughted and would not recover, a 25% water pulse was added to both ambient and droughted plants at three watering timepoints between Days 40 and 60. At Day 80 the final plants were harvested as described above.

For community analysis, DNA and RNA was co-extracted following the CTAB-phenol:chloroform method described by Griffiths et al. (2000). RNA products were treated with Zymo DNAse (Zymo Research, Irvine CA, USA) to remove any remaining DNA contaminant and all RNA were checked for DNA amplification and viewed on a 1% agarose gel. A minimum of 500 ng of RNA for n=3 replicates of each treatment group in live soil was sent to the Joint Genome Institute for metatranscriptomic analysis. 10 ng of each sample was converted to cDNA using the Protoscript II First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich MA,

USA). cDNA and dsDNA samples were then prepared for 16S amplicon sequencing as described in Chapters 2-5. Downstream bioinformatics of amplicon sequencing products was also conducted as described in Chapters 2-5. To analyze the active microbial community, we calculated 16S ratios as described by Bowsher et al. (2019).

Below are preliminary figures presenting the results of the plant physiology, root exudate chemistry and composition, and active vs. total community composition.

Results

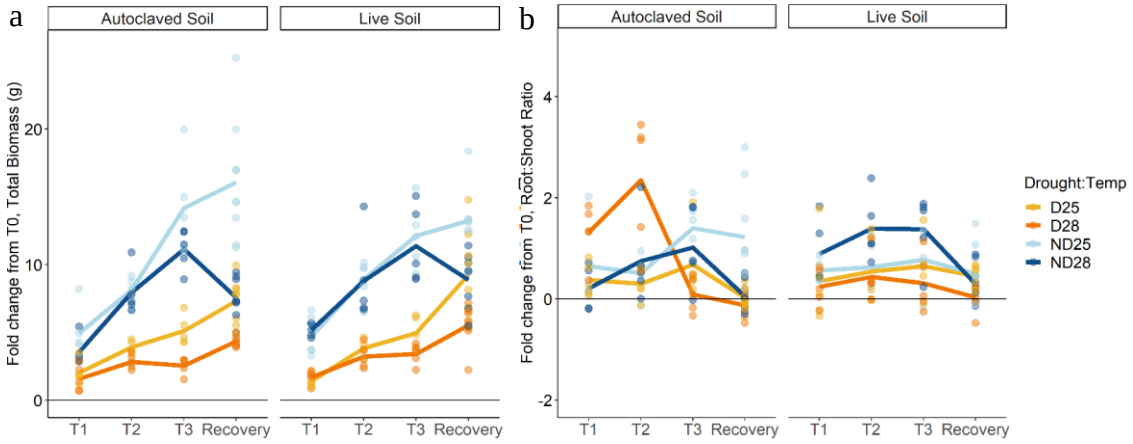


Figure 1. Fold change from T0 in total biomass (a) and root:shoot ratios (b) of blue grama.

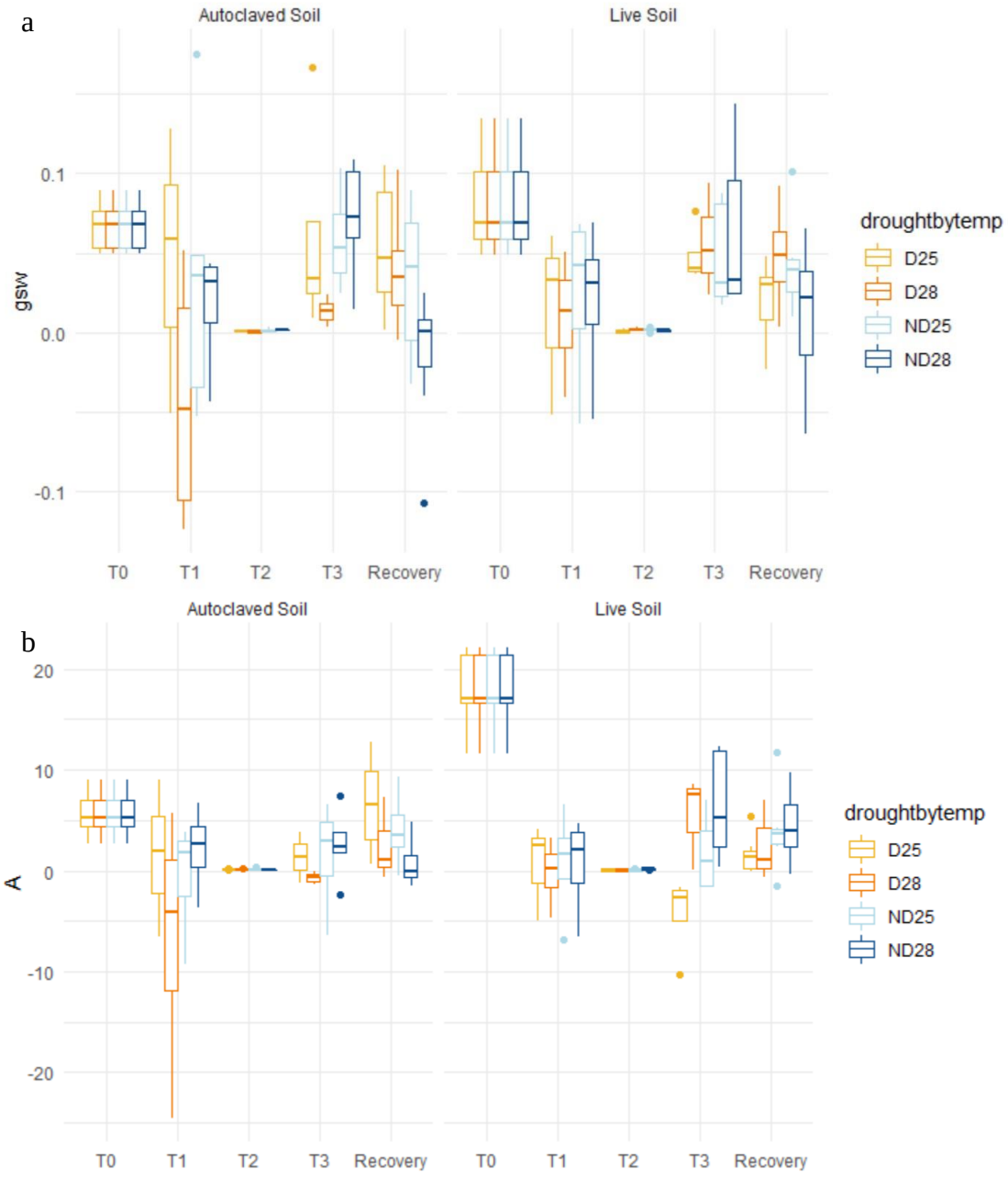


Figure 3. Stomatal conductance (a) and photosynthesis (b) across treatments and timepoints.

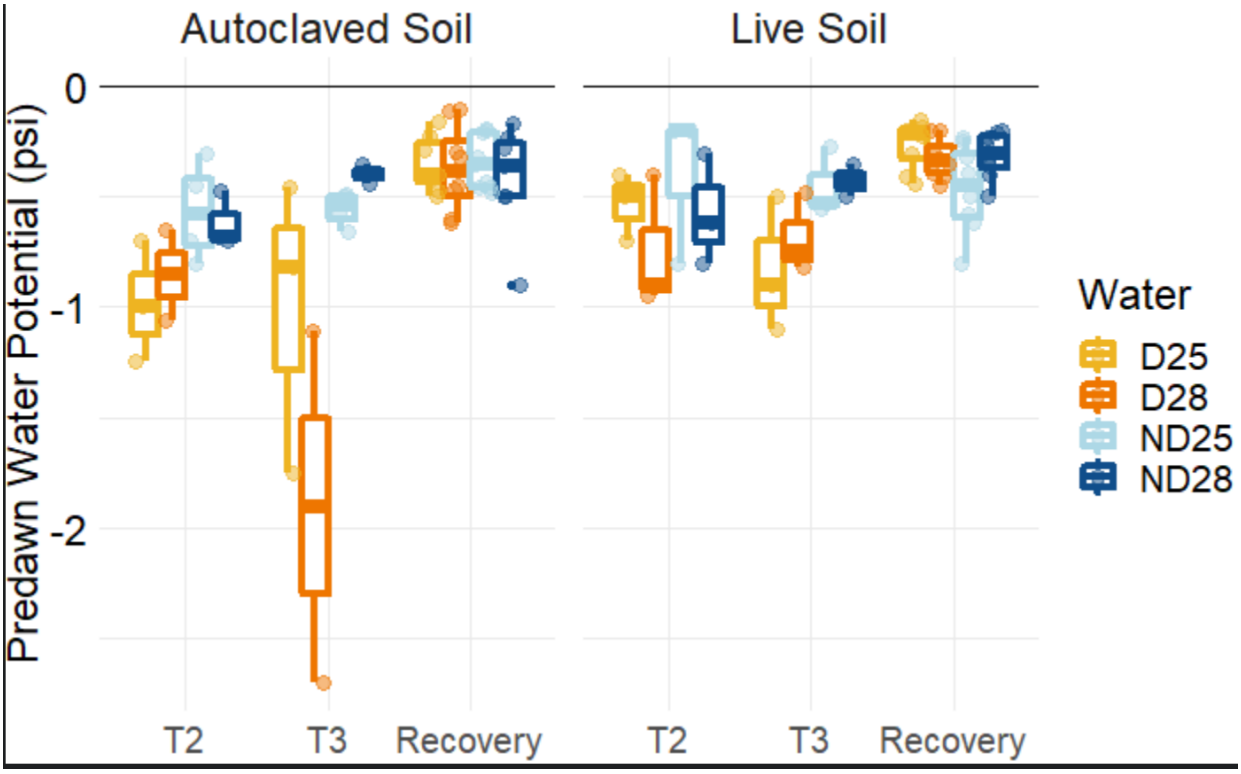


Figure 5. Predawn water potential across treatments and timepoints. Due to limited plant availability PDWP was not measured at T1.

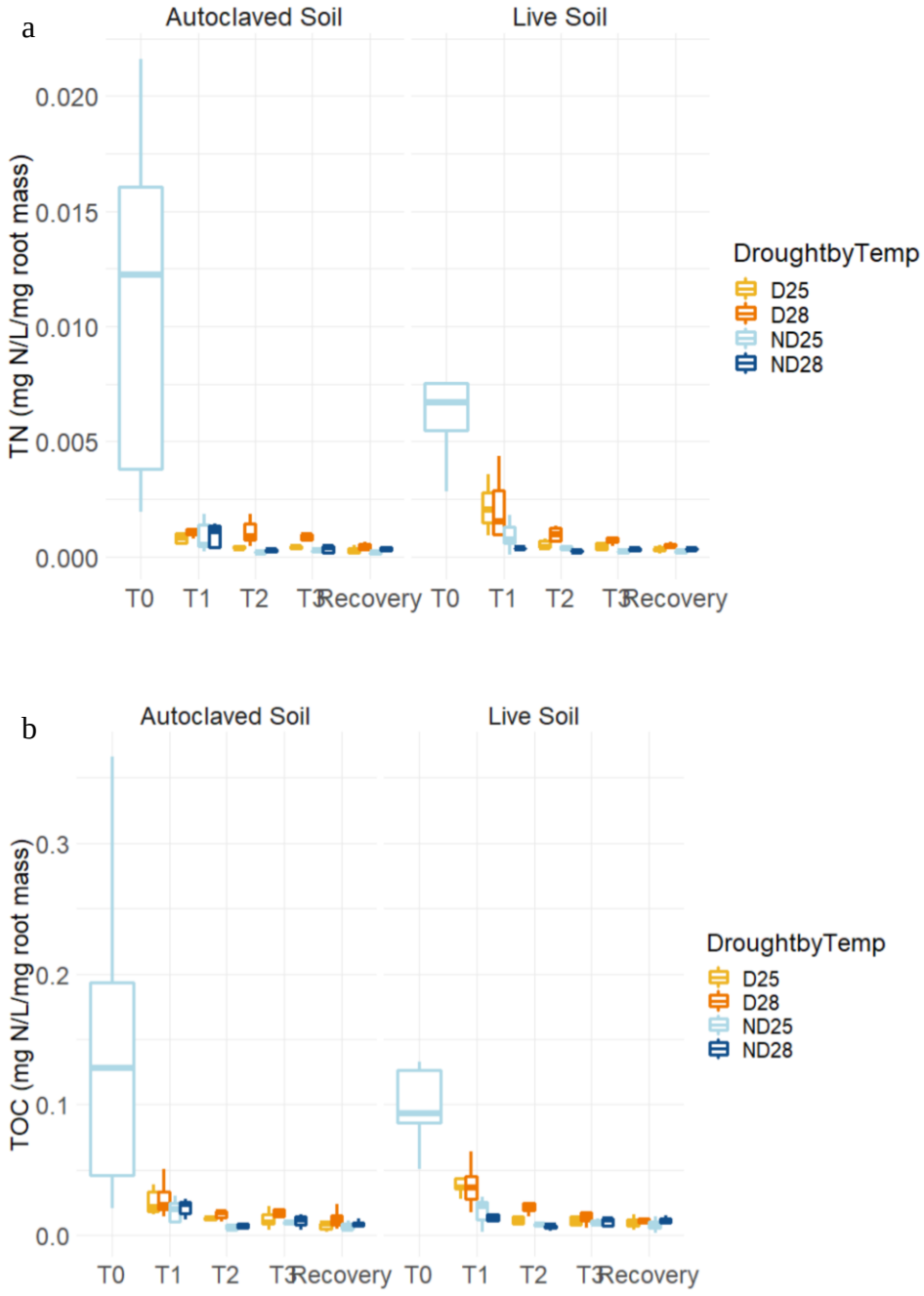


Figure 6. Root exudate total nitrogen (a) and total organic carbon (b) across treatment groups and timepoints.



Figure 8. Preliminary summary of changes in root exudate metabolites across timepoints and treatment groups. Colored boxes indicate significantly greater (green) or less (red) measured peak area ($p < 0.05$) between treatment groups. A = Ambient, D = Drought, H = Heat, DH = Drought*Heat. T2 = Day 40, T3 = Day 60 of drought.

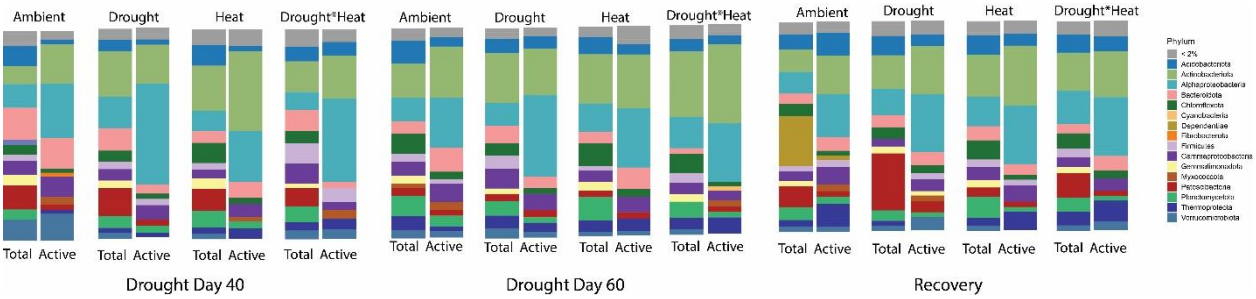


Figure 9. Active vs. Total community relative abundance of bacterial + archaeal phyla in the blue grama rhizosphere at three timepoints (Day 40, 60, and Recovery).

References

Bowsher A.W., Kearns P.J., and Shade A. 2019. "16S rRNA/rRNA Gene Ratios and Cell Activity Staining Reveal Consistent Patterns of Microbial Activity in Plant-Associated Soil." *mSystems* 4 (2): 10.1128/msystems.00003-19. https://doi.org/10.1128/msystems.00003-19.

Griffiths R.I., Whiteley A.S., O'Donnell A.G., and Bailey M.J. 2000. "Rapid Method for Coextraction of DNA and RNA from Natural Environments for Analysis of Ribosomal DNA- and rRNA-Based Microbial Community Composition." *Applied and Environmental Microbiology* 66 (12): 5488–91. https://doi.org/10.1128/AEM.66.12.5488-5491.2000.

APPENDIX G

DICHANTHELIUM LANUGINOSUM METAGENOMICS

Overview

In addition to the amplicon sequencing described in Chapter 5, extracted DNA was also sent to the Joint Genome Institute (JGI) for metagenomic sequencing. Preliminary investigation of the *D. lanuginosum* study metagenomes began with paired analysis of Heart Lake bulk soil and rhizosphere metagenomes. Note that HLGB5xRhiz60 does not have a bulk soil counterpart due to limitations in sample numbers available for sequencing at JGI.

Methods

Raw reads were assembled and quality filtered by the Joint Genome Institute following established protocols Clum et al. (2021). Assembled contigs were then uploaded to KBase (Arkin et al. 2018) for downstream processing. Contigs were binned using MetaBAT2 (Kang et al. 2019), MaxBin2 (Wu, Simmons, and Singer, 2016) and Concoct (Alneberg et al. 2014), and Das-Tool (Sieber et al. 2018) was used to optimize bins between the three separate binning methods. Bin quality was then checked with CheckM (Parks et al. 2015) and filtered following the guidelines suggested by Bowers et al. (2017). DRAM was used to annotate and create a functional profile of high-quality bins Shaffer et al. (2020). KEGG-Decoder (Graham, Heidelberg, and Tulley, 2018) was then used to visualize global metabolic pathway summaries for each sample of 11 metagenome samples from Heart Lake Geyser Basin. Below is the raw KEGG_Decoder output.

Results

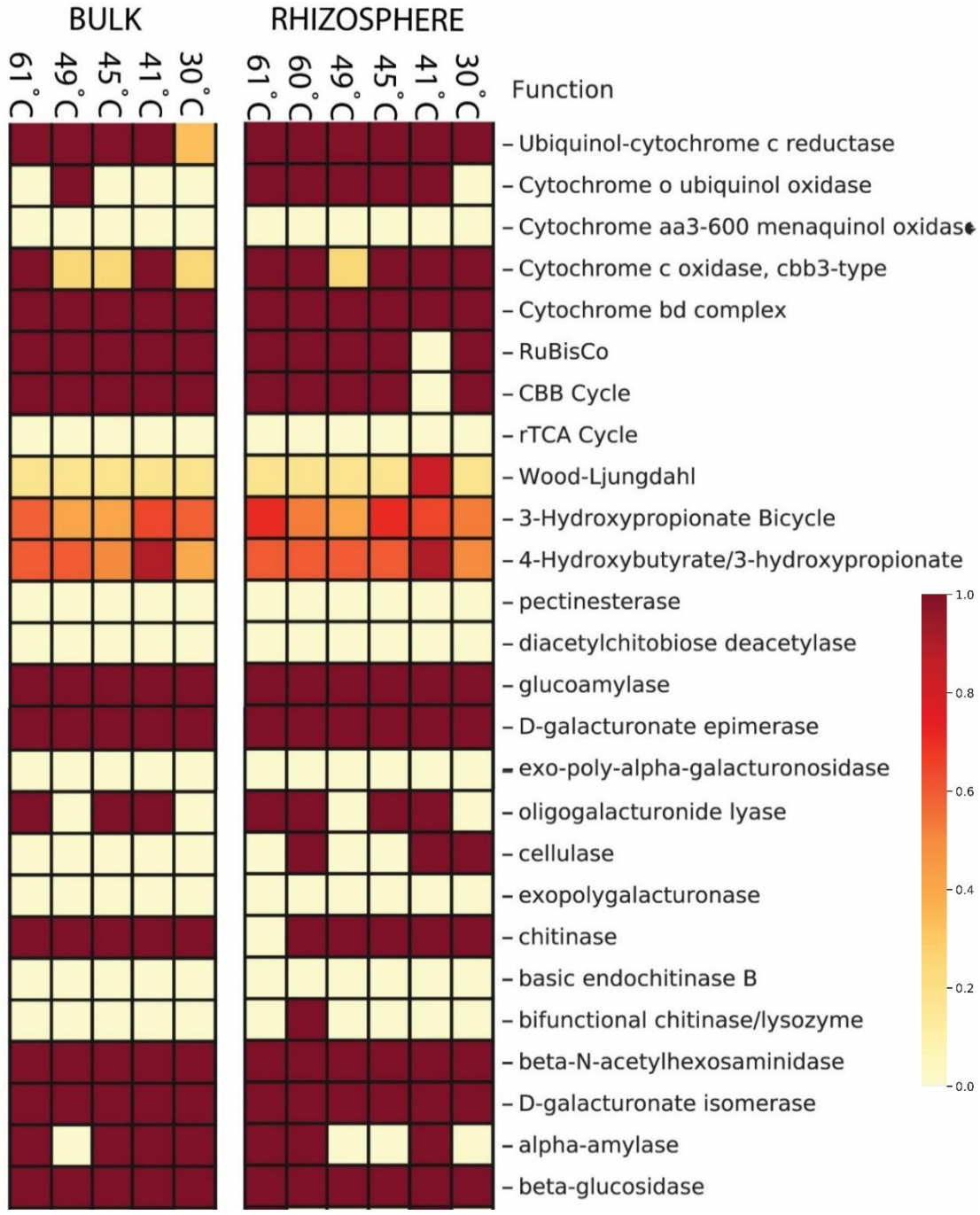


Figure 1. KeggDecoder heat map of enzymatic pathways present in rhizosphere and bulk samples at each temperature.

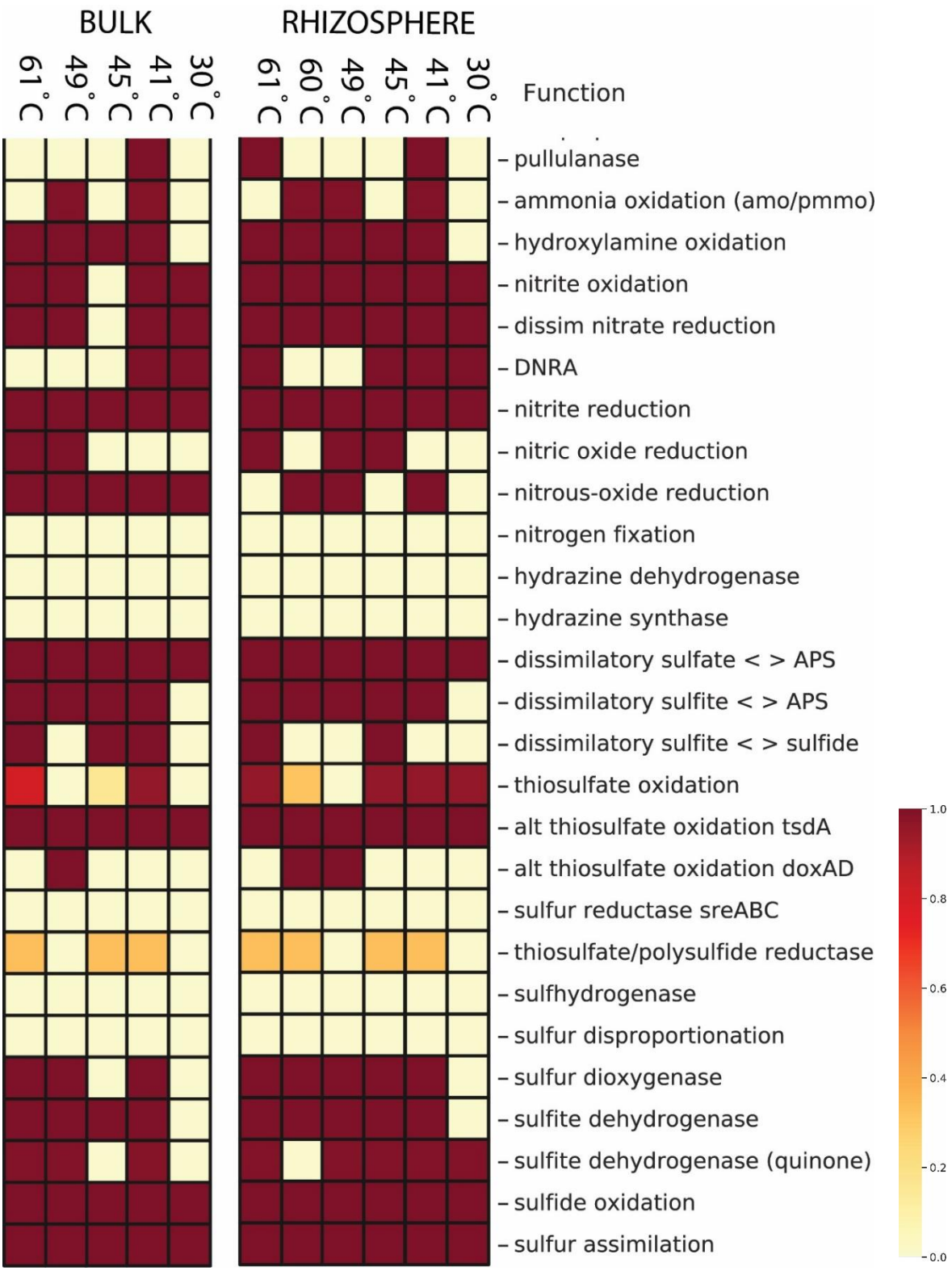


Figure 1. Continued

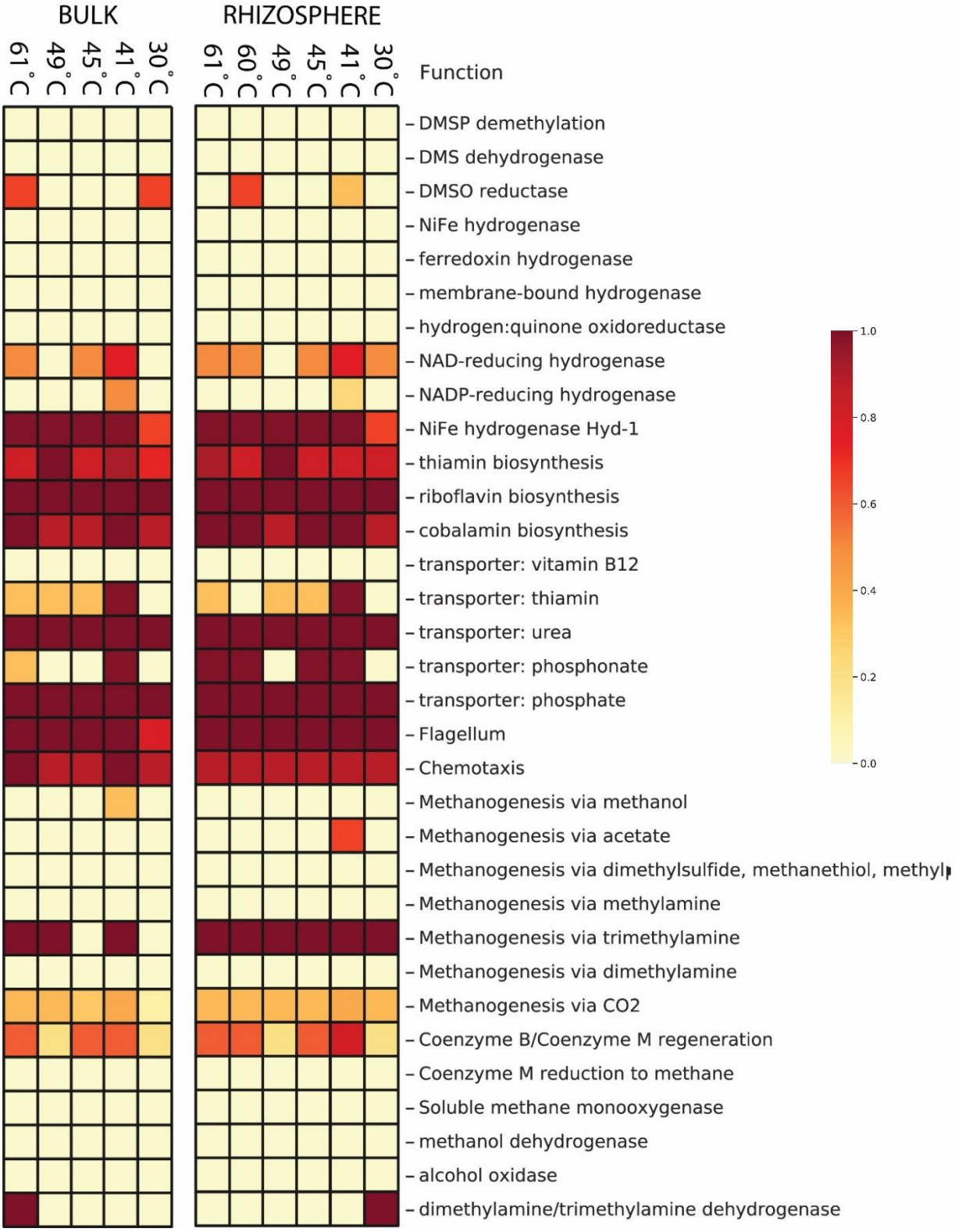


Figure 1. Continued

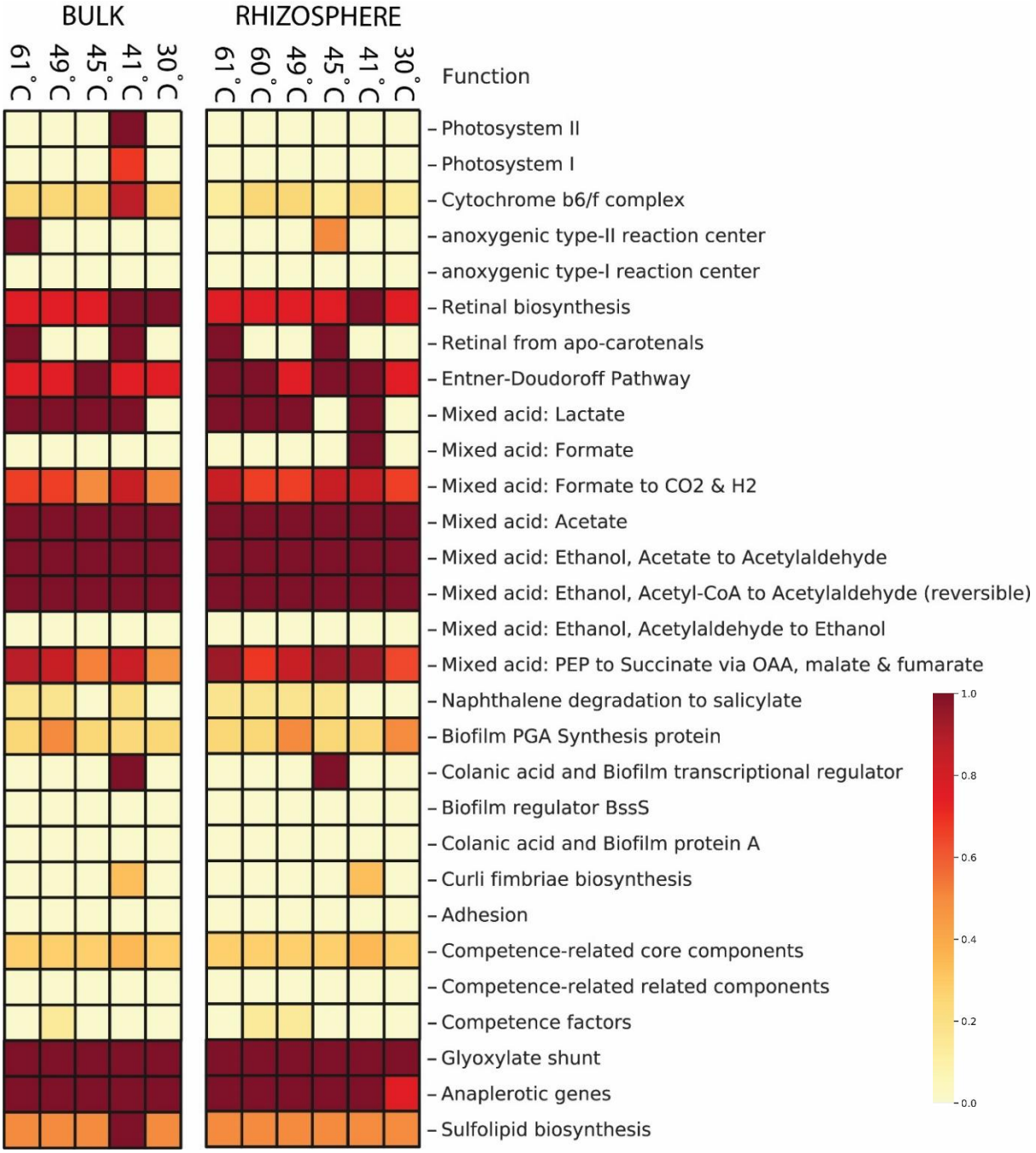


Figure 1. Continued.

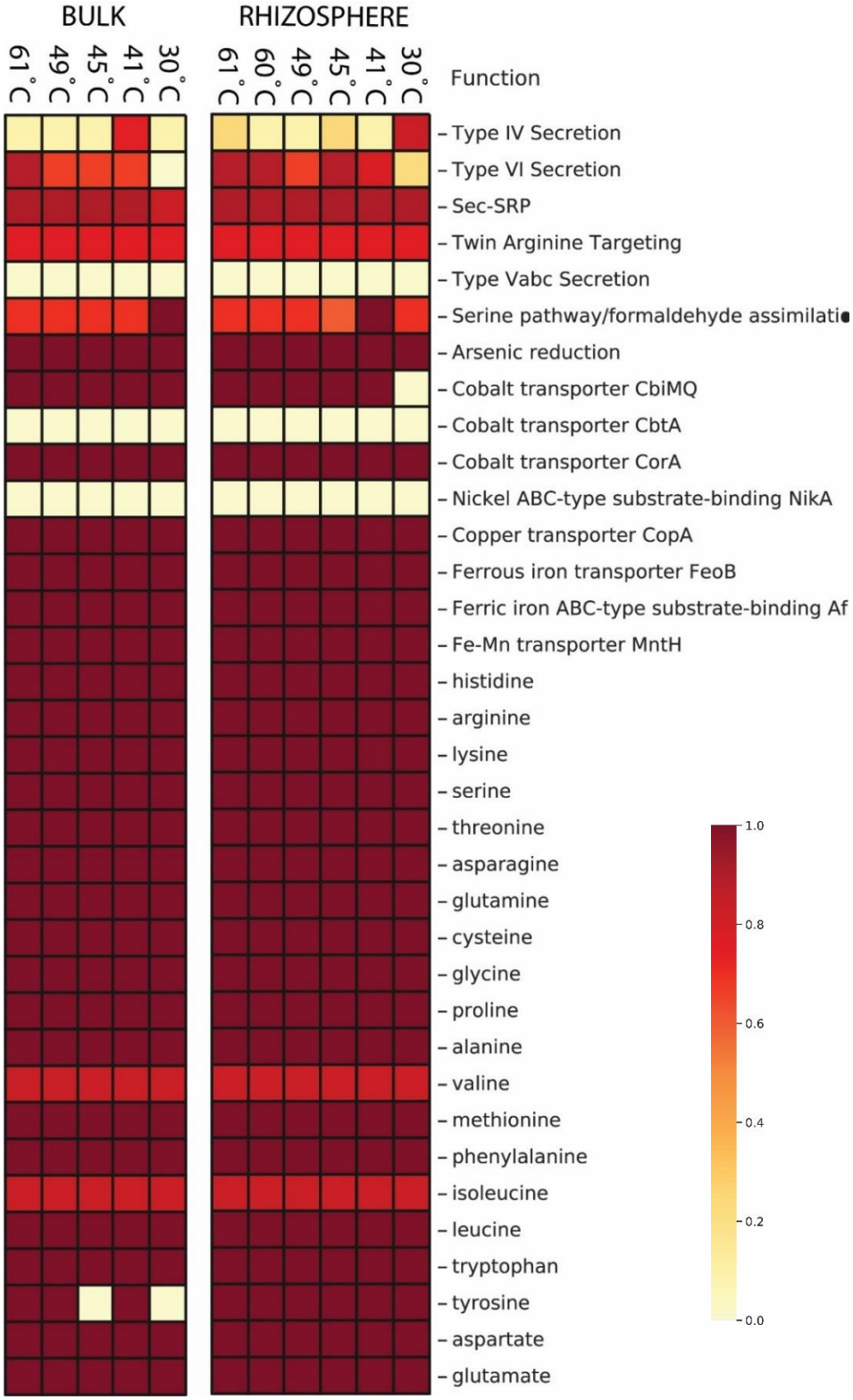


Figure 1. Continued

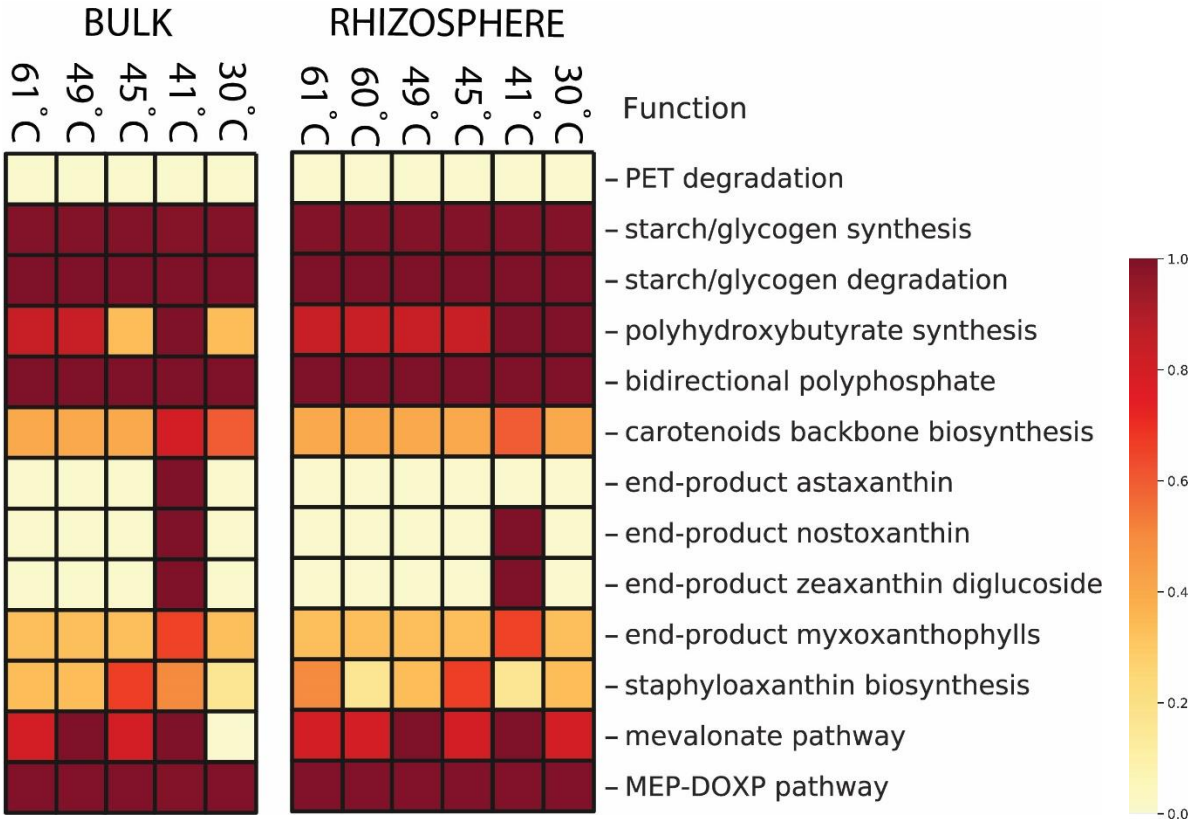


Figure 1 Continued.

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APPENDIX H

DATA AVAILABILITY SUMMARY

WAFERx Project

Published Sequencing Data:

Goemann et al 2021 (Chapter 2):

MG-RAST accession numbers mgm4916997.3 (LSU), mgm4916988.3 (16S)

Gay et al. 2022 (Referenced in Conclusions):

MG-RAST accession numbers mgs875156 (LSU), mgs875153 (16S)

Unpublished Sequencing Data:

Soil Crust (Appendix E):

MSU Blackmore: PeytonLab/HannahG/WAFERx/PostFarm_crust

2019 Post Farm Field Plots:

MSU Blackmore: PeytonLab/HannahG/ WAFERx/

2018-2019 Tall Wheatgrass Greenhouse Mini Study

MSU Blackmore: PeytonLab/HannahG/ WAFERx/PotsBackgroundSequenceData

Blue Grama Data

Published Sequencing Data:

Goemann et al. 2023 (Chapter 3):

NCBI SRA database under bioproject PRJNA1006788

Unpublished Sequencing Data:

Climate legacy (Chapter 4):

MSU Blackmore: PeytonLab/HannahG/BOGR/BG_predoc

Drought*Heat Study (Appendix F):

MSU Blackmore: PeytonLab/HannahG/BOGR/bluegrama_DH21

Dichanthelium Data:

Unpublished Sequencing Data:

MSU Blackmore: PeytonLab/HannahG/dichanthelium

APPENDIX I

LIST OF ADDITIONAL COAUTHORSHIPS

1. Mettler MK*, **Goemann HM***, Mueller RC, Vanegas OA, Lopez G, Singh N, Venkateswaran K, Peyton BM **2023**. Development of Martian saline seep models and their implications for planetary protection. *Biofilm.* 5

2. Gay, J. D., **Goemann, H. M.**, Currey, B., Stoy, P. C., Christiansen, J. R., Miller, P. R., Poulter, B., Peyton, B. M., & Brookshire, E. N. J. (2022). Climate mitigation potential and soil microbial response of cyanobacteria-fertilized bioenergy crops in a cool semi-arid cropland. *GCB Bioenergy*, *14*(12), 1303–1320. https://doi.org/10.1111/gcbb.13001

3. Ulrich DEM, Clendinen CS, Alongi F, Mueller RC, Chu RK, Toyoda J, Galleogos-Graves LV, **Goemann HM**, Peyton BM, Sevanto S, Dunbar J **2022**. Root exudate composition shifts with drought severity in blue grama (*Bouteloua gracilis*). *Scientific Reports*, 12:12581. 10.1038/s41598-022-16408-8

4. Goemann CLC, Wilkinson R, Henriques W, Bui H, **Goemann HM**, Carlson RP, Viamajala S, Gerlach R, Wiedenheft B **2022**. Genome sequence, phylogenetic analysis and structure-based annotation reveal metabolic potential of Chlorella sp. SLA-04. *Algal Research* 69. https://doi.org/10.1016/j.algal.2022.102943

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*Authors share co-first authorship

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