DISSERTATION

SOIL MICROBIAL COMMUNITIES ASSOCIATED WITH FOREST ROOT DISEASES AND ROCKY MOUNTAIN FORESTS

Submitted by

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ABSTRACT

SOIL MICROBIAL COMMUNITIES ASSOCIATED WITH FOREST ROOT DISEASES AND ROCKY MOUNTAIN FORESTS

The three research studies incorporated in this dissertation expand upon each other to enhance our understanding of the forest soil microbiome, at various spatial scales (tree, stand, and forest). In Chapter 2, we prepared a study to determine the interactions between soil microbial communities and two competing *Armillaria* species, *A. solidipes* (highly virulent) and *A. altimontana* (beneficial), and tree health (healthy, declining, and dead). Previous research conducted at the Ida Creek site within the Priest River Experimental Forest found that trees in association with *A. altimontana* were healthier than trees with no infection present, indicating a potential beneficial quality associated with trees and *A. altimontana* (Warwell et al., 2020). Our research resulted in a low sample size for *A. solidipes*, yet we found that soils associated with *A. altimontana* had greater bacterial richness and diversity than soils associated with *A. solidipes*. Although no significant differences were observed for bacterial β-diversity, certain taxa were more present for each species. Soils associated with *A. altimontana* had high abundances of Pseudomonadaceae, Chthoniobacteraceae, and Pyrinomonadaceae, all of which are highly likely to provide beneficial qualities such as, plant growth promotion, pathogen suppression, and nutrient cycling (Sah and Singh, 2016; Wust et al., 2016; Wang et al., 2019; Duan and Bau, 2021). In contrast, Enterobacteriaceae and Pseudomonadaceae were found in high abundance in soils associated with *A. solidipes*. Both families have a range of functions, that could consist of plant pathogens, as well as beneficial qualities (Sah and Singh, 2016; Koskella et al., 2017; Kumar et al., 2017; Rogers, 2020). To expand upon Warwell et al. (2020), the pathogenic qualities of *A. solidipes* may reduce the defense of host trees, making them

more vulnerable to secondary pathogens such as taxa in the Enterobacteriaceae and Pseudomonadaceae families. Additionally, in terms of tree health, soils associated with healthy and declining trees had greater richness and diversity than dead trees. Soils associated with healthy trees may incorporate a greater abundance of beneficial microbes, while soils associated with declining and dead trees may have a greater abundance of saprophytes or decomposers as the tree begins to die. Although more information is needed to fully determine if soil bacterial communities truly differ between *Armillaria* species and tree health, this study can infer that the soils associated with *A. altimontana* and healthy trees have a high abundance of taxa with beneficial qualities, while soils associated with *A. solidipes* and dead trees have a high abundance of taxa that function as potential contributing pathogens or linked pathobiome. Therefore, the association between soil microbial communities may play a key role in understanding the pathogenicity of root diseases and overall tree health.

Chapter 3 and Chapter 4 expand upon the understanding of soil microbial communities, at a stand level and landscape level, respectively, in association with forest species composition in the Rocky Mountains. The Pikes Peak Forest Dynamics Plot (Chapter 3) provided an extensive forest compositional study in coordination with research previous collected by the Colorado State University Department of Forest and Rangeland Stewardship. At this 17.6 ha site, there are six tree species that are frequently found throughout much of the Southern Rocky Mountains. The presence of differing forest types at one location allows our research to isolate the variable of forest type, without additional influences of elevation, latitude, and potential changes in climate. In the summer of 2018, 115 soil samples were collected in conjunction with previously established 1 m^2 plots at the site. We observed that dominant seedling species were correlated with soil fungal and bacterial communities. In contrast, the distance to the nearest mature tree and soil pH also influenced soil fungal communities. Although not statistically significant, understory and overstory forest type were trending to influence soil fungal communities. Mycorrhizal fungi, specifically ectomycorrhizal fungi are known to be host specific

(Berg and Smalla, 2009), which corresponds to our research that observed low core fungal communities with more unique taxa associated with forest type. The research concurs with additional studies that forest type has a greater influence on soil fungal communities, whereas soil edaphic properties may have a greater influence on soil bacterial communities (Fierer et al., 2012; Crowther et al., 2014).

To determine the relationship at a landscape level, Colorado and Wyoming P2+Soil Forest Inventory and Analysis (FIA) plots were utilized to understand the relationship among soil microbial communities, forest ecotypes, and stand, site, and climatic variables in Chapter 4. The FIA program is the most extensive forest inventory in the world, with plots established on public and private land throughout the entire United States and its territories (LaBau et al., 2007). This protocol provides a multitude of site and stand characteristics, which may be the key to fully understanding the dynamics between soil microbes and forest ecosystems. To date, we do not know of any other study that uses FIA plots to determine the variables that drive soil microbial community composition. Our research found that forest type had a direct influence on soil fungal and bacterial communities, as high elevation Engelmann spruce/subalpine fir and lodgepole pine were significantly different than low elevation deciduous oak and pinyon-juniper woodlands, as oak, aspen, and pinyon-juniper woodlands had the greatest fungal and bacterial richness and diversity. Additionally, elevation influenced fungal and bacterial richness and α-diversity, yet only influenced fungal β-diversity with the greatest richness and diversity observed from 5,000-7,000 feet. Site, stand, and climatic variables also influenced both soil fungal and bacterial communities. We found that stand age or variables of stand density and size (SDI and QMD) had a positive influence on fungal richness and diversity, as older, mature or highly dense stands provide greater deadwood and mortality, which increases overall fungal diversity. Additionally, slope had a positive influence on bacterial richness and diversity, while latitude and elevation had a negative influence on bacterial communities. For climatic factors, minimum and maximum temperatures had a positive relationship on bacterial Inverse Simpson’s diversity.

This could indicate that as temperatures increase, both minimum (winter) and maximum (summer), the relative abundance of dominant bacterial taxa also increased.

These studies enhance our knowledge of the relationship between soil microbial communities and forest ecosystems. The relationship between microbes and tree health allows for management techniques to expand outside of managing solely above-ground, and to incorporate the aspects that below-ground diversity may play on forest health. The goal of this research is to provide a baseline for forest microbial communities in Rocky Mountain forests, and to understand the dynamics that soil microbial communities play in the soil-plant-pathogen interaction in association to Armillaria root disease.

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DEDICATION

I am dedicating my dissertation to people that I did not even know when I began my graduate degree, my children. When Marilyn and I married in 2011, she knew that I was ready to become a father at that time, but we both decided to wait until the timing was right. Following the completion of my master's degree and her Doctorate, we knew it was perfect to expand our family. I did not how much love I could have for another person, other than Marilyn, until I met Ryely. He made me the father I always knew I wanted to be and drives me to better, not just myself, but my family. Your sweet sense of innocence and adventure is everything that I could have asked for in a son, and I am truly proud to call myself your Dadda. To our baby that we never met, it breaks my heart to have not been able to hold and cherish you, but I want you to know that you will forever be in my heart. And to our new sprout, I can't wait to meet you in the near future. Your momma and I love seeing and hearing you at each doctor's visit. You will be the perfect addition to our family and make us whole very soon. I truly hope that I can live up to the expectations that I give myself to support and love our family. I am completing this degree not just for myself, but to show my children that you can do anything you put your mind to no matter the difficulty.

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Chapter 1: INTRODUCTION AND BACKGROUND

1.1 Indicators of forest health

Words that often define forest health include resilience, resistance, and diversity (Teale & Costello, 2011). Resilience is the ability of a forest to recover following stress or disturbance, while resistance is described as the ability to resist change within a forest (Teale and Costello, 2011). Diversity can be thought of as in the number of species and the relative abundance of each species, and greater diversity has often been associated as a positive indicator of ecosystem health. These are all great buzzwords, yet categories that define forest health can differ depending on the governmental office or organization assessing forest health. This is partially because forest health can be determined in a multitude of ways, and the value associated with forest health is directly tied to the objectives of land managers (Crann et al., 2015). Depending on the focus, indicators can assist in determining the health of a forest at the landscape or individual tree level and either from management or ecological standpoint (Teale & Costello, 2011).

Forest Inventory and Analysis (FIA) is a national forest inventory program established in all 50 states and U.S. territories. FIA crews are tasked with the duty to collect forest measurements to report on the status and trends of all forest areas, including all aspects of land ownership. In the early 1990's, an emphasis on forest health prompted the collection of eight forest health indicators (crown condition, ozone injury, tree damage, tree mortality, lichen communities, vegetation, coarse woody debris, and soil health; Woodall et al., 2011).

While most forest health measurements are taken from above ground, much of the biological activity in the forest occurs under the surface (Buee et al., 2009). As defined by USDA-NRCS, soil health incorporates the ability for soil to serve as a vital function that sustains plants, animals, and humans (USDA-NRCS, 2021). Traditionally, soil health focused on identifying ways to enhance plant growth with agriculture as the primary application (O'Neill et

al., 2005; Fierer et al., 2021). As natural resource management strategies have expanded to overall sustainability, soils can be used as primary drivers of ecosystem functions in all environments (O'Neill et al., 2005). Therefore, soil health can be used as a proxy for forest and tree health, as soils provide physical support for infrastructure and plants, protect watersheds, filter and buffer pollutants, and act as the largest carbon sink (O'Neill et al., 2005; USDA-NRCS, 2021). The concept of soil health can be broken down into six categories of indicators: physical properties, chemical properties, biological indicators, environmental states and fluxes, agronomic responses, and general indicators (Stewart et al., 2018). Although certain indicators provide insight into soil health, it is all dependent on the context (Fierer et al., 2021). The key to determining soil health is identifying what aspects are important for your management goals to improve soil function (USDA-NRCS, 2021).

In terms of soil health, microbial diversity and species composition are increasingly studied as biological indicators because their analyses are relatively inexpensive and becoming more accessible in comparison to historic soil health measurements such as the Haney test (Stewart et al., 2018; Fierer et al., 2021). Microbes may act as bio-indicators, as they drive soil processes, vary temporally and spatially, and respond to biotic and abiotic conditions while providing a large diversity of biological species (Fierer et al., 2021). There is no known ideal microbial community, especially in forests, therefore more studies are needed to conceptualize the dynamics soil microbial communities play in the overall ecosystem. Distributing soil microbial measurements across landscapes would provide a baseline to assess the effects that forest management, pathogens, tree health, and climate may have over time.

1.2 Influence of pathogens and disturbance on forest ecosystem

To the untrained eye, any level of pathogens and disturbances may appear to disrupt or decrease forest health. However, at low levels, pathogens and other disturbances indicate a healthy forest (van Mantgem et al., 2009). The cycling dead and dying trees will assist in establishing a stable environment, allowing the health of the forest to flourish for future

generations. Manion and Griffin (2001) described a healthy ecosystem as having a stable or balanced size distribution as stands mature. As forests age, especially even-aged stands, they move from high-density, small-diameter trees to less dense with larger diameter trees (Teale & Costello, 2011). This process gives rise to the concept of baseline mortality, whereby not all trees survive to maturity. The ability to maintain a healthy, stable structure results in trees succumbing to competition, disease, insects, or other damage agents that act as natural thinning agents, allowing the remaining trees to utilize resources (van Mantgem et al., 2009). If mortality levels surpass this baseline, forests may not sustain adequate age and size classes, making them unhealthy (Teale & Costello, 2001).

The disease triangle can be used to understand how forest pathogens influence their ecosystem. The model highlights the interactions between a pathogen, host, and environmental factors. Changes to either one of these conditions affects the ability of a disease to survive or persist within a stand (Cobb and Metz, 2017). There is variability in virulence, rate and means of spread, and host specificity for pathogens. *Armillaria* species, causal agents of root disease, are examples of fungal species that may be highly virulent as they infect healthy hosts, act as weak pathogens, or facultative necrotrophs that persist in the soil until host conditions are suitable for infection (Baumgartner et al., 2011; Kedves et al., 2021). This variability in lifestyles of *Armillaria* spp. was identified by examining the interaction of highly virulent *A. solidipes* and nonpathogenic or weak pathogen *A. altimontana* (Warwell et al., 2019). Western white pine (*Pinus monticola*) colonized by *A. altimontana* during stand development had a greater survival rate and were larger in diameter at breast height (DBH) and height compared to trees in the absence of *Armillaria*. To understand the dynamics that *A. altimontana* has on western white pine, two conclusions were drawn; *A. altimontana* may act as a beneficial symbiont, or the soil microbial community could influence *A. altimontana* and western white pine growth and survival (Warwell et al., 2019).

These interactions prompt the need to think outside the disease triangle concept and instead use a square or diamond including microbial communities (Stewart et al., 2021). All plant organs interact with microbes, which vary by having positive and negative effects (Schirawski and Perlin, 2018). New technologies allow researchers to study plant-pathogen-microbial interactions to identify the driving factor to pathogenicity, inhibitory effects, plant growth, and induction of stress resistance (Schirawski and Perlin, 2018; Stewart et al., 2021). Plants exude compounds that attract microbial communities, especially in the rhizosphere, where beneficial microbes reduce the impact of infection and subsequently increase the uptake of vital nutrients (Schirawski and Perlin, 2018). Additionally, some microbes act mutualistically on pathogens, allowing for increased virulence (Schirawski and Perlin, 2018). These act as "bad" microbes, as they threaten plant health. This technology enhances our knowledge of the diversity and abundance of plant-soil-microbial interactions, whether considered good or bad.

1.3 Drivers of soil microbial communities

The innate heterogeneity of soils makes them one of the most challenging systems to perform metagenomic research due to the spatial scale of sampling, rhizosphere interactions, diverse soil characteristics, temporal variations, and differing techniques in processing samples (Fierer, 2017; Fierer et al., 2021). Bacteria and fungi are important factors for soil biogeochemical cycling, which directly relates to plant diversity and productivity (Li et al., 2015). Soil microbes provide many different ecosystem services as they decompose organic matter and litter into usable inorganic minerals (via degradation, mineralization, and humification), increase the uptake of water and minerals, provide resistance to stress, and act as competitors or antagonists to pathogenic microbes (Kile et al., 1991; Schloter et al., 2003; Leake et al., 2004; Davidson and Janssens, 2006; Chapman and Koch, 2007; Allison and Martiny, 2008; Azul et al., 2014; Horwath, 2014; Lee Taylor and Sinsabaugh, 2014; Robertson and Groffman, 2014; Cardenas et al., 2015; Chodak et al., 2016; Baldrian, 2017a; Yuan et al., 2020).

Many biotic and abiotic factors influence the ability of soil microbial communities to maintain these ecosystem services (Fierer, 2017). These can include direct factors such as moisture, temperature, pH, organic matter, and nutrient availability and indirect factors such as latitude, elevation, climate, soil texture, depth of sample, topography, and vegetation (Brockett et al., 2011; Lareen et al., 2016; Fierer, 2017). While numerous attributes may influence soil microbial diversity and richness, soil edaphic and climatic factors play the largest role in the presence of soil bacterial communities, while plant diversity is less influential (**Figure 1-1**; Fierer, 2017).

1.3.1 Soil edaphic properties and site factors

Chemical and physical properties are known to directly influence soil microbial communities (Brockett et al., 2011). The most influential chemical property effecting bacterial communities is soil pH (Fierer and Jackson, 2006; Rousk et al., 2010; Prescott and Grayston, 2013). Many acidic soils are dominated by Acidobacteria and Alphaproteobacteria (Lauber et al., 2009; Llado et al., 2018), whereas Bacteroidetes and Actinobacteria are more prevalent in basic soils (Baldrian et al., 2012; Llado et al., 2018). Similarly, pH is an important predictor of fungal richness (Dumbrell et al., 2010; Tedersoo et al., 2014). In particular, ectomycorrhizal (EcM) and arbuscular mycorrhizal fungi (AM) are positively influenced by pH (Dumbrell et al., 2010; Tedersoo et al., 2014).

Outside of pH, soil organic matter (SOM), including soil carbon (C) and nitrogen (N), have a direct effect on microbial community structure (Brockett et al., 2011; Kaiser et al., 2016). Carbon and nitrogen availability are directly related to the litter outputs, which can be labile or recalcitrant (Cotrufo et al., 2013). Typically, forest soils have a higher C:N ratio and are more acidic than soils associated with grasslands (Ostrowska and Prebska, 2015; Kaiser et al., 2016). Specifically, soils rich in organic carbon have more β-Proteobacteria and Bacteroidete*s* and less Acidobacteria comparatively to soils with less available carbon such as bulk soil (Fierer et al., 2007). In all environments in North America, the most abundant soil bacterial phyla are

Acidobacteria, Bacteroidetes, Firmicutes, Actinobacteria, α-Proteobacteria, and the β-Proteobacteria (Fierer et al., 2007). In a study looking at global fungal diversity, Tedersoo et al. (2014) identified that abundances of fungal communities differ between certain environments. Soils in grasslands have a higher proportion of Leotiomycetes, Dothideomycetes, and Chythdiomycota, whereas temperate coniferous forests have a higher proportion of Mortierellomycotina, Glomeromycotina, and Tremellimycetes (Tedersoo et al., 2014).

1.3.2 Climatic factors

At a continental scale, temperature and precipitation are considered key drivers of microbial communities (Lauber et al., 2009; Talbot et al., 2014; Lladó et al., 2018). Fungi are particularly sensitive to precipitation as community richness and biomass are limited by low levels of precipitation (Brockett et al., 2011). Fungal biomass greatly increased in comparison to bacterial communities with an increase in precipitation (de Vries et al., 2012). Precipitation has a large impact on soil drying and rewetting cycles, which increases the release of CO_2 and N_2 gases as activity is boosted following either drought conditions or freeze/thaw cycles (Fierer and Schimel, 2002). Temperature and solar radiation play a greater role in deciduous forests compared to coniferous forests, as there is greater below-ground allocation of resources in the growing season and no photosynthetic activity in the winter months. Further, the lack of foliage increases solar radiation, raising soil temperature in the winter months in deciduous forests, as there are no differences in coniferous forests (**Figure 1-2**; Baldrian, 2017b). In boreal and temperate forests, organic matter decomposition is greater in the summer, due to the increased topsoil temperatures, as 60-70% of the total activity occurs during warmer temperatures. Although activity is decreased in winter months, fresh litter from fall leaf drop allows for the release of vital nutrients when the release of photosynthates via roots is limited (Baldrian, 2017b).

1.4 Effect of forest structure on soil microbial communities

Forests are one of the most diverse environments in the world (Baldrian, 2017a). This diversity is highlighted with the abundance of plant and tree species above-ground, yet the biodiversity below-ground may be even more abundant. One tablespoon of soil can harbor thousands of individual organisms, including bacteria, fungi, archaea, and viruses (Buee et al., 2009; Fierer, 2017). The diversity exhibited within forest soils can vary from geographical distance, host species, within successional stages, and even at a local scale between the rhizosphere and bulk soil (**Figure 1-3**; Li et al., 2015; Lladó et al., 2018). High above-ground diversity may be linked to high soil microbial diversity (below-ground), especially with interactions within the rhizosphere (Aponte et al., 2013; Li et al., 2015). Li et al. (2015) determined that within a successional forest study, mixed results were found when linking high above-ground and below-ground diversity. They observed a negative relationship for α-diversity in late successional forests, while bacterial β-diversity had a positive relationship between above-ground and below-ground diversity. Alpha diversity explains diversity with an ecosystem, usually incorporating the number of species, while beta diversity compares between ecosystems (Wagner et al., 2018). Moreover, in a warmer and drier environment, high diversity in tree species increases forest resiliency to sustained drought (Gillespie et al., 2020). Further, soil microbial communities in mixed forests are more stable than monocultures, and therefore the soil may be able to withstand normal biogeochemical cycling as drought frequency increases (Gillespie et al., 2020).

At a local scale, above-ground diversity is a key driver of below-ground diversity as the composition of litter may directly or indirectly influence soil microbial communities (Ladygina & Hedlund, 2010; Thoms et al., 2010; Lladó et al., 2018: Gillespie et al., 2020). Tree physiochemical traits (i.e., litter quality) have an indirect relationship with soil pH, organic matter (OM), moisture, nutrient content, and cations (Lladó et al., 2018). Needles from coniferous forests decrease soil pH, while deciduous leaves increase pH, resulting in higher cation uptake

in the canopy (Kaiser et al., 2016; Lladó et al., 2018). Litter decomposition is key in forest soil biogeochemical cycling, as high litter quality will result in faster nutrient cycling back into the soil. Recalcitrant litter (low quality) will persist without releasing valuable nutrients for plant uptake, especially in areas lacking moisture, while labile materials are easily broken down (Baldrian, 2017a).

In conjunction with bacteria, fungi have a direct relationship with many plant species in the world. In forests, Mycorrhizal fungi are split into two main categories of ectomycorrhiza (EcM) and arbuscular mycorrhiza (AM). Ectomycorrhizal fungi mineralize nutrients (Dong et al., 2018) and provide greater surface area, via a mantle on the outside of roots, to access water nutrients (Johnson and Gehring, 2008). Whereas AM, scavenge for available nutrients (Dong et al., 2018) and provide internal fungal structures within root tissues (Smith and Read, 2008). EcM and AM fungi have distinct interactions between specific tree species (Li et al., 2015; Baldrian, 2017b), with nearly 80% of plants associated with AM (Cameron et al., 2013), while specifically 90% of trees have a relationship with EcM (Markkola, 1996). These interactions infer that the trees and fungi may have coevolved to provide a mutualistic relationship for each other (Ferlian et al., 2018). The ability for mycorrhizae to increase the uptake of water and nutrients within the soil allows for greater plant productivity, which allows for a greater carbon influx from the tree to the soil (Futai et al., 2008; Baldrian, 2017a). While mycorrhizae actively increase plant health, plant diversity indirectly influences these fungal communities (Aponte et al., 2010; Grossman et al., 2018). As stated above, litter composition shapes below-ground communities, in particular, litter from certain tree species assists specific mycorrhizae to colonize roots (Morris et al., 2008; Aponte et al., 2010). In oak woodlands, an increase of calcium levels due to leaf fall causes a shift in pH to more acidic soils. The more acidic environment enhanced the shift from russuloid to tomentelloid EcM fungal communities (Aponte et al., 2010). This positive relationship is a cycle that provides carbon and litter to additional microbes, increasing the overall below-ground diversity and richness, especially within the rhizosphere (Cui et al., 2018).

The direct relationship between roots and soil microbes in the rhizosphere at the local scale enhance overall richness and diversity. The rhizosphere acts as a carbon-rich reservoir to power nutrient availability (Bartelt-Ryser et al., 2005; Chodak et al., 2016; Baldrian, 2017a). Therefore, hotspots are prevalent in the rhizosphere compared to bulk soil (Baldrian, 2017a). Yet, bacteria are generally less abundant in the rhizosphere than bulk soil, most likely due to competition from fungi (Cui et al., 2018). As plant communities reach their climax stages (late successional species), this established ecosystem may stabilize the soil microbial communities (Li et al., 2015). In highly dynamic ecosystems, mortality and disturbances can disrupt this relationship, potentially resetting the microbial communities in the soil (Li et al., 2015). In addition, increased light exposure following disturbances or succession can cause carbon loss in forest soils as a large influx of microbial activity may act as a CO_2 source following an increase in respiration (Mayer et al., 2017).

1.5 Effect of soil microbial communities on regeneration

Forest recruitment is key for the health of future forests. Without adequate regeneration following natural succession or disturbances, forests will not persist. Few studies have examined the effects of soil microbes on regeneration, but inference can be made via plant growth characteristics or plant-soil feedback (Bartelt-Ryser et al., 2005; Aponte et al., 2013). Since above-ground plant diversity may influence soil microbial communities, these effects may enhance or deter the germination and growth of regeneration. Understanding the dynamics of soils before and after disturbances may explain the potential suitability for trees to persist and regenerate. This may also affect the movement of trees via assisted migration as climate moves towards warmer, drier environments (Williams and Dumroese, 2014).

Plant species may increase soil fertility, creating a competitive advantage to increase recruitment (Aponte et al., 2013). Leaf and root litter accumulation provides many key nutrients to be recycled back into the soil. Plant-soil feedback can indirectly affect soil chemical and physical properties, which improve reproduction opportunities for their species (Aponte et al.,

2013). This can be in the form of changing the soil composition, establishing beneficial microbes, or inhibiting the growth of other species via allelopathy. The mutualistic relationship of EcM and many plant species can assist by increasing the uptake of essential water and nutrients, especially in drought environments (Aponte et al., 2013). Ectomycorrhiza in the soil can be key in the establishment and development of seedlings and saplings (Simard and Austin, 2010). The ability to increase nutrient availability and utilize resources is key for the seedlings to survive to maturity.

Examining grassland ecosystems, Bartelt-Ryser et al. (2005) assessed soil feedbacks of plant diversity and their effects on microbes and growth characteristics. They identified carry-over effects in soil microbial communities by taking field soils and growing plants of the same species. This carry-over of microbial communities was less evident for plants grown in soils with previously high plant diversity but still present. This study highlights the potential for microbiomes to be a key factor in soil “memory” that enable establishment of similar forests following disturbance or transplanting seedlings. The carry-over effect did fade over the 14-month study, yet the soil microbial communities may play a strong role in the initial establishment of regeneration in their first year.

Conversely, regeneration also influence soil microbial communities. Following disturbance in a forest, regeneration is vital to continue the carbon cycling performed by overstory trees, yet the future forest may impact nutrient cycling (Mayer et al., 2017). Mayer et al. (2017) focused on the effects of regeneration on decomposition following disturbance. In association with a forest gap disturbance such as a clear-cut or harvest, soil temperatures increased, thereby enhancing microbial activity and decomposition of slash. Temperatures were lower in areas with regeneration, as the seedlings intercepted light that would benefit microbial activity. The impacts of decreased decomposition may have adverse effects as regeneration continues to mature, especially shade tolerant species. Similar to above, the interaction

between trees and soil microbial communities is variable. Therefore, understanding additional aspects soil microbes have on forest disturbances is key to the resilience of forests.

1.6 Biocontrol of root pathogens within the soil microbiome

Although many microbes are beneficial to the establishment, development, and persistence of trees, a select few are detrimental to the health of forests. Most root diseases are fungi, other than *Phytophthora* species which are oomycetes or water molds. Many fungal and oomycete root diseases are considered the most damaging group of forest diseases in the United States (Lockman and Kearns, 2016). These diseases greatly impact forest productivity, modify forest structure by disrupting natural succession patterns, and pose a threat to recreational opportunities due to the potential of hazard trees (Lockman and Kearns, 2016; Kedves et al., 2021). It is proposed that root diseases will affect over 1 billion ft^2 of forest basal area by 2030 in the United States alone, which is more damage than bark beetles and other insect complexes (**Figure 1-4;** Krist et al., 2014). Unfortunately, all forests are susceptible to root disease. Root diseases persist for decades, and management strategies focused on eradication are not effective. The lack of effective long-term management strategies in forest settings emphasizes the need to understand the role that root diseases play in the entire ecosystem (Kedves et al., 2021).

Many studies have focused on the importance of the soil microbiome on the suppression of root pathogens (Fu et al., 2017; Futai et al., 2008; Kope and Fortin, 1989; Mesanza et al., 2016; Trivedi et al., 2017; Xiong et al., 2017). Bacterial and fungal microbes have been a focus to determine their efficacy in establishing disease suppressive soils. Similar to increased diversity above-ground, the identification of highly diverse microbial communities below-ground provides a competitive advantage in protecting roots from pathogens (Fu et al., 2017; Trivedi et al., 2017). The association between specific bacterial phyla (Actinobacteria, Acidobacteria, Bacteroidetes, Firmicutes, Proteobacteria, and Verrucomicrobia) may be attributed to the inhibition and suppression of *Fusarium* spp. (Fu et al., 2017; Trivedi et al., 2017; Xiong et al.,

2017). *In vitro* studies allow researchers to directly observe the inhibitory effects microbes have on root pathogens. Mesanza et al. (2016) harvested native rhizobacteria (*Pseudomonas*, *Bacillus*, and *Erwinia*) from the soil of radiata pine (*Pinus radiata*) to determine if there were inhibitory effects toward *Armillaria mellea* and *Heterobasidion annosum*. Results indicated that *Pseudomonas fluorescens* and *Bacillus simplex* inhibited the growth of both *Armillaria* and *Heterobasidion*, while *Erwinia billingae* only affected *Heterobasidion*.

In forest soils, the fungal composition increases biomass as there is a direct relationship with roots (Lee Taylor and Sinsabaugh, 2014). As stated above, mycorrhizal fungi, including ECM and AM, play an integral role in increasing forest health. In addition to providing resources to their host, mycorrhizal fungi also provide antagonistic properties toward root diseases (Kope and Fortin, 1989; Futai et al., 2008). In an *in vitro* study, Kope and Fortin (1989) identified that *Pisolithus tinctorius* and *Tricholomas pessundatum*, ECM, inhibited the growth of root pathogens (*Armillaria mellea*, *Fusarium oxysporum*, *Rhizoctonia* spp., among others) at an efficacy of 85% and 55%, respectively. Researchers have been able to identify key fungal microbes in soils that are suppressive toward root pathogens, including *Mortierella*, *Ceratobasidium*, and *Gymnopus*, in comparisons of suppressive soils and disease conducive soils (Xiong et al., 2017).

Trichoderma species are also known to have antagonistic properties against root disease pathogens, especially *Armillaria* infected stands (Kedves et al., 2021). Field trials have shown that using *Trichoderma* spp. as biocontrol agents can occur if population levels are maintained higher than natural populations to provide beneficial properties. Once populations decrease over time, their functions as biological control agents may diminish (Longa et al., 2008). Similarly, *Hypholoma fasciculare* has been identified as a possible soil inoculant to combat Armillaria root disease (Chapman and Xiao, 2000).

In combination with previous management techniques, the inoculation or use of native bacteria and fungi may reduce root disease inoculum (Modi et al., 2020). When the removal of

infected stumps is implemented, there may be a greater effect than just the removal of the pathogen. Modi et al. (2020) identified that beneficial bacterial communities increased in soil following stump removal and planting resistant species. Biological control agents and native bacterial and fungal communities will assist in managing root disease, especially in combination with silvicultural and mechanical treatments.

1.7 Conclusion and Hypotheses

Understanding forest health is key for the management of forest ecosystems. Forest health indicators assist in the ability of land managers to interpret how forests react to abiotic and biotic pressures. Expanding on our knowledge of what indicators can be used for soil health is needed. Current protocols highlight ranges of chemical and physical properties underlying soil health. Yet, the interactions between microbial communities may elicit greater insight into the soil environment's diversity, richness, and functionality. Soils play an integral role in many ecosystem services that intertwine above and below-ground processes, such as disease suppression, tree and regeneration health, and plant biodiversity. Enhancing the appreciation for this unknown microbial world will allow land managers to increase our knowledge of forest management further.

Understanding the interactions between and among competing root fungi, *Armillaria solidipes* (highly virulent) and *A. altimontana* (beneficial) and their bacterial microbial communities, could help in the development of novel management strategies to manage Armillaria root disease in a forest setting (Chapter 2). My research objectives were to characterize soil bacterial communities associated with *Armillaria* species (*A. solidipes* and *A. altimontana*) and tree health status (healthy, declining, and dead). I hypothesize that soils associated with *A. altimontana* will have greater bacterial species richness and diversity than soils associated with *A. solidipes*. Although each species provide differing lifestyles, both have similarities between their genomes, yet the abundance of secreted and non-orthologous proteins differ (Ibarra-Cabellero et al., 2022). Specifically, the cytochrome p450 gene was in

higher abundance in association with *A. altimontana* than *A. solidipes*. The higher abundance of cytochrome p450 genes may be provide an inference to the saprophytic nature (beneficial) to *A; altimontana*, while less genes are associated with the pathogenic lifestyle of *A. solidipes* (Ibarra-Cabellero et al., 2022). Similarly, I hypothesize that there will be a shift in soil bacterial communities associated with tree health. Healthy trees will have the greatest richness and diversity, as photosynthate exuded in the rhizosphere will enhance microbial abundance and biomass. Conversely, the shift in exudate production may change the composition of bacteria as dead tissue increases within the soil.

It has been proposed that high plant species diversity increases below-ground microbial community diversity (Aponte et al., 2013). This hypothesis was tested by examining bacterial and fungal soil communities associated with local forest composition in the Rocky Mountain region (Chapter 3). The Pikes Peak Forest Dynamics Plot, near Woodland Park, Colorado, is the ideal location for investigating the interactions between soil microbial communities and mature and regenerating forest structures. The forest is composed of six main tree species with a mixture of regeneration, understory plants, and a range of soil pH. My objectives for the study were to: 1) determine what fungal and bacterial soil microbial communities are associated with forest habitat type (dry mixed, wet mixed, open) and forest ecological properties (i.e., regeneration, soil edaphic properties, understory vegetation, and tree spatial patterns). I hypothesize that there will be distinct shifts in microbial communities across the forest habitat types, whereby wet mixed forests will produce greater richness and diversity for fungal communities, and dry mixed forests will be greater in bacterial richness and diversity. These assumptions are due to the soil moisture availability in the wet mixed and the increase in soil light interception in the dry mixed. Additionally, greater above-ground diversity will positively influence below-ground microbial communities. In addition, bacterial communities will be greatly influenced by pH shifts. This information will provide greater insight into the variability of soil microbial communities at a stand level while elucidating differences within forest type changes.

The use of Forest Inventory Analysis plots, which is the most extensive forest monitoring program in the United States, will be used to expand our understanding of shifting forest soil microbial communities and forest type. These data also include various forest health indicators and stand measurements, providing greater insights into the relationship between forest health and microbial diversity (Chapter 4). My research objectives were to identify if there are distinct shifts in soil microbial community composition and diversity associated with forest ecotypes, elevational gradients, stand characteristics, and climatic variables. I hypothesize that there will be distinct shifts in soil bacterial and fungal communities regarding forest ecotypes, as litter composition indirectly links the richness and diversity of microbes. Additionally, spatial characteristics such as elevation may establish similar shifts in microbial communities, with lower elevations having greater richness and diversity. This research will expand our knowledge of forest health using a reliable forest inventory system, with the ultimate goal to assist in managing forests for future generations.

Figures

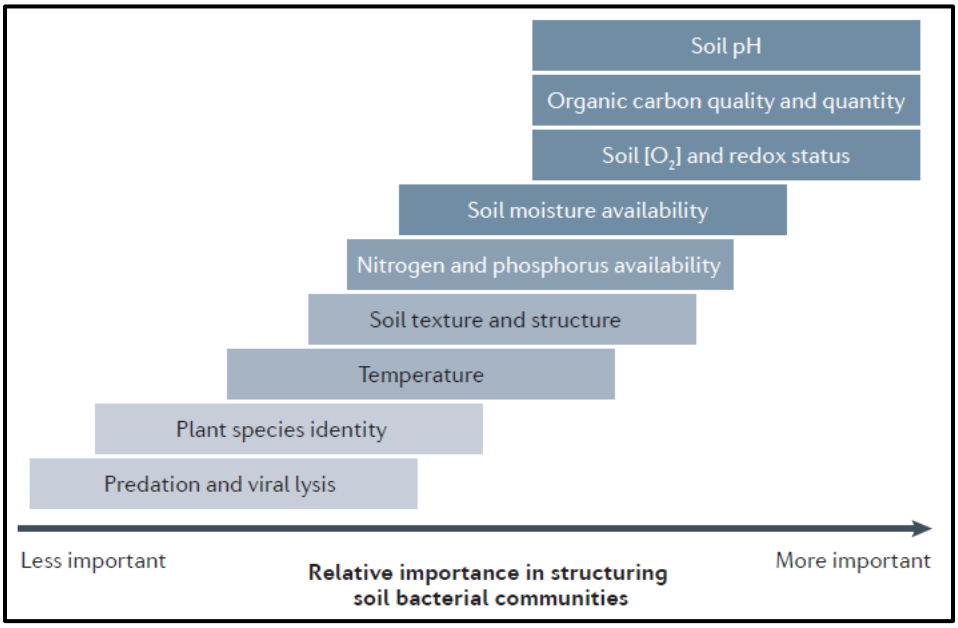

Figure 1-1: Biotic and abiotic factors that influence soil bacterial communities. The categories are broken down into the relative importance toward the structure of bacterial communities. The shading of each category determines how well they have been studied, with soil pH being the greatest and predation and viral lysis being the least (Fierer, 2017).

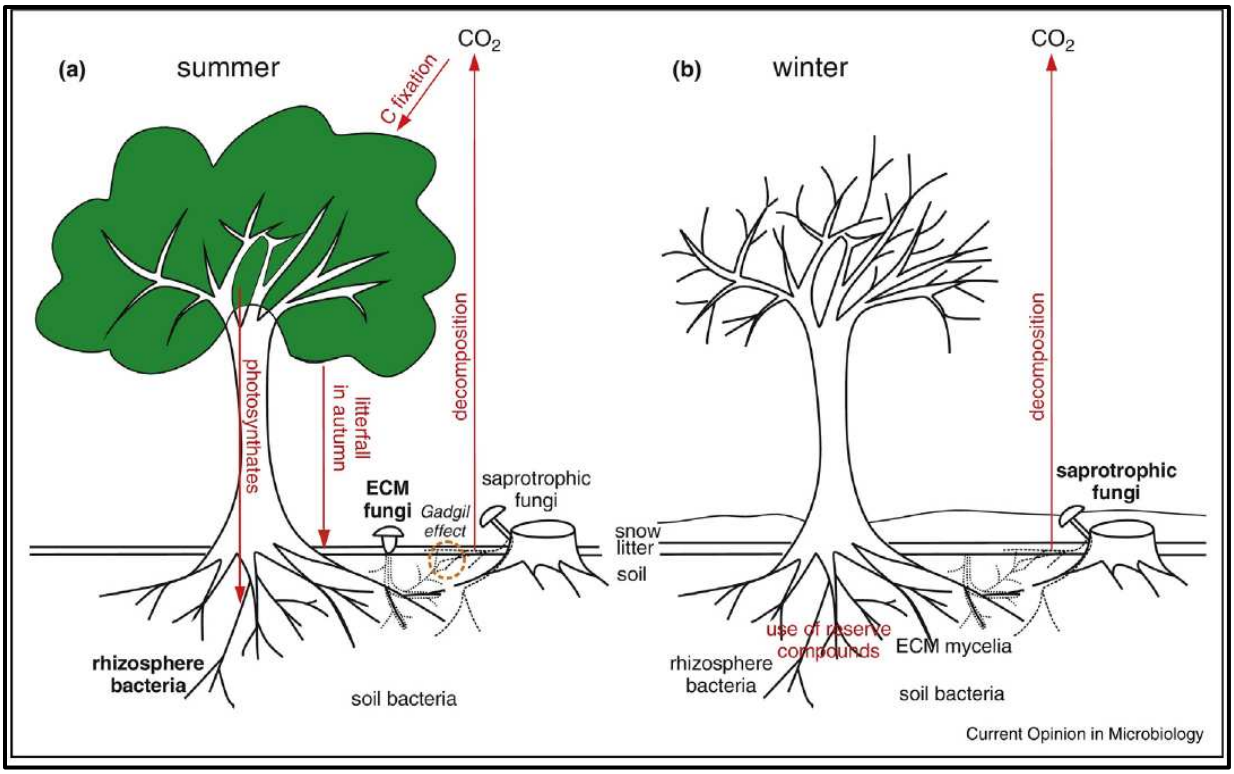

Figure 1-2: Seasonal and temporal changes in inputs and outputs of soil microbial communities. a) During summer months, photosynthate produced by trees is exuded in the soil, promoting rhizosphere bacteria. Additionally, litterfall allows for increased decomposition by fungal and bacterial communities. Ectomycorrhizal fungi are integral in the uptake of valuable nutrients and water, while saprophytic fungi emit CO_2 following the breakdown of litter on the soil surface. b) In the winter months, the use of reserve compounds in the soil allows microbial activity to persist. Additionally, litter accumulating below snow may still be a useful resource through the decomposition of nutrients (Baldrian, 2017b).

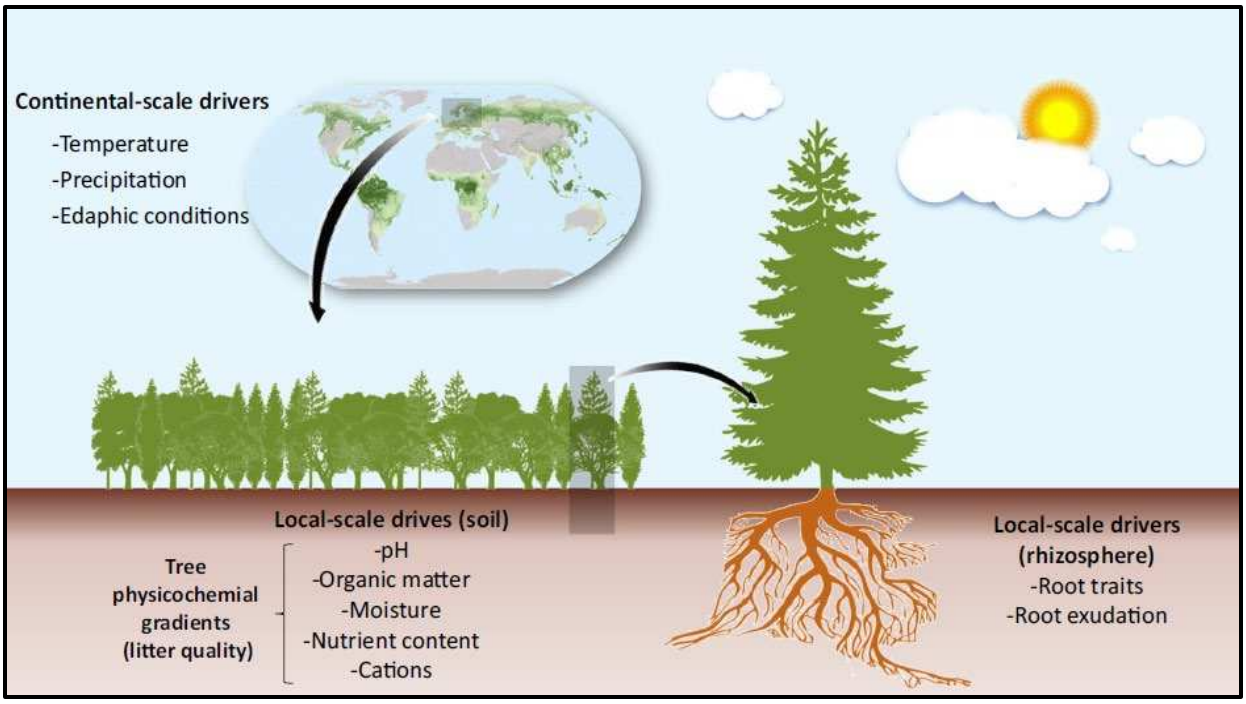

Figure 1-3: Major drivers of forest soil microbial communities in continental (landscape) and local scales (Llado et al., 2018).

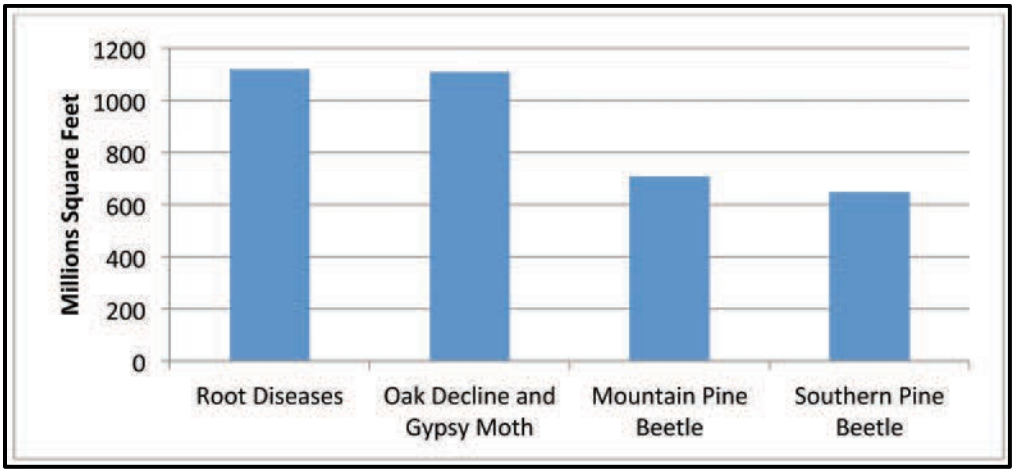

Figure 1-4: Prediction of potential acres affected by damage agents in the next 10 years. Root diseases are predicted to have the greatest impact to forests (Lockman and Kearns, 2016).

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CHAPTER 2: CHANGES IN SOIL BACTERIAL COMMUNITIES ASSOCIATED WITH ARMILLARIA ROOT DISEASE ON WESTERN WHITE PINE (*PINUS MONTICOLA*)

2.1 Preface

In North America, root diseases are one of the most damaging forest agents, with the potential to impact 15% of forest cover by 2027 (Krist et al., 2014). The most prolific root disease is caused by *Armillaria* fungal pathogens, with 10 North American species that infect the majority of tree species in our forests. When a root disease is present, it is often called “a disease of the site” due to its difficulty to manage. Frequently, *Armillaria* species can co-occur in the inland northwestern United States, most notably *A. solidipes* (highly virulent) and *A. altimontana* (less virulent) that have overlapping geographic ranges. The goals of this study were to identify the soil bacterial community composition of *A. solidipes* and *A. altimontana* infected western white pine (*Pinus monticola*) with varying degrees of tree health status. This field study showed significant differences between the soils associated with each *Armillaria* in bacterial species richness and Shannon’s alpha diversity. Similarly, there was a significant difference in Shannon’s diversity index among trees of varying health status. As bacterial community richness and diversity were greater for soils associated with *A. altimontana* and healthy/declining (alive) trees. Conversely, there were no differences among beta diversity between soils associated with the *Armillaria* species and trees of varying health status. Examining the most abundant bacterial families resulted in a combination of beneficial microbes, plant growth promoters, pathogen suppression, nutrient cycling, and potential pathogens. The most prominent bacterial taxa associated with *A. altimontana* was Pseudomonadaceae, which is known to be involved in disease suppressive soils. Additionally, soil moisture, pH, carbon, and charcoal were significant factors in the overall bacterial community composition, whereas soil moisture had a negative relationship to bacterial richness and diversity. This research suggests that utilizing soil microbial communities may be key in the

management of root disease. Understanding how to promote beneficial soil conditions to enhance bacterial communities to suppress root disease will increase forest resilience to detrimental pathogens.

2.2 Introduction

2.2.1 Impacts of Armillaria root disease

In the United States, root diseases are hypothesized to be the greatest damaging agent within forests, with the risk of losing 15% of the total basal area in the contiguous U.S. by 2027 (Krist et al., 2014). Root diseases have long-lasting impacts in forest settings as they persist for decades (Lockman and Kearns, 2016; Kedves et al., 2021). The most prolific root disease in North America is Armillaria root disease (*Armillaria* species), which are white-rot decay fungi that affect hundreds of different tree species (Kedves et al., 2021). The ecological and economic impacts of *Armillaria* are widespread as they degrade root tissue, affect growth characteristics, predispose trees to other biotic agents, alter forest species composition, and impact forest health, subsequently reducing carbon sequestration (Baumgartner et al., 2011; Lockman and Kearns, 2016). White rot fungi play a significant role in the decomposition of woody tissue, as they degrade both cellulose and lignin, increasing soil organic matter (Heinzelmann et al., 2019). Armillaria root disease is important in this process as it acts as a facultative necrotroph, in which it can actively colonize living hosts as a primary pathogen and as an opportunistic or weak pathogen, persisting in the soil as a saprophyte (Kile et al., 1991; Baumgartner et al., 2011; Heinzelmann et al., 2019; Kedves et al., 2021; Wojciech Przemieniecki et al., 2021).

Once *Armillaria* is established within a site, it can spread via the formation of root-like vegetative rhizomorphs, mycelial root-to-root contacts, and windborne aerial basidiospores from honey mushrooms (Baumgartner et al., 2011; Heinzelmann et al., 2019). Spread under the soil is the most prevalent means of dispersal as environmental conditions are not always suitable to form above-ground fruiting bodies (Ferguson et al., 2003). Expansion of rhizomorphs is a relatively slow process, as they are estimated to spread at a rate ranging from 0.22-1.5 m/year in suitable environmental conditions (Redfern and Filip, 1991; van der Kamp, 1993; Ferguson et al., 2003). *Armillaria* species that are weak pathogens are more prolific in their expansion, as

they tend to disperse to find nutrients. At the same time, pathogenic species actively seek susceptible hosts via root-to-root contact (Redfern and Filip, 1991).

Since *Armillaria* persists in the soil for decades, it is described as a disease of the site. Therefore, management techniques may suppress the spread, yet it doesn't ultimately eradicate the disease. Current management techniques include an integrative pest management perspective that uses silvicultural, biological, and chemical methods. Soil fumigants have been used to reduce the inoculum loads and impact of the disease. However, the adverse effects of the chemicals limit the use in the United States (Baumgartner et al., 2011). Silvicultural treatments fall into two main categories: partial thinning or clear-cutting. In partial thinning treatments, the stand is thinned to increase the vigor of residual trees despite adverse damages caused by wounding and soil compaction (Kile et al., 1991; Wargo and Harrington, 1991; Williams et al., 1986). Stress caused by soil compaction will exacerbate the impacts of *Armillaria*, resulting in more prevalence of the disease. Clear-cutting removes the potential host, therefore transitioning species composition to resistant hosts is needed for trees to persist (Kile et al., 1991). Leaving stumps in a clear-cut act as a reservoir for *Armillaria* to survive as a saprophyte. Stump removals can reduce the inoculum load, but it is costly and labor intensive (Baumgartner et al., 2011). Additionally, high-intensity fires burn hot enough to, again, reduce inoculum, while low-intensity fires have little effect (Kile et al., 1991). The central theme to all of these management techniques is to reduce the amount and spread of the disease. The lack of adequate management techniques prompts the need to develop more tools for managing Armillaria root disease.

2.2.2 Armillaria lineages and co-occurring species

Ten North American Biological Species (NABS) of *Armillaria* have been identified, with all falling in three main lineages; *Ostoyae*, *Mellea*, and *Gallica* (Klopfenstein et al., 2016; Kedves et al., 2021). Species in the *Ostoyae* lineage constitute primary pathogens including *Armillaria solidipes* Peck [as *A. ostoyae* (Romagnesi) Herink], *A. gemina* (Bérubé & Dessureault), and *A.*

sinapina (Bérubé & Dessureault). *Armillaria solidipes* is commonly found in conifer forests of inland regions of western North America, ranging from British Columbia to the southern Rocky Mountains (Klopfenstein et al., 2016; Kedves et al., 2021). The *Mellea* lineage consists of two species, *A. mellea* [(Vahl:Fr.) Kummer] and *Desarmillaria caespitosa* [(Berk.) Antonín, J.E. Stewart & Medel, comb. nov.], which infect a broad host range of over 500 tree species, most commonly infecting broadleaf species (Klopfenstein et al., 2016; Kedves et al., 2021). The *Gallica* lineage is the final group, highlighting weak or opportunistic pathogens such as *A. gallica* (Marxmüller & Romagn), *A. calvescens* (Bérubé & Dessureault), *A. nabsnona* (Volk & Burdsall), *A. cepistipes* (Velenovsky), and *A. altimontana* [Brazee, B. Ortiz, Banik, and D.L. Lindner (formerly North American Biological Species X)] (Klopfenstein et al., 2016; Kedves et al., 2021).

The interactions between species have been well-documented with *A. solidipes* and *A. altimontana* co-existing at the Priest River Experimental Forest (PREF) within the Inland Northwest conifer stands of the United States (Ferguson et al., 2003; Kim et al., 2010; Warwell et al., 2019). As stated above, *A. solidipes* acts as a virulent, primary pathogen in conifer stands (Kedves et al., 2021). *A. altimontana* has been proposed as a weak or opportunistic pathogen. Yet, a recent study found that *A. altimontana* infected western white pine (*Pinus monticola*) have larger diameters, heights, and rates of survival compared to trees not infected with *Armillaria* (Warwell et al., 2019). At the PREF site, *A. altimontana* is prolific throughout the stand, corresponding to the concept that weak, opportunistic pathogens expand their niche to decompose dead tissue for nutrients (Kedves et al., 2021). In turn, *A. solidipes* occupies relatively less area than *A. altimontana* on the site. This suggests that *A. altimontana* may be beneficial to hosts and suppresses the growth of *A. solidipes* (Warwell et al., 2019).

2.2.3 Influences and drivers of soil microbial communities

Soil microbial communities provide ecosystem services that directly impact forest health (Baldrian, 2017). Soils provide stabilization for roots, decompose litter, provide nutrient cycling, and mycorrhizal associations that increase water and nutrient availability (Nortclif et al., 2000).

The many ecosystem processes provided by soil are driven by factors including soil chemical and physical properties, climatic variables, above-ground plant species, and temporal variation (Brockett et al., 2011; Lareen et al., 2016; Fierer, 2017). These factors increase the overall heterogeneity of soils, as microbial communities differ spatially at local and continental scales. In proximity to a plant, soils vary from the rhizosphere to bulk soils, even micro-niches within these settings and soils that are in close proximity differ in microbial composition (Pinho et al., 2017). These characteristics are why soils are one of the most difficult ecosystems to study metagenomics yet identifying microbial community composition is vital for the management of soil and alike.

2.2.4 Soil bacterial communities as biocontrol for root pathogens

Bacterial and fungal microbes are essential for forests as they provide decomposition and nutrient cycling while also acting as antagonists to the growth and establishment of root pathogens (Modi et al., 2020). Many studies focus on overall bacterial communities to determine if high diversity in below-ground bacteria promotes disease suppressive soil. It has been proposed that the association between a suite of specific bacterial phyla (Actinobacteria, Acidobacteria, Bacteroidetes, Firmicutes, Proteobacteria, and Verrucomicrobia) may inhibit or suppress *Fusarium* species (Fu et al., 2017; Trivedi et al., 2017; Xiong et al., 2017).

Many native rhizobacteria naturally provide beneficial effects for plants when growing in inoculated soil. Mesanza et al (2016) performed an *in-situ* study to determine if abundant bacteria growing in the rhizosphere of radiata pine (*Pinus radiata*) provided inhibitory effects to *Armillaria mellea* and *Heterobasidion annosum*. In the study, harvested *Pseudomonas fluorescens, Bacillus simplex*, and *Erwinia billingiae* were all found to suppress *H. annosum*, while only *P. fluorescens* and *B. simplex inhibited the growth of A. mellea*.

To further understand the dynamics of how these bacteria establish within the soil, Mesanza et al. (2019) inoculated three-year old radiata pine seedlings using a bacterial suspension watering treatment. The bacterial suspensions of *B. simplex* and *E. billingiae,*

harvested in their previous study, were transformed with fluorescent plasmids to allow detection post-inoculation. Both bacterial strains were found colonizing the roots post inoculation with *B. simplex* identified 1-31 days after inoculation and *E. billingiae* found 1-16 days after incoulation. Although present, each inhibitory bacterium was found at a lower population later in the study compared to initial inoculations levels, indicating that inoculations of beneficial bacteria can successfully establish populations of emerging root tissue. Most notably, *E. billingiae*, which is known to be an epiphyte, was found clustered in high densities and in intracellular spaces of the roots (Mesanza et al., 2019).

Additionally, mechanical treatments alter soil microbial communities (Modi et al., 2020). Modi et al. (2020) found that following the removal of stumps, frequently performed in areas infected with root disease, bacterial diversity increased post-treatment, most notably, bacteria in the orders Pseudomonadales and Burkholderiales which are known to provide beneficial qualities in soil were more abundant in stumped soils. *Pseudomonas* species have been reported as mycorrhizae-helpers and antagonistic to *Armillaria* species (Modi et al., 2020). *Burkholderia* species provide ecological roles as saprophytes and nitrogen fixing mutualists (Hall et al., 2015). The combination of stumping and planting resistant species successfully prevented further infection by fungal root pathogens (Modi et al., 2020). Soil bacteria that inhibit fungal root pathogens, in combination with silvicultural and mechanical management techniques, maybe the key to suppressing root diseases (Mesanza et al., 2019; Modi et al., 2020).

2.2.5 Microbial community changes associated with tree health

The term tree health can be construed based on the perspective of the land manager, therefore “healthy” soils may vary based on management strategy, ecosystem, or forest setting (Fierer et al., 2021). Although soil health may be arbitrary, the use of tree health as indicators of soil microbial communities may elucidate the health of the soil, as microbial communities harbor beneficial (disease suppressive) and pathogenic taxa simultaneously, with dissimilarity

occurring in varying tree health status. Recent research has highlighted how microbial communities change as tree health degrades due to the impacts of disease, and how some soils withstand change due to their disease suppressive qualities (Feau and Hamelin, 2017; Koskella et al., 2017; Mercado-Blanco et al., 2018; Pinho et al., 2020; Wojciech Przemieniecki et al., 2021). Many aspects impact the ability of trees to withstand disease, such as declining or dead trees having less diverse microbial communities, potentially making them more susceptible to contributing pathogens (Koskella et al., 2017). Additionally, pathogens may suppress other taxa due to a competitive advantage once established, further decreasing diversity (Koskella et al., 2017). The concept of less diverse communities associated with symptomatic or diseased trees infers that protective benefits of soil microbial communities are associated with healthier trees (Feau and Hamelin, 2017).

This change in the microbial community may be induced by reduced tree root exudates or changing chemical signals in the roots due to a degradation of health (Pinho et al., 2020). According to Pinho et al (2020), oaks suffering from acute oak decline tended to be in more acidic soils and had distinct soil microbial communities when compared to healthy oaks. Additionally, healthy oaks had a higher presence of beneficial microbes with more stable soil conditions. This shift in microbial communities could also be explained by differences in soil chemical properties such as pH or nitrogen content (Pinho et al., 2020). In particular, root disease may have a greater impact on overall tree health because as the pathogen degrades root tissue, there is a reduction in the uptake of water and nutrients, potentially impacting the interaction between the soil, host tree, and microbial communities.

2.2.6 Objectives and hypotheses

Our research objectives were to characterize soil bacterial communities associated with *Armillaria* species (*A. solidipes* and *A. altimontana*), both of which have differing ecological behaviors (virulent pathogen and non-pathogen, respectively) and resultant tree health status (healthy, declining, and dead) on western white pine. We hypothesize that soils associated with

A. altimontana will have greater bacterial species richness and diversity than soils associated with *A. solidipes*, as the beneficial qualities of *A. altimontana* will increase the abundance of beneficial microbes. Similarly, we hypothesize that there will be a shift in soil bacterial communities associated with tree health. Healthy trees will have the greatest richness and diversity, as photosynthate exudes in the rhizosphere will enhance microbial abundance and biomass. Conversely, the shift in exudate production may change the composition of bacteria as dead tissue increases within the soil. Understanding the host-pathogen interactions between two species of *Armillaria* (high and low virulence), tree health, and their influences on soil microbial communities may assist in the management of Armillaria root disease.

2.3 Methods

2.3.1 Field Sampling

The study area was in northern Idaho at the USDA, Forest Service Priest River Experimental Forest (PREF). The field site was a historic western white pine seed provenance plot within the Ida Creek watershed at 760 meters elevation. In 1971, 2,372 seedlings from Idaho and Washington were planted in a common garden plantation (Warwell et al., 2019). In 1987, the 2,076 remaining trees were measured for diameter at breast height (DBH), height, tree health status, and association with *A. solidipes* and *A. altimontana*, as described by Warwell et al. (2019). Our 2016 sampling built on the 1987 inventory to further assess the health of the site and the spread of each species of Armillaria root disease. After the 30 years between data collections, around 600 trees remained due to the impacts of the pathogenicity of *A. solidipes*, white pine blister rust (*Cronartium ribicola*), and other abiotic and biotic mortality agents resulting in small openings being made over time.

The average temperatures at PREF range from 24.4 °F in the winter to 64.6 °F in the summer, as it receives an average of 31.4” of precipitation and 70-80 in of snowfall each year (Prism Climate Group, 2004; Tinkham et al., 2015). Although samples were collected in a site with high precipitation, June is historically the driest month of the year. In June 2016, the site

received 0.87 inches of rain, and temperatures were around 90°F during sampling (Prism Climate Group, 2004).

In 2016, 60 trees were randomly selected for sampling, ensuring that half of the trees were historically associated with either *A. solidipes* or *A. altimontana*. Three additional trees (ca. 63) were sampled due to the presence of needle discoloration and the formation of mycelial fans on the base of the trunk, indicating the presence of *A. solidipes*. Tree measurements included DBH and tree health status, based on the relative needle density, color of foliage, insect and disease presence, and dead/live status.

Soil samples were located 1 m from the main stem of each tree near the root zones, with duff and litter depths measured at each cardinal direction in a 30-cm diameter circle. The area was then cleared, and bulk soil samples were taken for each of the 63 trees using a 15-cm split soil corer with a 5/8” compact slide hammer (AMS, #400.99, American Falls, ID). Samples for each tree were homogenized and 2 g were placed in two 15 ml tubes (Qiagen Powersoil RNA Extraction Kit ®; Germantown, MD) with 5 ml of LifeGuard RNA preservation solution (Qiagen), and then samples were placed on ice for preservation. Samples were stored at -80°C freezer prior to DNA extractions. The remaining bulk soils from each tree were sent to the USDA Forest Service, Rocky Mountain Research Station, Soils Laboratory in Moscow, ID for soil characteristics measurements and chemistry calculations (moisture, rock, root, charcoal, other, pH, C, N, and OM).

Armillaria rhizomorphs adjacent to the roots were also excavated using a small Pulaski-like gardening tool and brushes. Rhizomorph collections occurred on the same side of the tree as the soil core, while an additional sample was collected 180° on the opposite side of the tree from the core. Rhizomorphs were placed in 15 ml tubes and placed on ice or 4°C until isolation.

2.3.2 Rhizomorph Isolation and DNA Mycelium Extraction

Rhizomorphs were plated for fungal isolation within 7 days of collection. Each rhizomorph was surface sterilized by initial rinsing with sterile-distilled water to remove the

attached soil particles, soaked in 20% Clorox® bleach solution (1.5% sodium hypochlorite, final concentration) for 6-10 minutes, then soaked in 3% hydrogen peroxide for 6-10 minute, and finally rinsed with sterile distilled water. Small 1-cm rhizomorph sections were plated onto *Armillaria* culture media (3% malt extract, 3% dextrose, 1.5% peptone, 1.5% agar) and incubated at 22°C in the dark until the formation of mycelium.

For DNA extractions of *Armillaria* cultures, mycelia were sub-cultured onto 0.22-µm, pore-size MF-Millipore ™ Membrane nylon filters (MilliporeSigma, Burlington, MA) that overlaid *Armillaria* media. After 2-3 weeks, mycelia were scraped, and DNA was extracted from > 50 mg of mycelia using Zymo DNA extraction kits (Irvine, CA), following manufacturer protocols with a few modifications. To maximize DNA quantity and quality, three 3-mm glass beads were added to the cell lysis step prior to homogenization (Thermo Savant FastPrep ® FP120 Cell Homogenizer; Qbiogene, Carlsbad, CA) at 6.0 speed with two 30-second cycles. DNA concentration and quality were quantified using a NanoDrop ™ 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

For species identification, DNA was amplified at the translation elongation factor-1α (*tef*1) locus using primers EF-983 and EF-2218 (Rehner and Buckley, 2005) with an Eppendorf Mastercycler pro Thermal Cycler (Eppendorf, Hamburg, Germany). The Polymerse Chain Reaction (PCR) cycle was 94°C for 2.5 minutes, 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1.5 minutes, ending with 72°C for 10 minutes, and maintained at 4 °C. PCR products were visualized using gel electrophoresis, and if successful, cleaned with ExoSAP-IT ™ PCR Product Cleanup Reagent (Thermo Fisher Scientific, Santa Clara, CA). Cleaned PCR products were sent to Eurofins Genomics (Louisville, KY) Sanger sequencing was performed in two directions. Sequences were edited and aligned in Geneious R11.1 (https://www.geneious.com). Aligned sequences identified by using BLAST to the National Center for Biotechnology Information Basic Local Alignment Search Tool (BLASTn) (Zhang et al., 2000).

2.3.3. Soil DNA extraction protocol

Soil extractions were completed using both MoBio Powersoil Total RNA Isolation and DNA Elution Accessory kits (Qiagen®, Carlsbad, CA). Preserved soil samples in 15 mL bead tubes were centrifuged to separate LifeGuard preservation solution from the two grams of soil. Following the removal of the LifeGuard preservation solution, extraction protocols were followed, resulting in 100 µL of DNA. Quantity and quality were measured using a NanoDrop ™ 2000. Eluted DNA was preserved in a -20°C freezer until all samples were extracted. All samples that resulted in less than 10 ng/µL of DNA were extracted using the second soil samples preserved in LifeGuard preservation solution. All DNA samples were prepped for sequencing by diluting the quantity to either 30/20/10 ng/µL depending on the initial quantity. A subsample of 30 µL of DNA was pipetted into a 96-well plate and sent to the Colorado State University Next-Generation Core Lab (CSU-NGS) for library preparation and Illumina Miseq sequencing. Fifty-five out of 63 samples were sent in for sequencing, while the remaining eight samples did not yield sufficient DNA. Libraries were prepared for the 16S region to sequence bacterial communities. Primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVHHHTWTCTAAT-3') (Walters et al., 2015) were used to amplify the v4 genomic region of the 16S rRNA gene.

2.3.4 Cleaning DNA sequence data

Data were cleaned to ensure base calling accuracy of ≥99.9% using the paired end mode in the program Trimmomatic (v0.36, Bolger et al. 2013). Sequences ≤100bp in length, low quality bases scores (≤15), and 4bp sliding window regions with low average quality scores (≤25) were removed from the data set. The software Mothur (v1.40.5, Schloss et al. 2009) was implemented utilizing the standard operating procedure (Kozich et al. 2013), with some adjustments, to call operational taxonomic units (OTUs) and classification of taxa. Following adjustments described in the SOP (https://github.com/Abdo-Lab/Microbiome-Analysis-Scripts/blob/master/PE-de-novo-processing.pl), UCHIME (Edgar et al. 2011) was used to de

novo identify and remove chimeric sequences, and USEARCH, utilizing the dgc (distance-based greedy clustering) option, was used for clustering. Groups at least 97% similar were classified as belonging to the same OTU. Sequences associated with chloroplast, mitochondria, archaea, and bacteria lineages were removed from the table of classified sequences. We utilized the 128 Silva database (Quast et al., 2013) for bacterial taxonomy, using Wang's Naïve Bayes classifier with a cutoff value of 80 (Wang et al. 2007). Rarefaction curves were generated using the package 'vegan' as implemented in R version 3.6.1 to assess diversity and suitability of depth of coverage per sample (Oksanen et al., 2013).

2.3.5 Statistical analysis of bacterial sequences

Using the RStudio (RStudio, 2015) interface to R (R Core Team, 2017), alpha diversity, including Shannon diversity index and Inverse Simpson, were calculated using the phyloseq package (McMurdie and Holmes, 2013) and rarefied richness (Richness) using the vegan package. Shannon's index is used to determine diversity utilizing the relationship between richness and rare microbes (Hill et al., 2006; Nagendra, 2002). Inverse Simpson relies on evenness and more dominant microbes to identify diversity (Nagendra, 2002). Richness is described as the number of individuals identified within a single sample, while evenness explains the relative abundance of the different individuals (Zhang et al., 2012).

The relative abundance of taxa was determined for the top bacterial taxa using a stacked bar graph for *A. solidipes* and *A. altimontana* using metagenomeseq package in R (Paulson et al., nd). Differences among communities associated with *Armillaria* species were assessed using a PERMANOVA. Principal coordinate analysis plots were completed in vegan to visualize bacterial soil differences associated with each *Armillaria* species.

Utilizing relative abundance data based on the resulting OTU table, bar graphs were generated using the ggplot2 package (Wickham, 2016) in R for observed taxa with relative abundance > 1% at the genus level to describe the microbial community structure per *Armillaria* species. The MetagenomeSeq package (Paulson et al., 2021) in R was used to fit a model that

identified those OTUs associated with significance of model fit at a 0.01 level and a minimum fold change of 2 (p values were adjusted for multiple testing). This was used to identify the driver of OTU differences between treatments and time points.

Core bacterial communities were created for each *Armillaria* species Counts were calculated in R to assess the presence of an OTU corresponding to each species of *Armillaria*. Venn diagrams were compiled using molbiotools.com (Čermák, 2020) to identify unique and shared bacterial taxa associated with *A. solidipes* and *A. altimontana*.

To identify what soil chemistry properties influenced soil bacterial communities, a PERMANOVA analysis was completed using the vegan package in R. The analysis identified significant predictors by completing a forward stepwise analysis based on the subset of variables that minimized the Akaike Information Criterion (AIC).

An ANOVA and PERMANOVA analysis were completed to determine which soil chemistry properties were significant to an *Armillaria* species, tree health, and a combination of both treatments. The ANOVA was analyzed to determine differences between each individual treatment, while the PERMANOVA included both treatments separately and simultaneously. The ANOVA used a linear model to determine significance for richness, Shannon's, and Inverse Simpson's diversity as the response variables. The variables for each linear model ANOVA included Treatment (*Armillaria* species or Tree health), moisture, pH, C, N, and OM. The PERMANOVA analysis completed for both treatments included all soil chemistry properties, including moisture, rock, root, charcoal, other, pH, C, N, and OM.

2.4 Results

2.4.1 Field sampling and rhizomorph species identification

Rhizomorphs were isolated from 51 total trees, yielding 87 rhizomorph samples that all produced pure *Armillaria* cultures. Sequencing the translation factor elongation factor 1-alpha (*tef1*) gene from the 87 rhizomorph samples resulted in 48 trees associated with *A. altimontana* and three trees associated with *A. solidipes*. Two of the three trees corresponding to *A.*

solidipes had *A. altimontana* rhizomorphs identified on the tree as well. Data for these trees were combined into the *A. solidipes* category due to the virulent characteristics of the pathogen. Sequences corresponding to both *A. altimontana* and *A. solidipes* resulted in 99% identity. Tree health status identified 38 healthy trees, 13 dead, and 12 in declining health. Trees with declining health were defined as any live tree with visible crown dieback.

2.4.2 Processing sequenced 16s and ITS2 libraries in Mothur

To identify bacterial communities within the soil at the Ida Creek location, total soil DNA from 55 of the 63 samples were sent to the Colorado State University NGS Core Lab for MiSeq Illumina sequencing of the v4 region of the 16S rRNA gene. The total number of reads prior to cleaning in Mothur was 2,437,256, with an average read depth of 44,314 reads/sample. Using screen.seqs, in Mothur, reads were screened to remove sequences longer than 275 bp and any with homopolymers larger than 8, resulting in the removal of 688,599 reads from the dataset. From the remaining 1,748,657 sequences, the use of unique.seqs identified 480,487 unique sequences. Following the identification of chimera sequences, 27,741 sequences were removed from the dataset. The mean sequencing depth after processing was 27,639 reads/sample, with a range from 6 to 107,582. Samples (18 ca) under 5,000 total reads were removed from analyses for the 16S dataset (Figure 2-1). After classifying all sequences to the reference Silva dataset, the 16S data resulted in 26,781 unique OTUs. Following the removal of low coverage OTUs of < 10 sequences from all samples in RStudio, 6,677 bacterial OTUs were identified. A rarefaction curve was prepared, which identified that the samples did not plateau. Since samples did not plateau, this indicated the data could be lacking much of the rare communities (Anonymous, 2021; Figure 2-2). For final data analyses in RStudio, 43 samples were used (41 *A. altimontana*, 2 *A. solidipes*, 27 healthy, 5 declining, and 11 dead), with 12 samples removed due to low sequence depth following sample cleaning in Mothur.

2.4.3. Data analysis associated with Armillaria species

Analysis of the bacterial community richness indicated that soils associated with *Armillaria* species were significant (p = 0.0563), with *A. altimontana* having greater richness. For both diversity indices, soils associated with *A. altimontana* had a greater diversity. Shannon's diversity was also significant (p = 0.0518), though the Inverse Simpson index was not significant between *Armillaria* species (p = 0.2448; Table 2-1).

Table 2-1: Richness and diversity indices calculated for *Armillaria* species and tree health status. Values based on samples within each group with standard errors.

	Richness	**Shannon**	**InvSimpson**
A. altimontana	593 ± 28.2	4.39 ± 0.18*	27.32 ± 4.1
A. solidipes	356 ± 116.2	2.85 ± 0.741*	6.71 ± 16.9
Healthy	607 ± 34.5	4.62 ± 0.21*	31.49 ± 4.85
Declining	658 ± 72.4	4.52 ± 0.44*	32.03 ± 10.18
Dead	468 ± 54.0	3.39 ± 0.33*	9.92 ± 7.59

* Significance based on p =0.05.

Both NMDS and Principal coordinate analysis (PCoA; p = 0.544) revealed no significant differences in the bacterial community diversity for the two Armillaria species. The NMDS identified overlap between bacterial communities of trees associated with *A. altimontana* and *A. solidipes*, indicating that soils associated with *Armillaria* species at the PREF Ida Creek Location have similar soil bacterial communities (Figure 2-3). The complete overlap in the PCoA plot for the two *Armillaria* species indicates no difference between their microbial communities (Figure 2-4).

Of the 6,677 total OTUs, the core bacterial communities for the two Armillaria species consisted of only 955 OTUs (14.3%; Figure 2-5). While a high abundance, 5,643 OTUs (84.5%), were uniquely associated with *A. altimontana*, whereas only 79 (1.2%) were only associated with *A. solidipes* (Figure 2-5). The results of the OTU variation provided similar results as the

NMDS and PCoA plots, which show overlap between *Armillaria* species with soils associated with *A. altimontana* having a greater diversity and richness than that of *A. solidipes*.

All 17 bacterial families were in soils associated with *A. altimontana*, and that quantitatively Pseudomonadadaceae was found in greatest abundance, followed by Chthoniobacteraceae and Pyrinomonadaceae. Within the two soil bacterial communities associated with *A. solidipes*, we observed the largest relative abundance of Enterobacteriaceae, followed by Pseudomonadaceae. Although not significant, there was a greater proportion of AD3, Burkholderaceace, Chthoniobacteraceae, Gemmatimonadaceae, Nitrosomonodaceae, Pseudomonadaceae, Pyrinomonadaceae, Acidobacteria subgroup 2 & 6, and Xanthobacteraceae observed in soils associated with *A. altimontana*, with a greater proportion of Enterobacteraceae in soils associated with *A. solidipes* (Figure 2-6).

Four bacterial taxa were identified that contributed significantly to the comparison between *Armillaria* species using the magnitude of OTU log-fold change (Figure 2-7). A proliferation, at 80% confidence, of Nitrosococcaceae (wb1-P19), Solirubrobacteraceae, Enterobacteriaceae and Gammoproteobacteria_PLTA13_fa were found in *A. solidipes*-associated soils; whereas, only uncultured bacteria were found to be significantly greater in *A. altimontana*-associated soils.

Soils associated with *A. altimontana* had greater soil moisture, root content, charcoal, organic matter, and carbon, while soils associated with *A. solidipes* had higher rock content. Both Armillaria species had similar levels of pH and nitrogen in the soils (Table 2-2).

Table 2-2: Average soil chemical and physical properties and standard errors associated with *Armillaria* species and tree health status.

	Moisture	Rock	Root	Charcoal	pH	OM	C	N
A.alt.	35.52±1.21	26.74±2.36	0.793±0.08	1.17±0.35	5.84±0.04	7.27±0.20	5.31±0.14	0.038±0.01
A.sol.	24.85±2.98	32.98±17.1	0.545±0.05	0.43±0.16	5.97±0.18	6.31±1.67	4.82±0.86	0.037±0.03
Healthy	33.71±1.62	23.62±2.98	0.772±0.11	1.47±0.51	5.83±0.06	7.36±0.08	5.48±0.19	0.041±0.01
Decl.	33.89±2.12	28.52±4.46	0.716±0.25	0.62±0.30	5.92±0.14	6.45±0.39	4.7±0.27	0.017±0.01
Dead	38.75±1.96	34.74±4.69	0.835±0.12	0.55±0.10	5.83±0.04	7.23±0.24	5.09±0.18	0.04±0.01

For the *Armillaria* ANOVA analysis, richness was not significant but there was a significant decrease in bacterial richness as soil moisture was reduced (Table 2-3). Shannon's diversity model was slightly significant across *Armillaria* species, and *A. solidipes* (with only two samples) and soil moisture both had a negative influence on diversity (Table 2-3). Inverse Simpsons was also significant across Armillaria species, as Inverse Simpson diversity had a negative influence on soil moisture.

Table 2-3: Bacterial ANOVA linear models of richness and diversity levels for *Armillaria* species.

Richness			Shannon's			Inverse Simpson's		
	t-value	p-value		t-value	p-value		t-value	p-value
A. alti	0.887	*p*=0.381	*A. alti*	1.123	*p*=0.269	*A. alt*	1.088	*p*=0.284
A. sol	-1.675	*p*=0.103	*A. sol*	-2.140	*p*=0.039*	*A. sol*	-1.614	*p*=0.115
Moisture	-2.038	*p*=0.049*	Moisture	-2.394	*p*=0.022*	Moisture	-2.365	*p*=0.024*
pH	-0.449	*p*=0.656	pH	-0.459	*p*=0.649	pH	-1.001	*p*=0.324
C	0.897	*p*=0.375	C	1.079	*p*=0.288	C	1.248	*p*=0.220
N	1.224	*p*=0.229	N	1.012	*p*=0.318	N	0.626	*p*=0.535
OM	-1.302	*p*=0.201	OM	-1.520	*p*=0.137	OM	-1.275	*p*=0.210
Model		*p*=0.128	Model		*p*=0.075	Model		*p*=0.043*

* Significance based on *p* =0.05.

2.4.4. Data analysis associated with tree health

For tree health, bacterial community richness was not significant (p = 0.0622), but soils associated with declining trees had the greatest richness, while soils associated with dead trees had the least richness. Similarly, for Inverse Simpson's diversity measure, soils associated with declining trees had the greatest diversity levels, whereas soils associated with dead trees had the least diversity (p = 0.0605). On the other hand, healthy and declining trees had significantly higher Shannon's diversity compared to dead trees (p = 0.0113; Table 2-1).

NMDS plots for tree health observed overlap for all three treatments with the greatest deviation between soils associated with declining and dead trees, while similarities are shown for soils associated with healthy and dead trees (Figure 2-8). Similarly, to *Armillaria* species, the PCoA plots have a complete overlap for all tree health statuses, indicating no difference in microbial communities (p = 0.936; Figure 2-9).

Due to the low sample size for *A. solidipes*, for the core communities among tree health status we only used tree health associated with *A. altimontana*. This resulted in a total of 6,598 total OTUs. The core bacterial communities for the three tree health statuses results in 2,129 OTUs (32.3%; Figure 2-5). Similar overlaps were found between health and declining and healthy and dead, while there was reduced overlap between declining and dead, which concurs with NMDS plots.

We found a clear trend in our samples associated with tree health as some taxa were greater in healthy trees, whereas others were greater in dead trees. In subsequent order of healthy, declining, and dead there was a greater proportion of Chthoniobacteraceae, Gemmatimonadaceae, Nitrosomondaceae, Pedosphaeraceae, Pyrinomonadaceae, Rokubacteriales, Solibacteraceae, Acidobacteria subgroup 2 & 6, and Xanthobacteraceae in soils associated with healthy trees (Figure 2-10). Conversely, there was a greater proportion of Enterobacteraceae and Pseudomonadaceae in soils associated with dead trees, while in the

figure there were no families more abundant for soils associated with declining trees (Figure 2-10).

All tree health log-fold analyses were performed at an 80% confidence, although significance is based on 95% confidence. For soils associated with healthy versus dead trees, two separate Pseudomonas taxa were significantly more abundant in dead trees and an unclassified Actinobacteria significantly more abundant in healthy trees. Additionally, although not significant, another Pseudomonas taxon and a Mucilaginibacter taxa were more abundant in dead trees, while an OTU identified as a Subgroup-5 (Acidobacteria) and an unclassified bacterial taxon were more abundant in healthy trees (Figure 2-11). There were no significant OTUs between soils associated with healthy and declining trees. Although not significant, there were more taxa identified as Solirubrobacteraceae, Pedospharaceae, Reyranella, one Alphaproteobacteria, Haliangium, GOUTA6 (Nitrosomonadaceae), RCP2-54, Candidatus Soilbacter, Chthoniobacteraceae, Microbacteriaceae, and Deltaproteobacteria in soils associated with declining trees. While taxa belonging to Chitinophagaceae, Subgroup 5 (Acidobacteria), IMCC26256, one unclassified Alphaproteobacteria, and Rhizobiales Incertae Sedi were identified in soils associated with healthy trees (Figure 2-12). For soils associated with declining versus dead trees, the declining trees had significantly greater abundance of taxa belonging to Pedospharaceae, Haliangium, and an unclassified bacterial, while no taxa were significantly more abundant in soils associated with dead trees. Although not significant, there was a greater abundance of taxa belonging to TRA3-20, GOUTA6 (Nitrosomonadaceae), and an unclassified Betaproteobacteriales in declining soils, whereas an OTU belonging to Chitinophagaceae and an uncultured Rhizobiales Incertae Sedis was more abundant in soils associated with dead trees (Figure 2-13).

Soils associated with healthy trees had greater charcoal and carbon concentrations, while soils associated with dead trees had higher moisture, rock, and root content. All treatments had similar levels of pH, organic matter, and nitrogen in the soils (Table 2-2). For

tree health, no models resulted in significance for richness, Shannon's diversity, or Inverse Simpson's diversity (Table 2-4). Further, no variables had significant effects on the richness or diversity measures (Table 2-4).

Table 2-4: Bacterial ANOVA linear models of richness and diversity levels for Tree health.

Richness			**Shannon's**			**Inverse Simpson's**		
	t-value	**p-value**		**t-value**	**p-value**		**t-value**	**p-value**
Dead	0.592	*p*=0.558	Dead	0.939	*p*=0.354	Dead	1.019	*p*=0.315
Declining	0.845	*p*=0.404	Declining	1.234	*p*=0.226	Declining	0.969	*p*=0.339
Healthy	0.256	*p*=0.799	Healthy	1.182	*p*=0.245	Healthy	1.185	*p*=0.244
Moisture	-1.372	*p*=0.179	Moisture	-1.278	*p*=0.210	Moisture	-1.460	*p*=0.153
pH	-0.387	*p*=0.701	pH	-0.602	*p*=0.551	pH	-1.152	*p*=0.257
C	0.955	*p*=0.346	C	0.791	*p*=0.434	C	0.880	*p*=0.385
N	0.759	*p*=0.453	N	0.536	*p*=0.596	N	0.309	*p*=0.759
OM	-0.982	*p*=0.333	OM	-0.958	*p*=0.345	OM	-0.812	*p*=0.423
Model		*p*=0.354	Model		*p*=0.259	Model		*p*=0.1033

* Significance based on p =0.05.

2.4.5 PERMANOVA analysis of soil factors associated with microbial community change

The PERMANOVA model for *Armillaria* species found that overall bacterial community composition was influenced by pH (p = 0.005), soil moisture (p = 0.010), and charcoal (p = 0.031; Table 2-5). Similar results were found for the PERMANOVA models using tree health and a combination of both treatments (*Armillaria* and tree health). Variables that significantly influenced bacterial community composition were pH (p = 0.005; p = 0.001), soil moisture (p = 0.017); p = 0.014), charcoal (p = 0.024; p = 0.030), and C (p = 0.044; p = 0.038), respectively (Table 2-5).

Table 2-5: PERMANOVA determining significant variables related to overall bacterial community structure. Species signifies differences within *Armillaria* species, while tree health indicates tree health statuses (healthy, declining, and dead).

Species Model		**Tree Health Model**		**Species/Tree Health Model**	
Species	$p = 0.629$	Health	$p = 0.635$	Species	$p = 0.664$
Moisture	$p = 0.010$*	Moisture	$p = 0.017$*	Health	$p = 0.61$
Rock	$p = 0.324$	Rock	$p = 0.271$	Moisture	$p = 0014$*
Root	$p = 0.692$	Root	$p = 0.645$	Rock	$p = 0.255$
Charcoal	$p = 0.031$*	Charcoal	$p = 0.024$*	Root	$p = 0.721$
Other	$p = 0.378$	Other	$p = 0.360$	Charcoal	$p = 0.030$*
pH	$p = 0.005$*	pH	$p = 0.005$*	Other	$p = 0.370$
OM	$p = 0.184$	OM	$p = 0.198$	pH	$p = 0.001$*
C	$p = 0.087$	C	$p = 0.044$*	OM	$p = 0.184$
N	$p = 0.588$	N	$p = 0.772$	C	$p = 0.038$*
				N	$p = 0.647$

* Significance based on p =0.05.

2.5 Discussion

2.5.1 – Significant predictors to bacterial communities associated with Armillaria species

We found that soils associated with *A. altimontana* had a greater bacterial community richness and diversity compared to those associated with *A. solidipes.* Similarly, Koskella et al. (2017) concluded that lower microbial diversity around host trees can result in greater susceptibility to pathogens or that pathogens may reduce diversity via a competitive advantage over other microbes. This is especially important as *Armillaria* species are known to have anti-bacterial properties during the breakdown of host tissue, further reducing bacterial richness and diversity (Dörfer et al., 2019).

Although there were not significant differences in bacterial community alpha and beta diversity between soils associated with different *Armillaria* species, specific taxa were highly abundant in both species. The most abundant family found in soils associated with *A.*

altimontana was Pseudomonadadaceae. The Pseudomonad family is one the most diverse Proteobacteria family identified in soils, as they are comprised of beneficial microbes, plant growth promoters and pathogen controls, as well as pathogens (Sah and Singh, 2016). We identified a high abundance of taxa within Pseudomonadadaceae, though we were not able to determine individual species, yet the inherent beneficial qualities known to be associated to these taxa may assist in the beneficial qualities that trees gain when associated with *A. altimontana*. Pseudomonadadaceae was also present in a high abundance in soils associated with *A. solidipes*, although at lower abundance. As species within Pseudomonadaceae vary in function, taxa could have included beneficial species in association with *A. altimontana,* whereas pathogens or saprobes associated with *A. solidipes*, though more data is required to verify this hypothesis. Another highly abundant family was Chthoniobacteraceae, which have been shown to enhance the breakdown of organic carbon and are associated with fungal symbionts known to increase yield in crops (Wang et al., 2019; Duan and Bau, 2021). As soil microbial communities assist in carbon cycling, the breakdown of organic carbons may act to increase soil health, making soils and trees more resilient to changing conditions. Chthoniobacteraceae are highly abundant soil microbes observed in 70% of soils used in the Earth Microbiome Project (O'Brien, 2016). Lastly, Pyrinomonadaceae are highly abundant in soils associated with *A. altimontana*. Pyrinomonadaceae are within the most abundant soil bacterial phyla, Acidobacteria. Some genera in the family Pyrinomonadaceae are able to withstand harsh environments such as drought or with nutrient limitations, therefore allowing soils to be more resistant to climatic changes or nutrient deficiencies (Wust et al., 2016).

The most abundant family within the soil samples associated with *A. solidipes* was Enterobacteriaceae. This family's functions range from pathogens to beneficials, increasing plant growth by improving nutrient uptake (P, N) most notably via nitrogen fixation (Kumar et al., 2017; Rogers, 2020). The high abundance of Enterobacteriaceae may be a result of *A. solidipes* influencing the soil microbial community, in which the decline of the host by virulent pathogens

may provide an opportunity for additional bacterial pathogens/saprobes to degrade tissue (Koskella et al., 2017). Although Enterobacteriaceae is found in conjunction with soils associated with both *Armillaria* species, this does not determine that they are the same species or OTUs. This is especially clear for *A. solidipes*, which had an 8-fold greater increase in unclassified Enterobacteriaceae over *A. altimontana*. The Enterobacteriaceae taxa in soils associated with *A. solidipes* could be potential contributing pathogens that further degrade the host, however, a finer resolution on the taxa present within Enterobacteriaceae is needed. In contrast, Enterobacteriaceae in soils associated with *A. altimontana* could play a role to increase plant growth via enhanced nutrient uptake, establishing that the interaction with *A. altimontana* and subsequent bacterial communities enhance growth, building off the results of Warwell et al. (2019), but again finer taxa resolution is needed to confirm our hypotheses.

The high abundance of Pseudomonadaceae observed in soils associated with *A. altimontana* implies these soils may be suppressive to pathogens and increase plant growth. Although we were not able to identify specific species within Pseudomonadaceae, Warwell et al. (2019) found that trees associated with *A. altimontana* have an overall increase in diameter and height. Pseudomonads found within the soils may increase overall soil health in soils associated with *A. altimontana* at the PREF site. The most common species within Pseudomonadaceae with pathogen suppression abilities is *Pseudomonas fluorescens*. Combinations of *Pseudomonas fluorescens, Erwinia billingiae*, and *Bacillus simplex* were identified to suppress both *A. mellea* and *H. annosus* in a study using native rhizobacteria (Mesanza et al., 2016). Further research by Mesanza et al. (2019) identified that harvested rhizobacteria, specifically *E. billingiae* and *B. simplex* could be applied during watering to artificially inoculate seedlings to protect against root pathogens. This study recognized that bacterial suspensions are an effective way to introduce beneficial bacterial communities to a soil environment, although there is a loss of efficacy overtime. The ability to identify native rhizobacteria within soils known to be suppressive to root pathogens will enhance our ability to manage forests.

2.5.2 – Significant predictors to bacterial communities associated with Tree Health

We found that soils associated with healthy and declining trees had greater bacterial community richness and diversity than dead trees, with declining trees having the greatest richness and Inverse Simpson's and healthy trees having the greatest Shannon's diversity. Shannon's diversity for tree health was significant, identifying that rare communities were significantly greater in soils associated with healthy and declining trees than dead trees. Although significantly higher Shannon's diversity was observed in healthy trees, the principal coordinate analysis was not significant, as there was overlap among points from all three tree health statuses, indicating that overall bacterial community composition did not differ (Figure 2-6).

Within tree health, there are clear transitions as certain bacterial families were more abundant in soils associated with healthy trees, then declining, and lowest in association to dead trees, and vice versa with dead trees having the greatest abundance for other taxa. Soils associated with healthy trees had the same taxa and community profiles as soils associated with *A. altimontana*, including Pseudomonadadaceae, Chthoniobacteraceae and Pyrinomonadaceae. These taxa function to suppress pathogens, increase carbon cycling, and enhance the resiliency of soils (Sangwan et al., 2004; Sah and Singh, 2016; Wust et al., 2016). All of these characteristics are vital to increase the health of trees to prevent disease and increase nutrient availability. Enterobacteriaceae, on the other hand, was identified in a greater abundance for both declining and dead trees. In association with tree health, Enterobacteriaceae may utilize the influx of organic materials and assist with the breakdown of recalcitrant materials following the death of woody tissue, especially the release of nitrogen and phosphorous.

2.5.3 Role of soil chemistry in bacterial communities

Soil chemistry plays a direct role on microbial activity, as a lack of resources within the soil may be limiting factors to the overall abundance and function of microbes. Soil pH, charcoal

content, soil moisture and carbon had the greatest influence, in order respectively, on overall bacterial community composition while carbon and nitrogen had a positive relationship, as pH, moisture, and OM had a negative relationship with bacterial richness and diversity. When analyzing tree health status, no variables significantly influenced bacterial richness or diversity indices, inferring that soil chemistry did not have a direct relationship with bacterial richness and diversity associated with tree health in our study.

The most significant soil chemical variable in our study was pH, which corresponds to numerous studies that highlight that pH may determine which bacterial communities are present within a site (Fierer and Jackson, 2006; Rousk et al., 2010; Prescott and Grayston, 2013). The Ida Creek sampling site had acidic soils ranging from pH 5.07 to 6.36, with little variation in pH among our treatments. Soil bacterial communities tend to be less diverse in acidic soil (Fierer and Jackson, 2006), yet soil pH at the PREF had a negative influence on bacterial richness and diversity. Typically, acidic soils have been shown to be dominated by *Acidobacteria* and *Alphaproteobacteria*, while *Bacteroidetes* and *Actinobacteria* are more prevalent in basic soils (Lauber et al., 2009; Baldrian et al., 2012; Llado et al., 2018). We identified a greater abundance of Acidobacteria associated with *A. altimontana* and healthy trees, and a greater abundance of Proteobacteria associated with *A. solidipes* and dead trees. Additionally, bacterial communities may be influenced by the presence of charcoal as it increases the pH of the soil, thereby enhancing bacterial richness and diversity. Charcoal can act as a means to store valuable nutrients and inhibits leaching of resources (Brockett et al., 2011). Soils associated with *A. altimontana* and healthy trees had the greatest amount of charcoal, as the myriad of bacterial communities are better equipped to breakdown charcoal and utilize the nutrients.

A negative relationship for soil moisture was identified with bacterial richness and diversity, indicating that an increase in moisture reduced bacterial richness and diversity which corresponds to Gömöryová et al. (2013), in which microbial diversity and activity decrease with soil moisture in old growth montane forests in Slovakia. Increased precipitation may result in an

increase in soil organic material breakdown, releasing nutrients into the soil (Cui et al., 2019). This may, in turn, elucidate a response for highly abundant microbial communities to utilize the resources more readily, decreasing more rare communities.

In the PREF, openings, caused by previous mortality or thinner canopies due to declining trees, may provide small niches for microbial communities as increased sunlight and less water interception would cause microsites that differ in moisture levels. Similar to Gömöryová et al. (2013), samples associated with dead trees had higher moisture levels which may have led to less richness and diversity, while soils associated with *A. altimontana* had higher moisture content most likely due to increased shade allowing for greater water retention during warm summer months. These locations may also be prone to drying-rewetting cycles during the drier months of June and July, which may affect soil microbial community structure. These cycles can cause an influx in activity if the soils encounter drought environments, then receive precipitation (Fierer and Schimel, 2002).

Carbon provides an energy source for both microbes and plants (Brockett et al., 2011). We observed greater bacterial diversity associated with high levels of carbon may result from the release of carbon into the roots to increase microbial activity in the rhizosphere. Healthy trees are likely to provide sufficient root exudates to these microbial communities, which may increase the overall richness and diversity, although our ANOVA model did not indicate that carbon was significant for richness or diversity, carbon was greatest in soils associated with *A. altimontana* and healthy trees. Fierer et al. (2007) found that soils rich in organic carbon have more β-Proteobacteria and Bacteroidetes and less Acidobacteria, which corresponds to our data as we observed a high abundance of Proteobacteria compared to Acidobacteria.

2.5.5 Limitations of the study

Since this study took a random sample of all known western white pine in the Ida Creek location, there was no known sample size for both *A. altimontana* and *A. solidipes*. The result of our study found only three trees with *A. solidipes* infections, yet the population of infected trees

is most likely far greater at the site. To mitigate this issue, a full survey for all trees would provide sufficient trees associated with both *A. solidipes* and *A. altimontana*. The ability to have a greater sample size for both *Armillaria* species may have resulted in more significant differences between soil microbial communities associated with a highly virulent and beneficial species of *Armillaria*.

Collecting soil DNA is a snapshot of all microbial communities, regardless of their activity, at a single time point. In future studies, the ability to utilize soil RNA can focus on active (live) communities rather than DNA which gives a broad scale of presence (alive and dead). This could also be accomplished in a temporal study to focus on how the soil microbiome evolves or transitions following the initial infection of a root disease. Although unsuccessful in causing infection, we attempted to expand on this theory by inoculating three-year old western white pine seedlings with *A. solidipes* and *A. altimontana* in a greenhouse setting at CSU. Although infections did not occur, the premise of this experiment may provide insight as to how soil microbial communities change over time in the presence of root disease. We identified that significant differences occurred based on the timing of soil samples, as pre inoculation treatments had higher fungal richness and diversity measurements. Further research is needed to identify the adaptation of soil microbial communities following the infection of root disease, yet this research may establish microbial community change in the development of seedlings.

2.6 Conclusion

Understanding the dynamics between plant-microbial-pathogen interactions will inevitably increase our knowledge of disease management. In this study, we identified bacterial communities associated with competing *Armillaria* species, *A. altimontana* (less virulent) and *A. solidipes* (highly virulent), and three levels of tree health. Though we had a small sample size for trees associated with *A. solidipes*, our analysis indicated slightly higher richness and diversity indices for trees associated with *A. altimontana*. These data suggest that distinct soil bacterial communities associated with the two *Armillaria* species, and with increased sample

size these differences may have been more apparent. Richness and alpha diversity were higher for soils associated with *A. altimontana* and healthy/declining trees. The most abundant bacterial taxa observed may function as beneficial microbes, plant growth promoters, pathogen suppression, and nutrient cycling, which increases the health of trees, directly or indirectly. Additionally, soil properties were observed to influence microbial communities. Most notably, soil moisture, pH, carbon, and charcoal may have a direct influence on the richness, alpha diversity, and overall microbial composition.

With these data we are not able to directly identify specific suppressive bacterial taxa; however, we did identify potentially beneficial bacterial families associated with *A. altimontana.* Our results infer that bacterial communities may enhance the growth and expansion of less virulent (potentially beneficial) *Armillaria* species. This research found a high abundance of Pseudomonadaceae, Chthoniobacteraceae and, Pyrinomonadaceae in soils associated with *A. altimontana*. These families represent a variety of potentially beneficial qualities that assist in the understanding as to why trees associated with *A. altimontana* are healthier than trees without the presence of *Armillaria*, as highlighted in Warwell et al., (2019). As *A. altimontana* has displaced *A. solidipes* at the Ida Creek location at the PREF, there is inherent beneficial qualities in the relationship between *A. altimontana* and soil microbial communities. Therefore, this research may play a direct role in the utilization of soil microbial communities to increase the management of root diseases. Further research is needed to understand the functional qualities of the microbial communities associated with *Armillaria* species at the PREF site.

Figures

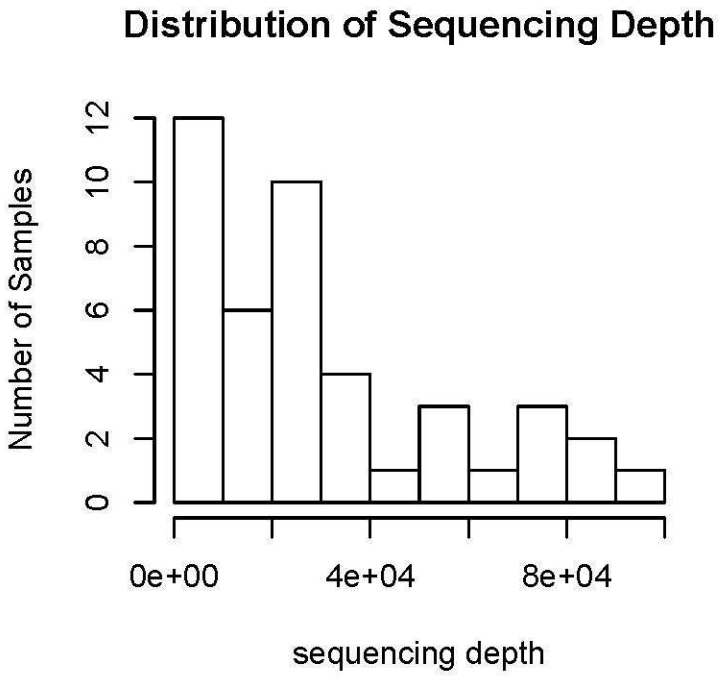

Figure 2-1: Sequence depth for all 16S data analysis following high-throughput sequencing.

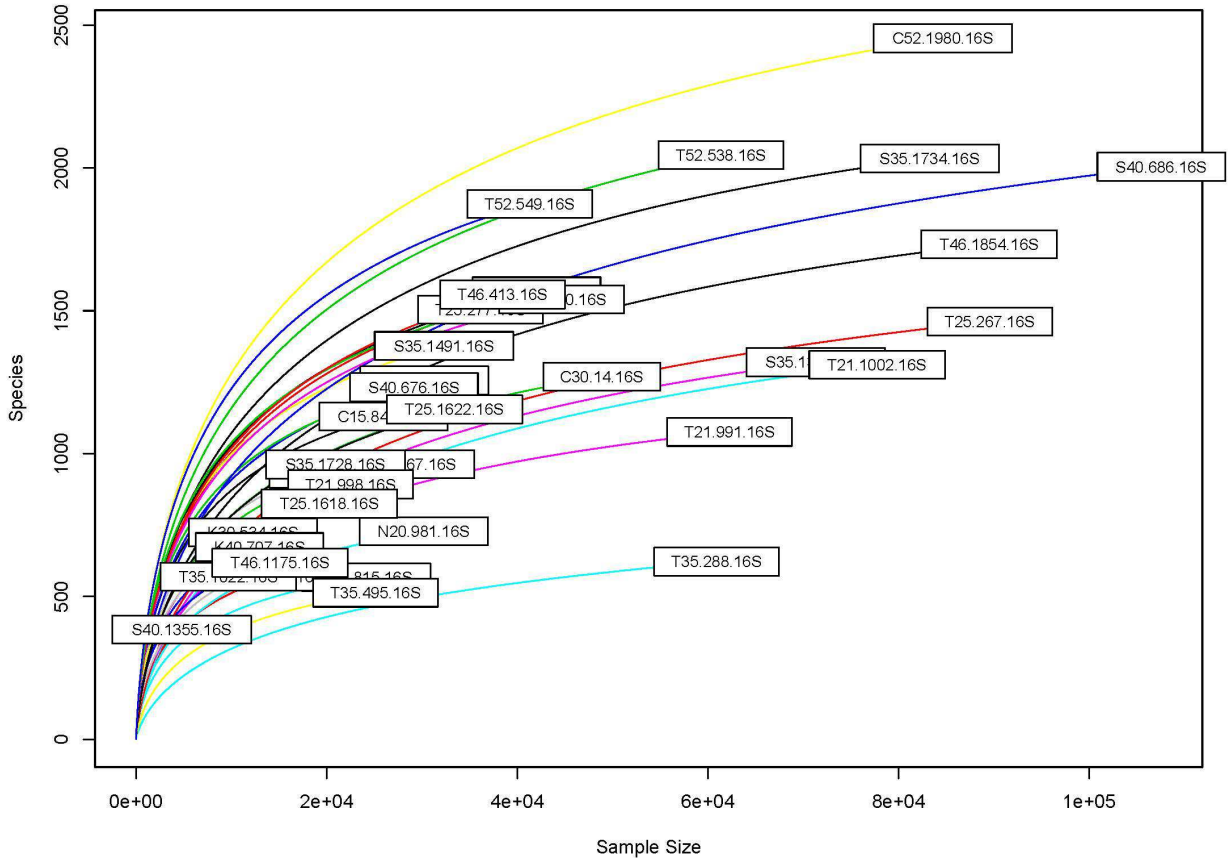

Figure 2-2: Rarefaction curve for all 16S microbial samples. Most samples continue to rise in their slope without a plateau, indicating greater sequence depth would have improved the study.

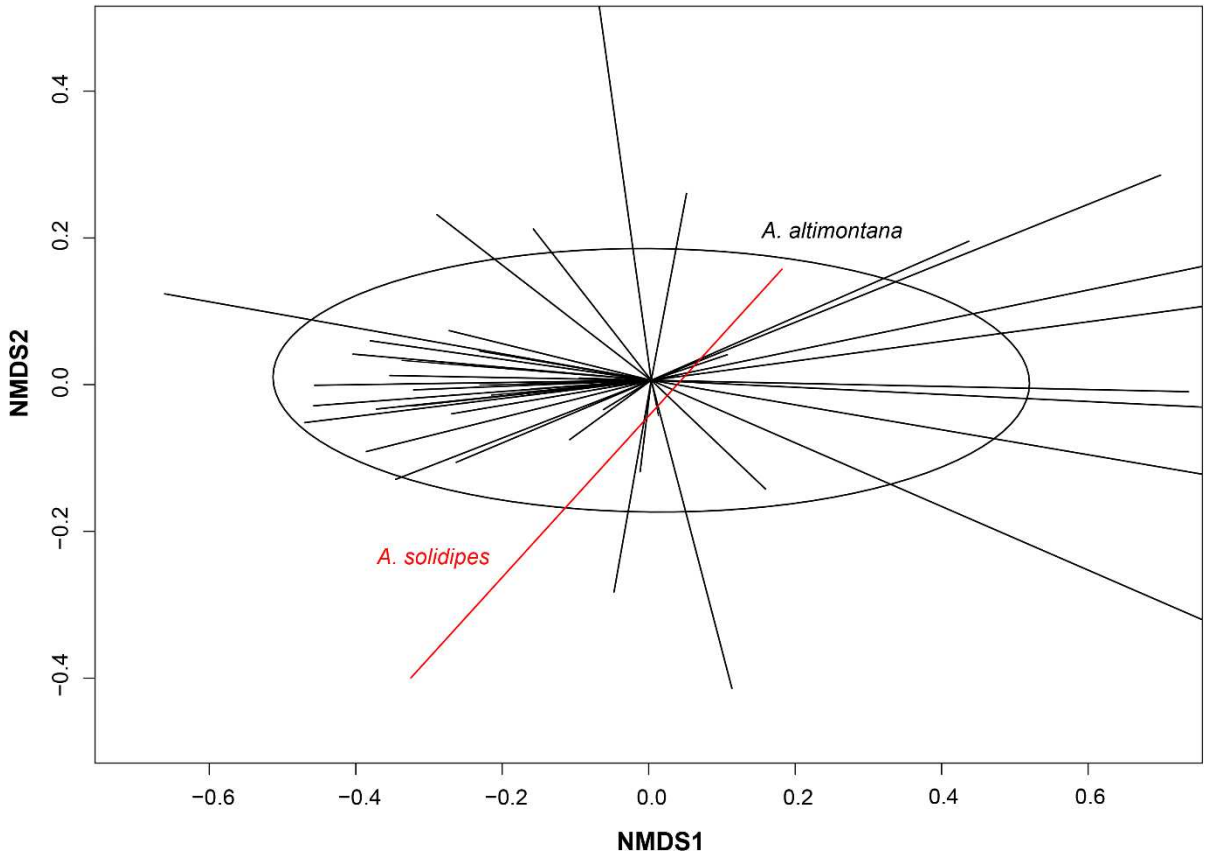

Figure 2-3: Non-Metric Multidimensional Scaling (NMDS) plot for bacterial communities associated with *Armillaria* species. Slight deviation occurs with *A. solidipes* with some overlap indicating microbial communities are similar with minor dissimilarities.

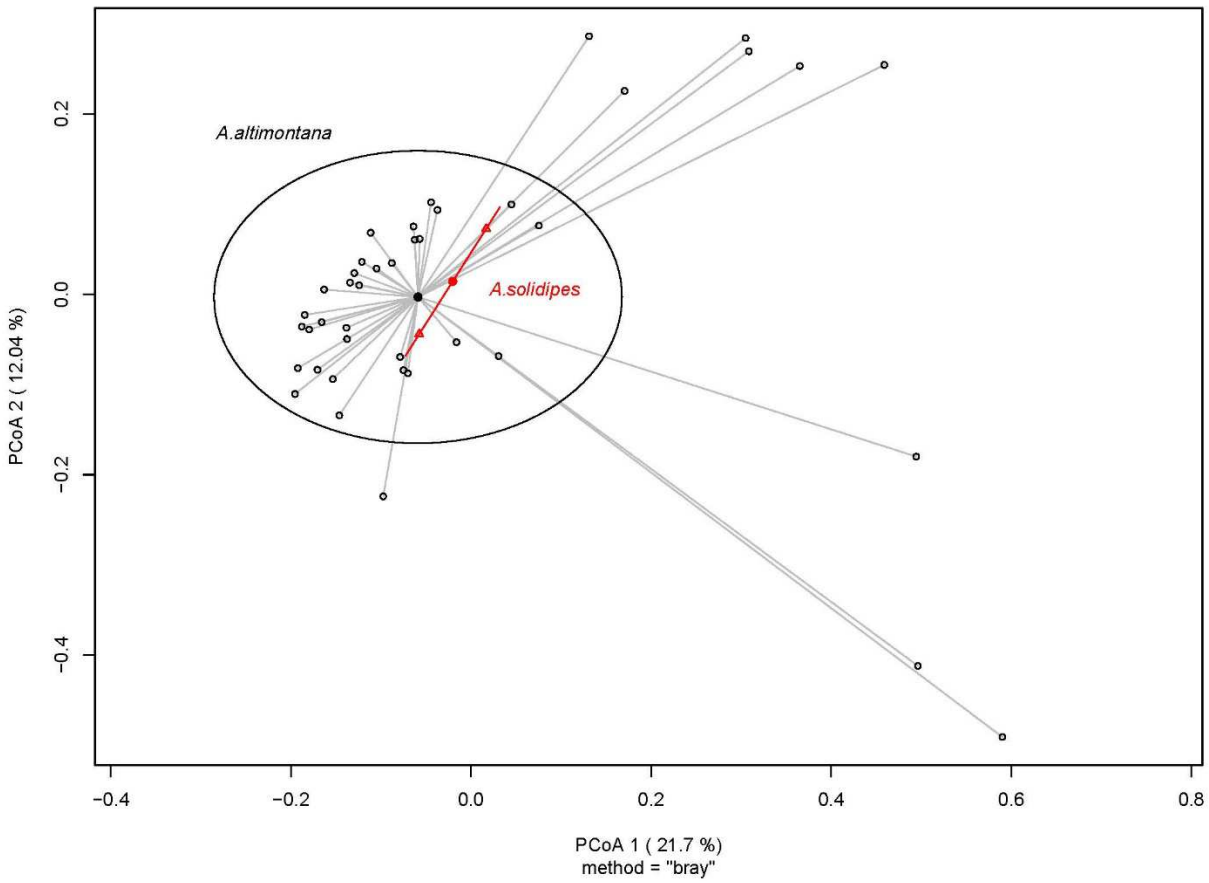

Figure 2-4: Principal coordinate analysis plot for bacterial communities associated with *Armillaria* species. There is complete overlap between treatments, which highlights the lack of significance (p = 0.544).

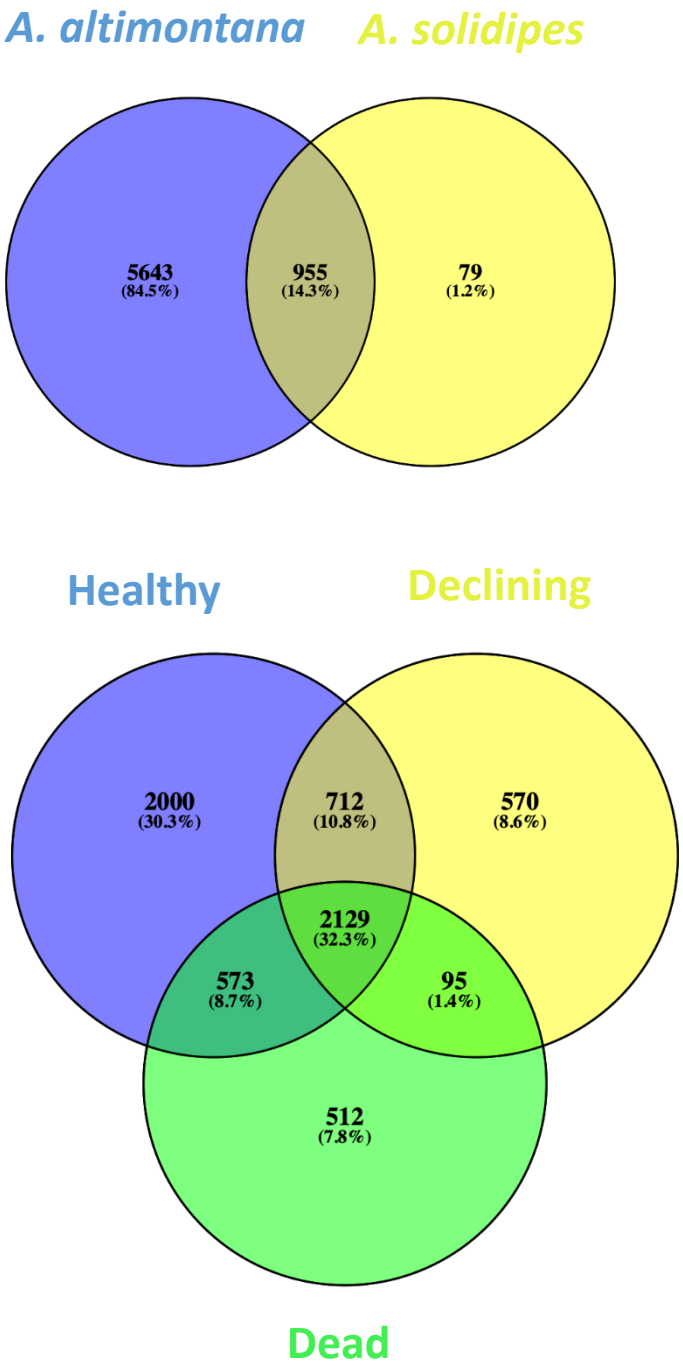

Figure 2-5: Microbial communities (OTUs) between *A. altimontana* and *A. solidipes*. Core microbiome encompasses overlap between both species, while unique OTUs occur within each circle (top). Microbial communities associated to tree health status (Healthy, moderate, and dead). Core microbiome encompasses overlap all three groups, while interacting OTUs occur between two groups. Unique OTUs occur within each of the three circles (bottom).

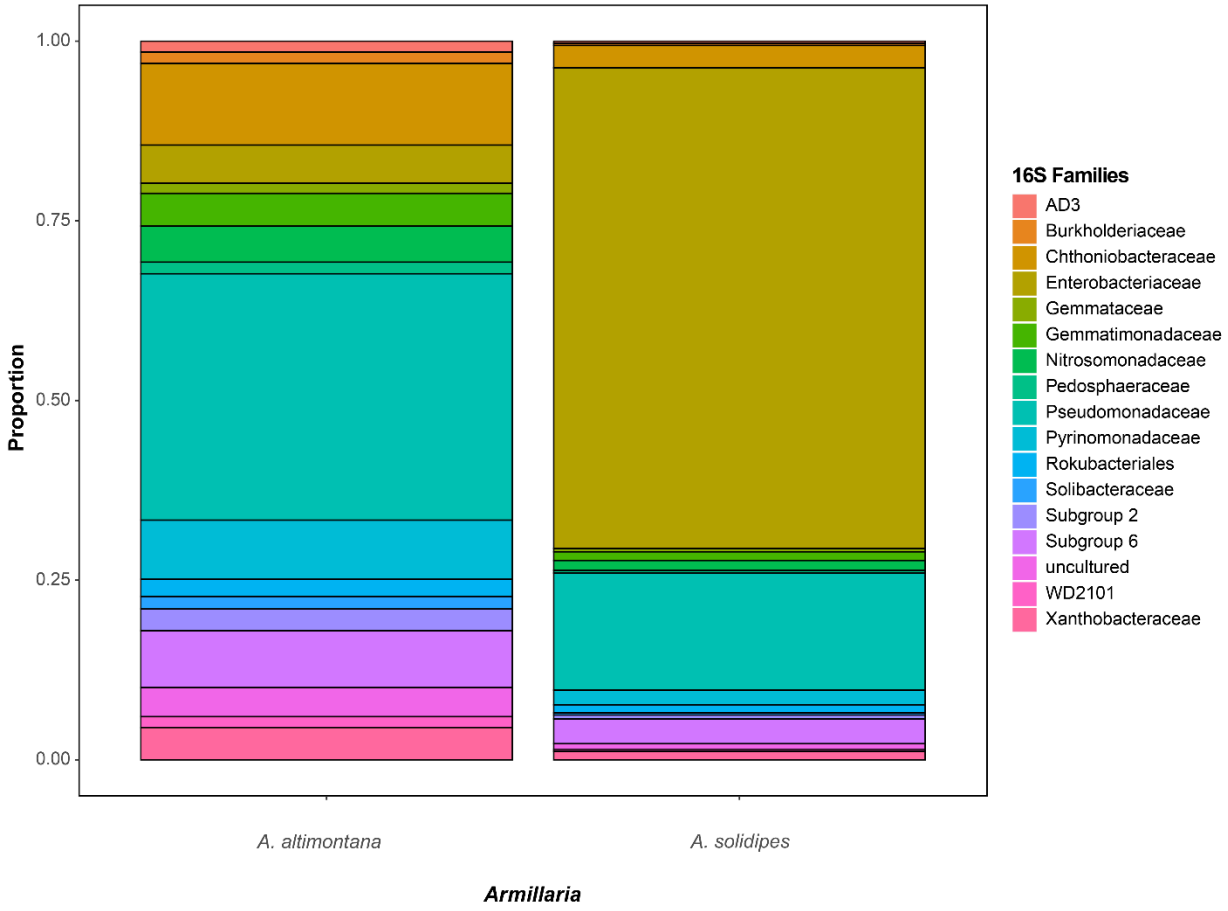

Figure 2-6: Stacked bar graphs of top 17 most abundant bacterial taxa for soils associated with *A. altimontana* and *A. solidipes*. Bar graph signifies bacterial taxa that result in greater than 1% overall taxa abundance.

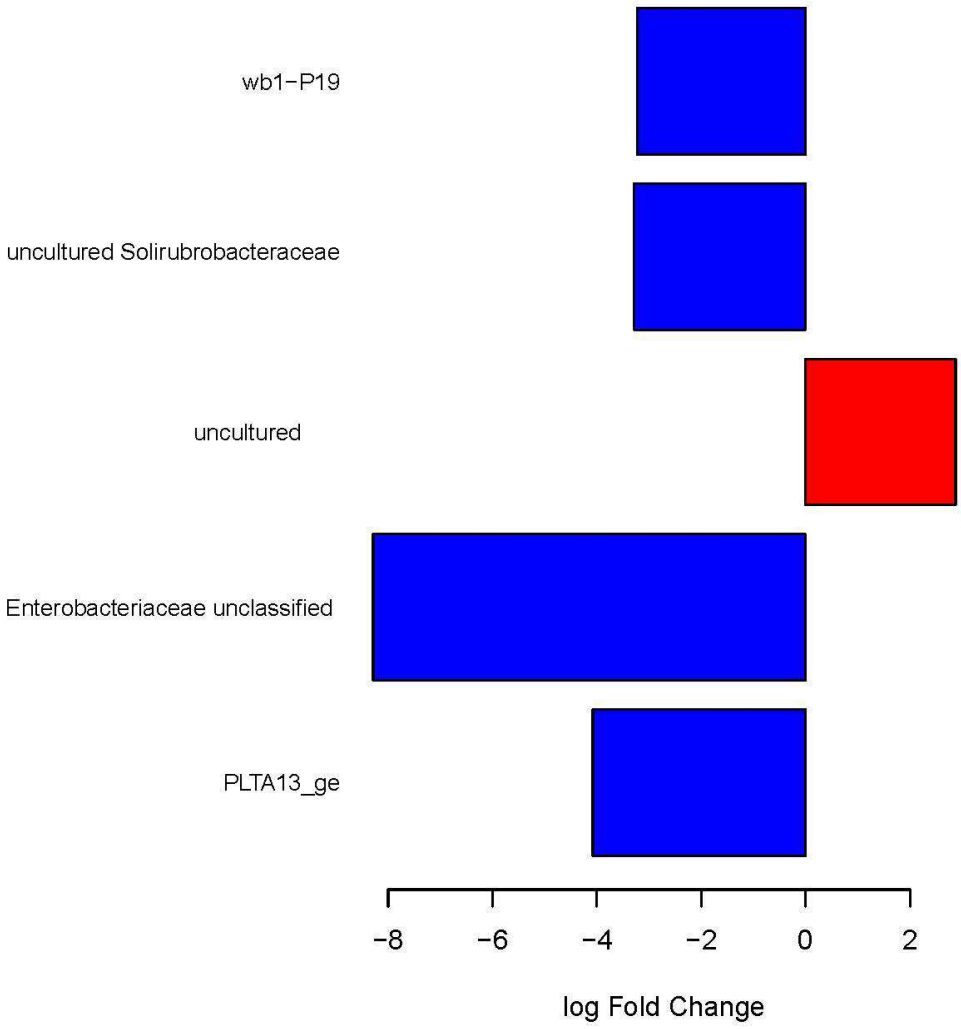

Figure 2-7: Log fold change determining OTUs differing in soils associated with *A. altimontana* (red) and *A. solidipes* (blue). Log fold change is analyzed at 80% significance as no OTU is significant at p = 0.05.

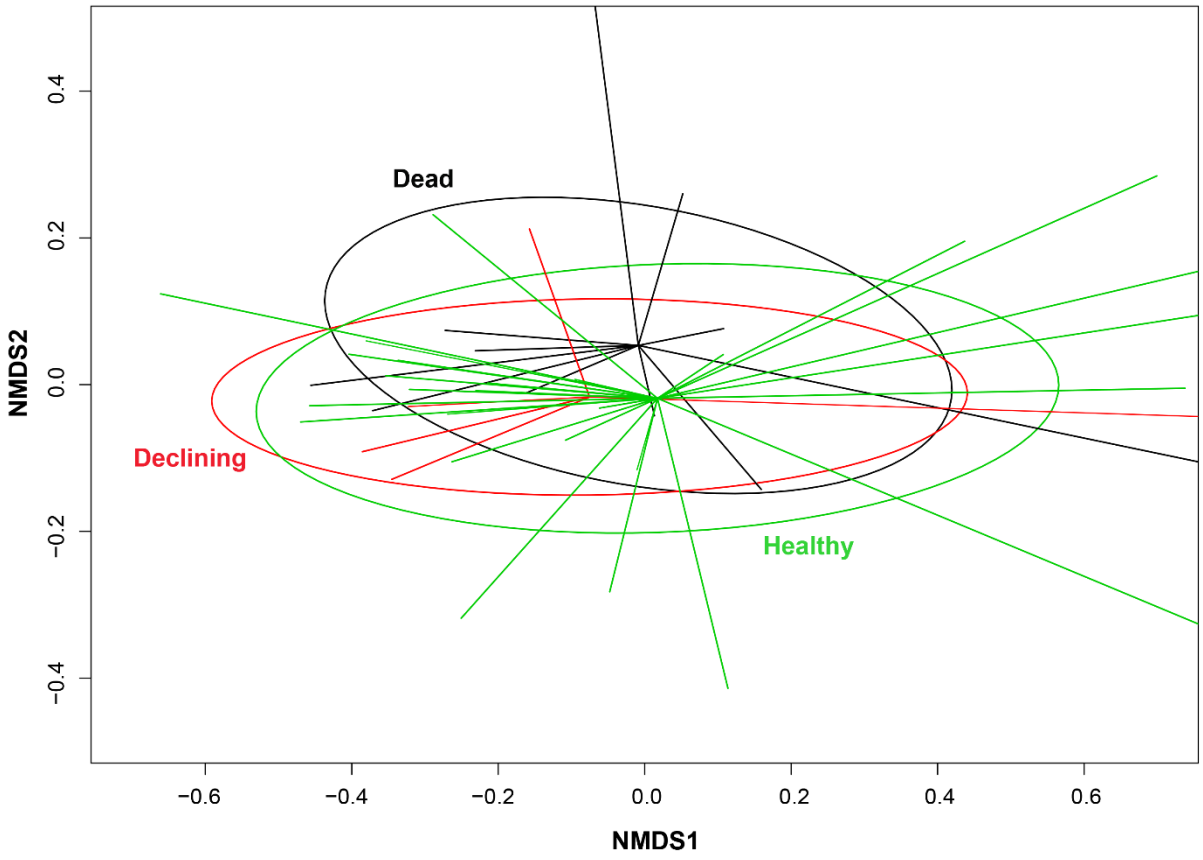

Figure 2-8: Non-Metric Multidimensional Scaling (NMDS) plot for bacterial communities associated with tree health. All complete overlap between treatments indicates that bacterial communities are similar.

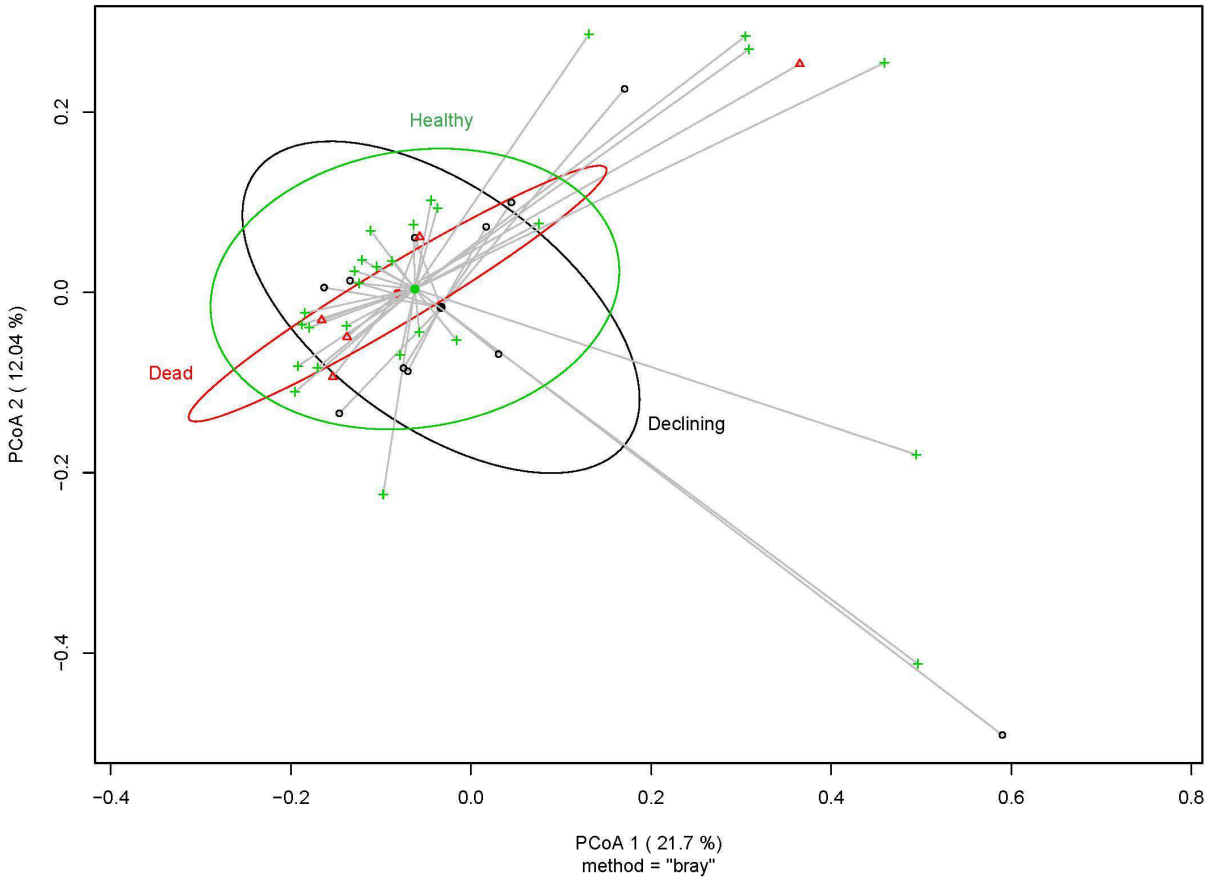

Figure 2-9: Principal coordinate analysis plot for bacterial communities associated with tree health. There is almost complete overlap between all treatments, which highlights the lack of significance (p = 0.936).

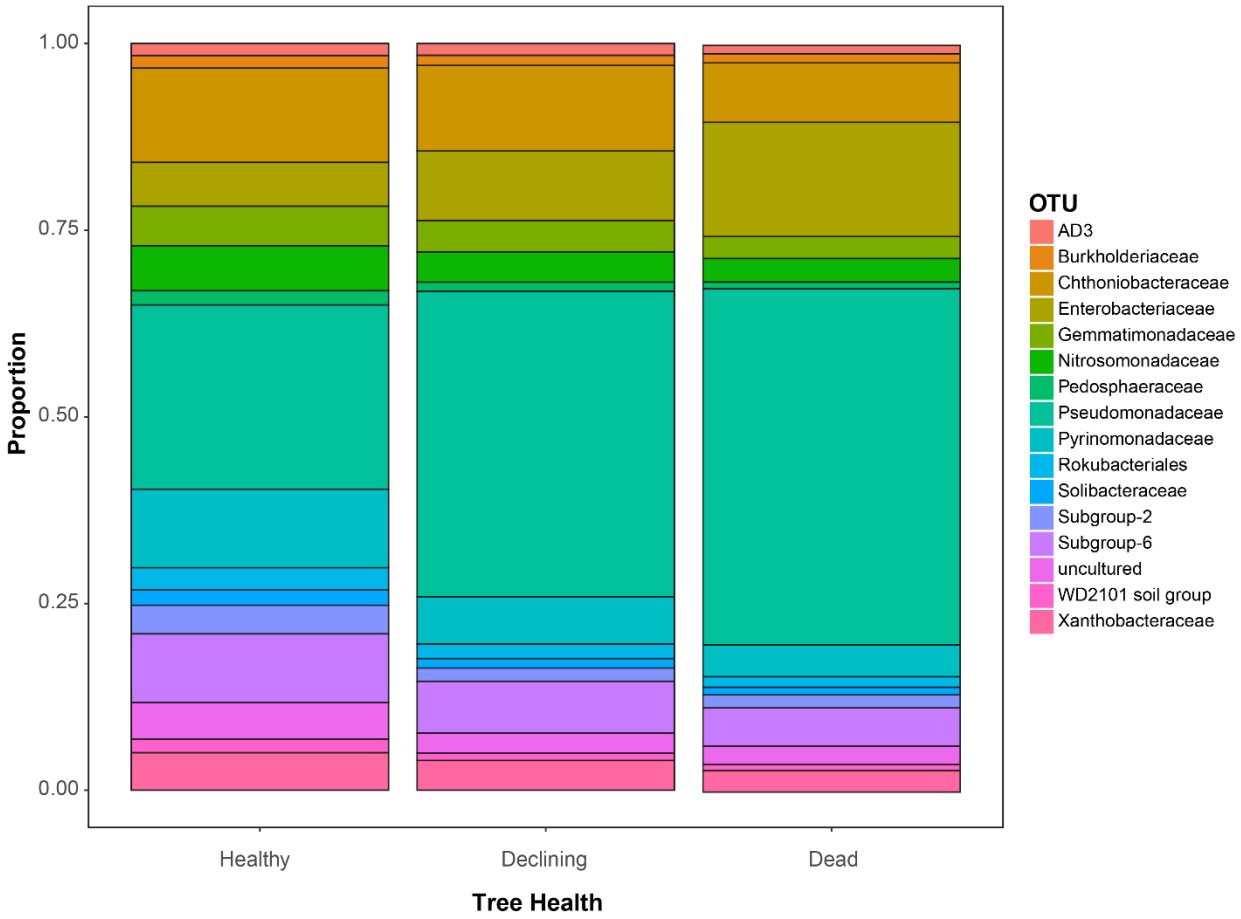

Figure 2-10: Stacked bar graphs of top 16 most abundant bacterial taxa for soils associated with tree health status (Healthy, declining, and dead). Bar graph signifies bacterial taxa that result in greater than 1% overall taxa abundance.

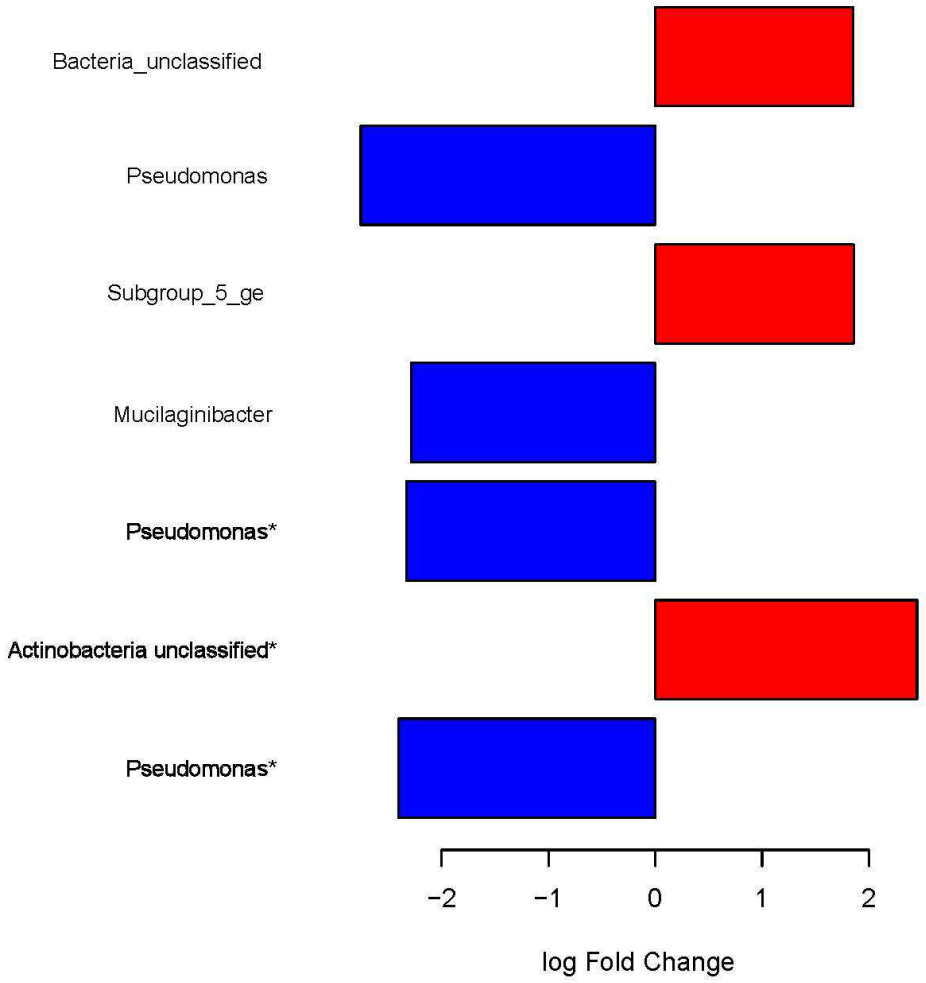

Figure 2-11: Log fold change determining OTUs differing in soils associated with healthy (red) and dead (blue) trees. Log fold change is analyzed at 0.2 significance to expand on difference outside of p = 0.05. Asterisks (*) indicates significance at p = 0.05.

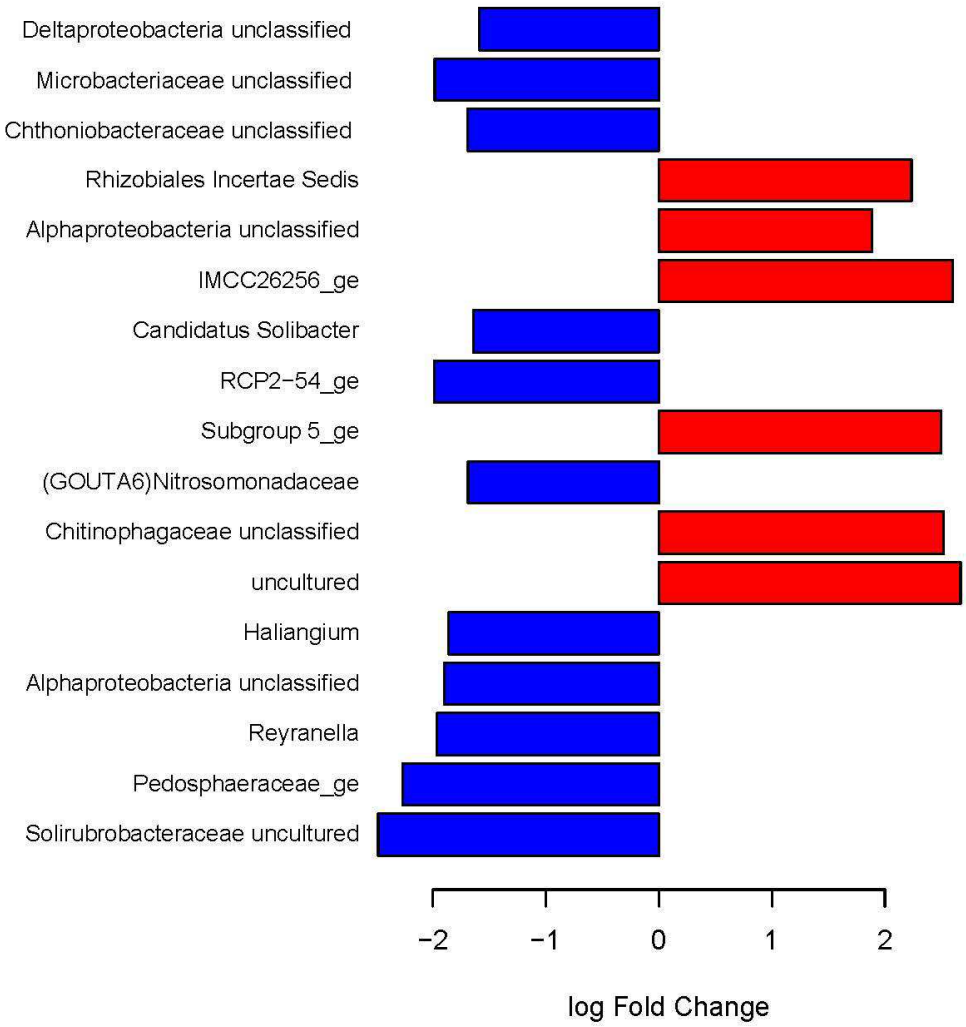

Figure 2-12: Log fold change determining OTUs differing in soils associated with healthy (red) and declining (blue) trees. Log fold change is analyzed at 0.20 significance as no OTU is significant at p = 0.05.

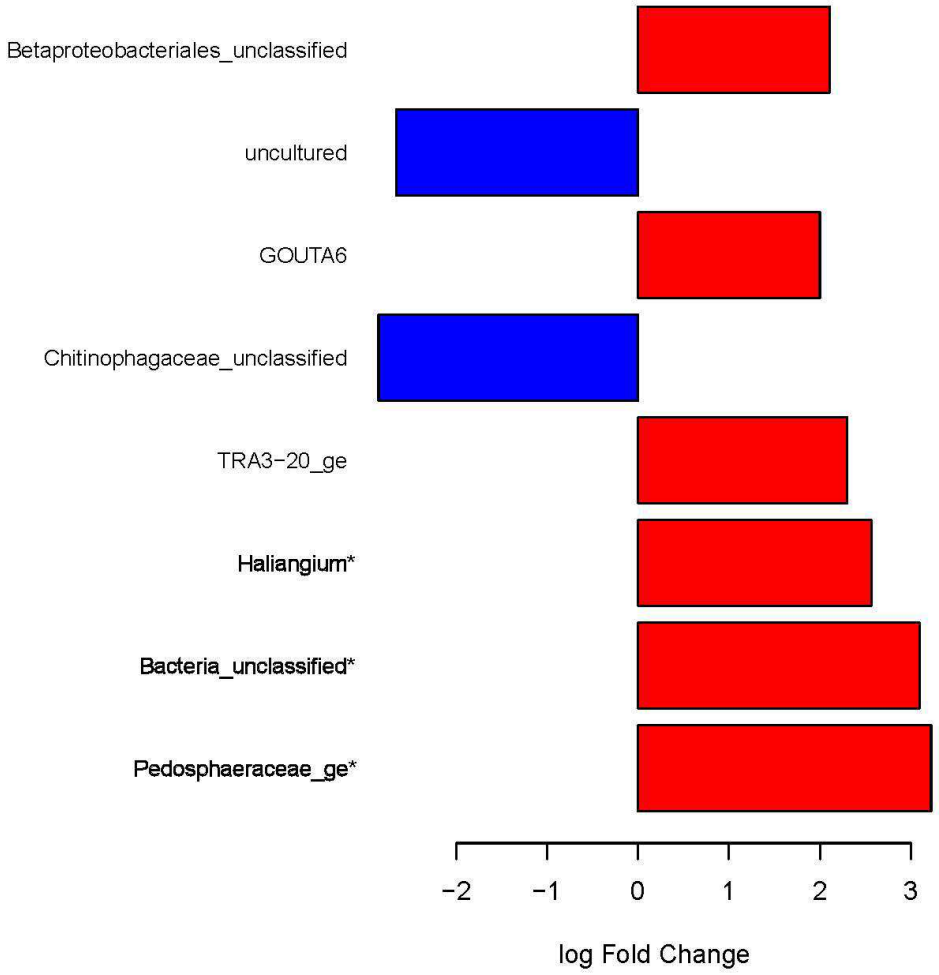

Figure 2-13: Log fold change determining OTUs differing in soils associated with declining (red) and dead (blue) trees. Log fold change is analyzed at 0.2 significance to expand on difference outside of p = 0.05. Asterisks (*) indicates significance at p = 0.05.

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CHAPTER 3: SOIL MICROBIAL COMMUNITIES USED TO DETERMINE DIFFERENCES IN SPECIES COMPOSITION AT THE PIKES PEAK FOREST DYNAMICS PLOT

3.1 Preface

Forest soils provide many essential ecosystem functions that impact tree life histories, some are beneficial such as nutrient cycling, decomposition, and symbiotic mycorrhizal interactions, while others are detrimental such as plant pathogens (Lladó et al., 2017). The understanding of these processes is well known yet the relationship as to which taxa are associated with tree species is less understood. The ability to identify baseline soil fungal and bacterial communities associated with forest types may allow land managers to enhance soil microbe richness and diversity to further increase forest health. The Pikes Peak Forest Dynamics Plot, near Woodland Park, CO is a 17.6 ha dry-mixed conifer site, on the Pike-San Isabel National Forest. There are six tree species that are frequently found throughout the Central and Southern Rocky Mountains. The ability to have numerous tree species in one location provides an opportunity to analyze the influence forest type may have on soil microbes, while reducing extenuating variables, such as elevation and climate, that influence the entire site similarly.

One-hundred and fifteen soil samples were collected and summarized to evaluate the influence that dominant seedlings, understory forest type, overstory forest type, pH and distance to the nearest mature tree had on the soil bacterial and fungal communities. Additional measurements were provided to determine the influence of site, stand, and soil edaphic properties. Our research found that dominant seedlings had a direct influence on both the soil fungal and bacterial communities with greater richness and diversity associated with dry-mixed species compared to wet-mixed. Distance to the nearest mature tree and soil pH also were correlated as driving differences in alpha diversity and community structure of the soil fungal

communities. Samples taken further away from trees and more neutral pH soils had the greatest richness and diversity for both bacterial and fungal communities.

Understory and overstory forest type, was trending to significant, with soil fungal communities, while no other categories influence soil bacterial communities. Additionally, fungal communities had a small core microbial community, in conjunction with understory and overstory forest type and distance to the nearest mature tree, indicating that unique fungal taxa were directly related to specific forest types and within the rhizosphere and bulk soil. Whereas soil bacterial communities had a highly abundant core community, with few unique taxa indicating that at a stand level forests soils generally have similar bacterial community composition. These results further the understanding that forest soil fungal communities tend to have host specificity, identified to direct tree species and forest type, whereas bacterial communities are more ubiquitous within a soil ecosystem (Crowther et al. 2014).

Additionally, soil pH was the most significant predictor to soil bacterial communities, while macro nutrients, carbon, phosphorous, nitrogen, iron, aluminum, and manganese also influenced bacterial communities, whereas phosphorous, nitrogen, aluminum, and potassium influenced fungal communities. Although edaphic variables were identified to provide an influence on soil and bacterial communities, there were not large differences in all soil edaphic properties. This study was able to isolate the factor of forest type at the Pikes Peak Dynamics Plot to further understand the influence that regeneration and understory and overstory forest type plays on fungal and bacterial communities. The research confirms additional studies that forest type has a greater influence on soil fungal communities, whereas soil edaphic properties may have a greater influence on soil bacterial communities. Yet, there is still a direct influence from dominant regeneration on both fungal and bacterial communities. These data provide baseline soil microbial communities for forest types frequently found in the Rocky Mountains, which establishes the need to understand if there are similarities or differences found at a landscape level, rather than stand level.

3.2 Introduction

Studying soil microbial communities within forest ecosystem is inherently difficult. This is especially due to the large diversity of soil microbes, as soil may harbor more than 5,000 organisms in just one tablespoon (Buee et al., 2009; Fierer, 2017). In highly diverse forest ecosystems, many aspects can influence change below-ground (Liu et al., 2020a). This is even more complex in forests in the Western United States as they encounter increased mortality due to abiotic and biotic agents (Cortés and Moltzan, 2020). A trend to warmer, drier climates may shift species composition and range (Williams and Dumroese, 2014). The dynamics of understory trees, as there is mortality in the older cohort, can provide context for future forests. Transitioning to younger stands can influence nutrient cycling and shift microbial communities (Mayer et al., 2017). Forests that are more diverse, above and below ground, may be more resilient to change (Gillespie et all. 2020). Comparatively, above-ground ecosystems are relatively low in diversity, as the greatest overall diversity in a forest ecosystem exists below-ground (Buee et al., 2009). Improving our knowledge of forest soil microbiome can facilitate precision forestry to promote resilience to disturbance and climate change.

Soil microbiomes, in a forest, act as decomposers, nutrient cycling, symbionts, and pathogens (Lladó et al., 2017), which result in the breakdown of litter and down woody material to release vital nutrients back into the soil, all of which enhance tree health and productivity (Chodak et al., 2016; Lladó et al., 2017; Habiyaremye et al., 2020). Fungi are important in forested environments as extracellular enzymes are able to degrade recalcitrant (woody) material, whereas bacteria also degrade cell walls and fix nitrogen to increase nutrient availability (Lladó et al., 2017). As forest microbial communities are primary drivers of ecological processes, there are inherent links between soil microbes and above-ground vegetation (Prescott and Grayston, 2013). Soil microbes have direct and indirect relationships with above-ground vegetation, especially with tree species, as the interaction between the rhizosphere and leaf litter influence soil microbial communities separately (Ladygina and Hedlund, 2010; Thoms

et al., 2010; Aponte et al., 2013; Prescott and Grayston, 2013; Li et al., 2015; Fierer, 2017; Lladó et al., 2018: Gillespie et al., 2020).

Current research on forest soil microbial communities is limited in the Western United States, especially within the Rocky Mountains. Furthermore, understanding whether understory or overstory forests drive changes in microbial communities is not clear. Therefore, the ability to utilize a study that focuses on tree species specific to the Central and Southern Rocky Mountains, using a large sample size, will enhance the understanding of soil microbial communities associated with varying forest types, with potential implications for future management.

Tree establishment leads to wide-spread interactions within the adjacent soil environment, as expansive roots reach out and deeply away from the stem (Brunner and Godbold, 2007). The rhizosphere of tree roots results in a carbon rich environment that increases microbial activity, while also selecting for specific microbes to inhabit the rhizosphere (Tedersoo et al., 2016; Habiyaremye et al., 2020). These tree-microbe relationships may vary depending on soil edaphic properties and climatic factors, therefore understanding local heterogeneity of soil microbial communities within locations with similar edaphic parameters may elucidate the influence trees have on soil microbial relationships (Bokulich et al., 2014; Gourmelon et al., 2016). Additionally, as observed in forest systems, greater above-ground plant species diversity increases below-ground microbial diversity and shifts in the above-ground community can be observed in the corresponding below-ground community (Steinauer et al., 2016). This involves a relationship among understory vegetation and overstory trees that play a role in microbial diversity. Root exudates from a suite of plant species can enhance the resiliency of trees, as diversity below-ground increases in response to above-ground diversity (Rivest et al., 2015; Tedersoo et al., 2016; Rivest et al., 2019). This is promoted by more diverse communities of mycorrhizal fungi that compete for vital nutrients, subsequently improving plant productivity and enhanced nutrient uptake (van der Heijden and Kuyper, 2003;

van der Heijden et al., 2006). These vital nutrients are a direct result of soil organic matter formed by leaf litter.

Leaf litter from various tree species can have differing effects on soil edaphic properties. The organic matter within the soil is a result of the decomposition of overstory plant litter, which indirectly influences soil microbial diversity and richness (Baldrian, 2017a). Additionally, leaf litter directly manipulates soil chemical properties, whereby conifer needles tend to reduce pH levels and deciduous leaves increase pH (Kaiser et al., 2016; Lladó et al., 2018). Although shifts in may occur in below-ground diversity via leaf litter, different plant species have distinct soil microbial communities when it pertains to mycorrhizal fungi and nitrogen-fixing bacteria (Fierer, 2017). Many of these microbes are considered as host-specific and have a direct relationship to certain plants species, especially mycorrhizae and tree species associates (Berg and Smalla, 2009). Trees are inherently connected to the communities below-ground, as a majority of trees have a symbiotic mycorrhizal association, with 90% of trees associated with ectomycorrhizal fungi (EcM; Markkola, 1996). This mutualistic relationship tends to favor fungal communities over bacterial, especially in the rhizosphere where dominant taxa saturate the biomass to reduce overall richness and diversity (Cui et al., 2018). In contrast, mycorrhizal fungi may act as a conduit to the release plant carbon rather than the plant directly exuding carbon (Frey, 2019). The mechanism is considered priming, which releases carbon toward soil organic matter for saprophytic bacteria and fungi to decompose nutrients needed by the mycorrhizae (Frey, 2019).

Forest soils exhibit a wide range of variability, in terms of geographic location, association to tree/plant species, within successional stages, and even at the local scale (Li et al., 2015; Lladó et al., 2018), therefore studies of forest soil microbiome is inherently difficult. This study explores the interactions of tree species, understory plant species and regeneration, site and stand characteristics, and soil edaphic properties with soil microbiome diversity at the 17.6-hectare stem mapped Pikes Peak Forest Dynamics Plot (PFDP) in the Southern Rocky Mountains of Colorado. This stand encompasses an intertwining of forest types, where high

elevation and low elevation merge with dry-mixed conifer and wet-mixed conifer. Specifically, our objectives for the study were to: 1) determine if fungal and bacterial soil microbial communities differ in association with forest habitat types (dry-mixed, wet-mixed, aspen, and open) and what forest ecological properties (i.e., regeneration and soil edaphic) influence richness and diversity, and 2) determine if overstory or understory plant species drive microbial community composition. We hypothesized that microbial community composition will vary across forest habitat types, whereby overstory tree species will have a greater influence on fungal community composition than understory tree species. Direct relationships with fungal symbionts and the increased light and temperature in dry-mixed stand will increase microbial activity and organic matter, enhancing richness and diversity. In addition, soil edaphic properties, specifically pH, carbon, and nitrogen, will directly influence bacterial and fungal communities.

3.3 Methods

3.3.1 Site Factors at Pikes Peak Forest Dynamics Plot

The PFDP was located in the Pike-San Isabel National Forest northeast of Woodland Park, CO. This site was established in 2016 to monitor forest dynamics at a critical ecotone between two forest habitats expected to undergo significant climatic changes over the coming decades. The 17.6 ha site consists of six main tree species: aspen (*Populus tremuloides*), Colorado blue spruce (*Picea pungens*), Douglas-fir (*Pseudotsuga menziesii*), Engelmann spruce (*Picea engelmannii*), limber pine (*Pinus flexilis*), and ponderosa pine (*Pinus ponderosa*). To provide a stem map for the site, the plot was split into a 20 m x 20 m grid, as all trees taller than 1.37 m, were measured for species, diameter at breast height (DBH), height, and crown base height. Overstory and understory interactions were characterized using 5 m x 5 m plots that were spatially distributed across the PFDP to sample the density of tree regeneration (less than 1.37 m tall). Additionally, 1 m^2 plots were nested within each 5 m x 5 m plot to characterize fine-down-dead-woody fuel loading and duff and litter depths. The fine-down-dead-woody fuel

loading was estimated in three fuel classes (particle diameters < 0.635 cm, 0.635-2.54 cm, and 2.54-7.62 cm) using the photoload estimation technique (Keane and Dickinson, 2007), with a double sampling process to correct any estimation bias (Tinkham et al., 2016). Duffer and litter depths were measured at three random points in each 1 m^2 plot and averaged to represent the plot. Coarse woody debris (> 7.62 cm diameter) was sampled using 289.68 m of line intercept within 60 m x 60 m blocks (Brown, 1974).

3.3.2 Bulk density soil sampling

Bulk density soil samples were randomly selected from 115 of the 1 m^2 plots. Soil samples were collected in a three-day time period, from June 13-15, 2017. A six-inch split bulk density soil core (Item # 41112900, AMS Inc. American Falls, ID) was used to sample soil. After removal of the duff and litter layers, the bulk density soil core was driven into the ground using a slide hammer (Item # 41113900, AMS Inc.). Collected soils were placed in one-gallon plastic bags and into a cooler to preserve samples on ice. Between each sample, the soil core was cleaned to remove excess soil and sanitized using 70% ethanol and wiped with KimWipes (Kimberly-Clark Professional, Roswell, GA) to prevent cross contamination of soil microbial communities. At the end of each day, soil samples were placed in a -20° C freezer to further preserve samples. All soils samples were transported back to Colorado State University in coolers and stored in a -20° C freezer until subsequent soil DNA extractions. A subsample of soil was sent to Ward Laboratories Inc. (Kearney, NE) to provide soil health analysis using the Haney Test. The Haney Test consists of analyzing for soil pH, excess lime rating, soluble salts, organic matter, soil respiration, total nitrogen, total organic carbon, total organic nitrogen, H3A extracts, nitrate-nitrogen, ammonium-nitrogen, inorganic nitrogen, total phosphorous, inorganic phosphorous, organic phosphorous, potassium, calcium, and magnesium.

The soil samples were summarized to evaluate the influence that dominant seedlings (highest abundant regeneration species in 5 m^2 plots), understory forest type (dry-mixed, wet-mixed, aspen, and open), overstory forest type (dry-mixed, wet-mixed, aspen, and open), pH

range (<5.0, 5.0-5.4, 5.4-5.9, 6.0-6.4, >6.5) and distance (meters) to the nearest mature tree had on the soil microbiome. Understory forest type was determined based on regeneration species, while overstory forest type was based on mature trees. Wet-mixed conifer consisted of Engelmann spruce, subalpine fir, and Douglas-fir on northern or eastern aspects, while dry-mixed consisted of ponderosa pine and limber pine in southern or western aspects.

3.3.3 Soil DNA extractions and microbial 16S and ITS tagged sequencing

Soil was thawed to reduce clumping of soil for extraction, then homogenized to thoroughly mix the entire sample. Soil samples were weighed, ranging from 100 – 250 mg, and placed into a disruptor tube included in the Omega E.Z.N.A. Soil DNA kit (Omega Bio-Tek Inc., Norcross, GA). Each soil sample was prepared for three DNA extractions to determine microbial communities associated with the sample. All steps from the DNA extraction kit were followed, with minor exceptions, as follows. Following the addition of soil to the disruptor tube, the SLX-Mlus lysis buffer was added to the tubes and placed at 4° C overnight. Samples were then resuspended in the SLX-Mlus buffer and homogenized (Thermo Savant FastPrep ® FP120 Cell Homogenizer; Qbiogene, Carlsbad, CA) for two cycles at 6.0 rpms for 30 seconds. All additional steps followed manufacturer instructions. DNA quantity and quality were determined using a NanoDrop ™ 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). DNA with a concentration above 20 ng/μL and a quality score above 1.7 was considered adequate. Any samples that yielded low quantity DNA were re-extracted using preserved soil. The three extractions for each sample were diluted at either 20 - 30 ng/μL and bulked prior to preparing samples for sequencing.

Subsamples of the 115 bulked soil DNA (30 μL) were sent to Macrogen USA (Rockville, MD) for Illumina Miseq 16S (bacterial) and ITS2 (fungal) tagged sequencing. The Bakt_341F (CCTACGGGNGGCWGCAG) and Bakt_806R (GACTACHVGGGTATCTAATCC) primers were used to amplify the v3-v5 region of the 16S for bacterial communities (Yu et al., 2005). The ITS3 (GCATCGATGAAGAACGCAGC) and ITS4 (TCCTCCGCTTATTGATATGC), which amplifies

the ITS2 region of the internal transcribed spacer region (White et al., 1990) was used for fungal communities. Paired end fasta files were received for all samples at both the 16S and ITS2 regions for further cleaning and analyses.

3.3.4 Cleaning DNA sequence data

DNA sequence cleaning was completed using the protocol from Lalande 2019, in which FastQC (Andrews, 2010), Trimmomatic (v0.36, Bolger et al. 2013) were used to assess and trim data prior to using Mothur to align with reference databases. The paired end sequences were cleaned and aligned using a modified Mothur SOP protocol to reference the sequences to known bacterial and fungal microbial communities (Schloss et al., 2019). The 16S paired end sequences were referenced to the Silva v 128 database (Quast et al., 2013) to determine soil bacterial communities for each soil sample. The ITS2 paired end sequences were referenced to the UNITE fungal reference database, version 02.02.2019 (Nilsson et al., 2019) to determine soil fungal communities for each sample. Rarefaction curves were generated using the package 'vegan' (Oksanen et al., 2013) as implemented in R version 3.6.1 (R Core Team, 2017) to assess diversity and suitability of depth of coverage per sample.

3.3.5 Statistical analysis of microbial sequences

Bulk density soil cores were evaluated based on dominant seedlings, understory and overstory forest type, pH range, and nearest mature tree. The dominant seedlings were split into eight factors: Engelmann spruce (*Picea engelmannii*; n=17), Colorado blue spruce (*Picea pungens*; n=5), limber pine (*Pinus flexilis*; n=6), ponderosa pine (*Pinus ponderosa*; n=23), quaking aspen (*Populus tremuloides*; n=32), Douglas-fir (*Pseudotsuga menziesii*: n=7), *Salix* spp. (n=1), and none (n=24). The dominant seedling characterized the regeneration forest type factors, in which soils were associated with dry-mixed (n=34), wet-mixed (n=25), aspen (n=32), and open (n=24) stands. Similarly, the overstory forest type resulted in dry-mixed (n=42), wet-mixed (n=46), aspen (n-19), and open (n=8) stands. Overstory forest type did not always correspond to the understory, therefore forest type was split to represent the differing stand

dynamics. The nearest overstory tree data were binned into factors of 0-1.0 (n=30), 1.0-2.0 (n=50), 2.0-3.0 (n=27), and >3.0 meters (n=8) from the nearest tree. The category for distance to nearest mature tree were split into one-meter factors to signify the influence that tree roots play on soil microbial composition. Additionally, soil pH ranged from 4.7 to 7.0, and were binned into 6 categories: <5 (n=6), 5.0-5.4 (n=15), 5.5-5.9 (n=28), 6.0-6.4 (n=43), and >6.5 (n=23), again to identify if there was deviation among microbial composition in more acidic to neutral soils.

All statistical analyses were conducted using the RStudio (RStudio, 2015) interface to R. The α-diversity analysis provided context into the diversity for each sample, which included Shannon and Inverse Simpson diversity indices using the 'phyloseq' package (McMurdie and Holmes, 2013), while richness was calculated using 'vegan'. Shannon's diversity incorporates richness and rare microbes, while Inverse Simpson utilizes dominant microbes and evenness (Hill et al., 2006; Nagendra, 2002). Richness is described as the number of individuals identified within a single sample, while evenness explains the relative abundance of the different individuals (Zhang et al., 2012). Richness and diversity measurements were calculated to determine if the differences within each category drive microbial composition. A linear model was used with richness, Shannon's, and Inverse Simpson as response variables and each category (dominant seedling, understory and overstory forest type, nearest mature tree range, and soil pH range) as predictor variables. The summary table was used to determine significance of the model, as averages and standard error were calculated using the 'lsmeans' package (Lenth, 2016). Differences between factors within each category were calculated using 'lsmeans' to determine similarities for bacterial and fungal richness and diversity indices.

Core fungal and bacterial communities were calculated for categories with four or less factors (understory forest type, overstory forest type, and nearest overstory tree range). Counts were calculated for each factor in R to determine the presence of all OTUs for each category. Venn diagrams displayed the communities using Venny 2.1

(https://bioinfogp.cnb.csic.es/tools/venny/) to identify unique and shared (core) fungal and bacterial OTUs.

A random forest classification model analysis (Liaw and Wiener, 2002) was used to build models for bacterial and fungal OTUs based on each stand and soil factor. For each model, 200 trees were used to grow the model. The number of variables randomly sampled at each split varied for each category analyzed. An out of bag (oob) error rate was calculated to determine the model error, based on a confusion matrix, which signifies how accurately samples reflect their category. OTU importance was measured based on mean decrease of the Gini coefficient to depict all OTUs that have significant influence (importance) for each category. The Gini coefficient represents purity of classification from 0 to 1, as in percent of importance (Menze et al., 2009). The higher mean decrease of Gini indicates a higher OTU importance to provide an accurate model for each parameter.

The relative abundance was calculated for fungal and bacterial families and bacterial phyla representing >1% abundance were displayed in a stacked bar graph for all categories (dominant seedling, understory forest type, overstory forest type, pH range, and nearest tree range) using the ‘metagenomeseq’ package in R (Paulson et al., nd). Differences were calculated within categories for diversity and richness using PERMANOVA. Beta-diversity was calculated using the variability of bacterial or fungal community composition between each sample.

A principal coordinate analysis in ‘vegan’ was used to statistically visualize the influence of each category has on fungal and bacterial composition. A log fold change analysis was performed for bacterial and fungal taxa using the ‘metagenomeseq’ package to identify difference at a 95% confidence among overstory and understory forest type and dominant seedlings factors.

Soil edaphic properties and stand and site variables (count of seedlings present, distance to nearest mature tree, trees per hectare and basal area per hectare for all overstory species (individually and total), fuels, slope %, aspect (cos/sin)), were compiled for each soil

sample. A spearmen's correlation was assessed, with all variables, to determine the relationship between variables. A threshold was upheld if variables were 90% correlated, and highly correlated variables were removed prior to performing a linear regression. A backward stepwise linear regression was used, with response variables of richness, Shannon's, and Inverse Simpson's diversity, and the final model was based on the minimized Akaike Information Criterion (AIC). The summary table for each model was used to determine significance based on the relationship of all variables simultaneously on the response variables.

3.4 Results

3.4.1 Processing sequenced 16S and ITS2 libraries in Mothur

The total number of bacterial reads was 4,515,568 with an average read depth of 39,266 reads/sample. The mean sequencing depth after processing was 13,613 reads/sample, with a range of 5,741 and 27,336. After classifying all sequences to the Silva v128 database, the 16S data resulted in 13,695 unique OTUs, 629 genera, 362 families, 221 orders, and 95 phyla. A rarefaction curve identified that no samples reached a plateau, indicating that the bacterial data could have been sequenced at a greater depth (Figure 3-1).

The total number of fungal reads was 4,465,378 with an average read depth of 38,829 reads/sample. The mean sequence depth after processing was 24,288 reads/sample, with a range of 12,612 and 42,859. After classifying all sequences to the reference UNITE dataset, version 02.02.2019, the ITS data resulted in 2,600 unique OTUs, 509 genera, 282 families, 128 orders, and 53 phyla. A rarefaction curve resulted in a plateau for all samples, indicating that the sample depth was sufficient for the fungal reads (Figure 3-1).

3.4.2 Differences in bacterial and fungal community α-diversity and richness

Bacterial richness was influenced by understory forest type and pH, while fungal richness was influenced by dominant seedlings, understory and overstory forest type. Bacterial Shannon's diversity was influenced by understory forest type and pH, whereas fungal Shannon's diversity was only influenced by overstory forest type. Additionally, bacterial Inverse

Simpson's diversity was influenced by dominant seedlings, pH, and nearest overstory tree, and fungal Inverse Simpson's diversity was only influenced by pH.

Table 3-1: Richness and diversity indices calculated for all categories of analysis; Dominant seedling species, Understory forest type (**U**), Overstory forest type (**O**), pH and distance to nearest mature tree (meters). Values represent within group mean and standard errors.

	Bacterial (16S)			Fungal (ITS)		
	Richness	**Shannon**	**InvSimpson**	**Richness**	**Shannon**	**InvSimpson**
Dominant Seedling			*	*		
None	1820 ± 42.2^{A}	6.59 ± 0.05^{A}	140 ± 8.45^{A}	146 ± 7.10^{A}	3.41 ± 0.11	16.12 ± 1.65
PIEN	1740 ± 50.5^{A}	6.43 ± 0.06^{B}	114 ± 10.04^{B}	115 ± 8.44^{B}	3.06 ± 0.13	12.85 ± 1.96
PIPU	1745 ± 93.2^{A}	6.51 ± 0.11^{B}	106 ± 18.52^{B}	114 ± 15.55^{A}	2.93 ± 0.24	11.53 ± 3.61
PIFL	1881 ± 85.0^{A}	6.56 ± 0.10^{A}	123 ± 16.91^{A}	119 ± 14.2^{A}	3.12 ± 0.22	10.46 ± 3.30
PIPO	1898 ± 43.4^{A}	6.70 ± 0.05^{A}	158 ± 8.64^{A}	146 ± 7.25^{A}	3.33 ± 0.11	13.79 ± 1.68
POTR	1796 ± 36.8^{A}	6.56 ± 0.04^{A}	132 ± 7.32^{A}	140 ± 6.15^{A}	3.22 ± 0.09	13.98 ± 1.43
PSME	1692 ± 78.7^{A}	6.49 ± 0.09^{A}	116 ± 15.65^{A}	106 ± 13.15^{B}	2.95 ± 0.20	10.64 ± 3.05
SALIX	1814 ± 208.3^{A}	6.57 ± 0.25^{A}	105 ± 41.41^{A}	123 ± 34.78^{A}	3.11 ± 0.54	8.88 ± 8.07
Understory	*	*		*		
Aspen-**U**	1786 ± 36.5^{A}	6.56 ± 0.04^{A}	132 ± 7.49^{A}	140 ± 6.24^{A}	3.22 ± 0.09^{A}	14.0 ± 1.41
Dry-Mixed-**U**	1875 ± 35.4^{A}	6.65 ± 0.04^{A}	143 ± 7.27^{A}	135 ± 6.06^{A}	3.23 ± 0.09^{A}	12.8 ± 1.37
Open-**U**	1820 ± 42.0^{A}	6.59 ± 0.05^{A}	140 ± 8.65^{A}	146 ± 7.21^{A}	3.41 ± 0.11^{A}	6.1 ± 1.63
Wet-Mixed-**U**	1726 ± 41.3^{B}	6.45 ± 0.05^{B}	116 ± 8.48^{B}	115 ± 7.06^{B}	3.03 ± 0.11^{B}	12.1 ± 1.6
Overstory				*	*	
Aspen-**O**	1828 ± 48.9	6.61 ± 0.06	138 ± 9.76^{A}	149 ± 8.16^{A}	3.32 ± 0.12^{A}	13.4 ± 1.82^{A}
Dry-Mixed-**O**	1817 ± 32.9	6.60 ± 0.04	144 ± 6.56^{A}	137 ± 5.49^{A}	3.32 ± 0.08^{A}	15.0 ± 1.22^{A}
Open-**O**	1813 ± 75.3	6.56 ± 0.09	129 ± 15.03^{A}	148 ± 12.57^{A}	3.46 ± 0.19^{A}	17.6 ± 2.80^{A}
Wet-Mixed-**O**	1786 ± 31.4	6.53 ± 0.04	122 ± 6.27^{B}	123 ± 5.24^{B}	3.05 ± 0.08^{B}	11.9 ± 1.17^{B}
pH	*	*	*	*	*	*
<5	1471 ± 72.4^{A}	6.13 ± 0.09^{A}	101 ± 16.93^{A}	92.3 ± 13.83^{A}	2.60 ± 0.21^{A}	7.14 ± 3.14^{A}
5.0-5.4	1698 ± 45.8^{B}	6.49 ± 0.06^{B}	116 ± 10.71^{A}	116.1 ± 8.75^{B}	2.97 ± 0.13^{B}	10.15 ± 1.98^{A}
5.5-5.9	1720 ± 33.5^{B}	6.52 ± 0.04^{B}	131 ± 7.84^{A}	126.0 ± 6.40^{B}	3.12 ± 0.10^{B}	12.48 ± 1.45^{A}
6.0-6.4	1903 ± 27.0^{C}	6.63 ± 0.03^{C}	134 ± 6.32^{A}	146.0 ± 5.17^{C}	3.41 ± 0.08^{C}	16.43 ± 1.17^{B}
>6.5	1888 ± 37.0^{C}	6.67 ± 0.05^{C}	156 ± 8.65^{B}	144.6 ± 7.06^{C}	3.31 ± 0.11^{C}	14.04 ± 1.60^{B}
Nearest tree			*			
0.0-1.0	1810 ± 38.6	6.56 ± 0.05	127 ± 7.63^{A}	139 ± 6.65	3.21 ± 0.10	14.4 ± 1.47
1.0-2.0	1775 ± 29.9	6.54 ± 0.04	133 ± 5.91^{A}	128 ± 5.15	3.12 ± 0.08	12.4 ± 1.14
2.0-3.0	1837 ± 40.6	6.59 ± 0.04	127 ± 8.05^{A}	135 ± 7.01	3.12 ± 0.10	14.7 ± 1.54
>3.0	1885 ± 74.7	6.72 ± 0.09	177 ± 14.78^{B}	151 ± 12.88	3.49 ± 0.19	15.5 ± 1.54

* Significance based on linear regression; p =0.05.
$^{A, B, C}$ significant differences within categories and richness and diversity indices, associated with lsmeans averages

3.4.3 Bacterial and fungal beta diversity

Principal coordinate analysis was completed to quantify beta diversity between bacterial and fungal communities associated with dominant seedlings, understory forest type, overstory forest type, nearest tree range, and soil pH range. Dominant seedlings had an effect on both bacterial and fungal beta diversity (p = 0.001; p = 0.001; Figure 3-2). Understory forest type trended towards significant for fungal beta diversity (p = 0.054) but did not have an effect on bacterial beta diversity (p = 0.366; Figure 3-3). Similarly, overstory forest type trended towards significant for fungal beta diversity (p =0.052) yet did not have an effect on bacterial beta diversity (p = 0.384; Figure 3-4). Likewise, the nearest overstory tree (>3.0 m) had an effect on fungal beta diversity (p = 0.044), whereas not on bacterial beta diversity (p = 0.372; Figure 3-5). Soil pH did not affect fungal or bacterial beta diversity (p = 0.07 and p = 0.234, respectively; Figure 3-6).

3.4.4 Core communities associated with bacterial and fungal communities

Venn diagrams showed that bacterial core communities for all categories consisted of at least 8,500 OTUs (62%), as unique communities for each factor resulted in less than 29 OTUs (0.2%; Figure 3-7). The opposite was identified for fungal communities, in which the core communities observed were less than 400 OTUs (15%), while unique communities were in greater abundance with as many as 595 OTUs (22.9%; Figure 3-8). Bacterial communities were more homogenous in all categories, as fungal communities had more unique taxa associated with each factor in all categories.

3.4.5 Random Forest identification of taxa driving microbial composition

The random forest analysis determined that pH had the lowest oob model error rate indicating that pH is the best indicator to predict soil bacterial community composition (Table 3-2). Whereas for fungal communities, overstory forest type had the lowest oob model error rate, indicating that overstory forests were the best category to predict soil fungal community composition (Table 3-3).

Table 3-2: Random Forest analysis for site factor categories in association to bacterial community composition, including the out of bag (oob) model error rate and most important bacterial taxa based on mean decrease in GINI value.

Site factors	Oob error	Important taxa (GINI values)		
Dominant seedling	59.13%	Archangiaceae (1.0)	*Kribbella* (0.997)	*Thermoleophilia* (0.997)
Understory forest type	70.43%	*Quadrisphaera* (1.0)	*Crossiella* (0.816)	Gemmataceae (0.816)
Overstory forest type	59.13%	Gaiellales (1.0)	67-24 (0.606)	Solirubrobacter (0.502)
Nearest tree range	59.13%	IMCC26256 (1.0)	*Candidatus Udaeobacter* (0.741)	Xanthobacteraceae (0.666)
pH range	51.3%	WD2101 (1.0)	*Chthoniobacter* 0.775)	Elsterales (0.752)

Table 3-3: Random Forest analysis for site factor categories in association to fungal community composition, including the out of bag (oob) model error rate and most important fungal taxa based on mean decrease in GINI value.

Site factors	Oob error	Important taxa (GINI values)		
Dominant seedling	59.13%	*Tricholoma triste* (1.0)	*Pseudogymnoascus* (0.5)	*Chaetothyriales* sp. (0.444)
Understory forest type	63.48%	*Helvella albella* (1.0)	Ascomycota (0.861)	*Neonectria candida* (0.764)
Overstory forest type	49.57%	Myxotrichaceae (1.0)	*Halokirschsteiniothelia maritima* (0.876)	*Chaetothyriales* (0.593)
Nearest tree range	56.52%	*Oidiodendron periconioides* (1.0)	Eurotiomycetes (0.879)	Hysteriales (0.703)
pH range	60.87%	*Penicillium* (1.0)	Helotiales 0.775)	*Umbelopsis changbaiensis* (0.638)

3.4.6 Microbial taxonomic trends and relative abundance

Eleven bacterial phyla exceeded the relative abundance of 1%. Variation occurred among all categories, although the most abundant phyla in all samples were Actinobacteria and Proteobacteria with Acidobacteria, Bacteroidetes, Chloroflexi, Firmicutes, and Verrucomicrobia occurring at a lesser abundance (Figure 3-9). There were 25 bacterial families that exceeded the relative abundance of 1%. Most families are associated with nutrient cycling or decomposition. The most abundant family was Xanthobacteraceae (Proteobacteria), followed by Chthoniobacteraceae (Verrucomicrobia) and Mycobacteriaceae (Actinobacteria) (Figure 3-10).

Twenty-five fungal families exceeded 1% relative abundance. The most abundant fungal families varied for all categories, therefore no clear trends occurred as fungi were unique for dominant seedlings, understory and overstory forest type, nearest tree range, and pH range.

Fungal families included a mixture of ascomycetes and basidiomycetes, with functions as mycorrhizae or saprotrophs. Aspergillaceae (Ascomycete), Atheliaceae (Basidiomycete), and Myxotrichaceae (Ascomycete) were the most abundant for all factors and categories (Figure 3-11).

3.4.7 MetagenomeSeq analysis associated with forest type and seedling species

In soils associated with tree species (overstory/understory forest type and dominant seedlings) mycorrhizal and saprophytic fungi were found in high abundance. Most notably, the differences between dry-mixed and wet-mixed stands resulted in greater *Cadaphora, Capnodiales, Cortinarius, Heleloma, Hygrophorus,* and *Tetracladium* species in association with dry-mixed forests, while *Basidiodendron, Cenococcum, Hygrophorus,* and *Trichoderma* species in association to wet-mixed forests. Expanding on the Random Forests analysis, in which *Tricholoma triste* was the most important fungal species in determining dominant seedlings, *T. triste* was four-fold greater in dry-mixed overstory than aspen stands, and two-fold greater in wet-mixed forests than aspen stands. Between coniferous forest types, *T. triste* was found 1.7-fold greater in dry-mixed forests than wet-mixed forests. For understory forest type, *T. triste* was not found in greater abundance between any forest types. Yet, when comparing dominant seedlings *T. triste* was in greater abundances in association with Colorado blue spruce and Engelmann spruce, contradictory to the overstory forest type comparisons.

3.4.8 Soil and stand factors associated with microbial community change

The backward stepwise multiple linear regression analysis determined different soil edaphic properties and site and stand characteristics that positively and negatively influenced soil bacterial richness and diversity (Table 3.4). Most notably, pH had a positive influence on bacterial richness, available nitrogen and potassium had a positive influence on Shannon's diversity, and phosphorous had both a positive and negative influence, depending on available or reserve status, on Inverse Simpson's diversity.

Table 3-4: Bacterial ANOVA linear models of richness and diversity indices. C = Carbon, N = Nitrogen, P = Phoshporous, K = Potassium, Org. & O. = Organic, Asp = Aspect, NutrVal = Nutrient Value.

Richness			Shannon's			Inverse Simpson's		
	t-value	p-value		t-value	p-value		t-value	p-value
10-hr fuels	-1.742	*p*=0.085	10-hr fuels	-3.181	*p*=0.002*	10-hr fuels	-1.529	*p*=0.130
Duff	1.948	*p*=0.055	Duff	2.363	*p*=0.020*	Org P Res.	-3.346	*p*=0.001*
Litter	-1.755	*p*=0.083	Litter	-2.011	*p*=0.047*	Slope	2.874	*p*=0.005*
Or N Res.	-1.798	*p*=0.075	Or N Res.	-1.388	*p*=0.169	Total N	2.151	*p*=0.003*
Cos Asp	1.540	*p*=0.127	Or P Res.	-2.133	*p*=0.035*	Org N	1.631	*p*=0.106
pH	2.958	*p*=0.004*	Slope	2.000	*p*=0.048*	Nitrate	1.591	*p*=0.115
CO_2:C	2.835	*p*=0.006*	CO_2:C	2.059	*p*=0.042*	Ammon.	2.158	*p*=0.033*
Total N	-2.740	*p*=0.007*	Org N	1.401	*p*=0.164	Total Phos.	2.923	*p*=0.004*
Org N	1.801	*p*=0.075	Tot Org. C	-2.093	*p*=0.039*	Calc.	2.212	*p*=0.029*
Tot Org. C	-2.781	*p*=0.007*	Tot Phos.	2.496	*p*=0.014*	Alum.	5.242	*p*=<0.001*
Nitrate	2.066	*p*=0.042*	Potas.	-1.440	*p*=0.153	Iron	-5.156	*p*=<0.001*
Tot Phos.	1.792	*p*=0.076	Alum.	2.599	*p*=0.011*	Mang.	-2.721	*p*=0.007*
Potas.	-1.993	*p*=0.049*	Iron	-4.196	*p*=<0.001*	Sodium	-1.585	*p*=0.116
Alum.	1.828	*p*=0.071	Mang.	-2.120	*p*=0.036*	Org. P Rel	-2.189	*p*=0.031*
Iron	-2.258	*p*=0.026*	MAC	-1.702	*p*=0.092	Avail K	2.463	*p*=0.016*
Zinc	-2.381	*p*=0.019*	Org. N Rel	-1.408	*p*=0.163	NutrVal	-2.509	*p*=0.014*
MAC	-2.593	*p*=0.011*	Avail N	2.438	*p*=0.016*			
Org. N Rel	-1.792	*p*=0.076	Avail K	2.461	*p*=0.016*			
Avail N	1.584	*p*=0.117	NutrVal	-2.408	*p*=0.018*			
Avail P	1.439	*p*=0.154						
Avail K	1.843	*p*=0.069						
NutrVal	-1.559	*p*=0.122						
Trad N	-2.019	*p*=0.046*						
Model		*p*=<0.001*	Model		*p*=<0.001*	Model		*p*=<0.001*

* Significance based on p =0.05.

Similarly, the backward stepwise linear regression analysis found different soil edaphic properties and plot characteristics that influenced soil fungal richness and diversity (Table 3-5). Highlighted by a positive influence on organic matter and phosphorus reserves and a negative influence of total phosphorous on fungal richness, a negative influence of available phosphorus and potassium on fungal Shannon's diversity, and a negative influence of total phosphorous, organic nitrogen:inorganic nitrogen, and available potassium on fungal Inverse Simpson's diversity.

Table 3-5: Fungal ANOVA linear models of richness and diversity indices. C = Carbon, N = Nitrogen, P = Phoshporous, K = Potassium, Org. & O. = Organic, I. = Inorganic, OM = Organic matter, Asp = Aspect, NutrVal = Nutrient Value.

Richness			**Shannon's**			**Inverse Simpson's**		
	t-value	**p-value**		**t-value**	**p-value**		**t-value**	**p-value**
100-hr fuels	-1.528	*p*=0.130	Litter	-1.861	*p*=0.066	Litter	-2.149	*p*=0.034*
Org P Res.	2.544	*p*=0.013*	Org N Res.	-1.557	*p*=0.123	Org N Res.	-1.605	*p*=0.112
Sin Asp	1.724	*p*=0.088	Org P Res.	1.611	*p*=0.110	Org P Res.	3.178	*p*=0.002*
OM	3.133	*p*=0.002*	pH	1.521	*p*=0.132	Slope	-1.680	*p*=0.096
Total Org. C	-2.198	*p*=0.030*	OM	1.895	*p*=0.061	pH	2.009	*p*=0.047*
Nitrate	-1.534	*p*=0.128	Organic N	1.535	*p*=0.128	OM	2.082	*p*=0.040*
Ammon	-1.651	*p*=0.102	Nitrate	-1.651	*p*=0.102	Org N	1.577	*p*=0.118
Total Phos.	-2.303	*p*=0.023*	Ammon	-1.758	*p*=0.082	Nitrate	-2.737	*p*=0.007*
Iron	-3.415	*p*=0.001*	Total Phos.	-1.817	*p*=0.072	Ammon	-2.820	*p*=0.006*
Sulfur	1.346	*p*=0.182	Calcium	-1.660	*p*=0.100	Total Phos.	-2.930	*p*=0.004*
Copper.	1.881	*p*=0.063	Iron	-1.966	*p*=0.052	Calcium	-1.812	*p*=0.073
Magnes.	1.691	*p*=0.094	Copper.	1.807	*p*=0.074	Iron	-1.519	*p*=0.132
MAC	-2.332	*p*=0.022*	Magnes.	2.288	*p*=0.024*	Copper.	2.414	*p*=0.018*
O.N:IO N.	-3.352	*p*=0.001*	MAC	1-.606	*p*=0.112	Magnes.	1.334	*p*=0.185
Org. N Rel	-1.455	*p*=0.149	O.N:IO N.	-2.879	*p*=0.005*	O.N:IO N.	-2.680	*p*=0.009*
Soil Health	1.386	*p*=0.169	Org. N Rel	-1.802	*p*=0.075	Org. N Rel	-2.011	*p*=0.047*
Avail N	-1.422	*p*=0.158	Avail N	-1.913	*p*=0.059	Avail P	-1.912	*p*=0.059

Avail P	-1.644	*p*=0.104	Avail P	-2.172	*p*=0.032*	Avail K	-2.692	*p*=0.008*
Avail K	-1.867	*p*=0.065	Avail K	-2.361	*p*=0.020*	NutrVal	2.691	*p*=0.008*
NutrVal	1.869	*p*=0.065	NutrVal	2.362	*p*=0.020*			
Model		*p*=<0.001*	Model		*p*=<0.001*	Model		*p*=<0.001*

* Significance based on *p* =0.05.

3.5 Discussion

The goal of our study was to identify how overstory and understory forest type drive change in bacterial communities associated with forest types commonly found in the Central and Southern Rocky Mountains and to determine other soil or site variables that influence microbial composition. Our study demonstrated that soil pH was the greatest driver of soil bacterial and fungal richness and α-diversity, which concurs with previous research (Fierer and Jackson, 2006; Dumbrell et al., 2010; Rousk et al., 2010; Prescott and Grayston, 2013; Tedersoo et al., 2014). Shannon's diversity and Inverse Simpson's diversity (alpha diversity) for bacterial communities were influenced by different factors. The relative abundance of dominant bacterial species (Inverse Simpson's) differed for dominant seedlings species and distance to the nearest mature tree, as dominant species were in higher abundances for drier species and further away from the roots, while rare bacterial communities (Shannon's) were affected by understory forest type. Fungal richness was influenced by forest type and Shannon's diversity (rare communities) was influenced by overstory forest type, as highlighted with a high percentage of unique fungal OTUs associated with each forest type.

Dominant seedling species drove β-diversity change in both soil bacterial and fungal communities. Additionally, proximity to the nearest overstory tree significantly changed fungal communities, as fungal communities were similar near roots (0-3.0 m from tree) compared to > 3.0 m, which had less influence from roots, resulting in a greater fungal richness and diversity. While trending toward significant, understory and overstory forest composition also influences fungal communities. The soil microbial communities in our samples include beneficial microbial

species that likely enhance plant growth and survival. These included mycorrhizae fungi, nutrient cycling bacteria, and decomposers. Though pathogens were likely present as well, we were not able to identify many taxa to the genus or species level.

Specifically for beta diversity, we found a greater difference in fungal communities compared to bacterial communities throughout all categories. The low number of core fungal community members suggests that trees are associated with species-specific fungi compared to bacteria that may associate with a wide range of plants (Crowther et al. 2014). We observed an influence of dominant seedlings on bacterial communities, and no other category was found to be significant, most likely due to a lack of differences occurring in similar soil edaphic properties within the site. As the stand at PFDP is relatively stable, it is highly likely that fungal communities would be more dominant, as they flourish in late successional stands with a lack of soil disturbance (Li et al., 2015). Under natural disturbance or stand treatments, bacterial communities may have been more prevalent; soil disturbance disrupts fungal connections thus providing new environments to enhance bacteria (Liu et al., 2020b). Fungal communities are more sensitive than bacteria to disturbances, as fungal biomass is reduced, allowing bacteria to increase during disturbances (Chen et al., 2021). The lack of disruption, especially in the soil, will allow trees and microbial communities to adapt to one another and create direct relationships that benefit each other, commonly observed in late successional forests (Li et al., 2015; Liu et al., 2020b).

At the PFDP a high diversity of overstory and understory trees was present which enhances the diversity of leaf litter on the forest floor. Litter accumulation plays a direct role in soil chemical and physical properties, as leaf litter can vary from easily decomposed leaves to recalcitrant needle and woody material (Chodak et al., 2016). The litter composition results in the accumulation of certain soil microbial communities to decompose this material, especially in conifer litter (Chodak et al., 2016) thus composition of leaf litter may manipulate soil chemistry (Prescott and Grayston, 2013). Although our samples were taken in the summer, fresh leaf litter

from fall leaf senescence can boost soil microbial community activity and diversity (Thoms and Gleixner, 2013). Soil and litter quality can affect the composition of fungal saprophytes and mycorrhizae, as seedling species richness have a positive correlation with soil fungi, especially ectomycorrhizal fungi (Tedersoo et al., 2016). Although, we analyzed seedlings to reflect the most dominant species, the interaction between numerous species may increase the richness and diversity below-ground, leading to plants that are more resilient in drought environments (Gillespie et al., 2020).

The most important taxa driving fungal community composition in association with dominant seedling species was *Tricholoma triste*. Outside of dominant seedlings, *T. triste* was found in association with all above- and below-ground forest types. *Tricholoma* spp. function as ectomycorrhiza commonly found in diverse temperate forests consisting of notably *Picea* and *Pinus* species (Sanchez-Garcia, 2016). Numerous mycorrhizal fungal taxa (Atheliaceae, Cortinariaceae, Inocybaceae, Myxotrichaceae, Pyronemataceae, Russulaceae, Suillaceae, Thelephoraceae, Tricholomataceae, and Trichomeriaceae) were found in high abundance with understory and overstory forests, especially dry-mixed and wet-mixed, and all seedlings. This is no surprise as mycorrhizae form a symbiotic relationship with plants, especially EcM which is associated with more than 90% of forest trees (Markkola, 1996), as mycorrhizae is linked to increased forest growth and establishment (Anthony et al., 2022).

The most abundant phyla observed in all samples at PFDP were Actinobacteria and Proteobacteria, which are commonly found in forests. These phyla are comprised of nutrient cyclers, and most notably include members associated with the breakdown of cellulose (Lladó et al., 2017; Zhang et al., 2019). Actinobacteria specifically function in the nutrient cycling of organic compounds, thereby increasing soil organic matter (Mhete et al., 2020). We found a greater abundance of Actinobacteria associated with dry-mixed and open stands, particularly in sites with ponderosa pine seedlings or with no seedlings. The open, dry, warm environments associated with ponderosa pine forests may attribute to the high bacterial diversity and richness,

as high understory vegetation such as labile leaf litter from grasses and forbs enhance copiotrophic (r-strategist) bacteria (Lladó et al., 2017). Copiotrophic species (r-strategists) are generalists, who spread rapidly with an increase in nutrients, frequently found in the rhizosphere or following a surplus of material to decompose (Koch, 2001).

Proteobacteria, on the other hand, cycle nutrients, specifically calcium and nitrogen (Mhete et al., 2020), which are frequently lacking in forests (Kaiser et al., 2016). The biomass and diversity of Proteobacteria increase as they rapidly decompose labile leaf litter (Lladó et al., 2017). We observed a greater abundance of Proteobacteria associated with wet-mixed and aspen stands and specifically Engelmann spruce, Douglas-fir, and aspen seedlings in our samples. This is especially important as bacterial communities may assist in decomposition in conjunction with saprophytic and increase the uptake of nutrients and water as mycorrhizal fungi helpers (Lladó et al., 2017).

The most important bacterial families driving bacterial community composition in dominant seedlings were Archangiaceae (Proteobacteria: Myxococcales), Kribbella (Actinobacteria; Nocardioidaceae), Thermoleophilia (Actinobacteria), and Quadisphaera (Actinobacteria: Kineosporiaceae). Although fungi draw more interest for the decomposition of recalcitrant material, both *Kribbella* and *Thermoleophilia* are important in nutrient cycling. Extracellular enzymes from both taxa can degrade lignin, cellulose, and other organic compounds, similar to their fungal symbionts, potentially releasing vital nutrients to seedlings at the PFDP site (Evtushenk and Ariskina, 2015; Zhang et al., 2019). *Kribbella* is also frequently found in the rhizosphere, as it plays a role as a sink of hydrogen (Osborne et al., 2010). Specifically, *Thermoleophilia* was found in greater abundance in association with dry-mixed or open understory, and dry-mixed overstory stands, and in locations with either no seedlings or ponderosa pine regeneration. As *Thermoleophilia* spp. have been observed decomposing recalcitrant (woody) materials, this taxon may assist in the breakdown of woody litter in open stands.

We observed that distance to the nearest tree drives fungal community composition, in which the greatest fungal richness and diversity was observed in soils > 3.0 m away from mature trees. Our analysis utilized mature trees as a reference of distance to the nearest tree, therefore this influence is based on the interactions of both fine and coarse roots in proximity to our soil samples. The rhizosphere is essential for plant nutrition and health as it acts as a hot spot for microbial communities (Berg and Smalla, 2009). Root exudates, from fine and coarse roots increase the carbon input into the soil, which saturates the area around the roots, as roughly 20% of carbon soil inputs occur in both types of roots (Brunner and Godbold, 2007). There is a common understanding that the rhizosphere environment has more abundant fungal taxa occupying niches thereby reducing bacterial richness and diversity (Cui et al., 2018). Whereas bulk soil has a greater diversity of bacteria due to less competition of fungi (Cui et al., 2018), concurring with our data as bacterial and fungal diversity and richness are greatest in the bulk soil rather than at the closer to roots. We sampled during the growing season, as increased carbon typically occurs during the growing season and roots exude provide more nutrients into the soil as they photosynthesize (Grayston et al., 1997). The concentration of beneficial microbial communities (mycorrhizae) reduces pathogen infection and increase the uptake of nutrients in the rhizosphere (Schirawski and Perlin, 2018). The influence of trees explained only one aspect to the drivers of soil microbial communities, therefore additional variables (i.e. soil edaphic properties) are needed to fully determine what prompts change in microbial community composition (Brockett et al., 2011).

In our study, soil edaphic properties were used as predictor variables for bacterial and fungal richness and diversity. Similar to other studies on soil bacterial communities, soil pH had the greatest effect on bacterial richness (Fierer et al., 2012). Additionally, soil pH had a positive influence on more abundant fungal species (Inverse Simpson's). Our data concurs with Fierer et al (2021) that documented that more neutral pH soils tend to have higher bacterial diversity than more acidic or basic soils. Outside of soil pH, macro nutrients, such as carbon and aluminum

had a positive influence and phosphorous, nitrogen, iron, and manganese had a negative influence on bacterial communities. Whereas nitrogen and potassium had a negative influence on fungi, as phosphorous had both a positive and negative influence on fungal communities. Organic phosphorous reserves tend to increase fungal richness and Inverse Simpson's diversity, while total and available phosphorous negatively influence Shannon's and Inverse Simpson's diversity. The negative relationship with available and total phosphorus may result in the relationship of arbuscular mycorrhizae, which function to increase phosphorous uptake (Dumbrell et al., 2010), further enhancing fungal dominance at the PFDP.

While not included in our study, climate may have a direct influence on both soil bacterial and fungal communities. Our samples provided an inference in climate, as we split forest type (understory and overstory) into dry-mixed and wet-mixed stands. These stands have differing environments based on slope and aspect, within the same stand with dry-mixed stands consisting of ponderosa pine, limber pine, aspen, and open meadows on south facing aspects and wet-mixed including spruce-fir with intermixed Douglas-fir on north facing aspects. These forests give an inference for soil temperature and moisture, as dry/open stands in south-facing aspects tend to have increased soil temperatures and less soil moisture than locations in north-facing aspects (Larsen, 1930). The amount of direct sunlight will differ seasonally, with south facing aspect receiving substantial sunlight all year, as north facing see minimal sunlight in winter months. Increased sunlight will result in higher soil temperatures for the dry-mixed or open stands, which enhances soil microbial activity (Krishna and Mohan, 2017). Similar to dominant seedling species, understory and overstory forest type are trending toward significant to drive change in soil fungal communities, which may correspond to the close relationship between forests and mycorrhizal fungi (Li et al., 2015; Baldrian, 2017b).

3.5.1 Limitations of the study

Although this study provides an expansive view of soil microbial communities using a stand level approach, the understanding of functional attributes would expand the depth of this research. Additionally, our study looked at a snapshot of samples in summer, providing a baseline for soil microbial communities within forest types at the PFDP. This information will enhance our knowledge of tree species diversity and soil microbial diversity, yet a temporal study will provide greater evidence of transitions following forest treatments at the location as well as seasonal variation occurring within the site. Moreover, we sampled at 25% of the established 1 m^2 locations in 3 days in the field. The ability to sample more locations would provide greater depth to our research and may expand upon correlations that we observed in our analysis.

3.6 Conclusion

The large sample size of soil cores collected at the PFDP allows for an enhanced view of how forest type influences soil bacterial and fungal microbial communities, while removing extenuating variables such as elevation and geographic changes. This research is one of the most robust soil microbial community sampling within a stand level study within the Southern Rocky Mountains. We observed that dominant seedlings influenced soil bacterial and fungal beta diversity, as a diverse suite of mycorrhizal and saprophytic fungi and nutrient cycling bacteria were associated with seedlings at the PFDP. For other response variables (nearest tree range and forest type), significant or slightly significant, respectively, differences were observed for fungal beta diversity. Additionally, fungal core communities were smallest for forest type and nearest mature tree range, indicating that interactions of unique fungal communities in association to tree species and root interactions occurred. Further, solidifying the understanding that mycorrhizae are species specific, and that above-ground diversity enhances below-ground diversity. This study improves our understanding of the dynamic interactions between trees and soil microbial communities and provides a snapshot of soil bacterial and fungal communities in

association to forest types in the Rocky Mountains, allowing land managers to understand the overall forest ecosystem to better manage for future forest.

Figures

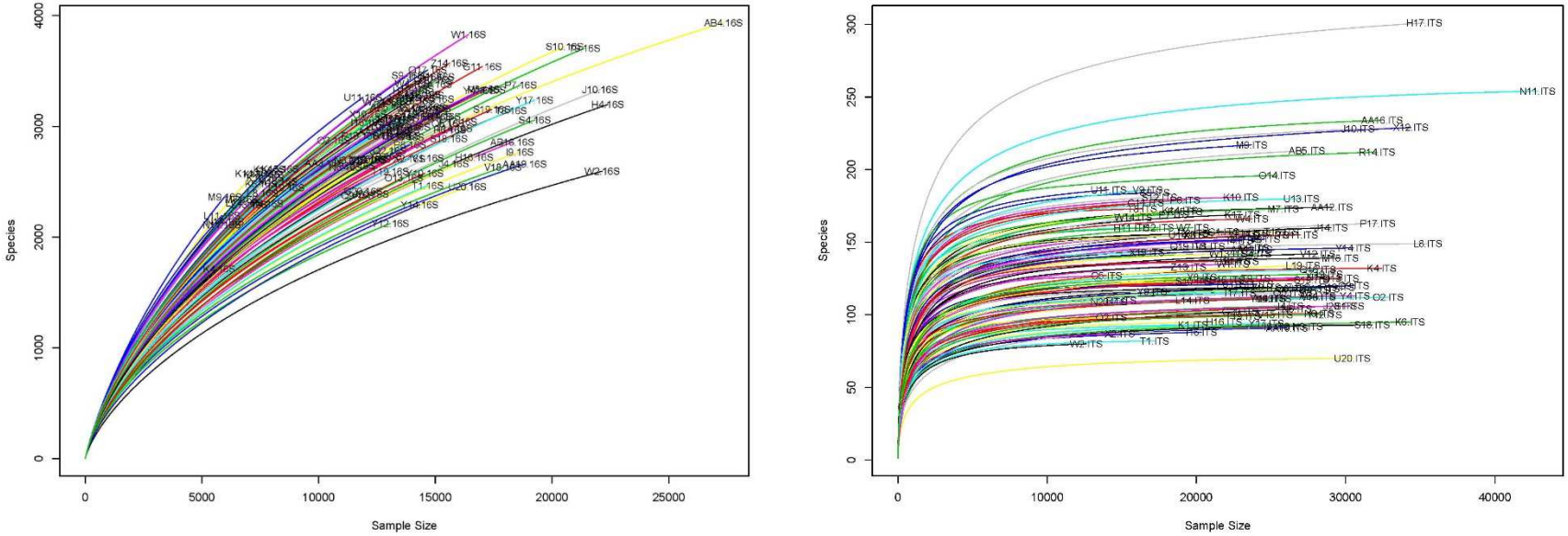

Figure 3-2: Left: Bacterial rarefaction curve with samples that did not plateau. This indicates that the bacterial samples could have been sequenced at a greater depth. Right: Fungal rarefaction curve with samples that plateaued, indicating that samples were sequenced at an adequate depth.

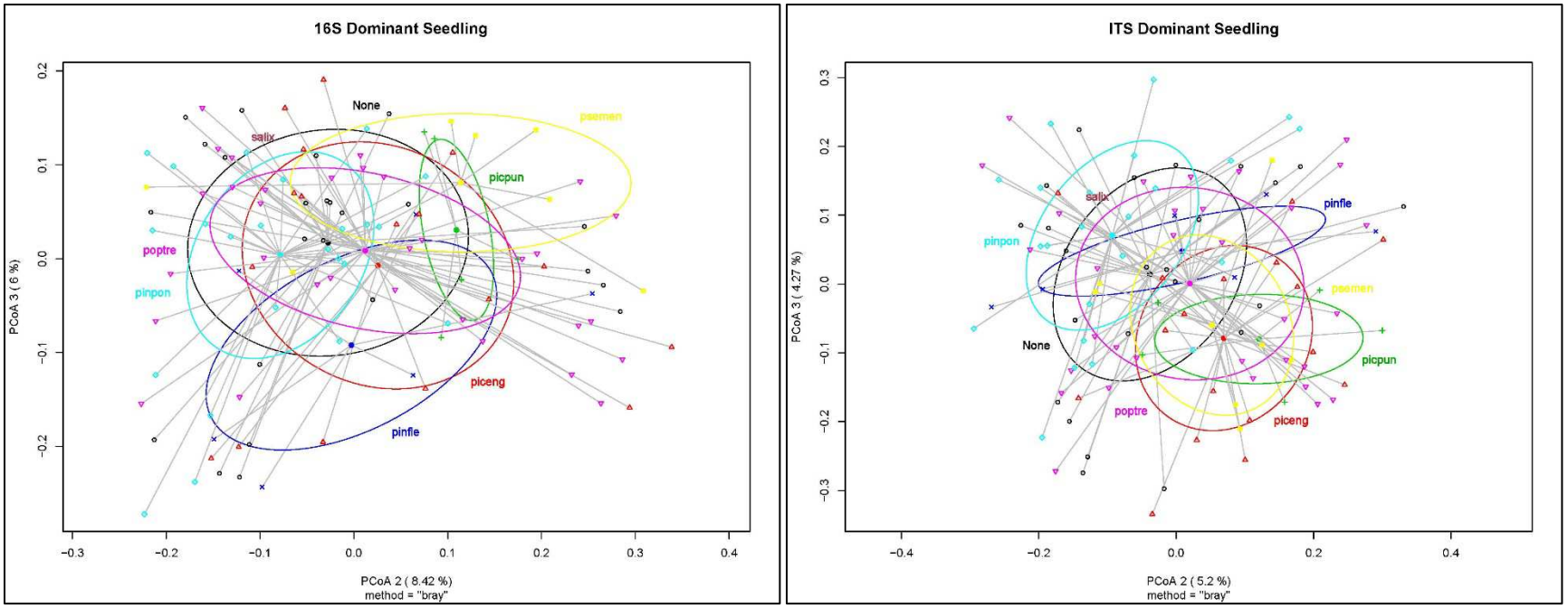

Figure 3-2: Principal coordinate analysis graphs for dominant seedlings. Left: Bacterial communities associated with dominant seedlings have high overlap, yet beta diversity is significant. Right: Fungal communities associated with dominant seedlings with spruce-fir separating from other species, as beta diversity is significant.

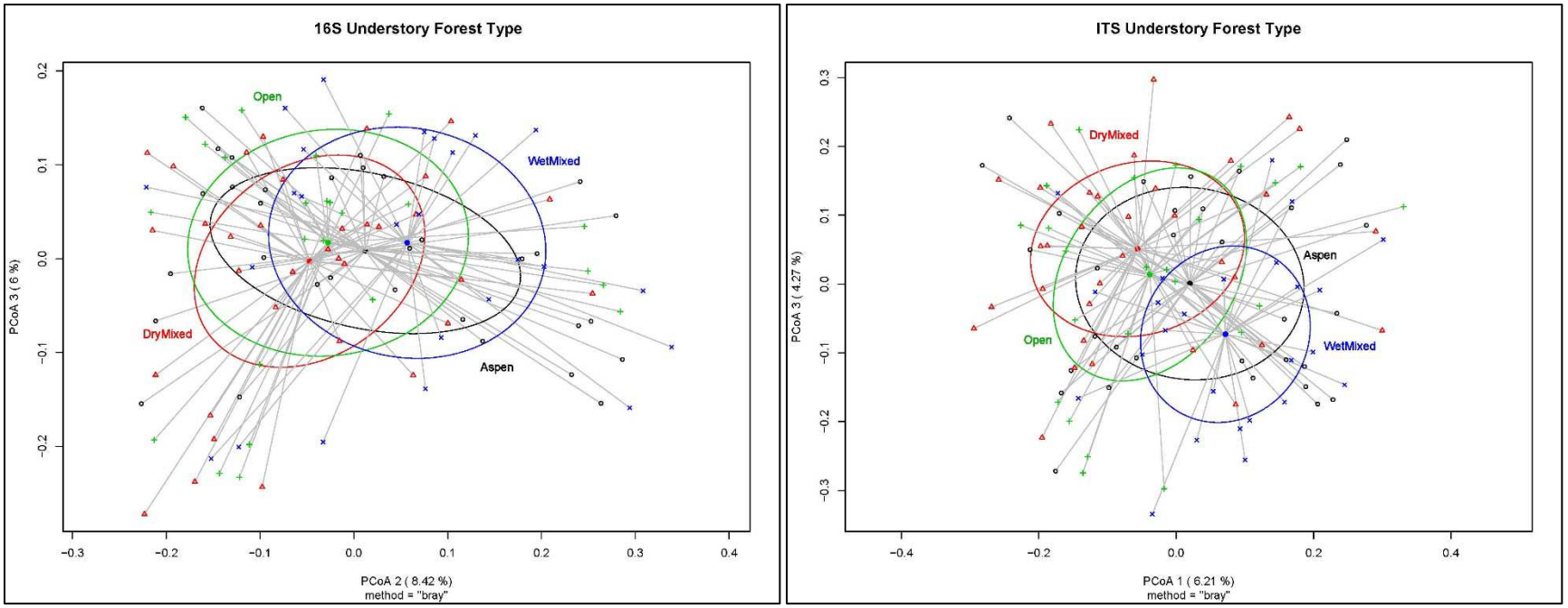

Figure 3-3: Principal coordinate analysis associated with understory forest type. Left: Bacterial communities reflect high overlap of all factors, as there is no significant difference. Right: Fungal communities with minor separation in factors, as the beta diversity is trending toward significant.

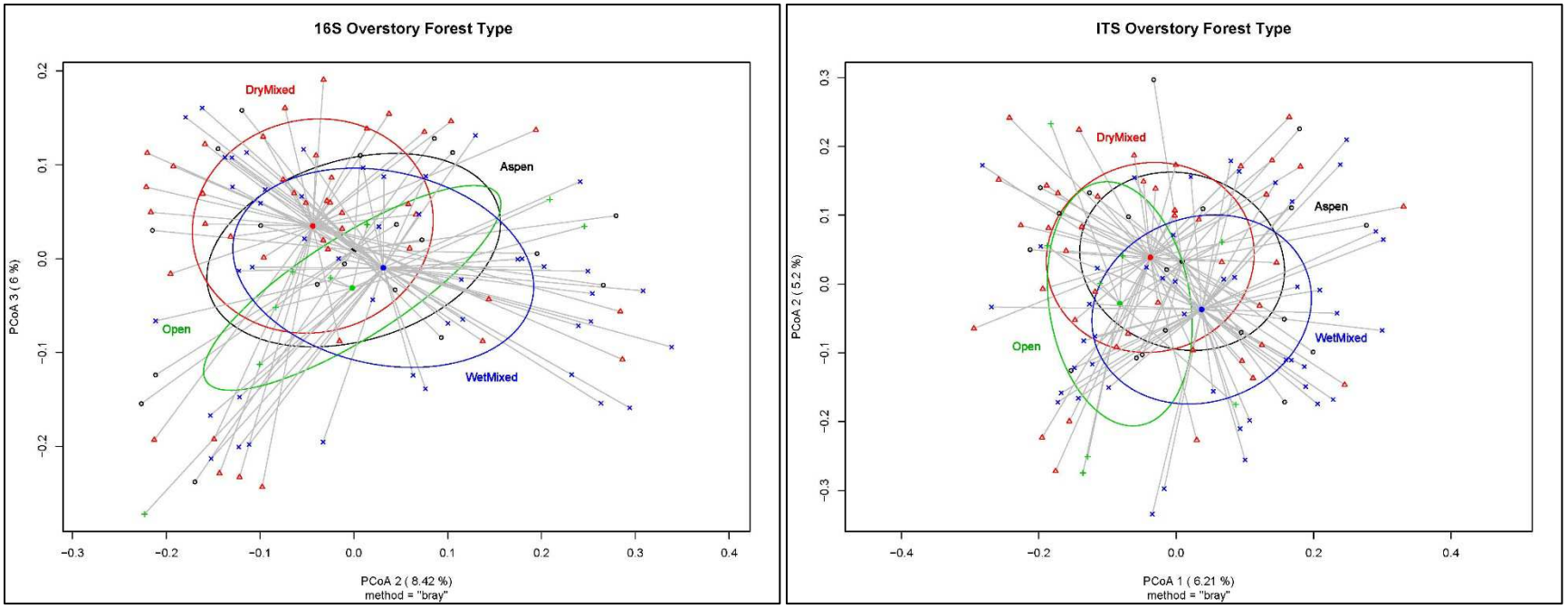

Figure 3-4: Principal coordinate analysis associated with overstory forest type. Left: Bacterial communities reflect high overlap of all factors, as there is no significant difference. Right: Fungal communities with minor separation in factors, as the beta diversity is trending toward significant.

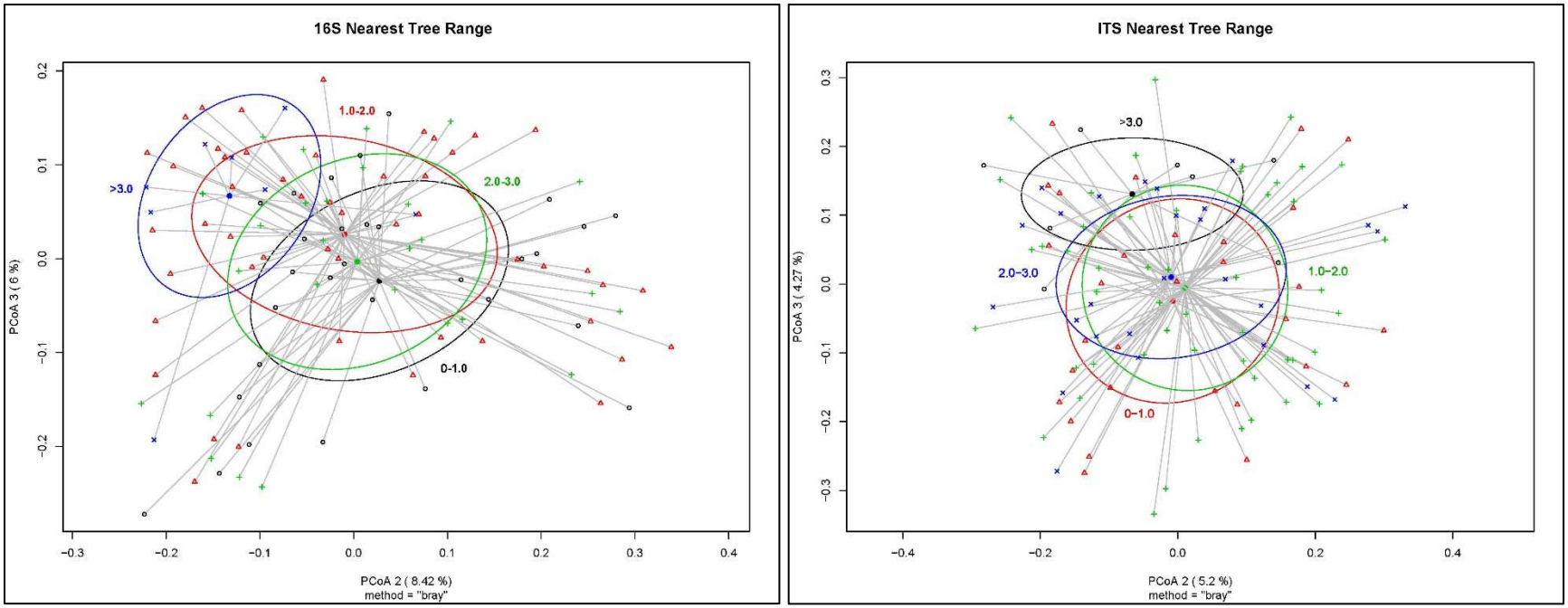

Figure 3-5: Principal coordinate analysis associated with nearest tree range. Left: Bacterial communities reflect high overlap of all factors with minor separation for >3.0 m, as there is no significant difference. Right: Fungal communities have high overlap for 0-3.0 m, while soils associated with >3.0 m (bulk soil) differ, as the beta diversity is significant.

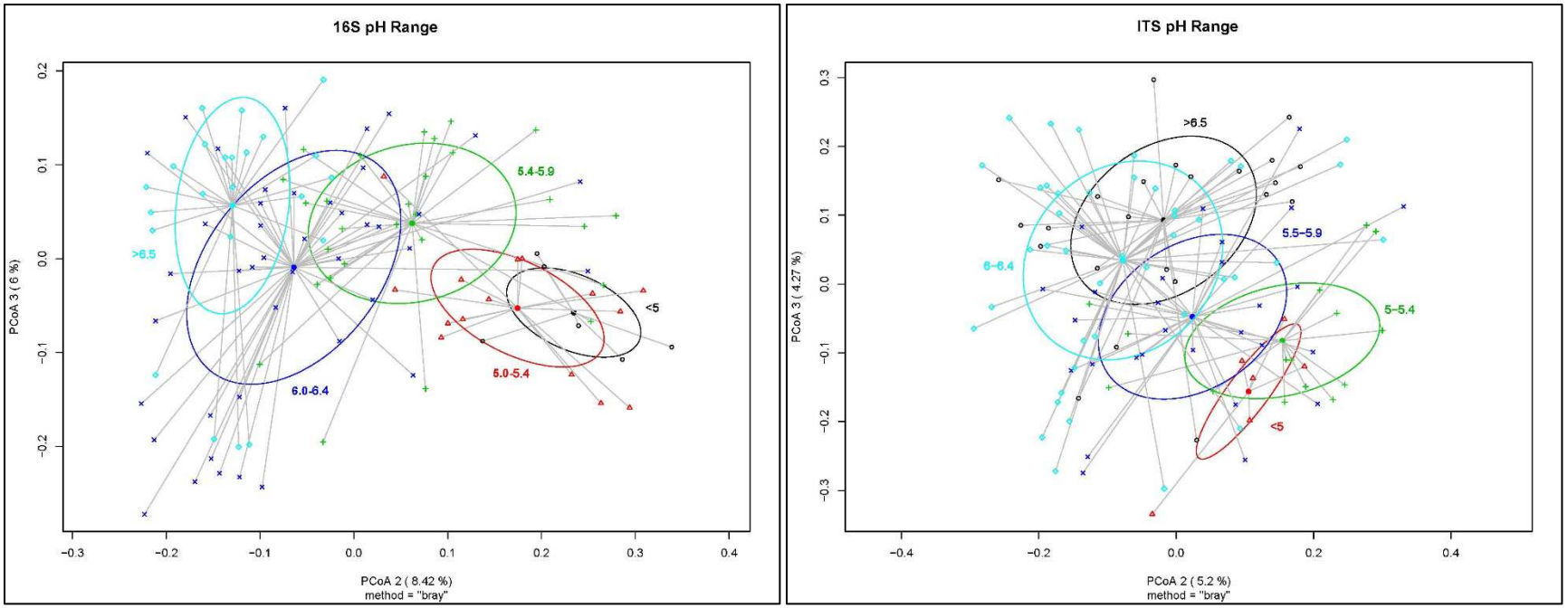

Figure 3-6: Principal coordinate analysis associated with pH range. Left: Bacterial communities reflect little overlap between factors, yet there is no significant difference. Right: Fungal communities have high overlap for more acidic and more neutral soils, as they are do not significantly influence beta diversity.

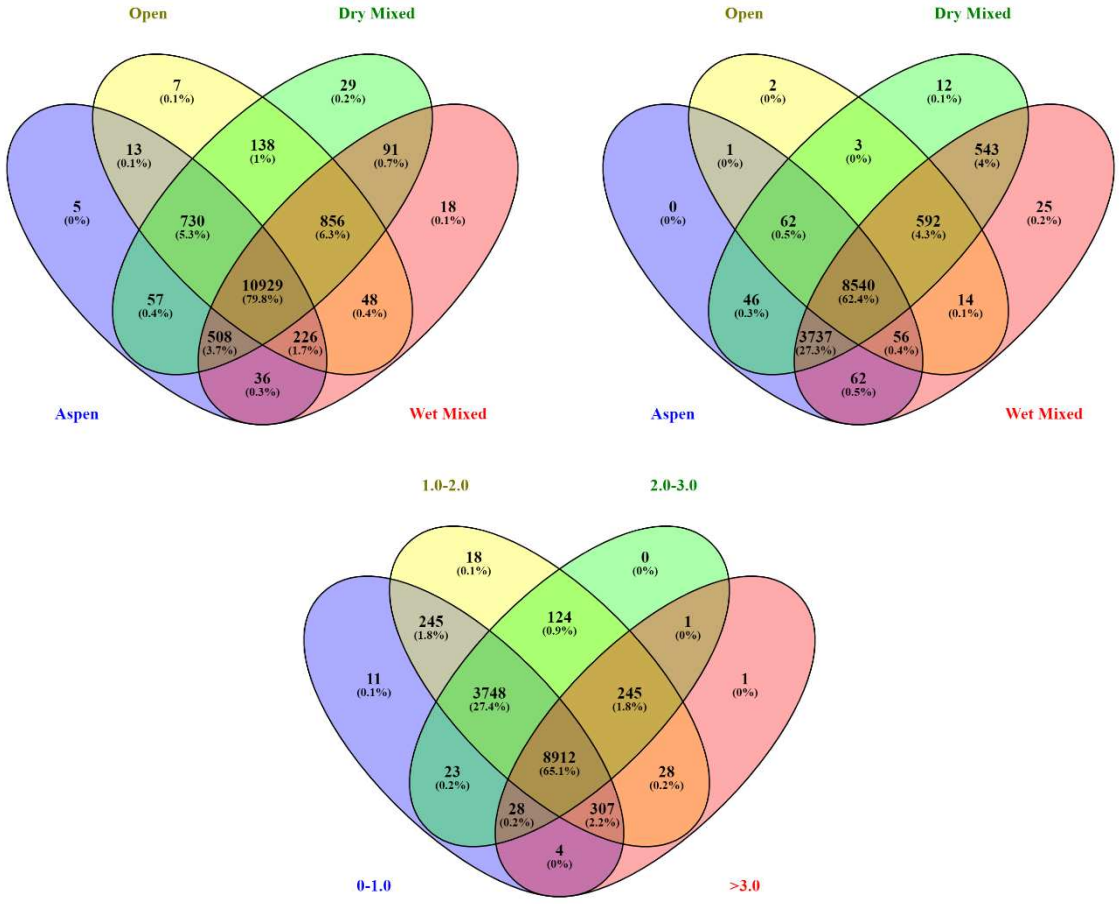

Figure 3-7: Bacterial core communities for understory forest type (top left), overstory forest type (top right), and nearest tree range (bottom). Bacterial core communities for all categories are large indicating similarities between bacterial communities in association with forest type and distance to the nearest tree.

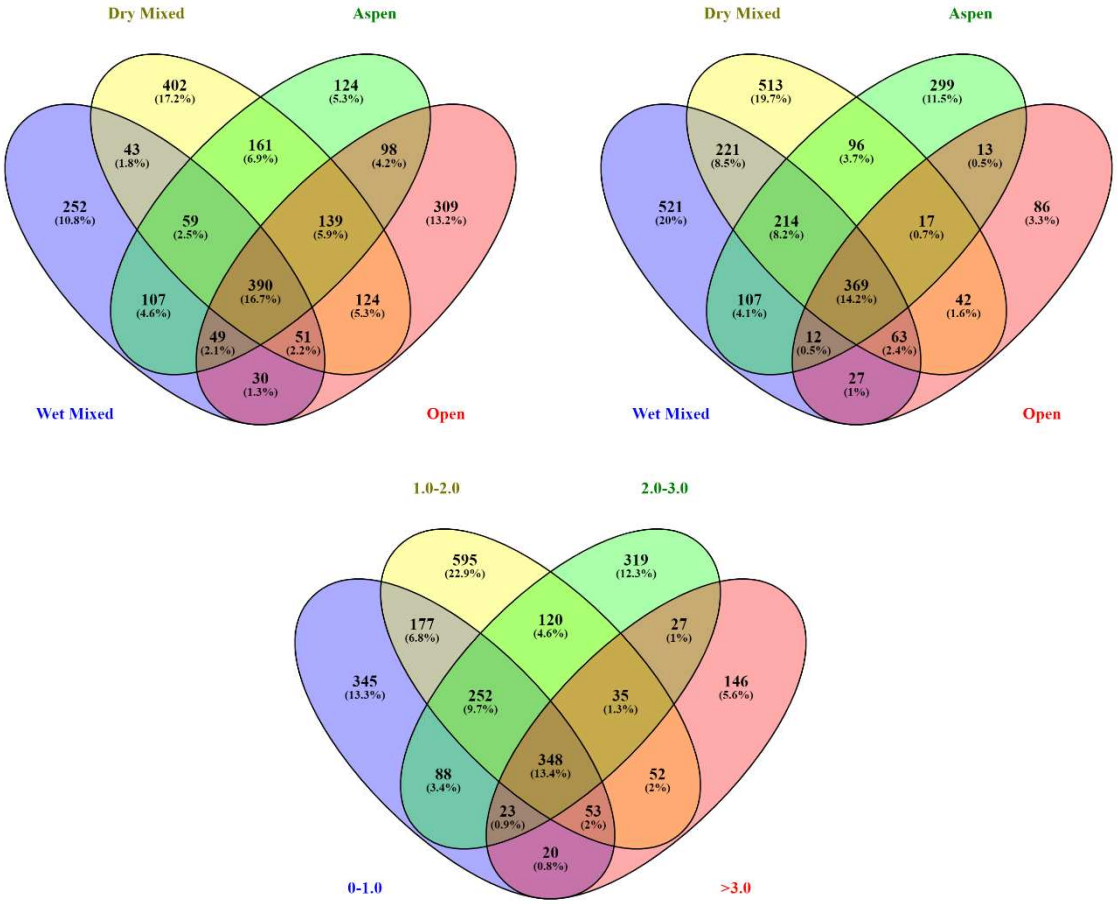

Figure 3-8: Venn diagram indicating fungal core and unique communities in association with understory forest type (top left), overstory forest type (top right), and nearest tree range (bottom). Core communities are small for all categories, as there is an increase in unique communities. This indicates that unique fungi are associated with different forest types and from the rhizosphere to bulk soil.

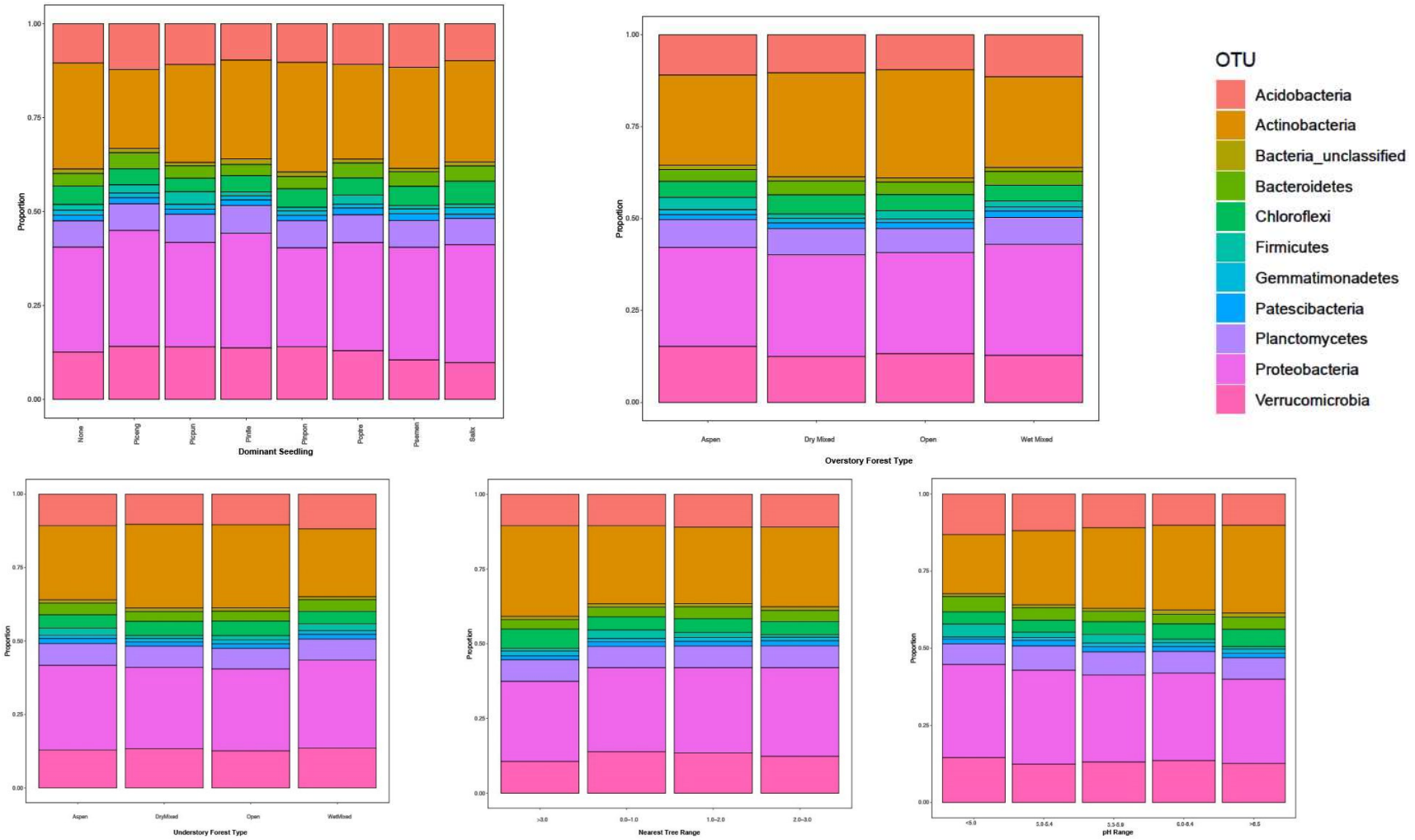

Figure 3-9: Bacterial communities' phyla relative abundance bar graph for soils associated with dominant seedlings (top left), overstory forest type (top middle), understory forest type (bottom left), nearest tree range (bottom middle), and pH range (bottom right). Eleven bacterial phyla are represented as their abundance is greater than 1% (top right).

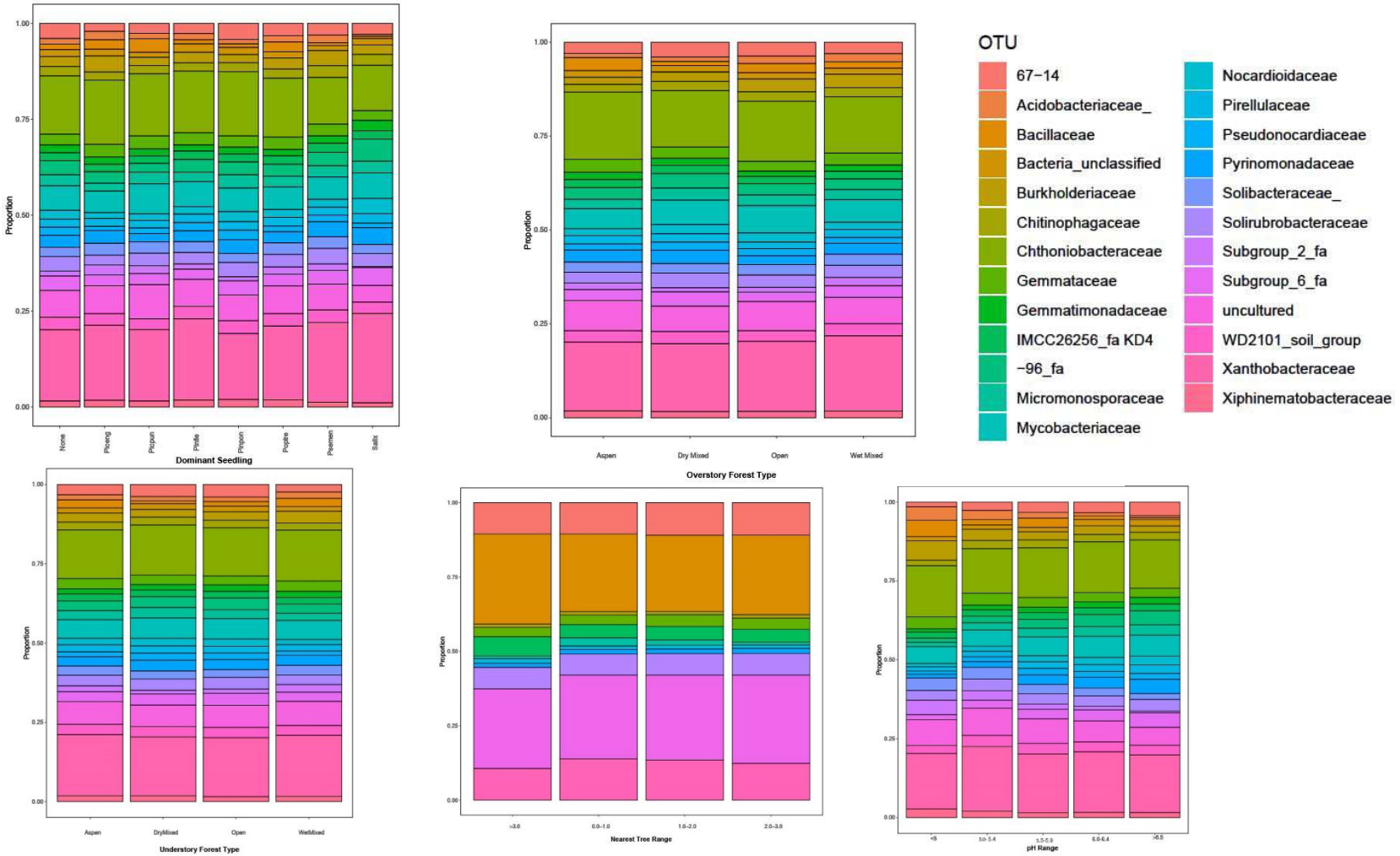

Figure 3-10: Bacterial communities' families relative abundance bar graph for soils associated with dominant seedlings (top left), overstory forest type (top middle), understory forest type (bottom left), nearest tree range (bottom middle), and pH range (bottom right). Twenty-five bacterial families are represented as their abundance is greater than 1% (top right).

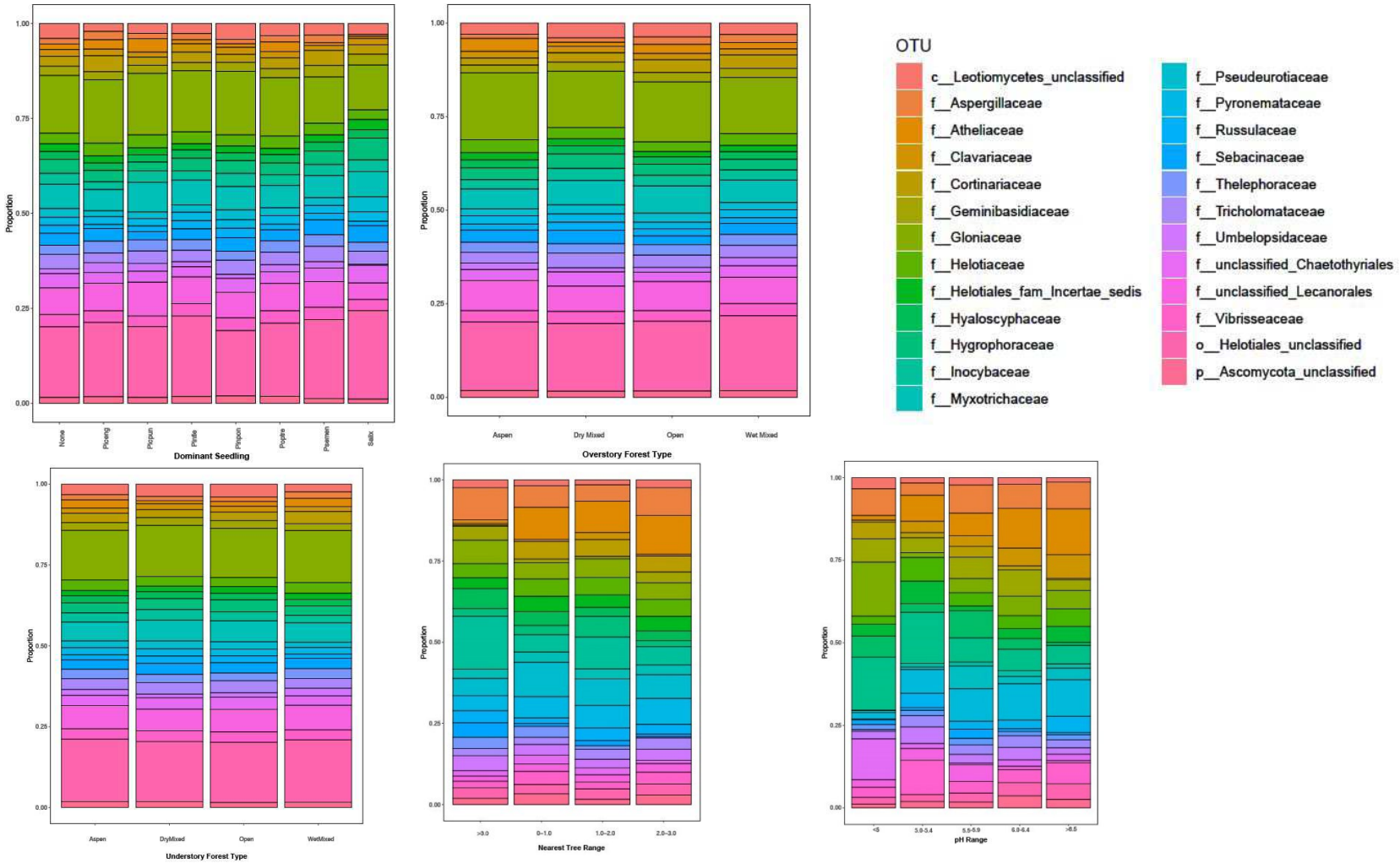

Figure 3-11: Fungal communities' families relative abundance bar graph for soils associated with dominant seedlings (top left), overstory forest type (top middle), understory forest type (bottom left), nearest tree range (bottom middle), and pH range (bottom right). Twenty-five fungal families are represented as their abundance is greater than 1% (top right).

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CHAPTER 4: INFLUENCE OF FOREST ECOYPES AND GEOGRAPHICAL DISTANCES ON SOIL MICROBIAL COMMUNITIES IN COLORADO AND WYOMING FOREST INVENTORY AND ANALYSIS PLOTS

4.1 Preface

Forest soils harness a multitude of biological diversity, that function to cycle nutrients , increase the uptake of water and nutrients, provide disease suppression, and as potential pathogens (Lladó et al., 2017). Yet, the knowledge of the specific connections between forest soils and above-ground plants and their distribution is still in the infancy of research. Current research has focused on specific geographic locations with a primary focus on forest management, yet an exhaustive study is needed to fully understand how forest type and elevation truly drive microbial community composition. To expand this research, the use of a Forest Inventory and Analysis (FIA) plots would provide the most robust sampling protocol within the United States. A subset of these plots already collects soil chemical and physical properties, therefore additional soil sampling for DNA analysis should be easy to implement at the program level. The use of soil microbial analysis will enhance the current use of soil as an indicator to forest health, by understanding the breadth of diversity that resides in a soil ecosystem.

Our study provided a pilot project from 54 Colorado and Wyoming P2+Soil FIA plots, consisting of 125 soils samples. We found that forest type drives change in fungal and bacterial, whereas elevation influenced fungal communities more so than bacterial communities. Additionally, site, stand, and climatic variables influence both fungal and bacterial differently. Stand age, stand density index, and quadratic mean diameter had a positive relationship with fungal richness and diversity, in which greater fungal richness and diversity are found in older, more dense stable forests, highlighted by a lack of disturbance in these stands. Slope had a positive influence with bacterial richness and diversity, as increased richness and diversity were

found in association to steeper slopes. Corresponding to the notion that bacteria are more prevalent in association to disturbance or high turnover (Liu et al., 2020). Climatic variables influenced bacterial communities, in which minimum and maximum annual temperature had a positive influence on bacterial Inverse Simpson's diversity.

All of this information greatly increases the knowledge of relationships between soil microbial communities and Rocky Mountain forests. As much of the information has been identified in different locations, the heterogeneity in both forests and soils makes regional specific connections important for future management practices. Therefore, the expansion of soil microbial analysis with the use of FIA plots is invaluable to expand our knowledge of all aspects of forest ecosystems to increase forest health for future generations.

4.2 Introduction

Forests are one the most productive ecosystems on Earth, which consist of a high level of diversity in terms of species and geographical locations (Buee et al., 2009; Baldrian, 2017a). Forests provide ecosystem services to conserve water, reduce erosion (Balloffet et al., 2022), aesthetics and recreation (Muth, 2016), and the reduction of greenhouses gases, as they are a vital aspect in global carbon sequestration (Lebeis, 2015). All of these services are connected below-ground by soils and microbial communities (Baldrian, 2017a). Soil microbial communities provide the greatest overall biological diversity in a forest ecosystem, consisting of as many as 5,000 organisms in one tablespoon of soil (Buee et al., 2009; Fierer, 2017), that sustain extensive biological processes including nitrogen fixation, increased uptake of water and nutrients, disease suppression, and potential pathogens (Lladó et al., 2017). These processes improve worldwide environmental quality as soil microbes act as a carbon sink, reducing carbon emissions, while increasing nutrient availability for plants and animals to utilize (Baldrian, 2017a). In fact, around 33-50% of all fixed carbon is exuded into the soil via roots, and stored as microbial biomass, while additional carbon, in recalcitrant litter and down woody material, will be decomposed and stored by soil microbes (Baldrian, 2017a). Understanding microbial communities and their ecosystem processes will inevitably enhance soil health, which provide vital nutrients for plants and trees to increase growth and productivity (Habiyaremye et al., 2020).

In forest ecosystems, researchers have identified microbes that are beneficial and detrimental to plant growth and productivity, while understanding the suite of microbes that assist in the protection of plants within the rhizosphere and bulk soil (Li et al., 2015). Studies have focused on differences between broadleaf and conifer trees, forests versus grasslands, and geographical isolation (Fierer et al., 2012; Aponte et al., 2013; Prescott and Grayston, 2013; Li et al., 2015). Tree species have direct and indirect influences on soil microbial communities (Ladygina and Hedlund, 2010; Thoms et al., 2010; Baldrian, 2017a; Lladó et al.,

2018: Gillespie et al., 2020). Mycorrhizal fungi exhibit host specificity in forests, especially ectomycorrhizal (EcM) and arbuscular mycorrhizal (AM) fungi, which promote the uptake of water and nutrients to plants, (Futai et al., 2008; Baldrian, 2017a; Lladó et al., 2017; Ferlian et al., 2018).

Although fungi are known to have a direct interaction with tree species, bacterial communities are less likely to be host specific (Crowther et al. 2014). Additionally, the relatively stable nature of forests promotes fungal communities over time, as disturbance enhances bacterial communities and overall microbial richness and diversity (Liu et al, 2020; Chen et al., 2021). Additionally, bacterial communities tend to be homogenous within forest soils, with more direct influences based on soil edaphic properties (Fierer and Jackson, 2006; Fierer et al., 2007; Crowther et al. 2014; Fierer, 2017).

Forests are generally diverse, as forest soils provide even more spatial heterogeneity (Buee et al., 2009). Trees and plants have a direct influence on microbes in the rhizosphere, with less interactions in the bulk soil. The carbon rich environment near the rhizosphere provides a hotspot for soil microbial activity (Tedersoo et al., 2016). A lack of resources in the bulk soil generally increases diversity of both fungal and bacterial communities, whereas the flush of resources in the rhizosphere can be saturated with r-strategists that quickly build biomass and utilize resources (Cui et al., 2018). Additionally, microbial community differences are also inherently driven by changes in plant communities associated with differing aspects, where spruce-fir tend to grow in northern or eastern aspects, while pine frequently grow in southern or western aspects, which encompasses differing influences of climate (Larsen, 1930). However, there is a huge unutilized resource in Forest Inventory and Analysis (FIA) plots that can enhance the analysis of forest soil microbial communities.

Since 1930, the FIA program has been the most important means to determine forest resources nationwide (LaBau et al., 2007), as it provides an understanding of the composition to our nation’s forests. The use of FIA field plots allows for the greatest diversity of sampling in the

United States as plots have been established in all states, as well as many U.S. territories (LaBau et al., 2007). Forest monitoring is key in maintaining overall tree health, as indicators of forest health were added to the FIA phase 2 protocol in the early 1990's to enhance the program's monitoring repertoire to include crown conditions, ozone, lichen, vegetation, down woody material, and soils (Woodall et al., 2011). Most of these forest health indicators still exist in the current inventory, while others (e.g. lichen and ozone) have been dismissed following sufficient collections. This study focuses on forest soils as an indicator of forest health.

According to the National FIA website, soil sampling, in conjunction with P2+Soils (formerly Phase 3) plots are collected to measure soil quality as an indicator of forest health. The P2+Soils plots are measured at a 1/16th subset of all FIA plots (Woodall et al., 2011). The three primary foci to the study determine the soil physical (compaction and erosion) and chemical properties to assess soil quality (O'Neill et al., 2005). The definition given on the National FIA site indicates that soil quality allows sustained biological productivity, environmental quality, and promotes plant and animal health. Yet, to fully determine these characteristics, greater insight may be provided with the use of soil microbial analyses, to expand outside of soil physical and chemical properties. To our knowledge, no other study has incorporated the use of soil microbial communities with FIA plots.

To understand the relationship between tree species and soil microbial communities, the ability to have direct interactions with trees is critical, making FIA plots a vital resource to expand knowledge of forest soil microbiome. Our main research objective for this study was to to identify if distinct shifts in soil microbial community composition and diversity are associated with forest ecotypes, elevational gradients, stand characteristics, and/or climatic variables. We hypothesize that distinct shifts in soil bacterial and fungal communities occurred along forest ecotypes, as litter composition indirectly links the richness and diversity of microbes. Additionally, microbial communities shift based on site characteristics, such as elevation, as greater richness and diversity are found at lower elevations. This research expands our

knowledge of forest health using a reliable forest inventory system and allows documentation of potential drivers of and changes to soil microbial communities across landscapes to assist in modeling, predicting, and managing forests for future generations.

4.3 Methods

4.3.1 FIA plot arrangement

FIA plots consisted of four permanent, fixed radius (24 ft.) subplots spaced by 120 ft apart arranged in the shape of triangle with a centralized subplot in the center (USDA-FS, 2022; Figure 4-1). Subplot 2 was located at 0° azimuth, subplot 3 was at 120°, and subplot 4 was at 240° from plot center of subplot 1. Forest site and stand characteristics were measured within the 24 ft. radius subplots, as four fixed radius (6.8 ft.) microplots were established 12 ft. due east of subplot center to measure seedlings and saplings. All plots were measured in the P2 protocol, whereas 1/16th of all plots had an expanded P2+Soils protocol to collect forest health indicators (USDA-FS RMRS, 2022). Specifically, soil samples and duff and litter were collected within P2+Soils plots (USDA-FS; 2010). Sampling locations were along the 120 ft. line transect from each sublot connecting to plot center (subplot 1). Each location was along the transect (heading back toward subplot 1) at 30 ft. from subplot center. The first soil sample was located along the line transect while subsequent samples would be offset 10 ft. alternating sides of the initial sample each measurement. Duff and litter were collected at all subplots (2, 3, & 4), while bulk density soil cores were collected only in conjunction with subplot 2. Bulk density soil cores consisted of sampling mineral soil from 0-to-8 inches in depth using an 8-inch soil core. The soils were split and bagged into 0-4- and 4-8-inch depth.

4.3.2 Supplemental soil sampling for soil microbial analysis

In conjunction with all 2018 P2+ soils plots in Colorado and Wyoming, subsamples of soil, ranging from 20 to 50 mL, were collected. No soil samples were collected on non-forest or denied access plots and subplots. Soil samples, in conjunction with bulk density samples, on

sublot 2 were collected by scooping soil within the walls of the hole provided by the bulk density sample from the top 0-4 inches of the mineral soil. Following the removal of duff and litter, soil samples in association with subplots 3 and 4, were collected by scooping mineral soil from the top layer of soil, without the use of the bulk density soil core. Similar to the excavation method for collecting soil cores on subplot 2, a small shovel was used to provide a hole to gather a soil sample with the metal tablespoon The metal tablespoon was sanitized between each sample using ethanol wipes to prevent cross-contamination. All soil samples were stored in 50 mL tubes and preserved either by placing in a cooler, when available, or by the use of instant ice packs when in remote locations. All soil samples were mailed overnight or two-day shipping to Colorado State University for DNA extractions and archiving.

4.3.3 Soil DNA extractions and Illumina Miseq sequencing

At Colorado State University, all 131 samples received were placed in the 4° C refrigerator for subsequent DNA extractions within one week of initial collection. A subsample of each soil was placed into a 5 mL tube and placed into a -80° C freezer to preserve the DNA. Three soil DNA extractions were completed for each sample, as described in Chapter 3 using Omega E.Z.N.A. Soil DNA kit (Omega Bio-Tek Inc., Norcross, GA). All samples that did not produce adequate quality or quantity of DNA were extracted again. All three duplicate samples were diluted to 30 ng/μL and bulked. DNA extraction efficiency varied depending on soil texture and composition. Most soil samples consisted of loam, while others were clay or sandy (Figure 4-2). Sandy soil was more difficult to extract DNA, which prompted numerous extractions to attempt to yield a sufficient sample.

A subsample of 30 μL the bulked DNA was pipetted into a 96-well plate and sent to the Colorado State University Next-Generation Core Lab (CSU-NGS) for library preparation and Illumina Miseq sequencing. One hundred and twenty-five total samples were sequenced, while the 6 samples did not yield sufficient DNA. Libraries were prepared for the 16S and ITS2 regions to sequence bacterial and fungal communities, respectively. Primers 515F (5'-

GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVHHHTWTCTAAT-3') (Walters et al., 2015) were used to amplify the v4 genomic region of the 16S rRNA gene. While the ITS3 (GCATCGATGAAGAACGCAGC) and ITS4 (TCCTCCGCTTATTGATATGC), which specifically amplified the ITS2 region with internal transcribed spacer region (White et al., 1990) were used to determine fungal communities. Paired end fasta files were received for all samples at both the 16S and ITS2 regions for further cleaning and analyses.

4.3.4 Cleaning DNA sequence data

As in Chapter 3, FastQC (Andrews, 2010), Trimmomatic (v0.36, Bolger et al. 2013), and Mothur (Schloss et al., 2019) were used to clean sequence data and reference to known fungal and bacterial databases. In the Mothur SOP protocol, all 16S paired end sequences were referenced to Silva database version 128 (Quast et al., 2013) to identify soil bacterial communities. While ITS2 paired end sequences were referenced to the UNITE fungal reference database, version 02.02.2019 (Nilsson et al., 2018), to identify soil fungal communities. Rarefaction curves were generated using the '*vegan*' package (Oksanen et al., 2013) in R version 3.6.1 (R Core Team, 2017) to compare the quality of read depth.

4.3.5 Statistical analysis of bacterial and fungal sequences

All data analyses were performed within RStudio interface to R (RStudio, 2015), based on methods in Ibarra Caballero and Lalande et al. (2022). After uploading the shared file of Operational Taxonomic Units (OTUs), all samples were further cleaned to remove low or potential error sequence. Each OTU read count was reduced by 2 and summed. All OTUs that resulted in ≤ 0 were removed, subsequently reducing the amount of OTUs. The shared and taxonomy files were merged to represent corresponding fungal and bacterial to OTU number. Site and stand variables were prepared into the metadata file and merged to corresponding plots for further analysis.

From FIA data collections, plots were categorized into forest type and elevational range. Forest type data was identified using the FIA coding for ForTypPCD, which is calculated using an algorithm basing its decision on dominant overstory canopy cover (USDA-FS, 2022). Forest type was split into 10 levels: aspen (fungal n=13, bacterial n=12), *Cercocarpus* woodland (fungal n=3, bacterial n=2), cottonwood (fungal n=1, bacterial n=1), deciduous oak (fungal n=12, bacterial n=11), Douglas-fir (fungal n=17, bacterial n=13), Engelmann spruce/subalpine fir (fungal n=19, bacterial n=16), lodgepole pine (fungal n=26, bacterial n=25), nonstocked (fungal n=2, bacterial n=3), pinyon-juniper woodland (fungal n=16, bacterial n=16), and ponderosa pine (fungal n=8, bacterial n=5). Elevational ranges were described as anything below 5,000 ft with factors of 1,000 ft until 10,000 ft. These categories were used for alpha and beta diversity analysis and creating relative abundance bar graphs. Elevational range was split into seven factors: 4000-5000 (fungal n=6, bacterial n=3), 5000-6000 (fungal n=5, bacterial n=5), 6000-7000 (fungal n=12, bacterial n=11), 7000-8000 (fungal n=26, bacterial n=26), 8000-9000 (fungal n=34, bacterial n=30), 9000-10000 (fungal n=20, bacterial n=18), and >10000 (fungal n=14, bacterial n=11).

Alpha diversity and richness analyses was summarized for forest type and elevational gradient and included Shannon and Inverse Simpson diversity indices using the '*phyloseq*' package (McMurdie and Holmes, 2013), as richness measurements were calculated using '*vegan*'. Numerous measurements were used to calculate alpha diversity since Shannon's diversity utilizes richness and rare microbes, while Inverse Simpson's diversity includes evenness and dominant microbes (Hill et al., 2006; Nagendra, 2002). Richness is calculated based on the number of individuals within a sample, as evenness calculates the relative abundance (Zhang et al., 2012).

Relative abundance for fungal families and bacterial families and phyla were developed for forest type, elevational gradient, and state using the '*metagenomeseq*' package (Paulson et al., nd). Beta diversity was calculated using principal coordinate analysis in '*vegan'* to

statistically visualize fungal and bacterial soil differences within each category above to determine the diversity between samples (Oksanen et al., 2013).

4.3.6 Statistical analysis of site and stand characteristics and climatic factors

Soil physical properties and stand variable were collected via FIA DataMart (https://apps.fs.usda.gov/fia/datamart/datamart.html) including down woody material (below ground carbon), litter, basal area, stand density index (SDI), basal area, quadratic mean diameter (QMD), crown cover, stand age, forest type, slope, elevation, aspect, latitude, and longitude. FIA soil chemistry analysis was not uploaded to DataMart, therefore down woody material was used as a proxy for above and below-ground chemical properties. Additionally, annual climatic variables (min/max temperature [C], precipitation [mm], and min/max vapor pressure deficit [vpd]) were collected for each plot location using Oregon State PRISM (PRISM Climate Group, 2021) to determine the influence climatic variables have on soil fungal and bacterial community richness and diversity. Climatic factors were calculated by averaging over 5 years prior (2014-2018) to the sampling. Data analysis incorporates the climatic differences. All soil physical properties and stand and climatic variables were used in a linear regression model, to determine which variables influenced fungal and bacterial richness and diversity. First, highly correlated variables were removed from the dataset to reduce redundancies in the predictor variables determined by variables that were 90% correlated. A linear model was designed to determine which predictor variables (soil physical properties, stand and climatic variables) influenced the response variables (richness, Shannon's, and Inverse Simpson's diversity). The summary table for each model was used to determine significance based on the interaction of all variables simultaneously on the response variables.

4.4 Results

4.4.1 Processing tagged ITS2 and 16S sequences and libraries in Mothur

A total of 125 samples were sent for sequencing and library preparation out of the potential 162 samples. Thirty-one samples were not collected due to extenuating circumstance,

such as non-forested plot, denied access, or hazardous conditions, and six samples, which consisted of sandy soils, were collected yet did not yield sufficient DNA to send for sequencing. Samples that yielded < 5000 reads were removed from analysis, as 8 fungal samples (n=117) and 21 bacterial samples (n=104) were removed.

The total number of fungal reads was 5,515,517 with an average read depth of 44,124 reads/sample. After classifying all sequences to the reference UNITE dataset, version 02.02.2019, the sequence depth ranged from 1,013 to 511,098 reads. The ITS data resulted in 13,579 unique OTUs, 1,279 genera, 558 families, 225 orders, and 21 phyla. A rarefaction curve plateaued for most samples, indicating that the fungal communities were sequenced at a good depth (Figure 4-3).

The total number of bacterial reads was 3,411,805 with an average read depth of 27,294 reads/sample. After classifying all sequences to the 128 reference Silva dataset the sequence depth ranged from 4 to 170,504 reads. The 16S data resulted in 14,675 unique OTUs, 804 genera, 441 families, 259 orders, and 29 phyla. Again, a rarefaction curve identified that most samples plateaued, indicating that the bacterial data was sequenced at a good depth (Figure 4-3).

4.4.2 Differences in fungal and bacterial community richness, α-diversity, and β-diversity

Fungal and bacterial communities were analyzed for richness and alpha diversity for forest type and elevational range. Forest type significantly influenced fungal and bacterial richness (p=<0.0001; p=0.0003), Shannon's (p = <0.0001; p=0.0003) and Inverse Simpson (p = <0.0001; p = <0.0001) diversity indices (Tables 4-1 & 4-2). Additionally, fungal and bacterial richness (p = 0.0004; p=0.009) and Shannon's diversity (p = 0.0003; p=0.006) were influenced by elevation, whereas elevation only influenced bacterial Inverse Simpson's diversity (p=0.001) (Tables 4-1).

Table 4-1: Fungal and bacterial richness and diversity indices calculated for all categories of analysis; Forest type and Elevational gradient. Spruce-fir = Engelmann spruce/subalpine fir. Values represent within group mean and standard errors.

	Fungal (ITS)			Bacterial (16S)		
	Richness	**Shannon**	**InvSimpson**	**Richness**	**Shannon**	**InvSimpson**
Forest Type	*	*	*	*	*	*
Aspen	199.9 ± 12.12^{A}	4.00 ± 0.168^{A}	28.72 ± 2.59^{A}	168 ± 9.54^{A}	5.00 ± 0.143^{A}	67.8 ± 6.34^{A}
Cercocarpus	163.5 ± 25.23^{B}	3.72 ± 0.351^{B}	19.79 ± 5.4^{B}	106 ± 23.36^{B}	4.34 ± 0.35^{A}	48.3 ± 15.53^{A}
Cottonwood	178.6 ± 43.7^{B}	3.95 ± 0.607^{B}	16.56 ± 9.34^{B}	184 ± 33.04^{A}	5.25 ± 0.495^{A}	97.4 ± 21.97^{A}
Deciduous oak	145.6 ± 12.6B^{b}	3.55 ± 0.175^{B}	19.44 ± 2.7^{B}	159 ± 9.96^{A}	4.93 ± 0.149^{A}	70.4 ± 6.62^{A}
Douglas-fir	104.1 ± 10.6^{B}	3.19 ± 0.147^{B}	13.45 ± 2.27^{B}	155 ± 9.16^{A}	4.86 ± 0.137^{A}	61.0 ± 6.09^{A}
Spruce-fir	89.2 ± 10.03^{C}	2.67 ± 0.139^{C}	8.14 ± 2.14^{C}	119 ± 8.26^{B}	4.26 ± 0.124^{B}	38.9 ± 5.49^{B}
Lodgepole	100.5 ± 8.57^{C}	3.13 ± 0.119^{B}	12.61 ± 1.83^{B}	129 ± 6.61^{B}	4.46 ± 0.099^{B}	44.2 ± 4.39^{B}
Nonstocked	138.5 ± 30.9^{B}	3.45 ± 0.429^{B}	14.87 ± 6.61^{B}	152 ± 19.08^{A}	4.77 ± 0.286^{A}	51.7 ± 12.68^{A}
Pinyon/Juniper	138.6 ± 10.9^{B}	3.37 ± 0.152^{B}	15.02 ± 2.34^{B}	163 ± 8.26^{A}	4.99 ± 0.124^{A}	76.5 ± 5.49^{A}
Ponderosa	107.5 ± 15.45^{B}	3.15 ± 0.215^{B}	13.22 ± 3.3^{B}	134 ± 14.78^{B}	4.57 ± 0.222^{A}	50.0 ± 9.82^{A}
Elevation	*	*		*	*	*
4000-5000	105.7 ± 20.38^{A}	3.14 ± 0.261^{A}	14.28 ± 4.27^{A}	110 ± 20.22^{A}	4.27 ± 0.301^{A}	40.3 ± 13.49^{A}
5000-6000	161.1 ± 22.33^{A}	3.69 ± 0.286^{A}	18.23 ± 4.68^{A}	152 ± 15.66^{B}	4.77 ± 0.233^{B}	54.2 ± 10.45^{B}
6000-7000	153.3 ± 14.41^{A}	3.49 ± 0.184^{A}	15.45 ± 3.02^{A}	155 ± 10.56^{B}	4.90 ± 0.157^{B}	74.1 ± 7.05^{B}
7000-8000	130.2 ± 9.79^{A}	3.33 ± 0.125^{A}	15.91 ± 2.05^{A}	159 ± 6.87^{B}	4,90 ± 0.102^{B}	65.1 ± 4.58^{B}
8000-9000	119.6 ± 8.56^{A}	3.39 ± 0.11^{A}	18.17 ± 1.79^{A}	145 ± 6.39^{B}	4.70 ± 0.095^{B}	54.8 ± 4.27^{B}
9000-10000	137.9 ± 11.16^{A}	3.33 ± 0.143^{A}	13.24 ± 2.34^{A}	140 ± 8.26^{B}	4.61 ± 0.123^{B}	58.2 ± 5.51^{B}
>10000	68.1 ± 13.34^{B}	2.47 ± 0.171^{B}	7.94 ± 2.79^{B}	112 ± 10.56^{A}	4.17 ± 0.157^{A}	30.8 ± 7.05^{A}

* Significance based on linear regression; p =0.05.
$^{A, B, C}$ significant differences within categories and richness and diversity indices, associated with lsmeans averages

A principal coordinate analysis was used to determine if fungal and bacterial community β-diversity was associated with forest type and elevation. Forest type had an effect on fungal β-diversity (p = 0.001), as there as little to no overlap between points across samples from the high elevation spruce-fir/lodgepole pine and low elevation species in the PCoA plots (Figure 4-4). Similar trends were observed for bacterial communities, as forest type had an effect on bacterial β-diversity (p = 0.021; Figure 4-4). Additionally, elevation had an effect on fungal β-diversity, with again, little to no overlap between higher elevations and lower elevations factors

(Figure 4-5). Elevation, however, had no effect on bacterial beta diversity (p = 0.229; Figure 4-5).

3.4.3 Fungal and bacterial taxonomic trends and relative abundance

Twenty-four fungal families resulted in >1% abundance of all samples. Across all samples Atheliaceae, Pyronemataceae, and Tricholomataceae were found in highest abundance for all forest types and elevation (Figure 4-6 & 4-7). Variation occurred among forest type and elevation separately, as some taxa were in greater abundance for specific categories (Figure 4-6 & 4-7).

Relative abundance for bacteria found 9 phyla and 28 families that resulted in >1% abundance. Forest type varied in phyla abundance, as Acidobacteria, Actinobacteria, and Proteobacteria were in high abundance for all categories (forest type, and elevation; Figures 4-8 & 4-9). Clear trends occurred for elevation, with some taxa more abundant in lower elevations compared to high elevations and vice-versa (Figure 4-9). For bacterial families, relative abundance analysis found that Chthoniobacteraceae (Verrucomicrobia), Solibacteraceae (Acidobacteria), TK10 (Chloroflexi), and Xanthobacteraceae (Proteobacteria) were at a high abundance for all categories (Figure 4-10 & 4-11).

3.4.4 MetagenomeSeq analysis associated with forest type and elevation

Soil edaphic properties, stand measurements and climatic variables were analyzed using a linear regression to determine which variables influenced soil fungal and bacterial richness and diversity indices. The aspen, slope, stand age, and quadratic mean diameter (QMD) had a positive influence on fungal richness, while Douglas-fir, lodgepole pine, pinyon-juniper woodlands, ponderosa pine, and latitude had a negative influence (Table 4-2). Whereas percent bare ground positively influenced fungal Shannon's diversity, while no variables had a negative influence (Table 4-2). Lastly, *Cercocarpus* woodlands and lodgepole pine had a negative influence on fungal Inverse Simpson's diversity (Table 4-2).

Table 4-2: Fungal linear regression models of richness and diversity indices. Std Age = Stand age, asp = aspect, DD = down dead, C = carbon, BG = below-ground, Precip = precipitation, VPD = vapor pressure deficit, BA = basal area, QMD = quadratic mean diameter, SDI = stand density index.

	Richness			Shannon's			Inverse Simpson's	
	t-value	p-value		t-value	p-value		t-value	p-value
Aspen	2.309	*p*=0.023*	Aspen	0.965	*p*=0.337	Aspen	-0.096	*p*=0.924
Cercocarpus	-0.961	*p*=0.339	Cercocarpus	-0.536	*p*=0.593	Cercocarpus	-2.027	*p*=0.046*
Cottonwood	-1.031	*p*=0.314	Cottonwood	-0.460	*p*=0.647	Cottonwood	-1.121	*p*=0.265
Oak woodland	-1.261	*p*=0.211	Oak woodland	-0.261	*p*=0.795	Oak woodland	-0.861	*p*=0.392
Douglas-fir	-2.565	*p*=0.012*	Douglas-fir	-1.574	*p*=0.119	Douglas-fir	-1.464	*p*=0.147
Spruce-fir	0.205	*p*=0.838	Spruce-fir	-0.497	*p*=0.620	Spruce-fir	-0.605	*p*=0.547
Lodgepole	-2.227	*p*=0.029*	Lodgepole	-1.355	*p*=0.179	Lodgepole	-2.068	*p*=0.042*
Nonstocked	-1.864	*p*=0.066	Nonstocked	-1.133	*p*=0.261	Nonstocked	-1.928	*p*=0.057
PJ	-2.581	*p*=0.012*	PJ	-1.332	*p*=0.187	PJ	-1.201	*p*=0.233
Ponderosa	-2.970	*p*=0.004*	Ponderosa	-1.532	*p*=0.129	Ponderosa	-1.081	*p*=0.283
Elevation	-1.939	*p*=0.056	Elevation	-0.595	*p*=0.554	Elevation	0.789	*p*=0.432
Latitude	-2.076	*p*=0.041*	Latitude	-0.749	*p*=0.456	Latitude	0.458	*p*=0.648
Longitude	-0.518	*p*=0.606	Longitude	-0.633	*p*=0.528	Longitude	-0.165	*p*=0.869
Std Age	2.239	*p*=0.028*	Std Age	1.185	*p*=0.239	Std Age	1.201	*p*=0.233
Slope	2.039	*p*=0.045*	Slope	1.269	*p*=0.208	Slope	-0.231	*p*=0.818
Sin asp	-0.281	*p*=0.780	Sin asp	-0.905	*p*=0.368	Sin asp	0.169	*p*=0.866
Cos asp	0.530	*p*=0.598	Cos asp	0.189	*p*=0.850	Cos asp	0.631	*p*=0.530
DD C	1.348	*p*=0.181	DD C	0.920	*p*=0.360	DD C	0.871	*p*=0.386
Litter	-1.567	*p*=0.121	Litter	-1.049	*p*=0.297	Litter	-1.054	*p*=0.295
BG C	0.094	*p*=0.926	BG C	-0.370	*p*=0.712	BG C	-0.029	*p*=0.977
Precip.	-0.300	*p*=0.765	Precip.	-0.543	*p*=0.589	Precip.	-0.772	*p*=0.442
Min (°C)	0.307	*p*=0.759	Min (°C)	0.216	*p*=0.830	Min (°C)	-0.311	*p*=0.757
Mean (°C)	-0.118	*p*=0.907	Mean (°C)	-0.093	*p*=0.926	Mean (°C)	0.408	*p*=0.684
Max (°C)	-0.156	*p*=0.876	Max (°C)	-0.079	*p*=0.938	Max (°C)	-0.497	*p*=0.621
VPD min	-1.564	*p*=0.122	VPD min	-0.850	*p*=0.397	VPD min	-0.533	*p*=0.596
VPD max	1.706	*p*=0.092	VPD max	1.065	*p*=0.290	VPD max	0.856	*p*=0.394
BA live	-1.151	*p*=0.253	BA live	-0.564	*p*=0.574	BA live	-1.326	*p*=0.188
Crown %	0.285	*p*=0.777	Crown %	0.225	*p*=0.823	Crown %	-0.142	*p*=0.888
% Bare	-1.886	*p*=0.063	% Bare	-2.229	*p*=0.029*	% Bare	-1.276	*p*=0.205
QMD	2.001	*p*=0.049*	QMD	1.275	*p*=0.206	QMD	1.573	*p*=0.120
SDI	0.249	*p*=0.804	SDI	-0.199	*p*=0.842	SDI	0.731	*p*=0.467
Model		*p*=<0.001	Model		*p*=<0.001*	Model		*p*=<0.001*

* Significance based on *p* =0.05.

Slope had a positive influence on bacterial richness, as spruce-fir and down dead carbon had a negative influence (Table 4-3). Again, slope had a positive influence on bacterial Shannon's diversity, as spruce-fir had a negative influence (Table 4-3). Additionally, slope, nonstocked forests, below ground carbon, minimum temperature, and maximum temperature had a positive influence, whereas cosine of aspect, down dead carbon, and mean temperature negatively influenced bacterial Inverse Simpson diversity (Table 4-3).

Table 4-3: Bacterial linear regression models of richness and diversity indices. Std Age = Stand age, asp = aspect, DD = down dead, C = carbon, BG = below-ground, Precip = precipitation, VPD = vapor pressure deficit, BA = basal area, QMD = quadratic mean diameter, SDI = stand density index.

Richness			**Shannon's**			**Inverse Simpson's**		
	t-value	**p-value**		**t-value**	**p-value**		**t-value**	**p-value**
Aspen	-0.842	*p*=0.403	Aspen	-0.731	*p*=0.467	Aspen	-0343	*p*=0.732
Cercocarpus	-0.565	*p*=0.574	Cercocarpus	-0.096	*p*=0.924	Cercocarpus	0.165	*p*=0.870
Cottonwood	-0.176	*p*=0.861	Cottonwood	0.195	*p*=0.846	Cottonwood	0.681	*p*=0.498
Oak woodland	-0.487	*p*=0.628	Oak woodland	-0.150	*p*=0.881	Oak woodland	0.082	*p*=0.935
Douglas-fir	-0.144	*p*=0.886	Douglas-fir	0.142	*p*=0.886	Douglas-fir	0.754	*p*=0.454
Spruce-fir	-2.048	*p*=0.044*	Spruce-fir	-2.041	*p*=0.045*	Spruce-fir	-0.864	*p*=0.390
Lodgepole	-0.539	*p*=0.591	Lodgepole	-0.553	*p*=0.582	Lodgepole	-0.217	*p*=0.829
Nonstocked	1.671	*p*=0.099	Nonstocked	1.599	*p*=0.114	Nonstocked	2.361	*p*=0.021*
PJ	0.079	*p*=0.937	PJ	0.446	*p*=0.657	PJ	0.644	*p*=0.521
Ponderosa	0.445	*p*=0.657	Ponderosa	0.771	*p*=0.443	Ponderosa	0.776	*p*=0.440
Elevation	-0.022	*p*=0.983	Elevation	0.313	*p*=0.756	Elevation	-0.870	*p*=0.387
Latitude	0.051	*p*=0.959	Latitude	0.292	*p*=0.771	Latitude	-0.755	*p*=0.453
Longitude	-1.193	*p*=0.237	Longitude	-1.241	*p*=0.219	Longitude	-1.146	*p*=0.256
Std Age	0.880	*p*=0.382	Std Age	0.752	*p*=0.455	Std Age	1.258	*p*=0.213
Slope	2.403	*p*=0.019*	Slope	2.430	*p*=0.018*	Slope	2.150	*p*=0.035*
Sin asp	-1.068	*p*=0.289	Sin asp	-1.092	*p*=0.278	Sin asp	-1.107	*p*=0.272
Cos asp	-1.802	*p*=0.076	Cos asp	-1.580	*p*=0.118	Cos asp	-2.037	*p*=0.045*
DD C	-2.145	*p*=0.035*	DD C	-1.946	*p*=0.056	DD C	-2.447	*p*=0.017*
Litter	0.676	*p*=0.501	Litter	0.497	*p*=0.621	Litter	0.444	*p*=0.658
BG C	1.964	*p*=0.053	BG C	1.742	*p*=0.086	BG C	2.045	*p*=0.045*
Precip.	0.438	*p*=0.662	Precip.	0.848	*p*=0.399	Precip.	0.508	*p*=0.613
Min (°C)	1.709	*p*=0.092	Min (°C)	1.595	*p*=0.115	Min (°C)	2.339	*p*=0.022*
Mean (°C)	-1.680	*p*=0.097	Mean (°C)	-1.576	*p*=0.119	Mean (°C)	-2.334	*p*=0.022*
Max (°C)	1.736	*p*=0.087	Max (°C)	1.644	*p*=0.105	Max (°C)	2.297	*p*=0.025*
VPD min	-0.059	*p*=0.953	VPD min	0.067	*p*=0.947	VPD min	0.549	*p*=0.591
VPD max	-0.850	*p*=0.398	VPD max	-0.829	*p*=0.410	VPD max	-0.314	*p*=0.754

BA live	1.023	p=0.310	BA live	0.913	p=0.364	BA live	-0.070	p=0.945
Crown %	-0.588	p=0.559	Crown %	-0.880	p=0.382	Crown %	-0.527	p=0.600
% Bare	0.240	p=0.811	% Bare	0.086	p=0.932	% Bare	-1.365	p=0.176
QMD	-0.910	p=0.366	QMD	-0.631	p=0.530	QMD	0.046	p=0.963
SDI	-0.552	p=0.583	SDI	-0.467	p=0.635	SDI	0.522	p=0.603
Model		p=<0.001	Model		p=<0.001	Model		p=<0.001

* Significance based on p =0.05.

4.5 Discussion

The main objective of this study was to determine the drivers of soil microbial communities at a landscape level, using FIA plots. Studies related to specific drivers of soil microbial communities in Rocky Mountain forests are limited, therefore the expansive use of FIA plots in Colorado and Wyoming allowed for greater insight into these stands. Using the current available FIA data from 2018, our results demonstrated that forest type was the main driver of change for both fungal and bacterial community composition, as soil edaphic properties were not available for analysis. Additionally, elevational gradient was a significant driver of fungal and bacterial richness and α-diversity, yet only influenced fungal β-diversity. We identified an abundance of beneficial microbes, that function primarily as mycorrhizal fungi and nutrient cycling bacteria, which are common in established forests (Li et al., 2015). Yet, many of the fungi found in high abundance were unclassified in the Unite database. Forest density and age influenced fungal richness; slope affected bacterial richness and diversity indices, whereas temperature had an effect on bacterial Inverse Simpson's diversity.

As soil microbial communities adapt within mature forests, they turn into stable communities (Ferlian et al., 2018). In our study, stand age and QMD had a positive influence on fungal richness, suggesting that as stands age, soils have more species of fungi present. Conversely, Lui et al. (2020) found that as forests reach late successional stages, fungal communities, especially mycorrhizal fungi, become more established, therefore fungi, specifically diversity, decreases over time. As stands age, microbial communities likely favor fewer fungal communities or oligotrophic bacteria which have higher biomass (Li et al., 2015;

Lui et al., 2020), although this concept may not incorporate the dynamics of older and dying forests as stands age. In our dataset, the average stand age was 109 years with a majority of stands over 80 years old, while 30% of plots were under 30 years old. Therefore, our high level of richness could be attributed to increased mortality and dead wood as trees age, in turn increasing saprophytic or decomposing fungi (Kyaschenko et al., 2017; Osburn et al., 2019).

Stand characteristics that influenced bacterial richness were highlighted by slope. Slope positively influenced richness and both α-diversity indices, which corresponds to a greater likelihood of soil disturbance with erosion in steep slopes (Shigyo et al., 2022). Bacteria tend to take advantage of areas with high disturbance as they can utilize the resources quicker than fungal microbes (Liu et al., 2020).

Forest type can directly or indirectly influence soil microbial communities, as the interactions between leaf litter and rhizosphere manipulate soil edaphic properties that are essential to the establishment of microbes (Baldrian, 2017a; Lladó et al., 2018). Trees at lower elevations and deciduous forests had high overlap in β-diversity (PCoA plots) of the fungal and bacterial communities whereas high elevation coniferous stands (spruce-fir & lodgepole pine) differed. This could have been influenced by soil edaphic properties, temperatures, or differing leaf litter types. Soil pH shifts depending on forest type, in which low quality coniferous leaf litter will decrease soil pH due to their chemical structure, and higher quality deciduous leaf litter typically reduces acidification (Kaiser et al., 2016; Lladó et al., 2018). Additionally, soil carbon, nitrogen, and phosphorous are frequently limited in forest soils, yet they can be increased through the breakdown of leaf litter and organic matter (Heuck et al., 2015). Soil microbial communities assist in this decomposition of leaf litter, converting usable nutrients for plants and microbes, while manipulating the soil pH, depending on litter type (Lladó et al. 2018). Soil pH is often the most influential driver of change in soil bacterial communities (Fierer and Jackson, 2006; Rousk et al., 2010; Prescott and Grayston, 2013), as well as mycorrhizal fungi are positively influenced by soil pH (Dumbrell et al., 2010; Tedersoo et al., 2014). We used down

woody material as a proxy for soil carbon, with no other soil edaphic properties, therefore further insight into all soil edaphic properties may elucidate other drivers attributed to forest type. At this resolution, we found that high levels of woody materials positively influenced fungal richness, while no other stand variables influenced fungal or bacterial communities. With this understanding, we utilized elevational gradients to grasp relationships outside of forest type alone.

Elevational gradients only influenced fungal β-diversity. The relative lack of elevational influence on bacterial communities corresponds to Fierer et al. (2011) which found that there were no distinct changes in bacterial diversity within elevational gradients. As for fungal communities, in the Northern Rocky Mountains an increase in elevation resulted in lower diversity. We also observed lower fungal diversity and richness in our extreme high and low elevations, most likely due to the sensitivity of fungi to more extreme environmental factors (Egan et al., 2017). The stressful conditions may provide niches for certain fungal taxa (i.e., mycorrhizae) to establish symbiotic relationships with their hosts (Bryant et al. 2008; Chagnon et al., 2013; Looby et al. 2016).

Forest type and elevation can be used synonymously for some species/forest types, such as spruce-fir and lodgepole pine in high elevation stands and pinyon-juniper or oak woodlands in lower elevation stands (Larsen, 1930). We observed that high elevation Engelmann spruce/subalpine fir and lodgepole pine had similar fungal beta diversity, and that low elevation species of pinyon-juniper, oak woodlands, cottonwood and *Cercocarpus* were also similar (Figure 4-4). Additionally, high overlap occurred between soils associated with low elevation species, ponderosa pine and aspen, which have large elevational ranges (Huckaby et al., 2003; CSFS, 2022). The similarities among low elevational species, like ponderosa pine, and aspen corresponds in which the highest fungal and bacterial richness occurred from 5,000 to 7,000 ft in elevation. This elevation zone is termed the montane mixed conifer forest and woodlands, consisting of a high diversity of trees combining dry-mesic and moist-mesic forest

types (Decker et al., 2020). Hence, within this elevational range forest diversity is likely to be relatively high, which we found associated with high microbial diversity.

Our observation of high overlap among overstory species at low elevations and that of ponderosa pine and aspen may suggest that these forest types may have similar microbial communities. This knowledge may be useful for the assisted migration of trees as a result of climate change. This is especially important as soils with more diverse overstory forests result in a greater likelihood of carry-over effects within soil microbial communities (Bartelt-Ryser et al., 2005), providing benefits for future forests. Additionally, soils with high above-ground diversity, provide inherent benefits to forest health, as they are more resilient to insects and diseases (Thompson et al., 2009) and increase below-ground diversity, which enhances plant productivity (Aponte et al., 2013; Li et al., 2015). The plant-soil feedback with certain microbial taxa will provide an adequate environment when the establishment of different species is necessary, as climate trend towards a warmer and drier environment (Williams and Dumroese, 2014)

Weather patterns can have a strong influence on microbial community structure (Brockett et al., 2011; de Vries et al., 2012; Baldrian, 2017b). Climatic factors directly influenced bacterial Inverse Simpson's diversity. We observed that minimum and maximum annual temperature had a positive influence on bacterial diversity, specifically greater relative abundance of more dominant communities. Therefore, climatic extremes enhanced the differences observed within our sampling. These warming trends may have influenced the composition of soil microbial communities, as an increase in soil temperature enhances microbial activity, especially in summer months (Baldrian, 2017b). More so, the increase in temperature may result in more resilient heterogenous forests comparatively to monocultures (Gillespie et al., 2020). Mixed stands or stands that have similar communities such as our low elevation forests, may be able to withstand climatic changes and maintain normal biogeochemical cycling during extreme droughts or weather events (Gillespie et al., 2020).

4.5.1 Limitations of study

The main duty for FIA crews were to collect field measurements at each plot, with a secondary task of collecting additional soil samples for our study. The procedures to fulfil our samples was another task to complete, in already busy inventory protocol. Therefore, the preservation of samples on ice, following collection may have not been ideal. We provided instant ice packs to preserve samples in field packs, until they reached a cooler or refrigerator. Yet, these ice packs only stay cold for a limited time and do not work as well as immediately placing samples in a cooler, as we normally do in our sampling protocol. With this understanding, we extracted DNA within one week of sampling. This allowed for the greatest quality DNA extractions, while reducing degradation of soil DNA. As most samples were kept cold following collection, while others were collected in more remote locations which could have degraded the DNA over time.

When analyzing data, we need to understand that FIA soil samples were taken adjacent to the subplots therefore forest type, density, and other characteristics were based on adjacent stand data. This provided inferences to the interaction between forest type in the stand, yet the heterogeneity within soil may be difficult to assess if these inferences were related to the forest type based on the plots or by neighboring trees or plants near the soil sampling site. Understanding that soil microbial composition is influenced by the interactions between adjacent plants and trees, our data analysis infers that the forest type within plots also extends to the location of each soil sample.

Soil edaphic properties were not available for the 2018 Colorado and Wyoming plots to download. Many studies present that soil edaphic properties ultimately influenced soil microbial communities, mainly bacteria (Fierer and Jackson, 2006; Fierer et al., 2007). Without the use of these data, it is difficult to determine if forest type had a direct influence or if soil edaphic properties at each site were manipulating microbial richness and diversity. The use of down woody material and subsequent carbon on the surface allowed our analysis to include some

aspects of soil chemical properties, albeit without the use of pH, which is frequently characterized as having the greatest influence on soil microbes (Fierer and Jackson, 2006; Rousk et al., 2010; Prescott and Grayston, 2013). Further analysis may entangle this connection when the data is available.

4.6 Conclusion

Our study found that forest type drives change in soil fungal and bacterial communities, while elevation more strongly influenced fungal rather than bacterial communities. Additionally, site and stand characteristics directly influenced fungal and bacterial communities separately. Stand age, SDI, and QMD had positive influences on soil fungal richness and diversity, while slope had a positive influence, while latitude and elevation negatively influenced bacterial richness and diversity. More so, climatic factors of minimum and maximum temperature (climatic extremes) positively influenced soil fungal and bacterial communities, while minimum vpd positively influenced fungi as precipitation positively influenced bacteria richness.

These data provide a better context as to the relationship between forested ecosystems in the Central and Southern Rocky Mountains and their soil microbial communities. The ability to utilize FIA as a conduit to study forest microbial communities, not only expands our knowledge of forest-soil interactions, but also allows researchers to harness the wealth of data that is encompassed in annual FIA plots. We fully believe that the expansion of soil microbial studies into the protocol of P2+Soils plots will, by far, expand the biological diversity known to inhabit forest soils while, increasing the repertoire of variables collected in FIA plots, as soil microbial communities are just one tool to use to increase forest health.

Figures

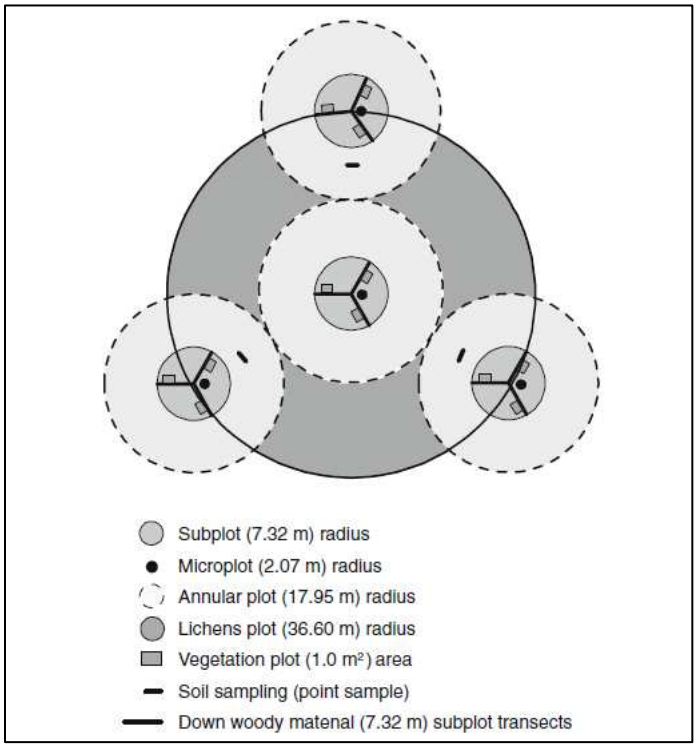

Figure 4-3: Depiction of FIA plot arrangement including four subplots and microplots, soil sampling location, down woody material transects.

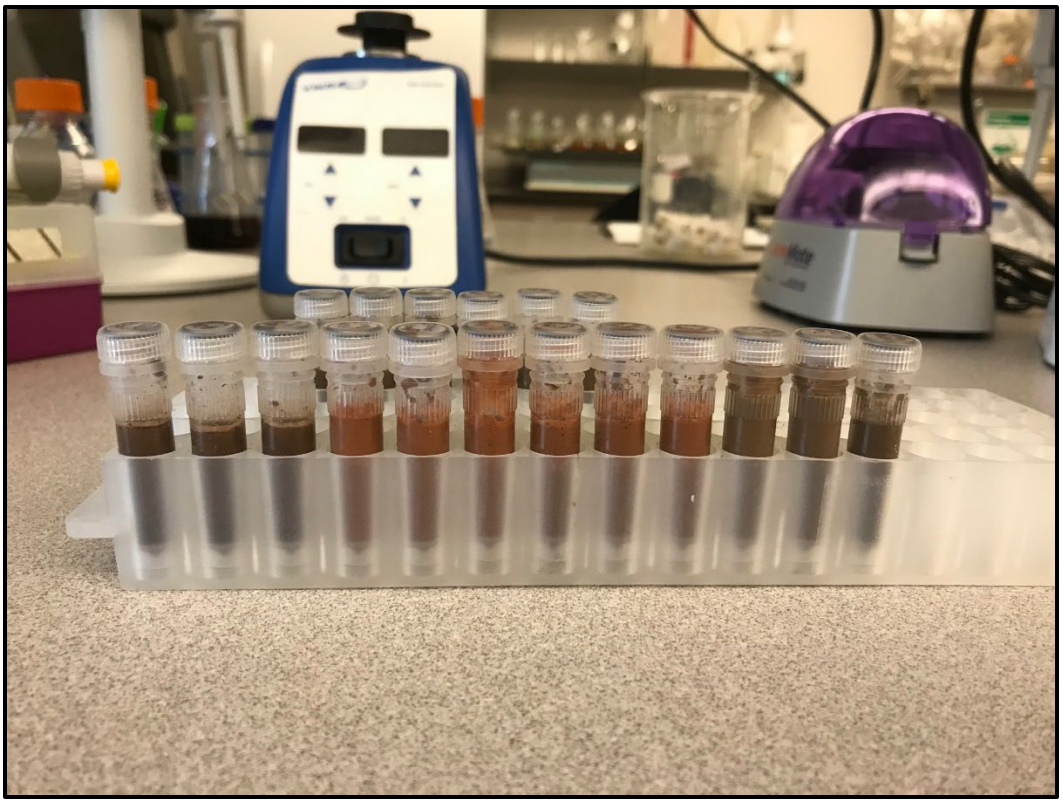

Figure 4-2: Variation in soils samples with loam samples on the outside and clay samples in the middle.

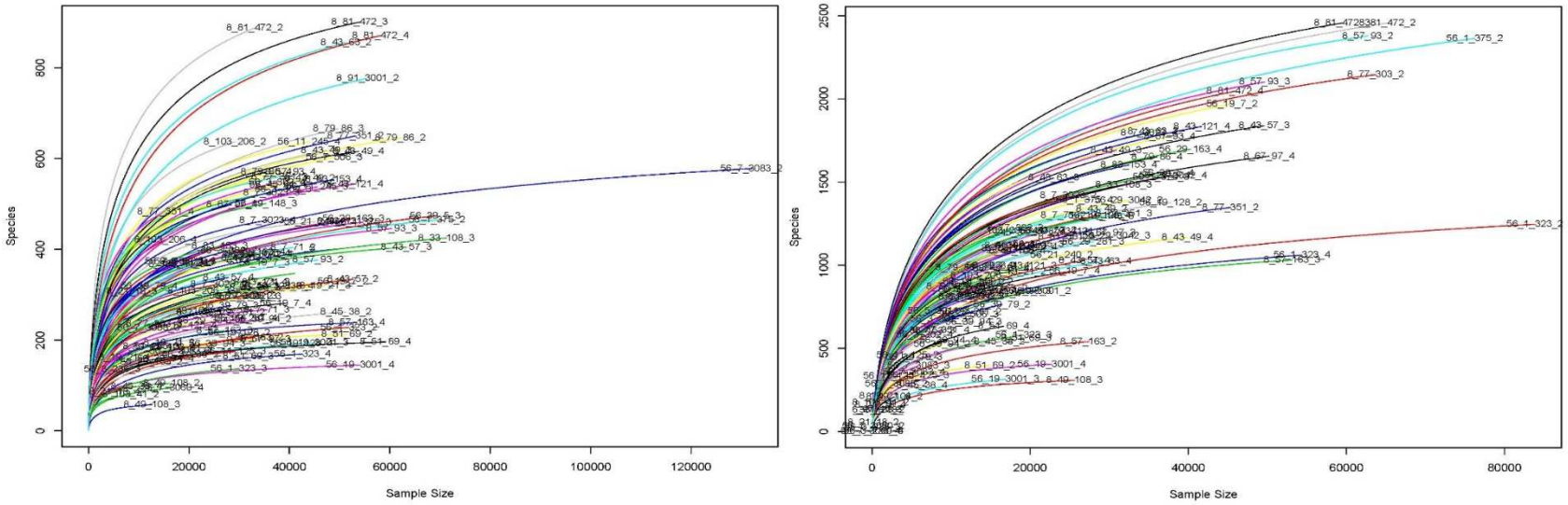

Figure 4-3: Rarefaction curve for fungal samples (left) and bacterial samples (right). Both graphs have a slight plateau occurring for samples, indicating that both fungal and bacterial sequencing was performed at an adequate depth.

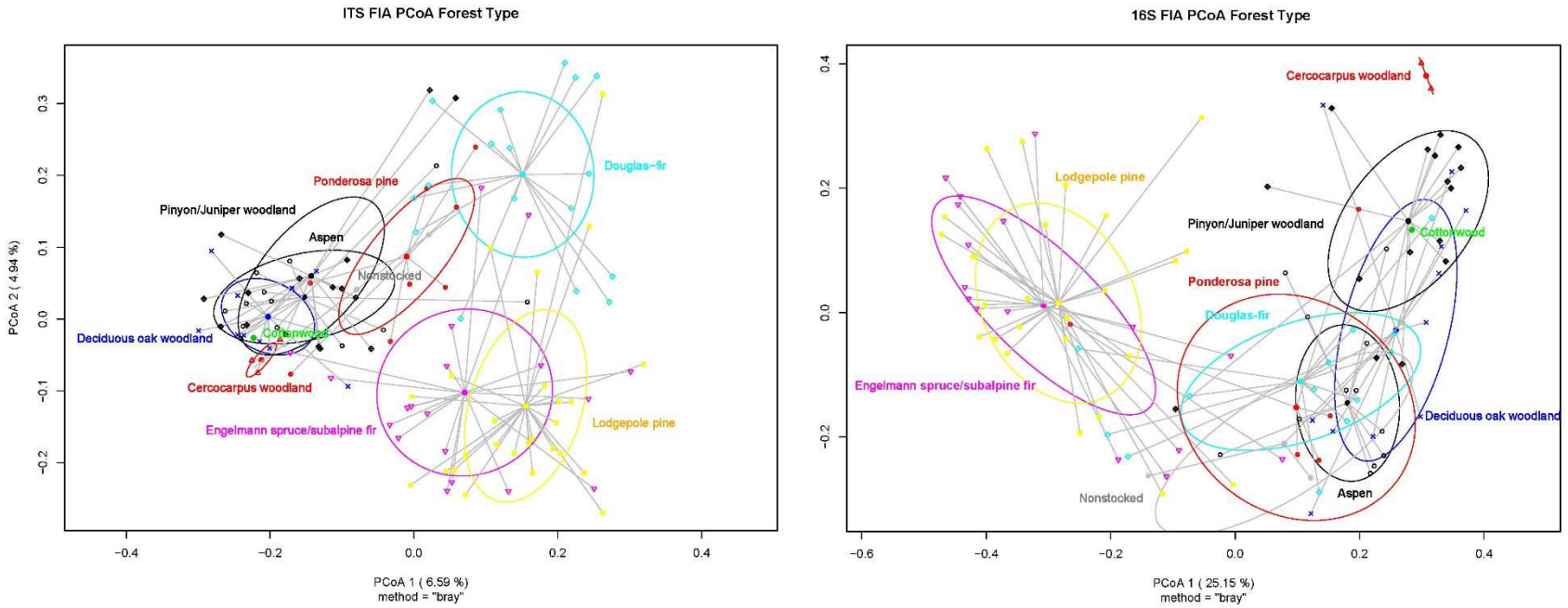

Figure 4-4: Principal coordinate analysis plot for forest types depicting fungal communities (left) and bacterial communities (right). Overlap signifies similar community composition, whereas no overlap means differences occur. Aspen (black), Cercocarpus woodlands (red), cottonwood (green), deciduous oak woodland (dark blue), Douglas-fir (light blue), Engelmann spruce/subalpine fir (pink), lodgepole pine (yellow), nonstocked (gray), pinyon-juniper woodland (black), and ponderosa pine (red).

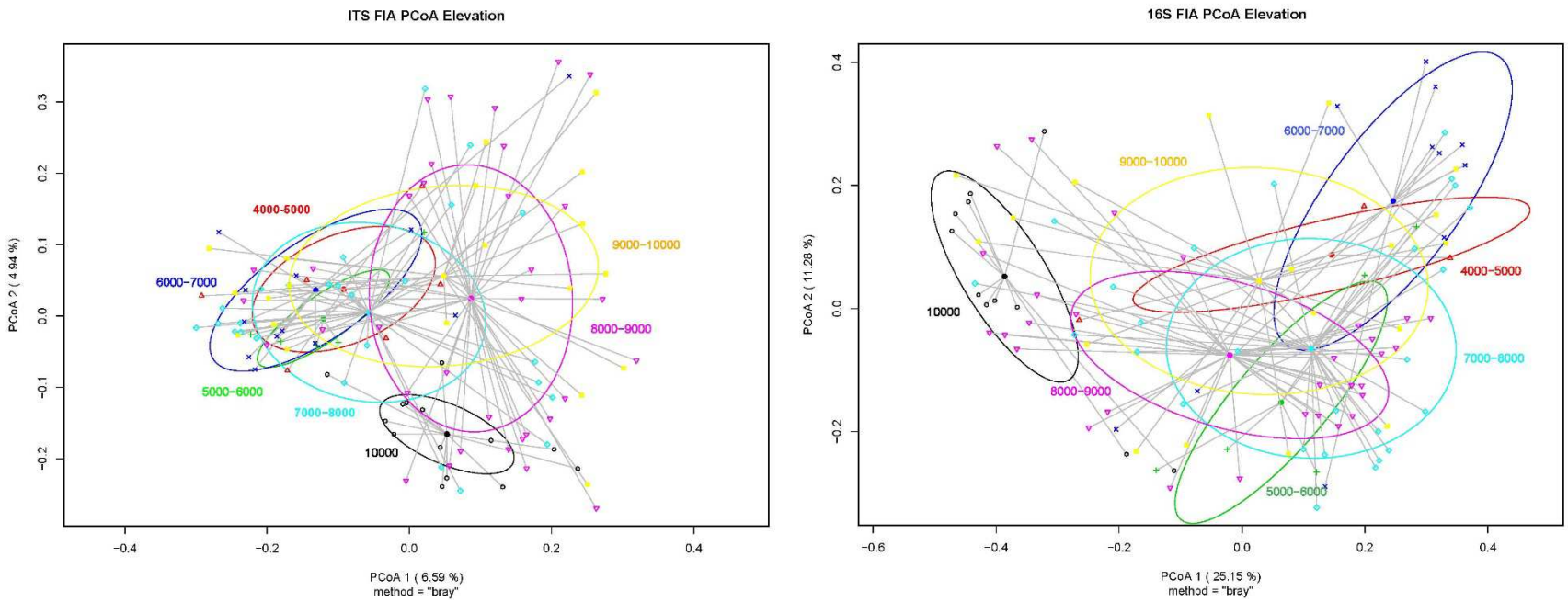

Figure 4-5: Principal coordinate analysis plot for elevational gradient depicting fungal communities (left) and bacterial communities (right). Overlap signifies similar community composition, whereas no overlap means differences occur. 4000-5000 (red), 5000-6000 (green), 6000-7000 (dark blue), 7000-8000 (light blue), 8000-9000 (pink), 9000-10000 (yellow), >10000 (black).

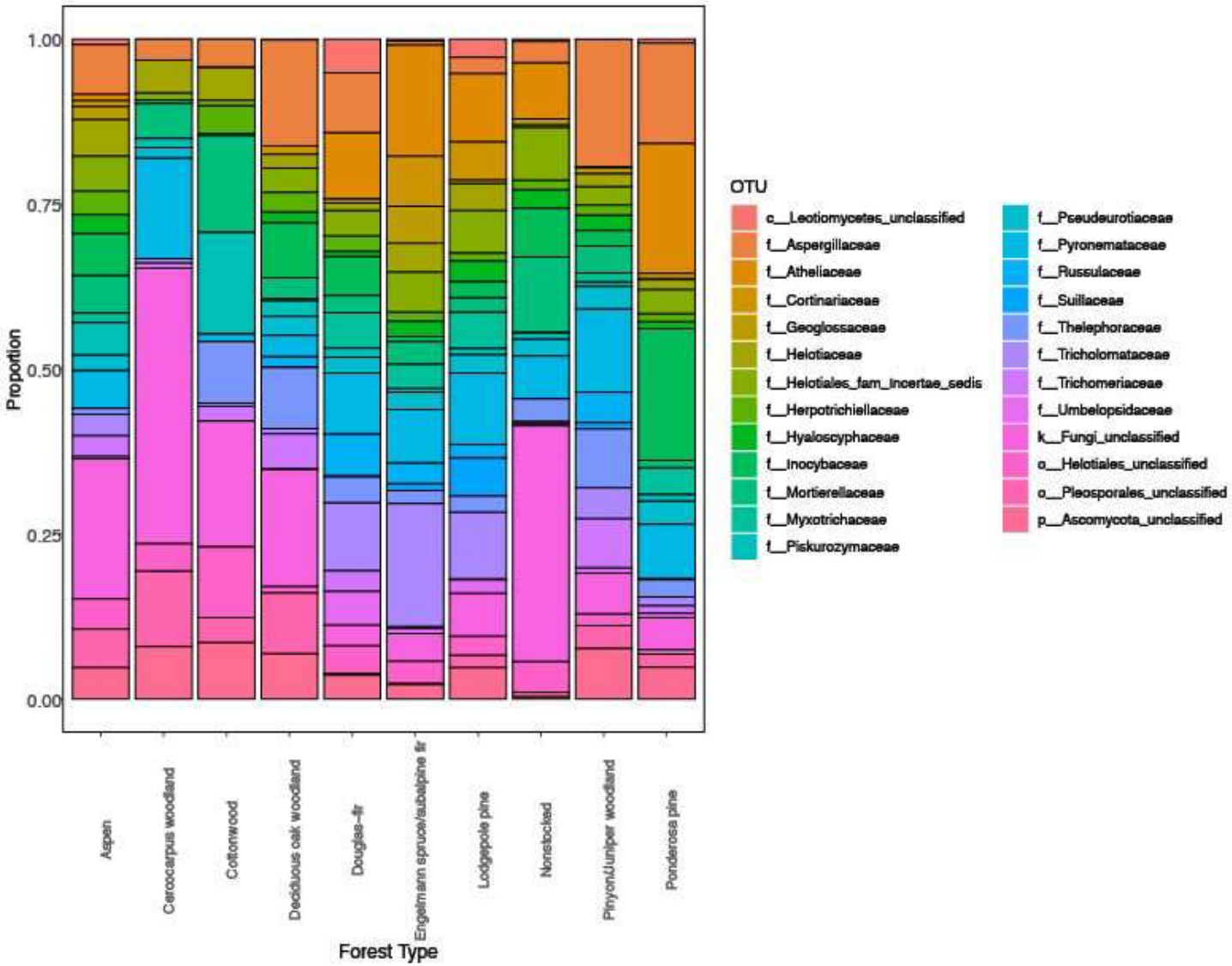

Figure 4-6: Relative abundance bar graph for fungal families >1% abundance associated with forest type.

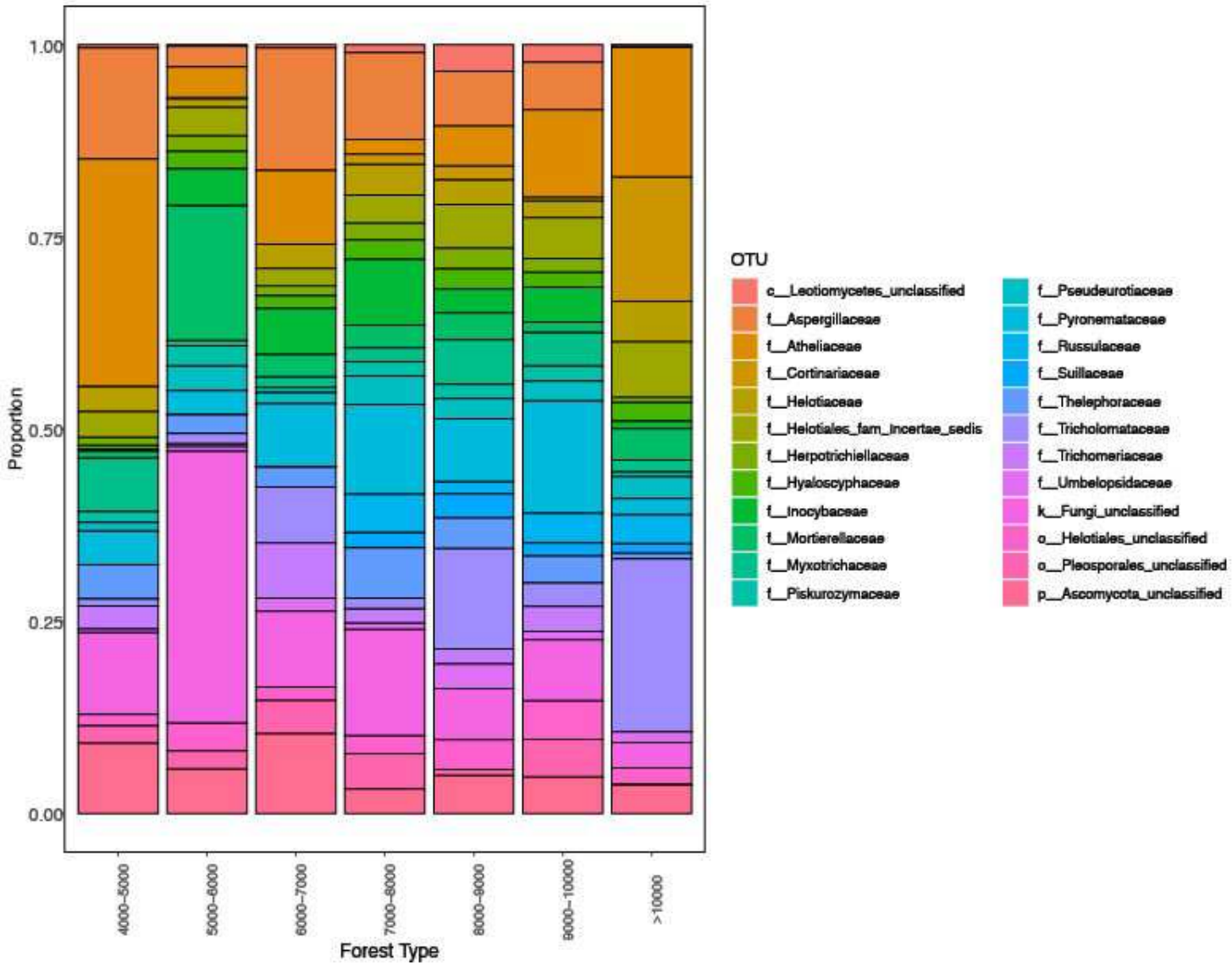

Figure 4-7: Relative abundance bar graph for fungal families >1% abundance associated with elevation.

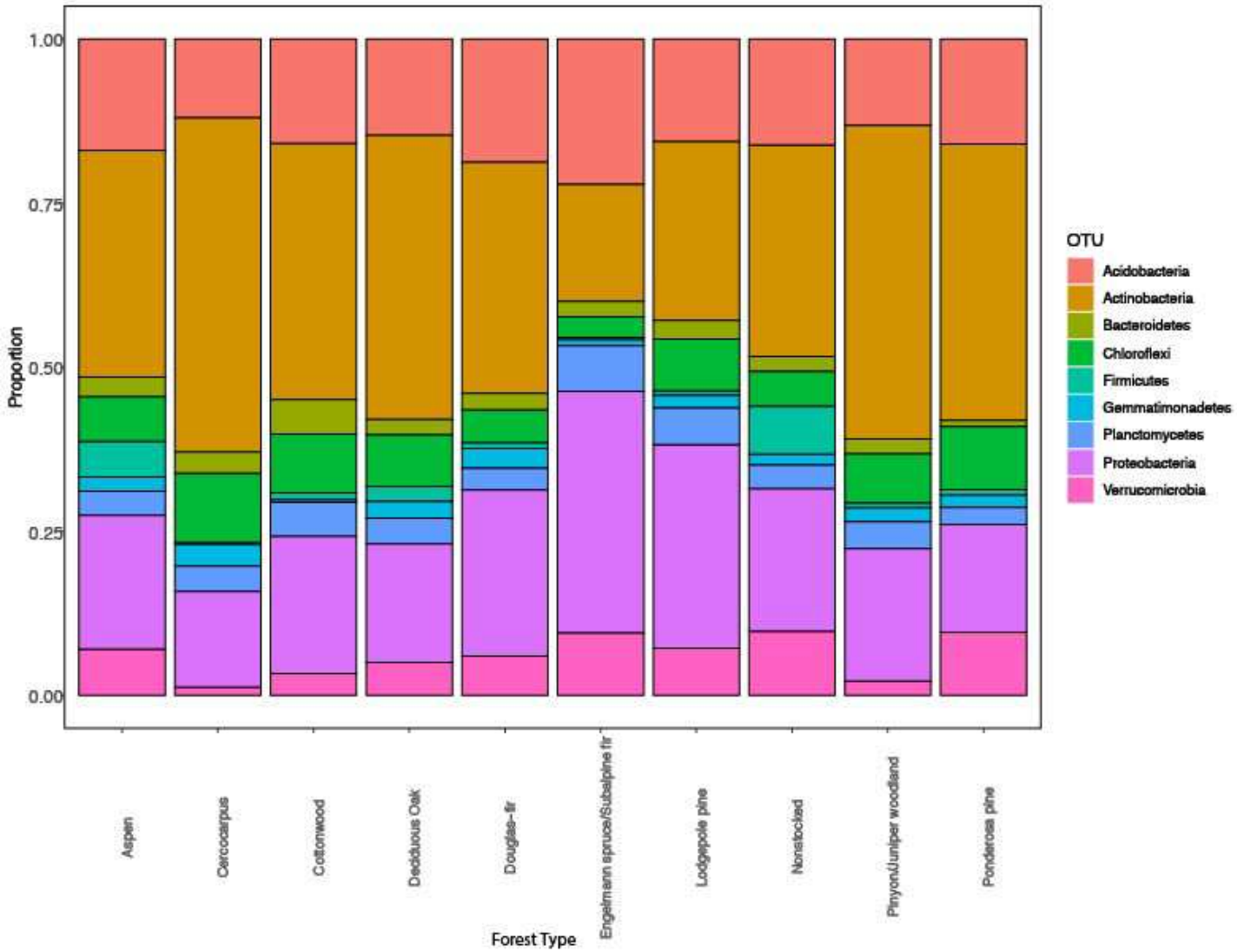

Figure 4-8: Relative abundance bar graph for bacterial phyla >1% abundance associated with forest type.

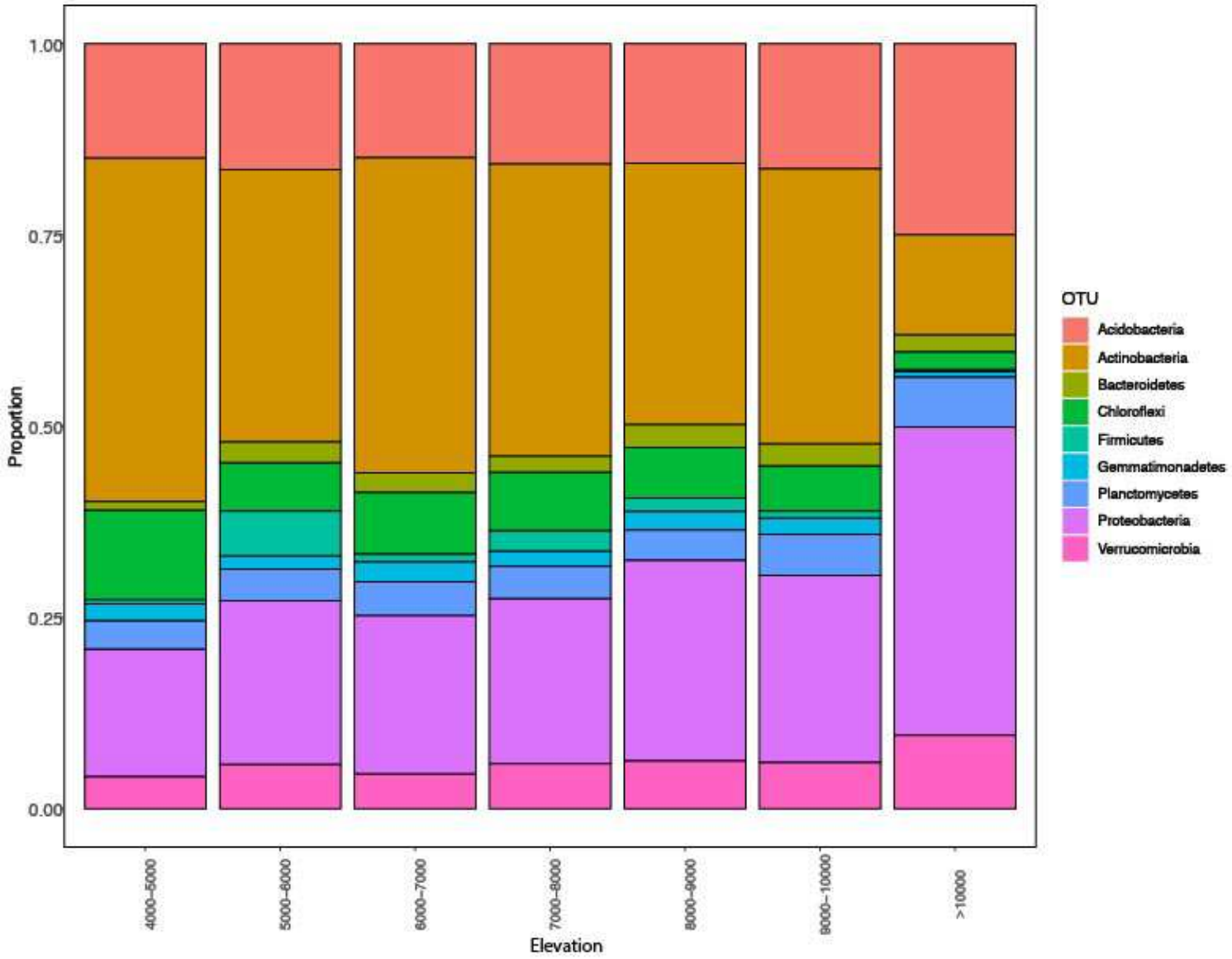

Figure 4-9: Relative abundance bar graph for bacterial phyla >1% abundance associated with elevation.

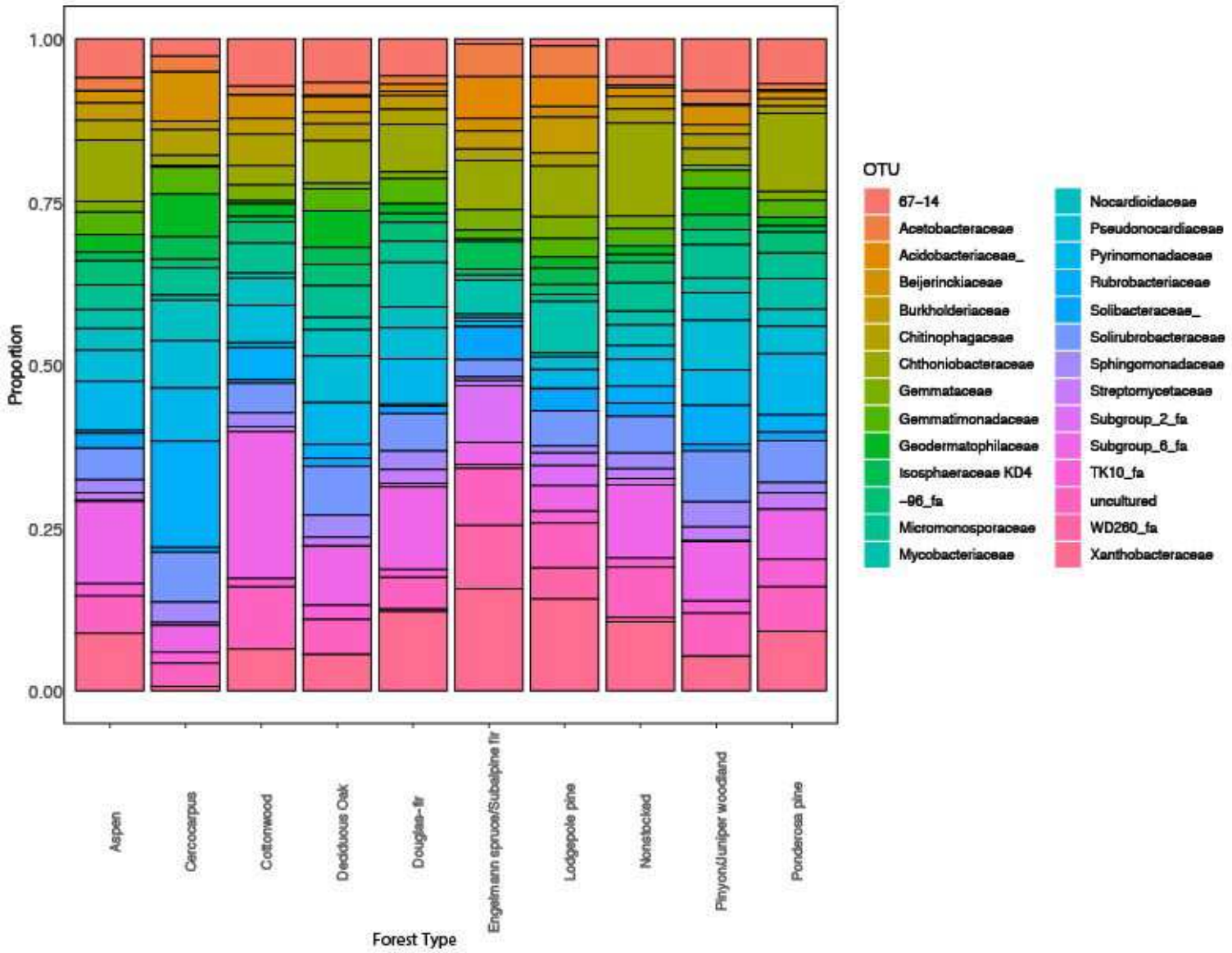

Figure 4-10: Relative abundance bar graph for bacterial families >1% abundance associated with forest type.

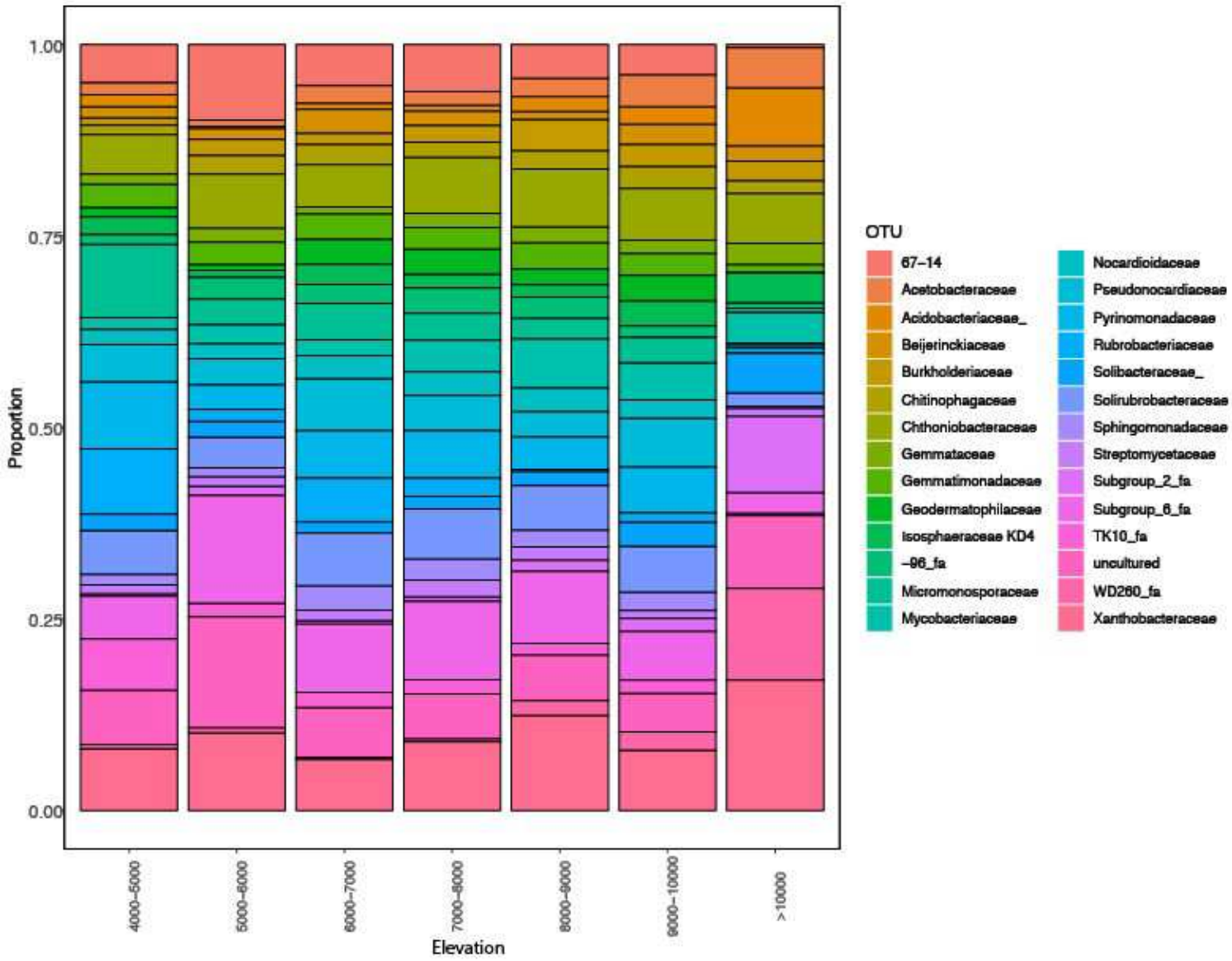

Figure 4-61: Relative abundance bar graph for bacterial families >1% abundance associated with elevation.

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Chapter 5: CONCLUSION AND FINAL THOUGHTS

Forest soil microbial communities may be the key to future management or movement of trees, as climate continues to change, and species ranges are reduced. Understanding the drivers of fungal and bacterial communities in a forest environment, especially in association with root diseases and forest type, will enhance our knowledge of the diverse interactions occurring below-ground that fuels plant health and productivity (Li et al., 2015; Habiyaremye et al., 2020). This dissertation focuses on three differing scales of studying soil microbial communities. At a tree level, Chapter 2 assesses if differing *Armillaria* species drive change in bacterial community composition. At a stand level, Chapter 3 determines if fungal and bacterial communities are associated with forest types, and other forest ecological properties, while differentiating if overstory and understory trees species drive microbial community composition. At a landscape level, Chapter 4 tests the usefulness of Forest Inventory and Analysis (FIA) to study soil microbial community composition, while understanding if there are distinct shifts in soil microbes associated with forest ecotypes, elevational gradients, stand characteristics, and climatic variables.

In the Chapter 2, I studied the direct interaction between soil microbial communities and Armillaria root disease and tree health. In this scenario, two competing root diseases, *Armillaria solidipes* and *A. altimontana*, inhabit similar forest niches in the Inland Pacific Northwest in the panhandle of Northern Idaho. The two species exhibit very different traits, as *A. solidipes* is highly virulent, while *A. altimontana* provides potential beneficial qualities and has supplanted *A. solidipes,* at the Ida Creek site in the Priest River Experimental Forest (PREF; Warwell et al., 2019). Although *A. altimontana* acts as a beneficial symbiont to the western white pine (*Pinus monticola*) at the PREF, the assessment of the genome determines that this species has the potential for pathogenicity (Ibarra-Caballero and Lalande et al., 2022). Our results highlighted the need for a more expansive study, as we only found *A. solidipes* in association with 3 of the

63 trees sampled, which is inevitable in a field study. The only clear way to determine *Armillaria* species is with the use of DNA-based analysis, therefore it is unknown which species is associated with the trees until after sampling. The small sample size could not differentiate if soil microbial communities differed in association with *Armillaria* species, while tree health also did not result in significance.

To further this research, it would be best to conduct a full sampling of the ~600 remaining western white pine at the Ida Creek field site. This would expand our ability to, hopefully, observe a greater sample size of *A. solidipes*, which would give greater strength to potential differences in relative abundance of taxa found between the *Armillaria* species. Establishing a large sampling size in one location allows for greater context into what is driving a shift in soil microbial communities, while eliminating other variables such as elevation, climate, and potential influence of differing tree species. Furthermore, the ability to extract RNA, as well as DNA would provide context into the active communities during sampling and determine their functional attributes. The common garden stand of white pine in PREF acts an ideal location to understand these dynamics, which can be fully harnessed with expanded research.

Chapter 3 removes the aspect of pathogen pressure to fully understand the baseline soil fungal and bacterial communities associated with forest types within a stand level study. The Pikes Peak Forest Dynamics Plot (PFDP) allowed me the ability to assess, on a fine scale, microbial communities difference associated with a multitude of forest types, as there are six overstory species that are found within Southern Rocky Mountains. This allowed for an in-depth analysis of the influence that forest type has on soil microbial communities with confounding elevational and climatic influences on our samples. Including a 115 soil samples at the site was by far, one of the most robust studies conducted at one location regarding forest soil microbiome within the Rocky Mountains.

In our study we found that dominant seedling species have a direct influence on both soil fungal and bacterial communities. Understory and overstory forest type had a slight influence on fungal communities, yet no influence on bacterial communities. Additionally, the distance to the nearest tree and soil pH directly influenced fungal communities, whereby decreasing distance and acidic soils decreased fungal diversity. At the PFDP, there were less overall fungal species (OTUs) identified, yet the fungal species were more diverse in association to forest type. The core fungal communities were low, as unique communities were associated with both understory and overstory forest type. This differed for bacterial communities, which had a high core community with relatively few unique communities associated with forest type. This directly relates to a stable, undisturbed forest ecosystem, as fungi tend to dominant with stand age and successional status (Li et al., 2015; Liu et al., 2020). This narrative may have changed at the PFDP, as the location had active treatments (thinning, etc.) conducted soon after our sampling.

This change in stand composition and potential disturbance to the soil may result in a shift in soil microbial community composition. Ideally, this study would have been a pre- and post-treatment analysis of soil microbes, yet this was not within our context of the research. As disturbance increases, fungal connections are severed being replaced by fast-growing, nutrient hungry copiotrophic bacteria (Lladó et al., 2017). Additionally stress to residual trees may provide an avenue for potential pathogenic taxa found in our study to inhabit roots of their hosts, which may change the dynamic from a stable forest to a forest in transitioning health. This is not guaranteed, as most of the taxa we observed are known to have functions that may benefit their hosts, yet taxa were also identified with the potential to become pathogenic. My study further developed our understanding of the influence that forest type has on soil microbial communities, yet this is the beginning to fully understand the dynamics of how trees and soil microbes interact over time.

In Chapter 4, the ability to utilize FIA plots within Colorado and Wyoming further expanded the research on forest soil microbial communities within the Rocky Mountains. This was a pilot study to piggyback off FIA protocols to enhance the current soil chemical and physical properties collected at 1/16th of FIA plots each year. The goal of this research was to expand upon the nationwide protocol as FIA plots are established throughout the entire United States and many territories and remeasured in 5-to-10-year increments (Woodall et al., 2011). I found that forest type (overstory dominant stands) and elevation directly influenced fungal and bacterial communities in the Rocky Mountains. Elevation was greater driver of fungal communities than bacterial communities as fungal richness, α-diversity, and β-diversity were all significant, whereas only bacterial richness and α-diversity were significant. This could provide an inference that overall forest type is a greater driver of change for both fungal and bacterial communities than elevation. This is shown as forest type deviates based on high and low elevation species, whereas there is high overlap in community composition from 5,000 to 10,000 feet elevation. Additionally, stand age and size (QMD) had a positive relationship on fungal communities, which corresponds to stable, late successional forests that are dominated by fungi.

One aspect that could be expanded upon for this research is the understanding that soil samples are taken off of each subplot, therefore forest type is an inference of adjacent plot data that could potentially differ from the location of the soil sample. From our research at the PFDP, in Chapter 3, we found that dominant seedlings, in close proximity to our soil samples had the greatest influence on soil microbial communities. Therefore, without direct understanding of trees and/or plants near our soil sampling location, these interactions may influence soil microbial communities differently than within plot. This is especially important if soil sampling sites were in closer proximity to nearby trees or plants, or if they resided in bulk soil, as soil microbial communities are generally more diverse in bulk soil comparatively to the rhizosphere (Cui et al., 2018). The use of FIA plots provides the greatest context into all forested lands in the

United States. The expansion of this research outside of Colorado and Wyoming will enhance our knowledge of soil microbial communities rather than focusing solely on soil edaphic properties as a forest health indicator.

All of these studies involve a specific detail on the interactions between soil microbial communities and their forest ecosystem. They enhance the knowledge of forest soils, in terms of potential disease suppression, and the idea that forest type/tree species does indeed influence soil fungal and bacterial communities. Forests in the Western United States are quite different than forests in the east, therefore specific research needs to continue to be studied in the west to understand all dynamics within a forested ecosystem. This may begin with the expansion of research conducted in soil ecosystems, as these environments are essential for the establishment, development, and persistence of forests for future generations to enjoy.

All of these projects highlighted the incorporation of both fungal and bacterial communities (Chapter 1 fungi in master's Thesis), which are typically not incorporate in most soil microbial studies. Forested settings tend to have a greater abundance and biomass of fungi (Ananyeva et al., 2010), yet bacteria tend to be key players in regenerating forests or areas that may have high disturbance (Chen et al., 2021), therefore it is important to study both organisms. As a whole, fungal communities were more directly related to our forest types, or tree communities, which is known as many fungi are species specific (Berg and Smalla, 2009). The hope is that this research enhances the knowledge of forest soil microbial communities, especially in Rocky Mountain.

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