EVALUATION OF BACTERIAL AND EUKARYOTIC DIVERSITY IN WILD BLUEBERRIES (*VACCINIUM ANGUSTIFOLIUM* AIT. AND *VACCINIUM MYTILLOIDES* MICHX.)

by

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Abstract

Plant and microbes have evolved interdependently enabling them to coexist and use symbiotic relations and interactions to survive abiotic and biotic stressors. In this study, the variations in bacterial and eukaryotic communities associated with wild lowbush blueberries within the soil system were examined. The changes in transition from unmanaged forest (multiple plant species) to managed field (single plant species) were found to influence bacteria and eukaryotic communities across sample types with root systems being the least influenced by the change in management. *Proteobacteria* and *Acidobacteria* were found to be the most abundant bacterial phyla with Proteobacteria increasing in relative abundance in root systems compared to rhizosphere and bulk soils and *Acidobacteria* showing the opposite trend. Ascomycota was the most abundant eukaryote order with consistent relative abundance across sampling locations except for being decreased in field rhizosphere. Further research is required to determine the exact structure and the stability of the microbiome of the root system within wild blueberries.

List of Abbreviations and Symbols Used

°C	Degree Celsius
A. thaliana	*Arabidopsis thaliana*
AM	Arbuscular mycorrhizae
B. circulans	*Bacilli circulans*
B. coagulans	*Bacilli coagulans*
B. sircalmous	*Bacilli sircalmous*
B. subtilis	*Bacilli subtilis*
BLAST	Basic local alignment search tool
bp	Base pair
C	Carbon
cm	Centimeter
Cu	Copper
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
EAA	ECM-associated Ascomycota
ECM	Ectomycorrhizae
ERM	Ericoid mycorrhizae
f	Family
Fl	Flavin
g	Gram
G	Genus
K	Potassium
kg	Kilogram

LB	Lysogeny broth
LCOs	Lippo-chitooligosaccharides
m	Meter
Mg	Magnesium
min	Minutes
ml	Milliliter
mm	Millimeter
MM	Minimal media
N	Nitrogen
N_2-BAP	Nitrogen blood agar plates
NF	Nitrogen-fixer
o	Order
OTUs	Operational taxonomical unit
P	Phosphorus
p	Probability or p-value
P. polymyxa	*Pseudomonas polymyxa*
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PGPMs	Plant growth promoting microbes
PGPR	Plant growth promoting rhizobacteria
PICRUSt	Phylogenetic investigation of communities by reconstruction of unobserved states
PNS	Plant nutrient solution
PS	Phosphate solubilizers
PVK	Pikovskayas Agar
q	Corrected p-value

R^2	Coefficient of determination
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
RPM	Rounds per minute
rRNA	Ribosomal ribonucleic acid
RSD	Reductive soil disinfectant
s	Species
sec	Seconds
STAMP	Statistically analysis of taxonomic and functional profiles
uL	Microliter
V. augustifolium Ait	*Vaccinium augustifolium* Ait
YMB	Yeast mannitol broth
Zn	Zinc
β-	Beta
γ-	Gamma

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Chapter 1: Introduction

This study was aimed to understand if the change in land-use from forest with multiple plants species to field with single plant species affects the microbial community in rhizosphere soils and root systems of wild blueberries. The immediate region of soil surrounding the plant roots (rhizosphere), is a microbial "hot spot" which is considered to be one of the most dynamic microbial habitats on the earth (Philippot et al., 2013). The microbial community which inhabits the rhizosphere participates in a complex food web through the acquisition and utilisation of nutrients released by the plant and found within the surrounding soil (Mendes et al., 2013). The rhizosphere is divided into three distinct zones which include: the endorhizosphere (root tissue); rhizoplane (outer region of roots); and ectorhizosphere (soil surrounding the roots). In many studies, the three regions are considered one region directly called the rhizosphere (Huang et al., 2014). Plant species and soil properties are major driving forces which regulate the microbial population, diversity, and activities within the immediate locality of the plant root system (Mendes et al., 2013). The primary source of species diversity and richness is the bulk soil (soil not penetrated by plant root systems). Alterations occurring in the bulk soil (such as land-use changes) will affect the assemblage and the final composition of the rhizosphere community (Mendes et al., 2014).

Plants and microbes have evolved symbiotic relationships that enable them to coexist and those interdependent interactions to survive abiotic and biotic stressors (Nihorimbere et al., 2011). Plant stressors can be divided into two categories abiotic and biotic. Abiotic stressors are physical

stressors which include but not limited to water, temperature, wind, and pH (Fig. 1). While biotic stressors are biological stressors which cause damage to the plant through living organisms such as prokaryotes (bacteria and archaea), Eukaryotes (fungi and weeds), and viruses (Fig. 1). In recent years, the plant microbiome has significant attention, as it influences both the plant's health and productivity (Lakshmanan et al., 2014). A soil microbiome is a distinctive collective term used to describe the microbes that inhabit soil including Bacteria, Archaea, microbial Eukaryotes such as fungi, amoebae, nematodes, and viruses (Bronkowski et al., 2009). A plant microbiome includes a diverse gene pool that has originated from viruses, prokaryotes, and eukaryotes, associated with various habitats on, within, and neighbouring the host plant (Lakshmanan et al., 2014). The interactions that occur between a host plant and neighbouring microbes are dynamic processes, in which the plant senses the adjoining environments and responds accordingly to the presence of abiotic and biotic stressors (Fig. 1) (Chaparro et al., 2012).

While many previous experiments have attempted to simplify plant-microbe interactions, these interactions include a chosen diverse assortment of microbes producing synergistic effects, which cannot be simplified (Mendes et al., 2011). Earlier studies have shown that the soil characteristics play an essential role in the determination of the diversity, structure and function of rhizosphere bacterial and fungal communities (Lauber et al., 2008). While experiments have highlighted these attributes, a comprehensive knowledge of the influence of the microbial communities on plant growth has yet to be achieved (Liang et al., 2012). The root system is one of the crucial components in the interactions between plants and microbes (Chaparro et al., 2012).

Plants and microbes communicate using chemical signalling. Microbes release chemical signals which are received by the plants which then respond through the release of root exudates (Chaparro et al., 2012). Chemical signalling is not solely accomplished by the microbes; plants also release chemical signals into the soils, which are then received by the microbes (Chaparro et al., 2012). Secretory compounds vary depending on the plant species and requirements; microbial species and abundances will vary depending on development stage or stressors. A diverse range of exudates released by the roots assists in the development of an environment within the rhizosphere through the release of sugars, amino acids, flavonoids, aliphatic acids, proteins, fatty acids and other components (Badri et al., 2009). The chemicals released from the roots can interact with the surrounding microbes and initiate communication, symbiotic or pathogenic interaction with the microbial population in the surrounding soil (Bais et al., 2006). The concentration and composition of root exudates will vary depending on the signals received by the rhizosphere microbiota, environment, soil type, abiotic, biotic, and age of the plant (De-la-Pena et al., 2010).

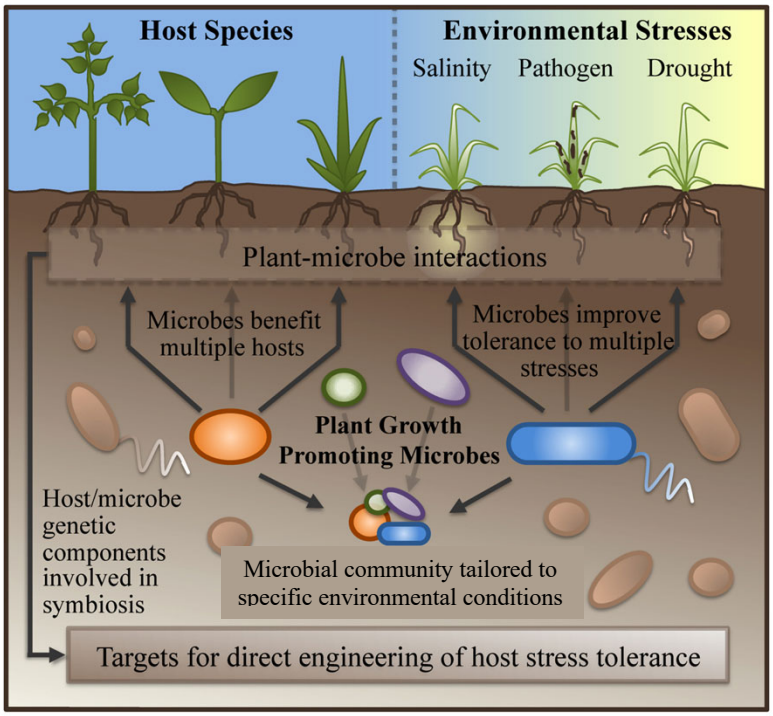


Figure 1: Visual representation of the plant-microbe interactions and complexity surrounding these interactions. Microbes can confer beneficial effects to plants through symbiotic relations assisting the plants in obtaining nutrients and increasing stress tolerance (CC by Coleman-Derr, D., & Tringe, S. G. (2014). Building the crops of tomorrow: advantages of symbiont-based approaches to improving abiotic stress tolerance. Frontiers in microbiology, 5, 283.)

While only some soil microbes can be cultured, recent advancements in "omics" research can help shed light on the taxonomy, function, and interactions within soil microbial communities (Morales and Holben 2011). Analysis of rhizosphere microbiota through "omics" techniques can provide a glimpse of the stressors which a plant is experiencing. Developing a better understanding of these microbial communities will provide comprehensive information for the use in agroecosystem's management including environmental impact assessments (Liang et al., 2012).

In this study, it was hypothesized that the shift in land-use from unmanaged forest blueberries with multiple plant species to managed field blueberries with single plant species would alter the

microbiome within the managed field blueberry sampling locations. The goals were to identify the most abundant bacterial phyla and eukaryotic orders within the bulk soil, rhizosphere and root systems, to determine if there were significant changes in the microbial population between field and forest sampling locations, and to isolate and identify beneficial bacteria within the root systems of wild blueberries. Using 16S and 18S rRNA gene sequencing, we aimed to determine if the transition from the unmanaged forest (with multiple plant species) to the managed field (single plant species) had significantly different bacterial and eukaryotic communities. A subset of potentially beneficial bacteria were isolated and cultured from field and forest root sampling locations and then were tested for their plant beneficial abilities on alfalfa seedlings.

Chapter 2: Background

2.1.0 Root System

Plants use their root system to explore the sub-terrestrial world, obtain water and nutrients to sustain growth and development, act as a reservoir for carbohydrates, and communicate with their environmental partners and rivals through the release of exudates (Vacheron et al., 2013; Rellan-Alvarez et al., 2016). Many of the factors affecting root growth and physiology arise from the structure of the soil at the microscopic level (Rellan-Alvarez et al., 2016). The roots release carbon-containing metabolites into the soil matrix resulting in rhizodeposition (Doornbos et al., 2012). These carbon-containing metabolites on average 5-21% of a plant's photosynthetically fixed carbon, and in some cases, up to 40% of the photosynthetically fixed carbon. This includes the shedding of root cells and exudates (the secretion and leakage of secondary metabolites sugars, organic acids, amino acids, and proteins) into the soil (Huang et al., 2014; Doornbos et al., 2012).

Previously, root exudation was believed to be a passive process. However, research has suggested the presence of ATP-binding cassette transporters in the cells of the roots are involved in the translocation of phytochemicals into the rhizosphere indicating that plant roots are actively secreting metabolites into the environment (Badri et al., 2008). A wide variety of plants have particular root cells, known as border cells (that contain mitochondria, golgi stacks, and golgi derived vesicles) whose function is the secretion of metabolites (Vicré et al., 2005). The proposed functions of the border cells are for attracting beneficial microorganisms, reduction of sensitivity to heavy metals, and entrapment of pathogenic bacteria and nematodes within the mucilage layer surrounding the roots (Hawe, 1990). There is increased evidence indicating the quantity and quality of the exudate released into the soil is determined by the following: species of the plant,

developmental stage of the plant, stress factors, and various environmental factors (pH, temperature, and presence of microbes) (Huang et al., 2014; Latour et al., 1996). A study conducted by Kamilova et al. (2012) on tomatoes, cucumbers and sweet peppers, grown under sterile, controlled conditions on rock wool showed that released exudates contained a higher amount of organic acids than sugars. Of the secreted organic acids, citric, succinic, and malic acids were the most abundant acids present, while fructose and glucose were the predominant sugars (Kamilova et al., 2006b).

2.1.1. Microbial Effect on Plant Root Exudates

Root exudates have close ties with the surrounding microbial population, and the composition of the root exudates is influenced by the rhizosphere microflora (Chaparro et al., 2014; Doornbos et al., 2012). The application of bacterial biocontrol strain *Pseudomonas fluorescens* WCS365 on tomato plants increased organic acid levels, whereas there was a decrease in succinic acid (Kamilova et al., 2006a). When the plants were inoculated with pathogenic fungus *Fusarium oxysporum f. sp. radicis-lycopersici* the fungus caused severe root rot which leads to a decrease in citric acid levels and increased succinic acid in comparison to control plants (Kamilova et al., 2006a). When plants were induced with *P. fluorescens* WCS365 and the pathogenic fungus, the effects of the disease were decreased, and the succinic acid content within the roots exudates was lowered compared to the pathogen-infected plants (Kamila et al., 2006a). The results of this study showed the availability and composition of the nutritional diet for the microbes within the rhizosphere is highly dynamic (Doornbos et al., 2012). The study also showed the root exudates are dependent on the interactions between microbes and the analysis of root exudates within a

gnotobiotic system. This type of research is the beginning of understanding the interactions and conditions of the rhizosphere (Doornbos et al., 2012).

2.1.2. Plant-Microbe Interactions

Although plant physiologists have simplified soil as a nutrient source for plants, it is a much more complex ecosystem with an intricate vast variety of bacteria, fungi, protists and animals (Bonkowski et al., 2009; Muller et al., 2016, Chaparro et al., 2012). Plants exhibit a diverse array of interactions with the organisms within the soil including competitive, exploitative, neutral, commensal, and mutualistic (Jacoby et al., 2017). Some of the many factors which affect the interactions include roots, microbes, surrounding plants, soil type, soil chemical composition and pH. Susanne C. Brink in her editorial "Unlocking The secrets of the Rhizosphere" describes the relationship between the plant and microbes within the soil as a single entity a "super-organism". This article is a critical approach to observing plant growth and development, allowing research into plant health, growth, and development from a different angle. Treating plant and its microbiome as a single entity will allow for a better understanding of each separate component, as the environment surrounding the plant is a complex entity, thus having more pieces to the puzzle will provide a clearer picture of "super-organism" (Rellan-Alvarez et al., 2016).

2.1.3. Effect of Root Exudates (Biotic Factors) on Diversity and Structure of the Microbial communities

The concentrations of microbes in the rhizosphere are 100-1000 times higher than that of bulk soil microbes (Goswami et al., 2016). Microbes can utilise root exudates resulting in an increase in

microbial biomass within the rhizosphere in comparison to bulk soil region. This allows for the generation of microbial communities modified for the plant during different developmental stages (Huang et al., 2014). Smalla et al. (2001) observed bacterial rhizosphere communities within strawberries, oilseed rape, and potatoes (using culturable-independent fingerprinting methods). In two years of growth, the seasonal shifts were seen in abundance and composition of the bacterial rhizosphere community (Smalla et al., 2001). The study showed that the plant-dependent shift was relative to the abundance of the bacterial population within the rhizosphere, which during the second year of growth became more pronounced (Smalla et al., 2001).

2.1.4. Effect of Abiotic Factors on Diversity and Structure of the Microbial Communities

Soil bacteria play an essential role in the biogeochemical cycles and have supported crop production for millennia (Hayat et al., 2010). The soil matrix is the primary reservoir for potential microbes creating each plant's unique rhizosphere microflora. Soil microbes compete for nutrients, and other resources which are sparsely found within the soil system, due to these resource limitations bacterial proliferation in the soil tends to be slow (Doornbos et al., 2012). A study conducted by Fierer and Jackson (2006) analysed close to 100 soil samples collected from across North and South America using DNA fingerprinting methods to compare composition and diversity of the bacterial communities across each site. This research team found that the diversity of the soil bacterial communities is more affected by soil properties, particularly pH, while site temperature and latitude were of little influence. They noted that in general, soils that were pH-neutral had a higher microbial diversity compared to that of acidic soils (Doornbos et al., 2012).

Soil scientists are beginning to have a better comprehension of the plant-microbe interactions and the effects of microbes on plant fitness and stress tolerance (East, 2013; Zolla et al., 2013). The use of microbiota transplantation (that colonise other species) has the potential to increase the stress tolerance of food crops (East, 2010; Zolla et al., 2013). Zolla et al. (2013) illustrated through using sympatric soil microbiomes (for example a soil having a history of exposure to *Arabidopsis*) are capable of helping plants increase the biomass significantly in moderate stress conditions. Sympatric soil microbiomes can increase stress tolerance during moderate drought conditions (Zolla et al., 2013). A recent study conducted by Zolla et al. (2013) demonstrated when *Arabidopsis thaliana* (seedling) were planted in soils previously exposed to *A. thaliana*, corn, and pine, the seedlings grew more vigorously in soils exposed to drought conditions (Zolla et al., 2013). The majority of the microbes which Zolla et al. (2013) identified belong to phyla *Actinobacteria* and *Proteobacteria,* previously reported to aid in abiotic stress conditions (Zolla et al., 2013; Mayak et al., 2004). However, more research is required to determine the mechanisms of the associated sympatric microbes with the host plant (Zolla et al., 2013).

2.2.0. Effects Soil Microbes Have on Soil Health and Plant Productivity

The distribution of microflora will vary depending on nutrient concentrations and locations, motility ability, and chemical signaling (from the plant and other microbes). Vessey (2003) indicated there are many species of soil bacteria which thrive in the rhizosphere (surrounding plants), and root system (grow within or on the plant roots). These bacteria can stimulate plant growth by numerous mechanisms. The collective term for these bacteria is plant growth promoting rhizobacteria (PGPR) (Jha and Saraf, 2015). Roughly 2-5% of the rhizosphere bacterial population

are considered PGPR (Antoun and Prévost, 2005). PGPR can create a beneficial effect on the plant upon inoculation, contributing to sustaining plant growth and development. PGPR beneficial effects are exhibited through the synthesis of specific compounds, facilitating of nutrients uptake from the soil, and aiding the plant in decreasing or preventing the effects of pathogens (Hayat et al., 2010). Gray and Smith (2005) suggested that the associated PGPR are dependent on the degree of bacterial proximity to the root and shoot systems. The majority of the PGPR to date belong the genera A*cinetobacter, Agrobacterium, Arthobacter, Azotobacter, Azospirillum, Burkholderia, Bradyrhizobium, Rhizobium, Frankia, Serratia, Thiobacillus, Pseudomonas*, and *Bacillus* (Vessey, 2003).

2.2.1. N2-Fixing Bacteria

Nitrogen is essential for all plants; it is a requirement for the synthesis of enzymes, proteins, amino acids, chlorophyll, DNA, and RNA. The canopy of a plant utilizes the majority of the plant's nitrogen resources, accounting for 1-2% of the dry matter content of the canopy. Although nitrogen may be found in abundance in the environment, it is a limiting plant nutrient, as the ampleness of nitrogen located within the environment is most commonly found as atmospheric di-nitrogen gas (an inert gas), containing a triple bond which consumes a great deal of energy to break. This limited nutrient has been overcome through the application of nitrogen-based fertilisers. The cost of implementing these fertilisers has an excessive price, both environmentally and economically. Although nitrogen fertilisers can provide plants with this difficult to obtain nutrient, the downfall of these nitrogen fertilisers is the negative impact they have on the environment through run-off (leaching) of excess nitrogen in the form of nitrates. The nitrate leached from agricultural land can

lead to acidification of water sources and hypertrophication (excess of nutrients in a body of water) which then can be accompanied by deficiencies in dissolved oxygen (Camargo & Alonso 2007). An alternative to the use of nitrogen fertilisers would be through the use of nitrogen-fixing bacteria. Stacey et al., (1992) reported nitrogen-fixing microbes within fertile soils are capable of fixing 20-30 kg per hectare per year.

Symbiotic Bacteria: Some bacterial strains have developed the ability to fix nitrogen; they can be classified as symbiotic bacteria or free-living bacteria (Goswami et al., 2016). Symbiotic bacteria, such as *Rhizobium, Sinorhizobium, Mesorhizobium, Brandyrhizobium, Azorhizobium*, and *Allorhizobium,* create symbiotic relations with legumes and fix nitrogen inside plant organs, nodules. This relationship is created through the secretion of the plant-derived signalling molecules (flavonoids), the bacteria respond to the chemicals through the secretion of Lippo-chitooligosaccharides (LCOs) (Oldroyd et al., 2011)

Free-living Bacteria: Conversely, free-living nitrogen-fixing bacteria do not require plant host to fix nitrogen. Free-living nitrogen-fixing bacteria can live near the plants the plants can use the nitrogen fixed from the atmosphere not utilised by the microbes. This type of relationship is described as a non-specific and loose symbiosis (Goswami et al., 2016). Some examples of free-living nitrogen-fixing bacteria include *Azospirillum, Azotobacter, Burkholderia, Herbaspirllum, Bacillus,* and *Paenibacillus* (Goswami et al., 2015). Species which belong to the genus *Azotobacter* and *Azospirillum* are the most commonly used nitrogen-fixing bacteria in agricultural trials, with the first reported use in 1902 and since then has been widely used around the world (Goswami et al., 2016; Bhattacharyya and Jha, 2010).).

2.2.2. Phosphate Solubilizing Bacteria

Phosphorus is one of the essential macronutrients for both, plant growth and development (Jha and Saraf, 2015). Much like nitrogen, phosphorus in its natural state is inaccessible to the plant, as plants are only capable of absorbing mono-, and dibasic phosphates (soluble forms of phosphates) (Jha and Saraf, 2015). Phosphates are reactive with calcium, iron, and aluminium. This reaction tends to lead to precipitation causing phosphates to become inaccessible to plants for uptake (Goswami et al., 2016). Thus, plant growth promoting microbes must convert the insoluble forms of phosphorus (both inorganic and organic forms) to available forms for the plants to increase plant yield.

Holford (1997) reported there are three essential soil components in controlling the supply of phosphorus from the labile pools to replenish the crop extractions (Jha and Saraf, 2015). These include the concentration of phosphorus in the soil solution, the frequency of phosphorus within the replenishment source that enters the equilibrium of the soil solution phase, and the phosphorus buffering capacity of the soil (Jha and Saraf, 2015). In wild unmanaged blueberry systems organic phosphorus is obtained through the symbiotic relations with mycorrhizal fungi. Maintaining plant-available phosphorus is essential for plants, it is crucial to avoid overexploitation of the soil natural phosphorus reservoirs, as this leads to deficiency and low plant yield (Jha and Saraf, 2015).

Microbes mineralise phosphorus in the soil by solubilising complex structured phosphates (tricalcium phosphate, rock phosphate, and aluminium phosphate) into usable forms for plant use (Goswami et al., 2016). Bacteria utilise multiple mechanisms to solubilise the insoluble phosphates

(Goswami et al., 2016). The primary mechanisms used by microbes in phosphate solubilization is using organic acid secretions, produces through sugar metabolism (Goswami et al., 2016). It has been estimated that 20-40% of the culturable population of microbes has been found to be phosphate solubilising microorganisms, with a significant proportion of these microbes being isolated from the rhizosphere (Chabot et al., 1993). The majority of the phosphate solubilising bacteria, separated from the rhizosphere of various plants tend to be metabolically more dynamic than those located in other soil regions (Jha and Saraf, 2015). Previous research has shown bacteria belonging to genera such as *Achromobacter, Agrobacterium, Bacillus, Enterobacter, Erwinia, Escherichia, Flavobacterium, Mycobacterium, Pseudomonas,* and *Serratia* are highly efficient at solubilising complex phosphates into accessible forms (inorganic phosphate ions) (Goldstein 2000). However, recent research on ectorhizosphere strains of *Pseudomonas, Bacilli,* and endosymbiotic *Rhizobium* has been determined as efficient phosphate solubilisers. While *Bacillus megaterium, B. circulans, B. coagulans, B. subtilis, P. polymyxa, B. Sicamous*, and *Pseudomonas striata* are referred to as the most critical and useful strains (Govindasamy et al., 2011).

2.2.3. Flavin

Flavins are known to be one of the most chemically diverse prosthetic groups of biochemistry (Manion, 2014). Flavins are organic compounds which are formed by tricyclic heterocycle isoalloxazine. Riboflavin, commonly known as vitamin B2 is essential to all organisms, playing a vital role in oxidative metabolism, as it comprises the precursors for flavin mononucleotide and flavin adenine dinucleotide, which are necessary for several flavoprotein-mediated redox reactions (Gutiérrez-Preciado et al., 2015; De Colibus, 2006). Riboflavin is the precursor of flavin adenine

dinucleotide and flavin mononucleotide, the cofactors in numerous oxidoreductases that transpires in all bacteria, plants, and animals. While in bacteria riboflavin is essential in processes such as quorum sensing, extracellular electron transfer and the establishment of the symbiotic association with plants (Gutiérrez-Preciado et al., 2015). Within plant systems, flavins and in particular, flavin monooxygenase are utilized in the defense against pathogens (Schlaich, 2007). These flavin monooxygenases catalyze specific steps in the biosynthesis of auxins (plant hormones) and the metabolism of glucosinolates (Schlaich, 2007).

2.2.4. Mycorrhizae

While bacteria play an essential role in plant growth, they are not the only microbial group which are beneficial to roots growth and development. Fungi are beneficial in organic matter decomposition and nutrient cycling as they provide a wide range of nutrients to both the surrounding soil and plants (Hannula et al., 2014). Mycorrhizal fungi form symbiotic relations with the roots of plants (Parniske, 2008; Bonfante and Anca, 2009). Mycorrhizal fungi require carbon from the host plant to execute beneficial effects on the host plant. Mycorrhizal fungi benefit the plant by increase roots surface area as the hyphae act as an extension of the root system allowing for the increase in nutrient and water uptake (Powell et al., 2009; Nadeem et al., 2014). Mycorrhizal fungi improve plant growth, by providing the host plant with phosphates and decrease adverse effects from pathogens, entangling the pathogens in hyphae (Powell et al., 2009; Sykes, 2010). The two most common mycorrhizal fungi are arbuscular mycorrhizae (AM) and ectomycorrhizal (ECM) fungi (Nadeem et al., 2014).

Arbuscular Mycorrhizae: AM fungi arendomycorrhizae, which means that the part of their hyphae resides intracellularly within the root. AM fungi live in the soil as spores until a host plant has been detected (Tkacz and Poole, 2015). AM fungi form symbiotic relationships with leafy green plants and commercially produced plants (Parniske, 2008). AM fungi are the most abundant and widely distributed fungi in agricultural soils (up to 70-90% of land species) (Nadeem et al., 2014; Parniske, 2008). AM fungal hyphae aid the structural stability of soil and increase the ability of the host plant to acquire nutrients and water from surrounding soil (Nadeem et al., 2014). AM fungal hyphae obtain approximately 80% of the phosphorus requirements of the host plant. The hyphae can also acquire the required macro- and micro-nutrients (K, N, Mg, Cu and Zn) in soils where the nutrients are present in a less soluble form (Nadeem et al., 2014).

Ericoid mycorrhizae (ERM): ERM fungi have evolved to reside in nutrient-limiting soils, with low pH, decreased available metals, and poor free drainage (Cairneey and Meharg, 2003). Among other plants, ERM fungi are associated with the *V. augustifolium Ait*. These endomycorrhizae fungi are beneficial to nitrogen uptake and supplying phosphorus to the host plant (Goulard et al., 1993; Jeliazkova and Percival, 2003b). ERM fungi have a unique ability to adapt to stressful environments. These fungi can enhance the host plant fitness by reducing stressors (Cairney and Meharg 2003). A study conducted by Jeliazkova and Percival (2003a) considering the effects of drought on the ERM fungi associated with wild blueberries suggested the distribution of ERM fungi stayed consistent throughout drought conditions. It was also found that the adaptive mechanisms of ERM fungi assisted the wild blueberries to withstand drought and other unfavourable conditions.

While mycorrhizal fungi may form symbiotic relations with plants, they also develop loose or tightly associations with bacteria, which are most likely to play a role in the functions of the mycorrhizal fungi (Bonfante and Anca, 2009). Garbaye's research in 1994 introduced the term "helper bacteria", in which the "helper bacteria" assist and support mycorrhizal establishment (Bonfante and Anca, 2009). Since the introduction of "helper bacteria" increased research has been completed describing bacteria-fungi interactions (Artursson et al., 2006). These interactions were determined to be more dynamic and complex than expected and these interactions may be crucial in the ecosystem (Artursson et al., 2006). Helper bacteria tend to colonise the extraradical hyphae, and in some fungal taxa reside as cytoplasmic endobacteria (Bonfante and Anca, 2009). Mycorrhizal-bacterial community studies have revealed that a vast of "helper bacteria" are predominantly from genera *Pseudomonas, Burkholderia,* and *Bacillus* (de Boer et al., 2005; Schrey and Tarkka, 2008; Bomberg and Timonen, 2007).

2.3.0. Management Practices and the Implications for Agriculture

Wild (lowbush) blueberries are a perennial, rhizomatous, cross-pollinating shrub, that is native to Atlantic Canada and one of Nova Scotia's most significant agricultural commodities (Debnath, 2009). Since the crops are derived from native wild plants, they are capable of advertising as their crops as "wild" blueberries. The two species of wild blueberries that are found in the majority of wild blueberry fields in Atlantic Canada are the sweet lowbush (*Vaccinium angustifolium* Ait.) which accounts for roughly 80-95% of the plants within a field (surface area basis), and *Vaccinium myrtilloides* Michx. (i.e., the "sour-top" or "Canada blueberry") (Drummond et al., 2009). In 1980 3.8 million kg were produced and in 2015 29.5 million pounds were produced in Nova Scotia

(Food Institute Report –January 21, 2016). The plants have adapted to handle the low fertile sandy, acidic soils of northeastern North America. Wild blueberries have been able to adapt to these conditions with the aid of the symbiotic relations it has formed with mycorrhizal fungi. The ideal conditions for growing blueberries are on sandy well-drained acidic soils, with sufficient organic matter (5-10%), with a pH range from 4- 5 (Yarborough, 2012). Blueberry plants are a long-living plant which can be found naturally in the forest (Drummond et al., 2009). The use of abandoned farmland with the natural wild blueberry plant is ideal for production, as farmers do not have to clear the land of stumps, roots, trees, weeds, and other plant material allowing the wild blueberries rhizome (runners) to give rise to new roots and shoots (McIsaac, 1997). The higher the density of the first wild, unmanaged plant the less time it will require producing new crops (McIsaac, 1997). Wild blueberry plants increase yield over many production cycles, after pruning (burning or mowing) plants grow more new shoots which allow for the growth of more flower and leaf buds (Drummond et al., 2009)

Wild blueberries have a massive, shallow fibrous root system that is typically found in the upper 10 to 15 cm of soil and constitutes 75 to 85% of the total dry weight of a plant . Thus, they thrive in soils that are loose and ideally with no competing weeds as they are not competitive for nutrients or sunlight (Drummond et al., 2009). Since mature plants only reach 0.5 m high, it becomes essential to increase plant density to ensure maximum yield (Yarborough, 2012). Unmanaged plant's rhizomes will grow roughly 8 cm per year, while in managed plant's rhizomes will grow roughly 38 cm in a single year (McIsaac, 1997). The rhizomes provide carbohydrate reservoirs and lateral water transport for the plant, allowing blueberries to adapt to unfavourable conditions such as drought (Jeliazkova and Percival, 2003a).

Blueberries typically grow on a two-year production cycle (sprout and crop year) most fields are divided into both vegetative and harvesting so ensure yearly crop production. Fields are developed from deforested land through the removal of competitive vegetation from native wild blueberry stands. Fields are pruned close to ground level in the late fall after harvest with the use of flail mowers, and fields are then fertilized in the vegetative year as shoot emergence commences (Chang et al., 2016). Fertilizers are typically applied in the form of soil applied granular fertilizer as the new growth of upright shoots is occurring with a N-P-K ratio of 1:1.8:0.8 being often used with fertilizer application rates in Atlantic Canada typically ranging from 20 to 40 kg N·ha-1 based on leaf tissue analysis results, removal of nutrients in the previous harvested crop and soil fertility. This may be supplemented with foliar applications of N-P-K nutrients while fungicide applications are being made throughout the production cycle. Bare spots and weed coverage can vary from 30-50% within growing wild blueberry fields (Zaman et al., 2008), however, most commercial fields typically have coverage that exceeds 70-80%. Removal of competitive weed pressures occurs with the application of pre-emergence and residual herbicides including hexazinone, and this is followed with postemergent herbicide applications for broadleaf and grass weed control.

Wild blueberries are typically produced using a 2-year production cycle to maximize production and ease of mechanical harvest. Shoot uprights are formed in vegetative phase of production with floral initiation and resulting floral bud growth and development occurring in the late summer and autumn.

Leaf and floral buds are produced during the vegetative year (McIsaac, 1997). On newly development shoots the ratio of floral buds to leaf buds is greater and more winter hardy in

comparison to that of older shoots. The weeds have become a major limiting factor, the use of specific herbicides has allowed for the increase of fertilisation, pollination, and irrigation, which overall has increased production four-fold (Yarborough, 2004). Previous studies have shown that the use of fertilisation can improve the yield within conventional fields when leaf tissue N and P levels are below 1.6% and 1.3% DW basis respectively (Drummond et al., 2009). Fertilize application when N/P levels are adequate can stimulate the growth of weeds and will not improve the overall yield of the blueberries (Drummond et al., 2009).

The application of soil-applied fertilisers (N, P, and K) is a routine procedure for crop management in the developed world and fertilisers are overused within the agricultural community (Tkacz and Poole, 2015). Nitrogen fertilisers are used in agricultural practices to provide the plant with nitrogen, but approximately 60% of the applied fertiliser is not absorbed by the plant (Tkacz and Poole, 2015). The unabsorbed nitrogen fertilisers can be utilised by other organisms within the soil or leach into groundwater, causing drastic changes to the marine microbial population, affecting the whole marine food chain in the process (Tkacz and Poole, 2015). Additionally, the application of nitrogen fertilisers halts the biological nitrogen fixation process (Omrane et al., 2009). It has become crucial to understand how to improve plant growth and development without becoming solely dependent on expensive and environmentally damaging fertilisers (Tkacz and Poole, 2015). Jeliazkova and Percival (2003a) studied the effects of N and P fertilisers on mycorrhizae fungi in wild blueberries. Interestingly, their data suggested that neither of the fertilisers used in the experiments significantly affected the ERM during vegetative and cropping cycles. They also noted nitrogen had marginal increased total stem length, stem numbers, and total dry weight (Jeliazkova and Percival 2003a).

The U.S National Association of Regional Councils is developing a farming system that is productive, profitable, energy conservative, environmentally sound, conservative of natural resource and ensures the food safety and quality (Lakshmanan et al., 2014). The Council has suggested using pre-selected beneficial microbes as a replacement for hazardous agrochemicals (Lakshmanan et al., 2014). These beneficial microbes show promising effects through nutritional benefit the soil, plant, and livestock as well as protecting from biotic and abiotic stresses (Lakshmanan et al., 2014). While the application of agrochemicals (fertilisers, herbicides and pesticides) may assist plant growth and reduce pathogens, the harm comes from the potential side effects of decreasing beneficial soil microbial diversity and create selection pressures for resistant pathogens (Krauss et al., 2011). An alternative method to traditional farming protocols would be to utilise naturally occurring microbiota available in the soil, using derivatives, secondary metabolite, and microbes for the protection of crops including pest, disease management, plant productivity enhancement, and increase fertility (Schäfar and Adams, 2015). Krauss et al. (2011) study revealed that organic farming protocols contribute to the preservation of biodiversity through enhancements of the selection of microbial communities. These enhancements provide beneficial influences on the microbiome and through improved control of pest species. It was also shown that the use of preventative insecticides on conventional fields resulted in undesirable effects on the plant's natural antagonists (Krauss et al., 2011). Some of the challenges that organic farmers and conventional farmers face when cultivating wild blueberries are, they cannot rotate crops, cannot use cover crops, or mechanically cultivate the soil for the reduction of weeds, as well as there is a limited amount of pesticides available for use on organic fields (Drummond et al., 2009).

2.4.0. Profiling Microbial Communities

Historically the discovery of a new microbial species was achieved through cultivation and subsequent characterisation of strains (Cardenas and Teidje, 2008). Recently, the methods developed for detection of non-cultivable or difficult to culture microbes has revolutionised the field of biology (Fadrosh et al., 2014). Recent advances in next-generation DNA sequencing methods such as pyrosequencing and shotgun metagenomics has enabled scientific studies of non-cultivable or difficult to culture microbial communities in a wide range of environments (Shokralla et al., 2012). Next-generation sequencing technology allows for sequencing of the whole genome, providing clarity in understanding the molecular genetics of microbial species (Gupta et al., 2014).

Culture-independent approaches to profiling bacterial and fungal communities rely on the amplification and sequencing of the 16S and 18S ribosomal RNA (rRNA) gene (Fadrosh et al., 2014). The 16S rRNA gene is present in all bacteria and archaea while the 18S rRNA gene within eukaryotic cells is a homolog of the 16S rRNA gene. The 16S rRNA is a component of the 30S ribosomal small prokaryotic subunits, and the 18S rRNA is a component of the 40S ribosomal small eukaryotic subunit (Ouvrard et al., 2000). Bacterial 16S and fungal 18S rRNA markers are frequently used to characterise the phylogenetic diversity and taxonomic composition of environmental samples (Chakravorty et al., 2007). The use of 16S rRNA gene profiling allows for large amounts of samples to be sequenced and examined in greater depth at a lower cost (Fadrosh et al., 2014). Sequencing cannot directly detect the metabolic or the functional capability of the organisms being studied (Langille et al., 2013). However, bioinformatic methods such as PICRUSt (Langille et al., 2013) can allow 16S gene profiling to provide a prediction of the

functional composition of the microbial community being studied similar to shotgun metagenomics, creating a more comprehensive picture.

Shotgun metagenomics provides a comprehensive list of the genes presents within the given sample. Unlike 16S rRNA gene profiling, shotgun metagenomics offers information about the functions of all the organisms being studied. Since the whole genome from each member of the microbial community is being sequenced, the cost per sample is much higher in comparison to a single gene approach such as 16S gene profiling. Metagenomics provides information about the microbes present and their genomic potential while metatranscriptomics provides information into the activity of the microbes (Mutz et al., 2013). The use of 16S profiling, metagenomics, and metatranscriptomics can be generated through several commercially available techniques such as Roche 454, Illumina and ABI SOLiD (Steward et al., 2010; Van Vliet et al., 2010). Each one of these techniques has positive and negatives, however, access to equipment, facilities, and cost tend to influence the final selection.

2.4.1. Alpha and Beta Diversity

Alpha diversity can be defined as the diversity within a particular area, habitat, or ecosystem, which is expressed by the number of taxon or species (i.e. species richness or evenness) (Whittaker, 1972). Alpha diversity can be calculated using several different metrics such as Shannon and Simpson indexes. Simpson's Index measures the diversity, taking into account the number of species present and the relative abundance of each species present. Simpson's index ($D = \sum (n/N)2$)

where n= the total number of organisms of a particular species and N = the total number of all organisms is used to measure the number of species present and the abundance of each species. As species richness and evenness increase so do species diversity. Shannon index ($H = \sum - (Pi * \ln Pi)$), where Pi = the fraction of the entire population calculates richness (number of species) and the evenness (proportion) of each species within the environmental sample. The index shows that as the richness increases or the distribution becomes more even, the result is an increase in biological diversity (Sea Grant Maryland, 2016).

Beta diversity can be measured as the variation in microbial communities' composition between areas, habitats, or ecosystems (Whittaker 1972). Beta diversity counts the total number of species which are unique to each ecosystem being compared. Beta diversity is calculated with phylogenetic diversity and non-phylogenetic diversity. Phylogenetic beta diversity uses a phylogenetic tree to measures the phylogenetic distances among the members of the microbial communities, while a non-phylogenetic beta-diversity metric does not require a tree and considers all organisms as independent entities (Graham and Fine, 2008). Phylogenetic beta diversity utilises temporal dimensions, these distances are known as the branch length between samples of individual organisms between any two locations (Graham and Fine 2008). Unique fraction metric (UniFrac) is a phylogenetic method that is used to measure the phylogenetic distances between sets of taxa in a given phylogenetic tree (Lozupone and Knight, 2005). UniFrac is a distance metric used in the comparison of biological communities which can be either weighted or unweighted, weighted provides appropriate weight (quantitative) to each sample, while unweighted considers each sample to be evenly weighted. UniFrac produces a similarity matrix which describes the pairwise phylogenetic distances between sets of sequences from the given specimens, which then

can be used with principal coordinate analysis (PCA) (Lozupone and Knight, 2005). Bray-Curtis is a non-phylogenic non-parametric multivariable method that is used to describe the dissimilarities between two samples by measuring the ecological distances (Anderson, 2001). Bray-Curtis assumes that the measurements used to characterise the samples are taken from the same physical size and are normalised to a scale of 0 to 1 where 0 equates to the samples which have the same composition while one means they do not share the same composition.

Chapter 3: Materials and Methods

3.1.0 Bulk Soil and the Rhizosphere

3.1.1. Soil and Root Sampling

Soil samples were collected from four different field locations for both high and low fruit yielding fields (Table 1). All sampled soil texture and drainage classification can be seen in Table 2. Soil chemical analysis was completed at the Nova Scotia Department of Agriculture & Food Operations Laboratory Services (Harlow Institute). Field bulk soil samples on average had a pH of 4.47, 0.28% nitrogen, 7.0% organic matter, 218.42 kg/ha P_20_5, 103.09 kg/ha K_20. Forest bulk soil samples on average had a pH of 4.46, 0.25% nitrogen, 7.5% organic matter, 105.38 kg/ha P_20_5, 91.71kg/ha K_20. Soil samples and root samples were collected during the second year of the growth cycle approximately two weeks before harvest (August 5^{th}-8^{th} 2015). Fields used in this study were on a two-year cropping cycle, plants were pruned using mowing, with fertiliser application occurring in the spring of the vegetative year fields were well established blueberry field. Samples were separated into managed field samples with solely wild blueberries within the fields (mono-crop) and unmanaged forest wild blueberry surrounding the field locations which included multiple different plant species.

Table 1: Geographical Location for Each Sampling Locations

Fruit Yield/Habitat	Geographical Location		
Low Fruit Yield	Debert 45°26’36” N, 63°27’ 1” W	Collingwood 45°36’53” N, 63°56’32” W	
High Fruit Yield	Debert 45°26’29” N, 63°27’2” W	Mt. Thom 45°29’23” N 63°59'40” W	Farmington 45°34’55” N, 63°54’43” W
Forest with Blueberry Plants	Debert 45°26’36” N, 63°27’ 1” W	Collingwood 45°36’53” N, 63°56’32” W	
Forest without Blueberry Plants	Debert 45°26’29” N, 63°27’2” W	Collingwood 45°36’53” N, 63°56’32” W	

Table 2: Soil Classification

Location	**Soil Characteristics**
Debert	gently slopped, sandy loam, poor moisture holding capabilities
Farmington	Developed from glacial drift stony sandy loam to clay loam, well-drained soil
Collinwood	Numerous slopes, dense with low water content, high shear resistance and low compressibility sandy loamy,
Mt. Thom	well drained, medium to light texture

Sampling: a total of 125 samples were collected for the analysis of this project. Samples were collected from bulk soil, rhizosphere, and root systems from both field and forest sampling locations (Table 1). Within field sampling locations 22 samples were collected from bulk soil, ten from rhizosphere, and 14 from root systems. While within forest sampling locations 35 samples collected from bulk soil, 20 from rhizosphere, and 20 from root systems.

Bulk soil sampling: Samples (~500 g) were taken from the top 20 cm topsoil layer in an X shaped pattern with at least 30 m of distance between each collection point creating a total of five collection points. The topsoil litter was removed to expose the bulk soil and collected using a coring device. To remove stones, gravel, and plant material samples were sieved (5 mm), placed within sterile bags, and transported into the laboratory on ice and then stored at -20° C. Additionally, 5 g soil samples were sieved (2 mm) and stored at - 86° C for DNA isolation.

Rhizosphere soil samples: Blueberry roots (with adhered rhizosphere soil) collected from the same field as the bulk soils were placed in a Falcon tube (50 ml) with 10% glycerol solution (40 ml) and vortexed (30 sec) until the roots were visibly clear from the adhering soil, the roots were removed, and the soil suspension was centrifuged at 3000 RCF for 15 min. The supernatant was decanted, and rhizosphere soil was collected were stored at -86° C for further analysis.

Root samples: Samples were collected from the corresponding field and forest bulk and rhizosphere soil collection sites. Samples were collected during the second year of the crop cycle to mirror the conditions of the wild samples, as they were not harvested. Roots systems (rhizomes included) were removed from collected plants from the corresponding bulk and rhizosphere soils sites. The roots systems were removed from the plants using sterile tweezers and washed several times with 10% glycerol solution, stored in 50 ml falcon tubes, and stored at -86° C for further analysis.

3.1.2. DNA Isolation from Soils

DNA was isolated from bulk and rhizosphere soils from both forest and field locations. DNA extraction was carried out at Dalhousie University Agricultural campus in the fall of 2015 using PowerSoil DNA Isolation Kit (Mobio Laboratories, Carlsbad, CA, USA) all required material (tubes, filters, and solutions) are provided within the kit. A total of 0.25 g of soil was placed into PowerBead Tube and gently vortexed. 60 µl of Solution C1 is added to the PowerBead Tube and sample is inverted several times to mix the solution. PowerBead Tube is then secured horizontally using the MO BIO Vortex Adapter tube holder and samples are vortexed at maximum speed for ten mins. After completion PowerBead Tubes were transferred to the centrifuge (ensuring tubes can rotate freely in the centrifuge without rubbing) and centrifuge samples at 10,000 x g for 1 min at room temperature. Afterwards, the supernatant was transferred (roughly 400 µl to 500 µl) to a clean 2 ml collection tubes (provided within the kit). A total of 250 µl of solution C2 was added, and vortex for 5sec then incubate samples at 4^{o} C for 5 mins. After incubation samples were centrifuged at room temperature at 10,000 x g for 1 min, the supernatant was transferred (upwards of 600 µl) to a clean 2ml Collection tube (provided in the kit), 200 µl of Solution C3 was added and vortex for 5 sec and incubate samples for 5 min at 4^{o} C. Samples were then transferred to the centrifuged and centrifuged for 1min at 10,000 x g. The supernatant was transferred (upwards of 750 µl) to a clean 2 ml Collection Tube. A total of 1.2 ml of Solution C4 (shake to mix solution C4 before use) was added to the newly transferred supernatants (be careful not to exceed the rim of the collection tube) and vortex for 5 sec. Approximately 675 µl of sample was transferred into a Collection Tube with a Spin Filter and centrifuge at 10,000 x g for 1 min at room temperature. The flow-through (liquid which passed through the filter), and the remaining supernatant was added to the Spin Filter and centrifuge at 10,000 x g for 1 min at room temperature. A total of

500 µl of Solution C5 was added and centrifuged at 10,000 x g for 1 min at room temperature; then the flow through was discarded from the collection tube. The samples were centrifuged again at 10,000 x g for 1 min at room temperature. The Spin Filter was transferred to a new clean 2 ml Collection Tube (carefully not to splash Solution C5 onto the Spin Filter) and 100 µl of Solution C6 was added to the central region of the white filter membrane. Samples were centrifuged at 10,000 x g for 1min at room temperature; then the spin filter was discarded. DNA quality and concentration were assessed as the ratio of A260/280 and A260/230 using NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, USA).

3.1.3. Sequencing of the Soil Microbiome

A total of 10uL of DNA from each sample was placed in 96 V-bottom PCR plates for 16S (V6-V8 region) and 18S sequencing (V4 region). In total, 125 DNA samples were sequenced (CGEB-IMR, Dalhousie University, Halifax) using the dual indexing approach for multiplexed gene sequencing of paired ends 300bp reads was accomplished using the Illumina MiSeq platform (Comeau et al., 2017).

3.1.4. 16S and 18S Data Analysis

The 16S fragments and 18S fragments were PCR-amplified from the DNA in duplicates or triplicates using a separate template dilution using a high fidelity Phusion polymerase. The 16S and 18S amplicon analysis were conducted using the standard operating procedure of the CGEB-IMR as outlined in the Microbiome Helper package

(https://github.com/mlangill/microbiome_helper). Sample sequences were filtered for low quality or probable chimeric reads. Sequences were clustered into 97% (for bacteria), and 99% (for eukaryotes) identify operational taxonomic units (OTUs) using an open-reference protocol as outline in QIIME (Caporaso et al., 2010) and assigned taxonomic labels using the GreenGenes database (McDonald et al., 2012). Alpha-diversity (Chao1, Simpson diversity, and Shannon diversity) was analysed within each sample (Lozupone et al., 2005). Functional capability of the samples was predicted using PICRUSt (Langille et al., 2013). Visualization and statistical comparisons were conducted within STAMP (Parks et al., 2014) including generation of PCA plots and bar plots while identification of the significantly abundant microbes was determined using Welsh's t-test and Benjamini-Hochberg with false discovery (FDR) rate correction. Tukey Method with a 95% confidence interval was used in ANOVA to determine the means that were significantly different. Anosim and Adonis test were used to determine the significant differences between the sampling locations as outlined in QIIME. Amplicon samples were sequenced using Illumina MiSeq, most often using the 300+300 bp paired-end V3 chemistry (Fadrosh et al., 2014). This allows for the overlap and stitching together of paired amplicon reads into one full-length read of higher quality. CGEB-IMR performed bacterial V6-V8 16S rRNA gene and fungal ITS library preparation and sequencing.

3.2.0. Root

3.2.1. Root Tissue Preparation

Root samples (~1g) were placed into 15 ml tube with 10 ml sterile water and sonicated for 60 sec (White et al., 2014). The roots were removed from the tube and placed into a new tube with and sonicated for 60 sec again. Finally, samples were placed into new sonication tubes and sonicated for 60 sec. The cleaned roots were cut into small five mm pieces using sterile scissors and placed into sterilised mortals. Liquid nitrogen and a pestle (sterile) were used to help grind the roots into a fine powder. A total of 0.250 g was set aside for DNA isolation, and 0.05 g was placed in 10% glycerol solution and stored in -86^{o} C for microbial isolation.

3.2.2. Sequencing Root Microbiome

Root DNA was isolated in winter of 2015 and early 2016 using the same kit and protocol as described for soil DNA isolation (Section 3.1.3.), 16S and 18S sequencing was completed as described for soil analysis (Section 3.1.5.)

3.2.3. Isolation of Root Microbiome

Prepared root tissues (50 mg) were placed into a 2 ml microcentrifuge tube and 0.5 mL of minimal salt base (Somerville et al., 1983) with no carbon and nitrogen source added. Serial dilutions up to 10^{-3} were placed on agar media, such as LB (Bertani, 1951), YMB (Somerville et al., 1983) and

potato dextrose agar (PDA) (Sigma Aldrich), and MM. Samples were incubated for seven days at 28° C. Samples were streaked on new plates for minimum three repetitions upwards of seven repetitions to ensure homogenous cultures were formed with no visual appearance of new colonies or contaminations.

3.2.4. Isolation of Beneficial Root Associated Microbes

Isolation of Flavin Secreting microbes: Microbial cultures were grown on minimal media; once pure cultures were obtained a sterile inculcation loop was used to transfer a loop full of microbes to 10 mL sterile falcon tubes with 4 mL of minimal medial broth. Samples were then grown at 28.5ºC and shaken at 250 RPM for seven days. A total, of 118 samples were tested for potential flavin secretion, 200 µL of each sample was placed into black 96 well microplates and tested for Flavin secretion. The excitation wavelength was set to 430 nm, and the emission wavelength was set to 530 nm. Absorbance was taken at a wavelength of 600 nm and used for the normalisation of the data. A total of three samples which had high readings from the initial 118 samples were selected for bacterial DNA isolation and sent for 16S sequencing.

Isolation of Phosphate Solubilizing Bacteria: For the determination of phosphate solubilising bacteria, isolates were grown on Pikovskayas Agar (PVK) media, pH adjusted to seven, and incubated at 28.5°C for seven days. Pure cultures were assumed once there was a similar growth of bacteria with no additional visual addition of cultures or contamination. Once pure cultures were formed, samples were given a number one-five or ALL depending on the size of their halo and their ability to solubilize/utilize the surrounding phosphate, where one was a halo of one-two

mm, two was two-three mm, three was four-five mm, four was majority of the plate, five was only small region of the plate not utilized, and ALL was the plate was clear and all phosphate was utilized in the plate. Number ranking was used instead of Marra et al. (2011) low, intermediate or high classification. The fast-growing plates which fell in the four-ALL range were selected for bacterial DNA isolation.

Isolation of Nitrogen Fixing Bacteria: For the determination of the presence of nitrogen-fixing bacteria, isolates were cultured initially with N2-BAP media and incubated at 28.5°C for seven days. To test the N2-BAP media *Sinorhizobium meliloti* 1021 was grown on N2-BAP media to ensure that no other microbes were capable of growing on the N2-BAP. Once pure cultures were obtained the pure cultures were re-streaked on minimal media for quick growth and then regrown on N2-BAP media to ensure no contamination had occurred. Once pure cultures were obtained, new cultures were grown and prepared for DNA isolation.

3.2.5. Bacterial DNA Isolation

The top 15 fast-growing and efficient phosphate solubilising bacteria were isolated for DNA isolation, and the top 15 fast-growing nitrogen-fixing bacteria were selected for DNA isolation. The bacterial cultures were isolated using the DNeasy Blood and Tissue Kit (Quagen). A loop full of pure culture was placed in a 2 mL Collection Tube with 200 µl of PBS then 20 µl of proteinase K was added to each Collection tube. A total of 4 µl of RNase A (100 mg/ml) was added to the Collection tube, samples were then vortexed, followed by incubation at room temperature for two mins. Once the incubation process was completed 200 µl of Buffer AL (without added ethanol)

was added to each collection tube then incubated at 56° C for 4 hrs. Once incubation was completed, 200 µl of ethanol (96-100%) was added to each collection tube. Samples were transferred to DNeasy Mini spin columns with attached 2 ml collection tube. Samples were centrifuged at ≧6,000 x g for 1 min at room temperature then flow through was discarded. DNeasy Mini Spin column was transferred to a new collection tube, and 500 µl of AW2 Buffer was added to the DNeasy Mini Spin column. collection tubes with DNeasy Mini Spin column were centrifuged at 20,000 x g for 3 min at room temperature after completion flow-through was discarded. DNeasy Mini spin columns were transferred to a Microcentrifuge tube and 100 µl fo AE Buffer was added to the spin column and incubated for 1 minute at room temperature. Samples were centrifuged at ≧6,000 x g for 1 min at room temperature. DNeasy Mini spin columns were, and DNA quality and concentration were assessed by the ratio of A260/280 and A260/230 using NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, USA).

3.2.6. 16S rRNA Sequencing

16S rRNA sequencing was performed on the single colonies. Single colony profiling was completed using the protocol as 16S Sequencing (Section 3.1.4.). The 16S sequences were compared to those in the GenBank database using BLAST.

3.2.7. Plant Growth Test

Growth tests were completed using alfalfa seeds purchased from Halifax Seed in Halifax, Nova Scotia; seeds were sterilised and initially grown on water agar in refrigeration for three days at 4° C. Seeds were then incubated at 28° C overnight or until germination. Plant nutrient solution (PNS) was used to moisten the vermiculite within the magenta vessels (V8505 SIGMA) ~100 mL per box. Seedlings were transferred to sterile magenta vessels using sterilised tweezers under a flame. Seedlings were grown for 24 hrs in magenta vessels afterwards inoculated with potential flavin secreting microbes. Potential flavin secreting microbes were mixed with PNS to ensure even distribution of potential flavin secreting microbes, ten mL solution of PNS and potential flavin secreting microbes were used on each sample. In the initial testing, the three potential flavin secreting microbes were used without and with urea (1 ml of a one molar solution) were tested in sub-triplicates. Two different controls were used in sub-triplicates, Control had PNS, and Control Urea had PNS and urea. Samples and controls were grown for four weeks, and then plants were harvested dry mass of shoots were collected.

During the second phase of testing the same protocols were used, however, at a larger scale a total of 60 magenta vessels (~five plants per box) were used. A total of nine magenta vessels per microbial inoculation (three potential flavin secreting microbes) and urea, nine magenta boxes per microbial inoculation (three potential flavin secreting microbes) without urea. While six magenta vessels were used as controls, three magenta vessels with PNS, and three magenta boxes with PNS + urea. Samples were grown for four weeks and harvested; dry mass of shoots was collected.

Statistical analysis - plant growth testing: once the four weeks were completed alfalfa plants were collected from the magenta vessels. Shoots of the plants were removed from the roots and air dried. All plants in each magenta vessel were massed (dry mass) together, and the number of plants was recorded. Average weight (dry mass) per magenta vessels was obtained. One-way analysis of variance with post-hoc Tukey test with a =0.05 was completed to determine significant differences.

Chapter 4: Results

4.1.0. Taxonomic Identities of Bacterial and Eukaryotic Populations

4.1.1. Bacterial and Eukaryotic Communities

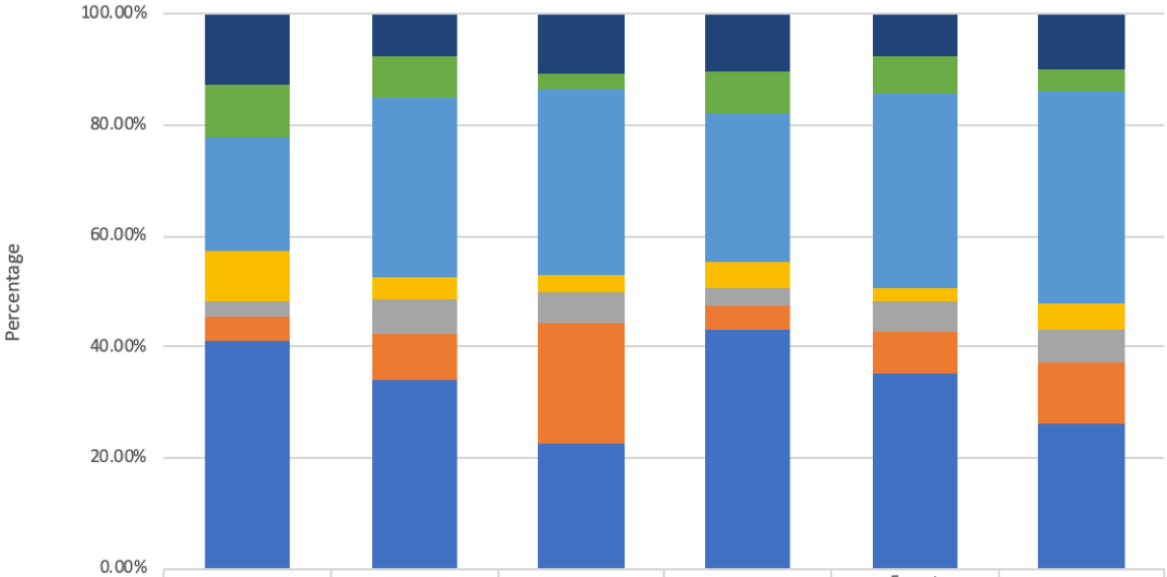
The total 16S and 18S sequencing reads generated were to be 2.57 million reads and 5.03 million reads, respectively. Due to a large amount of host amplification reads mapping the blueberries, we removed those associated with plant material leaving 1.21 million sequences with a median of 9,471 for 18S from the 125 samples. After merging the paired reads, quality control, chimera detection and taxonomic classification, samples were then normalized to 9,908 reads per sample for bacteria and 2,145 per sample for eukaryotes. Ten of the 125 samples with inadequate sequencing depth and were removed from further 16S and 18S analysis.

4.1.2. Structure of the Bacterial and Eukaryotic Community

On average, of the 29 phyla identified the top 6 most abundant bacterial phyla represented ~90% of the identified bacteria. These phyla included *Acidobacteria* (36%), *Proteobacteria* (29%), and *Actinobacteria* (8%), *Verrucomicrobia* (7%), *Chloroflexi* (5%), and *Bacteroidetes* (4%) (Fig. 2). Of the 78 eukaryotic orders identified the top 6 most abundant orders considered in this study represented ~72% of the identified eukaryotic communities. Of those Ascomycota (32%), *Dinophyceae* (11%), *Prymnesiophyceae* (6%), *Urochordata* (5%) *Arthropoda* (4%), and *Filosa-Sarcomonadea* (3%) represented most abundant eukaryotes (Fig. 11).

4.2.0. Effects of Field Management on Bulk Soils, Rhizosphere, and Root System Microbiome

4.2.1. Bacterial Communities



	Field Bulk	Field Rhizosphere	Field Root	Forest Bulk	Forest Rhizosphere	Forest Root
Remaining Phyla	12.6 ± 0.07	7.7 ± 0.06	10.5 ± 0.06	10.2 ± 0.08	7.6 ± 0.05	10.0 ± 0.08
Verrucomicrobia	9.5 ± 0.04	7.2 ± 0.03	2.9 ± 0.04	7.7 ± 0.02	6.6 ± 0.05	4.0 ± 0.04
Proteobacteria	20.6 ± 0.02	32.4 ± 0.02	33.4 ± 0.02	26.8 ± 0.02	35.0 ± 0.02	38.2 ± 0.02
Chloroflexi	9.1 ± 0.05	3.7 ± 0.02	3.2 ± 0.03	4.5 ± 0.04	2.6 ± 0.03	4.6 ± 0.05
Bacteroidetes	2.8 ± 0.05	6.6 ± 0.06	5.6 ± 0.06	3.2 ± 0.05	5.7 ± 0.06	6.1 ± 0.06
Actinobacteria	4.2 ± 0.002	9.2 ± 0.02	21.8 ± 0.02	4.4 ± 0.03	7.5 ± 0.02	11.0 ± 0.01
Acidobacteria	41.2 ± 0.002	34.1 ± 0.001	22.6 ± 0.001	43.1 ± 0.003	35.1 ± 0.003	26.2 ± 0.004

Figure 2: Six most relative abundant (%) of the major bacterial phyla present in bulk, rhizosphere, and root system located in both forest and field sampling locations. Only the six most relative abundant (%) bacterial phyla were considered.

Acidobacteria and *Proteobacteria* were determined to be the most abundant microbes within the all of the sampling locations (Fig. 2). *Acidobacteria* was determined to be the most abundant bacteria within field and forest bulk soils (Fig. 2) while *Proteobacteria* was found to be the most

abundant bacteria within field and forest root systems. *Acidobacteria* and *Verrucomicrobia* relative abundance decreased during the transitions from the bulk soil which can be seen in both field and forest sampling locations (Fig. 2). *Actinobacteria* and *Proteobacteria* exhibited an increase in relative abundance during the inward transition from bulk soil to root in both field and forest sampling locations. Corrected P-values (q-values) were calculated based on Benjamini-Hochberg FDR multiple test correction. Features with (Welch's t-test) q value <0.05 were considered.

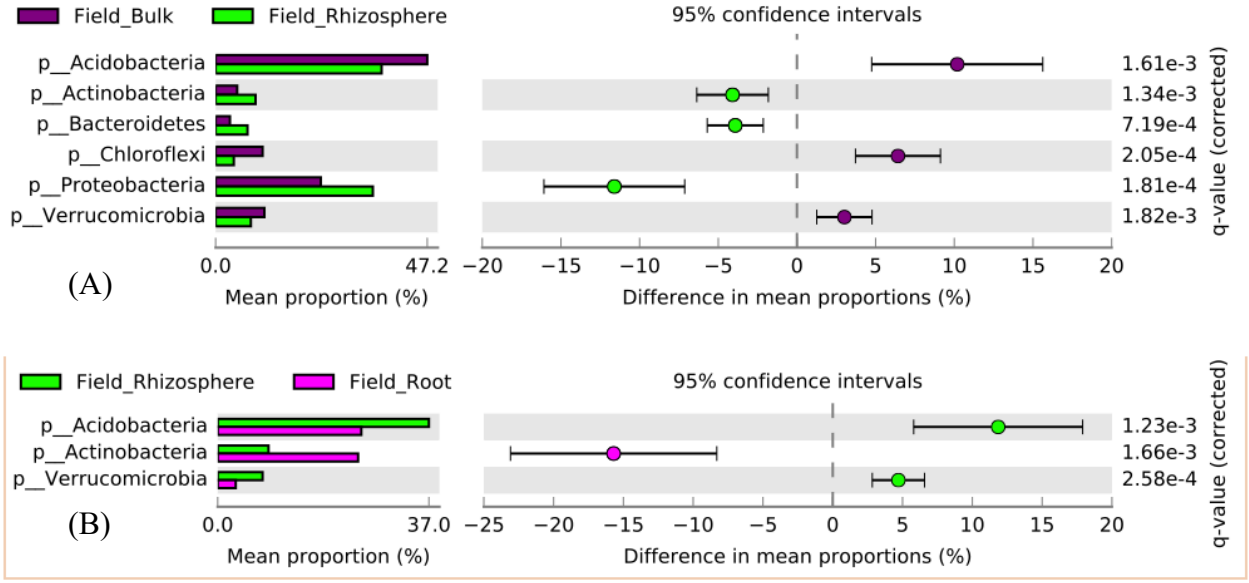


Figure 3: Comparison of the relative abundance of field bacterial sampling locations at the phylum level based on 16S sequencing: (A) field bulk and rhizosphere soils; (B) field rhizosphere soils and root systems. Only the six most abundant bacterial groups were considered. Corrected P-values (q-values) were calculated based on Benjamini-Hochberg FDR multiple test correction. Features with (Welch's t-test) q value <0.05 with a 95% confidence interval were considered significant and were thus retained, the analysis process showed apparent differences in the microbial communities.

When comparing field bulk soil to the rhizosphere the top 6 most abundant bacterial populations were considered significantly different (Fig 3A). Within the transition from bulk soil to the

rhizosphere, a significant increase in *Actinobacteria, Bacteroidetes* and *Proteobacteria* occurred (Fig 3A). The transition from field rhizosphere to root system *Acidobacteria* relative abundances was found to be significantly higher within the rhizosphere, while an increase in *Actinobacteria* and *Verrucomicrobia* were found to be significantly higher within the root system (Fig 3B).

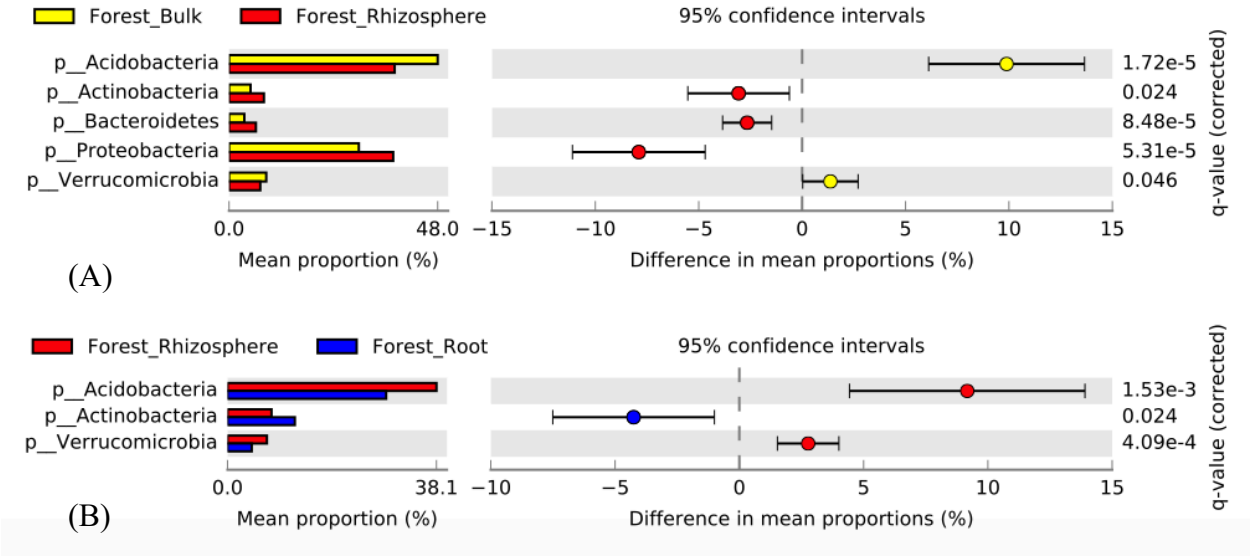


Figure 4: Comparison of the relative abundance of forest bacterial sampling sites at the phylum level based on 16S sequencing (A) forest bulk and rhizosphere soils; (B) forest rhizosphere soils and root systems. Only the six most abundant bacterial groups were considered. Corrected P-values (q-values) were calculated based on Benjamini-Hochberg FDR multiple test correction. Features with (Welch's t-test) q value <0.05 with a 95% confidence interval were considered significant and were thus retained, the analysis process showed apparent differences in the microbial communities.

When evaluating the transition from forest bulk to the rhizosphere (Fig 4A), forest sampling locations showed similar trends to that of the transition in field bulk to rhizosphere sampling locations and within the transition from rhizosphere to root systems. *Actinobacteria, Bacteroidetes* and *Proteobacteria* were found to be significantly higher in bulk soils when compared to the rhizosphere. Within the transition from the rhizosphere to the root system of forest locations,

Acidobacteria and *Verrucomicrobia* were determined to be significantly higher within rhizosphere.

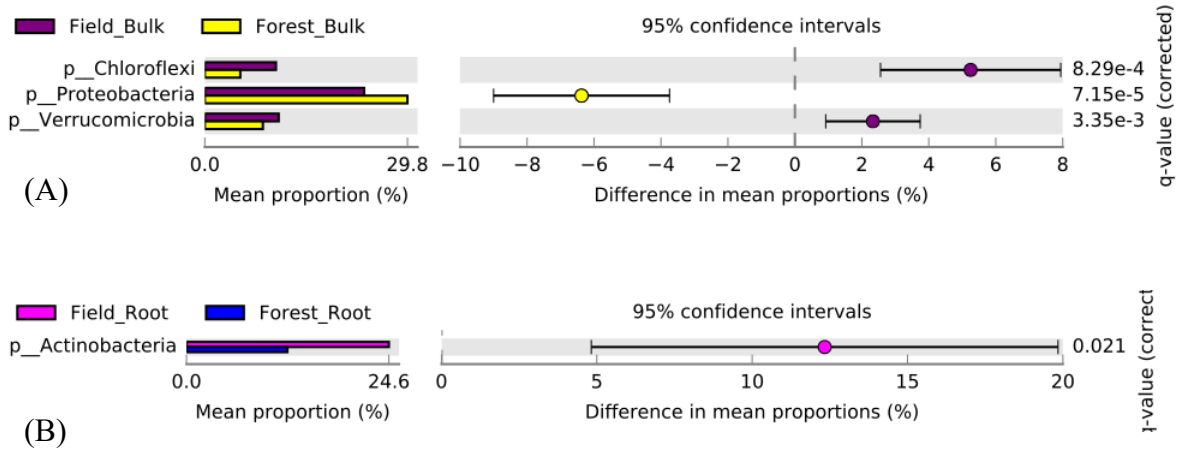


Figure 5: Comparison of the relative abundance of the corresponding field and forest bacterial sampling locations at the phylum level based on 16S sequencing. (A) Field and forest bulk soils; (B) field and forest root systems. Only the six most abundant bacterial groups were considered. Corrected P-values (q-values) were calculated based on Benjamini-Hochberg FDR multiple test correction. Features with (Welch's t-test) q value <0.05 with a 95% confidence interval were considered significant and were thus retained, the analysis process showed apparent differences in the microbial communities.

When comparing the corresponding field and forest locations *Chloroflexi* and *Verrucomicrobia* were determined to be significantly higher within Field bulk soils (Fig 5A). When comparing the corresponding root systems, *Actinobacteria* was found to be nearly double within field sampling locations (Fig 5B). There was no significant difference in bacterial relative abundances within corresponding rhizosphere sampling locations.

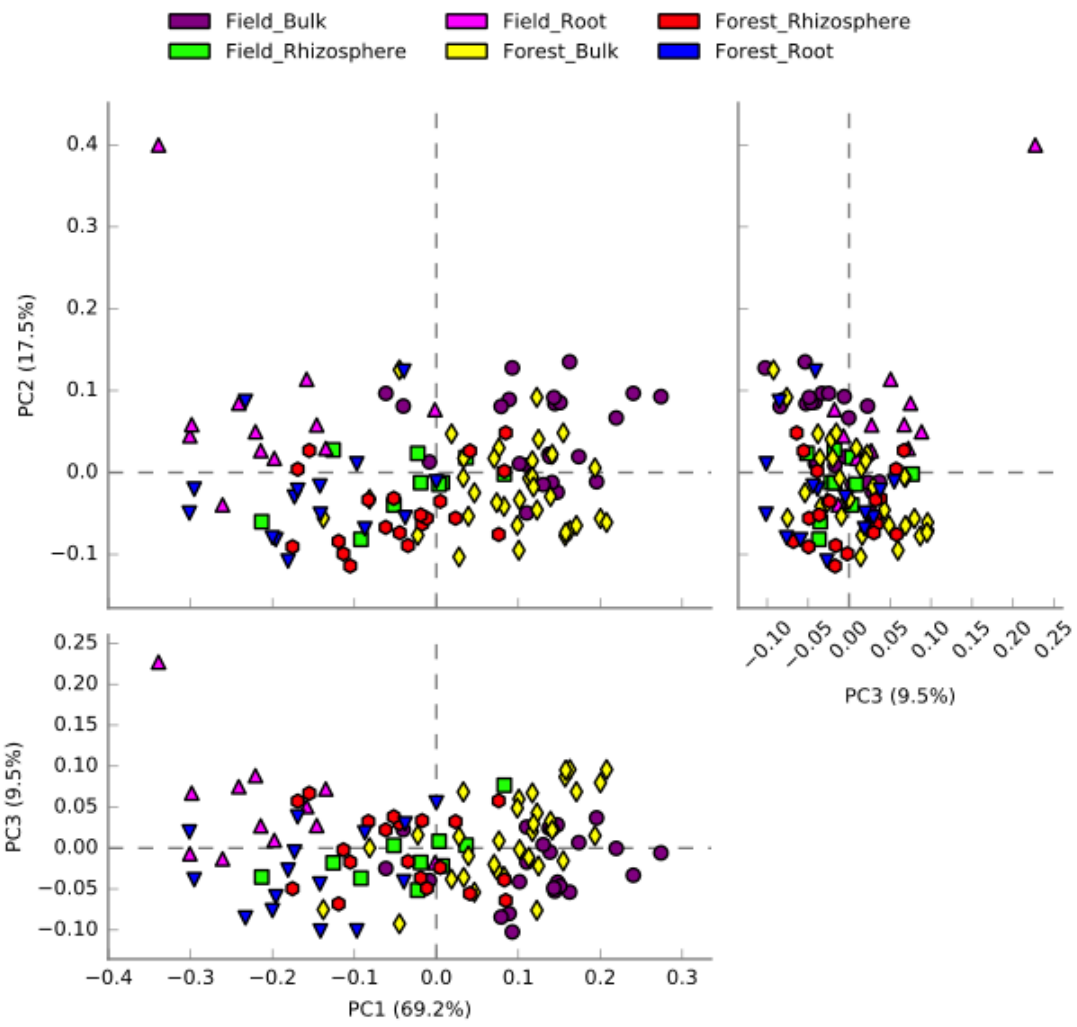


Figure 6: Principal Component Analysis (PCA) of bacterial communities based on 16S (bacterial) community. Each point is a different sample, and the colours indicate the different sampling locations. Samples which are closer together show fewer dissimilarities then samples which are further apart.

The results from the PCA plot revealed a linear transition of the bacterial populations of bulk, rhizosphere, and roots system within field and forest sampling locations (Fig. 6). Both the bulk field and forest soils along with field and forest roots are located in the same region of the PCA plot indicating some similarities between these two sampling locations (Fig. 6). While the field roots and rhizosphere are closer in proximity then that of field roots and bulk soils indicating they

are more closely similar to the rhizosphere soil then that of the bulk soil (Fig. 6). The Anosim test indicated that there was a significant difference (R2 =0.322, p< 0.001) between all 6 of the bacterial sampling locations. The Adonis test was used to indicate the significant difference between the bacterial sampling locations which can be seen below (Table 3) only significantly different sampling locations are shown.

BACTERIA		Forest			Field		
		Bulk	Rhizo	Root	Bulk	Rhizo	Root
Forest	Bulk	*	0.15	0.17	0.21	-	0.13
	Rhizo	0.15	*	0.20	-	-	0.15
	Root	0.17	0.20	*	0.08	-	0.29
Field	Bulk	0.21	-	0.08	*	-	0.07
	Rhizo	-	-	-	-	*	-
	Root	0.13	0.15	0.29	0.07	-	*

Legend

0.08 0.30

Figure 7: Adonis Test with a heat map of the bacterial sampling locations. Only significantly different (P-value <0.05) comparitive sampling locations were considered and were thus retained and the analysis process showed apparent differences in the bacterial communities. Heat map range was from 0.08 (low) to 0.30 (high).

The Adonis test of the bacterial sampling location was used to determine if there were significant differences between sampling locations. A heat map was used to aid in the visual of these significant differences. The test determined that there is significant difference between some of the sampling location (Fig. 7) which corresponds with the significant differences determined. The sampling location with the greatest R^2 value was determined to be forest root and field root sampling locations with and R^2-value of 0.29 indicating that these sampling locations are the most

significantly similar. While Forest bulk and field root sampling locations though significantly different had to lowest R^2-value of 0.07.

4.2.2. The Potential Functional Capability of the Bacteria Community Within Bulk Soils, Rhizosphere, and Root Systems

During the analysis, there was a broad range of significantly different potential functional groups within the six sampling locations. The four most relative abundant functional groups were selected for analysis. The highest proportion of potential functional groups belonging to carbohydrate metabolism which can be seen in all six sampling locations (Fig. 8). Other high proportions of potential functional groups belonged to energy metabolism, membrane transporters, and replication and repair.

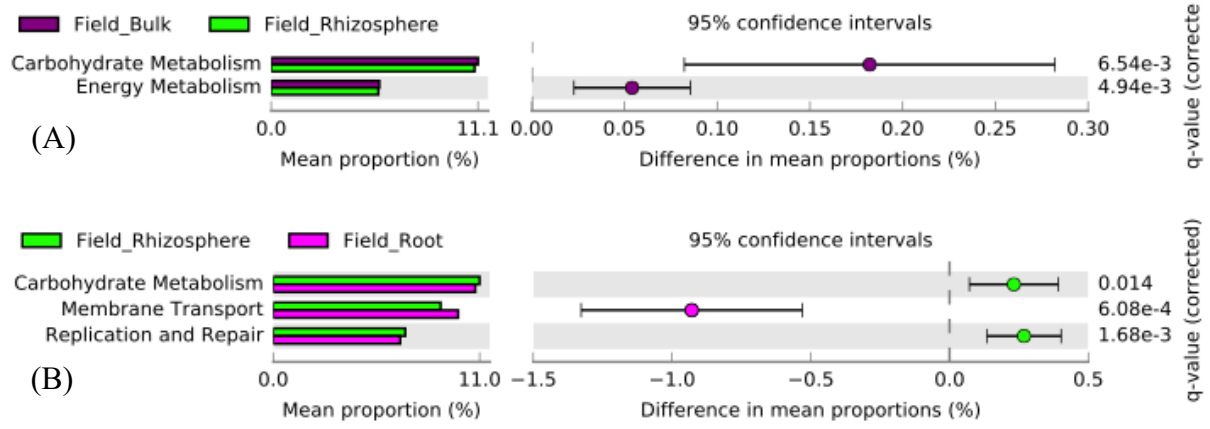


Figure 8: The relative abundance of potential functional groups (sub-system level 2) based on 16S sequencing. Percentage of total sequence reads in samples from field sampling locations. (A) Bulk soil and rhizosphere (B) rhizosphere and root systems. Corrected P-values (q-values) were calculated based on Benjamini-Hochberg FDR multiple test correction. Features with (Welch's t-test) q value <0.05 were considered significant and were thus retained, the analysis process showed apparent differences in the microbial communities.

The potential functional capabilities within the transition from field bulk soils to the rhizosphere showed a significant decrease in potential functional capabilities associated with carbohydrate metabolism (11.15% to 10.97%) and energy metabolism (5.83% to 5.78%) within the rhizosphere (Fig 8A). Within the transition from the rhizosphere to the root system, there is a significant increase in potential functional capabilities associated with membrane transport (8.90% to 9.82%) while there is a significant decrease in potential functional capabilities associated with carbohydrate metabolism (10.97% to 10.73%) and replication and repair (7.02% to 6.75%) (Fig. 8B).

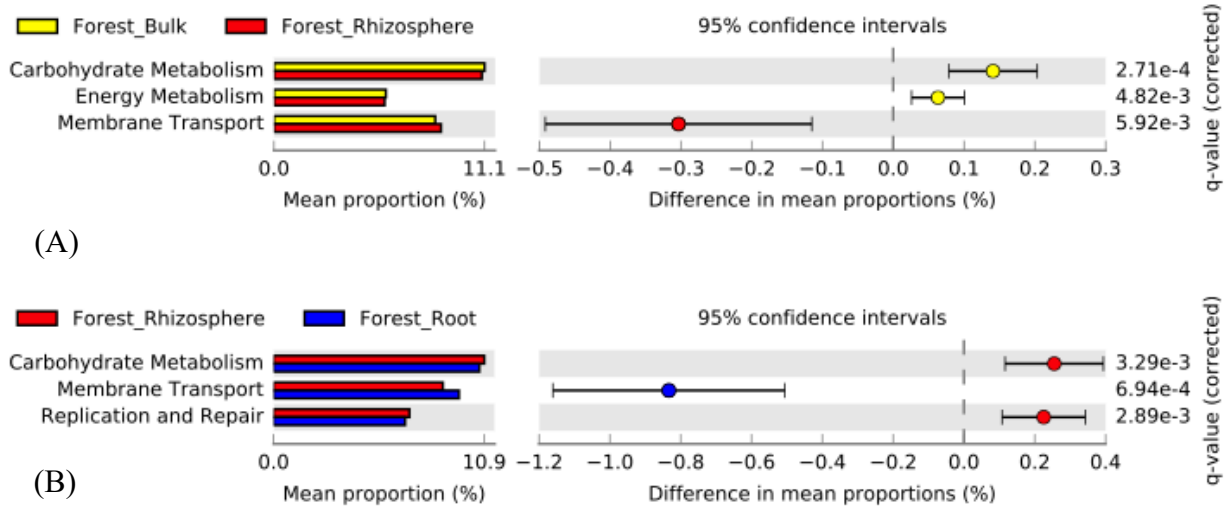
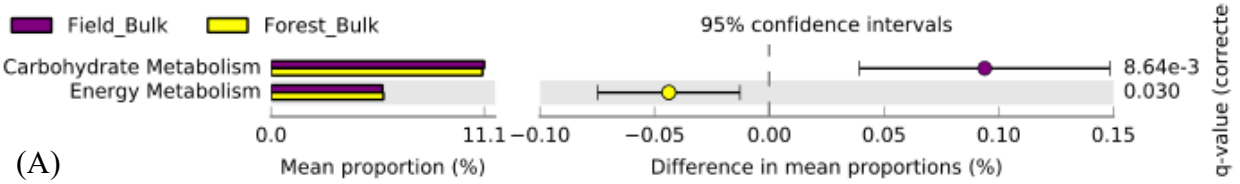


Figure 9: The relative abundance of functional capabilities (sub-system level 2) based on 16S sequencing. Percentage of total sequence reads in samples from forest sampling locations (A) bulk and rhizosphere (B) rhizosphere and root systems. Corrected P-values (q-values) were calculated based on Benjamini-Hochberg FDR multiple test correction. Features with (Welch's t-test) q value <0.05 were considered significant and were thus retained, the analysis process showed apparent differences in the microbial communities.

When evaluating the potential functional capabilities associated the transition within forest bulk soils to rhizosphere, there was a significant increase in membrane transport (8.47% to 8.77%) and

a significant decrease in carbohydrate metabolism (11.06% to 10.91%) and energy metabolism (5.88% to 5.81%) (Fig. 9A). The transition of forest rhizosphere to root system showed similar trends to that of the corresponding transition in field sampling locations with a significant increase in potential functional capabilities associated with membrane transport (8.77% to 9.60%) and a significant decrease in potential functional capabilities associated carbohydrate metabolism (10.91% to 10.66%) and replication and repair (7.04% to 6.81%) (Fig. 9B).

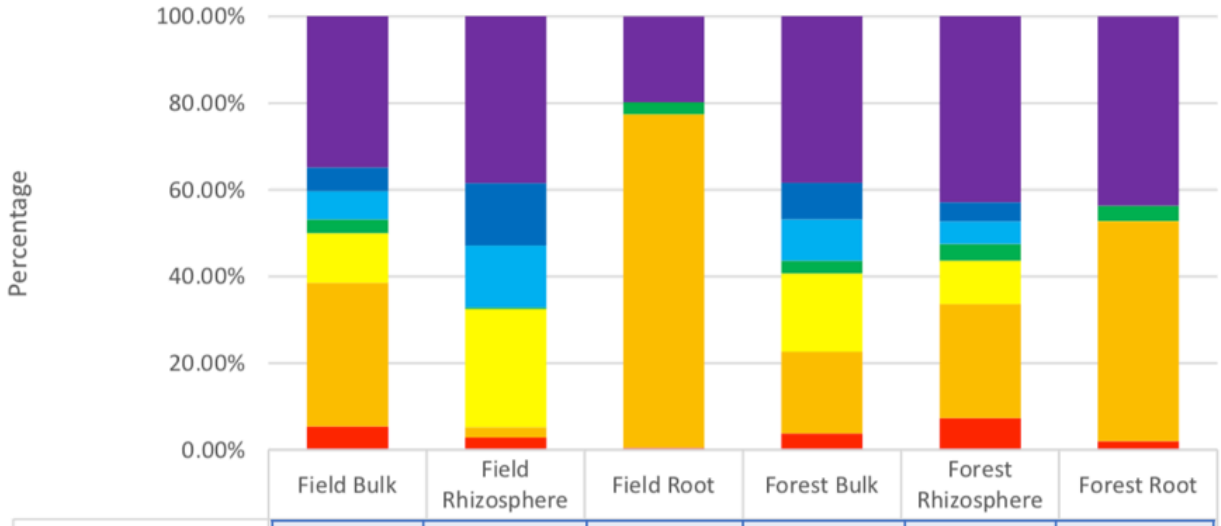


(A)

Figure 10: The relative abundance of functional capabilities (sub-system level 2) based on 16S sequencing. Percentage of total sequence reads in samples from the corresponding field and forest sampling locations (A) field and forest bulk soils. Corrected P-values (q-values) were calculated based on Benjamini-Hochberg FDR multiple test correction. Features with (Welch's t-test) q value <0.05 were considered significant and were thus retained, the analysis process showed apparent differences in the microbial communities.

When comparing the corresponding field and forest sampling locations only significant differences were found within bulk soils. Carbohydrate metabolism was found to be significantly higher within field bulk soils (11.14%) when compared to forest bulk soils (11.06%) (Fig. 10A). While energy metabolism was found to be significantly higher within forest bulk soils (5.88%) when compared to field bulk soils (5.83%) (Fig. 10B).

4.2.3 Soil Eukaryotic Community Composition



	Field Bulk	Field Rhizosphere	Field Root	Forest Bulk	Forest Rhizosphere	Forest Root
Remaining Orders	40.3 ± 0.005	52.9 ± 0.001	19.8 ± 0.009	46.8 ± 0.004	47.3 ± 0.006	43.65 ± 0.009
Urochordata	5.5 ± 0.06	14.4 ± 0.001	0.0 ± 0.0	8.4 ± 0.15	4.4 ± 0.08	0.0 ± 0.0
Prymnesiophyceae	6.5 ± 0.07	14.3 ± 0.01	0.0 ± 0.0	9.5 ± 0.16	5.2 ± 0.09	0.0 ± 0.0
Filosa-Sarcomonadea	3.2 ± 0.02	0.4 ± 0.002	2.8 ± 0.03	3.0 ± 0.005	3.9 ± 0.04	3.6 ± 0.03
Dinophyceae	11.5 ± 0.20	27.2 ± 0.02	0.02 ± 0.0009	18.0 ± 0.29	10.0 ± 0.18	0.1 ± 0.009
Ascomycota	33.2 ± 0.26	2.5 ± 0.01	76.9 ± 0.23	19.0 ± 0.04	26.3 ± 0.011	50.8 ± 0.23
Arthropoda	5.3 ± 0.06	2.8 ± 0.007	0.5 ± 0.03	3.7 ± 0.03	7.3 ± 0.07	2.0 ± 0.03

Figure 11: Six most relative abundant (%) of the major eukaryotic order found in bulk, rhizosphere, and root system located in both forest and field sampling locations. Only the six most relative abundant eukaryotic orders were considered.

Within the top 6 most abundant eukaryotes (excluding the accumulation of the remaining taxa) *Ascomycota* was determined to be the most abundant eukaryote within field bulk, forest bulk, and forest rhizosphere sampling locations. *Ascomycota* was determined to be the most abundant microbe within field and forest root systems (Fig. 11). There was a notable decrease in *Ascomycota*

within the field rhizosphere. The accumulation of the remaining orders was determined to account for the majority of the eukaryotic population within field rhizosphere.

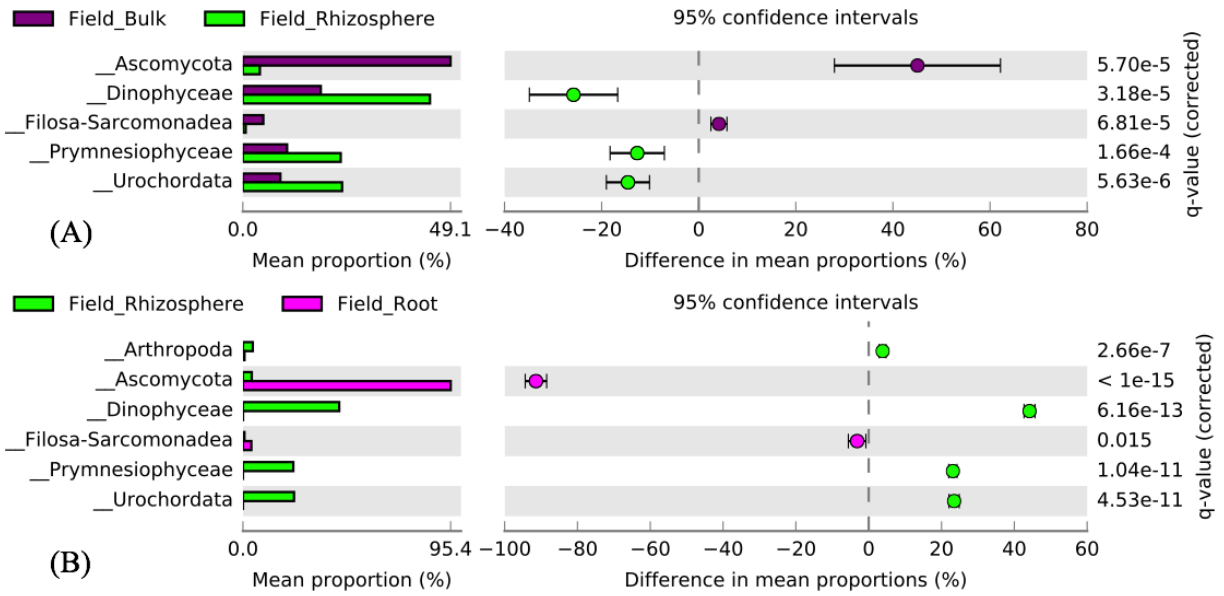


Figure 12: Comparison of the relative abundance of field eukaryotic sampling locations at the order level based on 16S sequencing: (A) field bulk and rhizosphere soils; (B) field rhizosphere soils and root systems. Only the six most abundant eukaryotic groups were considered. Corrected P-values (q-values) were calculated based on Benjamini-Hochberg FDR multiple test correction. Features with (Welch's t-test) q value <0.05 were considered significant and were thus retained, the analysis process showed apparent differences in the microbial communities.

When evaluating the transition from field bulk to the rhizosphere (Fig 12A), *Ascomycota* and *Filosa-Sarcomonadea* were determined to be significantly more abundant within bulk soils when compared to rhizosphere soils 33.18% to 2.46% and 3.18% to 0.41% respectively (Fig. 12A). While *Dinophyceae, Prymnesiophyceae* and *Urochordata* were found to be significantly more abundant within rhizosphere sampling locations. When evaluating the transition from the rhizosphere to the root system (Fig. 12B) the relative abundance of *Ascomycota* and *Filosa-Sarcomonadea* were significantly higher within the root system. While the relative abundances of

Arthropoda, Dinophyceae, Prymnesiophyceae and *Urochordata* were significantly higher within the rhizosphere soil (Fig. 12B).

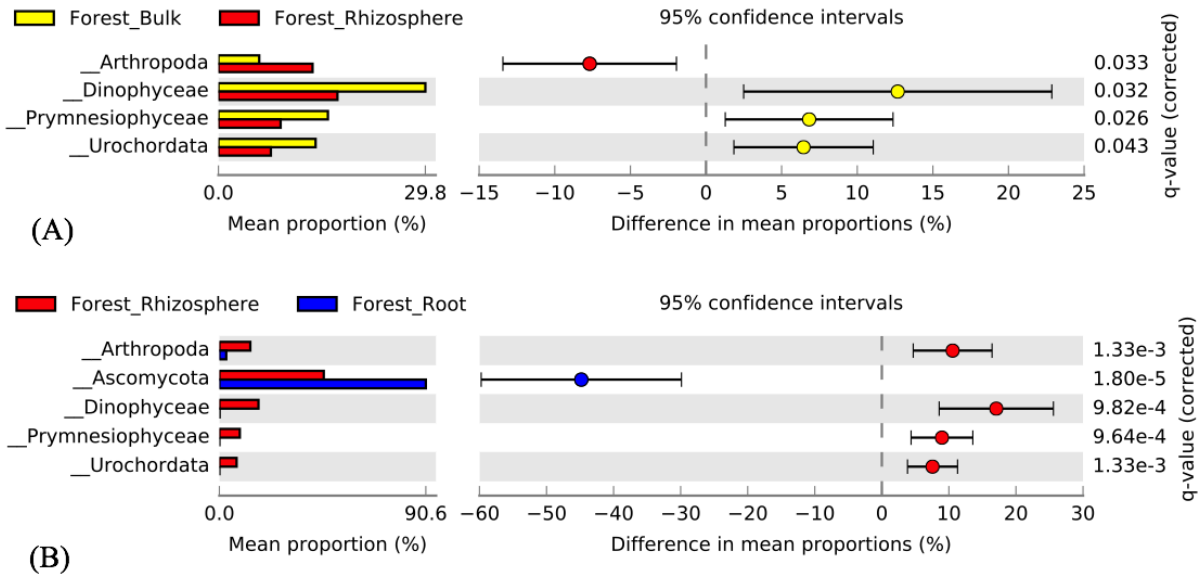


Figure 13: Comparison of the relative abundance of forest eukaryotic sampling locations at the order level based on 16S sequencing: (A) forest bulk and rhizosphere soils; (B) forest rhizosphere soils and root systems. Only the six most abundant eukaryotic groups were considered. Corrected P-values (q-values) were calculated based on Benjamini-Hochberg FDR multiple test correction. Features with (Welch's t-test) q value <0.05 were considered significant and were thus retained, the analysis process showed apparent differences in the microbial communities.

When evaluating the transition forest bulk and rhizosphere soils (Fig. 13A), there were significantly higher relative abundances of *Dinophyceae, Prymnesiophyceae,* and *Urochordata* within bulk soils, while Arthropoda was significantly higher within the rhizosphere. Within the transition from rhizosphere to root system (Fig. 13B) *Arthropoda, Dinophyceae, Prymnesiophyceae*, and *Urochordata* were significantly higher within rhizosphere soils, while *Ascomycota* was significantly higher within root system sampling locations.

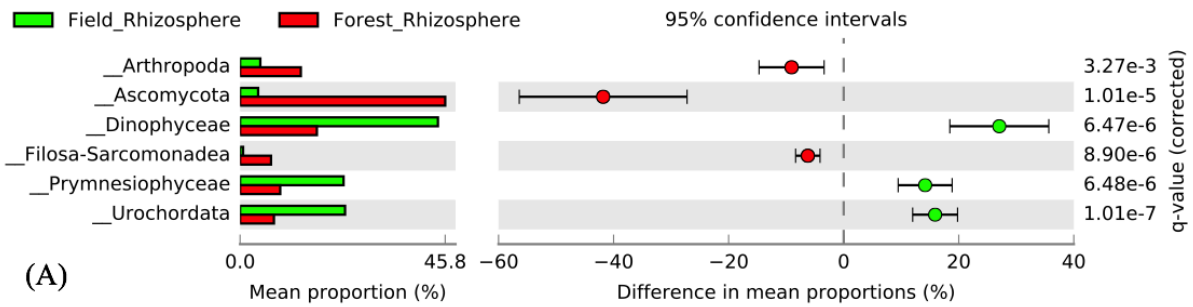


Figure 14: Comparison of the corresponding relative abundance of field and forest eukaryotic sampling locations at the order level based on 16S sequencing: (A) field rhizosphere and forest rhizosphere. Only the six most abundant eukaryotic groups were considered. Corrected P-values (q-values) were calculated based on Benjamini-Hochberg FDR multiple test correction. Features with (Welch's t-test) q value <0.05 were considered significant and were thus retained, the analysis process showed apparent differences in the microbial communities.

When comparing the corresponding field and forest locations, there were no significant differences within bulk and root systems (Fig. 14A). *Ascomycota* and *Arthropoda* were determined to be significantly more abundant within forest rhizosphere sampling locations, while *Dinophyceae, Prymnesiophyceae*, and *Urochordata* were determined to be significantly higher within field rhizosphere soil sampling locations (Fig. 14A).

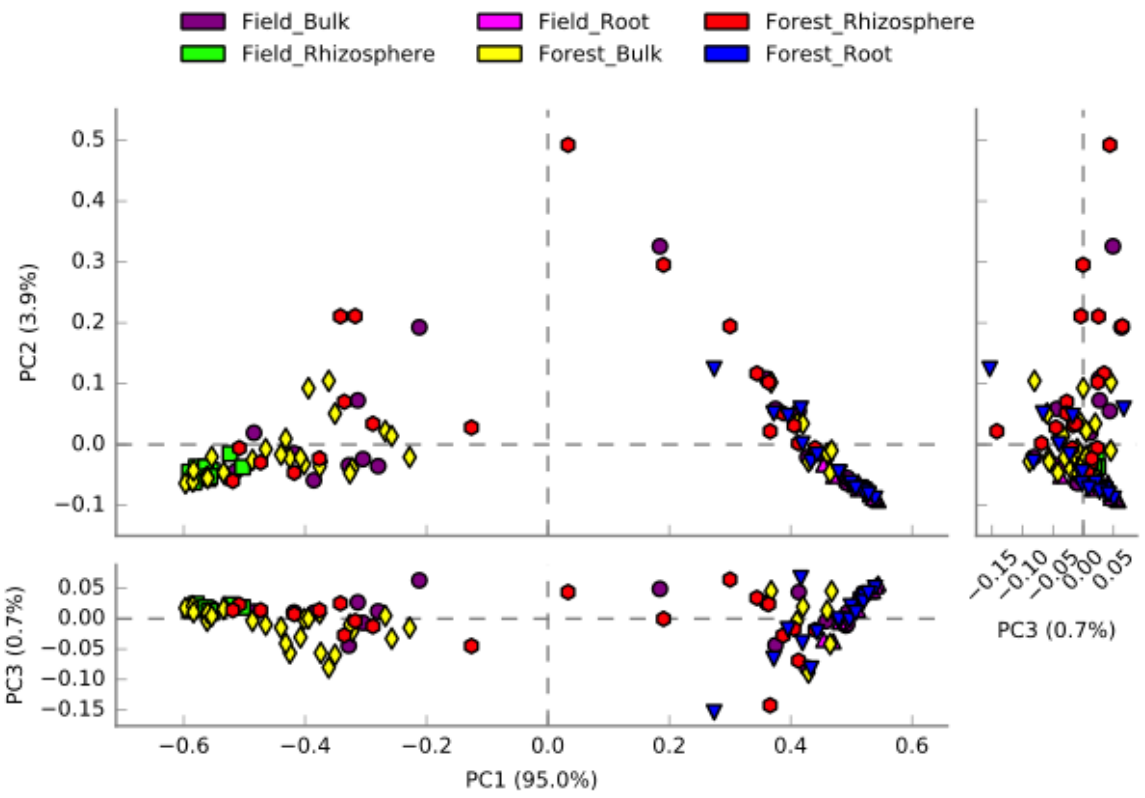


Figure 15: Principal Component Analysis (PCA) of microbial communities based on 18S (eukaryotic) community. Each point is a different sample, and the colours indicate the different sampling locations. Samples which are closer together show fewer dissimilarities then samples which are further apart.

The PCA plot of the eukaryotic community revealed less different separation between bulk, rhizosphere and root system sampling locations (Fig. 15). However, there is a district separation between the field and forest sampling locations. The Anosim test indicated that there is an overall significant difference in the field and forest blueberry eukaryotic sampling locations ($R^2 = 0.368$, $p<0.001$), however, the coefficient of determination was higher than that of the bacterial sampling locations (Fig. 15).

EUKAROYTIC		Forest			Field		
		Bulk	Rhizo	Root	Bulk	Rhizo	Root
Forest	Bulk	*	-	0.18	-	0.12	0.10
	Rhizo	-	*	0.20	-	-	-
	Root	0.18	0.20	*	0.25	0.26	0.54
Field	Bulk	-	-	0.25	*	-	0.13
	Rhizo	0.12	-	0.26	-	*	0.18
	Root	0.10	-	0.54	0.13	0.18	*

Figure 16: Adonis test with a heat map of the differences between eukaryotic sampling locations. Only R^2 values with P-value <0.05 were considered significantly different and were thus retained and the analysis process and showed apparent differences in the bacterial communities. Heat map rage was from 0.10 (low) to 0.54 (high).

The Adonis test of the eukaryotic sampling location was used to determine if there were significant differences between sampling locations. A heat map was used to aid in the visual of these significant differences. The test determined that there is significant difference between some of the sampling location (Fig 16) which corresponds with the significant differences determined in the Anosim test. The sampling location with the greatest R^2 value was determined to be forest root and field root sampling locations with and R^2 value of 0.54 which was nearly double to that of the corresponding bacterial sampling locations. While Forest bulk and field root sampling locations though significantly different had the lowest R^2 value of 0.10.

4.3.0. Isolation and Characterization of Root Associated Microbes

4.3.1. Nitrogen Fixing, Phosphate Solubilizing, and Flavin Secreting Microbes

Table 3: Flavin Florescence Testing

Sample ID	Florescence reading
FL45	1203.0^{BC}
FL59	1892.2^{A}
FL67	1368^{AB}
1021	717.4^{C}

For each mean, that does not share a letter(s) are significantly different according to Tukey's Pairwise test ($p<0.05$).

The isolation of potential nitrogen-fixing and phosphate solubilising bacteria was grown on specialised media N2-BAP and PVK respectively. A total of 30 bacteria were selected for their potential ability to fix nitrogen and solubilise phosphate (Table 4). Potential flavin secreting microorganisms were selected based on fluorescence reading (Table 3), which were later identified in Table 4. The genomic DNA from the microorganisms capable of fixing nitrogen, solubilise phosphate or secrete flavins was used for 16S rRNA PCR and sequencing.

The ability of root-associated microbes to secrete flavin was tested by measuring fluoresce of microbial liquid culture with 430nm excitation and 530nm emission wavelength. Of the 118 microbes, three potential flavin secreting microbes (FL45, FL59, and FL67) showed high levels of flavin production (Table 3). *Sinorhizobium meliloti* strains 1021, with high levels of flavin

secretion (Yurgel et al., 2014) was used as a control (Table 3). The identities of the three root associated root microbes can be seen in Table 4 to the lowest possible taxonomic rank.

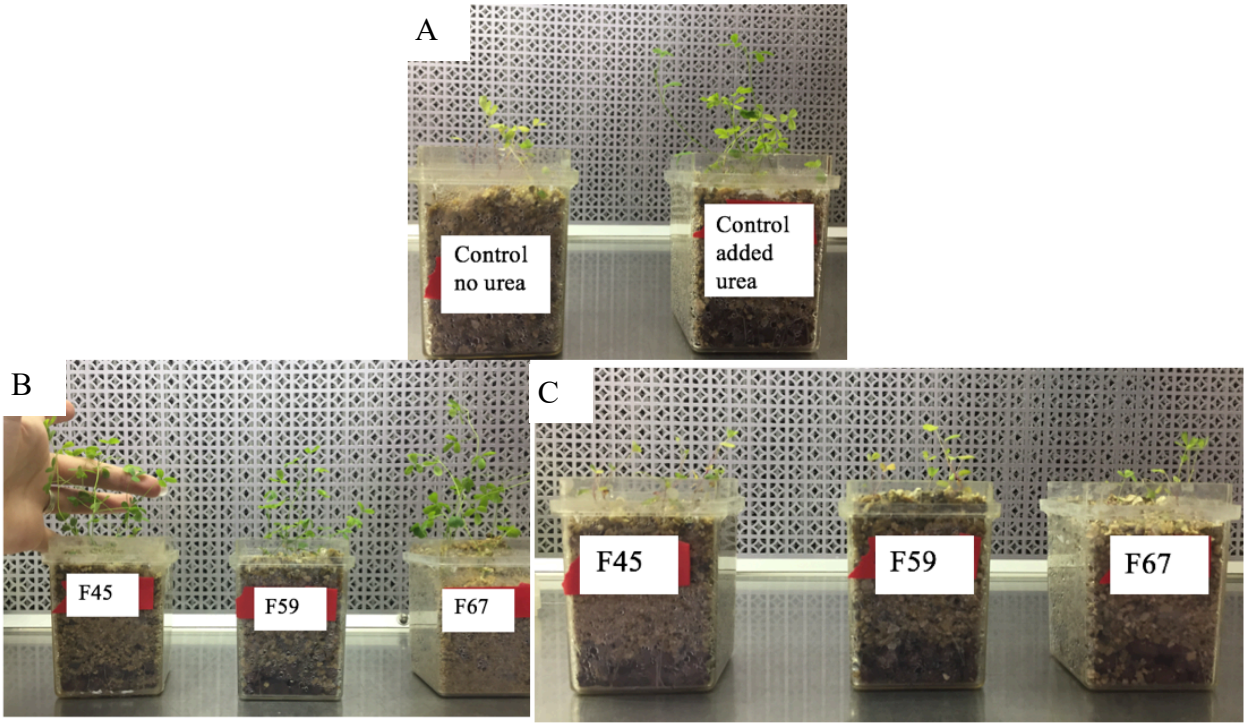


Figure 17: Plant growth trials. (A) Left to right control plants without added urea and control plants with added urea. (B) Left to right sample FL45, FL59, and FL69 with added urea. (C) Left to right sample FL45, FL59, and LF67 without added urea.

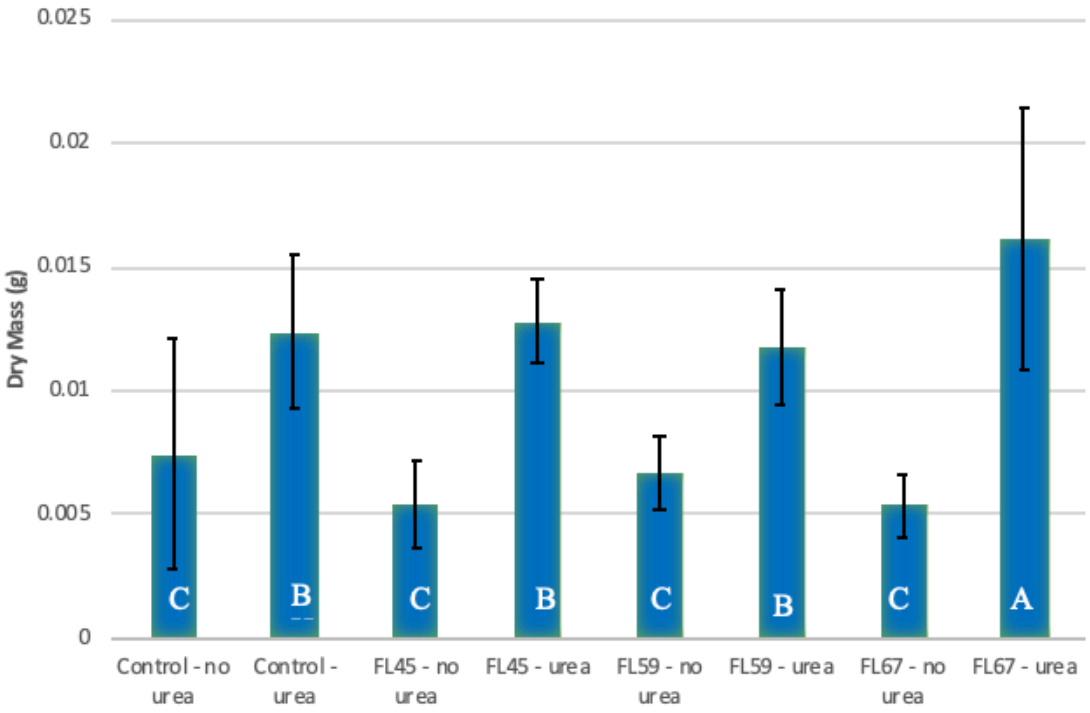


Figure 18: Plant growth test. For each mean, that does not share a letter(s) are significantly different according to Tukey's Pairwise test ($p<0.05$).

After four weeks of growth, the alfalfa control plants with added urea and the inoculated alfalfa plants with added urea were healthy and deep green colour (Fig. 17A and Fig. 17B). The inoculated alfalfa plants with added urea had longer shoots, and higher average shoot weight (dry mass) (Fig. 17A and Fig. 17C). The control plants without added urea developed a much smaller shoot system, and leaves were yellow due to low concentrations of chlorophyll (Fig. 17B). FL67 with added urea was significantly different from all other samples and controls (Fig. 17C and Fig. 18), while FL45 and FL59 with added urea were not significantly different from the control with added urea (Fig. 17C and Fig. 18).

Table 4: List of the identified Vaccinium angustifolium Ait root-associated microbes exhibiting potential ability to fix atmospheric nitrogen, solubilise phosphate, or secrete flavins.

SAMPLE ID [A]	CLOSES MATCH IN GENBANK [B]	ORDER [C]
NF01	f__Burkholderiaceae	o__Burkholderiales
NF02	f__Burkholderiaceae	o__Burkholderiales
NF04	f__Burkholderiaceae	o__Burkholderiales
NF07	f__Burkholderiaceae	o__Burkholderiales
NF09	f__Burkholderiaceae	o__Burkholderiales
NF12	s__glathei	o__Burkholderiales
NF13	g__Burkholderia	o__Burkholderiales
NF14	s__glathei	o__Burkholderiales
NF17	s__glathei	o__Burkholderiales
NF20	f__Burkholderiaceae	o__Burkholderiales
NF21	f__Burkholderiaceae	o__Burkholderiales
NF22	f__Burkholderiaceae	o__Burkholderiales
NF23	g__Burkholderia	o__Burkholderiales
PS26	g__Burkholderia	o__Burkholderiales
PS27	f__Burkholderiaceae	o__Burkholderiales
PS39	f__Burkholderiaceae	o__Burkholderiales
PS49	s__glathei	o__Burkholderiales
PS55	f__Burkholderiaceae	o__Burkholderiales
PS60	f__Burkholderiaceae	o__Burkholderiales
PS62	f__Burkholderiaceae	o__Burkholderiales
PS65	f__Burkholderiaceae	o__Burkholderiales
PS66	f__Burkholderiaceae	o__Burkholderiales
PS68	f Burkholderiaceae	o Burkholderiales
PS70	g__Burkholderia	o__Burkholderiales
PS71	f Burkholderiaceae	o Burkholderiales
PS74	s__glathei	o__Burkholderiales
PS77	f Burkholderiaceae	o Burkholderiales

FL45	s__Burkholderia caledonica	o__Burkholderiales
FL59	s__Burhholderia caledonica	o__Burkholderiales
FL67	s__Agrobacterium	o__Rhizobiales

[a] *NF: Nitrogen-fixers; PS: Phosphate solubilisers; FL: Flavin*
[b] *s: species, g: genus, f: family*
[c] *o: order*

Chapter 5: Discussion

5.1.0. Bacterial Phyla Within Field and Forest Sampling Locations

In previous studies, the most abundant species within soils belong to following phyla: *Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi,* and *Proteobacteria* (Yurgel et al., 2017; Feirer et al., 2009). In agreement with this concept, the most prevalent phyla detected in our study of wild blueberry forest and field habitats represented ~90% of all taxa were *Acidobacteria, Proteobacteria, Actinobacteria, Verrucomicrobia, Chloroflexi,* and *Bacteroidetes*. These bacterial phyla were observed in both field and forest sampling locations (bulk, rhizosphere and roots). We observed distinct differences in bacterial communities between the field and forest sampling locations (bulk soils, rhizosphere, and root system) (analysis of similarities R^2=0.322, $p < 0.001$). Adonis test was used to determine the significant differences between the sampling locations (Fig. 7). The forest and field root systems were the most similar (R^2=0.29) potentially indicating that the change from forest to field affected the root system the least. This could mean that the changes in from forest to field are less influential in comparison to the plant's selective ability with root-associated bacteria. These root-associated bacteria could potentially be more beneficial to the plant or that these bacteria have a higher affinity and preference to the conditions associated with the root system. Forest bulk and field bulk (R^2=0.21) sampling locations were the second most similar potentially indicating that these soils may also be least affected by the changes from forest to field. Forest bulk soil and rhizosphere (R^2=0.17) and forest rhizosphere and root system (R^2=0.15) sampling locations when compared had similar coefficients of determinations. However, the field bulk soil and rhizosphere and field rhizosphere and root systems sampling locations were not considered significantly different which may indicate that the transition from

forest with multiple plant species to field with single plant species affected these sampling locations.

5.1.1. Bacterial Phyla Associated with Wild Blueberry Systems

Proteobacteria

Proteobacteria are considered an evolutionary, geologically, and environmentally important phylum of bacteria, and are also considered the most diverse phylum of bacteria (Gargaud et al., 2011). Members of this phylum show extreme metabolic diversity, comprised of chemoautotrophic, chemoorganotrophic, and phototropic bacteria, this is especially important as they represent most of the recognised bacteria of medical, industrial and agriculture industry Gargaud et al., 2011). There is a total of five classes of *Proteobacteria (Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria*, and *Epsilonproteobacteria)* all are Gram-negative (Gargaud et al., 2011). Many of these bacteria rely on flagella for mobility while some are non-motile and use bacterial gliding for movement.

Proteobacteria was found to be the second most abundant bacterial phyla in this study. The proportion of *Proteobacteria* was not considered significantly different within the corresponding field and sampling locations this may indicate that this phylum may not be primarily affected by the fields with singular plant species (Fig. 5). Rampelotto et al. (2013) noted that as a whole phylum *Proteobacteria* may not be affected mainly by land-use however, some of the different subgroups were affected by land-use. Our data did agree with this team's research that

Proteobacteria as a phylum was not primarily affected by land-use our research. Rampelotto et al. (2013) also noted since *Proteobacteria* are considered necessary in global carbon, nitrogen, and sulfur cycling, the identification of the subgroups which are sensitive to land use is considered essential in understanding their potential roles in the microbiome. In agreement with the research completed by Rampelotto et al. (2013) of *Proteobacteria* more research would be required to determine if some of the subgroups of the Proteobacteria associated with wild blueberry systems and potentially other phyla are hypersensitive to changes in management and determine if their presence is beneficial or negative to the plant system. While we did not look at this at an in-depth level, this concept would be a useful area of research in a future analysis of the subgroups and how they were affected by the use of the land.

The proportions of *Proteobacteria* within forest showed a similar increasing transition towards the root system much like *Actinobacteria*. The only significant differences were found within the transition from bulk to rhizosphere soils within field and forest sampling locations (Fig. 3A and Fig. 4A). This change in transition showed that there was an increase in *Proteobacteria* within the rhizosphere relative to that of the bulk soils. Overall *Proteobacteria* was the most relative abundant bacteria in the root systems accounting for 33% within the field and 38% within the forest of the total percentages of microbial phyla discovered. These increases in transitions from bulk to rhizosphere soil potentially indicated that the bacteria prefer more pH neutral conditions however, it can also be due to the rise in available substrates within the rhizosphere based on the increase in relative abundance with the proximity to the root system (Fig. 2). In agreement with Santoyo et al. (2016) our study indicated that *Actinobacteria, Bacteroidetes, Verrucomicrobia,* and *Acidobacteria* were found associated with the root system, it was also noted like *Proteobacteria*

that *Bacteroidetes* and *Actinobacteria* preferred more pH neutral conditions (Fig. 2). The other phyla consistently found as root-associated bacteria were *Firmicutes* and *Actinobacteria* while *Bacteroidetes, Verrucomicrobia,* and *Acidobacteria* which are less commonly found as root-associated bacteria (Santoyo et al., 2016).

Despite being within forest locations, the soil microbial community associated with wild blueberries were not as unique as we initially believed. The transition from forest with multiple plant species to field with singular plant species had not substantially altered the bacterial community composition as initially believed. Based on the bacterial relative abundances and alpha diversity (Shannon diversity and Chao1 richness) the bulk soil and rhizosphere of both field and forest sampling locations were not significantly different from one another (Appendix Fig 2a and 3a). While the root-associated bacteria in field and forest sampling locations were significantly different from the bulk soil and rhizosphere, no significant difference was found when compared to each other. All of the above described phyla (Fig. 2) have been found to be as root-associated bacteria, while also ordinary dwellers of the rhizosphere (Santoyo et al., 2016). Within our study, the bacterial populations follow a similar inward increasing trend (increase in relative abundance as the bacteria transition within closer proximity to the plant) except for *Acidobacteria*. This could be attributed to its ability to survive in a broad range of environments, for its preference for acidic conditions and like *Proteobacteria* are less sensitive to field with singular plant species conditions.

Acidobacteria

Acidobacteria has recently been considered a newly recognised phylum; this bacterial phylum has been found to have a high relative abundance across a broad range of ecosystems (especially soil)

and can currently be subdivided into 26 subgroups (Kielak et al., 2016; Barnes et al., 2007). In recent studies, 16S rRNA gene-based approaches along with shotgun metagenomic analysis have revealed that *Acidobacteria* represents a highly diverse bacterial phylum which can inhabit a wide range of habitats around the world (Chow et al., 2002; Kushe et al., 2002).

Acidobacteria was found in all bacterial communities (bulk, rhizosphere and root system sampling locations), while it was the most abundant bacterial population in bulk soils and rhizosphere, it was found second most abundant to *Proteobacteria* in the root systems of field and forest sampling locations (Fig. 2). Within field and forest bulk soils, *Acidobacteria* accounted for 41% and 43% respectively of the total bacterial population (at the phylum level). While in field and forest rhizosphere soil and root system sample relative abundance were found to be 34% and 23% respectively in field sampling locations and 35% and 26% respectively in forest sampling locations, these transitions demonstrated that during the change in proximity to the root system there was a significant difference in the relative abundance of *Acidobacteria* (Fig. 3B and 4B). Studies have looked at the distribution of *Acidobacteria* within the soil system in relation to the proximity to the root system and found similar results. In agreement with our findings, these studies determined that there was a higher proportion of *Acidobacteria* within the bulk soil when compared to rhizosphere (Marilley and Argno, 1999; Sangiun et al., 2006; Fierer et al., 2007; Kielak et al., 2008). Our finding suggests that like *Proteobacteria,* the *Acidobacteria* identified in this study are potentially pH sensitive and unlike *Proteobacteria*, prefers more acidic conditions. This can be seen through changes in the relative abundances to the proximity of the root system in the field and forest sampling locations, the corresponding field and forest sampling locations were not considered significantly different (Fig. 2, 3B and 4B). A study conducted by Hartmann et al.

(2014) also showed similar results of the soil bacterial diversity. They noted that *Acidobacteria* had the most robust response, which suggests that this taxon prefers environments with low available nutrients and high acidity, which corresponds with our study of the soils within our study of *Acidobacteria* within wild blueberry soil systems.

The Naether et al. (2012) study was carried out on grasslands, and forest plots reinforced that *Acidobacteria* belonging to group 1 prefer nutrient limiting oligotrophic lifestyles within low-nutrient soils. *Acidobacteria* have been mostly considered as potassium strategist's oligotrophic bacteria having low growth rates which favour under resourced-limiting environments due to the high substrate affinities and that pH is the most reliable predictor of the *Acidobacteria* community (Fierer et al., 2007). A study conducted by Bakersman et al. (2014) on the bacterial populations within the permafrost of Antarctica noted that *Acidobacteria* belonging to group 6 were dominate in soils with a pH of 8.6. Chu et al. (2010) noted that in *Acidobacteria* belonging to group 1, 2, and 3 were found to be dominant in Arctic soils with a pH of 4-5. While high relative abundances of members belonging to group 5, 6 and 17 have been found in soils with high nutrient levels (Feirer et al., 2007). Based on Feirer et al. (2007) findings, it would be safe to assume that the majority of the *Acidobacteria* found within the fields used for this research belong to group 1-3. However, more research would be required to determine if other subgroups of *Acidobacteri*a phylum can be found within the acidic soils of the wild blueberries systems and whether or not those subgroups are beneficial or non-beneficial to the plant and the surrounding soil microbiome.

In 2009, Ward et al. (2009) were one of the first groups to sequence the genome of *Acidobacteria.* From these genomic studies, it was determined that carbon usage, nitrogen assimilation,

metabolism of iron, antimicrobials, and relative abundance of transporters were among the five aspects of the *Acidobacteria* physiology to be focused on (Kielak et al., 2016). Fierer et al. (2007) suggested that *Acidobacteria* populations were much more abundant within bulk soils in comparison to rhizosphere soils in which the soils have low carbon mineralization rates (low resource availability), these results correspond with our findings as bulk soils sampling locations which was significant greater from the rhizosphere sampling locations (Fig. 3A and 4A).

Many previous researchers have suggested that phylum *Acidobacteria* is sensitive to both inorganic and organic inputs (Navarrete et al., 2015). The *Acidobacteria* phylum has a broad, diverse range of bacteria and functions, in 2015 Huang et al. discovered *Acidobacteria* play a role within soil recovery following the occurrence of severe disturbances within the soil system, as this phylum is essential for nutrient cycling and plant growth. Huang et al. (2015) also noted during recovery that soils with *Acidobacteria* recovery occurred under aerobic conditions. During the experimental process conducted by Ward et al. (2009), *Acidobacteria* which were cultured on low-nutrient media excreted extracellular slime which suggests the ability to withstand harsh environmental changes (cycles of drying and rehydration) (Ward et al., 2009). The strains of *Acidobacteria* (*A. capsulatum*, Ellin345, and Ellin6076) which Ward et al. (2009) studied have the potential to utilise a broad variety of carbon substrates which rely on low-specificity and high affinity of sugar transporters. These strains of *Acidobacteria* and genomic traits show the ability to reside in nutrient-limiting environments because of their broad metabolic capability (Ward et al., 2009). From studying these strains, Ward et al. (2009) proposed that the isolates are long-living, divide slowly, demonstrate slow metabolic rates occurring under low-nutrient conditions, and are capable of surviving harsh environmental changes (cycles of drying and rehydration).

Studies similar to these begin to shed light on the elusive newly recognised phylum. Understanding the mechanics for which *Acidobacteria* can survive in low-nutrient and harsh conditions can potentially aid in the manipulation of soil microbes to better assist and stimulate the plant thrive in these same conditions.

Actinobacteria

Actinobacteria is a diverse group of bacteria which contains 18 significant lineages (phyla) (Ventura et al., 2007). *Actinobacteria* can interact with other organisms within the soil making them significant in human medicine, agriculture and food production (Maheshwari, 2014). Much like *Proteobacteria, Actinobacteria* followed a similar increasing trend and seemed to prefer more pH neutral conditions (Barka et al, 2016) (Fig. 2), as there was a significant difference in the relative abundance of *Actinobacteria* when comparing bulk soil to rhizosphere and rhizosphere to root systems of both of field and forest sampling locations (Fig. 3 and 4). Filamentous *Actinobacteria* can influence plant development along with protecting the roots system against soil-borne pathogens (Benizri et al., 2001; Maheshwari, 2014). Actinobacteria particularly Streptomycetes while have been known to be associated with bulk soils and rhizosphere and studies have suggested that they can associate with the plant either endophytically or mycorrhizal associated (Scherey et al., 2012; Shrey and Tarkka 2008), These bacteria are known to have the ability to produce secondary metabolites including antibiotic and antifungal used in controlling plant diseases (Shrey & Tarkka., 2008). The significant increase in field root associated *Actinobacteria* could potentially be triggered by soil-borne pathogens (Fig. 5B) as forest sampling locations have the added microbiota of other surrounding plants potentially suppressing the need for additional aid and support from the *Actinobacteria. Actinobacteria* within the field root systems

were nearly doubled in comparison to the forest root system, one possibility for this is forest with multiple plant species plays a role in the relative abundance and need for forest root-associated *Actinobacteria* (Fig. 2). A study conducted by Álvarez-Pérez et al. (2017) on the use of endophytic and rhizosphere *Actinobacteria* in grapevine plants to reduce fugal graft infections indicated that several *Actinobacteria* isolate had beneficial impacts when applied to the grapevine nurseries resulting in a decrease in infection rates by fugal pathogens. Thus, the increase in field root associated *Actinobacteria* could be the plants response to fungal pathogens, however, more research would be required to determine the exact cause in the increase.

5.1.2. Potential Functional Abilities of Soil Bacteria

In our study, specific potential functional were more significantly different in the rhizosphere and root systems when compared to bulk soils. Four functional groups which were found to be significantly different within the bulk soil, rhizosphere, and root systems of field and forest sampling locations were carbohydrate metabolism, energy metabolism, membrane transport, and replication and repair (Fig. 8-10).

There was a large of the of the identified potential bacterial function belong to metabolise carbohydrate. This function was the most abundant potential functional group in all sampling locations. In both field and forest sampling locations, there was a significant decrease in carbohydrate metabolism the closer the proximity to the root system (Fig 8 and 9). This indicates that there was a large proportion of heterotrophic bacteria residing in the field and forest sampling locations. Some species of *Acidobacteria* are known to be heterotrophic which could be

contributing to the high relative abundance of carbohydrate metabolism as they rely on carbohydrates as a source of energy. *Acidobacteria* belonging to group 1 is capable of utilising D-glucose, D-xylose, and lactose as carbon sources (Keilak et al., 2016). *Acidobacteria* is also known to play a role in the degradation of complex plant-derived polysaccharides such as lignin and cellulose (Ward et al., 2009). Carbohydrate metabolism is not solely completed by *Acidobacteria* each bacterial phylum has a diverse range of enzymes that enable them to utilise a wide range of carbohydrates. Carbohydrate metabolism was determined to be significantly lower within field and forest root systems when compared to the rhizosphere this could be due to an increased access to more readily available metabolized complex carbohydrates within the plant system.

Based on the functional capability of *Acidobacteria* and its high relative abundances within rhizosphere and root system, *Acidobacteria* may also be contributing to the high relative abundance of membrane transport (which control the route of small molecules and ions across the biological membranes of cells Fig. 8 and 9). *Acidobacteria* have a large proportion of genes encoded for transporters which was seen in *Acidobacteria* belonging to subgroup 1 (Challacombe et al., 2011; Kielak et al., 2016). The high quantity of transporters systems facilitates the acquisition of a broad range of substrate categories, which include amino acids, peptides, siderophores, cation, and anions (Kielak et al., 2016). *Acidobacteria* have adaptive abilities to deal with oligotrophic (nutrient limiting soil) conditions; this is made possible because of a broad substrate range of transporters for nutrient uptake (Kielak et al., 2016). High relative abundances of membrane transport can also be attributed to the symbiotic exchange of nutrients between the plant and the bacteria as membrane transport was found to be significantly higher within field and

forest root system (Fig. 8B and 9B) (Kielak et al., 2016).

5.2.0 Eukaryotic Communities Within Field and Forest Sampling Locations Within Wild Blueberry Habitats

In this study, we determined that *Ascomycota* was the most abundant eukaryotic order found in this study (Fig. 10), representing 32% of all taxa within the bulk soil, rhizosphere and root systems. The six most abundant eukaryotic orders (Fig. 10) represented 72% of the correlations with the other eukaryotic orders, *Arthropoda, Dinophyceae, Filosa-Sarcomonadea, Prymnesiophyceae,* and *Urochordata* were distinctive in field and forest bulk soils, rhizosphere, and root systems. The remaining order as a collective accounted for the large proportion of the identified orders.

When analysing the PCA plot of the eukaryote populations there was a less visually distinct separation within bulk soils, rhizosphere and root systems, (analysis of similarities $R^2 = 0.368$, $p<0.001$) (Fig. 15). However, there was a significant difference between the samples, and the coefficient of determination was higher than that of the bacterial community. While there were significant differences between all the sampling locations as a collective, Adonis testing was used to determine which of those sampling locations were considered significantly different (Fig. 16). The forest and field root systems were the most similar ($R^2=0.54$) potentially indicating that the change from forest to field affected the root system the least. The root systems of eukaryotic sampling locations were more similar then that of bacterial sampling locations potentially indicating that eukaryotic associated with root systems are less affected by changes in management when compared to bacteria associated with root systems. Forest rhizosphere and root systems

(R^2=0.20) and field rhizosphere and root system (R^2=0.18) sampling locations were considered significantly different.

5.2.1. Ascomycota

While traditionally the classification of *Ascomycota* has been based on the morphology of the sporocarp and ascus (Wang et al., 2006). Recent advancements in technology (molecular studies) have disproved classification based on morphology as it can be phylogenetically misleading (Wang et al., 2006). This is especially important as phylum *Ascomycota* contains approximately 64,000 known species, to date, it is the largest phylum of fungi and contains the most diverse and abundant phyla of eukaryotes (Kirk et al., 2008; Schoch et al., 2009).

While there was a greater relative abundance of *Ascomycota* within the field root system (77%) when compared to corresponding forest locations (51%) the relative abundance was not considered significantly different (Fig. 10). However, when comparing the rhizosphere soils to the root systems, the relative abundances of *Ascomycota* were significantly different in both field (2% and 77% respectively) and forest (26% and 51% respectively) sampling locations (Fig 11). Within the field rhizosphere, there was a significant decrease in *Ascomycota* (Fig 12). It is unclear the exact causes of this drastic decrease in field rhizosphere associated *Ascomycota* when compared to the forest rhizosphere as soil fertility has a substantial effect on eukaryotic microbes which could be the cause of the decrease in *Ascomycota*. *Ascomycota* form symbiotic relations with the roots in which they provide nitrogen and phosphates to the host plant. Tedersoo et al. (2009) found similar results in ECM-associated *Ascomycota* (EAA) species. Tedersoo et al. (2009) noted in his research

that the host preference among EAA, foliar endophytes, ECM symbionts, and arbuscular mycorrhizal symbionts implies plant diversity (through the niche complementarity effect) has the potential to promote the diversity of both commensal and mutualistic fungi above and below ground. They also noted that there is a relatively stronger effect from the plant host compared to the plot, site, and microbiotope effects on EAA species and communities propose that the interspecific alterations with the phytochemicals play a dominant role in constructing the distribution of EAA species when compared with quantitative differences in the soil matrix and geographical distances. They noted that during the phylogenetic analysis most of the EAA species were represented in the root endophytes much like was found in our study. One of the potential reasons for the significant decrease in *Ascomycota* within field rhizosphere sampling locations could be due to the pathogenic strains of *Ascomycetes* do not reside within the rhizosphere but within the plant system (root, shoots, berries, and leaves) which could also explain the high percentage (77%) of *Ascomycota* within the root system. *Monilinia vaccinii-corymbosi* is a disease of high concern within wild blueberry systems. *M. vaccinii-corymbosi* causes blight in leaf and shoots tissues along with mummification of berries also known as mummy berry (Batra 1983). There are ten species of *Monilinia* which attack *Vaccinium* species (Batra, 1983). When diving deeper into the 18S sequencing data to determine if *Helotiales* were present within the field sampling locations, it was determined they were present along with *Monilinia.* However, the relative abundances of *Monilinia* were not considered significantly different within field and forest rhizosphere and root sampling locations. Botrytis blight (*Botrytis cinerea*) also known as grey mould is a pathogenic fungus which causes floral blight, and which can be found in almost all wild blueberry fields (Hildebrand et al., 2001). When looking into the 18S sequencing data at the lowest level, it was determined that *Leotiomycetes* was present within field and forest rhizosphere and

root sampling locations, however, the relative abundances of *Leotiomycetes* were not considered significantly different in field and forest rhizosphere and root sampling locations. Further testing would be required to determine the exact cause of the extreme decrease in *Ascomycota*. Through the collection of additional samples, soil chemical analysis, 18S sequencing, and Fungal ITS sequencing to determine if the cause of the decline. It is unclear if this decline is because of one major abiotic or biotic factor or a cascading effect from multiple abiotic and biotic factors such as the eukaryotes being strongly affected by soil fertility, management practices, or other fungi suppressing the Ascomycota population.

5.3.0. Profiling Nitrogen-Fixing, Phosphate Solubilizing, and Flavin Secreting Microbes Within the Rhizosphere of Forest and Field Wild Lowbush Blueberry Systems

5.3.1. Nitrogen-Fixing Bacteria

Nitrogen is an essential macronutrient nutrient required for synthesis of enzymes, DNA, and RNA. Nitrogen-fixing bacterial were selectively grown on N2-BAP media and samples selected for analysis were chosen based high rate of growth. The 16S sequencing indicated that the bacterial strains were determined to be nitrogen-fixing and phosphate solubilising bacteria from the order *Burkholderiales* (Table 4). *Betaproteobacteria* comprises numerous groups of aerobic or facultative bacteria, which are versatile in their degradation capacities (Gargaud et al., 2011). Many of the species which belong to the class *Betaproteobacteria* play a role in nitrogen fixation, while several species are proficient in utilising ammonium, hydrogen, or methane as an energy source (Gargaud et al., 2011). *Burkholderia* is known to be plant growth promoting *rhizobacteria*

which have been used as biofertilizers or control agents for the improvement of agricultural practices which can fix nitrogen and solubilise phosphate (Sharma et al., 2011). *Burkholderia* is very adaptable organisms that can inhabit an extensive variety of ecological niches (Coenye and Vandamme, 2003; Kim et al., 2006).

The order *Burkholderiales* is known to be phenotypically, metabolically, and ecologically diverse. This order includes aerobic and facultative anaerobic chemoorganotrophs, obligate and facultative chemolithotrophs, nitrogen-fixing, plant and animal (including human) pathogens. Since all of the isolates which were tested for potential nitrogen-fixing abilities were capable of growing in a nitrogen free environment on the specialised media (N2-DAP), it is safe to assume that the potential nitrogen-fixing bacteria which were isolated are indeed nitrogen-fixing. During sequencing processes, a total of 5 samples (Table 4) were identified at the species level as *Burkholderia glathei. Burkholderia glathei* is a soil pathogen which is known to infect *Oryza sativa* (rice plant). While some species of *Burkholderia* are pathogenic, the majority of the *Burkholderia* genus are non-pathogenic soil bacteria (Coenye and Vandamme, 2003). While the majority of the research on this genus had been completed in health care, there is still a great deal which is unknown in respects to adaptability, diversity, and the factors that determine virulence in respects functions of *Burkholderia. Burkholderia spp*., such as *Burkholderia glathei, Burkholderia graminis, Burkholderia phenazinium, Burkholderia caribensis, Burkholderia caledonica, Burkholderia hospita, Burkholderia terricola* and *Burkholderia saccharii*, remains mostly unknown at this time (Coenye and Vandamme, 2003).

5.3.2. Phosphate Solubilizing Bacteria

The identification of phosphate solubilising bacteria was attempted during this research. A total of 14 isolates were found capable of rapidly solubilize phosphate on the PVK media. The identified bacteria (Table 4) all belong to order *Burkholderiales* shared by the nitrogen-fixing bacteria. However, it was determined after isolation that the media used was not ideal for bacteria residing in acidic conditions. PVK media utilises calcium phosphate as its source of phosphate, which is ideal for bacteria residing in alkaline conditions. The preferred media would have been to use one with which is ideal for acidic bacteria, containing iron and aluminium phosphates. Although these isolated bacteria did behave like phosphate solubilising bacteria in alkaline conditions, we cannot assume these bacteria would be able to solubilize phosphates in acidic conditions. The use of aluminium phosphate media would be required to determine if the identified bacteria had the capacity to solubilize phosphate at the pH of the bulk soil.

5.3.3. Flavin Secreting Bacteria

Fluorescence readings were used to determine if any of the bacteria associated with the root system showed potential readings for flavin secretions. Of the total of 118 samples tested three exhibited potential flavin secretion (Table 4). The control used in this testing was *S. meliloti* 1021 which possess the ability to secretes flavins, samples tested (FL45, FL59 FL67) were determined to not be significantly different from that of the control used (Table 3) indicating their ability to potentially secrete flavins.

The microorganisms with high levels of flavin secretion were sent for sequencing (16S). The microbial isolates FL45 and FL59 were identified as *Burkholderia caledonica,* and FL67 was identified as *Agrobacterium* (Table 4). *Agrobacterium* is a member of the order *Rhizobiales. Rhizobiales* had been extensively studied plant associates; they exert their plant-beneficial effects through providing various nutrients, phytohormones, and precursors for essential plant metabolites to their hosts (Ivanova et al., 2000). Within the order *Rhizobiales,* there is a wide variety of microbes with functions including nitrogen-fixing, methanotrophic, legume-nodulating, and micro-symbiotic bacteria (Jourand et al., 2004).

The three potential flavin secreting bacteria (FL45, FL59, and FL67) were then tested on alfalfa seedling for plant growth promoting properties. Samples were tested with the addition of urea and without the addition of urea (Fig. 17B-C). The addition of urea to the potential flavin secreting bacteria aided in the growth and development of the plants (Fig. 17B). Samples grown with urea were visually larger plants, healthier in appearance, and had much greener lusher leaves while the plants without the added urea were much smaller frail shorter plants. Of the bacteria tested in the growth trials, FL67 with added urea was found to have the greatest growth-promoting effects and was significantly different from the other samples (Fig. 17B and Fig. 18). The plants were determined to be significantly taller than the remaining plants within the trial, which could be seen visually as well (Fig. 17C). While sample FL45 with added urea was found to be not significantly different from the control with the added urea and was not significantly different from sample 59 with added urea (Fig 17B and Fig 18).

While currently, it is still unclear as to the exact mechanisms which aided the plants during the plant growth trials. Plants rely on many different mechanisms to deal with biotic and abiotic stressors. A study conducted by Chen et al. (2015) looked at the functions of flavin-containing monooxygenase 1 (FMO1) involved in the control of the root meristem size under DNA stress conditions of *Arabidopsis* plants. FMO1 (flavin containing monooxygenases) expression is increased in response to pathogenic attacks (Kosh et al., 2006; Chen et al., 2015). Chen et al. (2015) suggested that suppressor of gamma response 1 (SOG1) mediated control of the FMO1 may be involved in the immune response to pathogens. While FL67 could potentially play a role in defence against pathogens, another concept is they are a part of micronutrient acquisition. The plants secrete flavins in response to low iron availability. *Beta vulgaris* (sugar beets) is one of many plants which secret flavins from their roots when the plant becomes deficient in iron. Sisó-Terraza et al. (2015) study showed that flavins secreted from root systems were capable of improving the sugar beet iron requirements, allowing the beets to mine iron from iron (III)-oxides through reductive mechanisms. Sisó-Terraza et al. (2015) displayed that flavins were capable of dissolving iron (III)-oxide in the presence of NADH. Endogenous extracellular flavins as an electron transporter is a practical strategy for many bacterial species, allowing long-distance electron transportation between organisms and the acceptance of insoluble electrons (Marsili et al., 2008). Flavins secreted by *Shewanella oneidensi*s a gram-negative proteobacterium can transport 70% of the extracellular electrons (Marsili et al., 2008) with similar results found by Sisó-Terraza et al. (2015) of 80% by sugar beets.

With only preliminary testing completed on FL45, FL59 and FL67, from the data collected thus far we can assume that of the three samples only FL67 had beneficial properties. More testing is

required to determine which chemicals are being secreted by FL67. Since these chemicals had shown beneficial effects on the alfalfa seedlings (plant growth trial), they may serve potential beneficial purposes in the blueberry root system.

Chapter 6: Conclusions

There has been a lack of studies completed on the effects of land-use on the soil microbiome (Mendes et al., 2015). In this study, we examined the implications of the changes from forest to field on the plant root microbiome through examining the bacterial and eukaryotic communities associated with forest with multiple plants species field with single plant species within wild lowbush blueberry. In this study, microbial communities residing within the bulk soil, the rhizosphere, and roots systems across several sampling locations of the field and forest wild blueberry habitats were compared using 16S and 18S microbial profiling.

Based on the findings presented here, we concluded that overall the transition from the forest (unmanaged) to the field (managed) did affect the bacterial communities, however, upon closer inspection we noted that bacterial communities associated with the root systems were least affected by these changes in management. In agreement with previous studies, *Acidobacteria, Proteobacteria, Actinobacteria, Verrucomicrobia, Chloroflexi,* and *Bacteroidetes* were determined to be the most prevalent bacterial phyla. These bacterial phyla accounted for ~90% of the identified phyla. The bacterial phyla were observed in both field and forest sampling locations (bulk, rhizosphere and roots). We noted that most of the bacterial phyla followed a similar increasing trend when transitioning towards the root system, indicating that these bacteria phyla prefer more pH-neutral conditions. However, *Acidobacteria* were the only phyla not to support this transition (increase towards the root system trend) potentially indicating this phylum prefers more acidic nutrient limiting conditions. While the increase in *Actinobacteria* within the roots of field associated blueberry plants is believed to be due to an increase in pathogenic fungi though more research would be required to determine the exact cause for the increase.

Four potential functions were identified within field and forest sampling locations. The four identified potential functions: carbohydrate metabolism, energy metabolism, membrane transport, and replication and repair. In our study, potential functions were more significantly different in the rhizosphere and root systems when compared to bulk soils indicating that there potentially is more activity in the rhizosphere and root regions. A large proportion of the identified potential bacterial functions were from metabolising carbohydrate. There was a large proportion of heterotrophic bacteria residing within the field and forest sampling location as there was a significant decrease in carbohydrate metabolism, the closer the proximity to the root system.

We concluded that, while the eukaryotic communities overall were affected by the transition from the forest (unmanaged) to the field (managed), the root systems much like bacterial sampling locations were also least affected by these changes in the transition from forest to field. The root systems of eukaryotic sampling locations were more similar than that of bacterial sampling locations potentially indicating that eukaryotic associated with root systems are less affected by the transition from forest to fields when compared to bacteria associated with root systems. As expected, *Ascomycota* was the most abundant eukaryote identified within root systems accounting for ~32% of the determined orders, followed by the remaining taxa. While there was a significant decrease in *Ascomycota* within the field rhizosphere, this decrease is potentially due to *Ascomycota* having a more significant association with root system then rhizosphere. The six most abundant eukaryotic orders represented 72% of the identified orders, *Arthropoda, Dinophyceae, Filosa-Sarcomonadea, Prymnesiophyceae*, and *Urochordata* were distinctive in field and forest bulk soils, rhizosphere, and root systems. Eukaryotes followed different trends than that of the bacteria

as the majority of these microbes were not observed with the root systems and prevalent to remain in the bulk and rhizosphere indicating they may have a more neutral association with root systems.

We were able to isolate and identify potential bacterial capable of fixing nitrogen in a nitrogen-free environment. Through the use of 16S sequencing, we were able to identify nitrogen-fixing bacteria, which belong to the order *Burkholderiales*. While we were capable of isolated and identified potential phosphate solubilising bacteria which were identified also belong to order *Burkholderiales*. These bacteria showed the ability to solubilise phosphates in alkaline conditions, additional testing utilising an acidic based (iron or aluminium) media would be required to determine if these bacteria can also solubilise phosphate in acidic conditions. Of the 118 potential flavin secreting bacteria, three initial isolates showed promise. With additional testing, it was determined that one of the isolates was considered significantly different from the other two samples and had the potential to secrete flavins. Sequencing data revealed that two of the isolates were identified as *Burkholderia caledonica* and the other as *Agrobacterium*. These isolates were then used on alfalfa seedling to determine their plant growth abilities. *Agrobacterium* (with the addition of urea), was determined to have the most considerable growth-promoting effects and was considered significantly different from the other two bacteria tested. *Agrobacterium* (with the addition of urea) inoculated plants were visually larger, healthier in appearance and had much larger greener leaves. Agrobacterium is a member of the order *Rhizobiales* which has been extensively studied plant associates, which can exert their beneficial effects through various nutrients, phytohormones, and precursors of essential nutrients.

Future directions regarding our study includes, in the short term a more in-depth study of the causation of the decrease in rhizosphere-associated *Ascomycota* and the increase in root-associated *Actinobacteria* within field sampling locations. The use of soil chemical analysis increase in sample size, 18S sequencing, and fungal ITS sequencing may be used to determine why there is such a significant decrease in *Ascomycota*. While regarding the *Actinobacteria,* the use of Fungal ITS sequencing, additional 16S sequencing, bacterial isolation, and metagenomic analysis may be used to determine if there are pathogenic fungi which could be potentially causing the increase in *Actinobacteria* and the secretion of antimicrobial secondary metabolites. While in the long-term a more in-depth study of the potential flavin secreting microbes would be warranted. Some of the goals could include determining the chemicals, and physical mechanisms which the flavins use to stimulate the plant to promote plant growth. Being capable of identifying the chemicals and physical mechanisms would be the beginning of understanding the more complex larger scale interactions of the soil microbiome.

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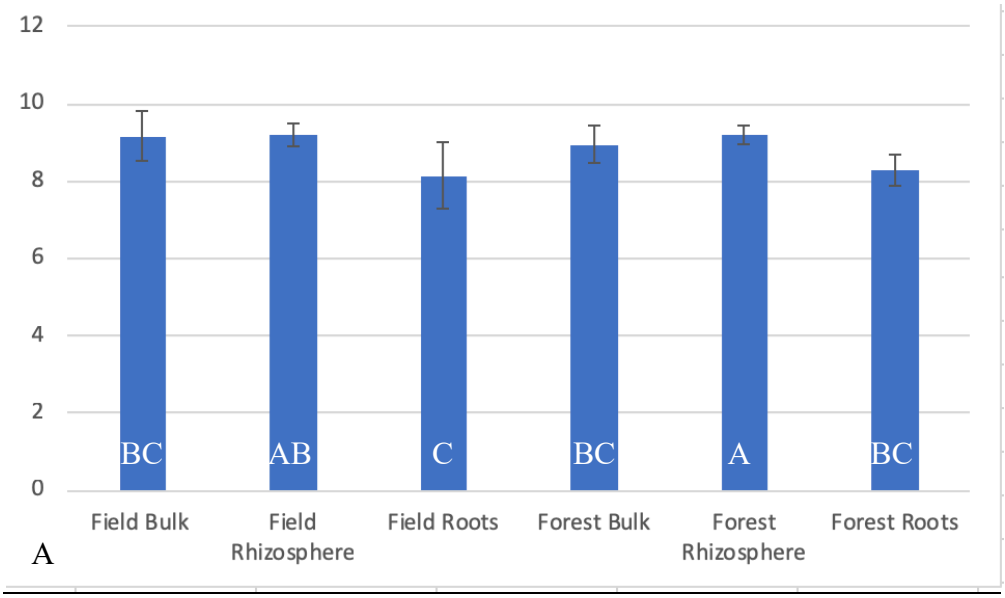
Appendix:

Alpha Diversity

When comparing the evenness of the bacterial and eukaryotic sampling locations the bacterial population had greater evenness (Appendix Fig. 1). Within the eukaryotic populations forest roots were the only sampling location with significantly different evenness. When comparing the diversity of the bacterial and eukaryotic sampling locations the bacterial population had greater diversity (Appendix Fig. 2. Within bacterial and eukaryotic populations field and forest roots were significantly different from the remaining sampling locations. When comparing the richness of the bacterial and eukaryotic sampling locations the bacterial population had nearly 4-fold the richness to that of the eukaryotic populations (Appendix Fig. 3). Within eukaryotic populations field and forest roots were significantly different from the remaining sampling locations.



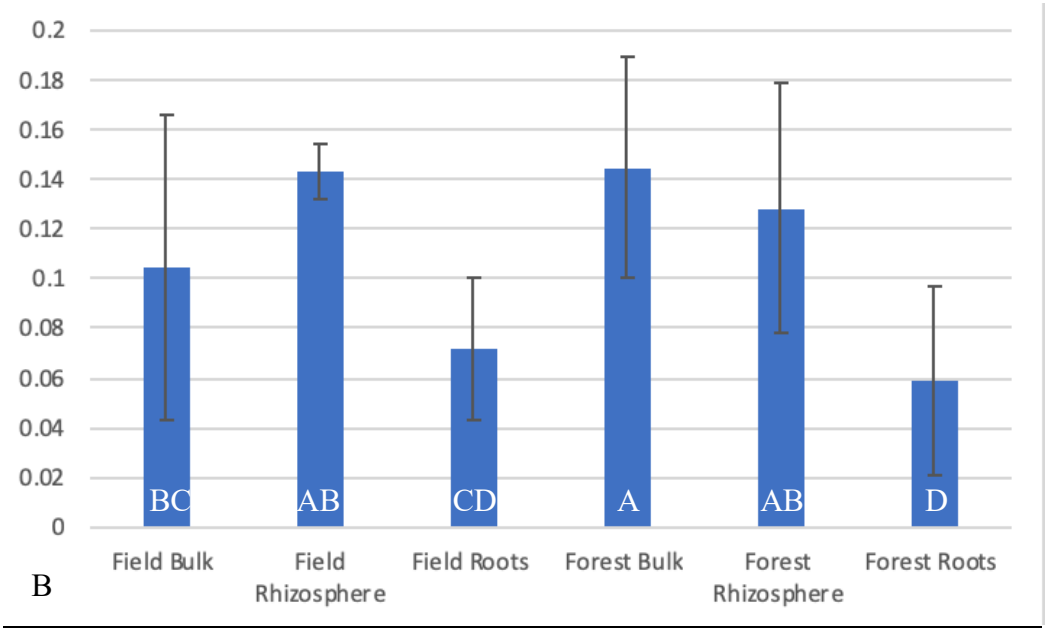


Figure 1: Bacterial and eukaryotic alpha-diversity (Simpson evenness) within bulk, rhizosphere, and root sampling locations. (A) Alpha diversity (Simpsons Evenness) of bacterial sampling locations and (B) Alpha diversity (Simpsons Evenness) of the eukaryotic sampling locations. For each mean, that do not share a letter(s) are significantly different according to Tukey's Pairwise test ($p<0.05$).

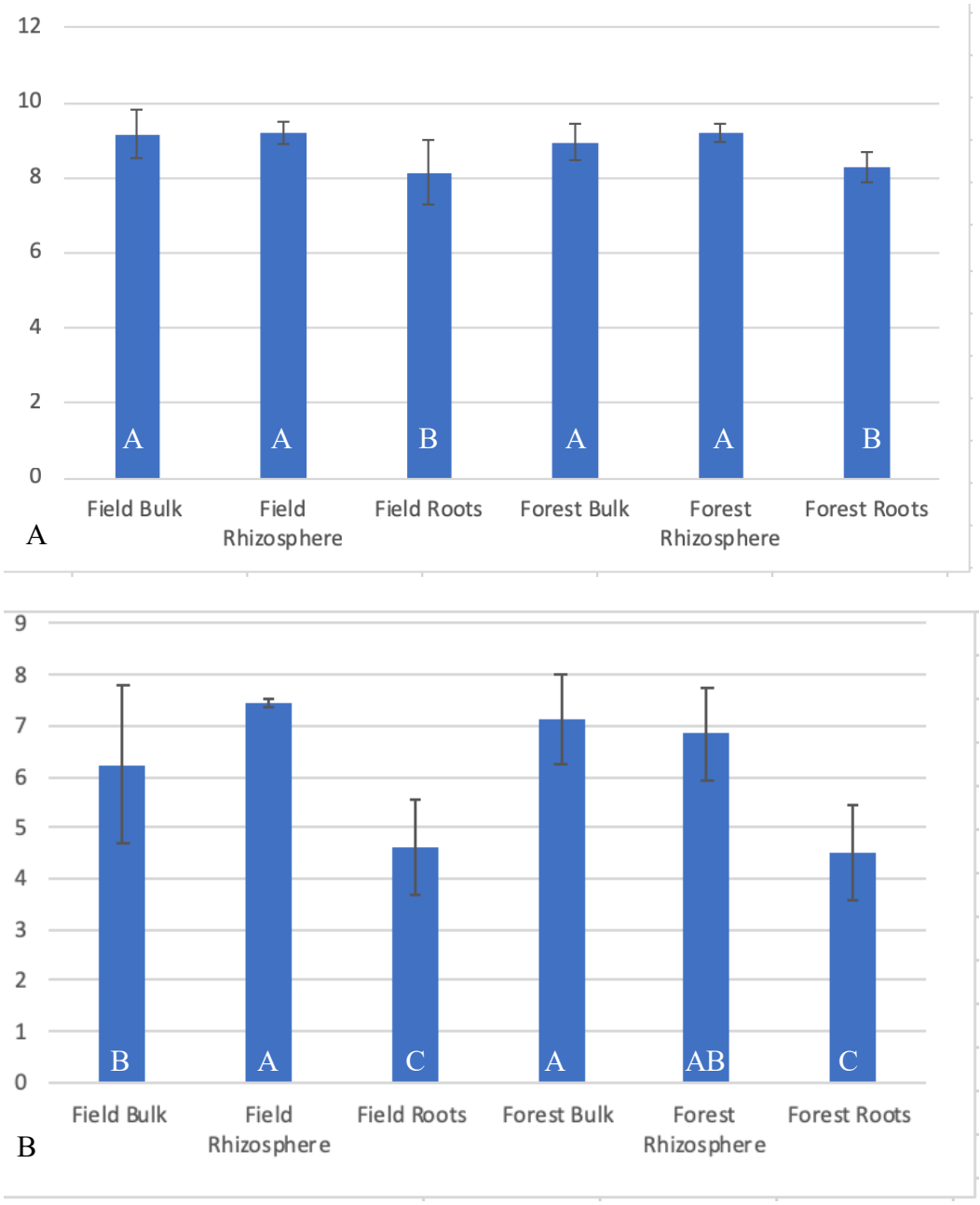


Figure 2: Bacterial and eukaryotic alpha-diversity (Shannon diversity) within bulk, rhizosphere, and root sampling locations. (A) Alpha diversity (Shannon diversity) of bacterial sampling locations and (B) Alpha diversity (Shannon diversity) of the eukaryotic sampling locations. For each mean, that do not share a letter(s) are significantly different according to Tukey's Pairwise test ($p<0.05$).

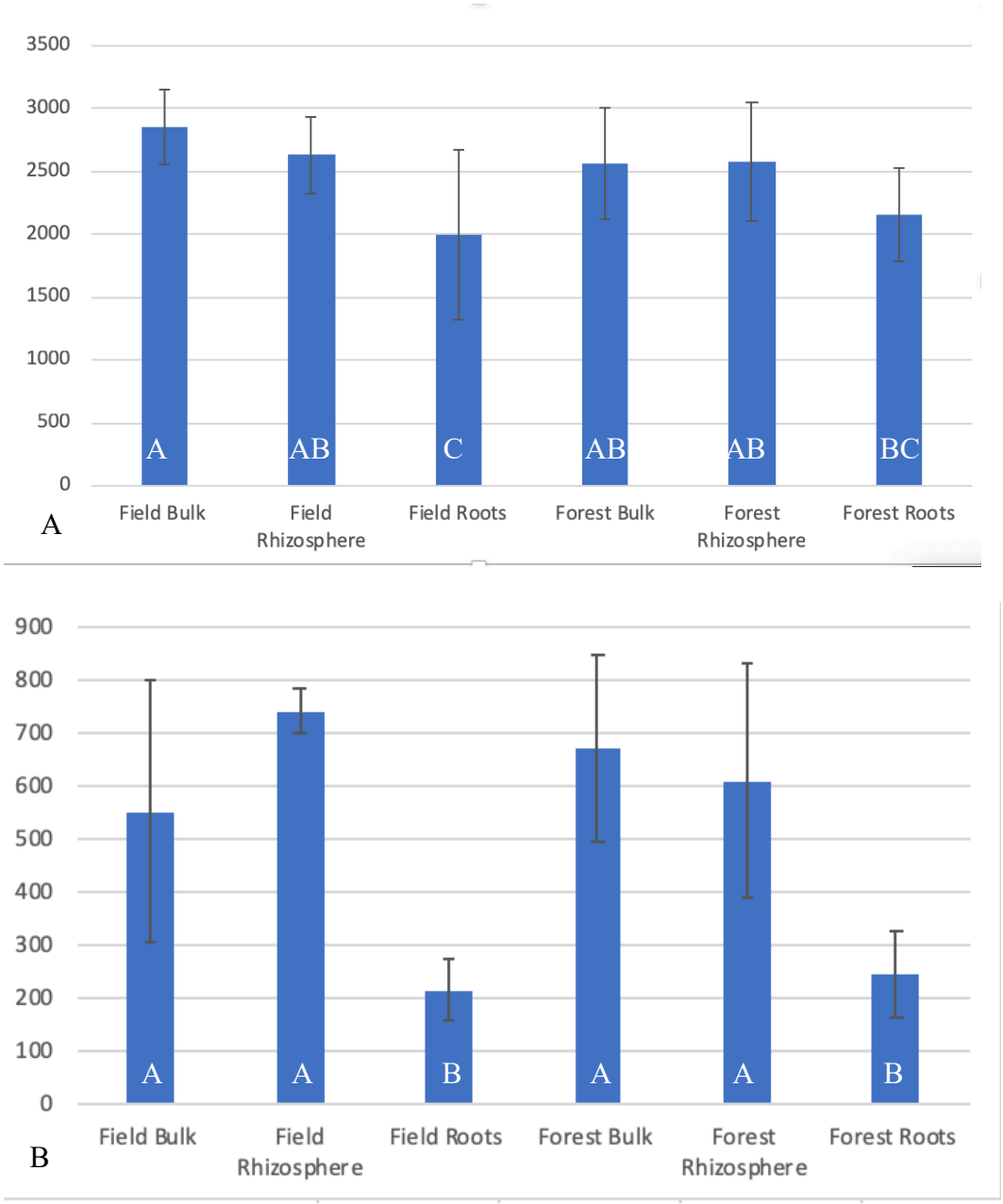


Figure 3: Bacterial and eukaryotic alpha-diversity (Chao1 richness) within bulk, rhizosphere, and root sampling locations. (A) Alpha diversity (Chao1 richness) of bacterial sampling locations and (B) Alpha diversity (Chao1 richness) of the eukaryotic sampling locations. For each mean, that do not share a letter(s) are significantly different according to Tukey's Pairwise test ($p<0.05$).