UNIVERSITÉ DU QUÉBEC À MONTRÉAL

# THE MICROBIOME OF *ACER SACCHARUM*: ENVIRONMENTAL DRIVERS OF MICROBIAL COMMUNITIES IN DIFFERENT PLANT STRUCTURES

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JESSICA WALLACE

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# LE MICROBIOME D'*ACER SACCHARUM*: L'INFLUENCE DES FACTEURS ENVIRONNEMENTAUX SUR LES COMMUNAUTÉS MICROBIENNES DES TISSUS VÉGÉTAUX

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JESSICA WALLACE

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ABSTRACT

Bacteria, Archaea, viruses and fungi can be found living on and within plant structures and tissues. These plant-associated microbial communities have many impacts on the host plant's fitness and function. While these microbes affect their hosts, in turn the plant's attributes and surrounding environment drive the structure and assembly of the microbial communities themselves. However the dynamics and interactions of these pathways and their causes are poorly understood. Using high-throughput DNA sequencing, we compared the structure of bacterial and fungal communities found on different plant surfaces and tissues of the deciduous tree species sugar maple (*Acer saccharum* Marsh). This included the surface of the leaves and roots, as well as their interior tissues. We also compared the microbial communities along environmental gradients of elevation, canopy composition, and soil type. Our results showed distinct microbial communities colonizing each plant surface or tissue. The plant-associated bacterial communities were dominated by the phyla Proteobacteria, Acidobacteria, Actinobacteria and Bacteroidetes while the main fungal phylum present was Ascomycota including the classes Dothideomycetes, Eurotiomycetes, Leotiomycetes, and Sordariomycetes. Also found were very high levels of the fungal genus *Mortierella* from the phylum Zygomycota. We found that all the bacterial and fungal communities of *A. saccharum* differed across elevational range edges. We found most of the bacterial communities to be affected by canopy composition and soil type. Our results imply that while these microbial communities and their diversity are driven by different environmental conditions, the microbial communities of each structure and tissue of the plant are affected separately and to varying degrees. These findings provide a greater understanding of the ecological processes and diversity of plant-associated microbial communities.

Keywords: plant-microbe interactions, sugar maple, forest ecology, high-throughput sequencing, environmental gradients

# RÉSUMÉ

Les bactéries, archées, virus et champignons peuvent être trouvés partout sur et dans les structures et tissus végétaux. Ces communautés microbiennes associées aux plantes ont de nombreux effets sur la croissance et le fonctionnement de leur plante-hôte. Bien que ces microbes influencent leurs hôtes, les traits fonctionnels de la plante et son environnement affectent également la structure et l'assemblage des communautés microbiennes. Les causes, les dynamiques et les interactions entre les communautés microbiennes et ces facteurs sont cependant encore mal comprises. Dans le cadre de notre projet, nous avons utilisé le séquençage d'ADN à haut débit afin de comparer la structure des communautés bactériennes et fongiques trouvées sur différentes surfaces et tissus de l'érable à sucre (*Acer saccharum* Marsh). Nous avons analysé les surfaces et les tissus internes des racines et des feuilles. Nous avons également comparé les communautés microbiennes le long de gradients environnementaux tels que l'altitude, la composition de la canopée, et finalement le type de sol. Nos résultats ont montré que différentes communautés microbiennes colonisent chaque structure ou tissu de la plante. Pour les bactéries, les communautés associées aux plantes ont été dominées par le phylum Proteobacteria, Acidobacteria, Actinobacteria et Bacteroidetes. Pour les champignons, le principal embranchement fongique était Ascomycota, le principal phylum fongique était Ascomycota ainsi que les classes Dothideomycètes, Eurotiomycètes, Leotiomycètes et Sordariomycètes. On trouve également une grande abondance du genre fongique *Mortierella* du phylum Zygomycota. Nous avons constaté que le gradient altitudinal a influencé toutes les communautés bactériennes et fongiques de *A. saccharum*. Nous avons également trouvé que la plupart des communautés bactériennes étaient influencées par le type de canopée et le type de sol. Ainsi, puisque les conditions environnementales affectent la structure et la diversité des communautés microbiennes, nous pouvons supposer que cet effet sera différent selon la location des différentes communautés microbiennes des plantes (surface ou tissu interne des feuilles ou des racines). Ces résultats nous permettent donc d'atteindre une meilleure compréhension des processus écologiques et de la diversité des communautés microbiennes associées aux plantes.



Mots-clés: interactions plantes-microorganismes, érable à sucre, écologie forestière, séquençage à haut débit, gradient environnemental.

# INTRODUCTION

Land plants including trees are hosts to a wide diversity of microorganisms both above and below ground including viruses, bacteria and fungi. Many new studies are focusing on defining the core microbiome of a plant species, defined as microorganisms common across certain habitats (Turnbaugh *et al.* 2007, Shade & Handelsman 2012). The plant-associated microbial communities that make up the plant's microbiome are very distinct from the microbiota found in the surrounding soil (Lundberg *et al.* 2012). The diversity and structure of these communities are influenced by many factors including the species of their hosts (O'Brien & Lindow 1988), the host's age (Di Cello *et al.* 1997), as well as the structure of the plant they colonize (Vujanovic & Brisson 2002). The microbes range from pathogenic to mutualistic, and can greatly alter a plant's health and functional traits (Gourion *et al.* 2006, Friesen *et al.* 2011). In turn the host plant also structures the assembly of the microbial community present on its surfaces and in its tissues (Bais *et al.* 2006). While plant-microbe interactions are ecologically important, they are poorly understood, as are the general diversity and dynamics of the microbial communities of most plant species.

Each structure of the plant such as the roots, leaves or stems, provides a different environment for microbes to colonize. The surfaces of a plant are colonized

externally by epiphytic microbes while interior tissues contain endophytic microorganisms which can be inter- or intracellular. While there have been a few studies looking at both epiphytic and endophytic microbial communities on a single plant species (Bodenhausen *et al.* 2013) most research has focused on a single community on either leaves or roots (Sessitsch *et al.* 2012). The differences and similarities between epiphytic versus endophytic and leaf versus root communities are poorly understood and investigation into the structure of the different communities could provide insight into species assembly and colonization of microbial communities on plant structures.

The microbial communities associated with plants are also highly influential to their host's survival, growth and fitness (Berendsen *et al.* 2012). At the same time the host plant's traits selectively drive microbial assembly in conjunction with environment (Schweitzer *et al.* 2008). Functional traits such as specific leaf area or plant height are an ecologically relevant way of classifying plant species in terms of their ecological functions as opposed to their taxonomy (Díaz & Cabido 2001). Previous studies have found links between plant-associated microbial communities and the host's functional traits such as chemical composition (Bailey *et al.* 2005) or growth and mortality rates (Kembel & Mueller 2014). The factors correlated with plant functional traits such as microbial communities can give insight into ecosystem dynamics (Friesen *et al.* 2011, Cornelissen *et al.* 2003), however very little is known about the effect of host functional traits on microbial communities on plants.

The sugar maple, *Acer saccharum* Marsh. is a deciduous hardwood tree species endemic to Eastern Canada and United States. This species is an ecologically important and economically valuable species in North America (Burns & Honkala 1990) and is considered environmentally sensitive (St. Clair *et al.* 2008) to both biotic and abiotic factors, for example soil nutrient levels (Mader & Thompson 1969) and soil compaction (Houston 1999). Sugar maples have been experiencing health declines in many regions including southern Quebec (Payette *et al.* 1996). One factor potentially influencing sugar maple declines that has not been investigated in depth is the importance of the plant microbiome. Given the importance of the plant microbiome for host health and function, we suggest that studies of sugar maple decline should include an evaluation of the microbial communities inhabiting sugar maple trees as these plant-microbe interactions may give insight into why this tree species expresses environmental sensitivities. Microbial communities on plants also contribute to plant composition, diversity and ecosystem functioning (van der Heijden *et al.* 1998). There have been studies indicating that biotic factors such as fungal pathogens may play a role in plant range edges and migration (Brown & Vellend 2014). The full effect microbes have on these ecological processes is unknown and research into these processes will help us understand the extent of the importance of microbial communities on plants along with what factors drive the structure of the plant microbiome. To date there have been no studies of the microbiome of sugar maple trees using high-throughput sequencing techniques. There

are also very few studies evaluating the microbial communities on this species using other methods such as culture-based techniques or microscopy.

New advances in high-throughput DNA sequencing technology make it possible to study microbial communities at a high resolution not previously available (Bartram *et al.* 2011, Degnan & Ochman 2012). The popularity of high-throughput DNA sequencing techniques has grown recently as the technology has become readily available and more affordable (Degnan & Ochman 2012). DNA sequencing techniques using molecular markers allow us to identify all the microbes present in a given environmental sample (Liu *et al.* 2012) using reference databases. These techniques are culture independent and allow for a greater representation of microbial diversity through their inclusion of taxa that cannot be grown in culture. This technology is advancing at a rapid pace but is still a new method of research and is therefore prone to many inconsistencies and biases (Claesson *et al.* 2010). There are many studies addressing these issues and suggestions to minimize biases. Factors such as DNA extraction methods (Morgan *et al.* 2010), PCR methods (Aird *et al.* 2011), sequencing technology (Kunin *et al.* 2010) and software analysis (Schloss & Westcott 2011) can affect the level of diversity and variation seen in samples.

Changes in microbial community structure can be observed using high-throughput sequencing of environmental microbial DNA. This involves targeted DNA amplification and sequencing of either bacterial or fungal marker genes. These

markers contain highly conserved sites that can be used for primer binding sites for amplification of the region (Liu *et al.* 2012). The 16S ribosomal RNA (rRNA) gene is a highly conserved region that codes for a part of the small subunit of bacterial ribosomes (Clarridge 2004). It has become the standard set of genes used in prokaryote identification and phylogeny studies since being developed in the 1980's (Woese *et al.* 1985). For fungal identification a similar molecular marker, the 28S rRNA gene can be used or the internal transcribed spacer (ITS) of the nuclear ribosomal coding cistron, which is a commonly used barcode for fungi (White *et al.* 1990, Schoch *et al.* 2012). This region is transcribed but spliced away before ribosome assembly and is composed of two highly variable regions ITS1 and ITS2 (Lindahl *et al.* 2013). Although ITS is too variable to allow sequence alignment or phylogenetic inference at higher levels taxonomic levels, ITS sequences are useful for identification of clusters of closely related sequences and for taxonomic identification of sequences, and currently have the best reference databases available for fungal sequencing (Begerow *et al.* 2010) leading to their proposal as the formal barcode for fungi (Schoch *et al.* 2012).

The amplified sequences are barcoded by sample and combined into a single library which is processed by a high-throughput DNA sequencer to produce a computer file containing millions of sequence reads. Through software processing these sequences are then clustered and binned into operational taxonomic units (OTUs). OTUs are a commonly used microbial diversity unit used to classify a species or group based on

DNA sequencing data. They consist of clusters of similar sequences (usually 97% similarity) binned together and assigned to a taxonomic group. Databases containing genomic information for assigning phylogeny based on ribosomal genes and other markers have been created for public use, for example the greengenes 16S rRNA gene database (DeSantis *et al.* 2006).

Many microbial DNA sequencing studies have been done on various plants and tree species since the technology became widely available in the late 90's (Clarridge 2004). These studies have looked at rhizosphere communities (Uroz *et al.* 2010), defined as the zone of soil surrounding the plant roots. Other studies available have been performed on the phyllosphere microbiome (Vorholt 2012, Redford *et al.* 2010), defined as the above ground parts of the plant, and on endophytic microbes of leaves and roots (Lundberg *et al.* 2012, Pedraza *et al.* 2009). The core microbiome is defined as the taxa shared among multiple communities sampled from the same habitat (Shade & Handelsman 2012). These taxa are considered to be potentially ecologically important due to their abundance and high frequency of occurrence. The core microbiomes of many plant structures often contain similar dominant taxa such as Proteobacteria and Acidobacteria in the rhizosphere (Hawkes *et al.* 2007). Despite the presence of a core microbiome of abundant taxa, a great deal of variation in microbiome structure is found inter- and intra-specifically (Redford *et al.* 2010), temporally or seasonally (Shakya *et al.* 2013, Ercolani 1991, Thompson *et al.* 1993) and under different environmental conditions (Kristin & Miranda 2013).

In this study we quantified the microbiome of *A. saccharum* seedlings and saplings in Quebec forests. We took samples from leaves and roots to determine what microbial communities are present on the exterior and the interior of these structures. We investigated changes in the microbiome over time and along environmental gradients. Our goal was to investigate what drives microbial community structure and assembly on this tree species and to compare the differences in microbiome structure between different plant structures and tissues using both bacterial and fungal data. We studied environmental gradients including elevation, soil type, and canopy cover that are known to affect sugar maple seedling performance (Babaei Soustani *et al.* 2014). We also measured host traits such as specific leaf area and leaf dry matter content to quantify correlations between microbial community structure and the tree's physical and functional traits. Previous studies of this kind using high-throughput sequencing have not been performed on sugar maple and the results will give insight into how environmental changes drive microbial community structure.

In Chapter I of this thesis we looked at the microbial communities on the surfaces and tissues of leaves and roots of sugar maple seedlings comparing two environments, the natural range of the species and the elevational range limit. These samples were taken from Mont-Mégantic, Quebec where a sugar maple elevational range limit occurs on the slope. From these samples we expected to see changes in the microbial community structure between the two environments such as lower diversity at the

elevational range limit. We looked at different structures and tissues of the plant individually in order to determine how the environmental change affected the different microbial communities.

In Chapter II of this thesis we investigated the microbial communities on the surfaces and tissues of leaves and roots of sugar maple saplings comparing two environmental factors. We looked at a change in canopy type (deciduous and conifer canopy species), and a change in soil type (natural soil and potting soil). These samples were taken from the Laurentian Biology Station near Montreal, Quebec. In this chapter we expected the environmental changes to drive microbial community variation but were interested in what changes would affect different structures and tissues of the plants. We also wanted to compare if changes in bacterial communities were correlated with variation in fungal communities in plant tissues. In both chapters we investigated these concepts by amplifying and sequencing DNA using both bacterial and fungal biomarkers to quantify the microbial community structure and examine drivers of variation

# CHAPTER I

# THE MICROBIOME OF *ACER SACCHARUM* SEEDLINGS: ELEVATIONAL RANGE LIMIT EFFECTS IN DIFFERENT PLANT STRUCTURES

## 1.1 INTRODUCTION

Microorganisms such as bacteria and fungi inhabit all surfaces and tissues of plants (Andrews & Harris 2000). The microbial communities that inhabit these plant structures have many beneficial effects on the host's functions including protecting against pathogens (Innerebner *et al.* 2011), synthesizing growth hormones (Gourion *et al.* 2006) and providing nutrients (Davison 1988). These plant-associated microbial communities harbour great biodiversity both on the leaves (Lambais *et al.* 2006) and roots (Lundberg *et al.* 2012). The dynamics, interactions and biodiversity of these microbial communities are poorly understood as well as the role and functions of most of the microbial species present. Recent advances in environmental DNA sequencing technologies allow us to investigate and quantify the structure of these bacterial and fungal communities and examine the driving factors behind their ecology and variation. These studies have found that microbial communities are correlated to host species (Redford *et al.* 2010), anthropological modifications of the environment (Sieber 1989) and host genotype (Bulgarelli *et al.* 2012) among other

factors, and distinct communities have been identified occurring between surfaces and tissues of the plant (Edwards *et al.* 2015). However currently there are few studies investigating the microbial communities from both above and below ground surfaces and tissues of a single plant species from different environmental conditions.

Sugar maple (*Acer saccharum* Marshall) is a deciduous tree species native to north-eastern North America where it is an important species both economically and ecologically (Burns & Honkala 1990). The species has a latitudinal range occurring from approximately 35-49 degrees north and is present in low to mid elevations. The species shows a range edge at both its latitudinal and elevational limit. At these limits the fitness of sugar maple trees decline and the composition of the forests transitions to dominance by other species. Elevational changes can create a gradient of variation in temperature, moisture and soil attributes. These changes affect the growth rate and survival of seedlings in many tree species along these gradients (Sa´enz-Romero *et al.* 2006). The upper-elevational range limit of sugar maple is thought to be controlled by climatic factors (Siccama 1974). However these limits have recently been found to have not only changes in abiotic factors, but a change in other biotic factors as well (Brown & Vellend 2014). It is expected that the plant-associated microbial community structure will also be affected by these environmental changes (O'Brien & Lindow 1988), but the variation in the sugar maple microbiome across elevation gradients is unknown.

Changes in global temperature are affecting plant ranges, allowing some species to increase their ranges while others are facing range contraction or high rates of extinction (Morin *et al.* 2008). It is predicted that many species will move upslope as climates warm, and some species distributions are already exhibiting this (Parmesan & Yohe 2003). However range expansion can be affected by many variables either slowing it down (Chen 2011) or preventing it (Zhu *et al.* 2012). The role of biotic interactions as a factor in range expansion has been understudied (Van Der Putten 2010) and recent research has found evidence suggesting that these interactions may be an important factor in sugar maple range expansion to higher elevations (Brown & Vellend 2014).

It is unknown how biotic factors vary across range limits. If biotic factors such as microbiome structure play a role in limiting sugar maple range expansion, they should vary consistently across range limits. By investigating the microbial communities and their variation from these elevational range edges in comparison to the variation found existing within the plant species' normal range, we can gain insight into the biotic limiting factors in sugar maple range expansion as well as how bacterial and fungal communities change under different environments and once the host plant species growing conditions become poor. It is expected that when trees exist in poor environmental conditions this will be reflected in their microbiome, with less biodiversity and beneficial microbes present. Through this study we investigated exactly how these microbial communities are affected at the plants' range limit and

evaluated if the stress of the plants' growing condition was reflected in their microbiomes.

In this study, we investigated the microbial communities found on the deciduous tree species *Acer saccharum* from samples taken along an elevational gradient at Mont-Mégantic, Quebec where a distinct sugar maple elevational range limit occurs between 600 and 1070 meters above sea level (Brown & Vellend 2014). At this site sugar maple is a dominant species of the deciduous forest below this range limit but the forest transitions into spruce (*Picea* spp.) and balsam fir (*Abies balsamea* (L.) Mill.) dominance at higher elevations. It has been predicted that as the climate warms sugar maples will expand their range north (Goldblum & Rigg 2005; Graignic *et al.* 2014) while decreasing abundance in the southern populations (Iverson *et al.* 2008). Due to the ecological importance of these species distribution shifts that are occurring, our study to investigate how biotic factors will covary along range limits and gradients is warranted.

The core microbiome is defined as the taxa shared among multiple communities sampled from the same habitat (Shade & Handelsman 2012). Next-generation sequencing techniques allow us to quantify the microbial communities from multiple samples and investigate the community structure and phylogeny from various environments. Here we used high-throughput DNA sequencing of amplified biomarker DNA regions to compare the bacterial communities of different structures

and tissues of sugar maple seedlings such as the surface of the leaves and roots, as well as their interior tissues. We also investigated the fungal communities of the interior root tissue in fine root samples. We used the hypervariable regions of the bacterial 16S ribosomal RNA gene (Claesson *et al.* 2010) and the fungal internal transcribed spacer (ITS) region (Schoch *et al.* 2012) as barcodes for identification. By examining both the fungal and bacterial communities of the same samples, we investigated if the two communities vary differently with elevation by comparing samples from the elevational range limit to samples from below this limit. This made it possible to determine if and how microbiome structure, a key biotic factor potentially influencing sugar maple range edge dynamics, varied along this gradient.

We compared microbial communities from seedlings found at either the elevational limit of the sugar maple range or from just below the limit. We separated the structures and tissues of the host plants to examine bacterial communities from each part individually. We expected to find significantly different communities inhabiting each part of the host plant and expected the structure or tissue type to have a greater effect on microbial community structure compared to elevation. We expected our leaf-associated microbial communities to show higher biodiversity and variability in the endophytic communities compared to epiphytic as this has been found in previous studies (Bodenhausen *et al.* 2013). We also expected higher diversity in the below ground structures compared to above ground structures due to the high abundance of microbes present on plant root surfaces (Berendsen *et al.* 2012). By investigating

each structure and tissue separately, we can see if variation in the bacterial communities caused by range edge effects is equally occurring in the communities of the leaves, roots and tissues. Sugar maple has not been previously studied with regards to its microbial communities using high-throughput DNA sequencing or comparing different structures and tissues and the information will give insight into the plant-microbe interactions and the driving factors of microbial community structure on sugar maple seedlings.

In this study our objectives were (1) to investigate and identify the microbiome of sugar maples by comparing microbial communities from the surfaces of the leaves, roots and from the interior tissues of the plant, (2) to investigate if community structure, variability, biodiversity, or assembly differ by plant structure or tissue. For example leaf-associated communities compared to root-associated communities and interior plant tissues compared to exterior plant surfaces. Also (3) does elevational range limit effect drive microbial community structure and is this effect occurring in every structure of the plant; and finally to investigate (4) are functional traits of sugar maple seedlings correlated with their microbial communities in sugar maples natural range?

## 1.2 MATERIALS AND METHODS

### 1.2.1 Specimen collection

*Acer saccharum* seedlings were collected in July 2013 from the eastern slope of Parc national du Mont-Mégantic, Quebec, Canada (45°26'51"N, 71°06'52"W). Ten whole seedlings from 4 plots were collected for a total of 40 seedlings (Table 1.S1). All seedlings were under 10 cm in height and between the ages of 2 to 7 years. The samples were taken from 2 zones on the slope between 720 and 830 meters above sea level (m a.s.l) with 2 replicates each. The first 2 plots occurred in the upper zone (Edge) between 790 and 830 m a.s.l where the sugar maple range limit occurs and the forest transitions into balsam fir stands. The second zone (Within) also contained 2 plots occurring between 720 -750 m a.s.l and located just below the range limit where sugar maple trees dominate the stands (Figure 1.S1). All samples were collected using gloves and placed immediately in sterile roll bags. Samples were transported on ice within the day and frozen at -80° C until processing.

### 1.2.2 Functional traits and biometrics

When necessary slight modifications were made but otherwise functional traits were measured according to protocols provided in Cornelissen *et al.* 2003. The age of the seedlings was found by counting the number of nodes present on the stem. The seedlings collected at Mont-Mégantic all had 2 leaves present. For these plants all leaves were included in the analysis and the total leaf area for each seedling was measured using WinFolia software to analyze scanned images of the leaves. The

leaves were weighed without drying and the weight and area were used to calculate a modified specific leaf area (SLA). It was not possible to obtain correct measurements for SLA using dried leaves as the drying process would have disturbed the microbial communities and affected the sequencing results so only SLA using fresh weight was measured. Stem length from the top of the roots to the top of the meristem was measured along with the diameter of the stem at the top of the roots. Stem weight was taken once stems were air dried for 96 hours at room temperature. Water displacement techniques were used to determine the volume of the stem by placing the whole stem suspended in water and recording the weight of water change. Stem volume along with the stem dry weight was used to calculate specific stem density (SSD).

### 1.2.3 Sample preparation and DNA extraction

We collected four sample types from different plant structure surfaces or tissues. These were the rhizosphere, defined as the surface of the roots and the soil within 1 mm from the roots (Clark 1949) and the phyllosphere defined as the above-ground surface of the plant (Ruinin 1965) for which we used leaf surfaces. The other two sample types were tissue samples from the interior of the roots and leaves. Leaf and root samples were processed separately through a series of steps in order to first remove all epiphytic bacteria and fungi. After the surface of the tissues were sterilized and washed to remove all remaining microbial cells. The tissues were then

finely sectioned and agitated in a bead beating tube to release as many endophytic microbes from the tissues as possible.

The epiphytic microbial communities were removed with a 5 minute agitation wash in 30 mL of 1:50 diluted solution of buffer [1M Tris-HCl, 0.5 M Na EDTA, and 1.2% CTAB] (Kadivar & Stapleton 2003). The plant tissues were then removed from the buffer solution and the samples were centrifuged at 4000 rpm for 20 minutes at 4° C to form a pellet. The supernatant was removed with a sterile pipette and the pellet was transferred to a bead beating tube from the MoBio PowerSoil DNA Isolation kit (Carlsbad, CA). The protocol was followed with the exception that the samples were vortexed for 15 minutes instead of 10.

The tissue samples from the first wash were place in 30 mL of ethanol and vortexed for 5 minutes. The ethanol was removed and the samples were then washed with DNA free water for 3 minutes. The water wash was repeated 3 times. The tissues were then sectioned finely using sterile techniques. The resulting samples were then transferred to a bead beating tube from the MoBio PowerSoil DNA Isolation kit (Carlsbad, CA). The protocol was followed with the exception that the samples were vortexed for 45 minutes instead of 10. The isolated DNA samples were frozen at -80° C until further processing.

1.2.4 PCR and multiplexing for 16S rRNA gene sequencing

The samples were amplified and barcoded using a two-step PCR process to prepare them for Illumina sequencing, this molecular protocol was taken from Kembel *et al.* (2014). The first PCR step used primers which target the V5-V6 region of the bacterial 16S rRNA gene [799F and 1115R (Redford *et al.* 2010)]. The primers exclude cyanobacteria in order to exclude plant chloroplast DNA. These primers are modified with a 5' tail which adds a 6-bp barcode and partial Illumina adaptor sequence to the 16S fragments during PCR (modified 799F: 5'-CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTxxxxxxAACMGGATTAGATACCCKG; modified 1115R: 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTxxxxxxAGGGTTGCGCTCGTTG, where "x" represents barcode nucleotides).

Twenty-five μL PCR reactions were run containing 5 μL 5xHF buffer (Thermo Scientific), 0.5 μL dNTPs (10 μM), 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), 0.25 μL Phusion Hot Start II polymerase (Thermo Scientific), 4 μL of genomic DNA, and 14.25 μL molecular-grade water. The reaction was performed using: 30 s initial denaturation at 98°C, 20 cycles of 10 s at 98°C, 30 s at 64°C, and 30 s at 72°C, with a final 10 minute elongation at 72°C. This was performed in triplicate for each sample and the products were pooled and cleaned using the Bio Basic EZ-10 Spin Column kit (Markham, ON) and resuspended in 40 μL of solution elution buffer.

The second stage of the PCR amplification was performed using this first stage PCR product as a template. The primers used were custom HPLC-cleaned primers to further amplify 16S products and complete the Illumina sequencing construct (PCRII_for: 5′-AAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGC ;PCRII_rev: 5′-ATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG).

Single reactions were run for each sample with the same reagents and conditions as the first PCR step with the exception that the cycle amount was changed to 15 instead of 20. A ~445-bp fragment was isolated by electrophoresis in a 2% agarose gel and DNA was recovered with the Bio Basic EZ-10 Spin Column kit. A multiplexed 16S library was prepared by adding equimolar concentrations of DNA from each sample. The resulting DNA library was sequenced on an Illumina MiSeq 250-bp paired –end sequencing platform at the University of Montreal, Quebec.

1.2.5 PCR and multiplexing for ITS fungal sequencing

We used sequencing of the fungal ITS (Schoch *et al.* 2012) region on environmental DNA samples from the root tissues of the samples to investigate endophytic fungal communities present in the fine roots of the sugar maple seedlings. The ITS1 primer (Gardes & Bruns, 1993) was chosen as it discriminates against plants (Lindahl *et al.*

2013). ITS2 (White *et al.* 1990) was chosen as it shares properties with ITS1 and can obtain similar results (Mello *et al.* 2011, Bazzicalupo *et al.* 2013).

The DNA samples were amplified for fungal sequencing using a one-step PCR step and normalization with primers designed to attach a 12 base pair barcode and Illumina adaptor sequence to the fragments during PCR (Fadrosh *et al.* 2014). The primers amplified the regions ITS1 and ITS2 of the internal transcribed spacer of the nuclear ribosomal coding cistron (Schoch *et al.* 2012). (ITS1 Forward: 5' - CAAGCAGAAGACGGCATACGAGATGTGACTGGAGTTCAGACGTGTGCTCT TCCGATCT xxxxxxxxxxxx CTTGGTCATTTAGAGGAAGTAA ITS2 Reverse: 5' - AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCT TCCGATCT xxxxxxxxxxxx GCTGCGTTCTTCATCGATGC – 3'). Where x represents barcode nucleotides.

One 25 μL PCR reaction was run for each sample. This reaction contained 5 μL 5xHF buffer (Thermo Scientific), 0.5 μL dNTPs (10 μM), 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), 0.75 μL DMSO, 0.25 μL Phusion Hot Start II polymerase (Thermo Scientific), 1 μL DNA, and 16.5 μL molecular-grade water. The reaction was performed using: 30 s initial denaturation at 98°C, 35 cycles of 15 s at 98°C, 30 s at 60°C, and 30 s at 72°C, with a final 10 minute elongation at 72°C. The samples were processed with an Invitrogen Sequalprep PCR Cleanup and Normalization Kit (Frederick, MD) to give all samples a finished concentration of

~0.55 ng/µl. The samples were pooled with equal amounts and sequenced on an Illumina MiSeq platform at the University of Montreal, Quebec.

1.2.6 DNA sequencing processing and data analysis

Data returned from the sequencing centre was processed using the fastx_toolkit, PEAR (Zhang *et al.* 2014) and QIIME version 1.8.0 (Caporaso 2010) software to trim and combine paired-end sequences to single sequences of approximately 300 - 350 bp in length (PEAR; default settings). Sequences with a quality score of less than 30 or with a quality window score of less than 5 were removed. The reads were de-multiplexed into samples using barcode sequences (QIIME; default settings). This involved combining the forward and reverse barcodes from each combined read into a 12-bp barcode for 16S samples or 24-bp barcode for ITS samples which could then be matched to a sample ID (Hamady 2008).

Sequences were chimera checked and all chimeras were removed using the Uclust and Usearch 6.1 algorithms (Edgar 2010). Sequences were then binned into operational taxonomic units (OTUs) at a 97% similarity cut-off rate using Uclust (Edgar 2010). The OTUs were assigned taxonomy using the Ribosomal Database Project (RDP) classifier (Wang *et al.* 2007) as implemented in QIIME, with a minimum support threshold of 80% for bacterial OTUs and 50% for fungal OTUs. Rare OTUs occurring below a count of 20 were removed. For 16S bacterial samples

each sample was rarefied to 4500 sequences. This resulted in a total of 116 usable samples from 37 seedlings (Table 1.S2) with ~6,800,000 bacterial sequences and a total OTU count of 3785. For ITS fungal samples each sample was rarefied to 10,000 sequences. This resulted in a total of 28 samples from 28 seedlings (Table 1.S2) with ~ 780,000 fungal sequences and a total OTU count of 2044. Missing samples were due to low sequence read amounts either as a result of extraction, PCR or sequencing errors. All subsequent analyses were performed on both the bacterial and fungal sequencing data.

### 1.2.7 Indicator species analysis

We tested for the significant association of indicator taxonomic groups present using the LDA Effect Size platform (LEfSe) (Segata *et al.* 2011). The LEfSe algorithm searches for biomarkers specific to sample groups, such as genes, pathways, or taxa using the Linear Discriminant Analysis (LDA) to approximate the effect size of each biomarker identified (Segata *et al.* 2011). This allows us to compare the structures and tissues or environments in order to identify any significant host-microbe relationships and their strength between the different environments. We compared the bacterial communities of each surface or tissue type of the plant separately with an LDA cut-off of 2. We compared the Edge and Within samples at the phylum level using all four plant parts to investigate if certain taxa were present on the seedlings from the elevational range limit or from below. We also compared the bacterial

communities of the structures and tissues at the phylum and class levels to find if certain taxa were associated with roots or leaves using combined surface and tissue samples. Finally we combined the two tissue samples in comparison to the two surface samples to test for taxa association with endophytic or epiphytic communities.

### 1.2.8 Statistical analysis

PCR and high-throughput sequencing techniques are known to cause errors and are subject to reagent contamination (Salter *et al.* 2014). Therefore we eliminated OTUs from our dataset which were represented by less than 20 sequences as this is a commonly used cut off for rare OTUs (Zhan 2014). Data analysis and plotting was performed using ape (Paradis *et al.* 2004), ggplot2 (Wickham 2009), picante (Kembel *et al.* 2010), and vegan (Oksanen *et al.* 2007) statistical packages for R (R Development Core Team; http://www.R-project.org).

We used the Bray-Curtis dissimilarity index to measure variation in the bacterial community structure between the structures and tissues and between the elevational range limit and below. We also used the UniFrac index to measure phylogenetic distance between sets of taxa using branch length (Lozupone *et al.* 2006). We investigated phylogenetic variation in the bacterial community structure among the different structures and tissues. We measured variation in the bacterial communities

using both the weighted and unweighted methods. The weighted method takes an abundance-weighted measure of the phylogenetic diversity among the microbial communities while the unweighted method measures variation independent of abundance.

For the fungal results we used the Bray-Curtis dissimilarity values to investigate variation between the root endophyte samples from the elevational range limit and from below. Analyses using the UniFrac index were not performed on the fungal community data due to the fact that ITS sequence data is too variable to assign phylogeny at higher levels.

Using nonmetric multidimensional scaling (NMDS) ordinations of the Bray – Curtis dissimilarity distances for the bacterial and fungal data and the UniFrac distances for the bacterial data we visualized dissimilarity and phylogenetic similarity between the structures and tissues and from the elevational range limit and below. Using the community matrix data of OTU counts, we performed permutational multivariate analysis of variance tests (PERMANOVA, Anderson 2001) to identify relationships between the microbial communities, elevation (Edge/Within) and structure or tissue. To test for associations between host functional traits and microbial communities we fit correlations between the host's traits and the microbial community ordination using the envfit function in R. Finally alpha bacterial and fungal diversity for each structure and tissue from both elevations were measured using the Shannon index for

each community. An ANOVA and subsequent post-hoc Tukey's tests were performed to measure the difference in diversity from the sites and between the different structures and tissues.

## 1.3 RESULTS

### 1.3.1 Taxonomic composition of bacterial communities

With OTUs below a count of 20 removed we identified a total of 3785 bacterial OTUs (sequences binned at a 97% similarity cutoff) from the 116 samples. Our collector's curve of the number of OTUs per sample appeared to reach a plateau, revealing that we sampled the majority of the diversity in the maple microbiome (Figure 1.S2a). From each structure or tissue and in the overall dataset, the OTU counts were consistently higher in the Within samples compared to the Edge samples. Highest OTU counts were found in the rhizosphere and lowest were found in the interior tissue of the leaves. An average of 446 ± 17 OTUs (mean ± SE) per sample with averages from each structure or tissue separately of 645 ± 16 OTUs per rhizosphere sample, 393 ± 26 OTUs per phyllosphere sample, 438 ± 17 OTUs per root tissue sample, and 206 ± 9 OTUs per leaf tissue sample.

From our data we detected a core microbiome of each structure of the plant as well as for all structures and tissues together by looking at all bacterial OTUs from the

Within samples. The microbial communities showed similar taxonomic groups but with highly varying levels between the structures and tissues (Table 1.1). Edge samples of all types were excluded from this analysis since the seedlings were outside the species normal range and our objective was to characterize the core microbiome of sugar maple in its natural range.

The microbiome of sugar maple including all structures and tissues was composed of four main phyla and 11 major classes. Four of these classes were from Proteobacteria (59.4%): Alpha- (23.1%), Beta- (23.0%), Delta- (2.9%) and Gammaproteobacteria (10.2%). Three of the class were from Acidobacteria (10.6%): DA052 (3.7%), Acidobacteriia (3.6%), Solibacteres (3.0%). Three from Bacteroidetes (15.4%): Cytophagia (9%), Saprospirae (3.2%), Sphingobacteriia (2.7%). Finally, the phylum and class Actinobacteria (7.8%): Actinobacteria (6.4%) were also abundant (Table 1.1) (Figure 1.1a).

### 1.3.2 Indicator species analysis of bacterial taxa

We performed tests on the bacterial communities for biomarker taxa using the LEfSe platform. First we compared epiphytic or endophytic communities in the abundant phyla and classes and found several associations including 2 major phyla associated with epiphytic communities: Acidobacteria and AD3 (Table 1.1) (Figure 1.2a). We compared leaf-associated bacterial communities to the root-associated communities

and found that most of the abundant phyla and classes were associated with either leaves or roots (Table 1.1) (Figure 1.2b). We also found several non-dominant bacterial phyla and classes to have significant associations with either epiphytic or endophytic communities as well as to leaf or root communities (Table 1.S3).

We also used LEfSe to investigate if specific bacterial phyla were associated with the elevational range limit or below (Edge or Within). We analysed each of the four parts of the plant separately from each elevational range. We found that there were many associations with the most occurring in the bacterial communities of the rhizosphere and root endophytes from below the elevational range limit (Table 1.2).

### 1.3.3 Differences in bacterial community structure among structures and tissues

Tests using the analysis of variances on the Bray-Curtis distances were used to investigate variation between bacterial community structures in the different structures and tissues and between samples from either the Edge or Within sites. The plots from the same sites but different replicates were not found to be significantly different ($p=0.355$) therefore these samples were grouped together into either Edge or Within groups for further analysis. PERMANOVA tests showed that each of the four parts of the plant had a distinct bacterial community structure ($p=0.001$) (Figure 1.3). Distinct bacterial communities were also found between seedlings from the

elevational range limit (Edge) and from below the limit (Within) ($p=0.006$) in each of the four bacterial communities (Figure 1.4a) (Table 1.3).

### 1.3.4 Differences in bacterial community phylogenetic structure

Distinct communities were also found between the sites in the root-associated bacterial communities using PERMANOVA tests on both the weighted and unweighted UniFrac values (Table 1.3). Both of the root-associated bacterial communities showed significant variation between the two sites using both UniFrac methods. The leaf-associated bacterial communities did not show a significant difference between the sites using UniFrac with the exception of the unweighted method in the leaf endophyte samples ($p=0.004$). Distinct bacterial communities were also found using the unweighted method in the combined dataset of all samples, but were not found with the weighted method.

### 1.3.5 Taxonomic composition of fungal communities

With OTUs below a count of 20 removed, the ITS sequencing results gave 2044 OTUs from the 28 samples of root tissue with an average of 258 ± 3 OTUs (mean ± SE) per sample (Figure 1.S2b). From these 28 seedlings, 18 were from the Edge group and showed 952 OTUs, the other 10 seedlings were from the Within group and

gave 818 OTUs. Therefore the fungal samples did not show a lower OTU count at the elevational range limit compared to below unlike the bacterial communities.

Taxonomic analysis of the Within samples showed that the most abundant phyla were Ascomycota (40.1%), Basidiomycota (12.4%), and Zygomycota (46.4%) (Figure 1.1b). The most abundant Ascomycota classes included Dothideomycetes (7.7%), Eurotiomycetes (2.6%), Leotiomycetes (7.5%), and Sordariomycetes (10.2%). Another abundant class was Agaricomycetes (11.5%) from Basidiomycota (Table 1.4). Similar to the bacterial results the plots from the same elevational ranges were not found to be significantly different using an ANOVA test on Bray-Curtis values ($p=0.7$) and were grouped together into either Edge or Within classification for further analysis. The fungal communities from the fine root tissue of the sugar maple seedlings showed significant variation between the Edge and Within samples ($p=0.001$) (Table 1.3) (Figure 1.4b).

### 1.3.6 Shannon diversity of bacterial communities

Shannon diversity values were compared using ANOVA tests with a linear model and subsequent post-hoc test of Tukey multiple comparisons to test for differences in alpha diversity between each structure and tissue and between elevations. While overall there was no significant difference between the sites for the leaf-associated bacterial communities or the fungal communities of the root tissue, there was a

significant difference between the Edge and Within samples in the rhizosphere ($p=0.01623$) and root endophyte ($p<0.001$) bacterial communities (Table 1.5) (Figure 1.5).

Shannon diversity was calculated by structure or tissue and was found to be highest in the rhizosphere and lowest in the interior of the leaves. Higher diversity was shown by the two root-associated bacterial communities compared to the two leaf-associated and by surface samples compared to tissue samples (Figure 1.5). The diversity between structures and tissues was found to be significantly different ($p<0.05$) between each pair but was not significant between the two sites (Edge, Within) in any of the bacterial communities except the endophytes of the roots ($p<0.05$) following a post-hoc test of Tukey multiple comparisons of means at a 95% family-wise confidence interval. In the fungal communities the root endophytes did not show a significant difference in the Within samples compared to the Edge samples ($p=0.1$).

### 1.3.7 Correlations of microbial communities with functional traits of host plant

Host functional trait data included leaf area, fresh leaf weight, specific leaf area (SLA) modified using fresh weight, stem weight, stem length, stem diameter, and specific stem diameter (SSD). Correlation tests were performed using the envfit function in R on the NMDS ordinations. Functional traits were measured in the Within samples only. Host traits that were correlated to bacterial community structure

included SSD in the phyllosphere samples along with stem weight and leaf area in the combined leaf-associated samples. In the combined dataset of all bacterial communities, correlations were found with stem weight, fresh leaf weight and leaf area. The fungal community samples of the root tissues were correlated with leaf weight only (Table 1.6).

## 1.4 DISCUSSION

### 1.4.1 Taxonomic distribution of bacterial and fungal communities

This research defines a core microbiome as all microorganisms commonly present across a habitat (Turnbaugh *et al.* 2007). Our study quantified and characterized the microbiome of sugar maple seedlings using four parts of the plant. We used samples from below the elevational range limit (Within) for our taxonomic analysis to investigate the microbiome of sugar maples in their natural range. We found our taxonomic results to be consistent with previous studies in other plant species (Kembel *et al.* 2014, Shakya *et al.* 2013, Davey *et al.* 2012). The bacterial communities were dominated by the soil or plant-associated phyla Proteobacteria, Actinobacteria, Acidobacteria and Bacteroidetes. We found many differences between the parts of the plant and the two environments including differences in relative abundances, taxa present and OTU counts.

The fungal communities were dominated by high levels of the phylum Ascomycota (40.1%) including high abundances of the genera *Capnobotryella* (5.8%) and *Lecythophora* (7.7%). Little is known about the genus *Capnobotryella* and the effect it may have on plant species, while the genus *Lecythophora* is a soil-associated fungal genus which contains 3 species and is known to be pathogenic to woody hosts (Damm *et al.* 2010). We also found high levels (46.4%) of the fungal phylum Zygomycota, where all of these sequences were identified as belonging to the genus *Mortierella*. This was the highest level of a specific genus identified in either the bacterial of fungal communities. *Mortierella* is a common saprotrophic soil-associated fungal genus which contains 85 species. Many of these species are known to produce polyunsaturated fatty acids (Shimizu & Jareonkitmongkol, 1995) which have the ability to protect plants from phytopathogens (Eroshin & Dedyukhina, 2002). Therefore it is possible that a high presence of this genus is a beneficial advantage for the host plants. In comparison to the elevational range limit samples to the natural range, there was no significant difference between the relative abundances of *Mortierella*.

### 1.4.2 Microbial associations with different plant structures and tissues

By testing for biomarker bacterial taxa using the LEfSe platform with a LDA cutoff of 2, we investigated which taxa at the phyla level were associated with each elevation. We also compared leaf-associated samples to root-associated and epiphytic

to endophytic communities at the phyla and class levels. We found that most of the abundant bacterial phyla and classes were significantly associated with either leaf or root samples with some phyla and classes associating with endophytic or epiphytic communities (Table 1.1). We found an association of the phylum Proteobacteria and the class Alphaproteobacteria to be associated with leaf bacterial communities. This is not surprising; previous research has found high amounts of this class on leaf surfaces (Kembel *et al.* 2014). This class is known to be able to acquire alternative energy sources by using phototrophy, nitrogen fixation, methylotrophy or methanotrophy (Brenner *et al.* 2005). This makes them adapted to living on leaf surfaces where carbon sources are in low availability (Lindow & Brandl 2003). We have provided a list of all bacterial phyla and classes found associated with either epiphytic communities compared to endophytic communities or between leaf or root associated communities (Table 1.S3).

We also found several associations of bacterial phyla between the two elevations. This shows that there is a significant difference in the presence of these phyla in the two environments, which may have an impact on the fitness and function of the host plant. A higher amount of phyla were associated with the natural range compared to the elevational range edge. The two root-associated communities showed higher amounts of indicator phyla compared to the leaf-associated communities (Table 1.2). This implies that the below ground communities are affected greater by this

environmental change than the above ground communities which is consistent with our other findings.

1.4.3 Analysis of variance between structures, tissues and environments

Our results showed that each surface and tissue of the host plants was colonized by distinct bacterial communities ($p=0.001$) (Figure 1.3). This was not surprising as each of these parts of the plant provides a unique environment with its own selective pressures and driving factors on microbial communities such as moisture levels and nutrient availability (Badri *et al.* 2009). Therefore microbes adapted to living on leaf surfaces may not be adapted to colonizing root surfaces.

The bacterial communities found on the rhizosphere of the plants had higher similarity to the communities found in the interior of the roots compared to the other structures. Also the interior of the leaves had higher similarity to the phyllosphere communities than the root structures (Figure 1.3). This implies there is a higher level of migration between endophytic and epiphytic bacterial communities than between the above and below ground structures of the host plant. This is not surprising as microbes from below ground would be limited in their ability to migrate upwards. Also while microbes from leaves and canopies are easily transferred downwards through rainfall, the high abundance of microbes living in the root-associated communities would most likely negate the impact of influx from the leaf-associated

microbes. Overall, this suggests a model of community assembly where microbes are progressively filtered as they colonize either the leaf surfaces followed by leaf tissues, or rhizosphere followed by root tissues, with decreases in diversity moving from the exterior to interior of the plant.

We found a high degree of variability in the phyllosphere samples in comparison to the other bacterial communities (Figure 1.3). This variability was present in bacterial community structure as well as alpha diversity (Figure 1.5). This may be explained by the fact that the leaf surfaces or seedlings are unprotected by a surrounding canopy and subjected to a high amount of migration from their surrounding environment through wind and rainfall.

We found a change in the bacterial communities at the plants' elevational range limit in every structure and tissue as well as in the combined dataset ($p<0.05$). We also found a change in the fungal communities of the root endophytes ($p=0.001$) (Table 1.3). This shows that microbial communities are affected by elevational range edges which may contribute to environmental stress on the plants. We found greater differences between the environments in the two-root associated bacterial communities in comparison to the leaf-associated communities (Figure 1.4a) (Table 1.3) indicating higher stability in above ground plant structures under poor environmental conditions. These results support the hypothesis that bacterial and

fungal communities have the potential to be driving range edge dynamics through differences in belowground interactions with plants at and beyond range edges.

We used the UniFrac method to test for phylogenetic differences between different structures and tissues and between the two elevations in the bacterial communities. The UniFrac method is a comparison between the two communities using phylogenetic distances where the weighted method accounts for abundance and the unweighted method does not take abundance into consideration. Our results showed significant differences between the two elevations in the two root-associated bacterial communities using both the weighted and unweighted values and in the leaf endophytes using the unweighted method (Table 1.3). Our results indicate that the difference found between the two environments is primarily driven by differences in the abundance of particular OTUs (measured by Bray-Curtis distance) and not in the overall phylogenetic composition of the communities (measured by UniFrac distance).

### 1.4.4 Microbial richness and diversity differed between plant structure and tissue

The highest OTU counts and alpha diversity were present in the rhizosphere; this was expected as exudates from roots provide nutrient for bacterial growth leading to high abundance and diversity on plant roots (Mendes *et al.* 2011). The root endophytes showed the second highest diversity and OTU counts, followed by the phyllosphere

and finally the leaf endophytes (Figure 1.5). Both endophytic samples showed lower diversity than the epiphytic communities of the same plant structures. While previous research has found that leaf endophytes were higher in diversity than leaf surfaces (Bodenhausen *et al.* 2013) our data showed the opposite. This may be due to the presence of high variability in phyllosphere samples that we found, it may also be due to a filtering effect on community assembly as the bacteria migrate onto plant surfaces through wind transfer and rain runoff. Plant tissues may act as an ecological filter, with only bacteria that are able to colonize interior tissues able to enter the plant structures while bacteria which are not specially adapted remain on the plant surfaces. We also found leaf-associated communities were consistently lower in diversity than root-associated communities. This was also expected as the phyllosphere contains high selective pressures for bacteria such as exposure to UV radiation, low nutrient availability and low moisture (Lindow & Brandl 2003) while the rhizosphere has relatively high resource diversity, nutrient levels and moisture availability (Badri *et al.* 2009).

When looking at the bacterial communities from the elevational range edge compared to the natural range, we consistently found higher OTU counts and higher alpha diversity in the samples from the natural range. This was present using data from all parts of the plant separately as well as in the combined dataset. Lower diversity in the bacterial communities at the elevational range edge suggests that the bacterial communities in the natural range may provide more benefits to the host plants since

bacterial communities with higher diversity are more stable under variable environments (Yachi & Loreau, 1999) or during abiotic and biotic perturbations (Awasthi *et al.* 2014, Eisenhauer 2012).

### 1.4.5 Correlations between microbial communities and host functional traits

We measured the host plants' functional traits including specific leaf area (SLA), stem length, width and diameter, and specific stem density (SSD). We also measured leaf area and weight. We looked for correlations between these traits to the microbial communities using the envfit function on R on the NMDS scores from the community matrices. We found no correlations between the bacterial communities of the rhizosphere, root tissue or leaf tissue to any of the functional traits. We found correlations between the bacterial communities of the phyllosphere, combined leaf data and combined data to some of the functional traits. For example bacterial community structure using the combined dataset was correlated with leaf area, leaf weight and stem weight. This indicates there is a connecting factor between the leaf and stem traits and the bacterial communities, which is being driven by an unknown cause on either side. We also found a correlation between the fungal communities of the root tissue and leaf weight (Table 1.6), which suggests a possible link between plant age or health and fungal community structure.

Previous studies have found similar correlations between phyllosphere bacteria and wood density and leaf mass per area when looking at interspecific trait variability (Kembel *et al.* 2014). We are unable to know which side of the plant-microbe association would be driving these correlations. They may be driven by the actions of the microbial communities, such as the bacterial production of plant growth hormones which can affect leaf traits (Glickmann *et al.* 1988). They may also be driven by the traits of the plants, creating effects on the microbial communities. In order to begin to understand these associations, experimental manipulations would need to be performed, but our results suggest that an understanding of plant functional traits may make it possible to better predict microbial community composition along environmental gradients.

### 1.4.6 Comparison of bacterial and fungal results in root endophytic communities

By examining both the fungal and bacterial communities of the same samples, we can investigate if the two communities vary differently with changes in environment. The bacterial and fungal endophytic communities of the root tissue were both affected by change in environment. This implies that the root tissue communities are sensitive to environmental changes and an impact in one community may drive changes in the other microbial community. The bacterial community showed lower diversity and OTU counts at the elevational range limit while the fungal community had no significant difference in alpha diversity between the environments and a higher OTU

count at the elevational range limit. This suggests that bacterial communities may be less stable under environmental changes compared to fungal communities.

## 1.5 CONCLUSION

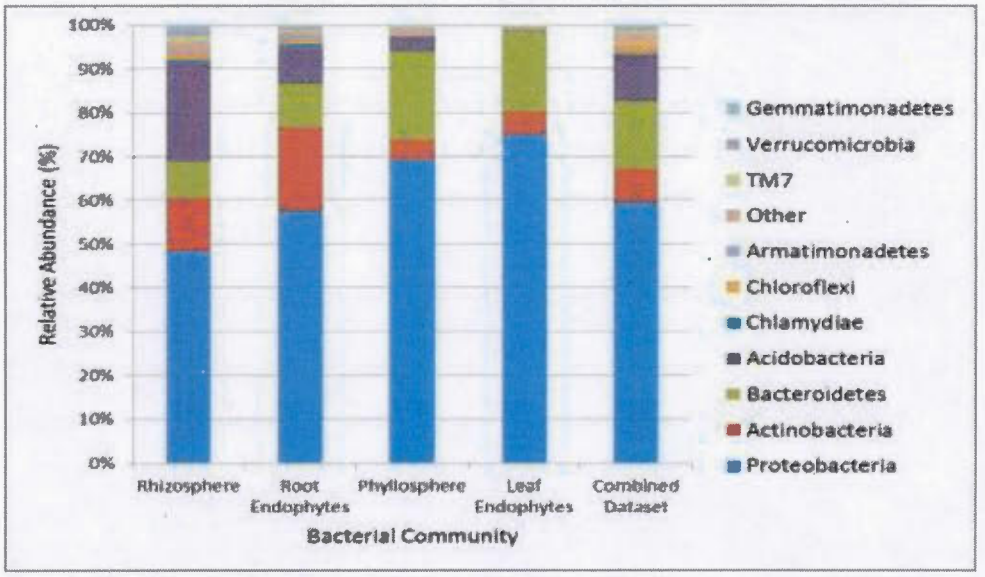
In this study we used high-throughput DNA sequencing of bacterial and fungal molecular markers to compare the microbial communities of *Acer saccharum* seedlings from different plant parts and along an elevational gradient where a distinct sugar maple elevational range limit occurs. In summary, *Acer saccharum* seedlings were found to have distinct bacterial communities inhabiting their leaves, roots and within different tissues. The communities between the exterior of the leaves and roots and their interior tissues were found to have greater similarity than between different structures of the plant. We also found there are significant differences in the bacterial communities at the plants elevational range limit in every structure and tissue and in the fungal endophytes of the roots. This study expands our knowledge of the ecology of plant-microbe interactions and the structure and assembly of microbial communities found on sugar maple trees. It gives an in depth look at the bacterial communities found in each structure and tissue of a host plant and provides insight into assembly of each of these communities.

The roles of most plant-associated microbial species are still unknown but it has been found that higher diversity creates a more stable community (Awasthi *et al.* 2014)

and can improve plant resistance to pathogens (Innerebner *et al.* 2011). Therefore since we found higher bacterial diversity in the host's natural range in the root-associated communities, it implies that below-ground changes in microbial structure could potentially play a role in elevational edge effects on host plant growth or health. The importance of microbes for their hosts' fitness and function is becoming apparent (e.g. Zamioudis & Pieterse 2012) and further research into these dynamics is needed to improve our understanding of plant-microbe interactions.

By identifying the abundant bacterial and fungal taxa present on sugar maples in these environments, this study provides a baseline for future research into the connections between microbiomes and plant fitness and function. From these results future research can investigate the ecological impact of these taxa on the host using experiments. These experiments could target specific taxa such as the taxa associated with the natural range or the elevational range limit to determine the positive and negative effect they have on sugar maple seedlings and to identify taxa that may influence the ecological distribution of sugar maples.

a)




b)

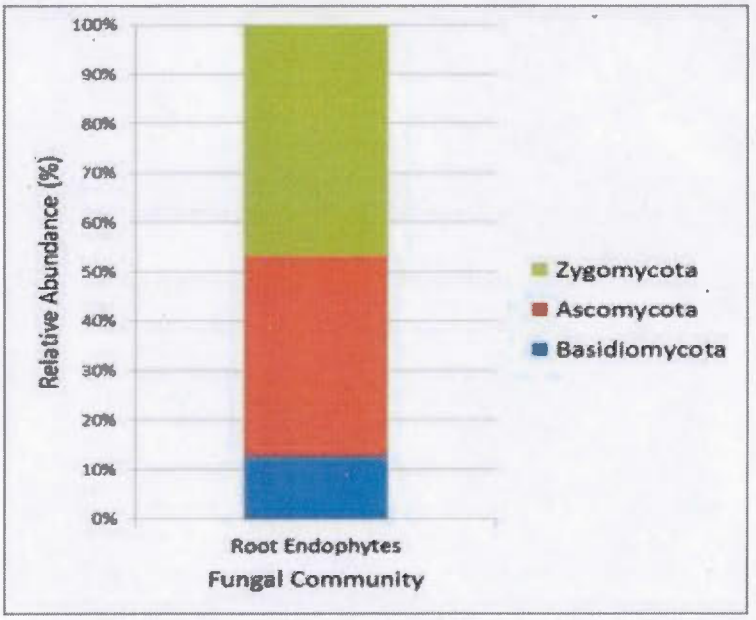
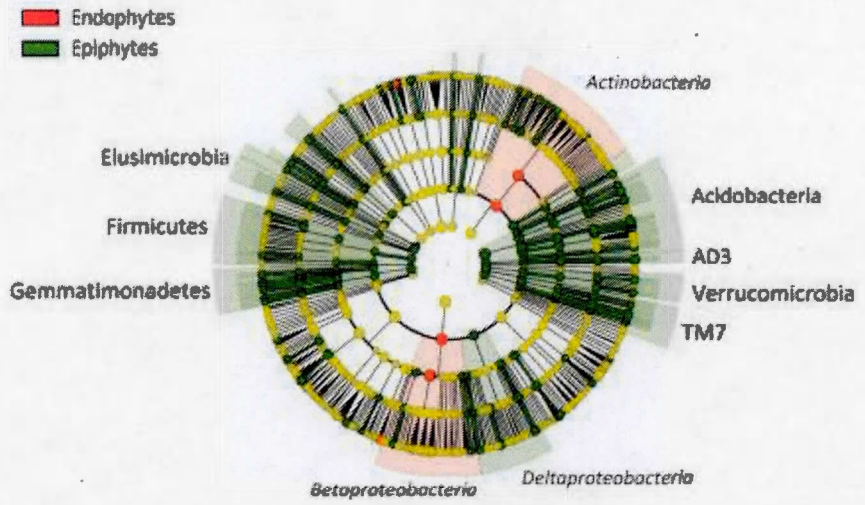



Figure 1.1: Relative abundances (%) of bacteria (a) and fungi (b) phyla in the different plant tissues and surfaces of sugar maple seedlings as well as the average for all structures and tissues combined using the samples from below the elevational range limit.

a)




b)

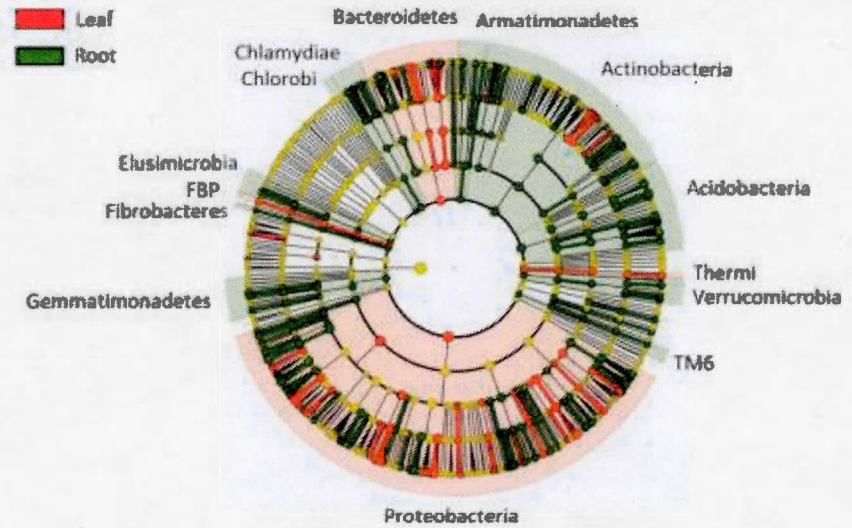



Figure 1.2: Cladograms of LEfSe results showing bacterial indicator taxa at the phyla levels comparing a) epiphytic to endophytic communities where red indicates a high presence in the leaf or root endophytic samples while green indicates epiphytic and b) root-associated to leaf-associated communities where red indicates a high presence in leaf samples while green indicates root samples.

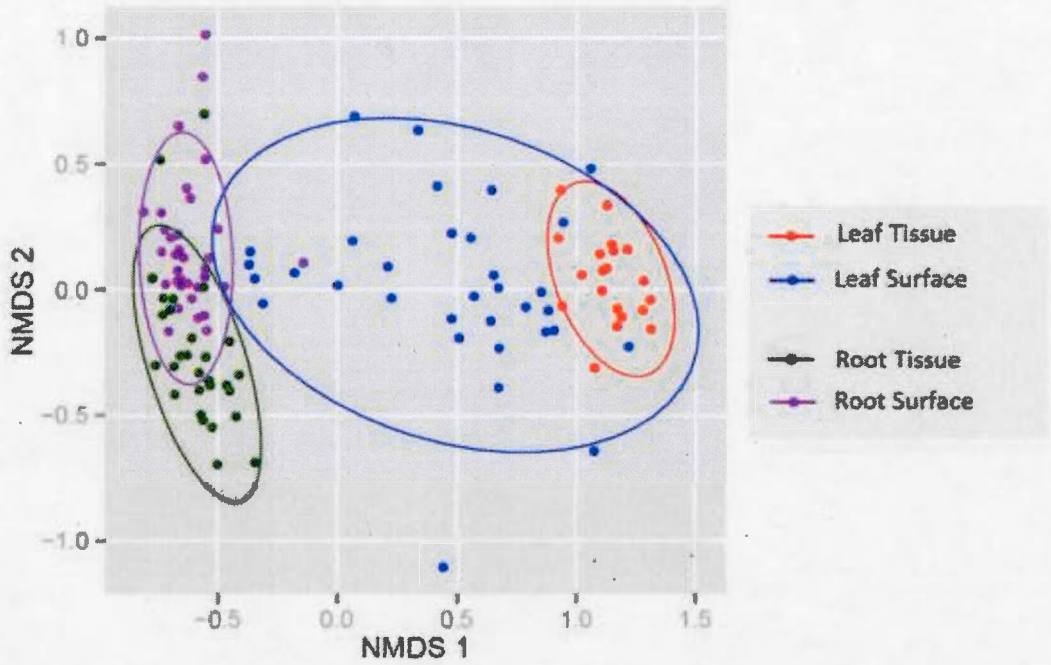
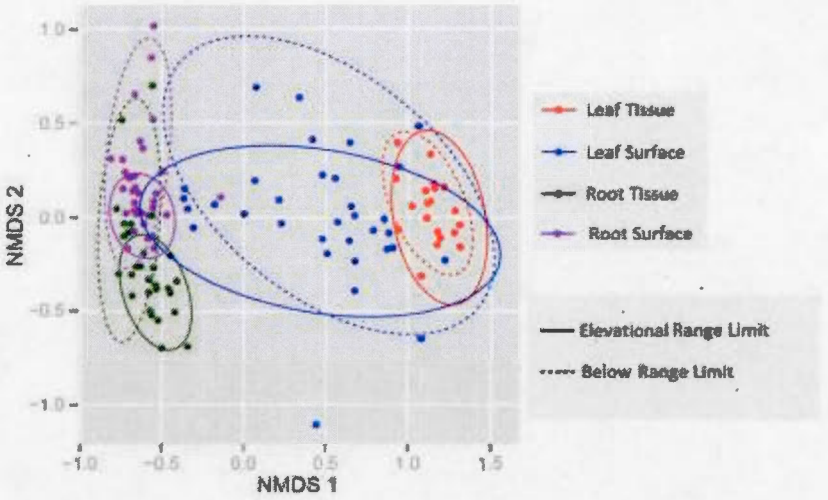



Figure 1.3: Non-metric multidimensional scaling (NMDS) ordination using Bray-Curtis distance of the dissimilarity between bacterial communities from different plant surfaces and tissues. Permutational analysis of variance showed significant differences (P=0.001) among all categories. Ellipses indicate 95% confidence intervals around samples from each category.

a)




b)

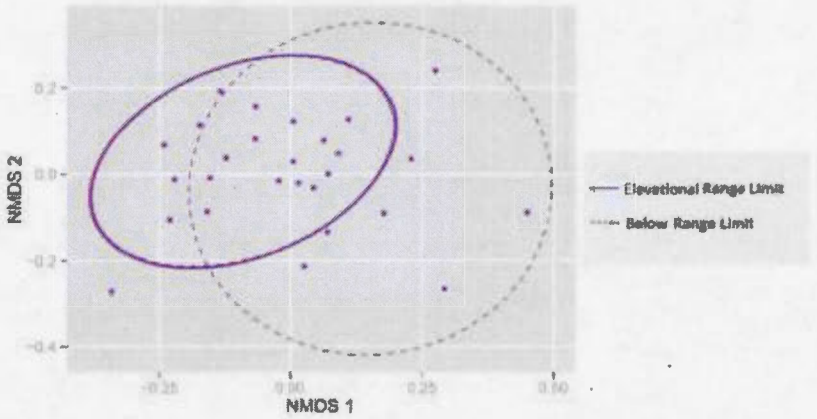



Figure 1.4: Non-metric multidimensional scaling (NMDS) ordination using Bray-Curtis distance of the dissimilarity between communities from seedlings taken from sugar maple's normal range and from the elevational range limit. Permutational analysis of variance showed significant differences between a) the bacterial communities in all structures and tissues (p=0.001) and b) the fungal communities of the root tissue (p=0.001). Ellipses indicate 95% confidence intervals around samples from each category.

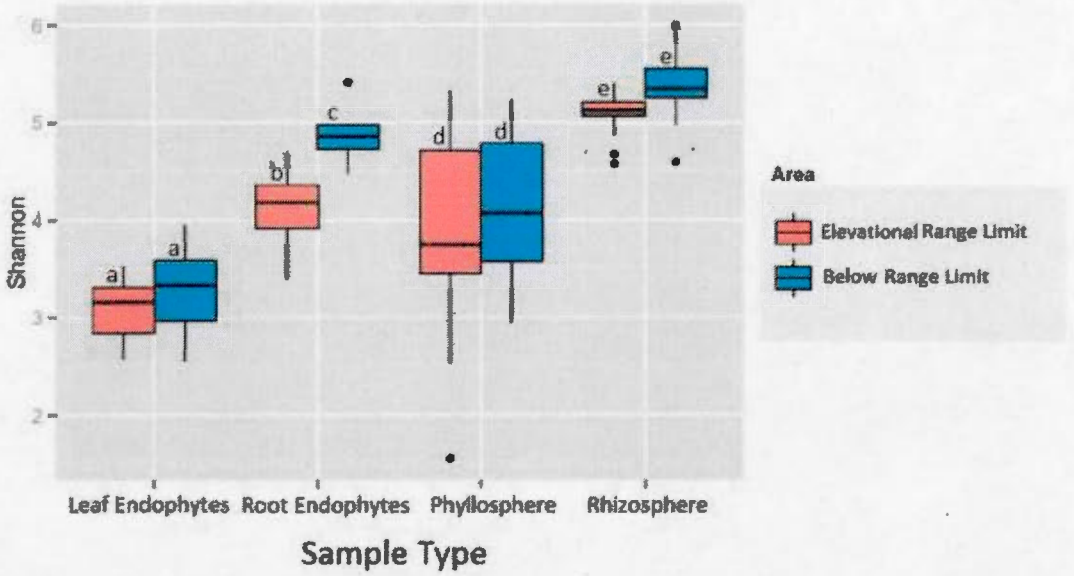



Figure 1.5: Boxplot of bacterial Shannon diversity by sugar maple structure. ANOVA test showed significant differences between above and below ground and surface to tissue communities ($p<0.001$). The diversity between parts of the plant (Rhizosphere, Phyllosphere, Leaf Endophytes, Root Endophytes) was found to be significantly different ($p<0.05$) between each pair but was not significant between the two sites (Edge, Within) in any of the bacterial communities except the endophytes of the roots ($p<0.05$) following a post-hoc test of Tukey multiple comparisons of means at a 95% family-wise confidence interval. Samples types that do not share a letter (indicated above boxplots) were significantly different according to the Tukey post-hoc test.

Table 1.1: List of relative abundances (%) of the most abundant bacteria phyla and classes associated with sugar maples, for different structures and tissues and from the combined dataset, along with the significant associations taxa showed using the LDA Effect Size platform (LEfSe). Bacterial phyla are represented in bold text while classes are represented in italics.

| Taxa | Rhizosphere | Root Endophytes | Phyllosphere | Leaf Endophytes | Combined Dataset | Taxa is an indicator of: |
|---|---|---|---|---|---|---|
| **Acidobacteria** | **24.7%** | **10.4%** | **2.0%** | **0.2%** | **10.6%** | **- Epiphytes**<br>**- Roots** |
| *- Acidobacteriia* | *9.04%* | *4.19%* | *1.43%* | *0.13%* | *3.6%* | - Roots<br>- Epiphytes |
| *- DA052* | *7.05%* | *1.63%* | *0.91%* | *0.01%* | *3.7%* | - Roots<br>- Epiphytes |
| *- Solibacteres* | *6.28%* | *2.03%* | *0.81%* | *0.02%* | *3.0%* | - Rhizosphere<br>- Epiphytes |
| **Actinobacteria** | **10.4%** | **16.3%** | **3.6%** | **8.6%** | **7.8%** | **- Roots** |
| *- Actinobacteria* | *8.85%* | *17.52%* | *4.18%* | *5.35%* | *6.4%* | - Roots<br>- Endophytes |
| **AD3** | **1.9%** | **0%** | **0%** | **0%** | **1.1%** | **- Epiphytes** |
| **Bacteroidetes** | **9.3%** | **9.2%** | **20.5%** | **20.5%** | **15.4%** | **- Leaves** |
| *- Cytophagia* | *0.43%* | *0.36%* | *14.42%* | *16.48%* | *9.0%* | - Leaves |
| *- Saprospirae* | *4.86%* | *6.43%* | *1.28%* | *0.22%* | *3.2%* | - Roots |
| *-Sphingobacteriia* | *2.98%* | *3.29%* | *3.80%* | *1.11%* | *2.7%* | |
| **Chloroflexi** | **3.7%** | **2.3%** | **0.3%** | **0%** | **1.6%** | **- Roots** |
| **Proteobacteria** | **41.9%** | **55.8%** | **71.3%** | **68.9%** | **59.4%** | **- Leaves** |
| *- Alpha* | *19.3%* | *22.4%* | *26.5%* | *21.9%* | *23.1%* | - Leaves |
| *- Beta* | *7.3%* | *14.8%* | *31.1%* | *40.5%* | *23.0%* | - Endophytes |
| *- Delta* | *5.0%* | *4.0%* | *1.9%* | *1.3%* | *2.9%* | - Epiphytes |
| *-Gamma* | *10.0%* | *13.9%* | *11.8%* | *5.2%* | *10.2%* | |
| **TM7** | **2.6%** | **1.1%** | **0.4%** | **0.7%** | **1.0%** | |

Table 1.2: Bacteria phyla which showed a significant association with sugar maples in either the bacterial communities of the elevational range limit (Edge) or below (Within) using the LDA Effect Size platform (LEfSe).

| Taxa | Rhizosphere | Root Endophytes | Phyllosphere | Leaf Endophytes |
|---|---|---|---|---|
| Acidobacteria | - | Within | - | - |
| Actinobacteria | Edge | - | - | Within |
| Armatimonadetes | Edge | Edge | - | - |
| Bacteroidetes | - | - | - | - |
| Chloroflexi | Within | Within | - | - |
| Chlamydiae | - | Within | - | - |
| Elusimicrobia | Within | Within | - | - |
| Gemmatimonadetes | Within | Within | - | - |
| Nitrospirae | - | Within | Within | - |
| Planctomycetes | Within | - | - | - |
| Proteobacteria | Edge | Edge | - | Edge |
| Spirochaetes | Within | - | - | - |
| Thermi | - | - | Within | Within |
| TM6 | - | Within | - | - |
| Verrucomicrobia | - | Within | - | - |

Table 1.3: Bray-Curtis /PERMANOVA tests and UniFrac results investigating if a significant difference occurred between the two elevations (Edge/Within) in each of the microbial communities of sugar maples and in the combined dataset.

| | **Bray-Curtis** | | **Unweighted UniFrac** | | **Weighted UniFrac** | |
|---|---|---|---|---|---|---|
| Bacterial Communities | $R^2$ | P-Value | $R^2$ | P-Value | $R^2$ | P-Value |
| **Rhizosphere** | 0.13861 | 0.001 | 0.14578 | 0.004 | 0.21317 | 0.001 |
| **Root Endophytes** | 0.1992 | 0.001 | 0.12574 | 0.011 | 0.45355 | 0.001 |
| **Phyllosphere** | 0.05806 | 0.045 | 0.04638 | 0.234 | 0.05615 | 0.157 |
| **Leaf Endophytes** | 0.12119 | 0.002 | 0.28477 | 0.004 | 0.07577 | 0.223 |
| **Combined Dataset** | 0.03881 | 0.006 | 0.0422 | 0.013 | 0.01962 | 0.091 |
| Fungal Communities | $R^2$ | P-Value | $R^2$ | P-Value | $R^2$ | P-Value |
| **Root Endophytes** | 0.18358 | 0.001 | NA | NA | NA | NA |

Table 1.4: List of relative abundances (%) of the most abundant fungal phyla and classes associated with the root endophytic communities of sugar maples. Fungal phyla are represented in bold text while classes are represented in italics.

| Taxa | Root Endophytes |
|---|---|
| **Ascomycota** | **40.1%** |
| *- Dothideomycetes* | *7.7%* |
| *- Eurotiomycetes* | *2.6%* |
| *- Leotiomycetes* | *7.5%* |
| *- Sordariomycetes* | *10.2%* |
| **Basidiomycota** | **12.4%** |
| *- Agaricomycetes* | *11.5%* |
| **Zygomycota** | **46.4%** |

Table 1.5: Results of ANOVA tests on Shannon diversity index values for the bacterial communities of each structure and tissue showing mean ± SD for each structure and tissue and comparing the microbial communities of sugar maples from two elevations.

| | Bacterial Communities | | |
|---|---|---|---|
| | Mean ± SD | F-Value | P-Value |
| **Rhizosphere** | 5.221 ± 0.308 | 6.439 | 0.016 |
| **Root Endophytes** | 4.398 ± 0.847 | 33.948 | p<0.0001 |
| **Phyllosphere** | 4.004 ± 0.454 | 0.844 | 0.3653 |
| **Leaf Endophytes** | 3.216 ± 0.411 | 1.069 | 0.3148 |
| **Combined Dataset** | 4.323 ± 0.887 | 1.821 | 0.1799 |
| | Fungal Communities | | |
| **Root Endophytes** | 2.678 ± 0.230 | 2.9361 | 0.09899 |

Table 1.6: Correlations between sugar maple seedling functional traits and microbial community structure. Functional traits correlation tests performed with envfit function on NMDS scores for Within samples only. Significance levels for each variable are given by: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

| | Rhizosphere | | Root Endophytes | | Combined Root | |
|---|---|---|---|---|---|---|
| | $R^2$ | P-Value | $R^2$ | P-Value | $R^2$ | P-Value |
| Leaf Area | 0.2279 | 0.315 | 0.3590 | 0.410 | 0.1661 | 0.223 |
| Leaf Weight | 0.1072 | 0.573 | 0.6685 | 0.163 | 0.2328 | 0.112 |
| Specific Leaf Area | 0.1626 | 0.452 | 0.2726 | 0.558 | 0.0208 | 0.834 |
| Stem Length | 0.1257 | 0.541 | 0.8262 | 0.055 | 0.1207 | 0.354 |
| Stem Weight | 0.0332 | 0.878 | 0.7452 | 0.092 | 0.1365 | 0.307 |
| Stem Diameter | 0.0120 | 0.946 | 0.4911 | 0.253 | 0.0523 | 0.650 |
| Specific Stem Density | 0.0729 | 0.691 | 0.4165 | 0.343 | 0.0473 | 0.667 |

| | Phyllosphere | | Leaf Endophytes | | Combined Leaf | |
|---|---|---|---|---|---|---|
| | $R^2$ | P-Value | $R^2$ | P-Value | $R^2$ | P-Value |
| Leaf Area | 0.2396 | 0.298 | 0.2743 | 0.329 | 0.2714 | 0.050* |
| Leaf Weight | 0.1334 | 0.559 | 0.0161 | 0.949 | 0.1319 | 0.268 |
| Specific Leaf Area | 0.1074 | 0.601 | 0.4471 | 0.139 | 0.1121 | 0.329 |
| Stem Length | 0.0289 | 0.873 | 0.4548 | 0.125 | 0.0079 | 0.932 |
| Stem Weight | 0.2173 | 0.345 | 0.3789 | 0.198 | 0.2688 | 0.048* |
| Stem Diameter | 0.1483 | 0.476 | 0.3795 | 0.188 | 0.0818 | 0.421 |
| Specific Stem Density | 0.5014 | 0.046* | 0.5315 | 0.073 | 0.1795 | 0.163 |

| | Combined Dataset | | Fungal Root Endophytes | |
|---|---|---|---|---|
| | $R^2$ | P-Value | $R^2$ | P-Value |
| Leaf Area | 0.1872 | 0.018* | 0.4524 | 0.287 |
| Leaf Weight | 0.1346 | 0.049* | 0.9422 | 0.004** |
| Specific Leaf Area | 0.0495 | 0.382 | 0.2237 | 0.614 |
| Stem Length | 0.0053 | 0.903 | 0.3791 | 0.400 |
| Stem Weight | 0.1475 | 0.048* | 0.6414 | 0.124 |
| Stem Diameter | 0.0392 | 0.482 | 0.3396 | 0.403 |
| Specific Stem Density | 0.0355 | 0.495 | 0.0250 | 0.955 |

Figure 1.S1: Plot and site locations on Mont-Mégantic, Quebec showing the different elevations from which sugar maple seedlings were sampled along with the approximate elevation where the sugar maple elevational range limit occurs.

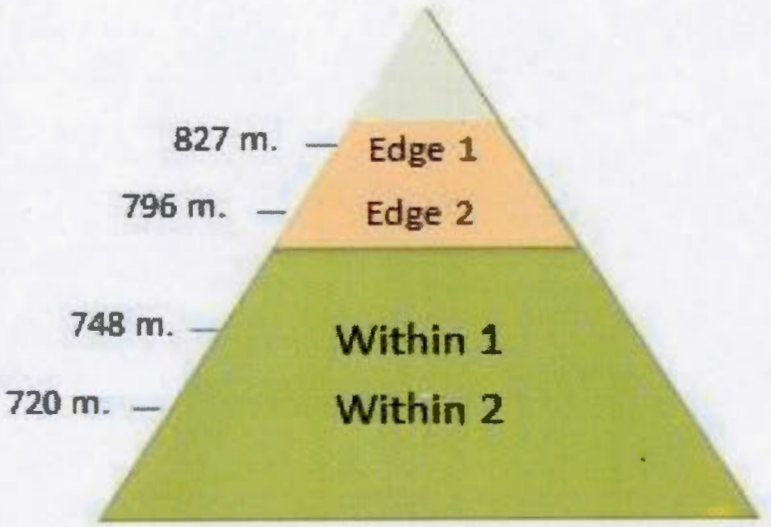
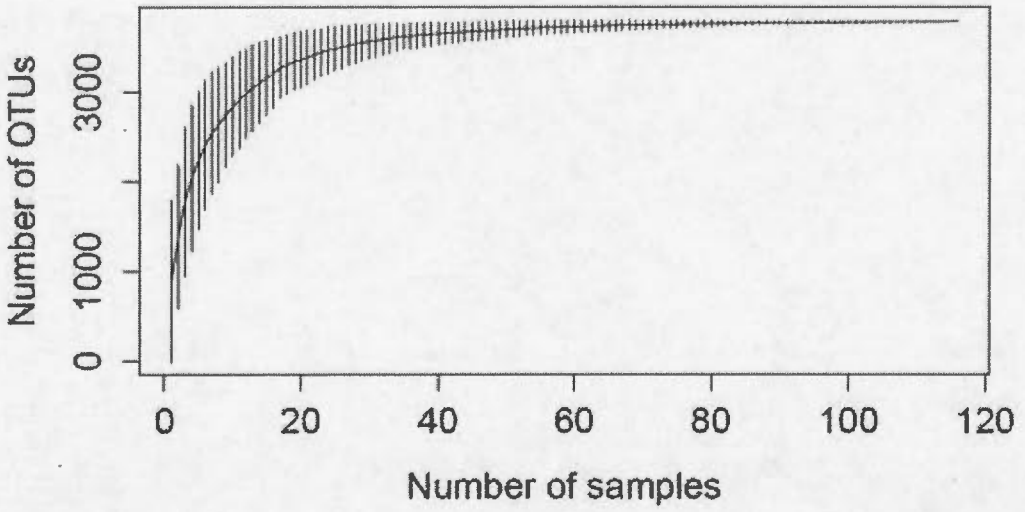


Figure 1.S2: Operational taxonomic unit (OTU; 97% sequences similarity) collector's curve (mean with 95% confidence intervals) based on random sampling of data before rarefaction with no singletons a) bacterial samples and b) fungal samples.

**a) 16S**




**b) ITS**

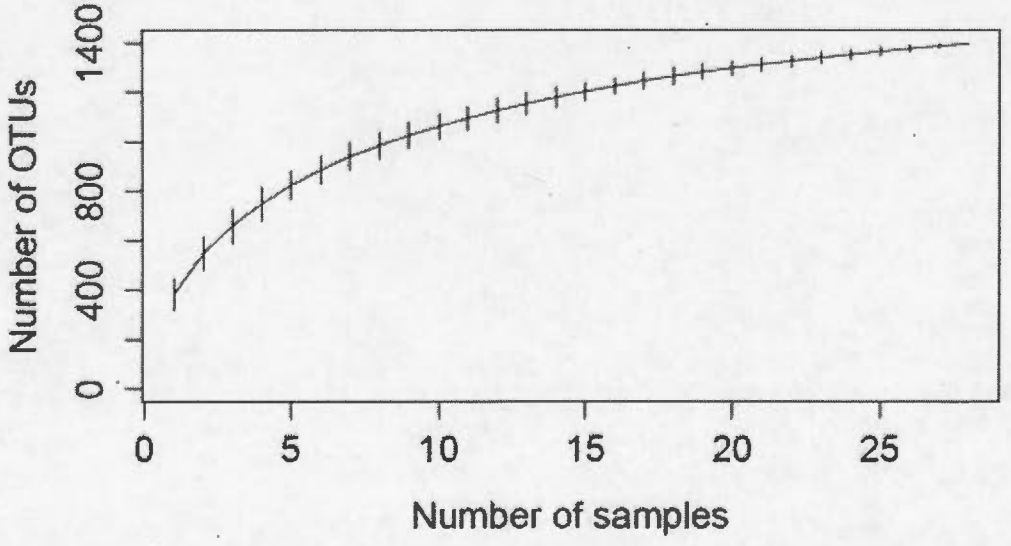


Table 1.S1: Elevations of plots and number of sugar maple seedlings taken from the four plots at Mont-Mégantic, Quebec.

| Zone Name | Plot | Elevation (m a.s.l) | Seedling Samples | From Elevational Range Limit |
|---|---|---|---|---|
| Edge | 1 | 827 | 10 | Yes |
| Edge | 2 | 796 | 10 | Yes |
| Within | 1 | 748 | 10 | No |
| Within | 2 | 720 | 10 | No |

Table 1.S2: 16S rRNA and ITS samples obtained from Mont-Mégantic after sequencing which were used for analysis.

| | **Primers** | **Within** | **Edge** | **Total** |
|---|---|---|---|---|
| Rhizosphere | 16S | 15 | 19 | 34 |
| Root Endophytes | 16S | 10 | 19 | 29 |
| Phyllosphere | 16S | 15 | 18 | 33 |
| Leaf Endophytes | 16S | 12 | 8 | 20 |
| **Total Bacteria** | **16S** | **52** | **64** | **116** |
| Root Endophytes | ITS | 10 | 18 | 28 |

Table 1.S3: Complete list of indicator taxa found at the phyla and class levels for comparisons between epiphytic bacterial communities to endophytic and between leaf and root-associated bacterial communities of sugar maple. Bacterial phyla are represented in bold text while classes are represented in italics.

| | Epiphytic | Endophytic | Leaf | Root |
|---|---|---|---|---|
| **PHYLA** | **Acidobacteria** | | **Bacteroidetes** | **Acidobacteria** |
| | **AD3** | | **FBP** | **Actinobacteria** |
| | **Elusimicrobia** | | **Proteobacteria** | **Armatimonadetes** |
| | **Firmicutes** | | **Thermi** | **Chlamydiae** |
| | **Gemmatimonadetes** | | | **Chlorobi** |
| | **TM7** | | | **Elusimicrobia** |
| | **Verrucomicrobia** | | | **Fibrobacteres** |
| | | | | **Gemmatimonadetes** |
| | | | | **Spirochaetes** |
| | | | | **TM6** |
| | | | | **Verrucomicrobia** |
| *CLASSES* | *ABS 6* | *Actinobacteria* | *Alphaproteobacteria* | *Acidimicrobiia* |
| | *Acidobacteriia* | *Betaproteobacteria* | *Cytophagia* | *Acidobacteriia* |
| | *Acidimicrobiia* | | *Deinococci* | *Actinobacteria* |
| | *Bacilli* | | *Flavobacteriia* | *Armatimonadia* |
| | *Clostridia* | | | *At12OctB3* |
| | *DA052* | | | *Chlamydiia* |
| | *Deltaproteobacteria* | | | *DA052* |
| | *Elusimicrobia* | | | *Elusimicrobia* |
| | *Ktedonobacteria* | | | *Fibrobacteria* |
| | *Gemm 1* | | | *Fimbriimonadia* |
| | *Gemmatimonadetes* | | | *Gemmatimonadetes* |
| | *Pedosphaerae* | | | *Pedosphaerae* |
| | *SC3* | | | *Saprospirae* |
| | *Solibacteres* | | | *SC3* |
| | *TK17* | | | *SJA 4* |
| | *TM1* | | | *Solibacteres* |
| | *TM7 3* | | | *Spirochaetes* |
| | | | | *Thermoleophilia* |
| | | | | *TM1* |
| | | | | *TM7 1* |

# CHAPTER II

# THE MICROBIOME OF *ACER SACCHARUM* SAPLINGS: EFFECTS OF DIFFERENT SOILS AND CANOPY COVERS ON ENDOPHYTIC AND EPIPHYTIC BACTERIAL AND FUNGAL COMMUNITIES

## 2.1 INTRODUCTION

The plant-associated microbial communities that make up the plant's core microbiome, defined as microorganisms common across certain habitats (Turnbaugh *et al.* 2007, Shade & Handelsman 2012) are ecologically important due to their abundance and frequent occurrence in microbial communities. These microbes include pathogens, commensals, and mutualists, and can greatly alter a plant's health and functional traits (Gourion *et al.* 2006, Friesen *et al.* 2011). While the importance of canopy cover and soil conditions for maple ecology has been quantified (St. Clair *et al.* 2008), their influence on the structure of the sugar maple microbiome is poorly understood. The bacterial and fungal communities inhabiting the host plants are most likely highly susceptible to changes in environment such as canopy or soil type and the variation caused by these changes may give insight into the performance of sugar maples in these environments. In this study we address this issue by quantifying

variation in sugar maple microbiome structure across plant tissues, canopy types, and soil types.

The diverse communities of microorganisms that colonize plants inhabit both the interior tissues and exterior surfaces of the hosts. Epiphytic microbes live on the surfaces of plant tissues while endophytic microbes live within plant tissues and may be intercellular or intracellular. Endophytic and epiphytic microbes are non-parasitic or disease causing and many have been found to provide beneficial effects to their hosts such as producing plant growth hormones (Gourion *et al.* 2006) or providing nutrients (Davison 1988). While some of these microbes have been shown to affect the plant's health, many microbial roles are still unclear along with knowledge of the environmental drivers of variation in these communities.

Sugar maple (*Acer saccharum* Marsh.) is an important deciduous tree species in North America both economically and ecologically (Burns & Honkala 1990) but recent health declines have caused concern for the species (Lachance *et al.* 1995). Sugar maple trees are sensitive to many environmental factors both biotic and abiotic (St. Clair *et al.* 2008) especially soil conditions such as soil nutrient levels (Mader & Thompson 1969) and soil compaction (Houston 1999). Sugar maples often grow in pure stands but are also found mixed with other hardwoods and occasional conifers (Hosie 1969). They are able to grow on many soil types (Godman *et al.* 1990) but are

considered to grow best on deep, moist, well-draining soils (Hosie 1969) with a pH of from 5.5 to 7.3 (Godman 1965).

Functional traits are an ecologically relevant way of classifying plant species on functional grounds (Díaz & Cabido 2001). Functional traits are defined as morphological, physiological or phenological characteristics which affect fitness or ecosystem function (Violle *et al.* 2007). Previous research found correlations between plant microbiomes and their functional traits such as chemical composition (Bailey *et al.* 2005), however very little is known about the interactions between host traits and plant-associated microbial communities. In this study we looked at two leaf traits, specific leaf area and leaf dry matter content and investigated if there were correlations between these traits and the bacterial or fungal community structures.

The community ecology of microbes has been revolutionized recently by the use of high-throughput DNA sequencing technology and has become an important area of research (Turnbaugh *et al.* 2007). Next-generation sequencing techniques allow us to quantify the microbial communities from multiple samples and investigate the community structure and phylogeny from various environments. To our knowledge, sugar maples have not been previously studied in regards to their microbial communities using high-throughput DNA sequencing or comparing different structures and tissues. Here we compared the bacterial communities of different structures and tissues of saplings such as the surface of the leaves and roots, as well

as their interior tissues separately. We also investigated the endophytic fungal communities of the interior root and leaf tissue. Finally we compared the microbial communities found in the surrounding soil to the plant-associated bacterial and fungal communities.

We used high-throughput Illumina sequencing of the bacterial 16S rRNA gene (Claesson *et al.* 2010) and fungal ITS region (Schoch *et al.* 2012) to quantify the microbial community structure from soil samples and from four different plant parts from sugar maple saplings. The microbial communities were investigated from leaf and root samples taken from saplings growing in the temperate forests of southern Quebec. We quantified microbiome structure for sugar maple seedlings growing in different soil conditions and under different canopy covers. For this study we separated the structures and tissues of the host plants to examine bacterial and fungal communities from each structure or tissue individually as well as from the surrounding soil. By investigating each structure and tissue separately, we could compare the microbial communities of the different structures of the plant along with the exterior and interior tissues to each other. This allowed us to investigate if variation in the microbial communities caused by environmental changes is equally occurring in the communities of the leaves, roots and tissues. We expected to find significantly different communities inhabiting each structure and tissue. We also expected the microbial community structure to be driven more by the structure or

tissue than by soil or canopy type, since each part of the plant provides a unique environment for microbial colonization with different nutrient availability.

In this study our objectives were (1) to investigate and identify the core microbiome of sugar maple saplings by comparing microbial communities from the surfaces of the leaves, roots and from the interior tissues of the plant, (2) to investigate if community structure, variability, biodiversity, or assembly differ by plant structure or tissue. For example leaf-associated communities compared to root-associated communities and interior plant tissues compared to exterior plant surfaces, (3) do environmental changes such as soil type or canopy cover drive microbial community structure and are these effects occurring in every structure of the plant; and finally to investigate (4) are functional traits (leaf dry matter content and specific leaf area) of sugar maple saplings correlated with their microbial communities.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Specimen collection

Leaf and root samples were collected from sugar maple saplings in the area surrounding the Laurentian Biology Station near Saint-Hippolyte, Quebec, Canada (45°55'52" N, 74°1'29" W). The samples were collected from trees approximately 5 years in age that were planted the previous year under different canopy species. The

trees had been observed over 1 year during an experiment investigating if canopy species or soil type affected sapling survival and growth. Half of the trees were planted in the natural soil occurring in the area while the other half were placed in pots inserted into the ground containing potting soil with fertilizer and mycorrhizae added to create an optimal soil condition. Overhead canopies were classified as either "Hardwood" which consisted predominantly of sugar maple (*Acer saccharum*) and yellow birch (*Betula alleghaniensis*), or classified as "Conifer" which were composed mostly of balsam fir (*Abies balsamea*) and cedar (*Thuja occidentalis*).

Fifty-four trees were sampled from 16 different sites which included 28 trees growing in natural soil and 26 growing in potted soil. Twenty of these samples were taken from under canopies classified as "Hardwood" while 34 came from "Conifer" canopy sites (Table 2.S1). Leaf samples were taken by removing 2 leaves from different branches of the tree for DNA extraction and 2 more for measurement of functional traits. Fine root samples were taken from a depth of approximately 6 cm. Twenty-eight soil samples were also taken from a depth of 4 cm. Seven of the soil samples came from potted soil under a hardwood canopy, seven from potted soil under a conifer canopy, seven came from natural soil under a hardwood canopy and the final seven came from natural soil under a conifer canopy. All samples were taken using gloves and sterile techniques to minimize contamination with human-associated microbes. The leaf, root and soil samples were placed in sterile roll bags immediately and frozen within 6 hours. They were stored at -80°C until processing.

### 2.2.2 Functional traits and biometrics

Functional traits were measured according to protocols provided in Cornelissen *et al.* 2003. The saplings which were sampled at the Laurentian Biology Station were approximately 50 cm in height. Several leaves of varying ages were collected from each sapling from various heights of the plant. For these plants 2 leaves were included in the analysis for specific leaf area (SLA) and leaf dry matter content (LDMC).

The leaf area of the 2 leaves was measured using WinFolia software to analyze scanned images of the leaves. The leaves were dried for 48 hours at room temperature and then weighed. The weight and area were used to calculate SLA and LDMC. Using leaves sampled from the plants, host functional trait data was measured for specific leaf area (SLA) and leaf dry matter content (LDMC). This included samples from both soil and canopy types. With the exception of the soil samples which were excluded, the communities from each structure and tissue was analysed separately along with the combined datasets for correlations to functional traits using both the bacterial and fungal community samples. We also combined data from epiphytic samples, endophytic samples, leaf-associated samples and root-associated samples to test for correlations.

2.2.3 Sample preparation and DNA extraction

We collected four sample types from different plant structure surfaces or tissues. These were the rhizosphere, defined as the surface of the roots and the soil within 1 mm from the roots (Clark 1949) and the phyllosphere defined as the above-ground surface of the plant (Ruinin 1965) for which we used leaf surfaces. The other two sample types were tissue samples from the interior of the roots and leaves. Leaf and root samples were processed separately through a series of steps in order to first remove all epiphytic bacteria and fungi. After, the surface of the tissues were sterilized and washed to remove all remaining microbial cells. The tissues were then finely sectioned and agitated in a bead beating tube to release as many endophytic microbes from the tissues as possible.

The epiphytic microbial communities were removed with a 5 minute agitation wash in 30 mL of 1:50 diluted solution of buffer [1M Tris-HCl, 0.5 M Na EDTA, and 1.2% CTAB] (Kadivar & Stapleton 2003). The plant tissues were then removed from the buffer solution and the samples were centrifuged at 4000 rpm for 20 minutes at 4° C to form a pellet. The supernatant was removed with a sterile pipette and the pellet was transferred to a bead beating tube from the MoBio PowerSoil DNA Isolation kit (Carlsbad, CA). The protocol was followed with the exception that the samples were vortexed for 15 minutes instead of 10.

The tissue samples from the first wash were place in 30 mL of ethanol and vortexed for 5 minutes. The ethanol was removed and the samples were then washed with DNA free water for 3 minutes. The water wash was repeated 3 times. The tissues were then sectioned finely using sterile techniques. The resulting samples were then transferred to a bead beating tube from the MoBio PowerSoil DNA Isolation kit (Carlsbad, CA). The protocol was followed with the exception that the samples were vortexed for 45 minutes instead of 10. The isolated DNA samples were frozen at -80°C until further processing.

2.2.4 PCR and multiplexing for 16S rRNA and ITS sequencing

All 16S and ITS samples were amplified using the same one-step PCR step and normalization with primers designed to attach a 12 base pair barcode and Illumina adaptor sequence to the fragments during PCR (Fadrosh *et al.* 2014). The primers for bacterial sequencing used primers which amplify the V5 – V6 region [799F and 1115R (Redford *et al.* 2010)] of the 16S ribosomal rRNA (rRNA) gene and contained a heterogeneity spacer along with the Illumina linker sequence (Forward (799F): 5' - CAAGCAGAAGACGGCATACGAGATGTGACTGGAGTTCAGACGTGTGCTCT TCCGATCT xxxxxxxxxxxx HS AACMGGATTAGATACCCKG – 3', Reverse (1115R): 5' – AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACA CGACGCTCTTCCGATCT xxxxxxxxxxxx HS – AGGGTTGCGCTCGTTG - 3'). Where x represents barcode nucleotides and HS represents a heterogeneity spacer

between 0 and 7 basepairs in length. The bacterial 16S primers exclude cyanobacteria in order to exclude plant chloroplast DNA.

The primers for fungal sequencing amplified the regions 1 and 2 of the internal transcribed spacer (ITS) of the nuclear ribosomal coding cistron (ITS1F-ITS2; Schoch *et al.* 2012). (ITS1 Forward: 5' - CAAGCAGAAGACGGCATACGAGATGTGACTGGAGTTCAGACGTGTGCTCT TCCGATCTxxxxxxxxxxxxCTTGGTCATTTAGAGGAAGTAA, ITS2 Reverse: 5' - AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCT TCCGATCTxxxxxxxxxxxxGCTGCGTTCTTCATCGATGC – 3'), where x represents barcode nucleotides.

One 25 μL PCR reaction was run for each sample. This reaction contained 5 μL 5xHF buffer (Thermo Scientific), 0.5 μL dNTPs (10 μM), 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), 0.75 μL DMSO, 0.25 μL Phusion Hot Start II polymerase (Thermo Scientific), 1 μL DNA, and 16.5 μL molecular-grade water. The reaction was performed using: 30 s initial denaturation at 98°C, 35 cycles of 15 s at 98°C, 30 s at 60°C, and 30 s at 72°C, with a final 10 minute elongation at 72°C.

The samples were processed with an Invitrogen Sequalprep PCR Cleanup and Normalization Kit (Frederick, MD) to give all samples a finished concentration of

~0.55 ng/μl. They were then pooled with equal amounts and sequenced on an Illumina MiSeq platform at the University of Montreal.

2.2.5 DNA sequencing processing and data analysis

Data returned from the sequencing centre was processed using the fastx_toolkit, PEAR (Zhang *et al.* 2014) and QIIME version 1.8.0 (Caporaso 2010) software to trim and combine paired-end sequences to single sequences of approximately 336 bp in length (PEAR; default settings). The reads were de-multiplexed into samples using barcode sequences (QIIME; default settings). This involved combining the forward and reverse barcodes from each combined read into a 24-bp barcode which could then be matched to a sample ID (Hamady 2008).

Sequences were chimera checked and all chimeras were removed using the Uclust and Usearch 6.1 algorithms (Edgar 2010). Sequences were then binned into operational taxonomic units (OTUs) at a 97% similarity cut-off rate using Uclust (Edgar 2010). The OTUs were assigned taxonomy using the Ribosomal Database Project (RDP) classifier (Wang *et al.* 2007) as implemented in QIIME, with a minimum support threshold of 80% for bacterial OTUs and 50% for fungal OTUs. Rare OTUs occurring less than 20 times were removed (Zhan 2014). After removing rare OTUs, the 16S bacterial samples for each sample were rarefied to 1000

sequences. This resulted in a total of 226 usable samples from 16 soil and 54 saplings with a total OTU count of 3288 after rarefaction.

For ITS fungal samples rare OTUs occurring less than 20 times were removed as well and each sample was then rarefied to 2700 sequences. This resulted in a total of 110 samples from 26 soil and 48 saplings with a total OTU count of 2071. Missing samples in the bacterial or fungal datasets were due to low sequence read amounts either as a result of extraction, PCR or sequencing errors.

### 2.2.6 Indicator species analysis of bacterial taxa

We tested for the significant association of indicator taxonomic groups present in different structure and tissue of the host plant using the LDA Effect Size platform (LEfSe) (Segata *et al.* 2011). The LEfSe algorithm searches for biomarkers specific to sample groups, such as genes, pathways, or taxa using the Linear Discriminant Analysis (LDA) to approximate the effect size of each biomarker identified (Segata *et al.* 2011). We compared the bacterial communities of each soil, surface or tissue type of the plant separately. This was performed at the phylum and class level with an LDA cut off of 2.0 in each of the five bacterial communities. This allows us to compare the structures and tissues or sites in order to identify any significant host-microbe relationships and their strength between the different canopy types, soil types and between the different structures and tissues in order to identify any significant

host-microbe relationships and their strength. We also compared the microbial communities of the different plant parts at the phylum and class levels to find if certain taxa were associated with roots or leaves using combined surface and tissue samples. Finally we combined the two tissue samples in comparison to the two surface samples to test for taxa association with endophytic or epiphytic communities.

### 2.2.7 Statistical analysis

PCR and high-throughput sequencing techniques are known to cause errors and are subject to reagent contamination (Salter *et al.* 2014). Therefore we eliminated OTUs from our dataset that were represented by less than 20 sequences as this is a commonly used cut off for rare OTUs (Zhan 2014). Data analysis and plotting was performed using ape (Paradis *et al.* 2004), ggplot2 (Wickham 2009), picante (Kembel *et al.* 2010), and vegan (Oksanen *et al.* 2007) statistical packages for R (R Development Core Team; http://www.R-project.org).

Using the UniFrac index to measure phylogenetic distance between sets of taxa using branch length (Lozupone *et al.* 2006) we investigated phylogenetic variation in bacterial community structure among the different structures and tissues. A phylogeny was created using QIIME software, we then measured variation in the bacterial communities using both the weighted and unweighted UniFrac methods. The

weighted method takes an abundance-weighted measure of the phylogenetic diversity among the microbial communities while the unweighted method measures variation independent of abundance. These analyses using the UniFrac index were not performed on the fungal community data due to the fact that ITS sequence data is too variable to assign phylogeny at higher levels.

We also used the Bray-Curtis dissimilarity index to measure variation in OTU counts between the structures and tissues and between the different canopy and soil types in both the bacterial and fungal communities. Using nonmetric multidimensional scaling (NMDS) ordinations of the Bray – Curtis dissimilarity distances, we visualized dissimilarity between the four structures and tissues and between the different canopies and soils. Using the community matrix data, we performed permutational analysis of variance tests (PERMANOVA, Anderson 2001) to identify relationships and assign variance found between the microbial communities, parts of the plant, soil and canopy. To test for associations between host functional traits and microbial communities we fit correlations between the host's traits and the microbial community ordination using PERMANOVA tests on the community structures. Finally alpha bacterial and fungal diversity for each structure and tissue from the different soil types and canopy covers were measured using the Shannon index. This was done using ANOVA tests with a linear model and subsequent post-hoc tests of Tukey multiple comparisons of means at a 95% family-wise confidence interval to

measure the difference in diversity between the different structures and tissues from each soil and canopy type.

## 2.3 RESULTS

### 2.3.1 Taxonomic composition of bacterial communities

With rare OTUs occurring fewer than 20 times removed and samples rarefied to 1000 sequences per sample we identified a total of 3288 bacterial OTUs (sequences binned at a 97% similarity cut-off) from the 226 samples. Our collector's curve of the number of OTUs per sample revealed a plateau in the number of additional bacterial taxa with each new sample (Figure 2.S1a), indicating that we sampled the majority of the diversity in the sugar maple microbiome. The highest OTU richnesses were found in the rhizosphere while the lowest richnesses were found in the interior tissue of the leaves. An average of 202 ± 5 OTUs (mean ± SE) per sample was found with averages from each structure and tissue separately of 253 ± 15 per soil sample, 299 ± 6 OTUs per rhizosphere sample, 155 ± 7 OTUs per phyllosphere sample, 222 ± 6 OTUs per root tissue sample, and 98 ± 6 OTUs per leaf tissue sample.

We detected a core microbiome of each structure of the plant as well as for all structures and tissues together by looking at all bacterial OTUs from the samples. The different structures and tissues showed similar taxonomic groups but with varying

relative abundances among the structures and tissues (Table 2.1). The relative abundances of the bacterial phyla were calculated using the combined dataset including the four structures and tissues. The sugar maple microbiome was dominated by four main phyla and 10 major classes. Four of these classes were from Proteobacteria (59.19%): Alpha- (35.41%), Beta- (9.53%), Delta- (1.69%) and Gammaproteobacteria (12.13%). Two of the class were from Acidobacteria (6.79%): DA052 (1.35%) and Acidobacteria (4.45%). Three were from Bacteroidetes (9.37%): Cytophagia (5.87%), Saprospirae (2.04%), Sphingobacteriia (1.18%). Finally, the phylum and class Actinobacteria (15.85%): Actinobacteria (13.95%) were also abundant (Table 2.1) (Figure 2.1a).

### 2.3.2 Indicator species analysis of bacterial taxa

We performed tests on the bacterial communities for biomarker taxa using the LEfSe platform. We used LEfSe with a LDA cutoff of 2 to identify discriminant bacterial taxon between the structures and tissues. First we compared epiphytic or endophytic communities in the abundant phyla and classes and found several associations including 3 major phyla associated with epiphytic communities: Acidobacteria, Firmicutes, and Gemmatimonadetes. We also found the abundant phylum Actinobacteria to be associated with endophytic communities (Figure 2.2a). We compared leaf-associated bacterial communities to the root-associated communities and found that most of the abundant phyla and classes were associated with either

leaves or roots (Figure 2.2b) (Table 2.1). We also found several non-dominant bacterial phyla and classes to have significant associations with either epiphytic or endophytic communities as well as leaf or root communities (Table 2.S2).

We also used LEfSe to investigate if specific bacterial phyla were associated with the different soil or canopy types. We analysed each of the soil, structures and tissues separately from each soil and canopy type. We found that there were many associations overall with the most occurring in the bacterial communities of the rhizosphere and root endophytes when undergoing a change in soil type. There were numerous phyla associations in the soil and rhizosphere communities under the Hardwood canopies when comparing the two canopy types (Table 2.2).

### 2.3.3 Differences in bacterial community structure among structures and tissues

Each of the five bacterial community samples from the tree structures and tissues, along with the soil samples, had distinct community composition (PERMANOVA tests on Bray-Curtis distances). These differences were also visible as compositional differences in a NMDS ordination of the Bray-Curtis dissimilarity values (Figure 2.3a).

Distinct bacterial communities were also found in the soil samples as well as in the two root-associated bacterial communities and the combined dataset when sampled

from different soil types (Table 2.3). From the two canopy types, significant differences in the bacterial communities were found in the combined dataset and also found to be present in every structure and tissue of the host plant except the rhizosphere samples along with the surrounding soil samples (Table 2.3). The effects of changes in soil or canopy type on each bacterial community individually are illustrated using NMDS plots of the Bray-Curtis values (Figure 2.4).

An analysis of variance in community structure (PERMANOVA on Bray-Curtis distances) showed that of the 48.9% explained variance in bacterial community structure, 35.6% ($p=0.001$) was explained by structure or tissue type, 3.8% ($p=0.001$) was explained by soil type and 1.5% ($p=0.001$) was explained by canopy type (Table 2.4).

### 2.3.4 Differences in bacterial community phylogenetic structure

Distinct communities were also found among the different structures and tissues in the bacterial communities using PERMANOVA tests on both the weighted and unweighted UniFrac values ($p=0.001$). Significant differences between soil types were found for all structures and tissues except the phyllosphere using both the unweighted and weighted values. Canopy type showed a significant effect on leaf endophytes using both values, in the phyllosphere with the weighted value and in the root endophytes with the unweighted values (Table 2.3).

### 2.3.5 Taxonomic composition of fungal communities

We detected 2071 fungal OTUs from the 110 samples (Figure 2.S1b). This was after removing singletons of less than 20 and rarefying to 2700 sequences per sample. Similar fungal OTU richness was found in the interior tissue of the leaves and the roots. There was an average fungal OTU richness of 152 ± 2 OTUs (mean ± SE) per sample, with averages from each structure and tissue separately of 158 ± 2 OTUs per root tissue sample and 159 ± 3 OTUs per leaf tissue sample.

The dominant phyla present in the fungal communities were Ascomycota (43.00%), Basidiomycota (11.80%), and Zygomycota (44.40%) (Figure 2.1b). The dominant classes were Dothideomycetes (5.00%), Eurotiomycetes (1.60%), Leotiomycetes (5.70%), and Sordariomycetes (12.00%) from phylum Ascomycota along with Agaricomycetes (10.20%) from Basidiomycota (Table 2.5).

### 2.3.6 Differences in fungal community structure among tissues and soil samples

PERMANOVA tests on the Bray-Curtis distances between fungal community structures showed significant differences between the tissues from the leaf and root samples and the surrounding soil ($p=0.001$) (Figure 2.3b). However we did not find

any effect in any of the communities from different soil types or canopy covers in any of the fungal communities individually or in the combined dataset (Table 2.3).

2.3.7 Shannon diversity of fungal and bacterial communities

The Shannon diversity of the different bacterial communities was significantly different ($p<0.001$) between each pair of the soil, structures, and tissues with the exception of the rhizosphere and the surrounding soil. While there were significant differences between all parts of the plant with the exception of the soil and the rhizosphere, there was no significant difference between the two soil types using the combined dataset ($p=0.718$). When comparing bacterial communities individually between soil types, only the root endophytes showed a significant difference in Shannon diversity ($p=0.01$). When comparing canopy covers, there was no difference found in Shannon diversity in the combined dataset or in any structure, tissue, or soil sample.

Shannon diversity was highest in the rhizosphere and lowest in the interior of the leaves. Soil and the two root-associated bacterial communities contained higher Shannon diversity in comparison to the two leaf-associated bacterial communities ($p<0.001$). We also measured epiphytic communities in comparison to endophytic communities and found that the epiphytic communities contained higher diversity ($p<0.001$) (Figure 2.5). Fungal alpha diversity was similar between both soil and

canopy types in all fungal communities including leaf and root endophytes and the surrounding soil.

### 2.3.8 Correlations of microbial communities with functional traits of host plant

Analysis to find correlations between microbial communities and traits were performed using PERMANOVA tests on the community structure. Host traits that were correlated with bacterial community structure included both SLA and LDMC in the bacterial communities of the phyllosphere samples and the leaf-associated samples (Table 2.6). No other correlations were found between host traits and microbial communities.

## 2.4 DISCUSSION

### 2.4.1 Taxonomic distribution of bacterial and fungal communities

We found most of our samples to be dominated by the same four bacterial and three fungal phyla. The bacterial communities were dominated by taxa known to be soil or plant-associated: Proteobacteria, Actinobacteria, Acidobacteria and Bacteroidetes. While composition at the phylum level was very similar across samples, when looking at finer taxonomic ranks and using OTU abundances we found significant differences between the different plant surfaces and tissues and between the different

environments we sampled from. The Alphaproteobacteria were the most abundant bacterial class in the sugar maple microbiome. This class was highly abundant especially in the two leaf-associated communities (45% – 54% relative abundance). This is not surprising as this class contains many phototrophic and methylotrophic members (Brenner *et al.* 2005) giving them an advantage in colonizing leaf surfaces where simple carbon sources are scarce.

Previous studies have found plant-associated fungal communities to be dominated by Ascomycota (Davey *et al.* 2012) with common classes including Sordariomycetes, Dothidiomycetes and Eurotiomycetes (Kembel *et al.* 2014). We found a similar pattern of high abundances of Ascomycota (43.0%) which included high levels of the soil-associated fungal genera *Lecythophora* (9.8%) which has been found to be pathogenic to wood species (Damm *et al.* 2010). We also found high levels of Zygomycota (44.4%) occurring in both root and leaf samples. These Zygomycota were identified at the genus level as *Mortierella*, a soil fungi which contains 85 species. Some of these species are known to produce fatty acids which may protect plants from phytopathogens (Eroshin & Dedyukhina, 2002).

2.4.2 Microbial associations with different plant structures, tissues and environments

We found that most of the abundant bacterial phyla and classes were significantly associated with either leaf or root samples along with several taxa associated with

epiphytic or endophytic communities (Table 2.1). These included a strong association (LDA>4) of the dominant phylum Acidobacteria to epiphytic samples and the phylum Actinobacteria to endophytic samples. We found the phylum Proteobacteria along with the class Alphaproteobacteria to be associated with leaves although it was dominant in every structure and tissue; this class is known to grow on leaf surfaces as it can survive under low nutrient levels. This association is consistent with other studies, which have found high levels in the relative abundances of Alphaproteobacteria in bacterial communities on leaf surfaces (Kembel *et al.* 2014). We have provided a list of all bacterial phyla and classes found associated with either epiphytic communities compared to endophytic communities or between leaf or root associated communities (Table 2.S2).

We also found several associations of bacterial phyla between the two soil types and canopy types (Table 2.2). Actinobacteria was found associated with natural soil in root endophyte samples and with the two leaf-associated communities in the hardwood canopies. Since some members of endophytic Actinobacteria are able to suppress fungal pathogens (Conn *et al.* 2008) and produce antimicrobials (Golinska *et al.* 2015), this may give an advantage to the trees growing in the natural soil and in the hardwood canopies. However there were more phyla associated with the potted soil over the natural soil in the rhizosphere and root endophyte samples including Proteobacteria, Fibrobacteres, and Gemmatimonadetes. An analysis of the two

canopy types showed several associations of taxa to the hardwood canopy in the soil and rhizosphere samples including the phyla Spirochaetes and Verrucomicrobia (Table 2.2). This higher number of associations is not surprising as conifer forests are known to have higher acidity in their soils (Augusto *et al.* 2002), which is associated with lower bacterial diversity (Lauber *et al.* 2009), along with lower bacterial biomass and activity compared to soil derived from deciduous tree litter (Bauhus *et al.* 1998).

### 2.4.3 Analysis of variance between structures, tissues and environments

Our results showed that each surface and tissue of the host plants was colonized by distinct bacterial and fungal communities in comparison to each other and to the surrounding soil (Figure 2.3). This is not surprising as each of these plant parts would require unique adaptation from the microbes colonizing them for community assembly, for example bacteria colonizing leaf surfaces need to be adapted to low nutrient availability and high UV exposure (Lindow & Brandl 2003). There was less similarity between the two leaf-associated bacterial communities to the three root and soil communities in comparison to each other. This implies that migration of microbes from surfaces to interior tissues is a higher contributor to microbial community structure than migration between leaves and roots.

Bacterial communities of the two leaf-associated samples were correlated with canopy composition. This difference could be driven by differences in the microbes colonizing sugar maples under different canopy types. The microbes of the canopy migrate to the saplings through rain runoff which then colonize the leaves. Microbes may also colonize plants via soil particles. Our results suggest that bacterial migration through rain runoff or other colonization mechanisms is a major potential contributor to community assembly in small undergrowth trees. The change in canopy from deciduous to conifer also affected the endophytic bacterial communities of the roots. It is unlikely that this effect is from rain runoff transfer but it may be caused by changes in soil which occur between deciduous and conifer forests, such as change in soil pH (Augusto *et al.* 2002). The effect was not seen on the communities of the rhizosphere indicating that endophytic bacterial communities of the roots are affected more by changes in canopy type. The effect of the canopy on root endophytes but not on rhizosphere communities was surprising; our prediction was that endophyte communities are more strongly filtered by the plant than rhizosphere communities. This pattern could possibly be explained as a result of the fact that rhizosphere communities will include some taxa from surrounding soils that are not directly interacting with the plant host, whereas endophyte communities will be under more direct control of the plant host which may be responding strongly to canopy conditions, but a test of this hypothesis will require additional experiments.

Changes in soil type created changes in the root-associated bacterial communities but not in the leaf-associated communities or the fungal communities. This suggests that short-term changes in plant growth and colonization from soil immediately below maple seedlings are not sufficient to modify aboveground microbial community structure.

2.4.4 Microbial richness and diversity differed between plant structure and tissue

We investigated alpha diversity and OTU counts for the bacterial communities and found the highest amounts in the rhizosphere samples. This was expected due to the high level microbial diversity found on plant roots relative to other plant parts (Mendes *et al.* 2011). The soil samples showed the second highest OTU counts and alpha diversity, followed by the root endophytes, the phyllosphere and the leaf endophytes with the lowest OTU counts and alpha diversity (Figure 2.5). It is interesting that rhizosphere samples contained higher richness and diversity than the surrounding soil, suggesting that there are microbial taxa adapted specifically to this habitat, rather than the root microbiome containing only a subset of the taxa found in surrounding soils.

We compared leaf-associated microbial communities to root-associated communities by combining surface and tissue data. We also compared endophytic communities to epiphytic by combining data from surfaces or tissues. Previous research has found

higher biodiversity and higher variability in epiphytic bacterial communities compared to endophytic communities in root-associated samples (Edwards *et al.* 2015) while some research has found that this is opposite for leaf-associated bacterial communities (Bodenhausen *et al.* 2013). However we found higher biodiversity in both the epiphytic bacterial samples compared to the endophytic samples of the same plant structure. Epiphytic microbial communities of plant surfaces are exposed to a high level of migration as wind and rain transfer microbes from the surrounding environment onto the plants. Therefore it is not surprising that the diversity was higher in these communities compared to the communities of the tissues. Also selective pressures may be higher in the tissues and the bacteria and fungi that are able to colonize the interior of the plant will need to be specially adapted to gain entry into the tissues.

We found the leaf-associated samples to be lower in alpha diversity compared to root-associated samples. This was not surprising as there is higher environmental stability and nutrient availability below ground due to the exudates from roots which attract beneficial microorganisms (Badri *et al.* 2009). The phyllosphere also contains high selective pressures such as exposure to UV radiation, low nutrient availability and low moisture (Lindow & Brandl 2003).

2.4.5 Correlations between microbial communities and host functional traits

We found correlations between the two leaf functional traits we measured and the bacterial communities of the phyllosphere and combined leaf data (Table 2.6). Correlations between phyllosphere bacteria plant traits have been found when looking at interspecific trait variability (Kembel *et al.* 2014), but this pattern has not been previously documented within a single plant species. This implies there is a driving factor connecting the leaf bacterial communities with the leaf traits, however without further experimentation using manipulation of the bacterial taxa present we are unable to determine what processes are creating these correlations.

While it is thought that host traits drive fungal colonization (Saikkonen *et al.* 2004) and previous studies have found correlations between epiphytic leaf fungi and leaf traits (Kembel & Mueller 2014), we did not find any correlation between the endophytic fungal communities and the leaf traits we measured.

### 2.4.6 Comparison of bacterial and fungal results in endophytic communities

We investigated individually both fungal communities using ITS sequencing and bacterial communities using 16S rRNA sequencing in the two endophytic communities of the leaves and roots along with samples from the surrounding soil. By looking at these endophytic communities in comparison, we can investigate how they vary differently with changes in environment. While most of the endophytic bacterial communities varied with changes in soil or canopy, the fungal communities

did not show any significant differences between environments. This indicates that changes in fungal communities are not correlated to changes in bacterial communities and that they remain more stable under environmental changes than bacterial communities. Future studies will be required if this is due to broader environmental tolerances or slower growth rates of fungi, or some other factor.

## 2.5 CONCLUSION

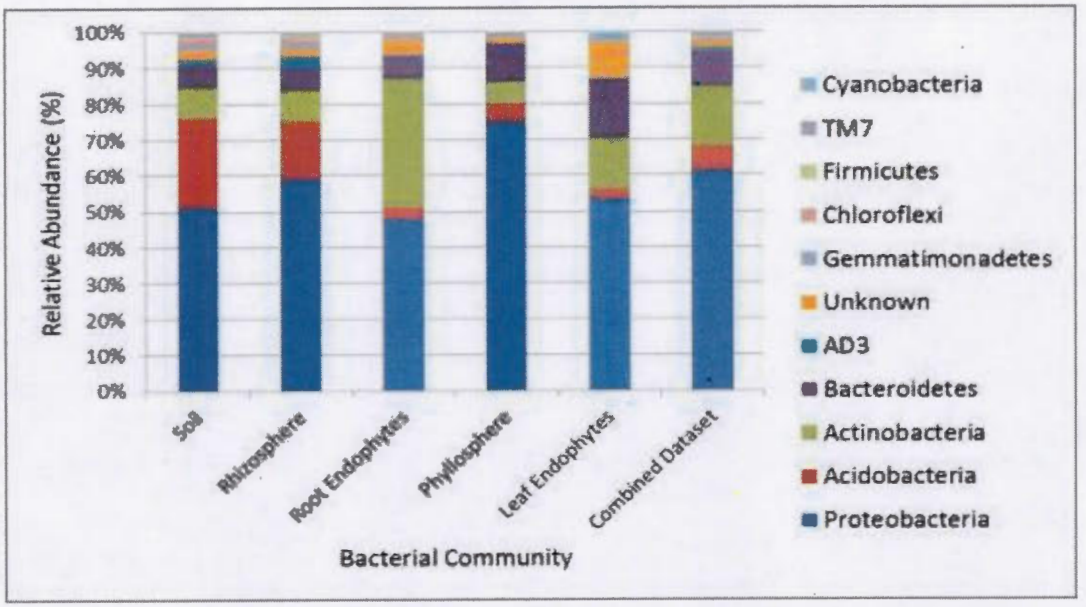
In this study we used high-throughput DNA sequencing of bacterial and fungal molecular markers to compare the microbial communities of sugar maple saplings from different structures and tissues and from different canopies and soil types. In summary, sugar maple saplings were found to have distinct bacterial and fungal communities inhabiting each of the structures and tissues we analyzed. We found greater similarity between interior tissues to their exterior surfaces than between above and below ground part of the plants.

We found that a change in canopy from hardwood to conifer species affected bacterial leaf-associated communities on undergrowth sugar maple saplings along with the bacterial communities of the root tissue. We also investigated different soil types and found that soil changes affected bacterial communities both on the surface and in the tissues of the roots of sugar maple. Soil changes did not cause variation in leaf-associated bacterial communities. Fungal community data from the soil, leaf

tissues and root tissues showed that all fungal communities were unaffected by changes in both soil and canopy composition.

In this study we have identified the dominant taxa of the bacterial and fungal communities of each plant part separately on a deciduous tree species under different environments. This research furthers our understanding of these associations and the structure and assembly of microbial communities found on sugar maple trees. This study also provides a baseline for future study into sugar maple microbiome research using experimental manipulations of the microbiome and its effects on host health and functional traits. The importance of microbes in the role of their host's fitness and function is becoming apparent by recent studies (Zamioudis & Pieterse, 2012) and further research into these dynamics is needed to improve our understanding of plant-microbe interactions.

a)




b)

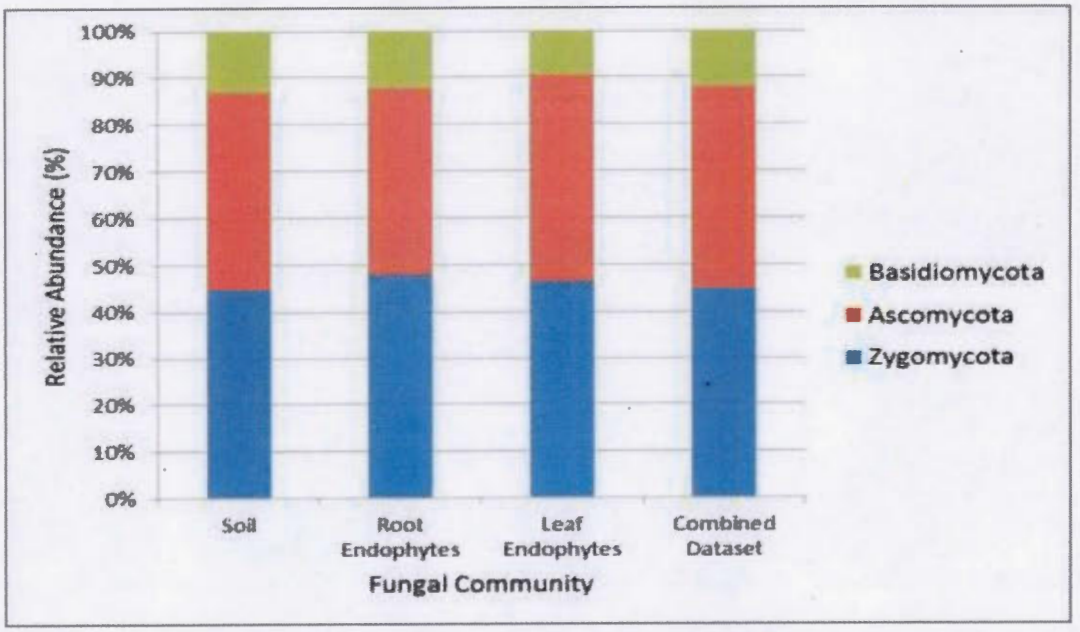
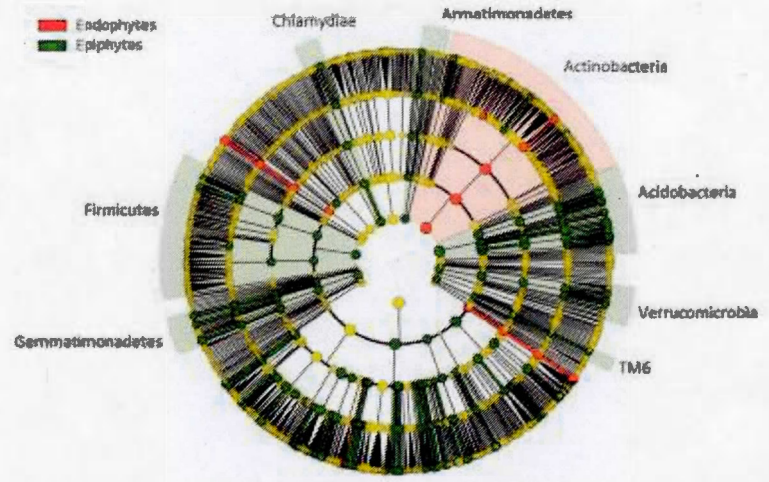



Figure 2.1: Relative abundances (%) of bacteria (a) and fungi (b) phyla found in the different plant tissues and surfaces of sugar maple saplings in a Quebec forest.

a)




b)

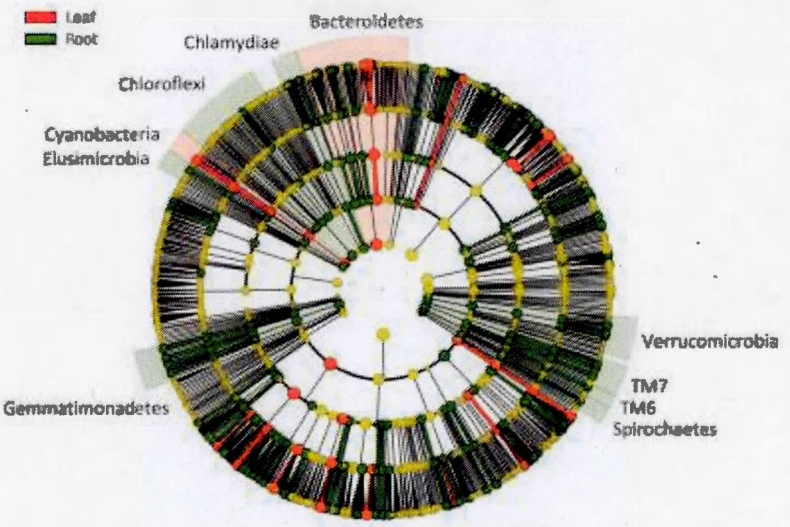
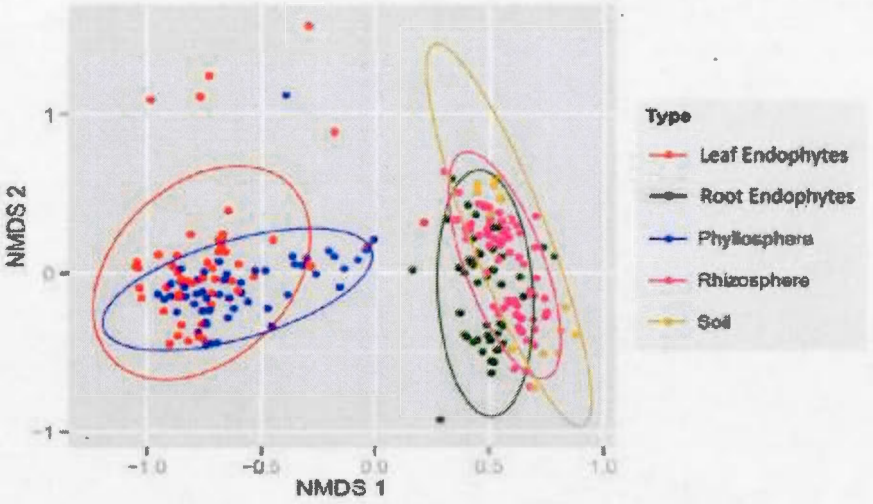



Figure 2.2: Associations between bacterial taxa and sugar maple seedlings. Results are cladograms of LEfSe results showing bacterial indicator taxa at the phyla levels comparing a) epiphytic to endophytic communities where red indicates a high presence in the leaf or root endophytic samples while green indicates epiphytic and b) root-associated to leaf-associated communities where red indicates a high presence in leaf samples while green indicates root samples.

a)




b)

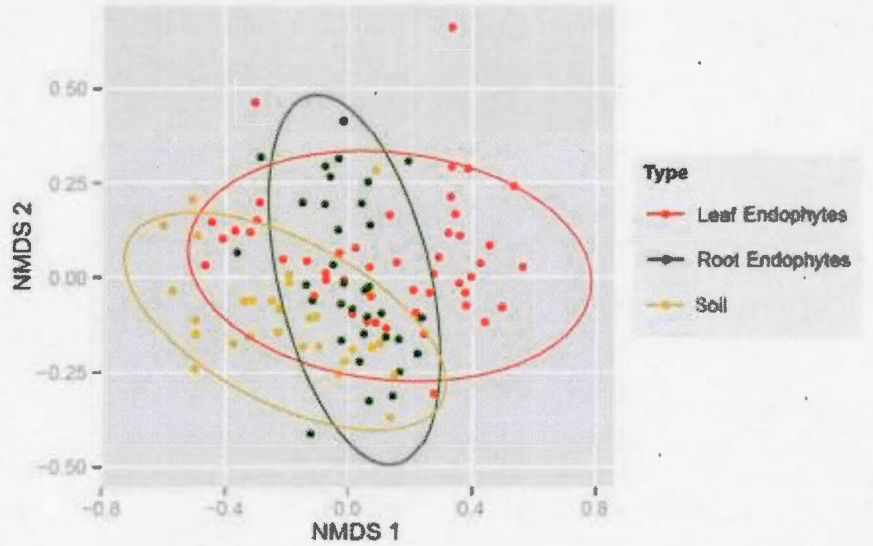
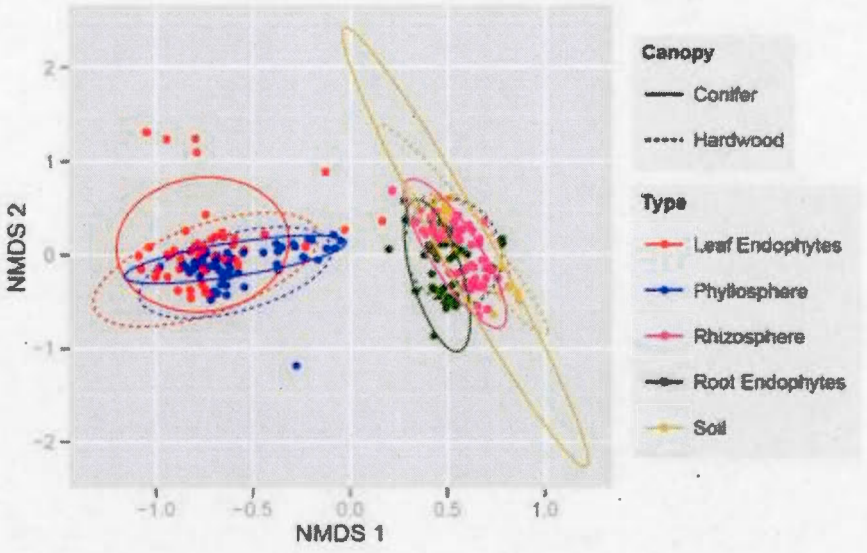



Figure 2.3: Non-metric multidimensional scaling (NMDS) ordination using Bray-Curtis distance of the dissimilarity between a) bacterial communities and b) fungal communities from different plant surfaces and tissues of sugar maple saplings. Permutational analysis of variance showed significant differences in bacterial and fungal communities amongst all groups (p=0.001). Ellipses indicate 95% confidence intervals around samples from each category.

a)




b)

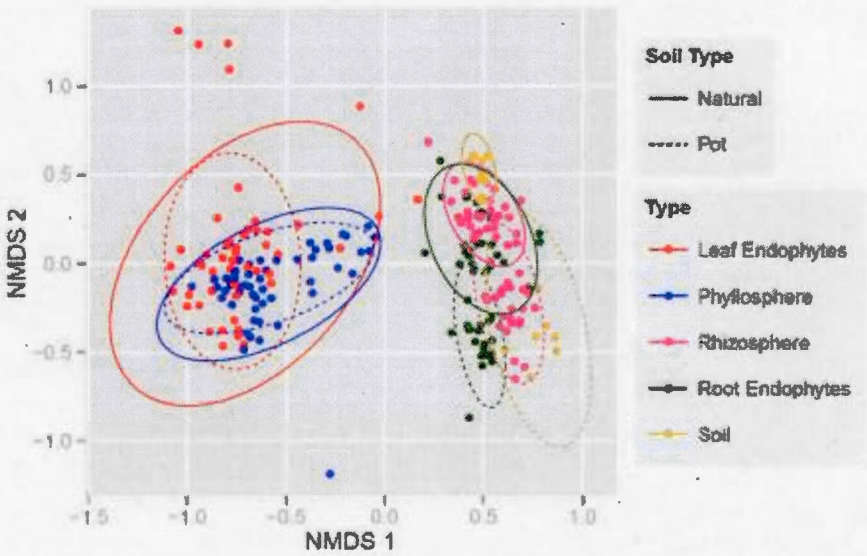



Figure 2.4: Non-metric multidimensional scaling (NMDS) ordination using Bray-Curtis distance of the dissimilarity between bacterial communities from different plant surfaces and tissues showing differences in a) canopy type and b) soil type. Ellipses indicate 95% confidence intervals around samples from each category.

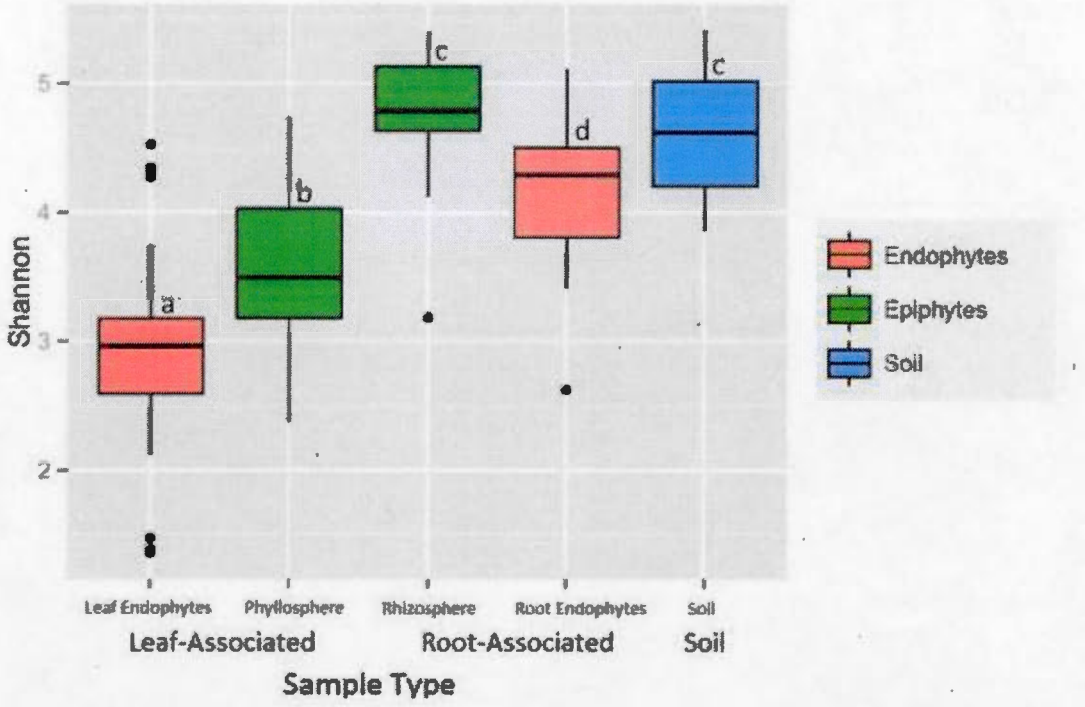



Figure 2.5: Shannon diversity values for bacterial communities of sugar maples. Soil, structure and tissues had significantly different diversity (ANOVA test and post-hoc test of Tukey multiple comparisons; $p<0.001$) with the exception of the rhizosphere and the soil samples. Leaf-associated and root-associated samples and epiphytic versus endophytic communities also had significantly different Shannon diversity (ANOVA; $p<0.001$). Bacterial communities that do not share letter indicate a difference according to the Tukey post-hoc test.

Table 2.1: List of relative abundances (%) of the most abundant bacteria phyla and classes associated with sugar maples, for different structures, tissues and from combined dataset, along with the significant associations taxa showed using the LDA Effect Size platform (LEfSe). Bacterial phyla are represented in bold text while classes are represented in italics.

| Taxon | Soil | Phyllosphere | Root Endophytes | Rhizosphere | Leaf Endophytes | Combined Dataset | Taxa is an indicator of: |
|---|---|---|---|---|---|---|---|
| **AD3** | **1.72%** | **0.23%** | **0.12%** | **3.21%** | **0.34%** | **0.95%** | |
| **Acidobacteria** | **24.40%** | **5.09%** | **3.34%** | **15.71%** | **2.82%** | **6.79%** | **Roots Epiphytes** |
| *- Acidobocteriio* | *7.68%* | *4.58%* | *2.45%* | *8.14%* | *2.30%* | *4.45%* | *Epiphytes* |
| *- DA052* | *8.90%* | *0.24%* | *0.46%* | *4.55%* | *0.24%* | *1.35%* | *Roots Epiphytes* |
| **Actinobacteria** | **8.47%** | **5.93%** | **35.09%** | **8.43%** | **14.12%** | **15.85%** | **Roots Endophytes** |
| *- Actinobacteria* | *5.16%* | *5.57%* | *31.25%* | *5.55%* | *13.78%* | *13.95%* | Endophytes |
| **Bacteroidetes** | **5.99%** | **10.53%** | **6.41%** | **6.02%** | **16.07%** | **9.37%** | **Leaf** |
| *- Cytophagia* | *0.66%* | *8.67%* | *0.80%* | *0.54%* | *15.48%* | *5.87%* | |
| *- Saprospirae* | *4.54%* | *0.38%* | *3.61%* | *3.77%* | *0.22%* | *2.04%* | *Roots* |
| *- Sphingobacteriia* | *0.72%* | *1.17%* | *1.51%* | *1.51%* | *0.29%* | *1.18%* | *Roots* |
| **Chloroflexi** | **1.68%** | **0.06%** | **0.50%** | **1.35%** | **0.11%** | **0.5%** | **Roots** |
| **Cyanobacteria** | **0.03%** | **0.21%** | **0.03%** | **0.02%** | **2.05%** | **0.46%** | **Leaf** |
| **Firmicutes** | **0.69%** | **0.22%** | **0.31%** | **0.95%** | **0.22%** | **0.42%** | **Roots Epiphytes** |
| **Gemmatimonadetes** | **1.19%** | **0.04%** | **0.26%** | **1.36%** | **0.05%** | **0.42%** | **Roots Epiphytes** |
| **Proteobacteria** | **50.44%** | **74.63%** | **47.11%** | **58.05%** | **53.06%** | **59.19%** | **Leaf** |
| *- Alpha* | *25.92%* | *54.03%* | *15.50%* | *26.86%* | *44.86%* | *35.41%* | *Leaf* |
| *- Beta* | *9.43%* | *8.76%* | *9.05%* | *14.20%* | *5.39%* | *9.53%* | *Epiphytes* |
| *- Delta* | *3.90%* | *1.80%* | *1.76%* | *2.37%* | *0.53%* | *1.69%* | *Roots Epiphytes* |
| *- Gamma* | *10.26%* | *9.79%* | *20.33%* | *13.79%* | *2.12%* | *12.13%* | *Roots Epiphytes* |
| **TM7** | **1.34%** | **1.26%** | **1.05%** | **0.80%** | **0.71%** | **0.99%** | **Roots** |
| **Other** | **2.47%** | **1.22%** | **4.29%** | **2.11%** | **9.88%** | **1.57%** | |

Table 2.2: List of all bacteria phyla found to have significant associations with sugar maple from certain environmental conditions in each structure and tissue using the LDA Effect Size platform (LEfSe).

| | Potted Soil | Natural Soil | Conifer Canopy | Hardwood Canopy |
|---|---|---|---|---|
| **Soil** | Planctomycetes | | | Acidobacteria<br>Firmicutes<br>Gemmatimonadetes<br>Spirochaetes<br>TM6<br>Verrucomicrobia |
| **Rhizosphere** | Bacteroidetes<br>Chlorobi<br>Cyanobacteria<br>Fibrobacteres<br>FBP<br>Gemmatimonadetes<br>Proteobacteria<br>Thermi | Acidobacteria<br>Elusimicrobia | FBP | AD3<br>Elusimicrobia<br>Spirochaetes<br>Verrucomicrobia<br>WS6 |
| **Root Endophytes** | Chlorobi<br>Fibrobacteres<br>FBP<br>Gemmatimonadetes<br>Proteobacteria | Actinobacteria<br>Chloroflexi<br>Spirochaetes | Firmicutes<br>Proteobacteria | AD3<br>Spirochaetes |
| **Phyllosphere** | | | Proteobacteria | Actinobacteria<br>Bacteriodetes<br>TM7 |
| **Leaf Endophytes** | Proteobacteria | Chloroflexi | Cyanobacteria<br>Proteobacteria | Actinobacteria<br>Bacteriodetes |

Table 2.3: Results of PERMANOVA tests on distance matrices using Bray-Curtis values and UniFrac values using both weighted and unweighted methods. Differences between bacterial and fungal communities of sugar maple found in different structures and tissues from two soil types (potted soil versus natural soil) and two canopy type (conifer versus hardwood canopy species).

| | Environmental Factor | Bray-Curtis | | Unweighted UniFrac | | Weighted UniFrac | |
|---|---|---|---|---|---|---|---|
| Bacterial Communities | | $R^2$ | P-Value | $R^2$ | P-Value | $R^2$ | P-Value |
| **Soil** | Soil Type | 0.3491 | 0.001 | 0.36652 | 0.001 | 0.41418 | 0.003 |
| | Canopy Type | 0.04736 | 0.589 | 0.04824 | 0.526 | 0.02948 | 0.648 |
| **Rhizosphere** | Soil Type | 0.22382 | 0.001 | 0.20687 | 0.001 | 0.28191 | 0.001 |
| | Canopy Type | 0.03018 | 0.066 | 0.0246 | 0.193 | 0.03643 | 0.065 |
| **Root Endophytes** | Soil Type | 0.1975 | 0.001 | 0.15133 | 0.001 | 0.26462 | 0.001 |
| | Canopy Type | 0.06285 | 0.005 | 0.05579 | 0.045 | 0.0499 | 0.059 |
| **Phyllosphere** | Soil Type | 0.03523 | 0.062 | 0.02899 | 0.203 | 0.0322 | 0.148 |
| | Canopy Type | 0.1245 | 0.001 | 0.01562 | 0.437 | 0.15267 | 0.001 |
| **Leaf Endophytes** | Soil Type | 0.03678 | 0.073 | 0.06054 | 0.048 | 0.05528 | 0.047 |
| | Canopy Type | 0.09993 | 0.001 | 0.07158 | 0.033 | 0.05456 | 0.047 |
| **Combined Dataset** | Soil Type | 0.04127 | 0.001 | 0.01271 | 0.07 | 0.01632 | 0.031 |
| | Canopy Type | 0.0175 | 0.003 | 0.00569 | 0.258 | 0.01277 | 0.05 |
| Fungal Communities | | $R^2$ | P-Value | $R^2$ | P-Value | $R^2$ | P-Value |
| **Soil** | Soil Type | 0.02527 | 0.569 | NA | NA | NA | NA |
| | Canopy Type | 0.01804 | 0.695 | NA | NA | NA | NA |
| **Root Endophytes** | Soil Type | 0.00969 | 0.904 | NA | NA | NA | NA |
| | Canopy Type | 0.03601 | 0.285 | NA | NA | NA | NA |
| **Leaf Endophytes** | Soil Type | 0.01281 | 0.58 | NA | NA | NA | NA |
| | Canopy Type | 0.01392 | 0.506 | NA | NA | NA | NA |
| **Combined Dataset** | Soil Type | 0.00442 | 0.739 | NA | NA | NA | NA |
| | Canopy Type | 0.01132 | 0.268 | NA | NA | NA | NA |

Table 2.4: Bacterial community structure variation explained by different factors (PERMANOVA on Bray-Curtis dissimilarities). The total $R^2$ explained by the model was 48.9%. Significance levels for each variable are given by: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; $_{NS}$, $P > 0.1$.

| | | Bray-Curtis | |
|---|---|---|---|
| **Variable** | | **$R^2$ (%)** | **Pr(>F)** |
| Single Factor | Type | 35.57 | $0.001^{***}$ |
| | Soil | 3.78 | $0.001^{***}$ |
| | Canopy | 1.54 | $0.001^{***}$ |
| $2^{nd}$ order Interaction | Type*Soil | 4.51 | $0.001^{***}$ |
| | Type*Canopy | 0.34 | $0.001^{***}$ |
| | Canopy*Soil | 2.44 | $_{NS}$ |
| $3^{rd}$ order interaction | Type*Canopy*Soil | 0.72 | $_{NS}$ |

Table 2.5: Relative abundances (%) of the dominant fungal phyla and classes associated with the soil and tissues of sugar maple and in the combined dataset. Fungal phyla are represented in bold text while classes are represented in italics.

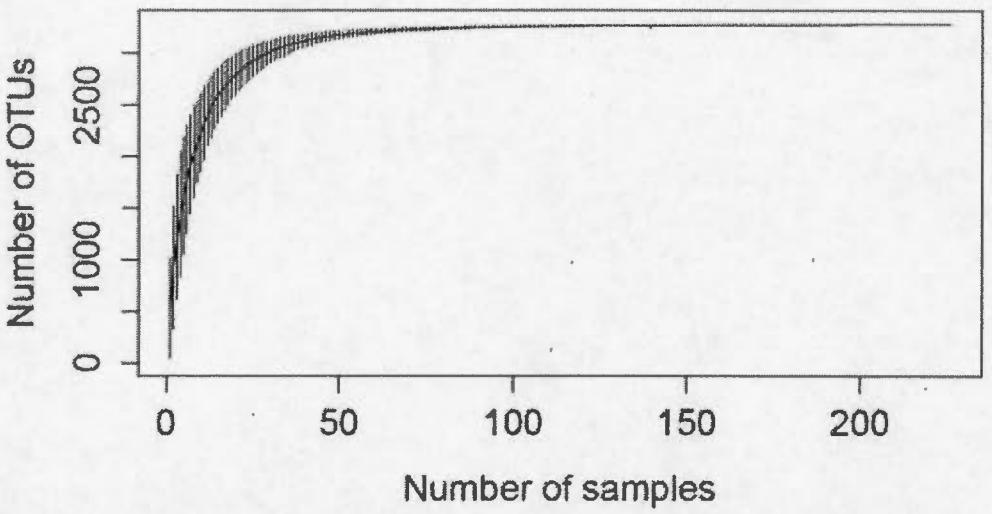
| Taxon | Soil | Root Endophytes | Leaf Endophytes | Combined Dataset |
|---|---|---|---|---|
| **Ascomycota** | **42.44%** | **40.00%** | **44.20%** | **43.00%** |
| *- Dothideomycetes* | *4.85%* | *4.62%* | *5.44%* | *5.00%* |
| *- Eurotiomycetes* | *2.07%* | *1.43%* | *1.36%* | *1.60%* |
| *- Leotiomycetes* | *5.89%* | *5.47%* | *5.92%* | *5.70%* |
| *- Sordariomycetes* | *10.98%* | *11.73%* | *12.67%* | *12.00%* |
| **Basidiomycota** | **12.93%** | **12.08%** | **9.42%** | **11.80%** |
| *- Agaricomycetes* | *10.43%* | *11.00%* | *8.13%* | *10.20%* |
| **Zygomycota** | **44.44%** | **47.66%** | **46.14%** | **44.40%** |

Table 2.6: Correlations between sugar maple sapling functional traits and microbial community structure. Functional traits correlation tests performed with PERMANOVA tests on Bray-Curtis dissimilarity values and functional trait values. Significance levels for each variable are given by: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

| | SLA | | LDMC | |
|---|---|---|---|---|
| **Bacterial Communities** | **$R^2$** | **P-Value** | **$R^2$** | **P-Value** |
| Rhizosphere | 0.02211 | 0.234 | 0.02109 | 0.280 |
| Root Endophytes | 0.03613 | 0.149 | 0.03180 | 0.221 |
| Combined Root | 0.01670 | 0.091 | 0.01613 | 0.100 |
| Phyllosphere | 0.04778 | 0.016* | 0.08467 | 0.001*** |
| Leaf Endophytes | 0.02354 | 0.401 | 0.03120 | 0.208 |
| Combined Leaf | 0.02331 | 0.032* | 0.04162 | 0.001*** |
| Combined Endophytes | 0.00972 | 0.574 | 0.01022 | 0.546 |
| Combined Epiphytes | 0.00948 | 0.399 | 0.01312 | 0.222 |
| Combined Dataset | 0.00705 | 0.239 | 0.00940 | 0.101 |
| | | | | |
| **Fungal Communities** | **$R^2$** | **P-Value** | **$R^2$** | **P-Value** |
| Root Endophytes | 0.01148 | 0.862 | 0.04942 | 0.213 |
| Leaf Endophytes | 0.02166 | 0.412 | 0.00727 | 0.956 |
| Combined Dataset | 0.01009 | 0.566 | 0.01882 | 0.221 |

Figure 2.S1: Operational taxonomic unit (OTU; 97% sequences similarity) collector's curve (mean with 95% confidence intervals) based on random sampling of data before rarefaction with no singletons a) bacterial samples and b) fungal samples.

a)




b)

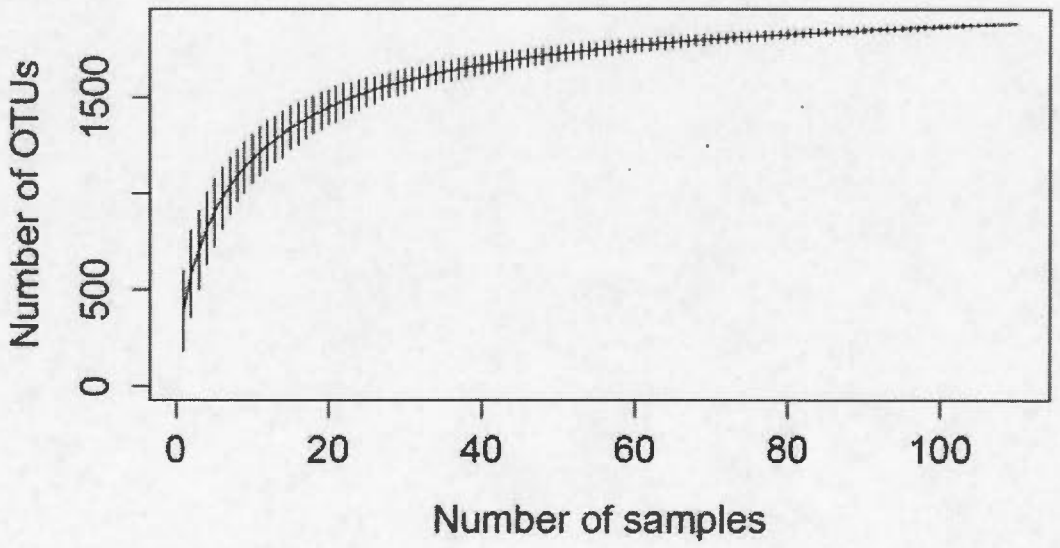


Table 2.S1: Number of sugar maple saplings sampled from different canopy covers and soil types at the Laurentian Biology Station sampling site.

| Canopy Type | Potted Soil | Natural Soil | Total |
|---|---|---|---|
| Hardwood | 9 | 11 | **20** |
| Conifer | 17 | 17 | **34** |
| **Total** | **26** | **28** | **54** |

Table 2.S2: Complete list of indicator taxa found at the phyla and class levels for comparisons between epiphytic bacterial communities to endophytic and between leaf and root-associated bacterial communities of sugar maple saplings. Bacterial phyla are represented in bold text while classes are represented in italics.

| | Epiphytic | Endophytic | Leaf | Root |
|---|---|---|---|---|
| **PHYLA** | **Acidobacteria** | **Actinobacteria** | **Bacteroidetes** | **Acidobacteria** |
| | **Armatimonadetes** | | **Cyanobacteria** | **Actinobacteria** |
| | **Chlamydiae** | | **Proteobacteria** | **Armatimonadetes** |
| | **Firmicutes** | | | **Chlamydiae** |
| | **Gemmatimonadetes** | | | **Chlorobi** |
| | **TM6** | | | **Chloroflexi** |
| | **Verrucomicrobia** | | | **Elusimicrobia** |
| | | | | **Firmicutes** |
| | | | | **Gemmatimonadetes** |
| | | | | **Spirochaetes** |
| | | | | **TM6** |
| | | | | **TM7** |
| | | | | **Verrucomicrobia** |
| *CLASSES* | *Acidobacteriia* | *Actinobacteria* | *Alphaproteobacteria* | *Acidimicrobiia* |
| | *Bacilli* | *Chloroplast* | *Chloroplast* | *Armatimonadia* |
| | *Betaproteobacteria* | | *Cytophagia* | *Bacilli* |
| | *Chlamydiia* | | | *Chlamydiia* |
| | *Clostridia* | | | *Chloroflexi* |
| | *DA052* | | | *Clostridia* |
| | *Deltaproteobacteria* | | | *DA052* |
| | *Fimbriimonadia* | | | *Deltaproteobacteria* |
| | *Gammaproteobacteria* | | | *Elusimicrobia* |
| | *Gemm 1* | | | *Fimbriimonadia* |
| | *Gemmatimonadetes* | | | *Gammaproteobacteria* |
| | *Pedosphaerae* | | | *Gemm 1* |
| | *SJA 4* | | | *Gemmatimonadetes* |
| | *Solibacteres* | | | *Pedosphaerae* |
| | *TK10* | | | *S085* |
| | | | | *Saprospirae* |
| | | | | *SJA 4* |
| | | | | *Solibacteres* |
| | | | | *Sphingobacteriia* |
| | | | | *Sva0725* |
| | | | | *Thermoleophilia* |
| | | | | *TK10* |
| | | | | *TK17* |

# CONCLUSION

In general the goal of this thesis was to obtain an understanding of the core microbiome of *Acer saccharum* in Quebec forests, and the relationships between sugar maple-associated microbial communities and their hosts. Using DNA sequencing and identification of the bacteria and fungi found on sugar maple plants we carried out an in-depth analysis of the assembly, structure and diversity of these communities. Also by examining changes in the microbiome under different environmental conditions, along with physical characteristics of the plants themselves, we acquired new insights into the driving factors in sugar maple-microbe associations.

In summary we found that there are distinct bacterial and fungal communities found inhabiting different plant surfaces and tissues of sugar maples, with similar broad taxonomic groups present in each surface and tissue, but with fine-scale taxonomic and phylogenetic differences at the level of OTUs. We found a high amount of variation between leaf and root-associated communities, and less variation between surface versus tissue communities of both roots and leaves. We found microbial alpha diversity measured as both OTU richness and Shannon diversity to be higher in root-associated communities compared to leaf-associated and in surface compared to

tissue communities. We also found several associations of bacterial taxa with either leaves or roots, as well as with either surface or tissue communities.

Elevational range edges caused significant differences in the bacterial and fungal communities of all parts of the host plant we investigated. When comparing these results to the data we found when looking at a change in canopy or soil, it implies that there are several factors created by the elevational limit that are driving variation in microbial communities in both the leaf and root-associated samples. These could include canopy species, soil pH or temperature among other changes. Regardless of the cause of these differences, our results suggest a potential role for biotic limits to the elevational range of sugar maples that will need to be investigated by future manipulative studies.

Changes in canopy from deciduous- to conifer-dominated did not affect fungal communities associated with sugar maple seedlings, but did affect bacterial communities of the sugar maple leaf-associated communities along with bacterial root endophytes. This suggests that bacterial colonization through rain runoff or soil particles could be a major contributor to leaf surface and leaf tissue community assembly in small undergrowth trees. Changes in soil from the natural soils found in the forests of Southern Quebec to a potting soil containing added fertilizer and mycorrhizae fungi created changes in the root-associated bacterial communities but not in the leaf-associated bacterial communities or the fungal communities. These

results show that short-term changes in below ground bacterial communities are unlikely to drive changes in above ground communities. These results plus the results of the other two environmental changes also shows bacterial communities to be highly susceptible to changes caused by the environment while fungal communities often remain unaffected.

This study was the first to document the microbial communities on sugar maple trees using high throughput DNA sequencing of bacterial and fungal biomarkers. However it was only able to explore a few of the environmental conditions that could drive variation in these microbial communities. Further research to explore the relationship between plants, microbes and environment on this tree species will be needed. Ultimately, this study has provided a baseline demonstrating the sensitivity of the sugar maple microbiome to plant attributes and environmental factors, and suggests several avenues for follow-up studies that can more directly test the importance of microbial diversity and individual microbial taxa for sugar maple growth and fitness in response to environmental change.

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