The effects of stumping and tree species composition on the soil microbial community in the Interior Cedar Hemlock Zone, British Columbia

by

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Abstract

Stump removal (stumping) is an effective forest management practice used to reduce the mortality of trees affected by fungal pathogen-mediated root diseases such as Armillaria root rot, but its impact on soil microbial community structure has not been ascertained. This study investigated the long-term impact of stumping and tree species composition on the abundance, diversity and taxonomic composition of soil fungal and bacterial communities in a 48-year-old trial at Skimikin, British Columbia. We used DNA metabarcoding targeting the fungal internal transcribed spacer (ITS) marker and the bacterial 16S rRNA gene to decipher the microbiomes. A total of 108 samples were collected from the FH (fermented and humus layers), 0-10 cm (A horizon) and 10-20 cm (B horizon) layers in 36 plots, 18 stumped and 18 unstumped, that were planted with pure stands and admixtures of Douglas-fir, western redcedar and paper birch. Fungal α-diversity in the A horizon increased with stumping regardless of tree species composition and had a tendency to increase in the FH and B horizons. In the FH horizon, the relative abundance of the saprotrophic fungal community declined while that of ectomycorrhizal (ECM) fungal community increased with stumping. Bacterial α-diversity in the B horizon declined with stumping, irrespective of tree species, and also tended to decrease in the A horizon. The B horizon of stumped plots was significantly enriched with potential plant growth-promoting bacteria (PGPR), such as rhizobia. Similarly, Pseudomonadales, known for their antagonistic role against pathogens, increased significantly in all three soil horizons with stumping and was especially observed in association with birch and its admixtures. The culture-based assessment focused on 16S rDNA substantiated the dominance of potential PGPRs in the stumped plots. Furthermore, molecular characterization of Armillaria using translation elongation factor-1 alpha (tef-1) and ITS revealed the occurrence of A. gallica, reported for the

first time at this site. Overall, we conclude that stumping along with plantation of resistant tree species with susceptible ones, led to a healthy fungal community structure and promotion of a beneficial bacterial microbiome, thus proves as a potent practice for the suppression of Armillaria root rot and promotion of forest health.

Lay Summary

Armillaria, commonly known as the humongous fungus, causes root rot in woody plants, reducing tree growth and may even lead to death. This fungus usually survives on dead woody stumps, but some species infect living trees, thus causing loss in timber production. For the management of this root disease, stumps are often removed in order to reduce the fungal inoculum load and risk of infection of live trees. Stump removal is performed using heavy machinery, and the loss of coarse woody debris reduces habitat quality and the source of energy for various microorganisms, and it is therefore important to know its impacts on the soil microbial ecosystem. This research shows that stump removal can have positive impacts on soil microbial communities, and when performed along with planting of mixtures of tree species such as Douglas-fir with paper birch, it increases the abundance of beneficial microbes thus protects against *Armillaria*.

Preface

For this study, Suzanne Simard, Les Lavkulich and Dixi Modi identified the key research questions and developed the general research design. Together with Suzanne Simard, Dixi Modi selected the specific research site. Sampling design and soil collection was done by Dixi Modi with the help of Jean Roach and Elana Evans. Soil analysis was done by Dixi Modi and the Analytical Chemistry Services Laboratory, B.C. Ministry of Environment and Climate Change Strategy, Victoria, B.C., Canada. All the laboratory work, data and statistical analyses was done by Dixi Modi. Bioinformatics analysis was done with the help of Dr. Jean Bérubé and under the advisement of Dr. Richard Hamelin.

For chapter 2, Dixi Modi planned the experimental design and did all the laboratory experimental work, bioinformatics and statistical data analyses. Jean Bérubé help with sequencing and bioinformatics work. Dixi Modi wrote the manuscript along with Suzanne Simard. Richard Hamelin, Sue Grayston, Les Lavkulich, and Jean Bérubé provided valuable suggestions and edits.

For chapter3, Dixi Modi planned the experimental design and did all the laboratory experimental work, bioinformatics and statistical data analyses. Dixi Modi wrote the manuscript along with Suzanne Simard. Richard Hamelin, Sue Grayston and Les Lavkulich provided valuable suggestions and edits.

For chapter 4, Dixi Modi planned the experimental design and did the fungal basidiocarp collection. Dixi Modi did the soil collection along with Eva Snyder. Dixi Modi performed the

bioinformatics analysis, statistical work, and wrote the manuscript. Suzanne Simard, Richard Hamelin provided the valuable suggestions and edits.

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List of Acronyms and Abbreviations

AM	Arbuscular Mycorrhiza
ANOSIM	Analysis of Similarity
ANOVA	Analysis of Variance
ASVs	Amplicon Sequence Variants
B.C.	British Columbia
bp	Base pairs
C	Carbon
CAP	Constrained Analysis of Principal Coordinates
CCMA	Combined Carbon Medium Agar
ECM	Ectomycorrhiza/l
FDR	False Discovery Rates
FH	Fermented and Humus layer
GLM	Generalized Linear Model
IGS	Intergenic Spacer
ITS	Internal transcribed spacer
LDA	Linear Discriminant Analysis
LEfSe	Linear Discriminant Analysis and Effect Size
MEGA	Molecular Evolutionary Genetics Analysis
ML	Maximum Likelihood
MOTU	Molecular Operational Taxonomic Unit

MP	Maximum Parsimony
MPB	Mountain Pine Beetle
N	Nitrogen
NA	Nutrient Agar
NGS	Next Generation Sequencing
OM	Organic Matter
OTU	Operational Taxonomic Unit
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PERMANOVA	Permutational Analysis of Variance
PGPR	Plant Growth Promoting Rhizobacteria
QIIME	Quantitative insights into Microbial Ecology
rDNA	Ribosomal DNA
RDP	Ribosomal Database Project
TBR	Tree-bisection-regrafting
Tef-1	Translation Elongation factor -1 alpha
TSS	Total Sum Scaling

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My thesis would have never been complete without the immense support of my loving husband, Dr. Basit Yousuf who has been patient with me throughout this journey.

To my Family,

To my best friend and husband, Basit,

To my little baby for whom I am eagerly waiting to arrive in this world.

Chapter 1: Introduction

1.1 Forest soils as a dynamic ecosystem

Forests are highly productive terrestrial ecosystems covering over 40 million km^2 and representing 30% of the land portion of earth (Keenan et al., 2015). Forests play essential ecological functions, ranging from carbon/nitrogen storage, water/air purification, and wildlife habitat conservation (Perry et al., 2009). There are around 3 trillion forest trees on Earth (Crowther et al., 2015), substantially contributing to the mitigation of climate change, health of the timber industry, as well as providing recreation, food, fuel, innumerable diverse bioproducts, and conservation of biodiversity. The forests are spread out globally, with Canada the second leading country, where they cover 35% of the country's total area (347 million ha) and represent 30% of the world's forest. Canada is rich in eight types of forests, including: (i) Deciduous, (ii) Acadian, (iii) Boreal, (iv) Montane, (v) Columbia, (vi) Subalpine, (vii) Great Lakes/St. Lawrence, and (viii) Coastal. British Columbia, the westernmost province of Canada, is divided into 14 bio-geoclimatic zones based on climate and vegetation, including: 1) Alpine Tundra, 2) Spruce-Willow-Birch, 3) Boreal White and Black Spruce, 4) Sub-Boreal Pine-Spruce, 5) Sub-Boreal Spruce, 6) Mountain Hemlock, 7) Engelmann Spruce-Subalpine Fir, 8) Montane Spruce, 9) Bunchgrass, 10) Ponderosa Pine, 11) Interior Douglas-fir, 12) Coastal Douglas-fir, 13) Interior Cedar-Hemlock, and 14) Coastal Western Hemlock. These zones are based on the classification system developed by V.J. Krajina and his students in the 1960's and the 1970's, which was adopted by the B.C. Ministry of Forests in 1976 and is widely used for forest, range and wildlife management (Pojar et al., 1987).

Forests have an intricate and interdependent relationship with forest soils, which act as an anchorage as well as source of nutrients and water, and in return, trees help in health and development of soils by the addition of litter, deadwood, microbial biomass and the photosynthate

rhizodeposits. Forest soils are referred as a C sink because 30 to 50% of C fixed by the forest trees is transported belowground via roots to the microbial communities (Zak et al., 1993; Högberg et al., 2001, Kaiser et al., 2010). Bacteria and fungi play an important role in the transformation of organic matter, as well as soil carbon (C), nitrogen (N), and phosphorus (P) cycling, linking forest and soil ecosystems (Koranda et al., 2013; Trivedi et al., 2013). These microbes inhabit multiple parts of forests, including different horizons of the soil, litter, roots and the rhizosphere, the wood of living trees, the bark surface, deadwood, litter, etc. (Baldrian, 2017). Each forest supports a unique below-ground soil microbial community with a structure that depends upon temporal dynamics (Li et al., 2014) as well as tree species composition, indicating a strong link between above- and below-ground processes (Hackl et al., 2005; Li et al., 2014).

This study was conducted in a temperate forest rich in coniferous and deciduous trees. Temperate forests are considered an important global C sink, storing 14% of global soil C, compared to 32% in boreal forests (Pan et al., 2011). Large amounts of organic material accumulate on the forest floor (organic layer), and the decomposition rate varies according to the forest type (Chapin et al., 2011). The difference in the substrate quality and quantity between organic and mineral soils leads to fluctuations in microbial growth and development (Yang et al., 2007), and shapes the microbial communities in different soil horizons. Furthermore, anthropogenic activities and environmental factors such as soil heterogeneity, nitrogen (N) deposition, pH, organic matter, and nutrient contents alter the microbial community abundance and composition (Barnard et al., 2013; Fierer and Jackson, 2006; Pajares et al., 2016), which consequently influence ecosystem processes. Despite pivotal and diverse roles of soil microorganisms in various ecosystem functions in the forests (Uroz et al., 2016), the response of these communities to anthropogenic/environmental changes is largely unknown. Recently, forest

soil ecology has become an active area of research due to the advent of next generation sequencing, and thus the soil known as “black box” is gradually being deciphered.

1.1.1 Fungal dynamics in forest soils

Fungi are ubiquitous inhabitants of soil with high adaptability to adverse environments due to their ability to produce a wide variety of extracellular enzymes for the breakdown of recalcitrant organic matter (Sun et al., 2005; Eichlerová et al., 2015). They play fundamental ecological roles, particularly functioning as decomposers, mutualists, biocontrol agents, parasites and/or pathogens. The interaction of fungi with each other and other organisms mediates soil carbon (C) dynamics and nutrient cycling processes, and consequently influence soil fertility and forest productivity (Burke et al., 2011; Heijden et al., 1998; Maron et al., 2011). Although there has been substantial research on the functional importance of fungi, scarce literature is available on spatial and temporal variation in soil fungal communities and the factors affecting them (Tedersoo et al., 2014; Liu et al., 2015). Globally, <2% of fungal species have been well characterized (Blackwell, 2011) and <5% have been cultured under laboratory conditions (Giri et al., 2005) due to limitations in the traditional cultivation-based methodology and the sporocarp morphology-based identification approach (Shi et al., 2014). Although DNA based high-throughput sequencing has recently been increasingly used to decipher soil fungal diversity of various terrestrial ecosystems, its use is still in its infancy in forests (Buée et al., 2009; Shi et al., 2014; Tedersoo et al., 2014). Nevertheless, taxonomic identification of fungal genera and species is vital for assigning ecological and functional roles, and thereby understanding their ecophysiology and diversity (Kõljalg et al., 2013; Jeewon and Hyde, 2016; Nguyen et al., 2016; Tedersoo and Smith, 2017). Molecular characterization has substantially improved morphology-based identification, phylogenetic

affiliations, divergence and classification of fungi (Hibbett et al., 2007; Zhao et al., 2017; Wijayawardene et al., 2018).

Temperate forests are known to be enriched with mycorrhizal fungi in symbiotic associations with trees. Mycorrhiza fungi help trees to absorb nutrients efficiently for growth in exchange of photosynthetically fixed carbon. Moreover, they play essential roles in mobilization and sequestration of N and P in the forest soil, as well as C transport and increasing tree tolerance against unfavorable soil conditions and pathogens (Heijden et al., 2015; Clemmensen et al., 2015).

Most woody plants form associations with ectomycorrhizal (ECM) fungi, and these constitute 80% of the fungal biomass in forest soils (Nehls, 2008). ECM fungi mostly grow on conifers, including pines, as well as oaks, whereas arbuscular mycorrhizal (AM) fungi grow mostly on deciduous trees such as maples (Zhu et al., 2018), as well as some conifers including western redcedar and Pacific yew. These fungi proliferate from root tips into the rhizosphere, and consequently to the bulk soil, and can form a network linking roots with the soil (Agerer 2001; Högberg et al., 1999; Simard et al., 2012). Soil water, nitrogen and phosphorus, and carbon synthesized through primary production of trees, are transported through these networks, and can even transmit those nutrients as well as defense signals from one plant to another (Simard et al., 2012). It has been observed that soil carbon to nitrogen ratios increase with greater ECM fungal abundance, implying they are important in the cycling of carbon and nitrogen (Zhu et al., 2018). Furthermore, Zhu et al. (2018) has emphasized that mycorrhizal relationships in ecosystems have implications for how forests will respond to global climate change. ECM fungi act as symbiotrophs as well as decomposers because they have an efficient enzyme system that breaks down recalcitrant organic matter in order to provide nutrients to plants (Bending, 2003; Fernandez and Kennedy, 2016). Besides mycorrhizal fungi, diverse communities of saprotrophs occur in temperate forests

and play fundamental ecological roles in decomposing logs, coarse woody debris, and litter for obtaining energy (Osono, 2007; Stenlid et al., 2008; Voříšková et al., 2014). Similarly, fungal pathogens play important ecological roles as they can reduce the growth or kill forest trees through diseases such as Armillaria root disease (caused by *Armillaria* spp.) and laminated root rot (caused by *Phellinus sulphurascens*.), thereby contributing to the nutrient cycling and structural diversity of forest.

The diversity and structure of fungal taxa is shaped by tree species identity, diversity, soil chemistry, litter type, land use, and climate (Prescott and Grayston 2013; Urbanová et al., 2015; Tedersoo et al., 2016; Liang et al., 2015). Each tree species and mycorrhizal fungus excretes unique and diverse arrays of exudates consisting of carbohydrates, amino acids, low molecular mass aliphatic and aromatic acids, fatty acids, enzymes and hormones (Prescott and Grayston, 2013).

1.1.2 Bacterial dynamics in forests soils

The majority of microbial studies on forest soils have focused on the fungal community, despite the pivotal role of bacterial populations in soil ecosystems (Lladó et al., 2017). Bacterial communities are actively involved in decomposition of dead biomass, carbon, nitrogen, and phosphorus cycling, and facilitating interactions between tree roots and mycorrhizal fungi through commensal relationships (Lladó et al., 2017). In one study, nitrogen fixing bacteria associated with mycorrhizal fungi provided more than 70% of the nitrogen and phosphorus to temperate and boreal forest trees (Heijden et al., 2008). Belowground microbial consortia thus play important roles in aboveground productivity and other ecological functions of tree species (Heijden et al., 2008; Schnitzer et al., 2011; Philippot et al., 2013; Wagg et al., 2014). Moreover, bacteria can act as indicators of climate change as their populations are modulated in response to global warming, increases in atmospheric carbon dioxide levels, and anthropogenic nitrogen deposition (Lladó et

al., 2017). However, studies on understanding the role of bacteria in complex forest ecosystem processes are scarce.

Forest soils represent one of the most diverse ecosystems wherein bacterial presence is widespread and abundant. Bacteria thrive in various habitats of forest soils, including the rhizosphere, bulk soil, litter, dead woody debris, etc., and community structure is fashioned according to the availability of nutrients, and interactions with the surrounding bacteria, fungi, soil properties, and other environmental factors, along with tree identity and diversity (Uroz et al., 2016; Baldrian et al., 2017). Although each habitat (e.g., different soil horizons) has a specific bacterial composition, there is a great overlap of taxa. The specificity of bacterial taxa to certain habitats is due to their preference for certain nutrients and quality of the organic matter (Fierer et al., 2003; López-Mondéjar et al., 2015).

Five bacterial phyla have been observed to be prevalent in most soils, including Acidobacteria, Actinobacteria, Proteobacteria, Bacteroidetes, and Firmicutes (Lauber et al., 2009). Amongst these phyla, Proteobacteria and Bacteroidetes are considered copiotrophic and are highly prevalent in organic horizons, whereas Acidobacteria, Proteobacteria, and Actinobacteria are abundant in organic as well as mineral horizons (Baldrian et al., 2012; Fierer et al., 2007; Uroz et al., 2013). Mineral soils are also known to harbor a high abundance of bacteria from the Firmicutes and Chloroflexi phyla, which are known to harness their energy from inorganic nutrients and recalcitrant carbon substrates (Uroz et al., 2013). Acidobacteria are metabolically versatile, slow-growing oligotrophs that decompose recalcitrant soil organic matter and are abundant in forest soils; however, their ecophysiology is obscure because only a few members of this phylum have been cultured under laboratory conditions (Lladó et al., 2016).

Various biotic and abiotic factors shape bacterial diversity and structure, including soil chemistry (pH, quality and amount of organic matter, quantity of N and other nutrients) (Fierer and Jackson 2006; Lauber et al., 2009; Rousk et al., 2010; Wang et al., 2018), land management practices (Jangid et al., 2008; Lauber et al., 2013), and aboveground tree diversity and density (Garbeva et al., 2006; Kulmatiski et al., 2011).

1.2 Disturbances in forest and its effect on soil microbial communities

Forest disturbances change the structure and functioning of forest ecosystems (Weber and Flannigan, 1997; Turner, 2010). Natural disturbances in forests include wildfire, windstorms, landslides, insect outbreaks, fungal infections, and other pathogenic outbreaks, whereas anthropogenic disturbances include pollution, introduction of invasive species, and forest management practices such as logging, clearcutting, stump removal etc. Changes in the community composition following disturbance depends on the intensity and severity of the event. The disturbances can have positive or negative effects on the forest ecosystems, including bacterial and fungal populations.

1.2.1 Natural disturbances and their impact on soil microbial communities

Fire is an important natural disturbance that is beneficial to the forest ecosystem, resulting in regeneration of species and changes in vegetation, soil properties, and microbial communities. Fires in North America can be severe, causing loss of organic matter (Goldammer and Stocks, 2000; González-Pérez et al., 2004) and increases in surface temperature above 1000 °C, which causes changes in soil structures as well as transformations of the nutrients (Ahlgren, 1974; Ulery et al., 1996). Severe heating is also a cause of concern for most of the microbial communities. Forest fires, depending on the severity, have shown to cause structural changes in the fungal communities mostly in the upper soil horizons but the functional implications are unknown

(Cairney and Bastias, 2007). Fungal richness and diversity declined with the severity of fire, especially those of mycorrhiza and saprotrophs (Day et al., 2019). Conversely, after a severe wildfire, the recovery of the forest depends on the ECM spore banks, but only specific fire-adapted fungi, such as *Rhizopogon* spp., were abundant and able to colonize the seedlings (Glassman et al., 2016). Soil microbial biomass was reduced by more than 50% in burned compared to unburned stands (Holden et al., 2016). Soil bacterial communities were more distinctly affected by wildfire than by harvesting, and indirectly due to changes in soil properties such as pH, moisture content, C/N ratio (Xiang et al., 2014; Whitman et al., 2019; Li et al., 2019). Bacterial groups belonging to Betaproteobacteria and members of Bacillus were abundant in fire-affected sites (Smith et al., 2008).

Outbreaks such as those caused by the mountain pine beetle, sudden oak death as well as root diseases such as *Heterobasidion* root rot, Laminated root rot, Armillaria root rot are also natural causes of disturbances. Mountain pine beetle (MPB) was studied in detail regarding its impact on soil microbial communities and was found to cause a decline in the symbiotrophic and ECM fungi with a relative increase in saprotrophic fungal taxa (Treu et al., 2014, Štursová et al., 2014, Pec et al., 2017). MPB has not generally affected the major bacterial communities, with the exception of active rare taxa decreasing with tree death (Mikkelson et al., 2016). MPB also increased the abundance of bacterial nitrifiers followed by lower C/N ratio (Mikkelson et al., 2017). However, the study by Ferrenberg et al., (2014) reported that bacterial communities remain fairly resistant to disturbance by MPB at larger spatial and temporal scales. No major studies have been done on impact of other forest pathogens on soil microbial communities.

1.2.2 Anthropogenic disturbances and their impact on soil microbial communities

Previously, research on the impact of forest management practices such as clearcutting, harvesting, thinning, etc., on soil microbial communities has been studied in detail (Hartmann et al., 2012; Kohout et al., 2018; Overby et al., 2015). ECM fungi and saprobic taxa such as ascomycetes (fungi) and actinomycetes (bacteria) are sensitive to disturbance by harvesting (Hartmann et al., 2012). Clearcutting in particular has resulted in diminished fungal activity and soil decomposition (Kohout et al., 2018). Forest thinning resulted in favorable conditions for understory grasses with increasing abundance and richness of AM fungi associated with soil bacterial communities (Overby et al., 2015). Dang et al. (2018) found that relative abundance of Proteobacteria was much higher following high intensity thinning whereas Acidobacteria was much higher in low intensity thinning and control plots. For fungal groups, they observed that Basidiomycota was lowest, and Ascomycota was highest in the high intensity thinning.

Soil Compaction caused by heavy machinery used in harvesting causes disruption of pore space thus reducing aeration, water infiltration rates and minimizing rooting space. Microbial communities are sensitive to lack of oxygen and altered water movement can favour Proteobacteria and Firmicutes which are adapted to anaerobic respiration and can carry out sulfate, sulfur and metal reduction (Wright et al., 1995; Schnurr-Pütz et al., 2006; Frey et al., 2011; Hartmann et al., 2014). Compaction also led to an increase in the saprobic and parasitic communities of fungi, thus leading to a structural shift in microbiota (Hartmann et al., 2014).

Stump removal leads to removal of organic matter like fire, harvesting, etc. and cause soil compaction due to the heavy machinery used, but the impact of stump removal on soil microbial communities has not been studied.

1.3 Armillaria root rot, a major disturbance and its impact on forest ecosystem

Armillaria root rot is the most concerning root disease of woody ecosystems worldwide (Shaw and Kile, 1991). The causal agent for this major disturbance is *Armillaria*, a genus of fungi belonging to the class *Agaricomycetes*, commonly known as "honey mushrooms". *Armillaria* species display diverse ecological roles ranging from beneficial saprophytes to virulent pathogens. This white-rot pathogen spreads among trees by root contacts and production of rhizomorphs and once trees are dead or close to dead the fungus switched to a saprophytic mode where it utilizes sapwood as well as heartwood. It then forms mats of dense mycelia (mycelial fans) under the bark. It attacks cambium of live trees and forms melanized rhizomorphs associated with the host tissue and surrounding soil, facilitating underground spread so that a single genet may actively connect to thousands of hosts (Ross-Davis et al., 2013).

Armillaria ostoyae is the most damaging and prevalent root pathogen among commercial conifer species in the forests of southern interior B.C. and the inland Pacific Northwest. The species is highly pathogenic to a number of commercial softwoods, notably Douglas-fir (*Pseudotsuga menziesii*), the true firs (*Abies* spp.) and western hemlock (*Tsuga heterophylla*). All of these conifer species are of great importance to forest ecosystems as well as the timber industry, and damage to the trees leads to losses in timber revenue and recreational values. Forest productivity is reduced through direct tree mortality and non-lethal infections, by this pathogen, that impair growth. Root diseases can also lead to increased stand heterogeneity and can have positive effects on biodiversity. Anecdotal evidence suggests that *Armillaria* root disease is increasing in prevalence, probably as a result of climate change and management practices (Kubiak et al., 2017; Kim et al., 2010). Even though Armillaria root disease is a natural disturbance agent in these forests and has positive effects on biodiversity, its negative impacts on timber productivity is increasing.

1.3.1 Prevalence of Armillaria species

Armillaria species occur in woody plants worldwide, especially affecting tree plantations, orchards, vineyards and natural forests. There are 40 officially described species reported from all over the world and six species have been reported in British Columbia (B.C.), Canada, mostly showing limited occurrence (Heinzelmann et al., 2019; Morrison et al., 1985). *Armillaria ostoyae* and *Armillaria sinapina* are common with *Armillaria ostoyae* being the predominant root rot pathogen in the interior B.C. *Armillaria sinapina* survives mostly as a saprotroph and is considered a weak pathogen. Species such as *A. gallica* and *A. nabsnona* have been reported on hardwoods on the lower mainland and southern Vancouver Island (Morrison, 1981). *Armillaria cepistipes* is rare and is thus less concerning for the forest managers. *A. altimontana* is a recently identified species found in the interior B.C. at higher altitudes and dryer climates. The Interior Cedar Hemlock zone, one of the most productive zones of B.C., suffers the greatest timber losses due to *Armillaria* infection.

1.3.2 Symptoms of *Armillaria* root rot and its disease cycle

Armillaria sp., causing the root disease, has two phases; one as a saprophyte in order to grow and the other as parasite that colonizes new hosts. *Armillaria* root rot spreads by basidiospores which give rise to new genets, and by contact with an infected root or rhizomorphs, i.e. via vegetative extension of a single genet. Basidiospores are airborne and have the ability to infect stumps and dead trees but rarely infect living trees (Shaw and Kile, 1991; Heinzelmann et al., 2019). Mushrooms are produced in late summer to fall especially during wet years. They mostly grow in clusters near the base of the trees. The caps of mushrooms are generally honey-brown in color thus, commonly known as honey mushrooms (Figure 1.2b). Most species also have annulus (ring) on the stem. Infection via contact with infected root or rhizomorphs is the most common means of

spread. Rhizomorphs are root-like dark-colored structures with a white core. They are usually 1 to 2 mm in diameter and run freely through soil while being in contact with the colonized base for nutrients. Thus, the root rot is also commonly known as “shoestring root rot” (Morrison, 1981). In severely infected trees, the fungus grows dense mycelial fans under the bark. Large and older trees may live for several years’ despite numerous lesions on the lateral roots, whereas young trees die quickly due to girdling of the cambium (Bloomberg and Morrison, 1989). The fungus causes speedy death of the infected trees during stress conditions such as, drought or high temperatures (Shaw and Kile, 1991) because it is an opportunistic pathogen and thus, relies on decreasing host defense capacity (Figure 1.1).

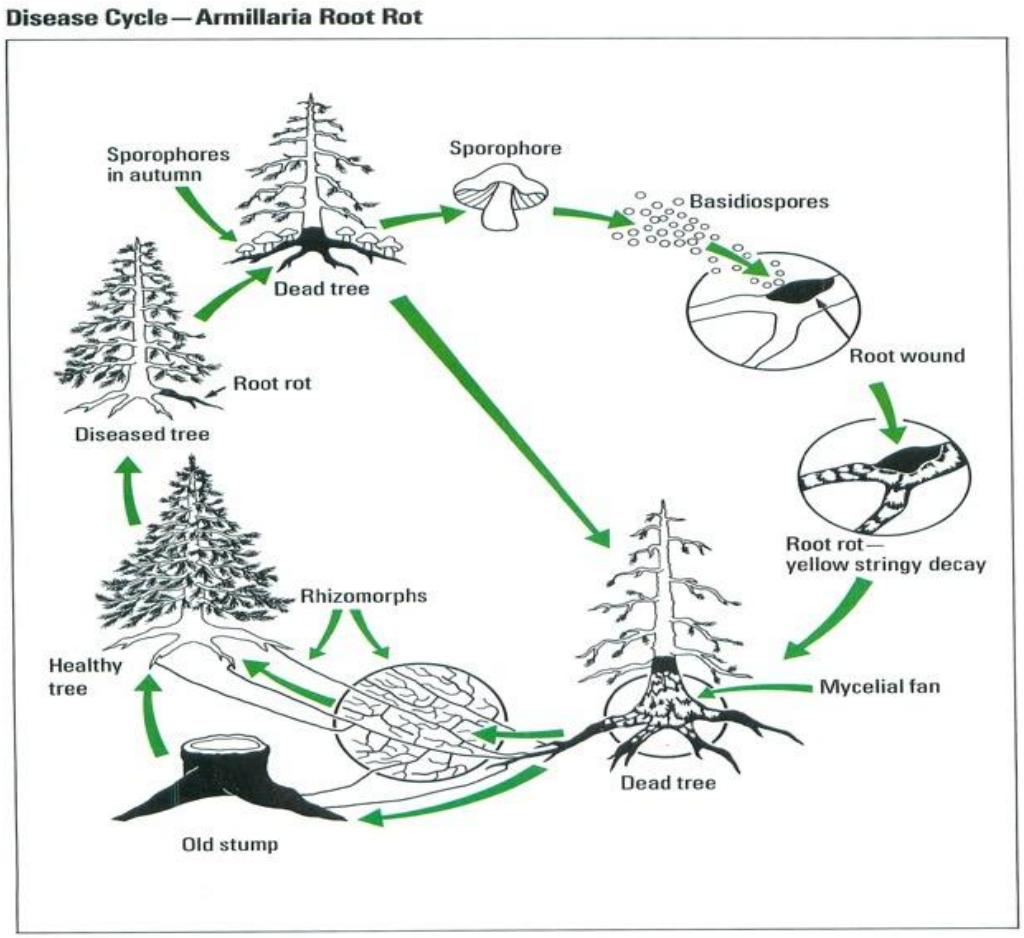


Figure 1.1 Disease Cycle of Armillaria root rot. (Source: R. D. Whitney, 1988, Government of Ontario)

Figure 1.2 a. Mycelial fan on the inner bark of a tree. b. Sporocarp of *Armillaria sp.*

1.4 Management practices for *Armillaria*

Several management practices have been proposed for controlling Armillaria root rot. In plantations, common approaches are fallow, palliative measures such as removal of infected roots, fertilizing of diseased trees, rotation of crop species, and planting mixed species (Morrison et al., 2014). Crop rotation works where the longevity of the crop is not an issue, such as in agricultural settings. All of the other practices are either too tedious, impractical at a larger scale, or had little to no impact on host mortality, with the exception of inoculum reduction. Inoculum reduction has been an effective management practice in forests, and can be carried out by ring barking, fumigation, poisoning of the stumps or mechanical stump removal. Ring barking of trees or

poisoning of stumps has been used to directly kill the root system quickly but was found ineffective in controlling *Armillaria* sp. (Garret, 1970; Redfern, 1968; Sokolov, 1964).

Some other silviculture practices for *Armillaria* root rot are biological control and maintaining a diversity of tree species. Biological control agents such as *Trichoderma* sp., or rhizosphere *Streptomyces*, *Pseudomonas* sp., have been effective in orchards and agricultural fields, but there have been no major studies performed in forest or timber plantations (Vasconcellos and Cardoso, 2009; Raziq and Fox, 2003; Otieno et al., 2003; Perazzolli et al., 2009). Maintaining the biodiversity of trees is one practice where susceptible trees are planted with tolerant/resistant species in order to reduce the incidence of root disease (Morrison et al., 2014).

1.4.1 Stump removal as an effective management practice

Hartig (1874) first suggested that infected stump should be removed to reduce inoculum and the likelihood of future infections. Since then, stumping has been widely used as a silviculture practice, and when applied with root raking, has been the most effective method for reducing root rot incidence. Stump removal is expensive; thus, it is mostly used on high value sites such as orchards or in high-priced timber plantations.

Numerous studies have reported on the effectiveness of stump removal and root raking at reducing Armillaria root rot. Stump removal successfully reduced root rot at numerous sites according to Vasaitis et al. (2008). Morrison and his coworkers monitored the effects of stumping on tree mortality at 20-year increments, and they observed a 100% reduction in Armillaria-caused mortality (Morrison et al., 1988; 2014). Cleary et al. (2013) also reported that stump removal reduced the Armillaria root rot incidence by 80-100% at various locations in Canada and Scandinavia. However, stump removal may lead to alterations in soil microbial communities due

to its impact on soil physicochemical properties (Hope, 2007; Kataja-aho et al., 2012; Kaarakka et al., 2016).

1.4.2 Tree species diversity

Planting diverse tree species helps maintain biodiversity and reduce the impact of any disturbance. Berendsen et al. (2012) said that microbial communities surrounding plant roots are a plant's second genome. Tree species are known to shape particular soil microbial communities through signaling via root exudates that contain sugars, amino acids, secondary metabolites, etc. (Leach et al., 2017). Tree species can also alter the composition and diversity of fungal and bacterial species in forest soils (Prescott and Grayston 2013; Bonito et al., 2014; Urbanová et al., 2015). Various tree species are known to be tolerant or resistant to Armillaria root rot and the mechanism of their resistance is not clearly known. Birch and western redcedar are regarded as tolerant species to Armillaria root rot (Cleary et al., 2008; 2011). Several mechanisms are thought to lead to resistance in mixed stands, including reduced probability of loss, gaps in pathogen inoculum continuum, and synergistic microbial interactions. In western redcedar, necrophylactic periderm formation around a site of penetration and thus, compartmentalization of root collar was effective in making it more resistant to *A. ostoyae* than Douglas-fir (Van Der Kamp, 2005). Paper birch support PGPRs such as fluorescent pseudomonads and may increase the resistance of Douglas-fir by enhancing nutrient turnover (Wargo and Harrington, 1991) or by producing higher concentrations of phenolic compounds (DeLong et al., 2002; Entry et al., 1992). It has also been reported that paper birch species provide higher C-rich root exudates, which support higher microbial consortia compared to Douglas-fir (Bradley and Fyles, 1995). Therefore, it is important to study the impact of tree species diversity on soil microbial communities with respect to stumping and its role against Armillaria root rot.

1.5 Site Background

The Skimikin experiment is a 48-year-old trial established in 1968 by L.C. Weir at Skimikin (50°48'N, 119°26'W) near Salmon Arm, British Columbia on a mesic site series in the Interior Cedar Hemlock (ICH) bio-geoclimatic zone. The original 80-year-old forest was comprised of 75% Douglas fir, 25% lodgepole pine, and minor proportion of western redcedar. The soil great group is a Eutric Brunisol developed over a glacial fluvial deposit. The experimental design was simple and reflective of the scale and limitations of stumping operations at the time, with one block stumped and an adjacent block left as an unstumped control. These two blocks were divided into thirty-two subplots that were planted with three replicates of single species and admixtures of Douglas-fir, western redcedar, lodgepole pine, and paper birch (Figure 2.1)

The trial was established 1.) to estimate the efficacy of inoculum removal by stumping and root raking in order to control root disease caused by *Phellinus weirii* and *Armillaria ostoyae* 2.) to determine the effect of the root disease on susceptible tree species planted together with resistant ones. The 20-year-results showed that the cumulative percent mortality of trees due to root disease was lower in the stumped plots compared with the unstumped control plots (Morrison et al., 1998). After 40 years, survival rate was still greatest in the plots that were stumped, especially those planted to Douglas-fir alone. Mortality was caused by several factors, including root disease (mainly *Armillaria ostoyae*), planting failure, and thinning. Most of the lodgepole pine was killed by mountain pine beetle. The average mortality of all tree species was 14% lower in the stumped than unstumped plots (Morrison et al., 2014).

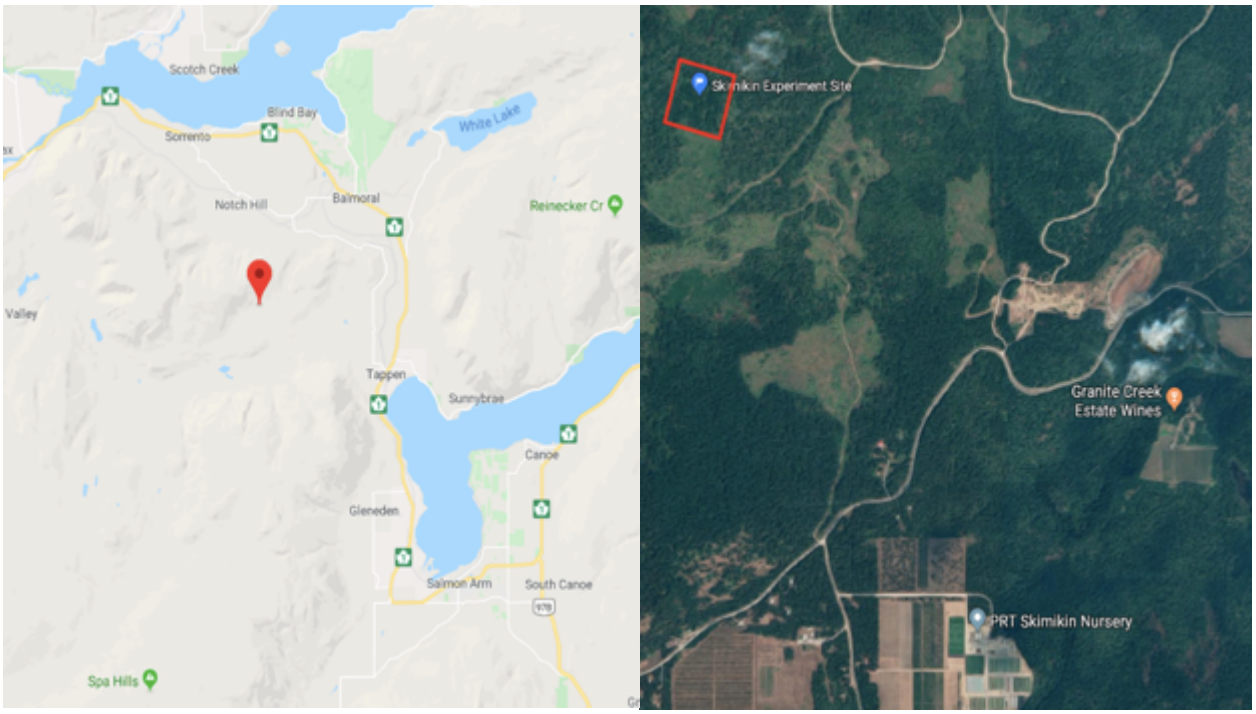


Figure 1.3 Maps showing the Skimikin trial plot located near Skimikin Nursery close to Tappen valley road. (Google Maps and Google Earth)

1.6 Objectives and hypothesis

The purpose of this study was to understand the effect of stump removal and planted tree species composition (resistant and susceptible species) on the soil microbial communities.

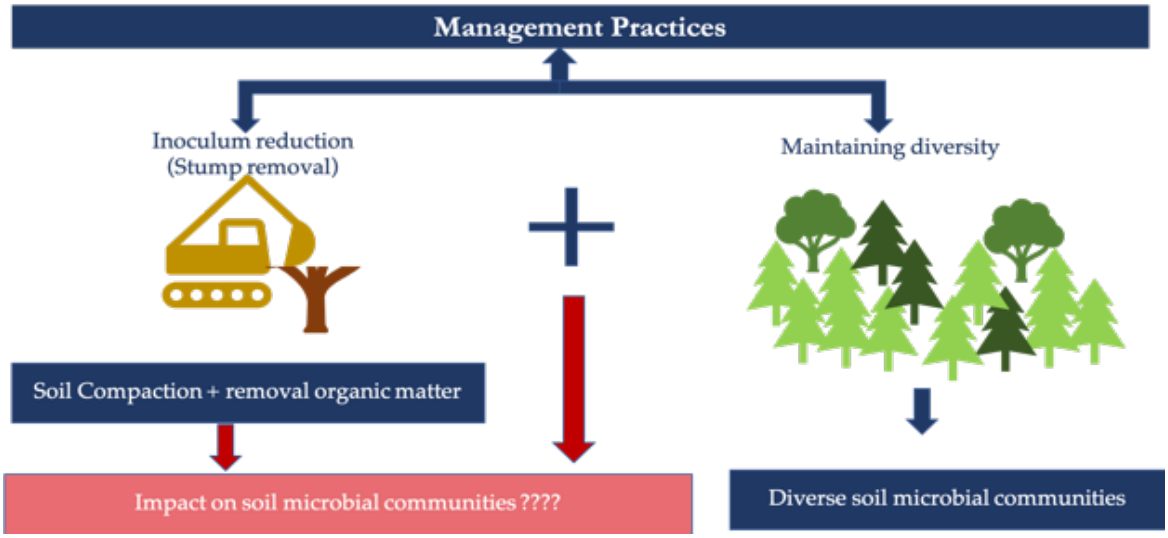


Figure 1.4 An overview of the purpose of the study

The main objectives were as follow:

- **Characterize** the soil microbial (fungal and bacterial) community among the stumping and tree species treatments using DNA Metabarcoding.
- **Compare and contrast** the different functional microbial guilds in the treated soil ecosystems.
- **Identify** key taxa of fungi and bacteria affected by stumping and/or tree species planting treatments. Identify tree species (single or admixture) that are resistant or susceptible to disturbance caused by stump removal**.**

The hypotheses were:

- **Stumping treatment effect:** Removal of organic matter by discarding the dead stumps and root raking will cause the stumped plots to have lower microbial diversity and abundance as compared to unstumped plots.
- **Tree species effect:** Tree identity and tree diversity will alter the composition of the soil fungal and bacterial communities (Prescott and Grayston, 2013; Bonito et al., 2014; Urbanová et al., 2015). The effect of stumping on microbial diversity will vary depending on the tree species planted.
- **Alterations in community structure:** Saprotrophic fungi and oligotrophic bacterial communities will differ in stumped than unstumped plots due to changes that will have occurred in carbon availability.

Chapter2: Long-term effects of stump removal and tree species composition on the diversity and structure of soil fungal communities

2.1 Introduction

Soil fungal communities are comprised of mycorrhizal, endophytic, saprotrophic and pathogenic fungi and they play crucial roles in the supply and cycling of nutrients in forest ecosystems (Finlay, 2004; Courty et al., 2010; Lafleur et al, 2018). Fungal species composition within functional groups is thought to affect tree productivity and forest ecosystem health. Fungi are sensitive to a range of abiotic and biotic factors, not least of which are disturbances, such as disease outbreaks, insect infestations, wildfire and wind storms, as well as anthropogenic activities such as logging, clearcutting, site preparation and planting of trees (Hartmann et al., 2009; 2014; Cairney and Bastias, 2007; Kohout et al., 2018). Armillaria root rot, caused by *Armillaria* sp., affects coniferous species in western North America, notably Douglas-fir (*Pseudotsuga menziesii*), but has a lesser impact on broadleaf tree species, particularly paper birch (*Betula papyrifera*). *Armillaria* sp. is an opportunistic pathogen that causes growth impairment and can lead to tree mortality, causing reductions in forest productivity and economic losses to the timber industry (Cruickshank et al, 2011; Bloomberg and Morrison, 1989). Stump removal, referred to hereafter as stumping, has been considered the most effective forest management technique in western Canada for reducing root disease incidence among susceptible conifers while increasing plantation productivity (Morrison et al., 1988; Morrison et al., 2014; Bogdanski et al., 2018). However, numerous studies have also shown that stumping can affect the physicochemical properties of soils, including short-term increases in bulk density, carbon concentration, and net N mineralization and nitrification (Hope, 2007; Kataja-aho et al., 2012; Kaarakka et al., 2016). Furthermore, stump removal has caused reductions in soil nitrogen due to loss of soil organic

matter which has also been shown to result in short-term losses of soil mesofauna and oribatid mites (Zabowski et al., 2008; Battigelli et al., 2004). Page-Dumroese et al. (1998) observed that stumping led to reduced diversity and number of ECM root-tips in Douglas-fir seedlings. Disturbances such as forest fire, logging and windthrow have been found to affect microbial biomass more negatively than pathogens and insect outbreaks due to their impact on soil physicochemical properties (Holden and Treseder, 2013), and it is thus plausible that stumping, in addition to logging, would further reduce microbial biomass.

Tree species help shape the soil fungal community through fluxes of photosynthetic carbon to the rhizosphere and their related effects on soil and litter chemistry. Tree species have fidelity to classes of mycorrhizal symbionts, endophytic fungi as well as pathogenic and saprotrophic taxa, and some fungi in turn have specificity for particular tree species (Molina et al. 1992; Prescott and Grayston, 2013; Urbanová et al., 2015). Thus, planting root disease resistant or tolerant tree species with susceptible trees may result in alterations in the soil mycobiome, which is important to study for the management of root disease. The effects of stumping and tree species composition on the structure and diversity of soil mycobiome has been not been investigated.

Describing and deciphering the mycobiome of the forest ecosystem is a crucial step in developing forest management techniques that sustain beneficial fungal communities and improve forest health over the long term. The exploration of fungal mycobiomes has been impeded by a lack of appropriate microbiological methods (Torsvik and Ovreas, 2002), but recent advances in metagenomics, meta-transcriptomics, and meta-proteomics, along with powerful computational and bioinformatics tools, provide new windows into the composition of the soil mycobiome (Nilsson et al., 2018).

The goal of this study was to determine the effects of stumping and tree species composition on the diversity, abundance and community structure of the fungal mycobiome several decades after treatments. We also assessed the soil physicochemical characteristics and correlated them with fungal community structure. We hypothesized that: 1) the stumped plots would have lower fungal diversity as compared to unstumped plots; 2) the effect of stumping on fungal diversity would vary depending on the tree species planted; and 3) saprotrophic fungi, in particular, would decline with stumping because of the reduction in available substrate.

2.2 Methods

2.2.1 Study site and sampling strategy

Soil samples were collected from the 48-year-old experimental site at Skimikin (50°48'N, 119°26'W) near Salmon Arm, British Columbia (Weir and Johnson, 1970). The trial was established in 1968, at which time all trees were push-felled from two adjoining 80 m X 80 m blocks (total of 80 m X 160 m). In the treated (stumped) block, trees were push-felled by bulldozer and yarded to the landing with roots attached. The roots were raked to depth of 45 cm using a toothed land-clearing blade attached to bulldozer. In the untreated (unstumped) control block, the trees were hand felled and skidded to the landing in a conventional way. A 10m-wide strip around the blocks was cleared and root-raked to reduce the chance of root disease entering the blocks from the surrounding forest. Each block was divided into thirty-two 20 m X 20 m plots and randomly assigned to three replicates each of single species and admixture plantings of Douglas-fir, western redcedar, lodgepole pine, and paper birch (Figure 2.1)

In April 2016, soils were randomly sampled from the three replicates of a subset of the tree species treatments (fir, cedar, birch, fir/birch, fir/cedar, and cedar/birch) in the stumped and unstumped blocks. The litter (L) layer was removed, and samples were then collected using

marked trowels from the FH (fermentation and humus) layer, A horizon (0 to 10 cm), and B horizon (10 to 20 cm).

Stumped				UnStumped			
1 Fir Birch	16 Fir	17 Cedar Birch	32 Larch	33 Cedar	48 Pine Cedar	49 Pine Birch	64 Fir Birch
2 Fir Cedar	15 Pine Birch	18 Pine Cedar	31 Birch	34 Pine Birch	47 Fir Pine	50 Pine	63 Spruce
3 Birch	14 Fir Pine	19 Pine	30 Fir	35 Cedar Birch	46 Cedar	51 Fir Cedar	62 Pine Cedar
4 Cedar	13 Cedar Birch	20 Pine Cedar	29 Fir Pine	36 Fir	45 Fir Birch	52 Birch	61 Fir
5 Spruce	12 Cedar	21 Fir Birch	28 Pine Birch	37 Fir Cedar	44 Birch	53 Fir Birch	60 Pine
6 Fir Cedar	11 Birch	22 Pine Cedar	27 Fir	38 Pine Cedar	43 Cedar Birch	54 Pine	59 Fir Pine
7 Cedar Birch	10 Pine	23 Pine Birch	26 Fir Birch	39 Fir	42 Fir Cedar	55 Cedar Birch	58 Birch
8 Fir Pine	9 Cedar	24 Fir Cedar	25 Pine	40 Fir Pine	41 Larch	56 Cedar	57 Pine Birch

Figure 2.1 The experimental design. Samples were collected from all the colored plots in the table.

Triplicate samples were taken randomly from each replicated treatment plot and then composited, resulting in three composite samples per tree species treatment. Soil samples were kept on ice before storage at -20°C and processed within 2 days for soil analyses and within a week for DNA extractions.

2.2.2 Soil analyses

Soils were processed directly after sieving through a 1 mm sieve, prior to determination of pH, in water and $CaCl_2$, soil moisture content, nitrogen (N) concentration (%), and carbon (C) concentration (%) were measured. C/N ratio was calculated from C and N concentrations (%). Soil moisture content was measured by weighing soil before and after drying soil at 105°C overnight. Total soil nitrogen and carbon concentrations were determined by dry combustion method using a Leco automated analyzer (Leco chn-600 Elemental Analyzer). These analyses were performed at

the Analytical Chemistry Services Laboratory, B.C. Ministry of Environment and Climate Change Strategy, Victoria, B.C., Canada.

2.2.3 DNA isolation, metabarcoding and sequencing

Total genomic DNA was extracted from 0.5 g of the sieved soil using a MoBio PowerSoil DNA extraction kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. All DNA extractions were performed in triplicate per sample, and extracts were combined into one sample. The polymerase chain reaction (PCR) was performed in triplicate to reduce PCR bias. PCR amplification of the fungal ITS1 region from DNA was performed using barcoded ITS1f and ITS7g primers (Bérubé et al., 2018). Each sample was tagged with differing indexes and then pooled in equimolar amount of 4 ng DNA per sample with DNA from samples of another experiment to fill up an Illumina run. Final quantification of pool, verification of primer artifact removal and amplicon quality check were done with the Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). Pooled DNA samples were sent to the Next-Generation Sequencing Platform, Genomics Centre, CHU de Québec- Université Laval Research Centre, Quebec City, QC, Canada, which performed paired-end 300 bp sequencing using MiSeq Reagent Kit v3 (600-cycles) through an Illumina MiSeq system.

2.2.4 Bioinformatics and statistical analysis

Sequence assembly was done using PANDASeq v2.7 (Masella et al., 2012). Illumincut (Gagné & Bérubé, 2017a) was used to filter and trim sequences. Sequences shorter than 120 bp after removal of barcodes, tags and primers; unambiguous positions and a maximum homopolymer length of 9 bp were removed with HomopRemover (Gagné & Bérubé, 2017b). Dereplication on the full-length of the set of sequences was performed before construction of clusters with MOTHUR v.1.28.0

(Schloss et al., 2009). The sequences set was then organized into clusters with USEARCH 64-bit v8.0.1623 (Edgar 2010) with a sequence similarity threshold of 97% to agglomerate reads and form the OTUs. Fungi with genus-level classification were assigned functional properties according to the trophic status, lifestyles, decay types, and growth forms designated by Tedersoo et al. (2014, Supplementary Table).

All statistical analysis was performed in R (v 3.5.1.). Fungal diversity (α-diversity) of each tree species treatment plot was measured by calculating the Shannon diversity index. Stumping (stumped versus unstumped) and tree species effects on diversity were then assessed using two-way analysis of variance (ANOVA) with a permutation test. A multiple-linear-regression model was used to identify variables that significantly contributed to α-diversity. Bray–Curtis dissimilarities matrix as an index of β-diversity was calculated using the "vegdist" function of the "vegan" package (Oksanen et al., 2016) and was used for constrained analysis of principal coordinates (CAP) to study between-plot diversity (β-diversity). CAP is similar to a redundancy analysis, but additionally it allows use of non-Euclidian dissimilarity indices such as Bray–Curtis distances. Statistical significance for CAP was determined by an ANOVA-like permutation test function with 1,000 permutations using "anova.cca" function in "vegan" package (Oksanen et al., 2016). Environmental variables were fitted into the CAP plot and their significant interaction was determined using "envfit" function of vegan package. Permutational multivariate analysis of variance (PERMANOVA) on Bray–Curtis dissimilarities was conducted to study the effect of different factors on the structure of fungal communities using the "adonis" function of the vegan package (at $P < 0.05$) (Oksanen et al., 2016). When significant PERMANOVA main effects were observed, pairwise Wilcoxon rank-sum tests were used to test whether the stumping by tree species treatment interaction was significant. Multivariate generalized linear models (GLM) were used to

evaluate fungal class and fungal guild responses to the experimental factors using the "mvabund" package (Wang et al., 2012). PERMANOVA was conducted on these models to study the overall multivariate response to these factors in addition to the univariate responses. All tests were conducted assuming a negative binomial distribution and resampled 999 times using the PIT-trap method, and the likelihood ratio was used as the test statistic. Hypothesis testing was conducted using the "anova.manyglm" function, and treatment contrasts were evaluated using the "summary.manyglm" function. Significant ($p<0.05$) effects are in bold. The top 10 most abundant OTUs were used for calculating Z-scores for detailed information on community structure at the species level and were visualized using "ggplot2". Pearson correlation tests were done in order to observe the correlation between ECM fungal and saprotrophic fungal functional groups. The raw sequence data have been deposited in the NCBI Sequence Read Archive (BioSample accession no. SAMN12885403 and BioProject accession no. PRJNA575258).

2.3 Results

2.3.1 Fungal community structure

A total of 8.7 million clean double-stranded Illumina DNA reads were obtained after filtering. Cluster analysis, with a sequence similarity threshold of 97%, produced 972,650 OTUs, which were regrouped into 8812 OTUs based on matching reference numbers in GenBank. A total of 8073 (92%) OTUs out of the 8812 were identified at the class level. We observed that sequences with >2% abundance in the entire data set were attributed to seven different fungal classes, including Agaricomycetes (54%), Archaeorhizomycetes (9%), Dothideomycetes (3%), Eurotiomycetes (3%), Incertae sedis (10%), Leotiomycetes (5%), and Pezizomycetes (6%), with 4% in an unclassified group. The 10 most abundant fungal species in the total data set were *Hodophilus smithii* (7.9%), *Rhizopogon rudus* (6.2%), *Hygrocybe pseudoconica* (5.4%),

Hygrocybe persistens (4.9%), *Wilcoxina rehmii* (2.9%), *Ramariopsis helvola* (3.1%), *Russula exalbicans* (1.8%), *Cortinarius diasemospermus* (1.7%), *Geomyces sp.* (1.6%) and *Suillus lakei* (1.5%).

2.3.2 Soil properties

Soil pH ($CaCl_2$) varied between 5.5 and 6.5 and was significantly lower in stumped plots compared to unstumped plots in the A and B horizons (Table 1). Soil moisture, and C and N concentrations of the A horizon were also significantly lower in stumped treatments. C/N ratio of the FH horizon varied significantly among tree species, with the highest ratio (45.33±7.37) in the Cedar treatment (Table 2.1). There were significant stumping X species interactions for soil moisture, and C and N concentrations in the FH layer (Table 2.2), where interactions were greatest in the Cedar/Birch mixture of the stumped plots according to univariate analysis (Figure. 2.2).

2.3.3 Alpha diversity

For determining diversity and abundance, the sequencing data for the three horizons (FH, A and B) was rarefied to a minimum library size (i.e., the minimum number of reads in a sample). The analysis resulted into 2240 OTUs in the FH horizon, 1771 OTUs in the A horizon and 1926 OTUs in the B horizon. α-diversity based on Shannon's index was significantly greater in the stumped than unstumped treatment in the A horizon (Figure 2.3, Table 2.3). Although not significant, alpha diversity also tended to be greater with stumping in the FH layer and B horizon (Table 2.3). No significant correlation was observed between Shannon's index and any soil property in any of the horizons.

Table 2.1. Physico-chemical properties of soil. The values in the table represent average ± SD and the level of significance *P<0.05; **P<0.01; ***P<0.001. (Fir = Douglas-fir, Cedar= western red cedar and Birch = paper birch)

		Stumped						UnStumped					
Tree/Species	**Horizon**	**Moisture**	**C/N ratio**	**pH**	**pH (CaCl2)**	**TotalC (%)**	**TotalN (%)**	**Moisture**	**C/N ratio**	**pH**	**pH (CaCl2)**	**TotalC (%)**	**TotalN (%)**
Fir	**FH**	10.69 ±0.39	26.67±0.58	6.31±0.3	5.89±0.39	44.38±3.6	1.64±0.13	12.01±0.19	26±1	6.13±0.6	5.79±0.48	48.3±1.71	1.83±0.09
Birch		10.34±1.4	29±0	6.64±0.17	6.05±0.32	38.44±9.89	1.32±0.33	9.87±1.07	29±1	6.34±0.32	6.32±0.14	35.84±7.93	1.23±0.29
Cedar		11.65±0.2	**45.33±7.3***	6.42±0.4	6.07±0.38	50.76±1.48	1.13±0.18	11.12±0.74	**33±9.17***	6.58±0.07	5.96±0.34	42.75±7.9	1.31±0.16
Fir/Birch		9.54±0.26	26.33±1.53	6.06±0.5	5.99±0.07	38.34±2.09	1.43±0.13	9.68±1.03	27.67±1.53	6.45±0.27	6.17±0.32	36.48±8.19	1.31±0.33
Fir/Cedar		10.69±0.2	27.67±3.21	6.29±0.2	5.9±0.15	46.5±4.49	1.65±0.03	10.29±1.61	28.33±1.53	6.39±0.14	6.02±0.52	39.61±11.28	1.37±0.38
Cedar/Birch		**12.94±0.5****	33.67±6.35	6.65±0.09	6.18±0.4	**51.93±0.9****	**1.56±0.3***	**6.21±1.08****	31.33±5.51	6.58±0.15	6.03±0.28	**22.56±10.4****	**0.72±0.34***
Fir	**A**	1.89±0.25	24±1	7.32±0.1	6.47±0.27	3.08±1.62	0.13±0.08	1.82±0.17	26.33±2.52	7.56±0.1	6.93±0.38	3.15±0.64	0.12±0.04
Birch		1.59±0.23	23±1	6.85±0.13	6.11±0.43	2.66±1	0.12±0.04	2.56±0.54	23.67±4.04	6.7±0.35	5.94±0.41	5.78±3.48	0.23±0.11
Cedar		1.8±0.32	29±9.54	6.35±0.4	5.65±0.29	3.18±2.39	0.1±0.05	1.83±0.19	23±1	7.14±0.19	6.64±0.68	3.06±1.6	0.13±0.07
Fir/Birch		1.6±0.08	26.67±4.04	6.83±0.2	5.99±0.35	2.41±0.55	0.09±0.01	2.03±0.26	25.67±3.06	6.72±0.28	6.63±0.49	3.64±1.15	0.15±0.06
Fir/Cedar		1.76±0.08	26.33±4.04	6.73±0.22	5.68±0.42	2.6±0.08	0.1±0.02	2.1±0.14	25.33±2.52	7.08±0.57	6.44±0.46	3.41±1.08	0.14±0.04
Cedar/Birch		1.45±0.07	22.33±2.08	6.9±0.21	6.08±0.28	1.77±0.58	0.08±0.02	1.89±0.24	28.67±8.02	6.81±0.87	6.57±0.5	4.66±0.82	0.17±0.07
Fir	**B**	1.49±0.04	32.33±8.74	7.36±0.13	6.52±0.3	2.2±0.72	0.07±0.01	1.41±0.08	29.67±5.69	7.66±0.39	6.75±0.32	2.18±0.17	0.08±0.02
Birch		1.36±0.01	23.33±3.51	7.1±0.42	6.26±0.32	2.01±0.25	0.09±0.01	1.43±0.23	26.33±6.81	7.24±0.1	6.51±0.43	2.55±1.07	0.1±0.06
Cedar		1.5±0.08	24±3.61	**6.45±0.2***	5.56±0.3	1.5±0.07	0.07±0.01	1.48±0.13	22.33±1.53	**7.22±0.4***	6.6±0.6	1.94±0.73	0.09±0.04

Fir/Birch	1.27±0.14	22.67±1.53	6.94±0.29	6.11±0.32	1.24±0.43	0.06±0.02	1.32±0.2	27.67±7.37	6.92±0.34	7.09±0.22	2.07±0.13	0.08±0.02
Fir/Cedar	1.59±0.08	26±5	6.7±0.17	5.97±0.34	1.69±0.38	0.07±0.01	1.46±0.01	26.33±6.03	6.55±0.72	6.24±0.88	2.08±0.95	0.08±0.02
Cedar/Birch	1.49±0.1	21.67±1.53	6.87±0.34	5.72±0.27	1.78±0.73	0.08±0.03	1.33±0.04	28.33±2.31	7.39±0.52	6.69±0.33	2.41±0.34	0.09±0.01

Table 2.2. ANOVA test for soil properties. The values in the table represent F values and the level of significance *P<0.05; **P<0.01; ***P<0.001

Main test	Moisture	pH in H_2O	pH in $CaCl_2$	C/N ratio	Total C	Total N
FH Horizon						
Species	1.64	1.53	0.77	**7.37*** **	**2.72***	**4.75****
Treatment	**4.62***	0.02	0.10	2.33	**10.61****	3.86
Species x Treatment	**5.02****	0.99	0.39	2.06	**4.22****	**3.64***
A horizon						
Species	0.61	**3.31***	2.05	0.34	0.55	0.96
Treatment	**6.28***	2.02	**13.76****	0.02	**6.82***	**8.75****
Species x Treatment	1.13	1.36	1.29	1.29	1.23	1.05
B Horizon						
Species	1.02	**3.94****	1.99	1.68	1.16	1.34
Treatment	0.39	4.25	**19.13*****	1.11	**5.77***	3.78
Species x Treatment	0.35	1.24	1.40	0.81	0.36	0.17

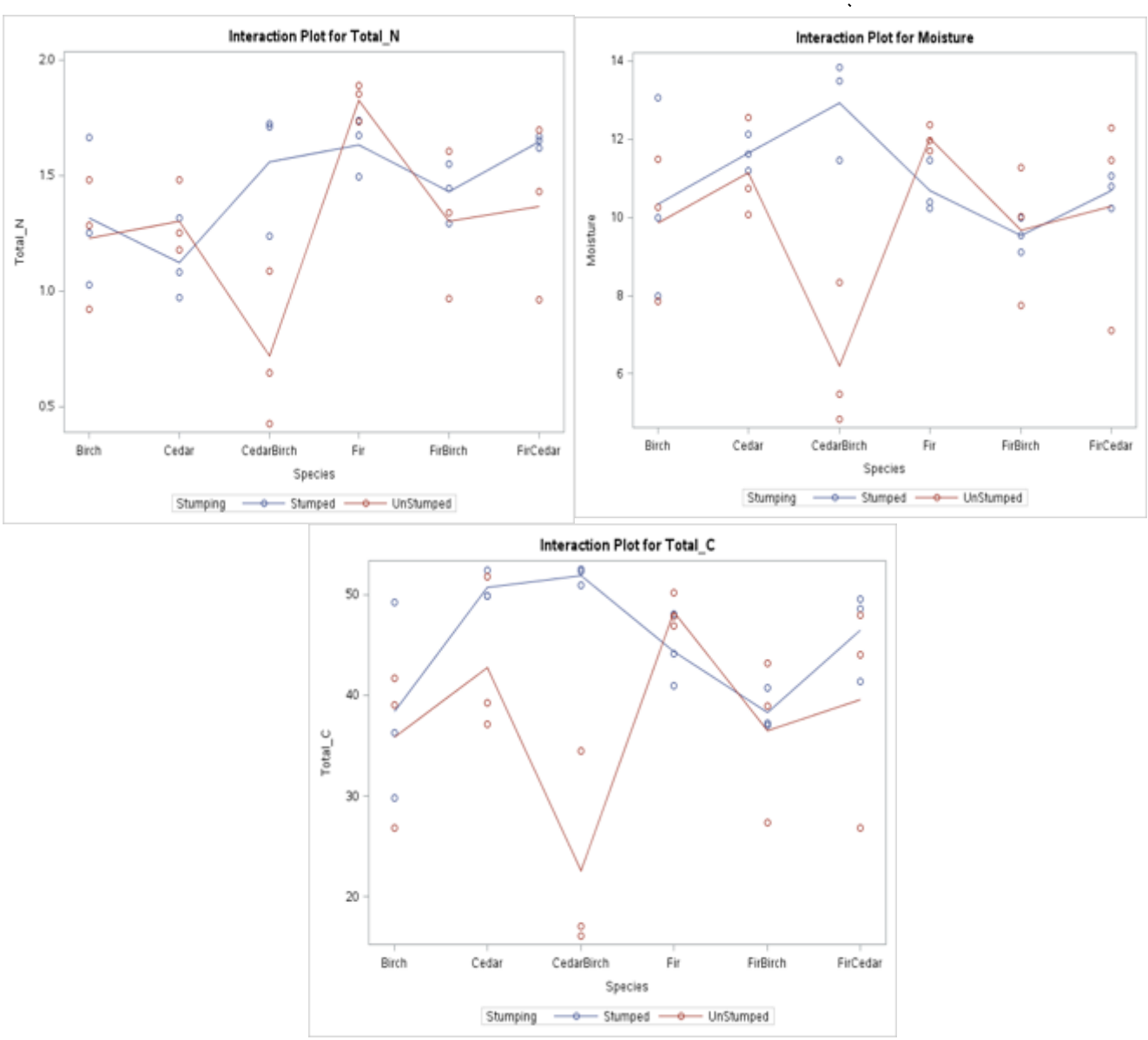


Figure 2.2 Interaction plots of total C, total N and Moisture in FH horizon. Cedar /birch mixture showing the highest of total C, total N and soil moisture content in the stumped plots.

Table 2.3 Effect of factors (tree species and treatment) and their interaction on α -diversity of FH, A and B horizons based on Shannon index measured by two-way ANOVA of multiple-linear-regression model. The P-value for the effect of factors and interactions is reported.

Factor	Df	Sum Sq	Mean Sq	F value	Pr(>F)
FH Horizon					
Species	5	0.365	0.073	0.494	0.777
Treatment	1	0.304	0.304	2.053	0.167
Species: Treatment	5	0.340	0.068	0.4600	0.801
Residuals	21	3.106	0.148		
A Horizon					
Species	5	1.229	0.246	1.237	0.323
Treatment	1	1.507	1.507	7.581	**0.011***
Species:Treatment	5	0.396	0.079	0.398	0.845
Residuals	24	4.770	0.198		
B Horizon					
Species	5	0.624	0.125	1.241	0.322
Treatment	1	0.284	0.284	2.829	0.106
Species: Treatment	5	0.329	0.066	0.654	0.661
Residuals	23	2.310	0.100		

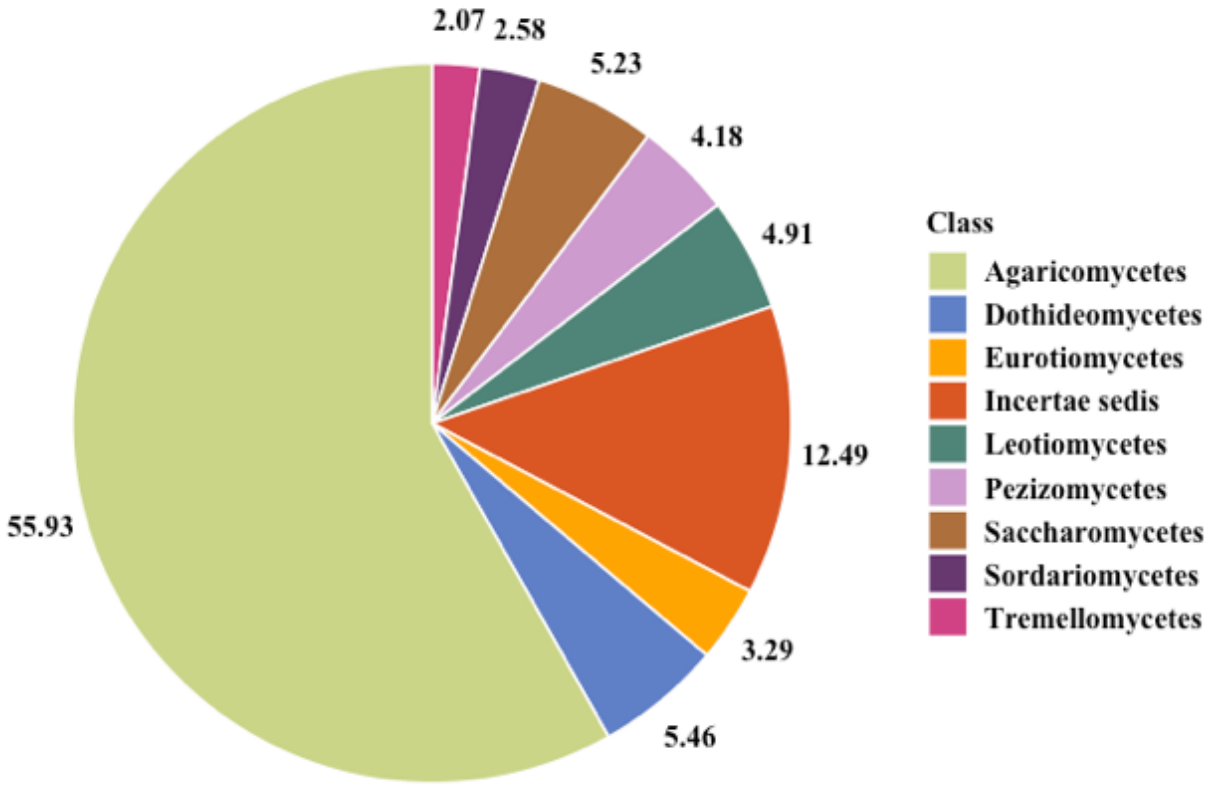


Figure 2.3 Overall proportion of fungal classes belonging to the most abundant phyla (> 2% of the sequences)

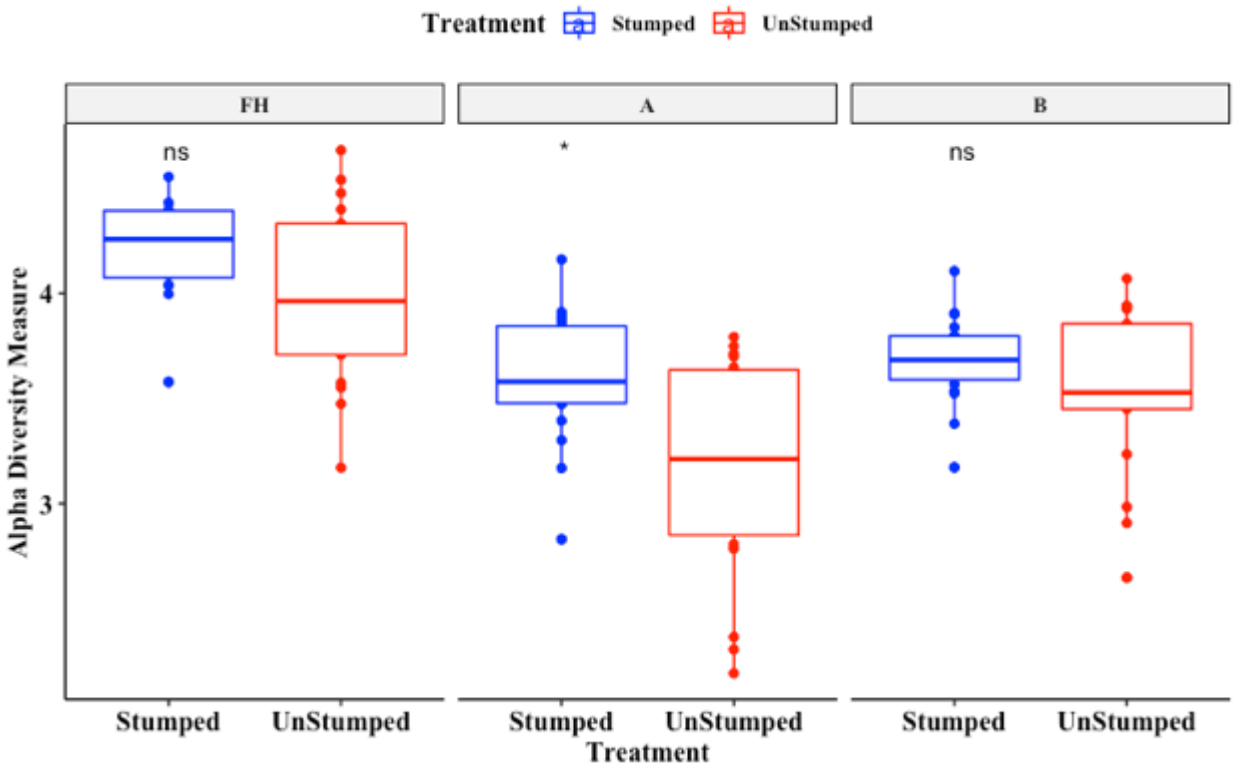


Figure 2.4 Shannon index showing α-diversity of all horizons (FH, A and B) vs treatment (Stumped and Unstumped). The significant effects are shown with asterisks (*P<0.05; **P<0.01; ***P<0.001).

2.3.4 Beta diversity

Bray Curtis dissimilarity index (representing β-diversity) was used to measure the dissimilarity in the community structure based on abundance between different planted plots. The constrained analysis of principal coordinates (CAP) showed reduced variability among tree species plots in the stumped versus unstumped treatment for all horizons, as these plots were clustered together along the CAP1 axis, whereas those in the unstumped plots were more dispersed, indicating high variability (Figure. 2.4).

Table 2.4 Effect of factors (tree species and treatment) and their interaction on fungal β-diversity of all three (FH, A and B) horizons assessed by PERMANOVA. Significant ($p<0.05$) effects are in bold.

Factors	Df	SumsOfSqs	MeanSqs	F. Model	R2	Pr(>F)
FH Horizon						
Species	5	1.338	0.268	1.414	0.192	**0.026**
Treatment	1	0.370	0.370	1.957	0.053	**0.033**
Species:Treatment	5	0.907	0.181	0.959	0.130	0.566
Residuals	23	4.352	0.189	0.625		
Total	34	6.968	1.000			
A Horizon						
Species	5	1.134	0.227	1.192	0.161	0.145
Treatment	1	0.485	0.485	2.549	0.069	**0.001**
Species:Treatment	5	0.859	0.172	0.902	0.122	0.672
Residuals	24	4.567	0.190	0.648		
Total	35	7.045	1.000			
B Horizon						
Species	5	1.123	0.225	1.538	0.199	**0.010**
Treatment	1	0.454	0.454	3.107	0.077	**0.002**
Species:Treatment	5	0.988	0.196	1.341	0.165	**0.044**
Residuals	23	3.360	0.146	0.577		
Total	34	5.916	1.000			

Stumping significantly reduced β-diversity in the FH and A horizons, and tree species affected beta diversity in the FH horizon only (Table 2.4), and there were no significant stumping X species interactions for these two horizons. In the B horizon, there was a significant stumping X species interaction (Table 2.4), where β-diversity was greater (Wilcoxon test, $p<0.05$) in the unstumped than stumped treatment in the fir, cedar, birch, cedar/birch and fir/cedar plots, but did not vary with stumping in the birch plot (Figure 2.5).

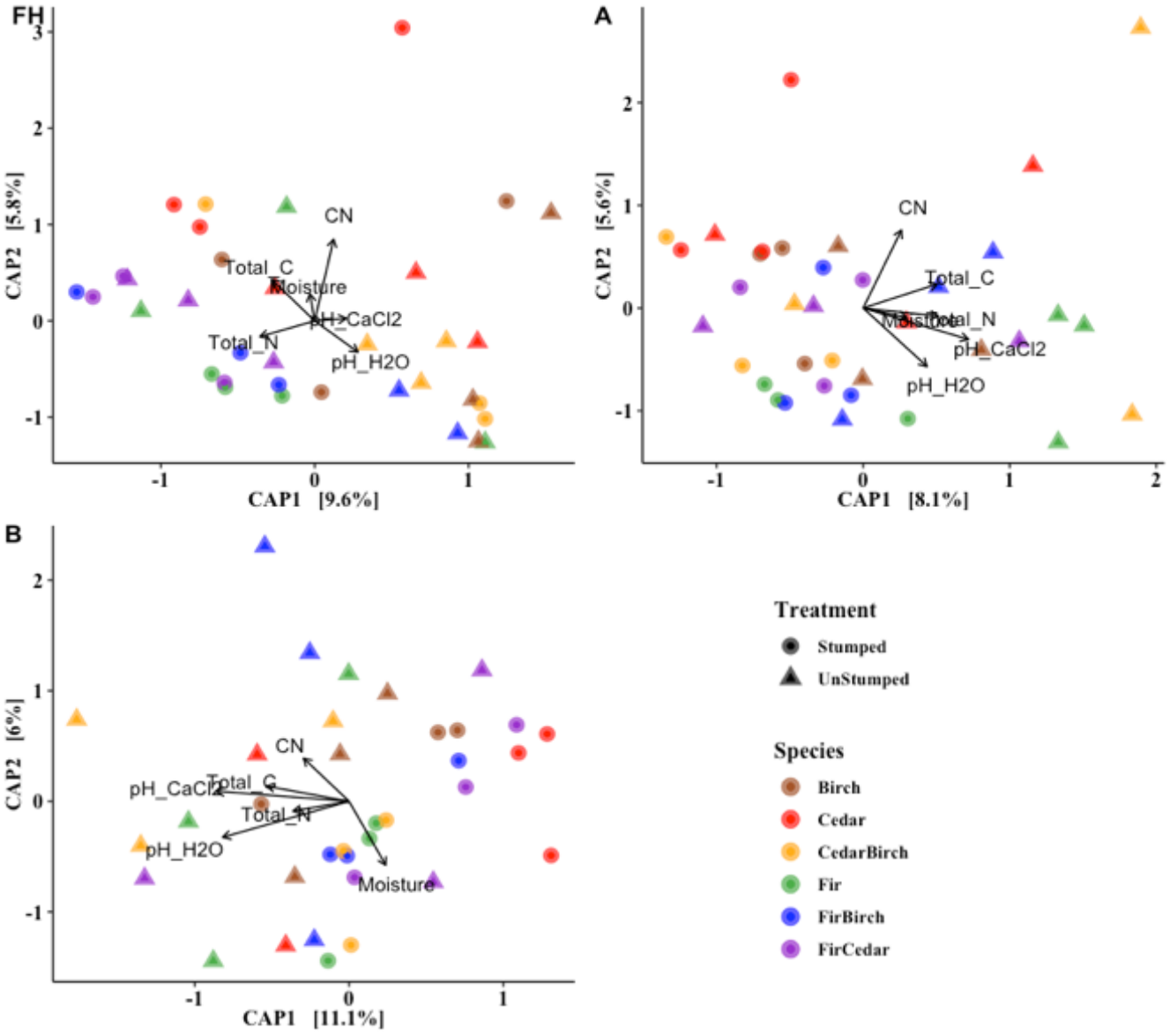


Figure 2.5 Bray–Curtis dissimilarities between samples were calculated using the "vegdist" function of the vegan package. Constrained analysis of principal coordinates (CAP) of Bray-Curtis dissimilarity matrix calculated based on relative OTU abundances of all horizons (FH, A and B) with environmental variables (pH_H_2O, pH_$CaCl_2$, Total_C, Total_N, C/N ratio, and Moisture).

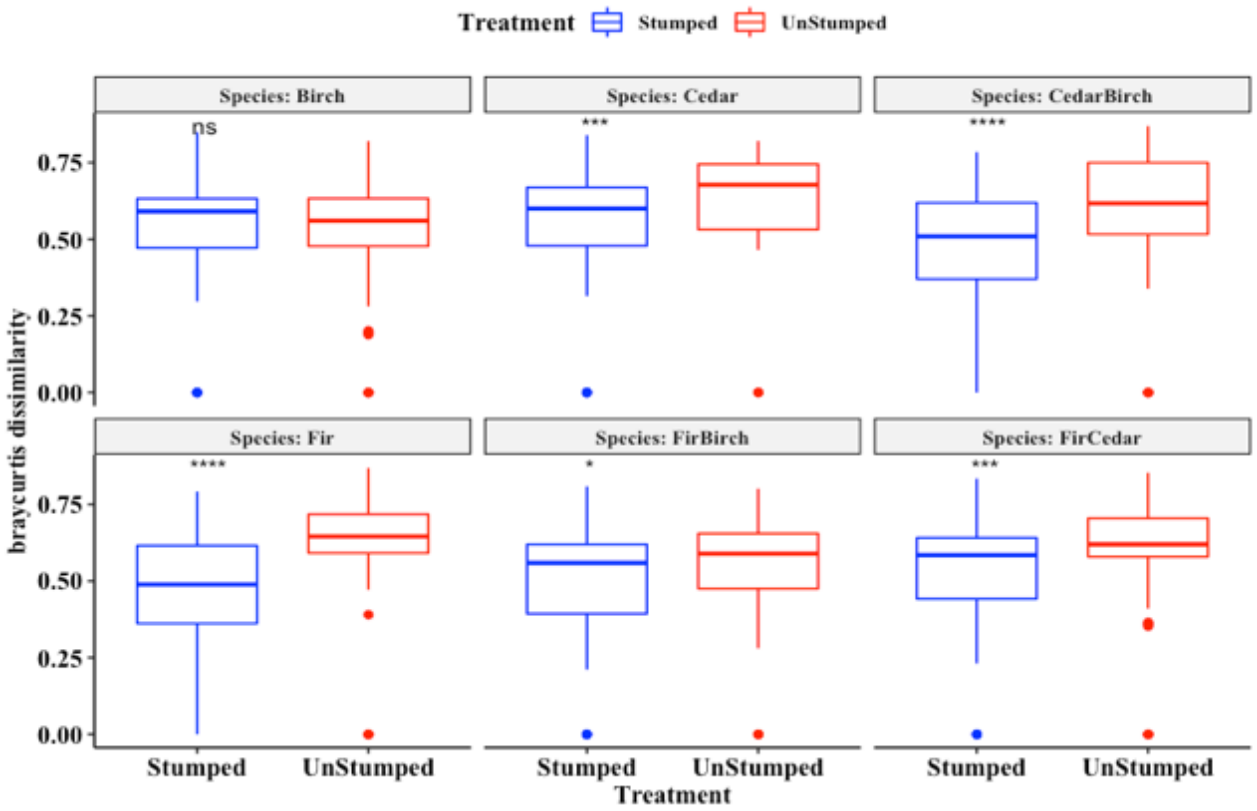


Figure 2.6 Bray Curtis Dissimilarity plots of B horizon. Bray–Curtis dissimilarities between samples were calculated using the "vegdist" function of the vegan package. Pairwise Wilcoxon rank-sum tests were used to test Bray–Curtis dissimilarities between tree species and treatment (stumped and unstumped) interaction significance to each other. The significant effects are marked with asterisks *P<0.05; **P<0.01; ***P<0.001.

2.3.5 Multivariate analyses of relative abundance of fungal classes and functional guilds

Fungal functional guilds that were assigned based on the ecological function of OTUs were affected by stumping in all three horizons, and by tree species in the A and B horizons (P<0.05) (Table 2.5) (Figure 2.6). In the FH horizon, the relative abundance of the symbiotroph/ECM guild was significantly greater in the stumped than unstumped plots (Figure 2.6). The major functional guilds, saprotrophs and ECM fungi, were not affected by stumping in the mineral horizons (A and B). Stumping affected fungal class abundances in the A and B horizons (Table 2.5, Figure 2.7) (P<0.05). Classes such as Leotiomycetes, Lecanoromycetes and Microbotryomycetes decreased in stumped plots, whereas classes such as Archaeorhizomycetes (only in B horizon), Saccharomycetes,

Dothideomycetes (only in B horizon), Tremellomycetes, and Wallemiomycetes increased in stumped plots ($P<0.05$) (Figure 2.5). Among the top 10 most abundant species, *Hodophilis smithii* and *Rhizopogon rudus* showed significant ($P<0.05$) positive correlation in all the three horizons (Figure 2.8).

Table 2.5 Multivariate generalized linear models were used to evaluate fungal classes and fungal functional guilds response to factors. *PERMANOVA* on these models was conducted to study the overall multivariate response to factors in addition to univariate response. All results were conducted assuming a negative binomial distribution and resampled 999 times using the PIT-trap method, and the likelihood ratio was used as the test statistic. Significant effects are in bold and the level of significance (*P<0.05; **P<0.01; ***P<0.001).

PERMANOVA multivariate GLM model FH horizon -Fungal Classes				
	Res. Df	**Df. diff**	**Dev**	**Pr(>Dev)**
(Intercept)	34			
Species	29	5	245.73	0.116
Treatment	28	1	59.86	0.114
Species : Treatment	23	5	192.08	0.359
FH horizon - Fungal Functional Guilds				
(Intercept)	34			
Species	29	5	210.86	0.248
Treatment	28	1	66.72	**0.017**
Species : Treatment	23	5	178.53	0.100
PERMANOVA multivariate GLM model A horizon -Fungal Classes				
(Intercept)	35			
Species	30	5	241.71	0.085
Treatment	29	1	87.29	**0.012**
Species : Treatment	24	5	152.98	0.537
A horizon - Fungal Functional Guilds				
(Intercept)	35			
Species	30	5	247.80	**0.025**
Treatment	29	1	74.71	**0.019**
Species : Treatment	24	5	75.95	0.323
PERMANOVA multivariate GLM model B horizon -Fungal Classes				
(Intercept)	34			
Species	29	5	233.78	**0.045**
Treatment	28	1	71.53	**0.013**
Species : Treatment	23	5	200.26	0.156
B horizon -Fungal Functional Guilds				
(Intercept)	34			
Species	29	5	251.03	**0.015**
Treatment	28	1	79.74	**0.003**
Species : Treatment	23	5	152.98	0.151

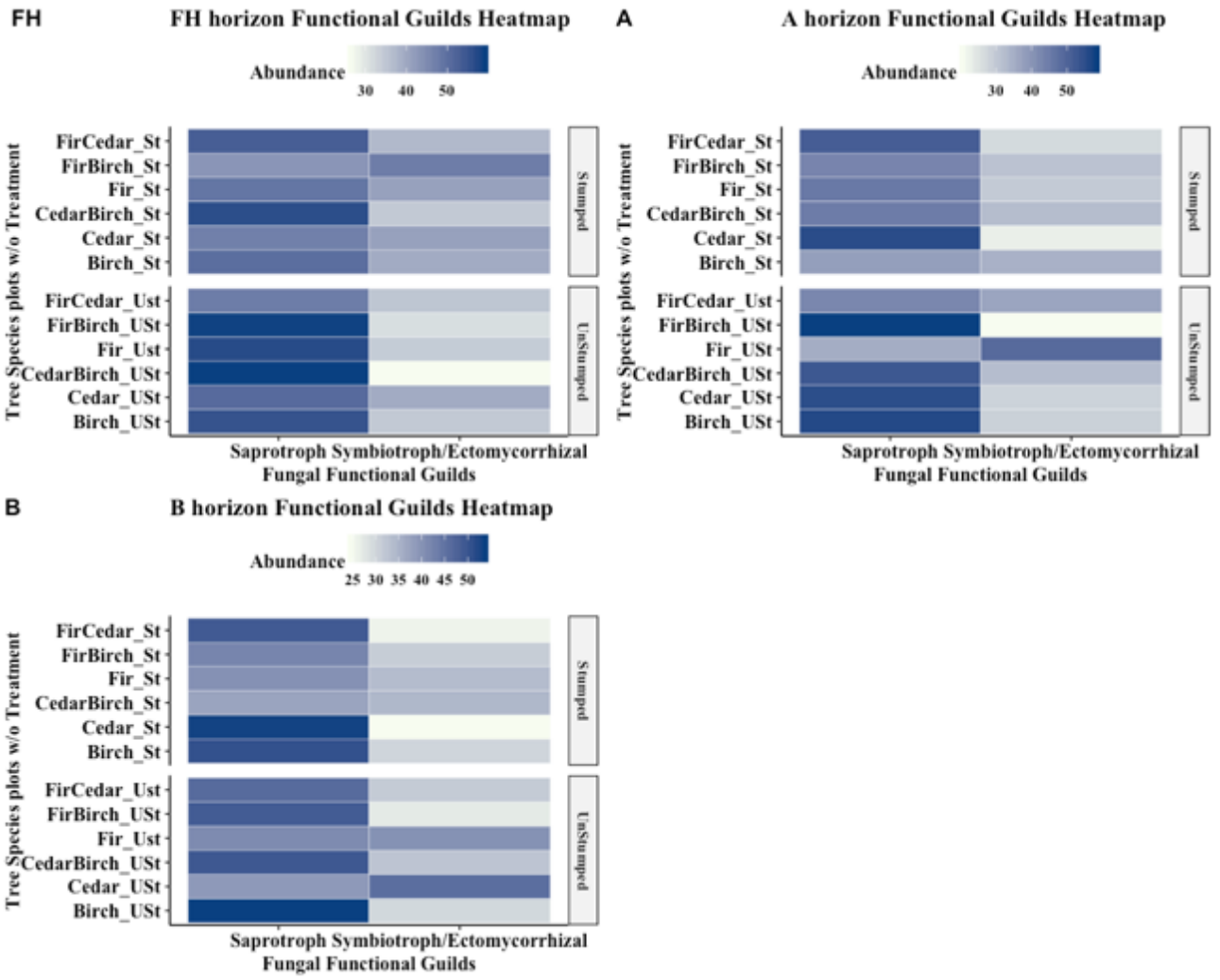


Figure 2.7 Mean relative abundances of the two most abundant fungal functional guilds (ECM and saprotrophs) in FH, A and B horizons of tree species and its ad-mixtures (fir, birch, cedar, fir-birch, fir-cedar, and cedar-birch) in stumped and unstumped plots.

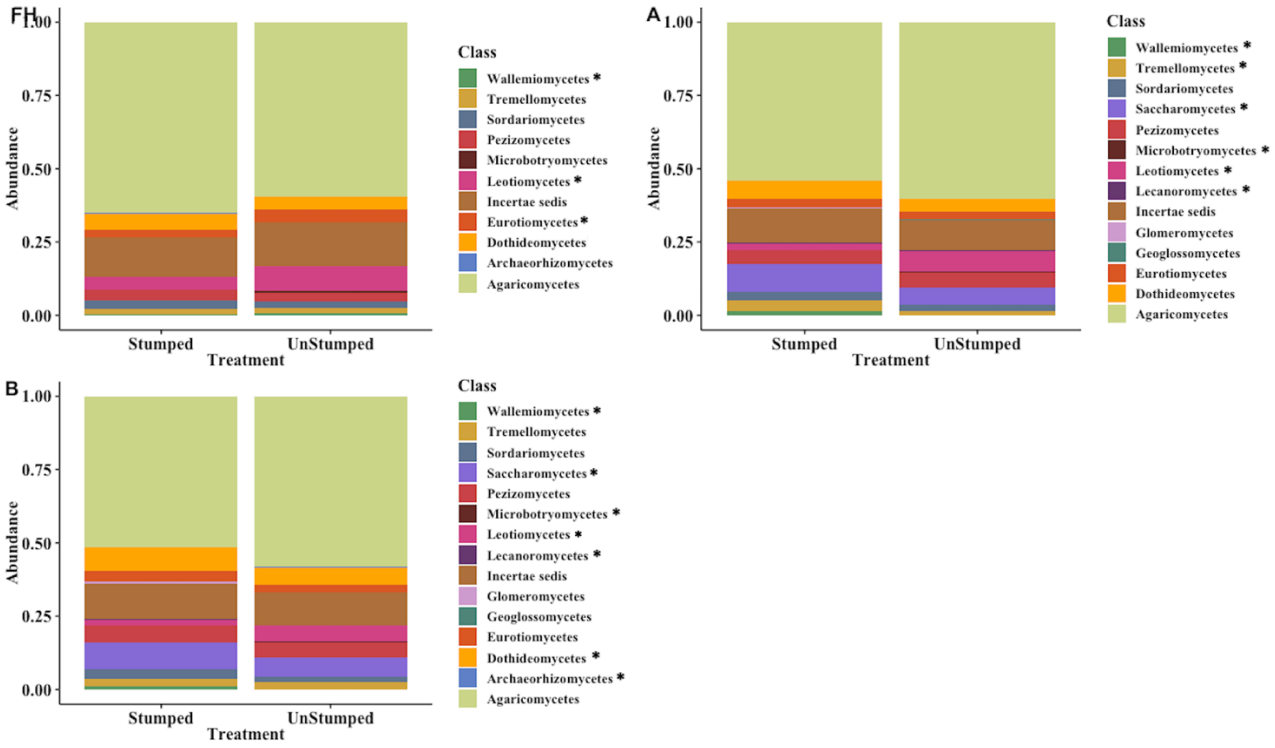
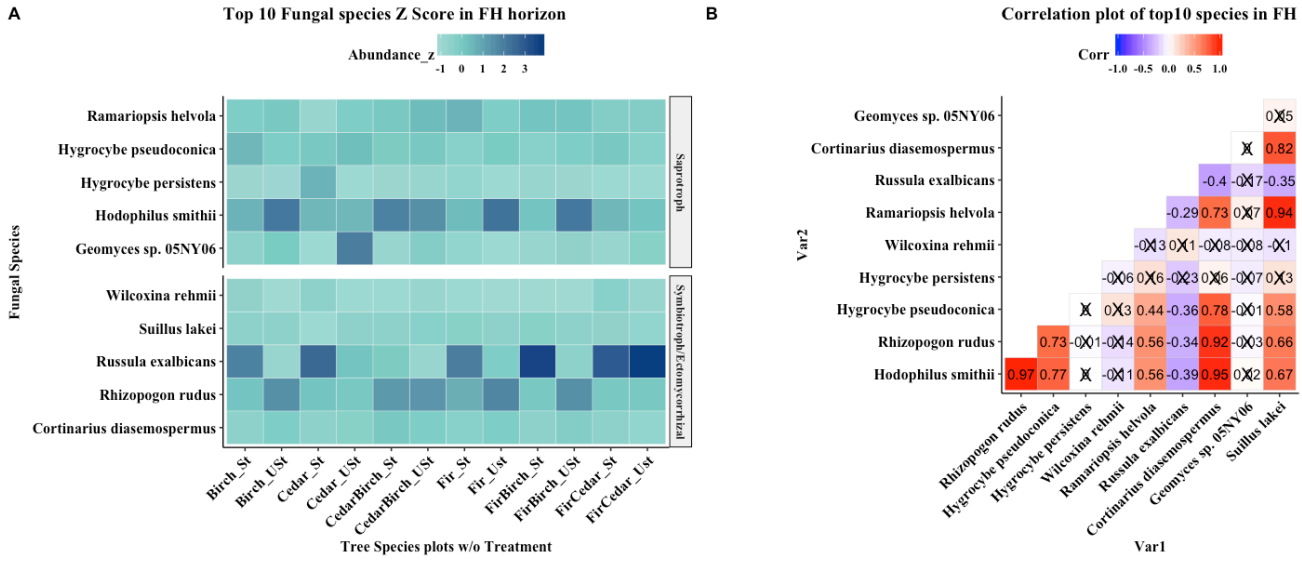


Figure 2.8 Mean relative abundances of fungal classes in FH, A and B horizons of tree species and its admixtures (fir, birch, cedar, fir-birch, fir-cedar, and cedar-birch) in stumped and unstumped plots. Fungal classes, having >2% abundance, were used. Asterisks (*) mark shows the significant classes that responded to stumping. (*P<0.05).



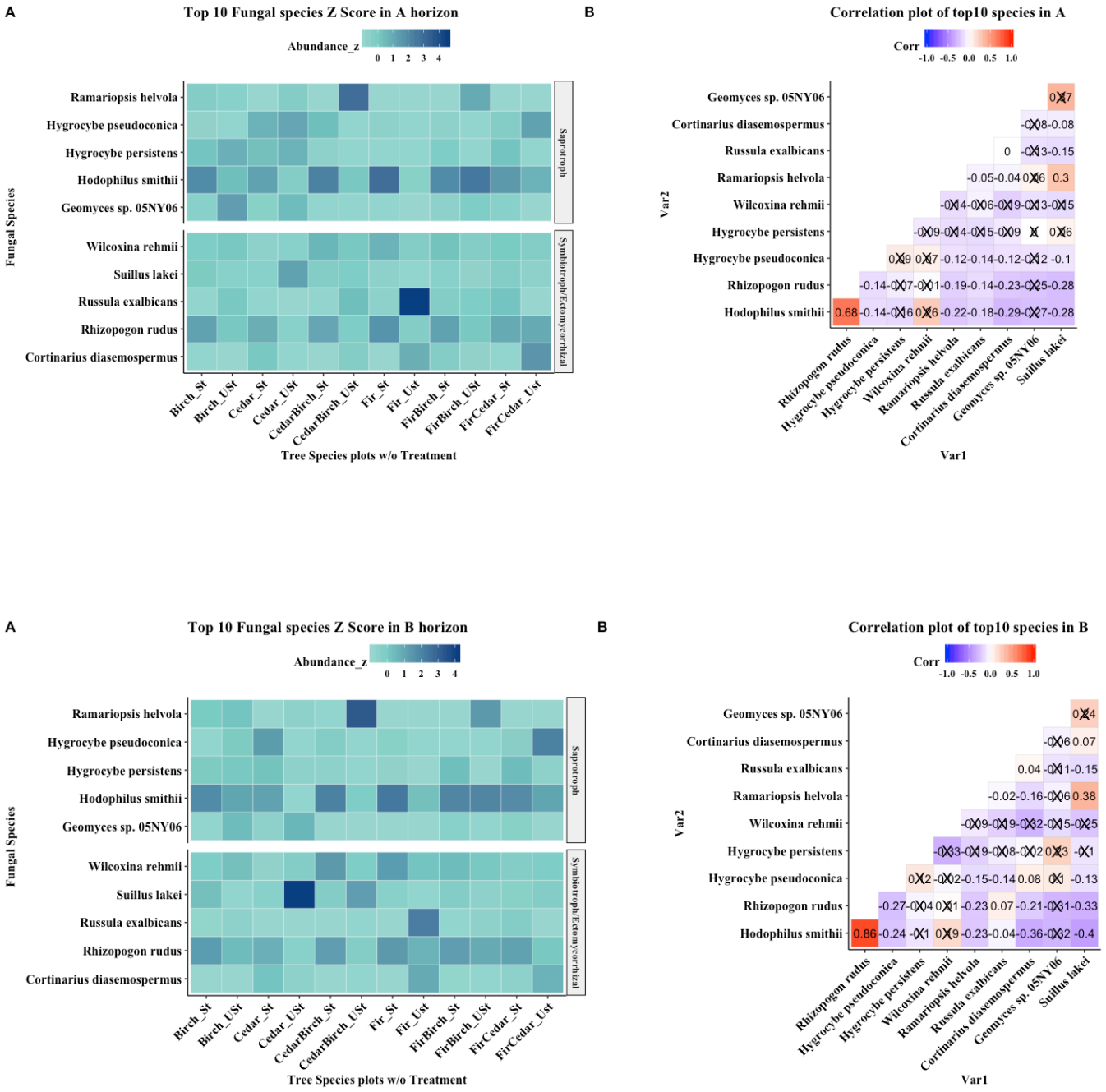


Figure 2.9 A. Z-scores of Top 10 most abundant fungal species in all three horizons (FH, A and B) of all tree species and their admixtures (fir, birch, cedar, fir-birch, fir-cedar, and cedar-birch) in stumped and unstumped plots. B. Correlation plots of the top10 fungal species of all the three horizons (FH, A and B) showing significant correlation coefficient values with P>0.05. The plots with values crossed were not significantly correlated (P>0.05).

2.4 Discussion

Forest trees are ubiquitously associated with consortia of fungal communities that include a variety of interactions, including parasitism, mutualism, and commensalism, and these play important roles in the health of the forest. There are numerous management practices developed to mitigate disease in forest trees such as stump removal, thinning and root raking. How these management practices affect the fungal mycobiome of the forest has not been deciphered in detail, although some studies have recently analyzed the effects of clear-cut harvesting on the fungal mycobiome (Hartman et al., 2014; Wilhelm et al., 2017; Kohout et al., 2018). In this study, we used the latest technologically advanced tool of metabarcoding to unravel the total fungal consortia (including both culturable and unculturable fungi) associated with different forest tree species and the effect of stumping on diversity and structure of fungal mycobiome.

2.4.1 Effects of stumping

We found that fungal species richness was greater in the stumped plots than unstumped control plots, leading us to reject our first hypothesis that fungal diversity will decrease with stumping. The practice of stump removal to reduce the spread of root rot could lead to either resistance, resilience or functional redundancy in the fungal communities, as suggested by Allison and Martiny (2008). Resistant fungal communities are considered insensitive to the disturbance because species composition largely stays the same, whereas resilient communities change, but then recover to their original composition. Alternatively, a community is considered to have functional redundancy when the composition of the community has been altered, but the functioning is the same as the original community (Allison and Martiny, 2008). In this study, we observed that stumping caused substantial alterations in the fungal mycobiome, confirming that the fungal communities were not resistant to the environmental disturbances. In general, rare fungal classes with low abundance were found to be

more sensitive to stumping than the more abundant generalists, the ECM and saprotrophic fungi. That the fungal mycobiome, regardless of tree species composition, was still affected by stumping after five decades, also suggests that the community lacked the capacity to fully recover. There was some evidence for functional redundancy in the fungal community: the ectomycorrhizas that replaced the saprotrophs following stumping are reported to have some capacity to break down the same substrates (Gadgil and Gadgil, 1971, 1975; Bödeker et al., 2016).

Stumping led to an increase in fungal α-diversity in the A horizon, in opposition to our first hypothesis. It is possible that the use of heavy machinery for stump removal led to soil compaction, disrupting the pore spaces between soil particles and allowing new species to flourish in newly formed micro niches (Hartman et al. 2014). It is also possible that the greater 40-year tree growth and survival that resulted from the stumping (Morrison et al. 2014) led to an increase in the photosynthetic carbon flux to the soil, fueling a more diverse fungal community.

2.4.2 Tree species effects

Fungal diversity associated with all tree species and admixtures was affected by stumping therefore our second hypothesis was confirmed. However, paper birch fungal mycobiome was found to be resistant to stumping. The constrained analysis of principal coordinates showed the fungal community structure in tree species plots within the stumping treatment were less variable than those in the respective unstumped control, indicating that stumping reduced the variability in the fungal community structure among tree species composition. This agrees with the Bray Curtis dissimilarity plots showing a reduction in β diversity among the various plantings. Stumping and root-raking removed the forest floor and at the same time could have compacted the soil and reduced the organic matter content, and these impacts may be responsible for the reduced heterogeneity and shift in fungal community structure with stumping. The reduced β diversity across species with stumping could also

have resulted from the decline in the saprotrophic community due to removal of woody debris, as well as disruption of ECM networks due to root-raking and compaction (Morrison et al., 1992; Berch et al., 2011; Hartmann et al., 2012). Thus, stumping and tree species appeared to favor the growth of fungal species that are resistant to disturbance, resulting in an overall decline in the heterogeneity of the total fungal community.

Although β diversity generally declined with stumping regardless of species, it was not reduced in any horizon in the soils planted to birch. This implies that the birch fungal mycobiome is the most resistant to stumping in all the three horizons, supporting our second hypothesis that stumping effects on fungal diversity depends on the tree species planted. As suggested by Bradley and Fyles (1995), birch trees have a greater affinity for soil microorganisms, possibly due to the larger surface area and length of root system (Priha et al., 1998) and greater belowground carbon allocation (Simard et al., 1997). Birch also has greater resistance to *A. ostoyae* than most conifers (Morrison et al. 1992) and can mitigate infection of Douglas-fir when the two species are planted in mixtures (Baleshta et al. 2005; Simard et al. 2005; Cleary et al. 2012).

2.4.3 Effects of stumping on fungal community composition

We observed a decline in abundance of saprotrophic community, and this was coincident with an increase in the ECM community in the FH horizon and thus our third hypothesis, that saprotrophic fungi, in particular, would decline with stumping because of the reduction in available substrate, was confirmed. Multivariate analyses showed that stumping affected the distribution of fungal classes in the A and B horizons, but to our surprise, not in the surface FH horizon. The univariate GLM analysis identified fungal classes responding to species x treatment interactions in all the horizons. The fungal classes most affected by stumping were the Leotiomycetes and Wallemiomycetes, and these effects occurred in all three horizons. The Leotiomycetes, a class of Ascomycota, declined with stumping in

all the horizons. This class was abundantly represented by OTUs belonging to saprotrophs, the most abundant species being *Geomyces* sp., supporting our third hypothesis that saprotrophic fungi would decline with stumping because of the absence of decayed stumps. Wallemiomycetes are a rare class of Basidiomycota having the characteristics of xero-tolerance (Zalar et al., 2005). This class increased in abundance in the mineral horizons of fir-cedar plots that had been stumped, but this did not correspond with changes in soil moisture content. It is quite possible that our soil moisture measurements were too coarse to detect changes in water content in the soil pores where these fungi exist. It is also possible that the ex-situ soil moisture measurements did not adequately reflect the in-situ conditions that were regulating the fungal community composition. The generalist fungi belonging to the most abundant fungal class, Agaricomycetes, was found to be resistant or resilient to the disturbance caused by stumping.

The most abundant fungal functional guilds in all the samples were the ECM fungi and the saprotrophs. Competition between these two functional guilds for nutrients could reduce decomposition rates of soil organic matter, contributing to the "Gadgil effect" (Gadgil and Gadgil, 1971, 1975). This was observed in FH horizon, where the abundance of saprotrophs was lower in the stumped than unstumped treatment where there was significantly higher total C, indicating probable slower decomposition rates of the SOM (Bending, 2003). Jones et al., (2003) observed that the major impact of clear-cut logging on the ECM fungal community was change in the species composition, rather than reduction in the percentage of roots colonized. Our results partially support this observation because we observed an increase in the abundance of the ECM fungal class in the FH horizon of the stumped over unstumped plots (Fig 6). The results also agree with greater tree survival in the stumped plots, which favored the growth of ECM fungal species at the expense of the saprotrophs and contributed to better health of the trees (Morrison et al., 2014).

Among the ten most abundant fungal species found, half were ECM fungi and half were saprotrophic fungi. The ECM fungi, *Rhizopogon rudus* and *Russula exalbicans*, and the saprotroph *Hodophilis smithii* were the most highly abundant species. There was a positive correlation in all the three horizons between abundance of *Rhizopogon rudus* and that of *Hodophillus smithi,* possibly leading to competition for nutrients from the same substrate (Bödeker et al., 2016). *Hodophilus smithii*, a recently identified saprotrophic species, has been found to be native to northeastern American forests (Adamčík et al., 2016). *Rhizopogon*, an ECM genus with disturbance resistant spores, is commonly associated with Pinaceae family trees especially with pine and firs (Molina and Trappe 1994; Luoma et al., 2006). *Rhizopogon rudus,* for example, was found to be abundant in ICH forests stands originating from wildfire (Twieg et al., 2007). In this study, we also found high abundance of *Rhizopogon rudus* in the mineral horizons of stumped plots, where stumping and root raking reduced organic matter content and fungal inoculum. We assume that *Rhizopogon rudus* fungal hyphae present in the deeper layers of the mineral soil aided in colonization of the forest trees, which led to its increased abundance in the mineral horizons (A and B) relative to the FH horizon in the stumped plots (Stendell et al., 1999; Dahlberg, 2002).

Armillaria ostoyae, the root rot-causing species on this site (Morrison et al., 1988), was found to occur in some samples but had negligible abundance. The other OTU, with ~200 reads, was affiliated with *Armillaria* cf. *calvescens* X-59, which is a species commonly found to co-occur with *Armillaria ostoyae.* However, these two species are difficult to differentiate in the absence of the basidiocarp (Bérubé and Dessureault, 1989). The possible reason for the low abundance of *Armillaria* sp. in our dataset could be that the soil samples were sieved, which might have resulted in the loss of rhizomorphs, the main source of *Armillaria* sp. identification. Further studies specifically targeting *Armillaria* sp. are needed to unravel the diversity and abundance of this species at this site.

In conclusion, stumping affected overall fungal diversity, but rare fungal classes with low abundance were more sensitive to stumping than the generalists with higher abundance, such as the ECM fungi. The replacement of saprotrophs with ectomycorrhizas with stumping indicates a shift in functional guilds without a concomitant alteration in the abundance of the fungal classes, which can be interpreted as a characteristic of resistance in fungal community structure to stumping. The fungal community of paper birch was resistant to stumping, whereas that of all other tree species including admixtures was significantly affected. We thus suggest that paper birch be included in mixtures with susceptible tree species, such as Douglas-fir, in order to maintain a healthy fungal community structure and reduce the negative effects of stumping on the mycobiome. In particular, mixtures with paper birch could promote the abundance of favorable fungal species, while reducing the inoculum load of fungi causing root disease (such as *Armillaria* and *Phellinus*). This study has a high significance as it unveiled major and minor fungal players associated with different tree species and this knowledge can ultimately be used to mitigate disease progression, promote a healthy mycobiome, and maintain forest health.

Chapter 3: Stump removal and tree species composition promote a bacterial microbiome that may be beneficial in the suppression of root disease

3.1 Introduction

Forests trees are dominant primary producers in the most highly productive terrestrial ecosystems globally. Bacteria and their interactions with fungi are indispensable for the functioning of many soil processes, including decomposition and nutrient cycling, as well as suppression of pathogens that affect the health of trees (Beneduzi et al., 2012; Lladó et al., 2017). Forest trees are known to shape microbial communities by providing photosynthate to fuel metabolic processes, in return for access to nutrients such as nitrogen and phosphorus for growth and development (Urbanová et al., 2015; Heijden et al., 2008). Tree species composition and diversity alter the composition of the fungal and bacterial communities in forest soils (Prescott and Grayston 2013; Bonito et al., 2014; Urbanová et al., 2015), and while fungi are well-studied (Voříšková et al., 2014; Baldrian, 2017; Shi et al., 2019), the bacterial community has been largely ignored (Lladó et al., 2017).

Forests face a multitude of natural biotic and abiotic disturbances as well as anthropogenic pressures from climate change, pollution and some management practices (Trumbore et al., 2015). Stump removal, commonly known as stumping, is a common forest management practice for reducing the spread of naturally occurring root diseases caused by *Armillaria* and *Phellinus* fungal species. This management practice can damage soil through compaction, pore disruption, and removal of organic matter (OM), potentially reducing microbial diversity and interfering with ecosystem functioning (Page-Dumroese et al., 1998, Hope, 2007; Kataja-aho et al., 2012; Kaarakka et al., 2016). For instance, soil compaction caused by stump removal can favor growth of anaerobic bacterial communities by disrupting pore space and reducing oxygen concentrations (Schnurr-Putz et al., 2006). Moreover, loss of OM with root-raking and removal of stumps can

lead to losses of copiotrophic bacteria (Fierer et al., 2007). The impact of stumping on soil bacterial diversity has not been well studied, whereas clear-cutting and tree planting effects on the microbial community has been widely quantified (Smith et al., 2008; Hartmann et al., 2009, 2012; Sun et al., 2017). Planting tree species resistant or tolerant to root rot in a mixture with susceptible tree species is a management practice to reduce the incidence of *Armillaria* and *Phellinus*. Tree species such as western redcedar and paper birch are resistant and immune to *P. sulphurascens*, respectively, and less susceptible to *A. ostoyae*, whereas Douglas-fir is highly susceptible to both pathogens (Cleary et al., 2008, 2011).

Forest soils provide highly diverse microbial habitats, harboring horizon-specific bacterial communities as well as many overlapping taxa (López-Mondéjar et al., 2015). The structure and diversity of bacterial communities are closely interrelated with soil physicochemical characteristics such as C/N ratio, soil pH etc. (Fierer et al., 2006; Hartman et al., 2008). Forest soils are reported to be dominated by five bacterial phyla, including *Acidobacteria*, *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* (Lauber et al., 2009). The recent development of next generation Illumina sequencing is a powerful tool to further explore the diversity and composition of the bacterial communities in forest soils with unprecedented resolution (Orgiazzi et al., 2015). Using this platform, groups of scientists are working across the globe on "The Earth Microbiome Project" to collect and catalogue information on microbial diversity (Gilbert et al., 2014).

The present study builds on our recent work examining the effect of stumping and tree species composition on fungal diversity and community structure using Illumina sequencing. Fungi showed increased diversity in the A horizon and tendency to increase in FH and B horizons due to stumping. Fungal study also showed a significant reduction in saprotrophic communities

with an increase in ECM communities due to stumping in FH horizon. In this study, we hypothesized that 1) stump removal will reduce the diversity of the bacterial community, and cause reductions in specific taxa associated with aerated and carbon-rich soils (i.e.) lignolytic bacteria helping saprotrophic fungi to decompose further; 2) tree species composition would shape bacterial community structure, particularly birch would show the abundance of beneficial bacteria that may promote recovery from the stump removal disturbance. We tested these hypotheses by comparing soil bacterial communities in different soil horizons between stumped and unstumped plots planted with different tree species compositions.

3.2 Methods

3.2.1 Study site and sampling strategy

Soil samples were collected from a 48-year-old experimental site at Skimikin (50°48'N, 119°26'W) near Salmon Arm, British Columbia (Weir and Johnson 1970). The site occurs on a mesic site series in the Interior Cedar Hemlock (ICH) bio-geoclimatic zone, where the original 80-year-old forest was comprised of 75% Douglas fir, 25% lodgepole pine, and minor amounts of western redcedar. The soil great group is a Eutric Brunisol developed over a glacial fluvial deposit.

During April 2016, soils were randomly sampled from the three replicates of a subset of the tree species treatments (fir, cedar, birch, fir/birch, fir/cedar, and cedar/birch) in the stumped and unstumped blocks. The litter (L) layer was first removed, and samples were then collected using marked trowels from the FH (fermentation and humus) layer, A horizon (0 to 10 cm mineral soil), and B horizon (10 to 20 cm mineral soil). Triplicate samples were taken from each planting treatment plot and composited, resulting in three composite samples per planting treatment.

3.2.2 DNA isolation, metabarcoding and sequencing

Total genomic DNA was extracted from 0.5 g of the sieved soil (<2 mm) using a MoBio PowerSoil DNA extraction kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. All DNA extractions were performed in triplicate, and extracts were combined into one sample. The polymerase chain reaction (PCR) was performed in triplicate to reduce PCR bias. The V4 region of bacterial 16S rRNA genes of the 108 soil DNA samples was amplified using the HotStarTaq Plus Master Mix Kit (QIAGEN, USA), the 515F (Parada et al., 2016)/806R (Apprill et al., 2015) primer set with barcodes in the forward primer, and following the Earth Microbiome project protocol for PCR. PCR products were purified using Ampure XP beads and were paired-end sequenced (2 × 300) in an Illumina MiSeq Sequencing platform at UBC's Sequencing and Bioinformatics Consortium (Vancouver, B.C., Canada).

Sequence processing was performed with QIIME 2 2017.4, a plugin-based system that, in some cases, wraps other microbiome analysis methods (Bolyen et al., 2019). Briefly, raw paired-end demultiplexed sequences were quality filtered, trimmed, denoised as well as merged using DADA2 (via q2-dada2) (Callahan et al., 2016, 2017). Chimeric sequences were filtered out using VSEARCH plugin q2-vsearch for open-reference OTU clustering (Rognes et al., 2016, Bokulich et al., 2013). Sequences were clustered into amplicon sequence variants (ASVs) (i.e., operational taxonomic units (OTUs) at 100% identity. Taxonomy was assigned to OTUs using the q2-feature-classifier plugin with the classify-sklearn Naïve Bayes taxonomy classifier against the Greengenes 13_8 97% OTUs reference database (McDonald et al., 2012). This classifier has been shown to achieve similar precision and recall to the RDP classifier at the genus level on 15 mock community data sets (Wang et al., 2007). The multiple sequence alignment of OTUs was performed with mafft

(via q2-alignment) (Katoh and Standley, 2013) and phylogenetic reconstruction was carried out using fasttree2 (via q2-phylogeny) (Price et al., 2010).

Further data analyses were performed in R ver. 3.5.1 using the phyloseq (McMurdie and Holmes, 2013). Bacterial diversity (α-diversity) of each planting treatment plot was calculated using the Shannon diversity index. Shapiro-Wilks test was performed on the Shannon diversity index to check the normality of the data, and this was followed by Wilcoxon rank sum to test for the significance of differences between stumping or tree species treatments. A Bray-Curtis dissimilarity matrix as an index of β-diversity was calculated using the "vegdist" function of the "vegan" package (Oksanen et al., 2016). A principal-coordinate analysis (PCoA) using a Bray-Curtis dissimilarity matrix on community data was performed to visually assess whether sites with different tree species and/or stumping treatments harbored different bacterial communities. Analysis of similarity (ANOSIM) on Jaccard distance and permutational multivariate analysis of variance (PERMANOVA) on Bray-Curtis dissimilarities was conducted to study the effects of different factors on the structure of bacterial communities using the "anosim" and "adonis" function respectively of the vegan package (at $P < 0.05$) (Oksanen et al., 2016).

Multivariate generalized linear models were used to evaluate bacterial phylum responses to the experimental factors using the "mvabund" package (Wang et al., 2012). PERMANOVA was conducted on these models to study the overall multivariate response to the experimental factors in addition to the univariate responses. The analysis was conducted assuming a negative binomial distribution and resampled 999 times using the PIT-trap method, and the likelihood ratio was used as the test statistic. Hypothesis testing was conducted using the "anova.manyglm" function, and treatment contrasts were evaluated using the "summary.manyglm" function. Treatment effects were considered significant at $\alpha = 0.05$. For in-depth understanding of *Proteobacteria* composition

and distribution, similar multivariate analysis was performed at the order level of *Proteobacteria* using "mvabund" package (Wang et al., 2012).

Heat trees, which use hierarchical structure of taxonomic classifications to quantitatively (median abundance) and statistically (non-parameteric Wilcoxon Rank Sum test) depict taxon differences among communities, were generated using the "metacodeR" package. These were used to observe differences in phyla and class abundances among treatments (Foster at al., 2018). Linear discriminant analysis (LDA) and effect size (LEfSe) analyses were performed using the LEfSe tool (Segata et al., 2011) in order to study the differences in bacterial abundance between stumping treatments at the genus level. LEfSe analysis was performed taking treatment (Stumped vs Unstumped) as a factor after normalizing data with total sum scaling (TSS). Kruskal-Wallis rank sum test with FDR (false discovery rates) adjusted p values < 0.05 was used to detect significantly different abundances between stumped and unstumped treatments, along with generating LDA scores to estimate the effect size (threshold: ≥ 2). All the graphs were made using "ggplot2" (Wickham, 2016) and "ggpubr" (Kassambara, 2017) packages. The raw sequence data have been deposited in the NCBI Sequence Read Archive (BioSample accession no. SAMN12881708 and BioProject accession no. PRJNA575258).

3.2.3 Soil sample collection for the isolation of diazotrophs

Soil samples were taken from the stumped plots of the Skimikin trial in order to isolate nitrogen fixing bacteria. Three replicates of soils samples were randomly taken with soil core near the base of all the three tree species fir, birch and cedar. The soil samples were kept on ice before storage at -20°C and processed within 2 days. The soil samples were serially diluted and aliquots (50µl) of serial dilutions were spread on nitrogen-free combined carbon medium agar (CCMA) (Rennie 1981). The CCMA plates were incubated at 30°C for 2-3 days and were screened for different

colonies morphologically. The screened colonies were then streaked on CCMA plates several times to achieve pure cultures. The pure cultures were then finally streaked on nutrient agar (NA) and also grown in nutrient broth for DNA extraction.

3.2.4 DNA isolation from bacterial isolates

Genomic DNA was isolated from 25 isolated pure cultures grown in nutrient broth using MoBio PowerSoil DNA extraction kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. All DNA extractions were performed in triplicate, and extracts were combined into one sample. The primers 27F and 1492R (Lane, 1991) were used for PCR amplification of the 16S ribosomal DNA. The polymerase chain reaction (PCR) was performed in triplicate to reduce PCR bias. PCR products were purified and sequenced at UBC's Sequencing and Bioinformatics Consortium (Vancouver, B.C., Canada).

3.2.5 Sequencing and phylogenetic analysis

All the DNA sequences were then edited using BioEdit and matched with GenBank sequences using BLAST. Phylogenetic analysis of the 16S rRNA gene sequences was performed using sequences of previously reported bacteria along with Acidobacterium species *Acidobacterium capsulatum* as a root. All the sequences were aligned using MAFFT version 7 using the FFT-INS-i option (Katoh & Standley, 2013). The phylogenetic trees were inferred using the Maximum Likelihood (ML) method with 1000 replicates for bootstrap analysis and the GTR+G model of evolution using RAxML (Stamatakis, 2006).

3.3 Results

3.3.1 Sequencing and community composition

A total of 7.5 million clean double-stranded Illumina DNA reads were obtained after denoising and filtering. Sequence clustering with a sequence similarity threshold of 97% produced 13733

bacterial OTUs. A total of 12973 (94%) OTUs out of the 13733 were identified at the phylum level, 12278 (89%) OTUs at the class level, 9664 (70%) OTUs at the order level, 5790 (42%) OTUs at the family level, 2023 (15%) OTUs at the genus level, and 166 (1.2%) OTUs at the species level. Sequences with >2% abundance were attributed to seven bacterial phyla, including *Proteobacteria* (34.5%), within which *Alphaproteobacteria* (15.9%) was the largest class followed by *Acidobacteria* (15.5%), *Actinobacteria* (13.6%), *Bacteroidetes* (12.6%), *Verrucomicrobia* (10%), *Gemmatimonadetes* (5.8%), and *Planctomycetes* (2.1%) (Figure 3.1).

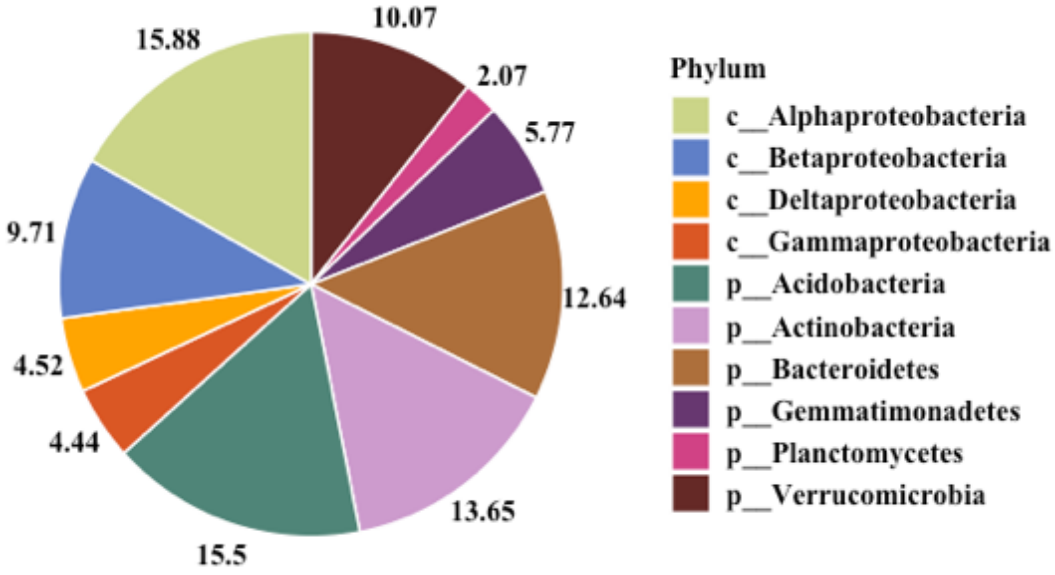


Figure 3.1 Overall proportion of bacterial classes (c_) belonging to the most abundant phyla (p_) (> 2% of the sequences)

3.3.2 Influence of tree species and stumping treatments on α and β diversity

For determining diversity and abundance, the sequencing data for the three horizons (FH, A and B) was rarefied to a minimum library size (i.e., the minimum number of reads in a sample). The

analysis resulted into 7750 OTUs in the FH horizon, 7162 OTUs in the A horizon and 5606 OTUs in the B horizon. Alpha diversity based on Shannon's index decreased significantly ($P < 0.01$) with stumping in the B horizon (Figure 3.2). Although not significant, alpha diversity also tended to decrease with stumping in the A horizon, with little to no change in FH layer (Figure 3.2).

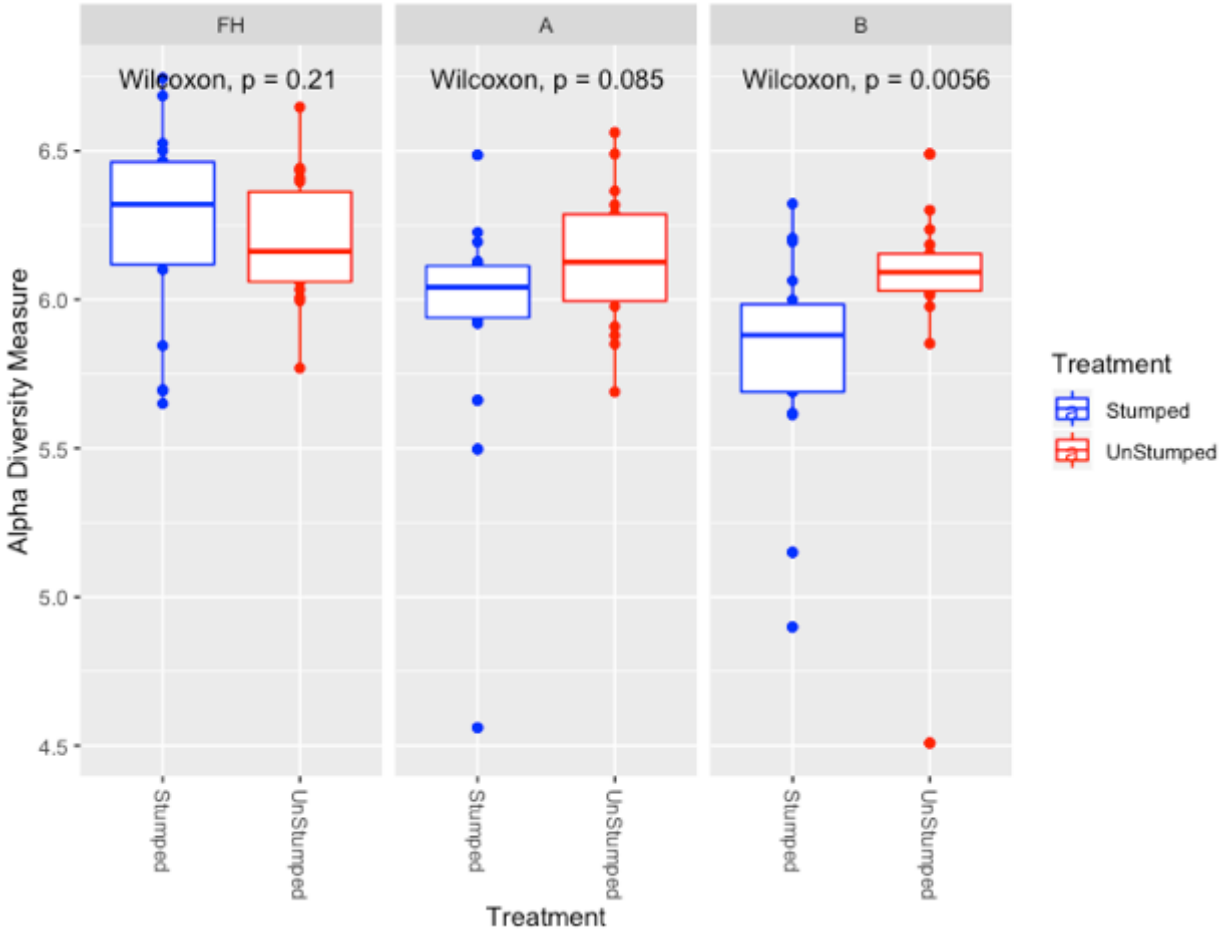


Figure 3.2 Shannon index showing α diversity of all horizons (FH, A and B) vs treatment (Stumped and Unstumped).

Table 3.1 Effect of factors (tree species and treatment) and their interaction on bacterial β-diversity of all the three layers (FH, A and B) assessed by PERMANOVA. Significant ($p<0.05$) effects are in bold.

Factors	Df	SumsOfSqs	MeanSqs	F. Model	R2	Pr(>F)
FH Horizon						
Species	5	0.9615	0.19230	1.4267	0.18059	**0.014**
Treatment	1	0.4371	0.43714	3.2434	0.08210	**0.001**
Species:Treatment	5	0.6906	0.13818	1.0252	0.12976	0.425
Residuals	24	3.2347	0.13478	0.60755		
Total	35	5.3242	1.00000			
A Horizon						
Species	5	1.1305	0.22610	1.44806	0.18609	**0.004**
Treatment	1	0.4512	0.45121	2.88974	0.07427	**0.001**
Species:Treatment	5	0.7459	0.14917	0.95535	0.12277	0.590
Residuals	24	3.7474	0.15614	0.61686		
Total	35	6.0750	1.00000			
B Horizon						
Species	5	1.1795	0.23589	1.5081	0.18861	**0.008**
Treatment	1	0.5829	0.58286	3.7263	0.09320	**0.001**
Species:Treatment	5	0.7372	0.14744	0.9426	0.11788	0.598
Residuals	24	3.7541	0.15642	0.60031		
Total	34	6.2536	1.00000			

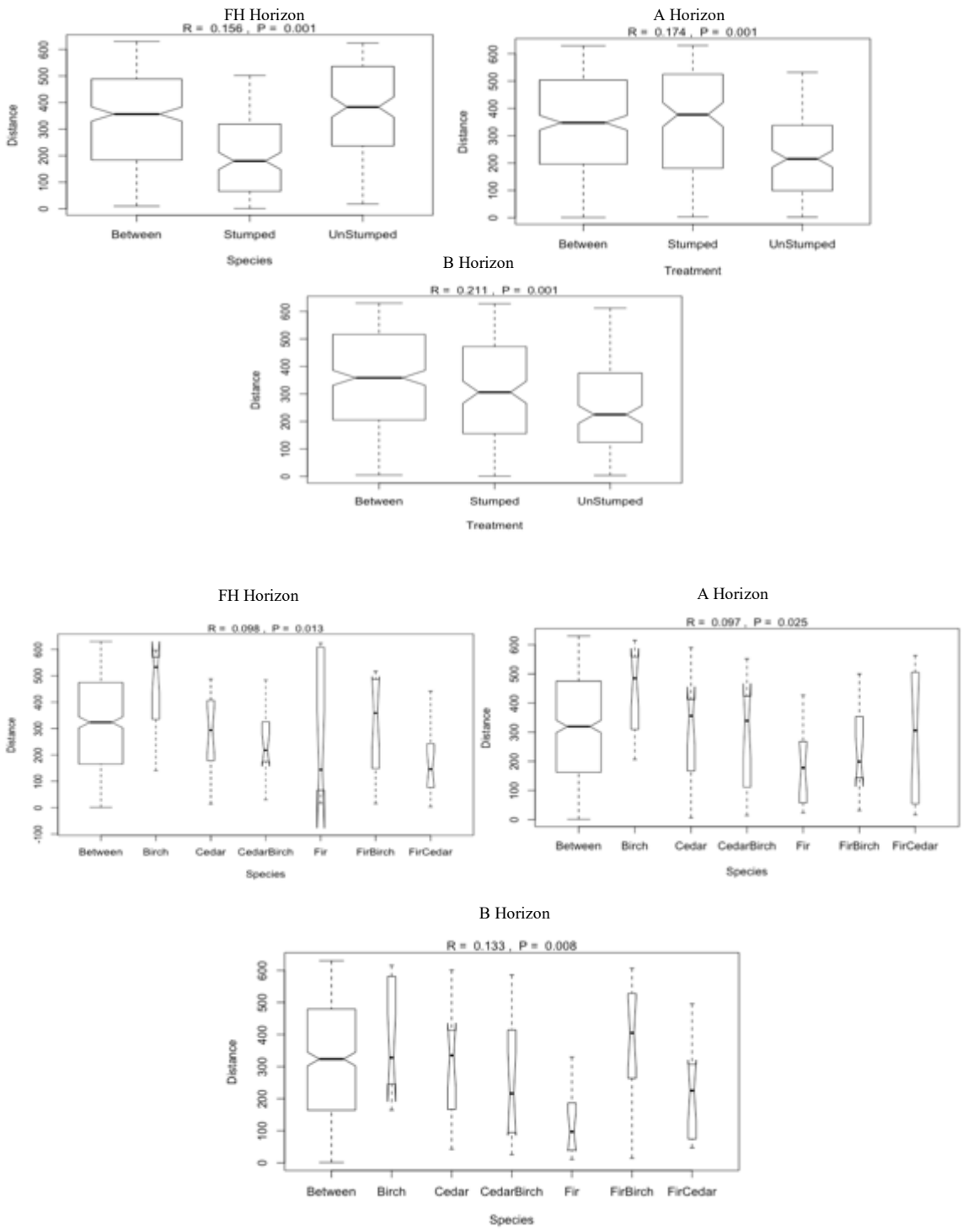


Figure 3.3 ANOSIM plots of between and within site dissimilarities of bacterial communities in all the three horizons (FH, A and B) of tree species and their admixtures (birch, cedar, fir, cedar-birch, fir-birch, fir-cedar) as well as stumping treatments.

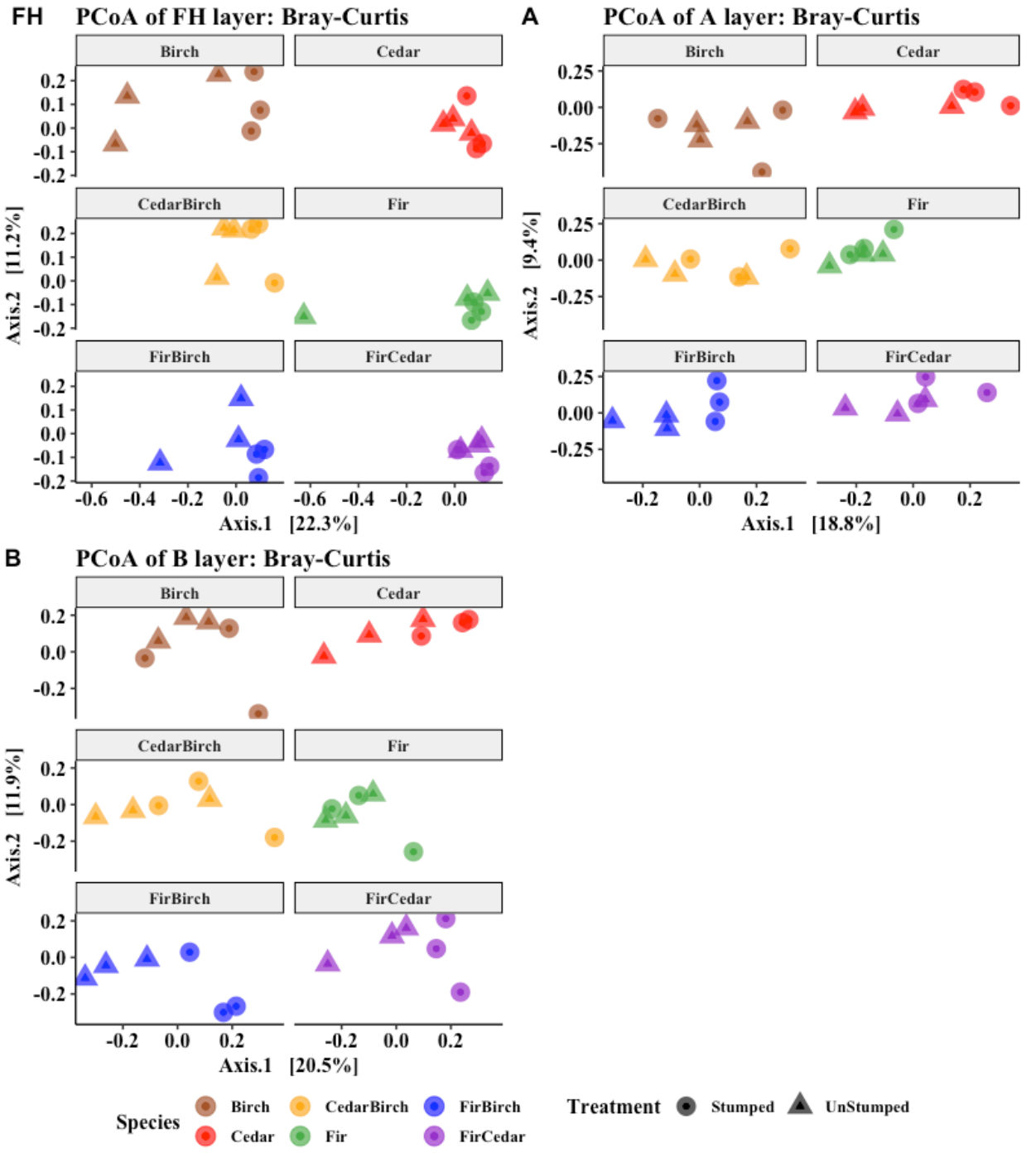


Figure 3.4 Bray–Curtis dissimilarities between samples were calculated using the "vegdist" function of the vegan package. Principal coordinates analysis (PCoA) of Bray-Curtis dissimilarity matrix calculated based on OTU abundances of all three horizons (FH, A and B).

Stumping and tree species composition altered the bacterial communities in all three horizons (Table 3.1). The ANOSIM plots of between and within plot dissimilarities showed clear distinctness in bacterial communities among tree species as well as between stumping treatments (Figure 3.3). The principal coordinates analysis (PCoA) distinguished the stumped from unstumped treatment for all three horizons, with the distinction tending to diminish with depth (Figure. 3.3). Stumping was particularly impactful on the bacterial community in the fir and birch mixture for all three horizons (Figure 3.4). Planting birch alone or in mixture with fir or cedar created greater distinction in the bacterial communities of the FH layer between the stumped and unstumped plots than did the other planting compositions (Figure 3.4). Dissimilarity plots show that the microbial community of fir alone was considerably different than that of the other tree species treatments regardless of horizon, but when fir was mixed with either birch or cedar, the microbial communities were more similar to the admixture species. Stumping distinctly affected the microbial community in the birch and cedar birch plantings in FH horizon, birch and cedar plantings in the A horizon, as well as cedar and fir-cedar plantings in B horizon (Figure. 3.4)

3.3.3 Comparative abundance of bacterial community at phyla and class levels

Multivariate analysis showed bacterial phyla were affected by stumping and tree species in the A and B horizons but not the FH layer, and the stumping X species interactions were significant for the FH and A horizons ($P<0.05$) (Table 3.2, Appendix A: Figure A.1). The heat trees show the comparative abundance of phyla between unstumped vs stumped plots. The taxa colored in red are more abundant in unstumped plots and the taxa colored in blue are more abundant in stumped plots (Figure 3.5). Appendix Table A1-3 lists the taxa that differed significantly between stumped and unstumped treatments and that are depicted in the heat trees of FH, A and B horizons, respectively.

The heat tree analysis revealed that the FH horizon had significantly greater abundance of major classes such as Bacilli (Firmicutes), Gemm_1, and Gemm_5 (Gemmatimonadetes) in unstumped plots whereas classes such as Deltaproteobacteria, Chloroflexi, Anaerolineae, Cytophagia, Opitutae, Verrucomicrobia, and some uncultured classes such as C0119 (phylum Chloroflexi), 0319_6E2 (Armatimonadetes), VC2_1_Bac22 (Bacteroidetes), and vadinHA49 (Planctomycetes) were greater in stumped plots (Figure 3.5, Appendix A: Table A.1).

In the A horizon, Nitrospira, Deltaproteobacteria, Betaproteobacteria, and Chloracidobacteria Gemm_1, Gemm_5 (Gemmatimonadetes), Fimbriimonadia, Cytophagia, Bacteroidia, Opitutae, and some uncultured classes were significantly greater in unstumped than stumped plots, whereas Solibacteres and Acidobacteriia classes were significantly enriched in the stumped plots (Figure 3.5, Appendix A: Table A.2).

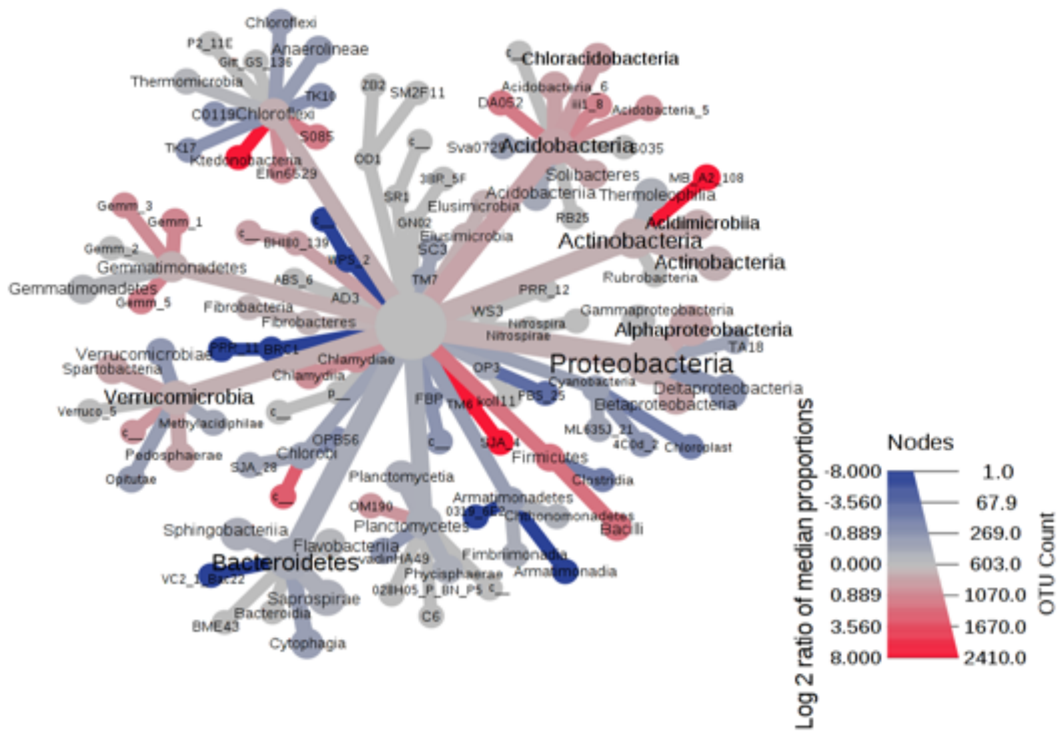
Cytophagia, Planctomycetia, Opitutae, Nitrospira, Gemm_1 (Gemmatimonadetes), Phycisphaerae, Chloracidobacteria and some uncultured classes were significantly higher in abundance in the B horizon of unstumped than stumped plots. Alphaproteopbacteria, Sphingobacteriia, Chthonomonadetes and Acidobacteriia classes were significantly more abundant in stumped plots (Figure 3.5, Appendix A: Table A.3)

Table 3.2 Multivariate generalized linear models were used to evaluate bacterial phyla and Proteobacteria orders' response to factors. *PERMANOVA* on these models was conducted to study the overall multivariate response to factors in addition to univariate response. All results were conducted assuming a negative binomial distribution and resampled 999 times using the PIT-trap method, and the likelihood ratio was used as the test statistic. Significant effects are in bold.

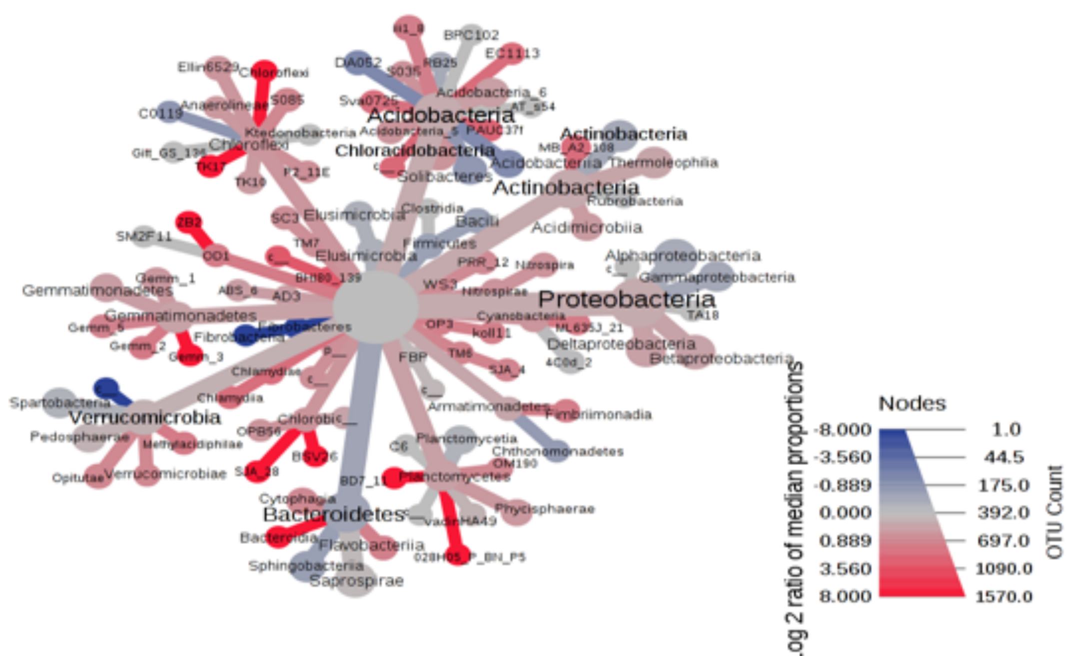
PERMANOVA multivariate GLM model FH horizon - Bacterial Phyla				
	Res. Df	**Df. diff**	**Dev**	**Pr(>Dev)**
(Intercept)	35			
Species	30	5	272.0	0.204
Treatment	29	1	136.3	0.292
Species : Treatment	24	5	1050.0	**0.001**
PERMANOVA multivariate GLM model A horizon - Bacterial Phlya				

(Intercept)	35			
Species	30	5	317.6	**0.025**
Treatment	29	1	96.4	**0.010**
Species : Treatment	24	5	395.2	**0.009**
PERMANOVA multivariate GLM model B horizon - Bacterial Phlya				
(Intercept)	35			
Species	30	5	309.9	**0.021**
Treatment	29	1	127.1	**0.025**
Species : Treatment	24	5	189.5	0.795
PERMANOVA multivariate GLM model FH horizon –Proteobacteria				
(Intercept)	35			
Species	30	5	301.4	0.141
Treatment	29	1	150.1	**0.001**
Species : Treatment	24	5	278.1	0.253
PERMANOVA multivariate GLM model A horizon –Proteobacteria				
(Intercept)	35			
Species	30	5	376.1	**0.005**
Treatment	29	1	1555.3	**0.001**
Species : Treatment	24	5	244.5	0.466
PERMANOVA multivariate GLM model B horizon –Proteobacteria				
(Intercept)	35			
Species	30	5	331.7	**0.013**
Treatment	29	1	188.6	**0.002**
Species : Treatment	24	5	246.8	0.398

UnStumpedFH_vs_StumpedFH



UnStumpedA_vs_StumpedA



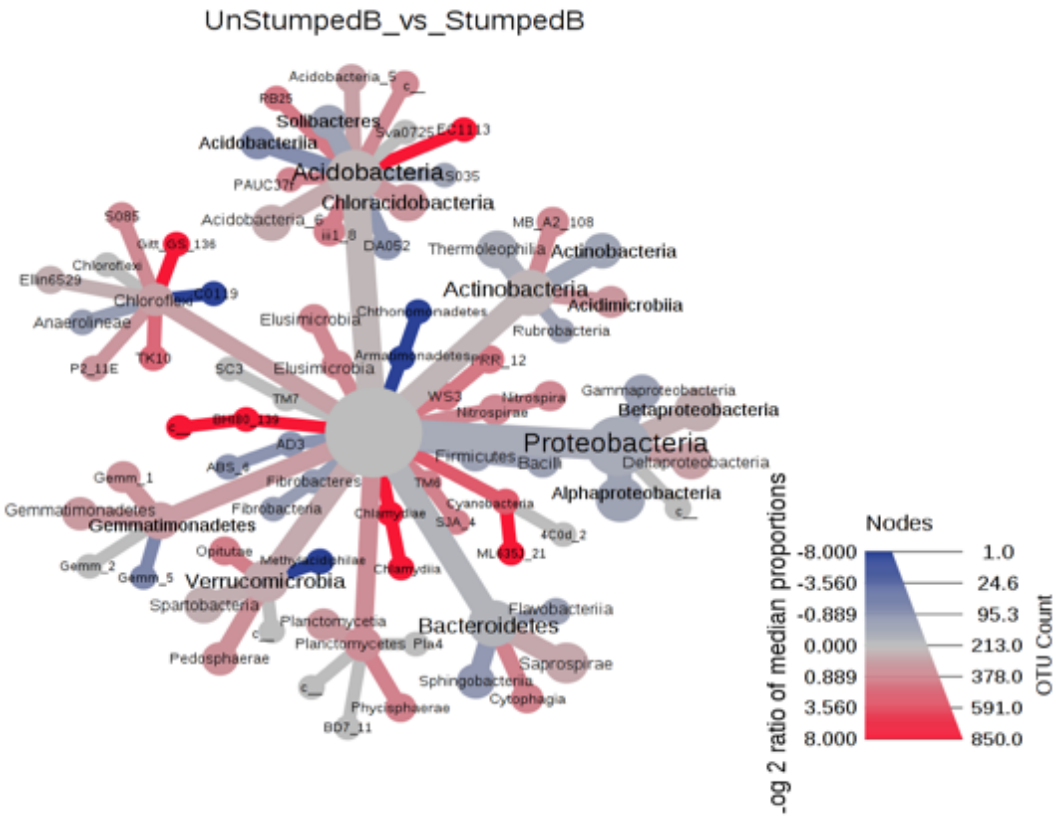


Figure 3.5 Heat trees were generated using metacodeR to observe the difference in abundance of taxa regarding treatments in all the three horizons (FH, A and B). The log2_median_ratio is the ratio of log2(unstumped/stumped), where the taxon has more counts in unstumped plots, when the ratio is positive (colored in red) and the taxon has more counts in stumped plots, when the ratio is negative (colored in blue).

3.3.4 Comparative community structure of proteobacteria phyla at the order level

The order levels of Proteobacteria were studied for detailed information on this highly diverse and abundant phylum. The Psuedomonadales order belonging to the Gammaproteobacteria phylum was substantially more abundant in all the three horizons in the stumped than unstumped plots (Figure 3.6). Deltaproteobacteria (Myxococalles) was more abundant in the FH horizon of stumped than unstumped plots, whereas Legionellales (Gammaproteobacteria) was more abundant in FH and A horizons of stumped than unstumped plots. Burkholderia belonging to Betaproteobacteria increased significantly in the A and B horizons with stumping (Figure 3.6).

Tree species composition also had significant effects on the Proteobacterial order in the A and B horizons, with the Pseduomonadales order particularly abundant in birch and its admixtures (Appendix A: Figure A.2). Conversely, the unclassified order belonging to Betaproteobacteria class was less abundant in birch plantings but was more prevalent with cedar and its admixtures also than fir or fir with birch.

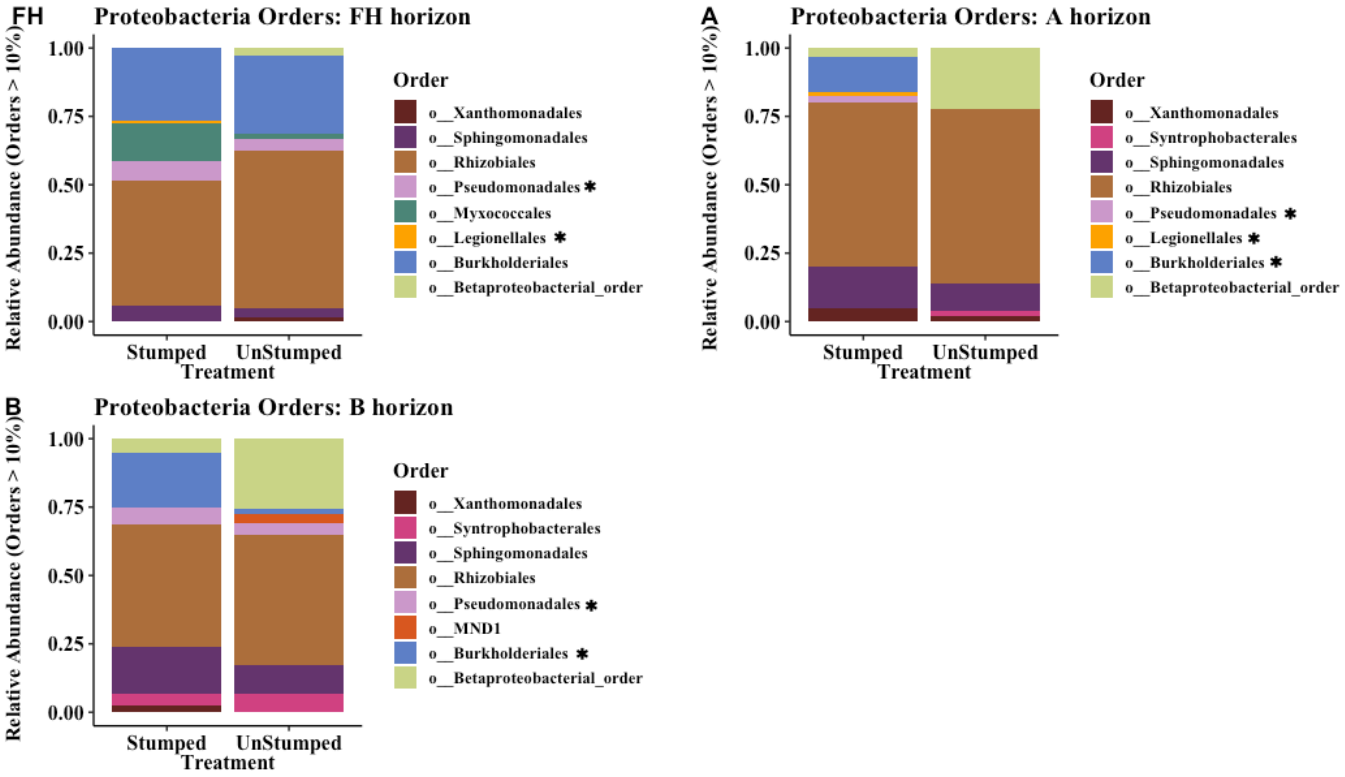


Figure 3.6 Mean relative abundances of orders (o_) of Proteobacteria in FH, A and B horizons. Orders having >10% abundance were used. Letters a, b, and c were used to show the significant orders that responded to stumping in FH, A and B horizons, respectively. (P<0.05).

3.3.5 Differentially abundant bacterial genera and/or OTUs at different soil horizons

LEfSe analysis showed significantly different abundances of OTUs representing specific genera in all three horizons of stumped and unstumped plots (Figure 3.7). Eight genera were more abundant in the FH horizons of stumped plots, including Zymomonas, Spirosoma, Sphingomonas, Rhodoferax, Methylibium, Dactylosporangium, Cystobacter and Chondromyces. By contrast, four

genera, which include Arhtrobacter, Bacillus, Rhodoplanes and Kaistobacter, were more abundant in unstumped than stumped plots. In the A horizon, only two OTUs were abundant in stumped plots (Candidatus_Koribacter genus and Acidobacteriaceae family), whereas the OTUs belonging to Sphingomonadaceae family and Planctomycetes phylum were abundant in the unstumped plots. In the B horizon, nine genera were more abundant in stumped than unstumped plots, including Rhizobium, Pedosphaera, Mesorhizobium, Kaistobacter, Janthinobacterium, Ellin506, Candidatus_Koribacter, Burkholderia, and Bradyrhizobium. On the other hand, Nitrospira, Niabella, Pedomicrobium and Aeromicrobium were more abundant in unstumped than stumped plots.

Overall, the unstumped plots harbored genera belonging to Alphaproteobacteria, Actinobacteria, Firmicutes, Bacteroidetes and Planctomycetes phyla, whereas stumped plots were abundant with genera belonging to paraphyletic Alpha/Beta and Deltaproteobacteria along with high abundance of Actinobacteria, Acidobacteria, Bacteroidetes and Verrucomicrobia phyla.

3.3.6 Identification of cultured diazotrophs and phylogenetic analysis

Twenty-five isolates of cultured diazotrophs were obtained and the 16S r-DNA sequencing showed that they belong to genera including *Arthrobacter*, *Bacillus*, *Brevibacterium*, *Burkholderia*, *Paenibacillus*, *Pseudomonas*, *Streptomyces*, and *Variovorax*. Phylogenetic analyses of 16S-rDNA showed that four major clades were formed, one each belonging to Firmicutes, Beta-Proteobacteria, and Actinobacteria with *Acidobacterium capsulatum* taken as root. (Figure 3.8, Appendix A: Table A.4).

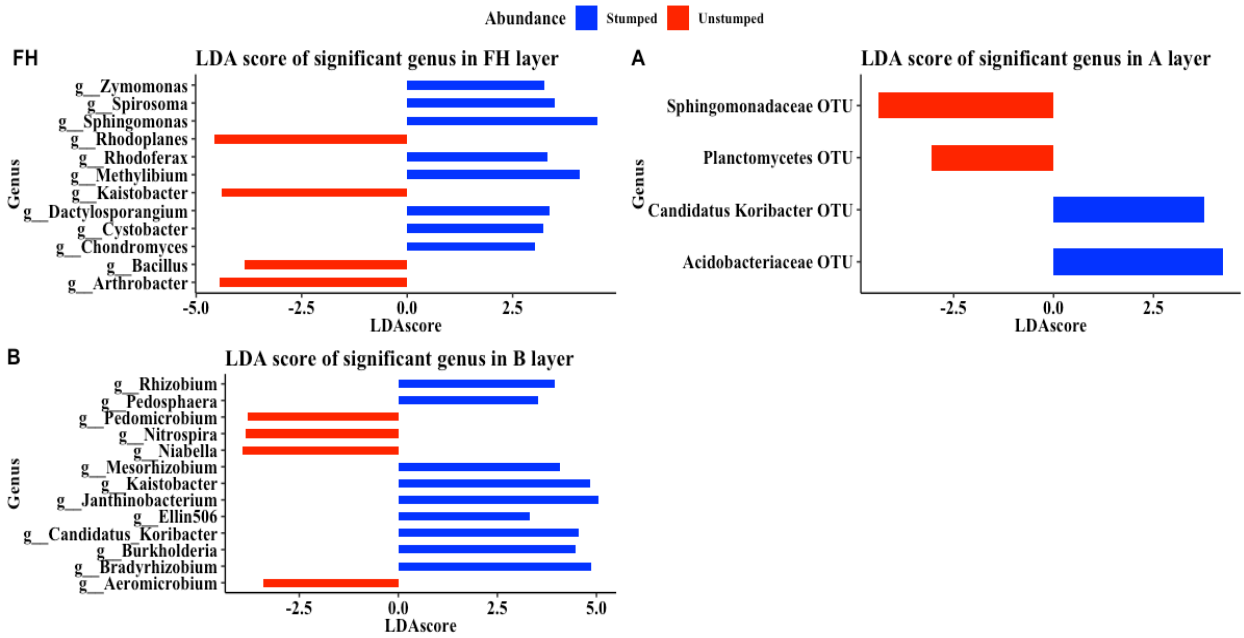


Figure 3.7 LEfSe analysis, performed taking treatment (Stumped vs Unstumped) as factor for all the three horizons, shows significantly abundant genera (g_). Kruskal–Wallis rank sum test with FDR (false discovery rates) adjusted p values < 0.05 was used to detect significantly different abundances along with generating LDA scores to estimate the effect size (LDAscore ≥2).

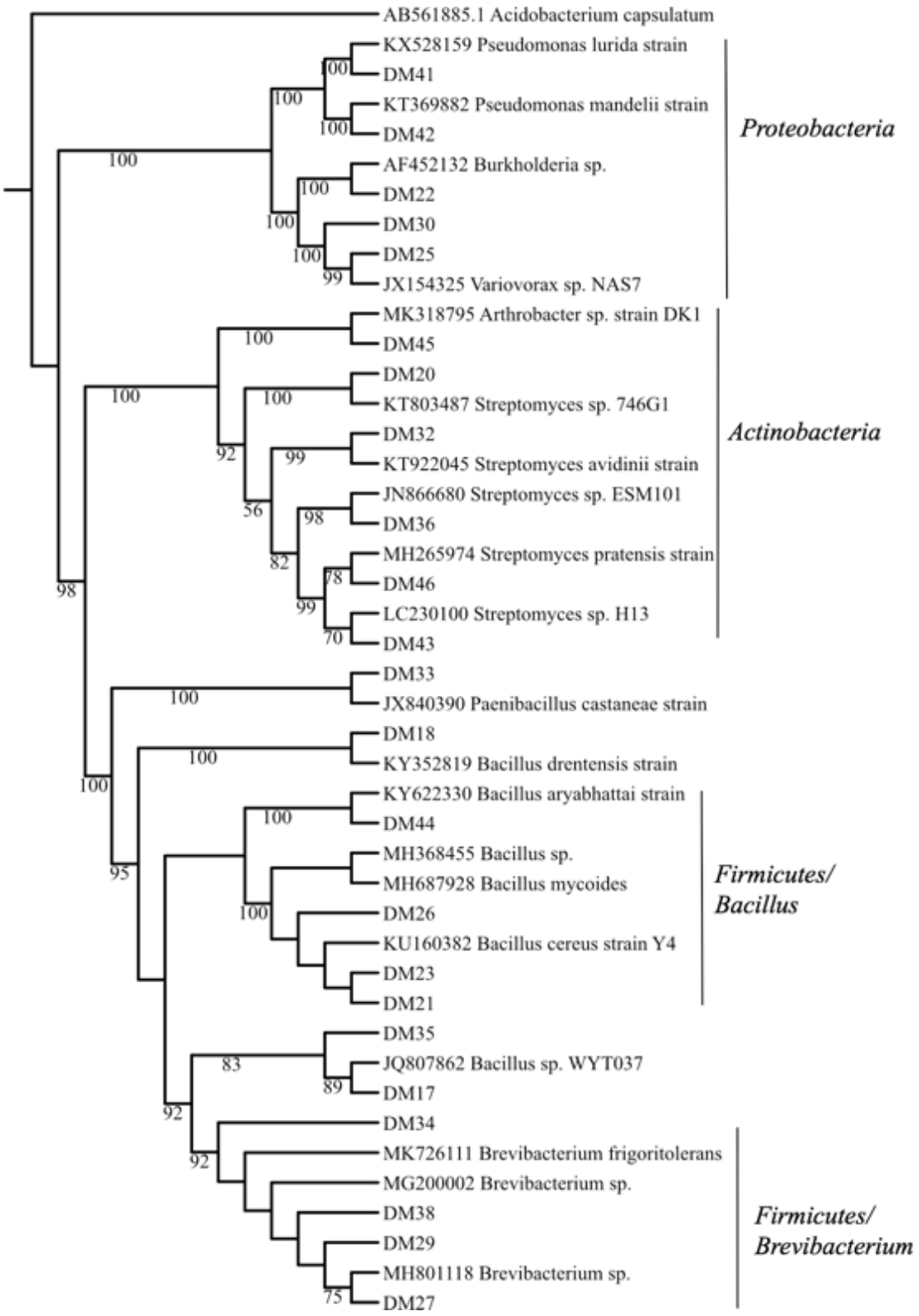


Figure 3.8 Phylogenetic tree based on 16S-rDNA sequences of cultured diazotrophic strains using Maximum Likelihood with bootstrap values on the branches.

3.4 Discussion

3.4.1 Stumping and tree species modulate bacterial diversity and community structure

Stump removal substantially altered the bacterial community structure in all the three horizons, with the greatest and most significant reduction in diversity in the B horizon, affirming our first hypothesis. Bacteria considered beneficial for tree growth were found to be more abundant in the stumped than unstumped plots, which is in concordance with the recent report that the stumping treatment at Skimikin significantly reduced root-rot incidence by 80-100% and promoted tree growth (Morrison et al., 2014; Bogdanski et al., 2018). The significant decline in alpha diversity of the B horizon with stumping could be attributed to the removal of dead stumps and the forest floor during the stumping operation. Similarly, the change in the soil bacterial community composition with stumping, as illustrated by the PCoA, PERMANOVA and ANOSIM analyses, could have been caused by changes in the belowground carbon pools (Figure 3.3 and 3.4).

Tree species composition also affected the bacterial community structure, providing support for our second hypothesis. The identity of the host tree species has been shown to alter bacterial communities through the release of unique root exudates, where distinct microbial associations form according to preferences by some fungi and bacteria based on their needs for nutrients and/or disease suppression (Yousuf et al., 2012; Prescott and Grayston, 2013; Bonito et al., 2014; Urbanová et al., 2015; Zhalnina et al., 2018; Dukunde et al., 2019). The root exudates released by the host tree consist of various molecular compounds such as sugars, secondary metabolites like antimicrobial compounds flavonoids, etc. that play a crucial role in shaping the soil microbiome along with preventing pathogens and pests and maintaining tree growth (Bais et al., 2006; Oldroyd, 2013). Trees release root exudates to the soil through the mycorrhizal network and the interactions between mycorrhizal fungi and bacteria shapes the bacterial community

structure, which is indirectly dependent on the tree species present (Twieg et al., 2007; Gorka et al., 2019; Dukunde et al., 2019). Burke et al. (2008) reported that Douglas-fir ECM tips belonging to the Russulaceae group were colonized with Alpha-proteobacteria and Bacteroidetes groups.

The Skimikin experiment was established in order to study the effect of resistant/tolerant tree species when planted with susceptible ones (Weir and Johnson, 1970). Birch is considered resistant to *Armillaria* root rot (Morrison et al., 1992) with a facilitative effect on Douglas-fir survival (Baleshta et al., 2005; Simard and Vyse, 2006) and long-term productivity (Sachs, 1996; Wang et al., 1996) through production of nitrogen- and carbon-rich litter (Bradley and Fyles, 1995). Mercado-Blanco et al. (2018) has suggested that the belowground microbiota affects the health and growth of trees. Mixing birch with fir resulted in distinct clustering of bacterial communities in stumped vs unstumped plots, and fir and birch dissimilarities were neutralized or tended towards birch when fir and birch were planted together. This finding is congruent with Delong et al. (2002), who found that pseudomonad populations were higher in mixed stands of fir and birch than where fir was planted alone. The results also suggest that birch may attract beneficial bacteria such as pseudomonads to the roots of neighboring firs, perhaps by providing richer carbon exudates or by harboring disease resistant microbial species, helping increase the resistance of fir to *Armillaria*.

Cedar is considered less susceptible to Armillaria root rot and is also known as a “soil improver”, having higher foliar calcium and soils with higher pH (Krajina, 1969). Cedar has also been reported to be enriched with ammonium-oxidizing bacteria and high nitrification rates (Turner and Franz, 1985). Mixing cedar with fir also promoted the growth of a bacterial microbiome that was more like that of cedar planted alone, potentially through enrichment of the acidic fir litter and forest floor. The Burkholderiales order was abundant in cedar alone and was

enhanced when fir was mixed with cedar. Bal and Chanway (2000) isolated *Burkholderia* spp. from western redcedar and it is well known as a nitrogen fixer and plant growth-promoting bacteria in agricultural and forest ecosystems (Puri et al., 2017a, b). In contrast, adding cedar to birch did not change the bacterial community, potentially because birch litter is already rich in nutrients. Overall, stumping had a positive effect on the bacterial microbiome in mixed stands, which may help explain why Morrison et al., (2014) found higher survival rates of Douglas-fir at Skimikin trial when it was planted with birch and cedar.

Soil physicochemical properties also play a pivotal role in shaping the bacterial diversity and community structure (Faoro et al., 2010; Liu et al., 2019; Lee et al., 2019). In this study, the tested soil properties did not vary considerably, and were not correlated with any bacterial group. Soil pH has previously been identified as the most important factor affecting soil bacterial communities (Ferrenberg et al., 2013; Shen et al., 2013), however, we found little variation in soil pH across the sites.

3.4.2 Horizons harbor signature taxonomic functional groups

Previous studies have reported high abundance of Acidobacteria, Proteobacteria, and Actinobacteria in forest soils (Uroz et al., 2013; Nacke et al., 2011; Kurth et al., 2013). We observed the same trend, with high abundance of these phyla along with Bacteroidetes, Gemmatimonadetes, Verrucomicrobia and Planctomycetes. Actinobacteria were abundant in the FH layer and less common in mineral horizons, whereas Acidobacteria and Verrucomicrobia abundance increased with depth (Bergmann et al., 2011; Eilers et al., 2012). The high abundance of Acidobacteria and Verrucomicrobia in the mineral horizon is potentially indicative of high functional importance. Acidobacterial members are difficult to culture under *in vitro* conditions, thus there is scarce information available about their ecophysiology (Kielak et al., 2016), but it has

recently been reported that Acidobacteria have high metabolic versatility and are able to decompose complex carbon substrates (Lladó et al., 2016). Acidobacteria are also known to improve soil health by actively participating in nutrient cycling and benefiting plant growth after a drastic disturbance (Huang et al., 2015). The importance of Verrucomicrobia is not clear, as it is unknown whether they prefer to live as oligotrophs or to settle in specific micro niches (Lladó et al., 2017).

3.4.3 Stumping promotes abundance of beneficial growth-promoting bacteria

We observed that the organic horizons of unstumped plots were populated with genera with diverse functions. The high abundance of photosynthetic bacteria such as *Kaistobacter* and *Rhodoplanes*, for example, reflects a high tolerance for nutrient- and carbon limited environments where root rot is present (Tang et al., 2018b). The presence of *Rhodoplanes,* found in wood at advanced decay stages, and Bacillus with its lignolytic properties, may be attributed to the prevalence of dead stumps (Bandounas et al., 2011; Chakravarthy et al., 2012; Tláskal et al., 2017). In contrast, stumped plots were enriched with genera affiliated with hemicellulolytic activity (Rhodoferax, Methylibium), and mycorrhiza-helper bacteria (Sphingomonas) (Dahm et al., 2005). In the B horizon of stumped plots, we also observed high abundance of *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Burkholderia*, *Janthinobacterium*, and *Kaistobacter* genera, and this was associated with greater tree survival and growth (Morrison et al. 2014). These genera possess beneficial traits essential for plant growth such as nitrogen fixation, phosphate solubilization, and the potential for disease suppression (Halder et al., 1990; Anderson and Habiger 2012; Gómez Expósito et al., 2017). Also, the nitrite-reducing *Koribacter* genera were abundant in the stumped plots and are known for reduction of nitrate and nitrite to ammonia (Ward et al., 2009). However, unstumped plots showed high abundance of nitrite oxidizing Nitrospira and Planctomycetes

genera. Notably, some of these genera have recently been shown to be anaerobic ammonium-oxidizing (anammox) bacteria, converting ammonium and nitrite into dinitrogen gas and playing a pivotal role in the global nitrogen cycle (Jetten et al., 2005; Daims et al., 2015).

Rhizobia species promote nodule formation when they live in symbiosis with leguminous roots, however, information about non-nodulating rhizobia species is very limited (Tanaka and Nara, 2009). We speculate that tree species in stumped plots may be forming tripartite symbioses with mycorrhizal fungi and *Rhizobia* sp., which would promote tree growth and improve soil health (Khetmalas et al., 2002; Chaer et al., 2011). These symbioses may improve the capacity for trees to grow quickly in the substrate-limited stumped plots and also help them to withstand the harsh conditions caused by the disturbances. Similarly, we also observed that Pseudomonadales and Burkholderia were highly abundant in stumped plots compared to unstumped ones. Pseudomonas sp. have been previously reported as mycorrhiza-helper bacteria and are also known for their biocontrol properties against soil borne pathogens (Duponnois and Garbaye, 1991a, b, Haas and Défago, 2005). Pseudomonads play an antagonistic role, potentially by producing antibiotics or other inhibitory compounds, against *Armillaria* and have been found predominantly in the mixed stands of Douglas-fir and birch, suggesting a mechanism by which birch promotes resistance of fir against *Armillaria* (Delong et al., 2002). Birch also increases N mineralization in the soil, potentially fostering higher growth rates of pseudomonads (Bradley and Fyles, 1995).

3.4.4 Diazotrophic bacterial isolates as potential PGPRs

The aim of isolating diazotrophic bacteria was to gain insight about cultured PGPR members from stumped plots, which could be used for future studies to understand the effect of PGPR's on *Armillaria* root rot under *in vitro* and *in vivo* conditions. Some of the isolates obtained such as *Brevibacterium*, *Paenibacillus*, *Streptomyces*, *Arthorbacter*, *Variovorax* have previously been

known as PGPR (Sun et al., 2018; de García Salamone et al., 2001). *Paenibacillus* is an endophytic diazotroph associated with lodgepole pine and western red cedar that has been studied by Chanway and his group for nitrogen fixation and growth promoting characteristics (Bal and Chanway 2012a, b; Bal et al., 2012; Anand and Chanway, 2013a, b). *Streptomyces*, *Pseudomonas* and *Burkholderia* species have been widely known for their role in antagonism against plant pathogens and have been extensively used in agriculture as biocontrol agents. *Streptomyces* has been reported as a PGPR for pine and a biocontrol agent against phytopathogenic fungi like *Heterobasidion*, *Fusarium* and *Armillaria* by inducing plant defense responses (Vasconcellos and Cardoso, 2009; Lehr et al., 2008; Golinska et al., 2013; Shekhar et al., 2006). *Burkholderia* has been reported to have various PGPR properties such as cellulase, N-fixing, and phosphate-solubilization (Tang et al., 2018a). *Pseudomonas* and *Bacillus* are also known to act as PGPR for spruce seedlings (Shishido and Chanway, 1998).

3.5 Conclusions

This study suggests stump removal along with planting resistant tree species with susceptible ones is an effective management practice for Armillaria root rot. This combined approach seems to have a synergistic effect in shaping beneficial a bacterial microbiome with the potential to enhance resistance against *Armillaria* root rot and promote tree productivity. This study detected bacterial groups unique to the B horizon of stumped plots, which included rhizobia bacteria and Acidobacteria members associated with non-leguminous forest trees. Unveiling the functional importance of these specific microbiome-tree associations could provide novel ways to control one of the most important tree diseases in the northern hemisphere.

Chapter 4: Molecular characterization and phylogeny of Armillaria species isolated from a 48-year-old plantation at Skimikin, British Columbia

4.1 Introduction

Fungal species belonging to the genus Armillaria are responsible for causing Armillaria root rot, one of the most destructive root diseases of woody plants. Armillaria species have ecologically diverse roles, from saprotrophic to parasitic, and with host specificity. Many commercially important tree species such as Douglas-fir, western redcedar, and lodgepole pine are affected by Armillaria root rot, leading to huge financial losses in the timber industry (Morrison et al., 1992; Cruickshank, 2000; Morrison et al., 2000; Cleary et al., 2008). *Armillaria ostoyae* is known as the most prevalent and destructive species of the Armillaria genus. There are about 70 species of Armillaria listed in Mycobank (http://www.mycobank.org/) and Index Fungorum (http://www.indexfungorum.org/) worldwide, but only ~40 are well characterized (Heinzelmann et al., 2019).

Species identification of Armillaria can be challenging. There are three ways to identify Armillaria species: morphologically, biologically and phylogenetically. For morphological identification, basidiocarps (fruiting bodies) are used. They are season-dependent and therefore are not a reliable diagnostic feature. The basidiocarps are somewhat similar among many species such as *A. ostoyae* and *A. gemina, A. gallica* and *A. cepistipes,* and *A. nabsnona* (Antonin et al., 2009; Park et al., 2018; Bérubé and Dessureault, 1989). Biological species identification is based on reproductive barriers, which can be tested by mating two species *in vitro*. This method also has limitations because the reproductive barriers are not complete (Klopfenstein et al., 2017). With the advent of DNA sequencing technology, however, species identification has become easy and precise. The ribosomal RNA gene family (rDNA)-based internal transcribed region (ITS) and the intergenic spacer (IGS) have been successfully used to identify some Armillaria species (Anderson

and Stasovski, 1992; Chillali et al., 1998; Coetzee et al., 2000; Dunne et al., 2002; Hasegawa et al., 2010; Keča et al., 2006; Keča and Solheim, 2010; Kim et al., 2006; Lima et al., 2008; Tsykun et al., 2013). However, some Armillaria species are not easily distinguishable based on molecular phylogeny of the ITS or IGS sequences, such as for *A. gallica*, *A. calvescens*, *A. cepistipes*, *A. nabsnona*, *A. sinapina, A. borealis*, *A. gemina* and *A. ostoyae* (Brazee et al., 2011; Kim et al., 2006; Tsykun et al., 2013; Hanna et al., 2007; Keča and Solheim, 2010; Pérez-Sierra et al., 2004). The translation elongation factor-1 alpha (tef-1) can provide another genome region that can be used successfully for species differentiation and can be useful when ITS and IGS sequences are not discriminant (Brazee et al., 2011; Ross Davis et al., 2012; Mulholland et al., 2012; Tsykun et al., 2013; Klopfenstein et al., 2017; Park et al., 2018).

We hypothesized that there is only one species, *A. ostoyae*, present at Skimikin, as reported in earlier studies on the basis of morphology. The objective of this study was to do molecular characterization of the Armillaria species present on the 48-yr old Skimikin trial, which is examining the effect of stump removal on Armillaria root rot caused by *A. ostoyae*.

4.2 Materials and Methods

4.2.1 Sample collection

A total of three clumps of basidiocarps were collected from the Skimikin plot at Skimikin (50°48'N, 119°26'W) near Salmon Arm, British Columbia. The specimens were originally identified as *A. ostoyae* based on morphology. The specimens were kept on ice before storage at -20°C and processed within 2 days.

4.2.2 Culture on PDA

Attempts were made to culture unexposed (sterile) basidiocarp tissue on Potato Dextrose Agar (PDA) prepared as per manufacturer's instructions. The plates were kept at room temperature for one month.

4.2.3 DNA extraction, PCR amplification, and sequencing

DNA was extracted from basidiocarp tissue after grinding it in liquid nitrogen using the DNeasy Plant Mini Kit as per the manufacturer's instructions. The ITS region (ITS1, 5·8S and ITS2) of the rRNA operon was amplified using primer set ITS1f/ITS4 (Gardes and Bruns, 1993). The partial *tef*-1 region was amplified using primers EF595F (Maphosa et al., 2006) and Arm EF1-a-REV (Mulholland et al., 2012). PCR amplification protocol for tef-1 primers was as follow: 94ºC for 2 min and 35 cycles of 94ºC for 30 s, 55ºC for 30 s, 72ºC for 45 s, followed by 10 min at 72ºC. Amplified ITS and *tef*-1 PCR products were visualized on 1% agarose gel using Ez-Vision Three™ DNA Dye Loading Buffer under UV illumination. PCR products were purified and sequenced at UBC's Sequencing and Bioinformatics Consortium (Vancouver, B.C., Canada).

4.2.4 Phylogenetic analysis

The sequences were edited and aligned using BioEdit 7.1 (Hall 1999) and then matched with sequences available in GenBank using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). ITS and *tef*-1 based phylogenetic analysis was performed on sequences of *Armillaria* sp. isolates (DM1, DM2 and DM3) and other previously reported North American *Armillaria* spp. whose sequences were retrieved from GenBank based on their accession numbers (Kim et al., 2006; Ross-Davis et al., 2012). All the sequences were aligned using MAFFT version 7 using FFT-INS-i option (Katoh & Standley, 2013). ITS and *tef*-1 sequences of *Strobilurus esculentus* were used as outgroups. For phylogenetic analysis, a neighbour-joining (NJ) tree was constructed using MEGA version X (Kumar et al., 2018) with bootstrap analysis (1000 replicates). Maximum Likelihood analysis were

also performed with 1000 replicates for bootstrap analysis and GTR+G model of evolution using RAxML (Stamatakis, 2006). In addition, Bayesian Inference based phylogenies was used to calculate posterior probabilities, performed in Mr. Bayes version 3.2, for 10 million generations, under Jukes Cantor model (Ronquist et al., 2012). FigTree v 1.43 and Interactive Tree of Life (iTOL) v4 was used to view and annotate the trees (Rambaut, 2010; Letunic and Bork, 2006).

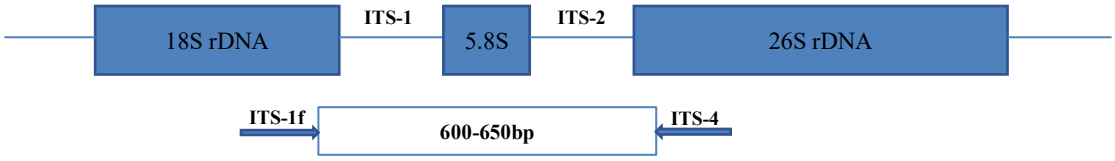


Figure 4.1 Schematic diagram of ITS regions amplified by primers ITS-1f and ITS4.

4.3 Results

4.3.1 Morphology of basidiocarps

The DM1 basidiocarps found in stumped plots were solitary, had a dark brown to blackish colored hairy pileus covered with scales, and had a stipe with a pronounced annulus. The DM2 basidiocarps were collected from unstumped plots; they were growing in clusters, the pileus of theses samples had a honey brown color, and distinct scales and spots in the center. The stipe was a lighter color and had a distinct annulus and cream-colored gills. The DM3 basidiocarps, found in unstumped plots, had a tan to light brown pileus with very light scales and light cream gills and grew in clusters. The basidiocarps were smaller sized than DM1 and DM2 (Figure 4.2).

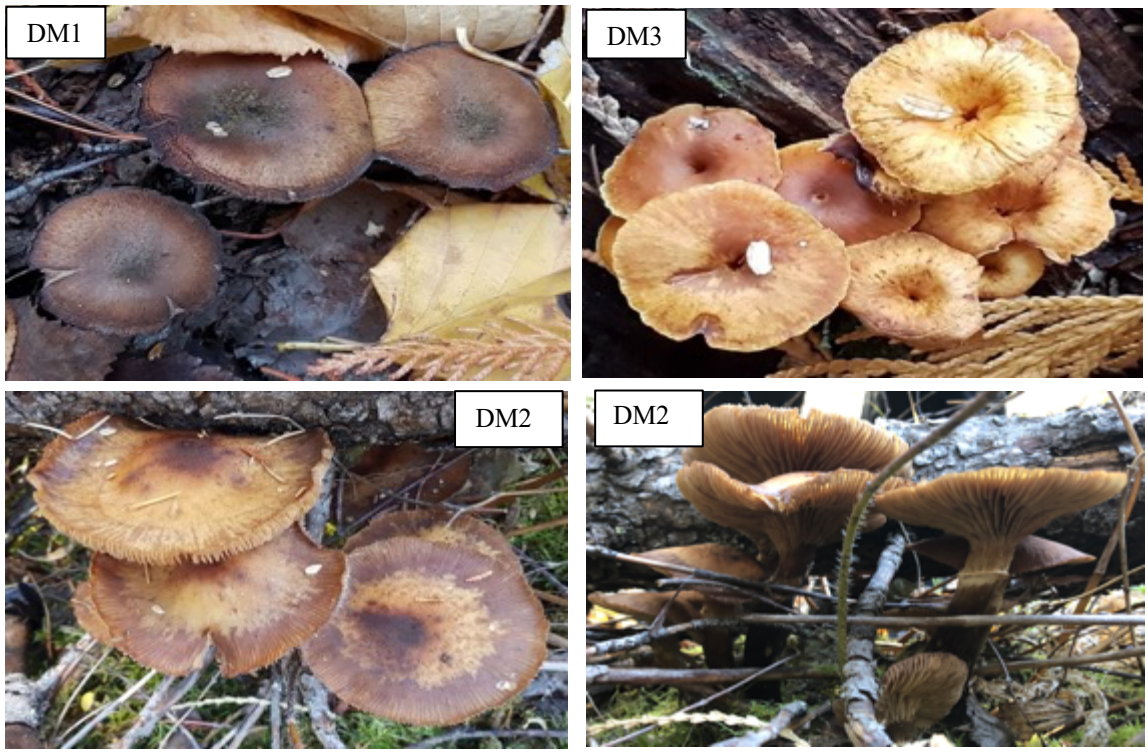


Figure 4.2 Pictures of basidiocarps of DM1, DM2 and DM3 collected from Skimikin plots.

4.3.2 DNA amplification

Single amplicons for each gene region were successfully generated for all specimens used in this study. Amplicons ranged in size from ~ 650 bp for ITS and ~500 bp for *tef*-1.

4.3.3 DNA sequencing and phylogenetic analysis

Partial DNA sequences of ITS1-5.8S-ITS2 from the DM1, DM2 and DM3 isolates showed 99-100% similarity and 100% coverage with the *A. gallica* strain DR-140 reported from the North American in-lab study (GenBank accession number FJ744699). DM3 also showed 99% similarity and 100% coverage with *A. calvescens* X-59 reported from Ontario, Canada (GenBank accession number KC176342). Partial DNA sequences of *tef*-1 from DM1 and DM2 showed 97% similarity with the *A. gallica* isolate CMW31093 reported from China (GenBank accession number KM20526 and 100% coverage), and DM3 showed 98% similarity with *A. gallica* isolate Nualolo-2 reported from North America (GenBank accession number KX772409 and 100% coverage).

Phylogenetic analyses performed for the ITS region showed six major clades each for *A. mellea; A. tabescens; A. ostoyae* and *A. gemina*; *A. sinapina* and *A. cepistipes*; *A. calvescens* and *A. gallica* forming the fifth clade with the isolates of this study (DM1, DM2 and DM3); and *A. nabsnona* with *Armillaria* sp. NABS X as the sixth clade. The three isolates (DM1, DM2 and DM3) were not well supported by NJ, ML and BI analysis and thus their species could not be resolved based on ITS sequences (Figure 4.3).

Phylogenetic analysis of *tef*-1 sequences demonstrated very distinct clades of all the species. Notably *A. calvescens* and *A. gallica* formed two separate clades with strong support of NJ, ML, and BI analysis and we observed that the three isolates (DM1, DM2 and DM3) also belonged to the *A. gallica* clade especially associated with *A. gallica* M70 isolate reported from British Columbia, Canada (Figure 4.4).

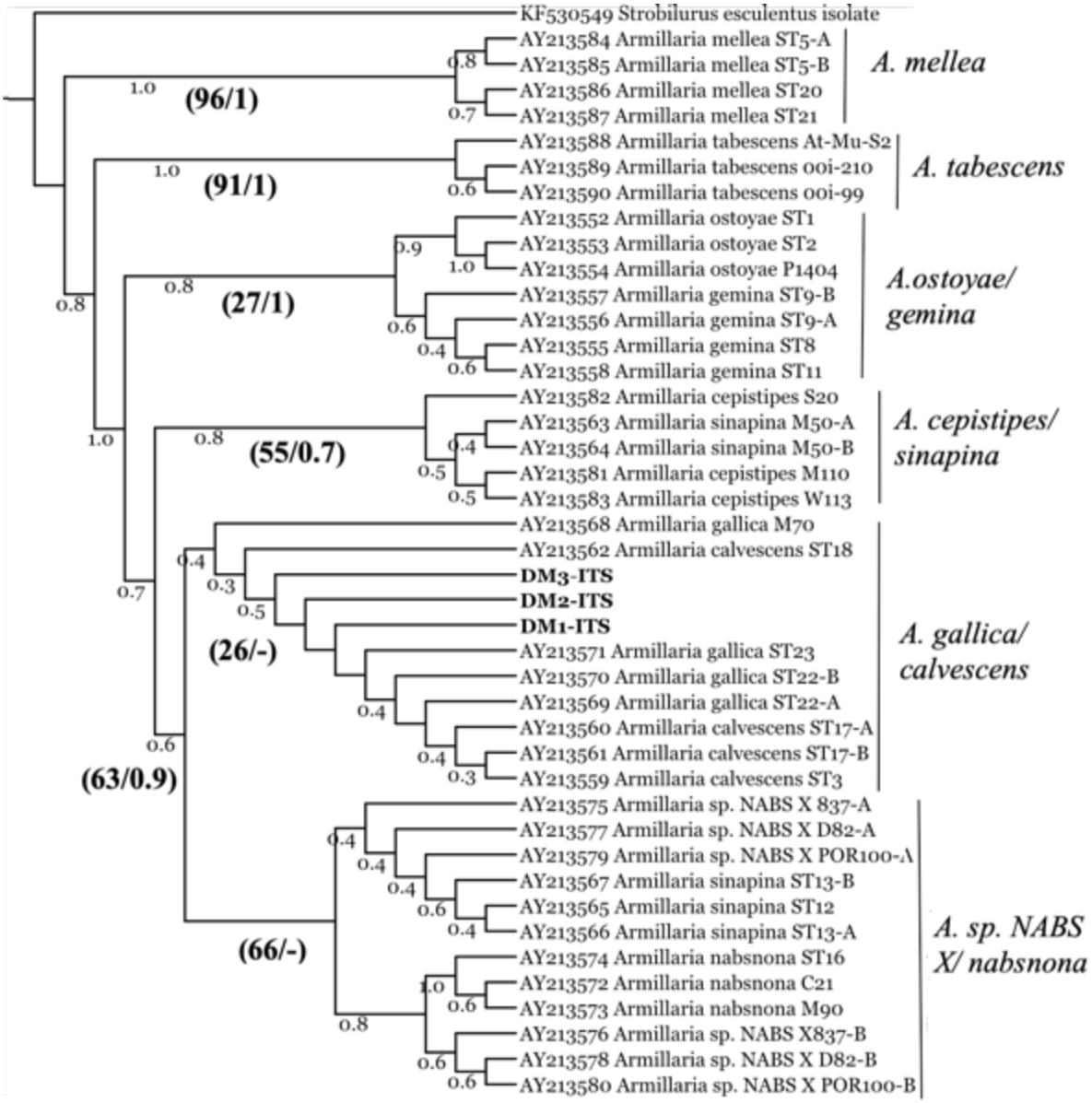


Figure 4.3 Neighbour-joining (NJ) based phylogenetic tree for the analysis of ITS region of *Armillaria* species of North America with sequences of the isolates (DM1, DM2 and DM3). Branch support values show neighbor-joining (NJ) bootstrap values (>50%) with maximum likelihood (ML) bootstrap values and Bayesian posterior probabilities in bold brackets.

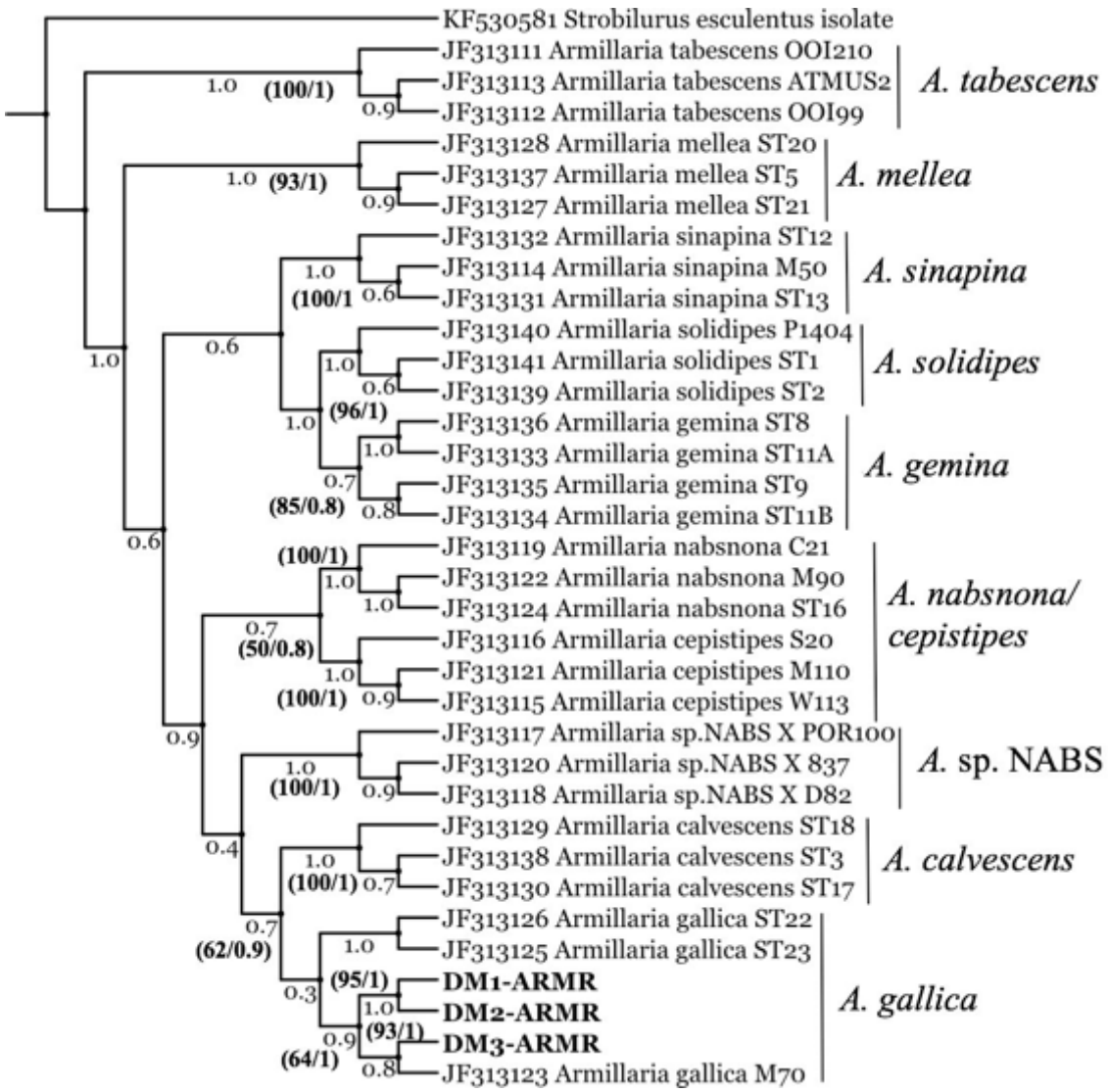


Figure 4.4 Neighbour-joining (NJ) based phylogenetic tree for the analysis of partial *tef*-1 region of *Armillaria* species of North America with sequences of the isolates (DM1, DM2 and DM3). Branch support values show neighbor-joining (NJ) bootstrap values (>50%) with maximum likelihood (ML) bootstrap values and Bayesian posterior probabilities in bold brackets.

4.4 Discussion

Characterization of Armillaria species is important in order to understand their ecological roles and calculate the risk factor for tree species growing in the vicinity. The difficulty in identification of *Armillaria* sp. based on their morphology alone has been well reported (Antonín et al., 2009; Bérubé and Dessureault, 1989). We also faced challenges in identifying the three isolates; although

they showed distinct morphological characteristics of *Armillaria* such as the annulus on the stipe and scales on the brown colored pileus, this was not enough to identify the species. For the DM3 isolate, we found some discrepancy between the ITS and tef-1 sequences, which showed 99 and 98% nucleotide similarity with *A. calvescens* and *A. gallica* respectively. Kim et al., 2006 has reported that *A. calvescens* and *A. gallica* are genetically very close and thus could not be distinguished based on ITS + 5.8S sequences. Also, *A. calvescens* has only been reported in Northeastern America and is known to cause butt rot in hardwood trees such as sugar maple and birch trees (Bérubé and Dessureault, 1989; McLaughlin, 2001), whereas *A. gallica* has been reported earlier from British Columbia, Canada and other sites from western North America.

The dual phylogenetic analysis (ITS and *tef*-1) was intended to provide a convincing basis for *Armillaria* species identification (Klopfenstein et al., 2017). From the phylogenetic analysis of the ITS, the identification of the isolates was not resolved substantially due to identification as *A. calvescens* or *A. gallica*. Phylogenetic analysis of *tef*-1 strongly supported by NJ and ML bootstrap values as well as Bayesian probabilities confirmed that the three isolates (DM1, DM2 and DM3) were *A. gallica* with strong support for the *A. gallica* M70 isolate. This isolate has only been previously reported on southern Vancouver Island, British Columbia, Canada (Morrison, 1981). Thus, this is the first ever report of *A. gallica* species from interior British Columbia (Skimikin trial).

In order to assess the disease risk for paper birch, the most beneficial tree species at this site (from chapter 2 and 3) when planted alongside Douglas-fir, it is extremely important to perfectly identify the *Armillaria* species. *A. gallica* is normally considered a weak pathogen and mostly survives as a saprobe on weak or dying trees. Based on the study of Denman et al. (2016) on oak death, however, *A. gallica* can cause stem bleeds in some trees and could be associated

with root rot in healthy trees. Other reports found that *A. gallica* (weak pathogen) acted as an opportunistic weak pathogen when it co-occupied host roots with *A. ostoyae* (highly virulent) (Rishbeth, 1982, 1985; Guillaumin and Legrand, 2013).

In conclusion, we found that *A. gallica,* closely related to the isolate M70, was found at Skimikin with previous reports of *A. ostoyae* (Morrison et al., 1988; 2014), where the efficacy of stump removal at reducing on tree mortality caused by Armillaria root rot was studied. *A. ostoyae* was the primary pathogen causing Armillaria root rot as reported previously. Recognizing that the role of *A. gallica* is unknown but may be a co-infecting pathogen with *A. ostoyae* thus, needs further research. This information, while unprecedented, could be useful to develop better forest management practices.

Chapter 5. Conclusion

This thesis explains the research that contributes new knowledge and supports perspectives from speculations made by previous studies on the effectiveness of stump removal in suppression of Armillaria root rot. The study also enriches understanding of the role of tree species composition in shaping soil microbial communities. New information was gained on the synergistic effect of stumping and tree species plantation on changes in soil fungal and bacterial communities. Next generation Illumina Miseq sequencing was used for the first time to determine the impact of Armillaria root rot management practices, stump removal and mixed tree species planting, on the structure of microbial communities and their possible influence on forest health, tree growth, and productivity.

5.1 Effects of stumping on soil microbial communities

Previous studies showing short term negative impacts of stumping and root raking on soil physicochemical properties are well documented but their impact on composition and structure of soil microbial communities is unknown. Previous reports based on long term studies about successful reduction (80-100%) in tree mortality caused by *Armillaria* due to stumping (Morrison et al., 1988; Cleary et al., 2013; Morrison et al., 2014; Bogdanski et al., 2018) also raises questions regarding the role of soil fungal and bacterial communities in improving tree health.

Chapter 2 was focused on stumping effects on diversity, abundance and structure of soil fungal communities. Our findings were complex, with an increase in fungal alpha diversity in the A horizon and a tendency for similar increases in the FH and B horizons. The increased richness of the fungal community with stump removal could not be attributed to a single taxa or trophic level, but we observed that highly abundant fungal generalists were more resistant to stump removal compared to the rare fungal classes with low abundance. The sensitivity of rare fungal

taxa to stumping could be attributed to the presence of a lower number of habitats specific to those taxa (Barberán et al., 2014). Thus, increased richness of rare fungal species could have led to increased alpha diversity due to stumping. This can be attributed to newly developed microhabitats due to increased bulk density or compaction at certain places caused by heavy machinery for stump removal (Hartmann et al., 2014). The other main finding was replacement of saprotrophic fungi by ECM fungal communities, indicating a functional shift in generalists' fungal classes. Allison and Martiny (2008) suggested that resistance, resilience and functional redundancy are the three ways to describe the changes in the microbial communities caused by disturbance, which can further result into changes in ecosystem functioning. They suggest that if disturbance alters microbial composition, it will either remain functionally similar due to functional redundancy or will function differently. Surprisingly, we found that the composition of the fungal community was unaltered, but there was a shift in functional guilds. This could be because fungi are classified based largely on the method of sexual reproduction/ spore production and not based on their ecological function. Thus, many fungi belong to the same fungal taxa but perform different ecological functions. There is also a fine line between saprotrophic fungi and ECM fungi as they both are known to depend on trees for their carbon needs, but ECM fungi are mutualists, providing nutrients to the trees from the soil. ECM fungi are also known for their ability to decompose organic matter similar to saprotrophs (Bending, 2003; Fernandez and Kennedy, 2016). Previous reports of reduced tree mortality and increased tree productivity due to stumping were corroborated by our findings as we observed increased abundance of ECM communities, which are known to increase flow of carbon through root exudates. Notably, saprotrophic communities, which largely depend on dead woody material and organic matter, were reduced in the stumped plots.

Chapter 3 examined changes in the composition, diversity, and abundance of soil bacterial communities due to stump removal. We observed a significant decline in the alpha diversity in the B horizon, and a tendency for a reduction in the A horizon. The loss of organic matter and dead woody debris could be attributed to the reduction in alpha diversity. The most important findings of this chapter were the significant increase in abundance of the Pseudomonadales order, known for its antagonistic role against pathogens, in all the three horizons of stumped versus unstumped plots, and increased abundance of other beneficial growth-promoting bacteria such as *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Burkholderia*, *Janthinobacterium*, and *Kaistobacter*.

The improved tree growth reported by Morrison et al. (2014) probably resulted from increased nutrient uptake via the ECM fungi and PGPR bacteria, as well as increased defense against pathogens and other disturbances via abundant Pseudomaondales and Burkholderiales. These results mark a very important addition to the knowledge on stump removal efficacy in management of Armillaria root rot which concludes that stumping had a profound effect overall on the fungal and bacterial communities, and this appeared to benefit trees through increased survival and growth rates.

5.2 Effects of tree species composition on soil microbial communities

Chapters 2 and 3 both examined the impact of tree species composition on bacterial and fungal communities, respectively. A more ecologically sensitive co-strategy to stumping may be establishment of native tree species mixtures that include susceptible and resistant species, such as mixes of interior Douglas-fir (highly susceptible) and paper birch (resistant). Plants can shape their own microbiome and microbial communities make plants capable of modifying their responses according to their needs in their established environment. The composition and activity of the microbial community can be plant species-specific, depending on root morphology as well as the

quality and quantity of rhizo-deposits. Soil microbes are stimulated by certain metabolites resulting into multiple responses. Therefore, tree species composition would play an important role in shaping the fungal and bacterial communities and thus would also interact with the changes caused by any disturbance. The main findings for this objective were: 1) fungal communities associated with paper birch were relatively unaffected by stumping, and 2) tree species composition had significant effects on the Proteobacterial order in A and B horizons, with the Pseduomonadales order particularly abundant in birch and its admixtures. Birch, known for its tolerance against Armillaria root rot, had beneficial bacterial consortia that might be helping it to guard against Armillaria infections. Thus, it can also be concluded that the microbial interactions between fir and birch microbiome help fir to modify its responses and increase its resistance against pathogenic as well as anthropogenic disturbances.

The above findings thus draw attention to the important observation made by previous studies on this site where Douglas-fir showed increased productivity when planted with birch, most prominently in stumped plots (Morrison et al., 2014). Thus, from all the above observations and reasonings, it can be concluded that stump removal and planting resistant species with susceptible ones, can work together to successfully mitigate root rot diseases and increase tree growth and productivity.

5.3 Characterization and identification of diazotrophs

From chapter 3, we were able to conclude that stumping promotes the growth of beneficial bacteria, and thus we also focused on characterizing the diazotrophs that were isolated from the Skimikin trial and cultured on nitrogen-free carbon combined media. The culture-based study revealed abundance of diazotrophs which could acts as potential PGPRs and biocontrol agents against Armillaria species such as *Pseudomonas*, *Burkholderia*, *Brevibacterium*, *Bacillus*,

Arthrobacter, *Panebacillus* and *Streptomyces* and were in corroboration with the aforementioned DNA metabarcoding results. The isolated bacteria can further be investigated for their PGPR characteristics such as production of phytohormones, or phosphate solubilization, production of antibiotics or their ability to stimulate plants defense mechanisms as well as evaluating their efficacy as biocontrol agents against Armillaria under *in vitro* and *in situ* conditions.

5.4 Characterization of Armillaria species

Chapter 4 was mainly focused on first-ever molecular characterization of Armillaria species present at Skimikin trial. *A. ostoyae* was previously reported species on that site, and we found *A. gallica* was present, which is a new species found at this site and never reported previously in the interior British Columbia. The presence of *A. gallica* at this site needs further investigation to understand its role and implications to the Skimikin trial. One of our specimens also showed 99% similarity with *A. calvescens* with ITS primers, which was further clarified by *tef*-1 as *A. gallica*. These results coincide with our NGS data where we observed using ITS primers in low abundance, *A. calvescens, A. gallica* and *A. ostoyae*, which were found particularly more in unstumped plots. Isolation and identification of Armillaria from the soil was indeed difficult because we sieved the soil, and this may have led to the loss of rhizomorphs and low abundance of *Armillaria* species in NGS data. It is absolutely necessary to collect rhizomorphs from this site and characterize them molecularly in order to confirm the presence of *A. ostoyae* at this site.

5.5 Caveats and future prospects

This study was performed on the samples taken from the 48-yr old Skimikin trial to study the effectiveness of stump removal on tree species mortality by Armillaria root rot. Despite our successful efforts in understanding the role of stump removal on soil fungal and bacterial communities, this study lacks replication of the stumping treatment plots. It also lacks pre-

treatment knowledge of the soil microbial communities of the 80-yr old forest that was cleared in order to establish this trial. This would have given us an insight on the initial microbial communities and the changes that they have gone through following stumping and planting of tree species.

Furthermore, more research and replication are required in order to clarify the ECM fungi increase in stumped plots, with a decline in saprotrophic fungi. This can be done by performing the same methods on samples from other stumping trial sites that exist. A very important aspect of this study was that stump removal along with planting birch in mixture with fir resulted in greater abundance of potential growth promoting bacteria compared to stumping or birch/fir mixture alone. This should be followed up with further investigations of plant-microbe interactions and possible changes in fir's defense mechanism when planted with paper birch and when planted alone in presence of Armillaria root rot. Our results agree with previous studies showing that fir when planted with birch reduces the incidence of Armillaria root rot and increases productivity due to the promotion of beneficial microbes such as ectomycorrhiza fungi, *Rhizobia* species, *Pseudomonas*, *Burkholderia*, thus enhancing nutrient uptake as well as defense mechanisms of fir.

One of the most important outcomes of this study is that, among all the three tree species (Douglas-fir, paper birch, western redcedar), the birch microbiome consistently behaved significantly differently in response to stumping practices. Birch can now be considered a species resistant not only to Armillaria but also to stumping. With the advent of transcriptomics, insights may be gained on the functional aspects of this specific microbiome-tree association in order to understand the mechanisms of *Armillaria* infection on trees and ways to control the most concerning root disease of North America. Thus, this thesis concludes that stump removal and tree species composition significantly impacted the microbiome of the belowground ecosystem.

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Appendices

Appendix A: Supporting information for Chapter 3

Table A1 Effect of treatment (Unstumped vs Stumped) on bacterial taxa of FH layer assessed by metacodeR. Non-parametric Wilcoxon Rank Sum test was used for statistically significant taxa. Only significant ($p<0.05$) taxa are shown.

Tax_rank	Tax_name	Treatment_1	Treatment_2	log2_median_ratio	Median_diff	Mean_diff	wilcox_p_value
phylum	Firmicutes	UnStumpedFH	StumpedFH	1.57295338	0.00613995	0.00627011	0.01289933
phylum	TM6	UnStumpedFH	StumpedFH	∞	0.00039648	0.00033432	0.00328673
phylum	Chlorobi	UnStumpedFH	StumpedFH	-0.3597435	-0.0003101	-0.0004382	0.04708483
phylum	WPS_2	UnStumpedFH	StumpedFH	−∞	-0.0005049	-0.0003585	0.00498211
class	Deltaproteobacteria	UnStumpedFH	StumpedFH	-0.8135205	-0.0212737	-0.0127063	0.0078738
class	Bacilli	UnStumpedFH	StumpedFH	1.6542195	0.0062622	0.0067793	0.00464486
class	SJA_4	UnStumpedFH	StumpedFH	∞	0.00039648	0.00033432	0.00328673
class	vadinHA49	UnStumpedFH	StumpedFH	-0.6901246	-0.0004151	-0.0006266	0.0050986
class	Cytophagia	UnStumpedFH	StumpedFH	-0.4829314	-0.0052316	-0.0047681	0.04022258
class	VC2_1_Bac22	UnStumpedFH	StumpedFH	−∞	-2.73E-05	-4.42E-05	0.03160595
class	Opitutae	UnStumpedFH	StumpedFH	-0.5189257	-0.0020949	-0.001689	0.0314917

class	Verrucomicrobiae	UnStumpedFH	StumpedFH	-0.7137338	-0.0018587	-0.0013546	0.04708483
class	Gemm_1	UnStumpedFH	StumpedFH	0.85488802	0.00112603	0.00423464	0.01870594
class	Gemm_5	UnStumpedFH	StumpedFH	1.02271412	0.00038474	0.00038471	0.0244013
class	c__	UnStumpedFH	StumpedFH	−∞	-0.0005049	-0.0003585	0.00498211
class	Chloroflexi	UnStumpedFH	StumpedFH	-0.4910746	-0.0003947	-0.0005339	0.005761
class	Anaerolineae	UnStumpedFH	StumpedFH	-0.5269057	-0.0009658	-0.0008228	0.03711403
class	C0119	UnStumpedFH	StumpedFH	-0.9308108	-0.000858	-0.0007639	0.00057796
class	S085	UnStumpedFH	StumpedFH	1.24248766	0.0003544	0.00048556	0.00309241
class	0319_6E2	UnStumpedFH	StumpedFH	−∞	-0.0001101	-6.61E-05	0.02266734
class	Ellin6529	UnStumpedFH	StumpedFH	0.65024697	0.00148337	0.00197888	0.00963088
class	MB_A2_108	UnStumpedFH	StumpedFH	∞	0.00066098	0.001274	0.00010655

Table A2 Effect of treatment (Unstumped vs Stumped) on bacterial taxa of A layer assessed by metacodeR. Non-parametric Wilcoxon Rank Sum test was used for statistically significant taxa. Only significant ($p<0.05$) taxa are shown.

Tax_rank	Tax_name	Treatment_1	Treatment_2	Log2_median_ratio	Median_diff	Mean_diff	Wilcox_p_value
phylum	TM7	UnStumpedA	StumpedA	0.59174373	0.00029685	0.00038578	0.0278762
phylum	WS3	UnStumpedA	StumpedA	0.73152928	0.00127302	0.0020021	0.0244013
phylum	Nitrospirae	UnStumpedA	StumpedA	0.49645929	0.00113171	0.00174952	0.03711403
class	Deltaproteobacteria	UnStumpedA	StumpedA	0.23560009	0.00619873	0.01038015	0.00144513

class	Fimbriimonadia	UnStumpedA	StumpedA	1.17014686	0.00037339	0.00048489	0.03362195
class	Cytophagia	UnStumpedA	StumpedA	0.77189899	0.00463524	0.00102479	0.02659809
class	Bacteroidia	UnStumpedA	StumpedA	∞	0.00031813	0.00050009	0.00046507
class	Opitutae	UnStumpedA	StumpedA	0.84525397	0.00249583	0.00157551	0.01418212
class	Betaproteobacteria	UnStumpedA	StumpedA	0.20040318	0.01424721	0.0116249	0.04354282
class	Gemm_1	UnStumpedA	StumpedA	0.38118059	0.00461917	0.00697073	0.0037232
class	Gemm_5	UnStumpedA	StumpedA	0.97174287	0.00103776	0.00079458	0.00184858
class	SC3	UnStumpedA	StumpedA	0.59174373	0.00029685	0.00038578	0.0278762
class	Solibacteres	UnStumpedA	StumpedA	-0.7000452	-0.0072011	-0.0066141	0.00963088
class	S085	UnStumpedA	StumpedA	0.83279129	0.00083904	0.00103686	0.03143177
class	TK17	UnStumpedA	StumpedA	∞	0.00034331	0.00024496	0.00557717
class	MB_A2_108	UnStumpedA	StumpedA	1.86724371	0.00440249	0.00277961	0.01557273
class	PRR_12	UnStumpedA	StumpedA	0.73152928	0.00127302	0.0020021	0.0244013
class	PAUC37f	UnStumpedA	StumpedA	2.2228619	0.00031759	0.00024113	0.00581349
class	Acidobacteriia	UnStumpedA	StumpedA	-1.2350597	-0.0147459	-0.0163458	0.00963088
class	BD7_11	UnStumpedA	StumpedA	∞	0.00025008	0.00023122	0.0001013
class	028H05_P_BN_P5	UnStumpedA	StumpedA	∞	0.00017427	0.0001781	0.0174792
class	c__	UnStumpedA	StumpedA	2.05681702	0.00039253	0.00060275	0.03404801
class	iii1_8	UnStumpedA	StumpedA	1.11090664	0.0039023	0.00299851	0.00144513
class	Chloracidobacteria	UnStumpedA	StumpedA	0.48428569	0.03269122	0.03296082	0.00416163
class	Nitrospira	UnStumpedA	StumpedA	0.49645929	0.00113171	0.00174952	0.03711403

Table A3. Effect of treatment (Unstumped vs Stumped) on bacterial taxa of B layer assessed by metacodeR. Non-parameteric Wilcoxon Rank Sum test was used for statistically significant taxa. Only significant ($p<0.05$) taxa are shown.

Tax_rank	Tax_name	Treatment_1	Treatment_2	Log2_median_ratio	Median_diff	Mean_diff	Wilcox_p_value
phylum	Planctomycetes	UnStumpedB	StumpedB	0.64734455	0.00504317	0.00482947	0.00032853
phylum	BHI80_139	UnStumpedB	StumpedB	∞	0.00032524	0.0002428	0.03099793
phylum	Armatimonadetes	UnStumpedB	StumpedB	−∞	-0.0004964	-0.0004442	0.00363395
phylum	WS3	UnStumpedB	StumpedB	1.35485488	0.0031768	0.00250608	0.00750344
phylum	Nitrospirae	UnStumpedB	StumpedB	0.8252929	0.00366066	0.00353409	0.00419018
class	Alphaproteobacteria	UnStumpedB	StumpedB	-0.3417189	-0.0306881	-0.0280739	0.02659809
class	ML635J_21	UnStumpedB	StumpedB	∞	0.00032446	0.00027589	0.04958686
class	Cytophagia	UnStumpedB	StumpedB	0.97853723	0.00483186	0.00353956	0.00517662
class	Sphingobacteriia	UnStumpedB	StumpedB	-0.6652529	-0.0088237	-0.0157198	0.00032853
class	Planctomycetia	UnStumpedB	StumpedB	0.38953651	0.00146692	0.00130465	0.04354282
class	Opitutae	UnStumpedB	StumpedB	1.11967599	0.00216132	0.00119682	0.04793754
class	Gemm_1	UnStumpedB	StumpedB	0.50188098	0.00853716	0.00821367	0.00208559
class	c__	UnStumpedB	StumpedB	∞	0.00032524	0.0002428	0.03099793
class	TK10	UnStumpedB	StumpedB	2.08769123	0.00075191	0.00070492	0.00318356

class	S085	UnStumpedB	StumpedB	0.75981316	0.00103491	0.00176206	0.03536952
class	Gitt_GS_136	UnStumpedB	StumpedB	∞	0.00042914	0.00050835	0.00235923
class	Chthonomonadetes	UnStumpedB	StumpedB	−∞	-0.0004964	-0.0004442	0.00363395
class	PRR_12	UnStumpedB	StumpedB	1.35485488	0.0031768	0.00250608	0.00750344
class	Acidobacteriia	UnStumpedB	StumpedB	-1.1685288	-0.0190061	-0.02208	0.0001807
class	Phycisphaerae	UnStumpedB	StumpedB	1.01611893	0.00325984	0.00328571	0.00024469
class	iii1_8	UnStumpedB	StumpedB	1.15307786	0.00364158	0.00238356	0.00184858
class	Chloracidobacteria	UnStumpedB	StumpedB	0.46180133	0.03223464	0.03185756	0.02659809
class	Nitrospira	UnStumpedB	StumpedB	0.8252929	0.00366066	0.00353409	0.00419018

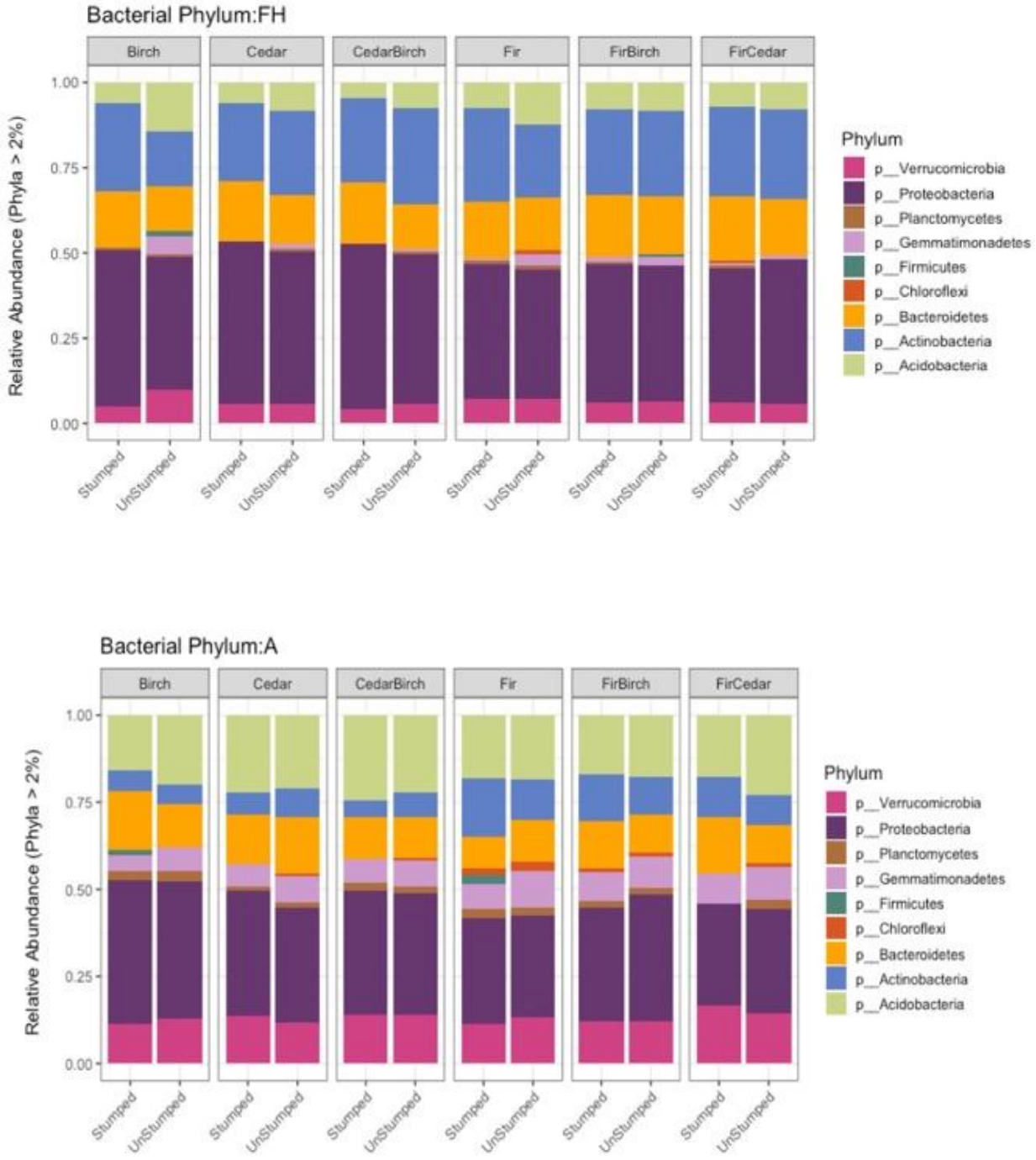

Figure A.1 Mean relative abundances of bacterial phyla in FH and A horizons. Orders having >2% abundance were used.

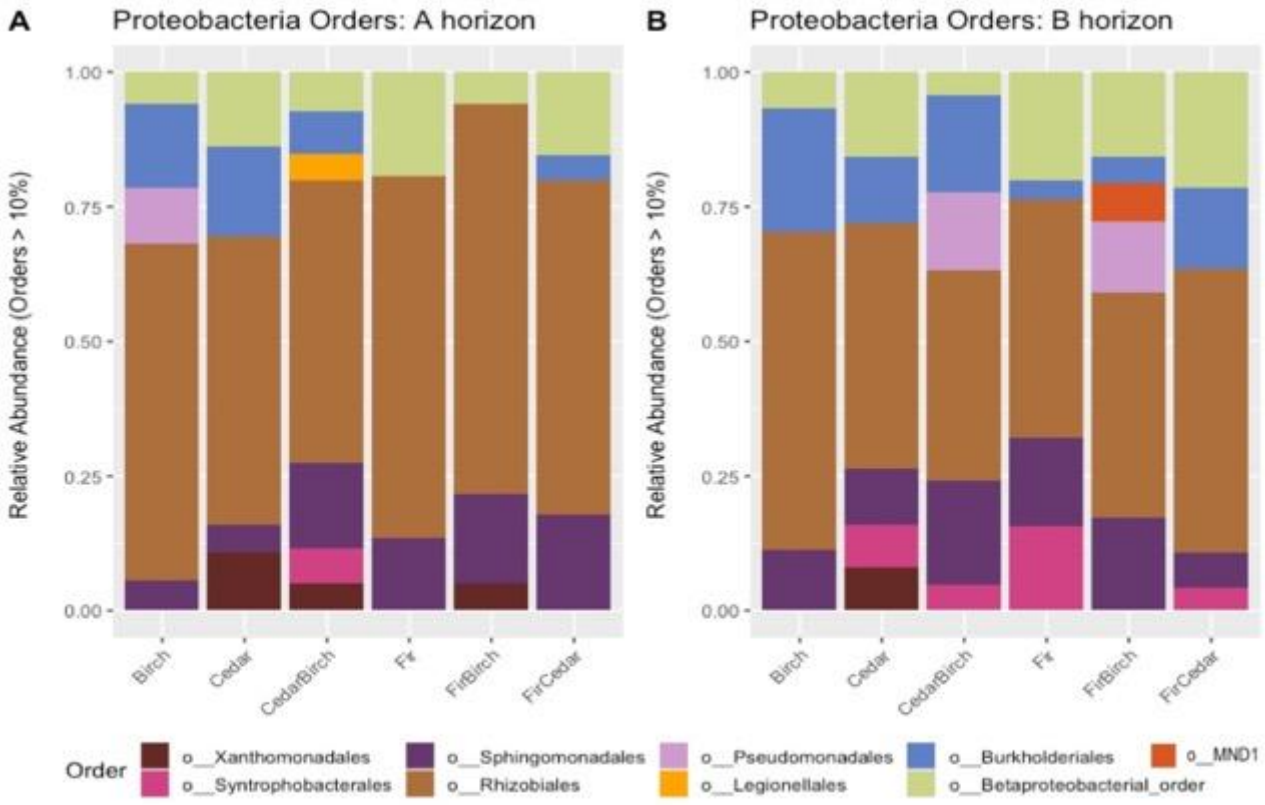

Figure A.2 Mean relative abundances of orders of Proteobacteria in FH, A and B horizons. Orders having >10% abundance were used.

Table A.4 Strains of isolated and cultured diazotrophs from the stumped plots.

Strain No.	Identified as
DM 17	Bacillus Sp WYT037
DM 18	Bacillus drentensis
DM 20	Streptomyces Sp. 746G1
DM21	Bacillus mycoides
DM22	Burkholderia

DM23	Bacillus mycoides
DM24	Bacillus mycoides strain 81
DM25	Variovorax sp
DM26	Bacillus mycoides
DM27	Brevibacterium Sp
DM28	Brevibacterium Sp
DM29	Bacterium BAB1 sp.
DM32	Streptomyces nojiriensis strain P4M83
DM33	Paenibacillus Sp.
DM34	Brevibacterium Strain
DM35	Bacillus sp. WYT037
DM36	Streptomyces sp.
DM37	Bacillus simplex strain 86%
DM38	Brevibacterium Strain
DM41	Pseudomonas lurida
DM42	Pseudomonas sp.
DM43	Streptomyces sp.
DM44	Bacillus sp.
DM45	Arthrobacter
DM46	Streptomyces sp.