

**Root-associated fungal diversity, nutrient mineralization and
micro-climatic interconnections:
Fundamental processes in canopy soils of old-growth *Acer macrophyllum*
in Washington State's temperate rainforests**

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Abstract

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The temperate rainforests of western Washington are commonly recognized for their extensive areas of old-growth forest and more recently their unique canopy soil environment. Over the last 40 years, fundamental studies on canopy soils have emphasized their importance as structural and functional components of these ecosystems; but there remain many unknowns on the biotic and abiotic processes in canopy soil environments, how these may be impacted by climate change, and the implications this may have on host tree resiliency. For example, old-growth bigleaf maple trees (*Acer macrophyllum* Pursh.) grow extensive adventitious roots that form fungal associations. However, no studies have explored the diversity of these adventitious canopy root associated fungal communities and how they compare to forest floor rooting networks. Further, no studies have explored the seasonal mineralization rates of plant available nutrients in canopy soils and how they enhance forest-level nutrient cycling. Therefore, this

study aimed to not only compare these biotic and abiotic processes between the two soil environments, but it also aimed to shed light on how these processes may be impacted by increased and decreased rainfall amounts to better understand how these trees may be affected by climate change.

Prior to identifying fungal communities associating with roots in canopy and forest floor soil environments, a methodological approach for long-read sequencing of fungi was designed and tested on the MinION Nanopore Sequencer. To assess the capabilities of the MinION, three fungal mock communities were sequenced. Each had varying ratios of 16 taxa. The MinION recovered all mock community members, when mixed at equal ratios. Highly accurate consensus sequences were derived and identified to species level, proving that the MinION was suitable as a practical alternative to gain insights on root-associated fungal communities.

After benchmarking this technology, roots were collected from canopy and forest floor environments to determine if there were any differences in the percent of fungal colonization. There was no significant difference between the percent of fungi colonizing adventitious canopy roots ($56.5\% \pm 5.4$) and forest floor roots ($65.1\% \pm 3.6$). Subsequently, a rainfall experiment was implemented and root associated fungal communities were identified seasonally (excluding winter) over the duration of one year. At ambient conditions, root associated fungal community composition was significantly different between the two old-growth sites and also between canopy and forest floor environments. However, these communities did not shift in response to seasonal changes. In canopy soil environments, the increased and decreased rainfall experiments and site differences also significantly affected fungal community composition; seasonality also had an effect. The MinION was able to identify a diversity of obligate mutualists and facultative

endophytes. There were several species associating with adventitious canopy roots that have never been reported to associate with bigleaf maple prior to this study.

Nitrogen (N) and phosphorus (P) mineralization rates were also determined seasonally during the rainfall experiment as well as annual N and P pools. In canopy soil environments, both the rainfall treatments and seasonality had a significant effect on N mineralization rates. Phosphorus mineralization rates were also impacted by the rainfall treatments. On a *per mass basis* at ambient conditions, canopy soils have higher rates of net N ($355.3 \pm 54.7 \text{ mg N kg}^{-1} \text{ yr}^{-1}$) and net P mineralization ($387.6 \pm 34.5 \text{ mg PO}_4\text{-P kg}^{-1} \text{ yr}^{-1}$) than forest floor soils ($58.2 \pm 3.9 \text{ mg N kg}^{-1} \text{ yr}^{-1}$ and $387.6 \pm 34.5 \text{ mg PO}_4\text{-P kg}^{-1} \text{ yr}^{-1}$). When converted to an *areal basis*, canopy soils enhanced the annual $\text{NO}_3\text{-N}$, $\text{NH}_4\text{-N}$, and $\text{PO}_4\text{-P}$ mineralization pools by 5.2%, 48.4%, and 3.7%, respectively. Additionally, some of these resources are leaching to the forest floor soil environment.

The first part of this study benchmarked a methodological approach that was utilized throughout the project and allowed the inference of genus and species-level resolution in canopy and forest floor environments. The other two parts of this study demonstrated that canopy soils provide an extra compartment for nutrients and that adventitious rooting systems are associating with a diversity of fungi distinct from forest floor environments. Further, higher and lower inputs of rainfall impact these biotic interactions, as well as the nutrient dynamics. Collectively, this research reveals that fungal communities associating with adventitious roots may be acting as adaptive facilitators to environmental extremes (e.g., climatic changes) and that biogeochemical cycles in canopy soils and their inputs to the ecosystem should not be overlooked.

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Chapter 1: Introduction

1.1 Prelude

When I was an undergraduate, I took several courses that opened my eyes to the wonderful world of soils. As soon as I started ‘digging’ in on the topic and seeking out research opportunities, I was awed by the vast unknowns regarding soil organisms, especially the cryptic nature of fungi. With a background in horticulture and restoration ecology, I was eager to learn more about how fungal organisms interact with plants. To my amazement, I learned that plant-fungal interactions dated back to when plants first evolved on the terrestrial landscape. It was inspiring to me to think about the fact that if it wasn’t for fungi ‘holding hands’ with plants, Kingdom Plantae may not have evolved to the complexity we see today. I quickly reflected on a quote from E.O. Wilson’s ‘Diversity of Life’, where he states:

“If all mankind were to disappear, the world would regenerate back to the rich state of equilibrium that existed ten thousand years ago. If insects were to vanish, the environment would collapse into chaos.”

I started to think about how that stood true for fungal organisms as well. In reality, we would never really know unless something of that sort happened to fungi. However, the fact remains that fungal organisms go mostly unseen to the human eye, but are responsible for so many ecological processes with which other organisms are interconnected. Yet, they are often overlooked in microbial studies and are not often incorporated into ecosystem-scale models. The realization that there were so many things *still to be discovered* regarding plant-fungal interactions which is what set me on my current research path.

As my interest on plant-fungal interactions grew, I obtained an undergraduate research position with a PhD student who was working in the old-growth forests of western Washington.

As if working in these forests was not already cool enough, she was doing research in the tree canopies! I can vividly remember my first time climbing up to a branch 15 m above the forest floor, seeing canopy soils for the first time, and having so many questions. It was like observing a mini-forest within a forest, and that is when I learned that certain trees grow adventitious roots from their branches into these organic soils. I started to ask questions like: 1) What fungi are they associating with; 2) What role do these canopy soil plant-fungal interactions play in host tree resiliency as we face climate change; 3) Would these trees even survive in this old-growth forest without the presence of canopy soils; 4) What is the mushroom diversity like up here? To my surprise, all these questions were yet to be answered - and that was the start of my PhD journey.

1.2 Plants and fungi

The origins of fungi are estimated to be between 760 million to 1.06 billion years ago (Lucking *et al.* 2009). However, the inconsistency of molecular clocks demonstrates that their origin is questionable, and their evolutionary pathways are complex (Naranjo-Ortiz and Gabaldón 2019). Both Kingdom Animalia and Kingdom Fungi diverged from the Kingdom Plantae, suggesting that plants evolved before animals and fungi, even if only by a short period of geologic time (Moreira *et al.* 2007, Yoon *et al.* 2004). However, over the last century, and especially during the recent decades, research has begun to emphasize increasing our understanding of the importance surrounding the diversity of fungi and the large role they held in the establishment of land-plants in terrestrial environments (see Rimington *et al.* 2018). For example, about 90% of plant species form mutualistic relationships with mycorrhiza in their rooting system (Wang and Qiu 2006, van der Heijden *et al.* 2015, Begum *et al.* 2019). It is the 'norm' and not the exception.

The Latin term '*mycor*' means fungus and the term '*rhizae*' means roots, so mycorrhiza literally translates to fungus-roots. These fungal mutualists are a key component in plant adaptation and survivability in their growth environment (Begum *et al.* 2019), providing nutrients such as phosphorus (P) and nitrogen (N) to plants in exchange for carbon to grow their tissues. In fact, some plant species depend on these associations for germination and subsequent growth (Swarts and Dixon 2009, Rasmussen and Rasmussen 2018, Feijen *et al.* 2018), and mycorrhiza can be responsible for providing up to 80% of the P and N acquired by the plant hosts (van der Heijden *et al.* 2015). They are also a key component of biogeochemical cycles, for example their global distribution is linked to terrestrial C stocks sequestered by vegetation (Soudzilovskaia *et al.* 2019); they also are drivers of the soil N cycle (Lin *et al.* 2017) and can enhance soil fertility (Basu *et al.* 2021).

There are several different groups of mycorrhizal fungi, including arbuscular mycorrhizal fungi (AMF), ectomycorrhizal fungi (EMF), as well as orchidaceous, ericoid, arbutoid, and monotropoid mycorrhiza (Smith and Read 2008). Orchidaceous, ericoid, arbutoid, and monotropoid mycorrhizas are host-specific; the first group associates with orchids, and the latter three groups with a diversity of plants in the Ericaceae family. Arbuscular mycorrhiza is the most widespread group of obligate plant mutualists, forming relationships with ~80% of land plants (Smith and Read 2008). These fungi form intracellular structures (e.g., vesicles, arbuscules, coils) within the plant's fine rooting system. Ectomycorrhiza associate with a broad diversity of plants, especially tall woody trees and shrubs. They form a sheath (or mantle) on the outside of a root-tip and form a Hartig net around the epidermal cells within a root, but they don't develop intracellular structures in fine roots. There are some fungal species, typically belonging to the class Helotiales or Pezizales, that form characteristics that overlap with EMF, but also form

intracellular coils. These are referred to as ectendomycorrhiza, and some of the host specific groups discussed earlier possess these characteristics (Smith and Read 2008).

The essential role that mycorrhizal associations hold in plant health and soil biogeochemical cycles are increasingly recognized as an integral part of ecosystem function and resilience. There remain many other root-fungal interactions, however, that are obfuscated due to their ambiguous nature, such as the role of dark septate endophytes (DSEs) in plant nutrient allocation and resiliency. For example, DSEs were often disregarded as dormant endophytes or facultative pathogens until the late 1990's. Then research started suggesting that some species may forming mutualistic associations similar to mycorrhiza (Jumpponen 2001). Studies have also showed that some DSEs have been found to improve the tolerance and growth of plants under various stressors, such as high salinity, metal contamination, and drought (Likar and Regvar 2013, Zhang *et al.* 2017, Mateu *et al.* 2020).

As research advances, it is becoming apparent that certain fungal species are facultative plant mutualists and further studies are needed to understand their complexity. For example, ectomycorrhizal species are often detected in ericoid roots and ericoid species are detected in non-ericoid tree roots (Bougoure *et al.* 2007). Dark septate endophytes often make up part of the fungal community in both EMF and AMF associated rooting systems (Wagg *et al.* 2008, Vohnik *et al.* 2013), and some DSEs form ericoid mycorrhiza (e.g., *Rhizocyphus ericae* [Leopold 2016]). Though some mycorrhiza are obligate mutualists (e.g., arbuscular mycorrhiza), studies have demonstrated that there is still much to be discovered when exploring root-associated fungal communities, and the implications these relationships have on ecosystem resiliency in the face of climate change.

1.3 Washington's old-growth temperate rainforests

Old-growth temperate forests represent the longest living and largest stores of carbon on earth on a per unit of terrestrial area (Urrutia-Jalabert *et al.* 2015). This makes these temperate zone forests the best remaining natural laboratory to study because of their low anthropogenic impacts (DellaSala *et al.* 2011). The temperate rainforests of western Washington are part of the Pacific Coastal Temperate Rainforest, the largest temperate rainforest ecoregion globally, stretching from the Eel River in California to the Alsek River in Alaska (Fig. 1.1). Many of the old-growth stands in these areas are located in alluvial floodplains, forming dynamic riparian habitats (Naiman and Bilby 1998), and the location of two of my study sites (Fig. 1.2). The high structural variability, rainfall regimes, and associated diversity of these forests are conducive for a wide array of niches for biotic relationships to form (Frank *et al.* 2009). Despite that fact, the role of abiotic and biotic interactions in the functioning and carbon dynamics of these forests has rarely been described.

Current research shows that the diversity of microorganisms living belowground contribute significantly to shaping aboveground biodiversity and functioning of terrestrial ecosystems (Bardgett and van der Putten 2014), but these forests have more than just a belowground, they have 'belowground' soil interactions located above ground in tree canopies. These are known as canopy soils. Yet, there is not a mechanistic understanding of how canopy soils, adventitious roots, and associated fungal biodiversity link into the functional dynamics of these ecosystems. In fact, the basic science of these unique canopy soil habitats is relatively unknown, as studies only date back to 40 years ago (Nadkarni 1981, Nakamura *et al.* 2017). Understanding the complexity of biotic interactions, abiotic processes, and how these ecological legacies evolve is

pivotal to understanding how these old-growth forests function, and how resilient they are to disturbances.

1.4 Canopy soils and adventitious roots

Canopy soils are thick organic mats that are found in cloud forests and temperate rainforests. They are prominent on tree branches in the old-growth forests of Washington because the climatic characteristics stimulate the formation of an epiphytic layer on tree branches that intercepts litterfall and atmospherically derived nutrients and moisture. As these layers of epiphytic and organic material increase in thickness, the bottom layers decompose, contributing to the ecosystem-level carbon and nutrient cycles (Nadkarni 1981, Coxson and Nadkarni 1995, Tejo *et al.* 2014). The species I chose to study for my PhD research was bigleaf maple (*Acer macrophyllum* Pursh.). I was fascinated by the canopy soils that reach depths >35cm on tree branches, and the extensive growth of adventitious root networks into the canopy soils.

Species within the *Acer* genus form mutualistic relationships with AMF, and evidence shows that adventitious roots have AMF structures (Nadkarni 1981). However, fungal community diversity associating with host tree adventitious roots has not been the focus of research in these canopy soil ecosystems. There is a lack of knowledge of the seasonal and annual fluxes of plant available nutrients in bigleaf maple canopy soils, and the role of these fungal communities in the nutrient dynamics of canopy soils and their responses to environmental changes. Some existing studies suggest that canopy soils in these forests contribute unique structural and functional components in these forests. For example, canopy soils were found to contribute as much as 20% and 25% of the total N and C found in storage pools, respectively, at the ecosystem level (Tejo *et al.* 2014). It is known that geomorphology,

hydrology, sediment, and riparian-derived large wood shape these riparian forests (Swanson and Lienkaemper 1982, Naiman *et al.* 2000, Abbe and Montgomery 2003), but it is crucial to understand the complexity of biotic and abiotic interactions in canopy soils because of their potential influence on the characteristics of organic matter and nutrient fluxes into streams, estuaries, and the atmosphere.

Understanding how disturbances impact canopy soils is especially important because global climate models predict the continuance of seasonal extremes, including wetter winters and drier summers (Mote and Salathé 2010, Bachelet *et al.* 2011). The increasing global prevalence of droughts and their impacts on the carbon cycle (Vogt *et al.* 2016) supports the need to understand these relationships and interconnections in canopy soils where host trees are forming these relationships with adventitious roots. Martinez-Garcia and others (2017) have shown that fungal mutualists are able to increase host resilience to disturbances, but this has rarely been considered from a canopy soil perspective.

1.5 Canopy soils as an experimental system

Understanding the role of the belowground environment, even if located aboveground, on ecosystem carbon and nutrient pools and fluxes is needed since these systems contain compounds that directly impact system-level fluxes. Especially because the resulting processes and abiotic stressors impact root-associated fungal communities, including mycorrhizal diversity (Millar and Bennet 2016). Part of the lack of research has reflected the need to develop tools and manipulative experimental systems capable of distinguishing the impact of multiple drivers, e.g., litter quality, Net Primary Productivity (NPP), fungal biodiversity, on ecosystem-level carbon sequestration and nutrient cycles (e.g., Vogt *et al.* 2016). Research has successfully tested the

effect of reduced rainfall levels on plant biomass (Henry *et al.* 2018), and climate and soils on the vulnerability of tropical forests productivity to drought (Vogt *et al.* 2016); but understanding how fungal diversity and nutrient pools are impacted under climate change scenarios remains a challenge, and has resulted in fungal processes being widely ignored in ecosystem models.

There have been studies that demonstrate that fungal communities respond directly to natural rainfall gradients (Hawkes *et al.* 2011) and experimental drought conditions (Ficken *et al.* 2019, Buscardo *et al.* 2021), but insights have been somewhat limited by the inability to design a field experiment capable of separately testing changes in the fungal diversity from the other drivers impacting productivity or soil carbon stabilization following a disturbance.

This is when I realized that canopy soils were a great testbed for *in situ* experimental manipulations. They featured soils and rooting systems but didn't have as many confounding factors that makes it difficult to conduct *in situ* soil studies and include fungal processes in ecosystem scale models. A majority of studies that explore the functional roles of fungi in decomposition and nutrients have been in artificial experimental systems where the fungi are not studied in an ecosystem context. Canopy soils provide an experimental system that allows us to test *in situ* shifts in fungal diversity, as well as nutrient dynamics.

Typical diagrammatic representation of the influence of climate on ecosystems and productivity show the belowground as distinct and separate from the aboveground (Suseela and Tharayil 2017). This creates a false dichotomy where plant-fungal interactions are studied separately from the rest of the ecosystem. When roots grow in the tree canopies, as found in many ecosystems globally, our understanding from a belowground focus is not clear. With this in mind, highly organic canopy soils – *separated* from mineral soils, but not completely decoupled (see Matson *et al.* 2014) – provide a unique experimental system to explore and

model biotic interactions and abiotic processes and further compare these to the forest floor soils.

1.6 Objectives

Canopy soils provide a unique study system to experimentally manipulate factors controlling biotic and abiotic processes without the confounding effects from the mineral soil. When I first started brainstorming my PhD research design, it was obvious that fundamental questions needed to be answered in canopy soil systems before any processes could be mechanistically linked to overall ecosystem function and incorporated into climate models. The science of canopy soil is still relatively young and only began to be a focus of research starting in the 1980's. With this in mind, this research project was designed to explore the diversity between root associated fungal communities and nutrient fluxes in canopy and forest floor soils of old-growth bigleaf maple at ambient conditions.

This research was designed to provide fundamental information needed to better understand how canopy soils may contribute to host tree resiliency and overall ecosystem function. Canopy soils are an ideal experimental system since the micro-climatic conditions can be manipulated without the confounding factors found in soils where changes are harder to detect. The canopy soils are amenable to experimental manipulation of micro-climatic factors to explore the impact of drought or high rainfall on nutrient pools and fluxes as well as the biodiversity of the fungal communities. Another benefit of utilizing canopy soils as a research system is that studies could be designed to compare it to the forest floor soils and belowground roots, which are more challenging to study because of the inability of separating the impacts of processes occurring at

a microscale and where the roots of a tree 30 m away can influence the micro-scale processes being measured.

To better understand the extent of plant-fungal interactions and nutrient dynamics in canopy soils, the objectives of this PhD research were:

1. Seasonally compare root-associated fungal communities in bigleaf maple canopy and forest floor soils.
2. Seasonally compare root-associated fungal communities in bigleaf maple canopy soils under a rainfall experimental system.
3. Determine the seasonal and annual pools of mineralized N and P in canopy soils and compare those to forest floor soils.
4. Seasonally compare rates of N and P mineralization in canopy soils under experimental rainfall conditions.

The first objective was an initial survey comparing the amount of fungal structures occurring in adventitious canopy and forest floor rooting systems. The remaining research was designed to measure the latter three objectives as temporally linked variables to expand our mechanistic understanding of these processes. This would not have been possible if studying the forest floor because many variables are auto-correlated and the results are an index of the processes that are occurring. Therefore, hypotheses cannot be refuted since they are based on descriptive knowledge where a paradigm shift has to occur to develop mechanistically understanding of an ecosystem and how it is impacted by a changing climate (Reason 1990).

At the start of my PhD, it was clear that the tools for assessing fungal diversity were evolving and did not provide the level of resolution needed for this research. For example, the high-throughput sequencing technology that was often used for fungal community analysis had high accuracy and worked well, but often did not provide species-level resolution. This was in part due to the short sequence reads, but also reflected the fact that a majority of fungal organisms remain unclassified. During this time, new molecular technologies were being

released to the scientific community, including the third generation of high-throughput sequencers. As an aspiring graduate student, I thought it would be great if we could use one of these newer technologies to get longer sequence reads and potentially better species resolution. This required my immersion in techniques and tools that were not robust, as well as determining if it was possible to refine these techniques to provide the level of resolution needed for this research.

Using the MinION Nanopore sequencer was of interest because fungal communities in these old-growth forests, let alone canopy soils, had barely been explored. Initially, I decided to pursue the use of the MinION to identify root-associated fungal communities in collaboration with researchers who were experts in these tools. It became apparent during the initial testing of this system that no paper had been published using the MinION to identify fungal communities. Therefore, testing if the MinION could accurately identify fungi to species level evolved into a methodology manuscript that was published in *Biotechniques*. This manuscript was later selected as an Editor's Top 5 Pick – it is featured as Chapter 2 of this dissertation. In this chapter, three fungal mock communities were created, and subsequently sequenced and processed through a bioinformatics pipeline that was specifically selected for MinION data. The results of this research led to using the MinION to conduct the fungal community analysis (see Chapter 3).

In Chapter 3, both the MinION and Illumina MiSeq were used to identify and compare root-associated fungal community diversity in ambient canopy soils and forest floor soils. Further, the percent of fungi colonizing adventitious roots in the canopy soils were compared to the forest floor roots. Also, this paper addresses how fungal communities found in the canopy soils are impacted by the different levels of rainfall by comparing root-associated diversity under

ambient conditions and experimentally manipulated rainfall levels. In Chapter 4, the seasonal and annual mineralization rates in canopy soils under ambient conditions were used to determine how much P and N is available for plant uptake in the canopy, and how much canopy soils enhance mineralized pools of N and P at the ecosystem scale. Further, it was tested if canopy soil mineralization rates are affected by the experimental rainfall manipulations. In Chapter 5, I discuss overall conclusions and future perspectives on why canopy soils should not be overlooked in the ecosystems where they prevail.

Figures

Figure 1.1. The coastal temperate rainforest region and subregions of North America. Image from Carpenter *et al.* (2014).

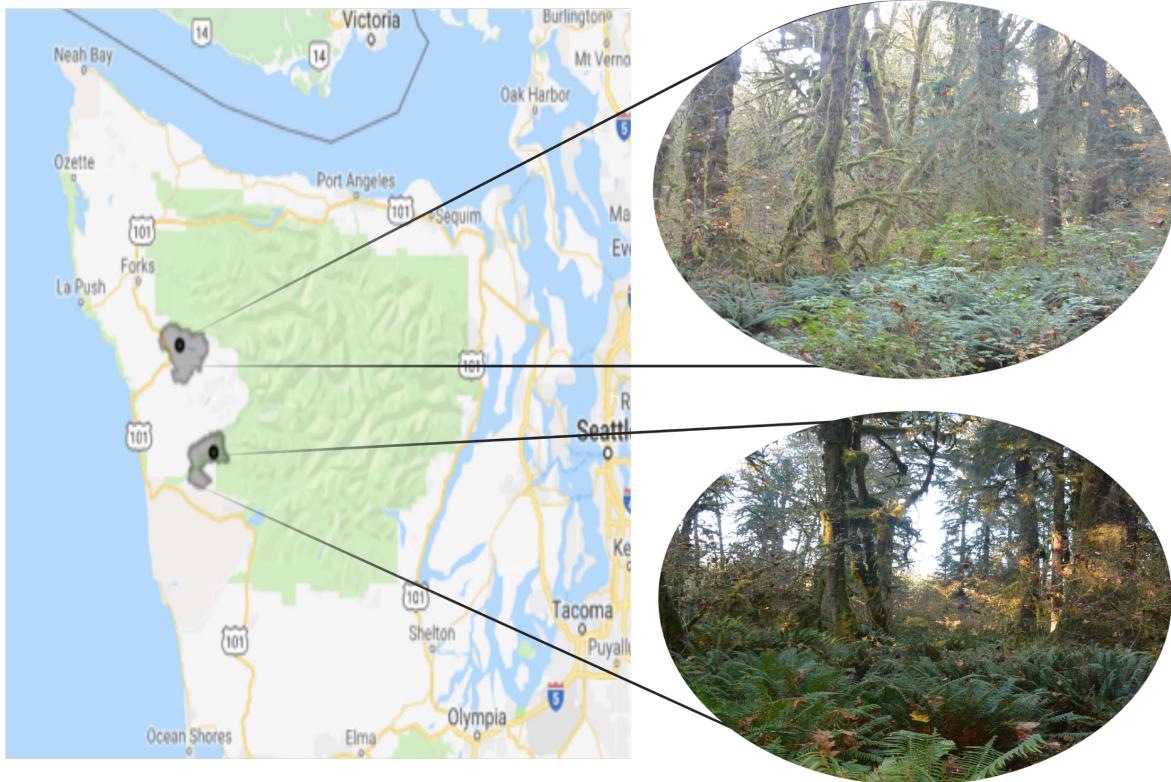


Figure 1.2. The location of the two old-growth rainforest stands used as study sites for this research. One of the old-growth stands was located in the Hoh River Valley and the other in the Queets River Valley. The grey outline delineates the part of the watershed and the dots are where the sites are located.

Chapter 2: Benchmarking a protocol for fungal community analysis on the MinION nanopore sequencer

Mafune *et al.* 2019. *A rapid approach to profiling diverse fungal communities on the MinION Nanopore Sequencer*. *BioTechniques* 68(2), 72-78

2.1 INTRODUCTION

High-throughput sequencing (HTS) provides the ability to multiplex and profile fungal communities across environmental samples, including but not limited to leaf tissue (e.g., Brown *et al.* 2018), soil (e.g., Glassman *et al.* 2017), and roots (e.g., Bergleson *et al.* 2019, Cheeke *et al.* 2015). Despite these capabilities, established HTS methods (e.g., Illumina and Pyrosequencing) are limited in taxonomic resolution because of their shorter sequence reads (Kennedy *et al.* 2018), and the need for expensive analytical technology. However, the ongoing improvements of third generation HTSs hold promise for diversity analyses of environmental samples. For example, they provide longer sequence reads, and despite the higher error rates (5-13%, [Jain *et al.* 2015, Rang *et al.* 2018]), high-quality reference data can be generated and species-level identification can be inferred by averaging out the errors with a consensus from multiple reads (Kilianski *et al.* 2015, Benítez-Páez *et al.* 2016, Brown *et al.* 2017, Ashikawa *et al.* 2018, Tedersoo *et al.* 2018, Wurzbacher *et al.* 2019).

Oxford Nanopore Technologies' (ONT) MinION sequencer is a small third generation HTS platform that became commercially available in 2015 (Reuter *et al.* 2015). It has the ability to generate longer reads, and is efficient in determining bacterial microbiomes (Benítez-Páez *et al.* 2015, Shin *et al.* 2016). It has also proved to be successful in genome assembly of fungal species such as *Leptosphaeria*, *Saccharomyces*, and *Candida* spp. [Giordano *et al.* 2017, Dutreux *et al.* 2018, Panthee *et al.* 2018, Rhodes *et al.* 2018). Although challenges still remain

with DNA/RNA extraction, polymerase chain reaction (PCR), and sequencing protocols in remote environments, the MinION has also been successfully executed in remote pop-up labs ranging from the Arctic Canada to the Amazon Rainforests [Goordial *et al.* 2017, Parker *et al.* 2017, Pomerantz *et al.* 2018]. Despite these advancements, there still remains a paucity of information regarding the MinION's capacity for identifying a broad range of fungal diversity using amplicon sequencing.

To test the MinION's ability in identifying fungi to the species level and distinguishing closely related species, three barcoded mock libraries were sequenced (Mock A, Mock B, and Mock C), and processed through our selected pipeline. The three libraries contained 16 taxonomically diverse species (Table 2.1). The libraries were assembled from DNA extracted from sporocarps or cultures directly isolated from leaf and root tissue. The aim of this experiment was to (1) develop an applicable pipeline to analyze MinION 1D sequence data; and (2) determine if 1D reads from the MinION™ could accurately identify a diversity of environmentally derived fungal taxa.

2.2 MATERIALS AND METHODS

2.2.1 Mock libraries

To design the three mock libraries (Mock A, Mock B, and Mock C), a total of 16 species from 12 genera representing 11 families from Basidiomycota, Ascomycota, and Mucoromycotina (see Spatafora *et al.* 2016) were collected or cultured from environmental samples (e.g., roots and leaves). These 16 members represented mycorrhiza, saprotrophs, pathogens, and endophytes. All DNA was extracted using an optimized protocol (see Appendix A) for the Synergy™ 2.0 Plant DNA Extraction kit (OPS Diagnostics). Extracted genomic DNA

(DNA extracted from organismal cells [gDNA]) was successfully amplified using the forward and reverse primers. Both primers were ordered with ONT's barcoding tags, which are required for subsequent barcode attachment. The forward primer selected for this experiment was ITS1f-kyo2 (5' TAG AGG AAG TAA AAG TCG TAA 3'), based on its greater taxonomic coverage of fungi (Toju *et al.* 2012). The reverse primer was a variant of the LR3 primer (5' CCG TGT TTC AAG ACG GG 3'), which binds on the 5'-end of the rRNA gene large subunit region (LSU), and also provides broad taxonomic coverage (Liu *et al.* 2012, Mueller *et al.* 2015). This primer pair targets the internal transcribed spacer (ITS) region and several hundred bases in the LSU region.

The variant used is referred to as LR3-I (5' TGG TCC GTG TTT CAA GAC 3'). LR3-I was created due to the high failure rate of the original LR3 primer during PCR amplification when ONT's barcoding tag was attached to the 5'-end. To design LR3-I, a sample of 1000 fungal sequences from GenBank were aligned using CLC Genomic Workbench (Qiagen [v8.5.1]) software to identify a highly conserved region near the LR3 binding site. The conserved site that was selected for the LR3-I primer overlapped part of the original LR3 primer, but shifted four nucleotides over to the 5'-end and excluded the last three nucleotides on the 3'-end (Fig. 2.1). The resulting LR3-I primer was tested in-silico against the complete National Center for Biotechnology Information's (NCBI) nucleotide database to confirm comparable coverage and specificity to the original LR3 primer.

Identities of the fungi used in the mock communities were confirmed by Sanger sequencing the purified PCR amplicons (GeneWiz, Seattle, WA). Contigs for the mock community taxa were assembled using MacVector (MacVector Inc. [v11.1.2]). See Table 2 for details on accession numbers, sequence length, GC content, and mock library ratios. After identities were confirmed with Sanger sequencing, the gDNA of the 16 species were mixed together in three

different mock communities. When working with environmental samples, the proportion of fungal DNA is expected to be low relative to plant/root/soil DNA. Therefore, a lower concentration of fungal gDNA was used in these mock communities. All of the species in Mock A were combined at an equal concentration of 0.028 ng/ μ l each. Mock B included .028ng/ μ l of 13 species along with a 1:10 dilution of *Boletus edulis*, *Trichoderma atroviride*, and *T. reesei*, respectively. Mock C included .028ng/ μ l of 15 species along with a 1:10 dilution of *Mortierella elongata*. After the gDNA from the 16 species was combined, respectively, the mock libraries were processed through PCR, barcoding, and library preparation prior to MinION™ sequencing.

2.2.2 PCR amplification

PCR was performed on the three mock communities in 50 μ l reactions. Each reaction contained 1 μ l template DNA, 17 μ l of PrimeStar GXL Taq Polymerase Master Mix (TaKaRa Bio Inc.), 1 μ l of 10 μ m tagged forward primer, 1 μ l of 10 μ m tagged reverse primer, and 30 μ l of molecular grade H₂O. The conditions for PCR were: 5 cycles of denaturation at 98°C for 10 seconds, annealing at 55°C for 30 secs, ramping up at 2°C/sec to 68°C for 2-minute extension, followed by 28 cycles at 98°C for 10 seconds, 55°C for 20 seconds, and 68°C for 90 seconds. Once completed, reactions were purified using AMPure magnetic beads (Beckman Coulter) and resuspended in 20 μ l molecular-grade H₂O. Concentrations of the amplicon libraries were determined using the Qubit Fluorimeter 2.0 (Thermo-Fischer Scientific).

2.2.3 Barcoding mock communities and library preparation

Using the barcoding expansion pack (EXP-PBC001), a respective barcode was attached to the three mock libraries. The barcodes were attached using LongAmp Taq 2x MasterMix (New

England Biolabs), as described in ONT's PCR barcoding protocol. Once complete, the barcoded amplicon libraries were purified with AMPure magnetic beads and re-suspended in 20µl molecular-grade H₂O. Concentrations were determined on the Qubit. The 1D Ligation Sequencing Kit (SQK-LSK108) was used to complete the library preparation, and the three libraries were pooled together in equal amounts to a total of 1µg, as specified in ONT's protocol.

Prior to loading the library, a quality control check was performed on the MinION Flowcell (R9.4.1) to confirm there were enough active pores (>800 pores). Following the successful check (1,238 pores), the barcoded library was mixed with ONT's loading beads and buffer, and pipetted into the flowcell. The run was executed on a Macbook Pro using ONT's MinKNOW software (v2.0).

2.2.4 Downstream processing

The raw reads were demultiplexed and sorted into barcode directories using ONT's Albacore software (v2.0.1). The fastq files (NCBI SRA #PRJNA565592) were filtered through NanoFilt v. 2.0.0 (De Coster *et al.* 2018) - <https://github.com/wdecoster/nanofilt>), at a Q-score of 9, corresponding to c. 90% accuracy. Individual barcodes were aligned using the global pairwise alignment option, -gins1, in MAFFT v7.407 (Katoh & Standley 2013). This alignment method, used with reduced gap penalties (Kazutaka Katoh, Pers. Comm.), was found to produce good consensus sequences from operational taxonomic units (OTUs) clusters produced by mothur v1.39.5 (Schloss *et al.* 2009). The alignments were processed on the University of Washington's high performance computer – Hyak. Upon completion, a distance matrix was calculated for each alignment, using the F84 model (Kishino and Hasegawa 1989, Felsenstein and Churchill 1996) in PHYLIP's (v3.695) DNADist executable with default parameters (Felsenstein 2005).

Mothur (Schloss *et al.* 2009) was used for *de novo* OTU clustering using the OptiClust method (Wescott and Schloss 2017). To determine optimal clustering thresholds, distance matrices were clustered at 92%, 90%, and 88% sequence similarity. OTUs were checked for chimeras (ChimeraSlayer [Haas *et al.* 2011, Schloss *et al.* 2011] and manually) and processed through a Python script that organizes sequences into individual OTU clusters for subsequent alignment using MAFFT. A second script processes the aligned OTUs and calculates an ungapped consensus sequence, a third reports the top BLAST hits for each consensus sequence, and assigns the corresponding sequence count to each OTU (<https://github.com/mycoophile/nanopore-ITS>). Species identities were assigned using the National Center for Biotechnology Information's (NCBI) database.

2.2.5 Data analyses

All data were imported into Rstudio v1.1.383 (Rstudio team, Boston, MA), and further computations were performed using R v3.4.4 (R core team 2018). Pearson's correlation test was performed on OTU data to determine if there was a relationship between the number of sequences in an OTU and percent identity match. If an OTU consensus did not match $\geq 97\%$ to a mock community member, the consensus was considered mixed or weak, and dropped from further analyses. Consensus sequences produced from OTUs with less than five sequences were also dropped from further analyses. All figures were generated using base R functions and the ggplot2 package (Wickham 2016).

2.3 RESULTS and DISCUSSION

2.3.1 Sequence output

The entire MinION sequencing process, including library preparation and the sequencing run, was completed within two working days. The sequencing run was terminated after 14 hours and generated 753,855 reads. The summation of reads includes three other barcoded libraries not related to this mock community experiment, and a barcoded negative control which had contaminants of some mock community taxa (see Table 2.3). Albacore basecalling and quality check sorted sequences into pass/fail directories for each barcoded mock library. From the three mock libraries, a total of 41,324 sequences failed initial basecalling and quality check (13,721, 19,851, and 7,752 sequences for Mock A, Mock B, and Mock C, respectively). A total of 131,665 sequences passed, producing 46,216, 59,688, and 25,761 sequences from Mock A, Mock B, and Mock C, respectively. The mean read quality from these three libraries was 8.4. After subsequent filtering at a q-score of nine, 12,657, 15,196, and 6,832 sequences remained from Mock A, Mock B, and Mock C, respectively (Table 2.4). To standardize subsequent alignment and clustering steps, Mock A and B were rarefied to 6,832 sequences, respectively.

The number of OTUs returned for the three mock communities (see Table 2.5) was dependent on the clustering similarity threshold. The amount of sequence data represented in OTUs with ≥ 5 sequences at 88% similarity is nearly two-fold that at the 92% similarity threshold. When clustered at a similarity $\geq 92\%$, a majority of sequences were represented in OTUs that had ≤ 5 sequences. When clustered at a similarity $\leq 88\%$, a majority of sequences were clustered into three to four large OTUs with the remainder consisting of mostly singletons. This variation in sequence similarities presumably reflects the inherent error rate of the 1D MinION™ reads (8-13%).

There is a positive correlation between the number of sequences in an OTU and the percent identity of the BLAST match found for all three sequence similarity thresholds (Fig. 2.2). For example, consensus sequences generated from OTUs with ≤ 20 sequences match to a mock community member, but often return weaker identity matches (88-96%). Pearson's correlation coefficient for clustering thresholds of 92%, 90%, and 88% are 0.44 (df = 170, $P < 0.001$), 0.41 (df= 213, $P < 0.001$), and 0.41 (df= 177, $P < 0.001$), respectively. This demonstrates that the MinION™ 1D reads may not be able to resolve smaller OTUs consistently to a species level, when working with environmental samples.

2.3.2 Mock community representation

In Mock A, all 16 taxa were recovered at the 92% and 90% similarity cut-off (Fig. 2). *Cantharellus formosus* was identified but excluded from analyses because all OTUs contained less than five sequences post chimera filtering. *Cantharellus* has been reported to amplify poorly with ITS primers (Buyck and Hofstetter 2011), which is assumed to be the issue here. Mock B had diluted concentrations of *Boletus edulis*, *Trichoderma atroviride*, and *T. reesei*. When diluted, *Boletus edulis* was not represented by any OTUs across all thresholds. In the other two mock communities where it was mixed equally, it consistently returned smaller OTUs. This may be owing to the fact that many members in Boletales have longer ITS amplicons, which can result in PCR biases [Bellemain *et al.* 2010, Irhmark *et al.* 2012]. In Mock B at 92% and 90% similarity, the relative abundance of *T. reesei* experienced a 20-fold and 26-fold, and 32-fold and 15-fold decrease when compared to Mock A and C, respectively. The relative abundance of *T. atroviride* at 92% and 90% similarity experienced an 8-fold and 12-fold decrease, and 15-fold and 13-fold decrease when compared to Mock A and C, respectively. It also did not return any

OTUs at the 88% threshold. *Mortierella elongata* was mixed at a smaller ratio in Mock C, and did not cluster into any OTUs at the 92% threshold. It was recovered at both the 90% and 88% threshold, and showed a 20-fold and 9-fold decrease, and a 12-fold and 6-fold decrease in relative abundances when compared to Mock A and Mock B, respectively.

2.3.3 Primer selection

For this experiment, we aimed to select primers that would amplify a large majority of fungi from environmental samples, but also sequence into the LSU region. Amplicons sequences that contain part of the ITS and LSU rRNA region have been gaining traction (Brown *et al.* 2014, Raja *et al.* 2017), as they offer some advantage in inferring evolutionary ecology, although it may have limited species resolution (Halwachs *et al.* 2017). ITS1F-KYO2 was chosen because it is highly selective for fungi and provides slightly better taxonomic coverage than ITS1f (Toju *et al.* 2012); and LR3-I because it is located in the LSU region and generates an amplicon of up to 1500 bases, enough for species-level identification for most fungi.

2.3.4 OTUs and consensus sequences

The relatively low raw read accuracy MinION has 1D reads warrants some skepticism concerning the ability to assign correct identities to individual reads. It was not possible to generate OTUs at high sequence similarity (e.g., $\geq 98\%$) or depend on a representative sequence from an OTU to accurately assign identity. When a representative sequence was used to assign identity to OTUs clustered at lower thresholds (88%, 90%, and 92%), all members in the mock communities were recovered, although match quality was consistently lower than 97%. This was expected for a sequence that contains up to 13% of incorrectly assigned bases that was filtered at

a q-score of 9. However, extracting a consensus sequence from a multiple sequence alignment has the potential to eliminate the random errors (Rang *et al.* 2018) This methodological approach had high success in producing a species-level ($\geq 97\%$) identification of mock community members, and showed positive correlation between number of sequences per OTU and percent match at all similarity cut-offs (Fig. 2).

There is almost a two-fold increase in number of sequences represented at the 88% similarity threshold, but the 92% threshold provided an advantage in distinguishing closely related species. For example, all four *Trichoderma* spp. were resolved to species at 92% and 90% similarity cut-off, but only two were resolved at 88% similarity and the relative abundance of *T. harzianum* showed a four-fold increase when compared to the relative abundance at 92%. This increase demonstrates that closely related taxa may cluster together at lower similarity thresholds, limiting species-level resolution. At all similarity cut-offs, consensus sequences from OTUs with ≤ 5 sequences consistently dropped under the species-level identity threshold (97%). With consideration that smaller OTUs may not provide enough sequences to average out a strong consensus, any OTU that had ≤ 5 sequences or that matched lower than 97% to one of the mock community members was considered a weak consensus. Therefore, those were filtered out and not assigned species identity.

2.3.5 Considerations

It was common for certain species to be over-represented and under-represented in these mock communities. This is presumably due to the variable number of repeats of the rRNA genes in different fungal species and strains, and the fact that initial mixed concentrations of gDNA were not standardized by qPCR but rather mixed at equal or staggered gDNA concentrations.

Other factors such as inaccuracies in DNA quantity measurements and priming efficiency may also contribute, emphasizing the importance of stringency in experimental design, extraction, and library preparation techniques (Lindhahl *et al.* 2018). It also supports that a mock community standard should be used in all fungal community analyses of environmental samples, as suggested by Bakker (2018) and Taylor *et al.* (2016), and others.

Sequencing depth should also be considered when designing the experiment, and needs to be further explored with mock amplicon communities on the MinION platform. This flowcell had seven barcoded libraries (three mock communities, three barcodes unrelated to the mock community report, and a negative control). Several species were recovered at low relative abundances in the even community, and one was not recovered when included at a 1:10 ratio. It is possible that these would have been better represented if e.g., only one to three barcodes were sequenced on a flowcell. However, this introduces a limitation regarding the amount of samples that can be multiplexed.

This methodology will work for amplicon sequencing of environmentally derived samples, with primer selection dependent on the targeted group of organisms. We do note, that this was successfully executed on amplicons ranging from 929-1385 bases from communities rarefied to 6,832 sequences. There could be computational limitations in the alignment steps if working with larger sequence datasets and/or longer amplicons. When this experiment was being designed, ONT was in the process of discontinuing the 2D sequencing kit and pursuing the release of the 1D² sequencing kit. Both Calus *et al.* (2018) and Wurzbacher *et al.* (2019) provide insights on sequencing fungal amplicons using ONT's 2D and 1D² kits, using Nano-AmpliSeq and a tandem repeat barcoding workflow, respectively. Loit *et al.* (2019) recently reported that although the MinION may not suffice for metabarcoding of complex samples, it does identify

dominant pathogens and other associated fungi from plant tissues. To our knowledge, this is the first experiment that explores the MinION's ability to sequence a broad range of fungal taxa using the 1D kit. The 1D kit may not provide a complementary strand to create a low-error contig, however the amount of sequences yielded from 1D² kits is much lower, and contigs may have high failure rates (i.e., one of the barcoded strands will sequence, but software may fail at constructing the contig due to missing complementary strand).

2.4 CONCLUSION

This experiment showed that ONT's MinION identified all 16 species from 12 genera in an ecologically based fungal mock community, when gDNA was mixed at an equal concentration of .028 ng/ μ l. Although *C. formosus* was recovered in all barcoded libraries, it was not accounted for in downstream comparisons, due to a majority of sequences being chimeric and the non-chimeric sequences clustering into very small OTUs. In all cases, species that were mixed in smaller ratios decreased in relative abundance when compared to the equal ratio mock library. *Boletus edulis* was the only species that was not recovered, when diluted 1:10. Overall, these results demonstrate that this methodology can be used as a practical alternative to identify a broad range of taxa from an environmental sample, when detecting rare taxa is not the main focus. It also may be capable of providing inferences on shifts in community patterns. To determine this, further experiments that quantitatively measure gene copy numbers (e.g., qPCR) prior to mixing the mock communities are necessary.

2.5 FUTURE PERSPECTIVES

Identifying microbial communities associating with environmental samples has contributed significantly to our understanding of fungal diversity and ecosystem functional dynamics. Established platforms, such as Illumina and Pyrosequencing, remain highly accurate, but require expensive equipment and cannot be executed in remote locations. Oxford's MinION technology has continued to progress since the commercial release in 2015. Error rates remain higher than established high-throughput platforms. However, it holds promise as a pragmatic alternative for assessing fungal diversity from environmental samples. The main advantages reflect the relative cost when compared to other HTSs, longer reads, feasibility of use, and rapid real-time processing. If chemistry and protocols continue to improve and error rates continue to decrease, it could provide an alternative HTS platform for microbial community analyses in the future.

Tables

Table 2.1: Taxon details, sample location/source, and extraction material for all 16 taxa in the mock communities. All samples were collected from various outdoor locations in Washington State, except *T. harzianum* and *T. reesei*, which were obtained from the University of Washington’s Biology Teaching Lab and the American Type Culture Collection (ATCC), respectively.

Family	Genus	Species	Location/Source	Extraction Material
Cunninghamellaceae	<i>Absidia</i>	<i>cylindrospora</i>	Queets, WA	Leaf isolate
Boletaceae	<i>Boletus</i>	<i>edulis</i>	North Cascades, WA	Sporocarp
Cantharellaceae	<i>Cantharellus</i>	<i>formosus</i>	Belfair, WA	Sporocarp
Saccharomycetaceae	<i>Debaryomyces</i>	<i>polymorphus</i>	Queets, WA	Root-tip isolate
Marasmiaceae	<i>Gymnopus</i>	<i>dryophilus</i>	Queets, WA	Sporocarp
Nectriaceae	<i>Ilyonectria</i>	<i>destructans</i>	Queets, WA	Root-tip isolate
Agaricaceae	<i>Lycoperdon</i>	<i>perlatum</i>	Queets, WA	Sporocarp
Morchellaceae	<i>Morchella</i>	<i>exuberans</i>	North Cascades, WA	Sporocarp
Mortierellaceae	<i>Mortierella</i>	<i>minutissima</i>	Queets, WA	Root-tip isolate
Mortierellaceae	<i>Mortierella</i>	<i>elongata</i>	Queets, WA	Root-tip isolate
Physalacriaceae	<i>Strobilurus</i>	<i>trusillatus</i>	Edmonds, WA	Sporocarp
Hypocreaceae	<i>Trichoderma</i>	<i>spirale</i>	Queets, WA	Root-tip isolate
Hypocreaceae	<i>Trichoderma</i>	<i>atroviride</i>	Queets, WA	Root-tip isolate
Hypocreaceae	<i>Trichoderma</i>	<i>harzianum</i>	Seattle, WA	UW lab culture collection
Hypocreaceae	<i>Trichoderma</i>	<i>reesei</i>	ATCC	ATCC 13631
Boletaceae	<i>Xerocomellus</i>	<i>zelleri</i>	Queets, WA	Sporocarp

Table 2.2: GC content, Sanger sequence length, and ratios of mock community members. Ratios of mock community members are based on standardized DNA concentrations. Mock A included all species mixed at equal ratios of .28ng/ul each. In Mock B, *B. edulis*, *T. reesei*, and *T. atroviride* were added at a 1:10 ratio. In Mock C, *M. elongata* was added at 1:10 ratio.

Accession number	Taxon	GC content (%)	Sequence Length (bp)	Mock A	Mock B	Mock C
MK674163	<i>Absidia cylindrospora</i>	47.6	1,216	10	10	10
MK674159	<i>Boletus edulis</i>	50.8	1,279	10	1	10
MK673251	<i>Cantharellus formosus</i>	48.5	929	10	10	10
MK674166	<i>Debaryomyces polymorphus</i>	40.8	1,140	10	10	10
MK673252	<i>Gymnopus dryophilus</i>	42.8	947	10	10	10
MK674164	<i>Ilyonectria destructans</i>	50.4	977	10	10	10
MK674159	<i>Lycoperdon perlatum</i>	46.2	1,317	10	10	10
MK674160	<i>Morchella exuberans</i>	51.6	1,240	10	10	10
MK674168	<i>Mortierella minutissima</i>	42.3	1,270	10	10	10
MK674167	<i>Mortierella elongata</i>	42.7	1,328	10	10	1
MK674156	<i>Strobilurus trullisatus</i>	45	1,385	10	10	10
MK673510	<i>Trichoderma harzianum</i>	54.4	1,138	10	10	10
MK673509	<i>Trichoderma reesei</i>	55.3	961	10	1	10
MK674165	<i>Trichoderma spirale</i>	54.3	1,064	10	10	10
MK674162	<i>Trichoderma atroviride</i>	54.9	1,062	10	1	10
MK674157	<i>Xerocomellus zelleri</i>	52.4	1,277	10	10	10

Table 2.3: The amount and percentage of contaminant sequences in the negative control that passed through quality filtering and clustered into operational taxonomic units (OTUs). The negative control contained trace contaminants from nine of the 16 mock community members. This is presumably due to cross-contamination during PCR and library preparation steps, despite surface sterilization and stringency in protocols.

Taxa	Amount of Contaminant Sequences	Percent of Total Contaminant Sequences
<i>Lycoperdon perlatum</i>	714	16.38%
<i>Trichoderma harzianum</i>	632	14.50%
<i>Mortierella minutissima</i>	596	13.67%
<i>Trichoderma atroviride</i>	454	10.42%
<i>Absidia cylindrospora</i>	409	9.38%
<i>Gymnopus dryophilus</i>	356	8.17%
<i>Ilyonectria destructans</i>	339	7.78%
<i>Debaryomyces polymorphus</i>	554	12.71%
<i>Strobilurus trullisatus</i>	305	7.00%
Total	4359	100%

Table 2.4: Amount of sequences passed through built-in Albacore quality filtering, through subsequent filtering at Q9 using NanoFilt (De Coster *et al.* 2018), and the percent of sequences remaining. On average, 26.36% of sequences were recovered, which showed high consistency among samples with a standard deviation of .008.

Barcoded Library	Sequences Passed through Albacore	Sequences Passed at Q9	Percent Remaining
Mock A	46,216	12,657	27.39%
Mock B	59,688	15,196	25.46%
Mock C	25,761	6,832	26.52%

Table 2.5: Amount of Operational Taxonomic Units (OTUs) before and after filtering. For the mock libraries (A, B, and C). OTUs that were chimeric were removed, and OTUs that matched $\leq 95\%$ to one of the mock community members were considered to be a weak consensus match and were also removed.

Barcoded Library	Similarity Cut-off (%)	OTUs before filtering	OTUs after filtering
Mock A	92	61	41
Mock A	90	83	51
Mock A	88	67	37
Mock B	92	61	41
Mock B	90	70	48
Mock B	88	61	38
Mock C	92	53	41
Mock C	90	65	38
Mock C	88	58	36

Figures

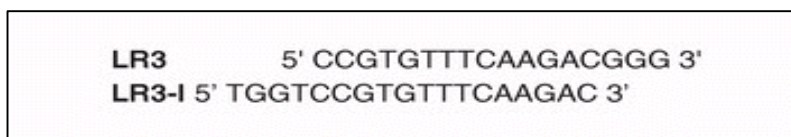


Figure 2.1: Comparison of the original LR3 primer to the LR3-I primer used for this experiment. When selecting a variant of LR3, LR3-I covered 100% of 1000 randomly selected aligned fungal sequences from Genbank. To further confirm coverage, a primer BLAST search was limited to fungi, and the ITS1f-Kyo2 and LR3-I primer pair returned 56,307 hits compared with the 56,034 hits from the ITS1f-Kyo2 and LR3 primer pair.

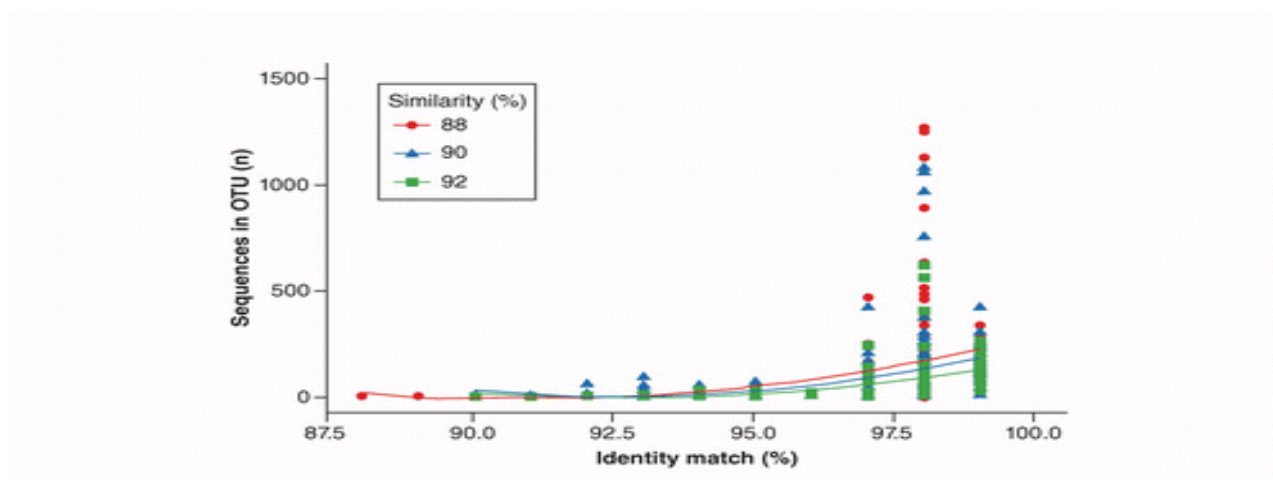


Figure 2.2: Number of sequences in each operational taxonomic unit clustered at 92, 90 and 88% sequence similarity, fitted with quadratic polynomial linear regression lines. Correlation test results for 92% are $r = 0.44$, $p \leq 0.001$, $df = 170$. At 90% are $r = 0.41$, $p = <0.001$, $df = 213$. At 88% are $r = 0.41$, $p = <0.001$, $df = 177$. OTU: Operational taxonomic unit.

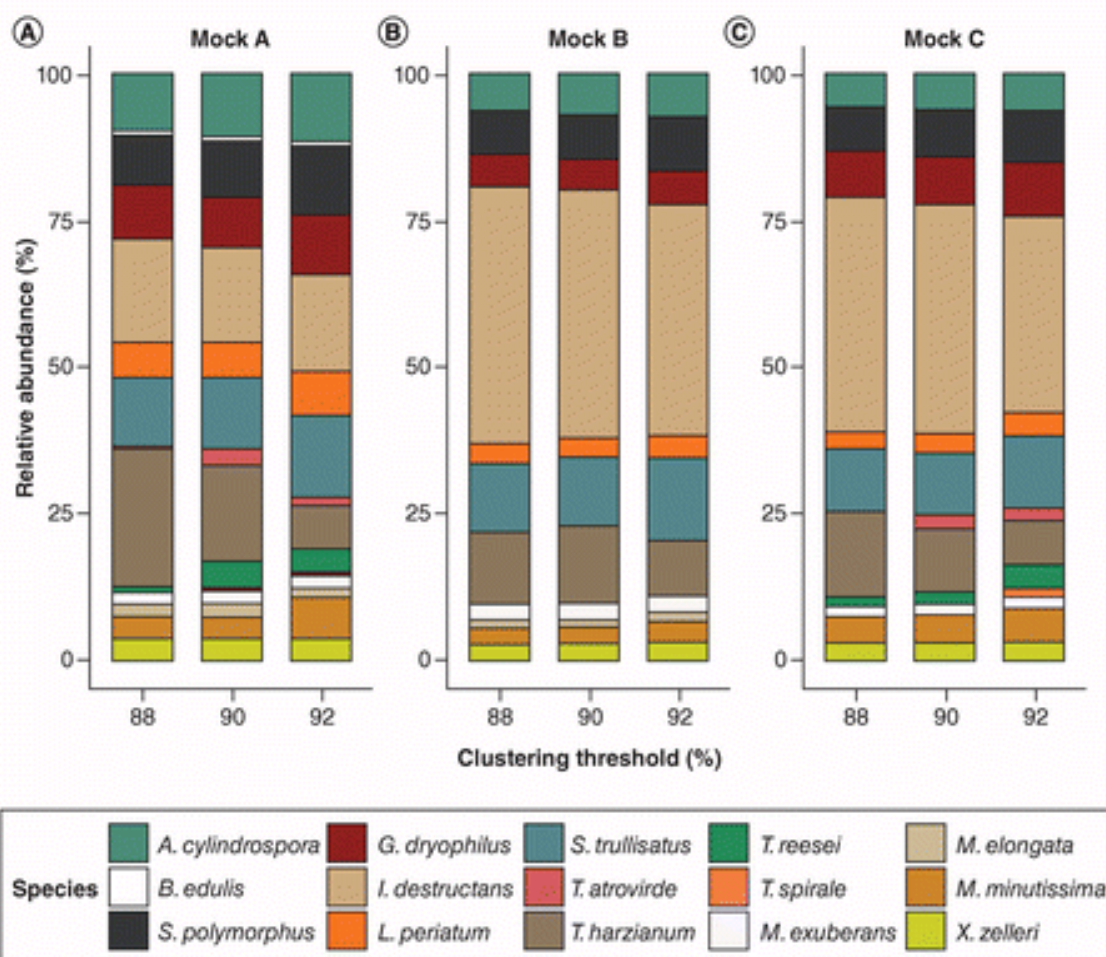


Figure 2.3: Relative abundance (%) of species in each barcoded community type (Mock A, B and C) at each respective similarity threshold (88, 90 and 92%). Mock A included the 16 taxa combined at an equal concentration of 0.028 ng/ μ l each. Mock B included 0.028 ng/ μ l 13 species along with a 1:10 dilution of *Boletus edulis*, *Trichoderma atroviride* and *T. reesei*, respectively. Mock C included 0.028 ng/ μ l 15 species along with a 1:10 dilution of *Mortierella elongata*.

Chapter 3: Root-associated fungal Community dynamics in canopy coils and forest floor soils of old-growth *Acer macrophyllum* in the temperate rainforests of Washington State.

1.1 INTRODUCTION

Fungal organisms are abundant in forest soils, fundamentally shaping both the structure and functioning of these ecosystems; including, but not limited to, their ability to move, transform, and store carbon (C) and nutrients (Treseder and Holden 2013). There is a broad diversity of fungi (e.g., ecto- and endo-mycorrhiza) that can engage in nutritional mutualisms with plant roots, enhancing the growth and fitness of the plant (Begum *et al.* 2019, Unuk *et al.* 2019, Barea *et al.* 2002), while helping them adapt to a changing climate (Bennett and Classen 2020). These plant-fungal mutualisms are common, dating back to when plants first evolved on the terrestrial landscape; today ~90% of land plants form mycorrhizal associations (Smith and Read 2008, van der Heijden *et al.* 2015). Root fungal endophytes are also globally prevalent and can indirectly benefit plants by increasing their resistance to environmental stressors (Molina-Montenegro *et al.* 2020, Brundrett 2006), and studies have demonstrated that a diversity of root-associated endophytes have mycorrhizal attributes with their plant host (Jumpponen 2001, Hoysted *et al.* 2018, Hoysted *et al.* 2019, Terhonen *et al.* 2019).

Advances in sequencing technologies over the last two decades have enabled researchers to focus more comprehensively on biotic interactions and abiotic conditions that drive fungal diversity, community dynamics, fungal response to climate change, and their role in ecosystem function; as a result, fungi are increasingly recognized as an integral part of the soil C cycle, ecosystem-level nutrient dynamics, and how plants respond to climatic extremes (Schimel 2013, Treseder and Holden 2013, Sahu *et al.* 2017). Despite this knowledge, the diversity of root-associated fungi in forested ecosystems remain widely unexplored at local and temporal scales.

This is in part because many existing studies focus on bulk soil community composition and spatial sampling to explore the mechanisms that drive community assembly (e.g., Beck *et al.* 2015), and also because our current understanding remains limited by geographical study bias (Bennet and Classen 2020).

This especially holds true for the old-growth temperate rainforests of western Washington, which represent some of the longest-living carbon stores on earth (Urrutia-Jalabert *et al.* 2015) and host some the region's highest biodiversity (Brandt *et al.* 2014, Franklin 1988). The high storage of carbon and vegetation speciation in these forests are linked to fluvial disturbance regimes and the presence of large old-growth trees (Fjeldsa and Lovett 1997, Van Pelt *et al.* 2006). Historically, forests with these characteristics are temporally stable and have trivial or slow responses to transient disturbances (Thom *et al.* 2017). The resulting high structural variability, rainfall regimes, and associated flora, fauna, and fungi (Kuhar *et al.* 2018) are conducive for a wide array of niches for biotic relationships to form (Frank *et al.* 2009); including, but not limited to, plant-fungal interactions.

There are some fundamental studies on fungal diversity in Washington's old-growth rainforests. For example, large woody debris is a defining characteristic of these old-growth ecosystems, and a study identified 88 fungal taxa on decaying logs (Edmonds and Lebo 1998). A temporal study collected 150 ectomycorrhizal species over a two-year period from a ~2 ha sample plot, and reported that ectomycorrhizal species abundance was partially dependent on moisture gradients (O'Dell *et al.* 2000). Further, a spatial study demonstrated that epigeous macrofungi differed between two old-growth stands with similar tree species composition, due to soil moisture and nitrogen (N) conditions (Trudell and Edmonds 2004). These studies demonstrate that these old-growth temperate rainforests have high fungal richness and diversity,

but local studies on root-associated fungal communities remain extremely limited; especially when considering fungi that are more enigmatic (e.g., arbuscular mycorrhiza) or ambiguous (e.g., root endophytes) in nature.

Most of the existing studies on fungal diversity in these forests focus on ectomycorrhizal and saprophytic fungi, plausibly because many of these old-growth stands are dominated by large woody conifers (e.g., Sitka spruce [*Picea sitchensis* (Bong.) Carrière], western hemlock [*Tsuga heterophylla* (Raf) Sarg.], and Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] that are obligate ectomycorrhizal associates. Historically, ectomycorrhizal and arbuscular mycorrhizal root-fungal mutualisms have been explored separately and root-associated endophytes have been widely overlooked (Toju and Sato 2018). However, many of the old-growth riparian stands have mixed tree dominance with an established community of old-growth bigleaf maple (*Acer macrophyllum* Pursh.), which can account for up to 34% of total forest cover (Canfield 1941).

Species within the *Acer* genus are known to associate with arbuscular mycorrhiza (Helgason *et al.* 2014, Brundrett *et al.* 1990), and the understory is often dominated by salal (*Gaultheria Shallon*), salmonberry (*Rubus spectabilis*), sword fern [*Polystichum munitum* (Kaulf.) C. Presl], and vine maple (*Acer circinatum* Pursh.). Salal associates with ericoid mycorrhiza (Xiao and Berch 1995), and the other species associate with arbuscular mycorrhiza (Berch *et al.* 1988, Brundrett *et al.* 1990, Hocking and Reynolds 2011).

It becomes important to research root-associated community dynamics in these ecosystems, not only from a below ground perspective, but also from an above-ground perspective. This is because a defining characteristic of these old-growth rainforests is the presence of thick organic mats of canopy soil on tree branches. Canopy soils are categorized as arboreal Histosols (Nadkarni 2002, Enloe *et al.* 2006) and contribute unique structural and functional components

where they prevail. For example, some trees host copious amounts of canopy soil on their branches and grow extensive adventitious rooting networks. Nadkarni (1981) observed arbuscular mycorrhizal structures in adventitious roots of bigleaf maple in Washington's temperate rainforests and Orlovich *et al.* (2013) sequenced ectomycorrhizal species in adventitious roots of silver beech (*Nothofagus menziesii*) in a New Zealand temperate rainforest. These studies demonstrate that root-fungal interactions are not limited to the forest floor soil environment.

In Washington's temperate rainforests, old-growth bigleaf maples can host copious amounts of canopy soil (Fig 3.1a) and they form extensive adventitious rooting networks (Fig. 3.1b). Due to the branching structure and deciduous nature (i.e., high litterfall return [Tejo *et al.* 2015]) of bigleaf maples, canopy soils often reach depths >35 cm (Tejo *et al.* 2014). In these forests, they were found to contribute as much as 20% and 25% to the total nitrogen (N) and carbon (C) storage pools (Tejo *et al.* 2014). Canopy soils also have higher concentrations of plant available N and phosphorus (P) than forest floor soils (see Ch. 4). Arbuscular mycorrhizal vesicles were briefly reported in adventitious roots of bigleaf maple in this region (Nadkarni 1981). However, the root-associated fungal community diversity and dynamics in these old-growth temperate rainforests remains widely unexplored in both canopy and forest floor soil environments. It becomes timely to explore root-associated fungal community diversity on a temporal scale, as these forests face wetter winters and prolonged summer drought because root-associated fungal communities can differ in how they respond to seasonal extremes (Deveautour *et al.* 2018, Ficken and Warren 2019), which in return can affect processes involved in C and nutrient cycling.

In order to understand how these plant-fungal interactions in canopy soils may be contributing to host tree health and overall ecosystem function, fundamental information on the

extent and diversity of these relationships is needed. Additionally, research needs to explore how these communities temporally respond to change. Therefore, this study aimed to apprehend the extent of fungal root colonization, diversity of root associated fungi in canopy and forest floor soils of old-growth bigleaf maple trees, and further explore if root-associated fungal community composition is impacted by higher or lower rainfall inputs.

We chose to focus this study on old-growth bigleaf maple trees because although old-growth Sitka spruce can harbor more canopy soil biomass due to size and age (Van Pelt 2012, unpublished data), bigleaf maples have much thicker mats (Tejo *et al.* 2014) and form more extensive adventitious rooting networks. Also, studies on arbuscular mycorrhizal associated trees are often overlooked in these ecosystems. Further, bigleaf maple canopy soils are excellent testbeds for *in situ* soil studies because 1) they are not confounded by the different physical and chemical mineral soil layers and the extensive root grafting that is found at the forest floor level; and 2) they are amenable to experimental manipulations due to their large branches and isolated organic soil volume.

To conduct this study, two old-growth riparian rainforest stands in the Hoh and Queets watersheds were selected (Fig. 1.2). Six trees in each stand (n = 12 trees) were rigged for canopy access. Prior to implementing the rainfall experimental manipulation in April 2018, adventitious roots and terrestrial roots were sampled from all 12 trees for clearing and staining to determine the extent of fungal colonization and examine fungal structures. Immediately following baseline sampling, custom designed experimental structures that either block roughly half of the rainfall or increased it by two-fold were fixed to branches of four of the twelve trees, respectively, leaving four of the branches at ambient rainfall conditions. Adventitious canopy roots were collected seasonally (excluding the winter dormant season) from the manipulated canopy soil

area in May 2018, August 2018, November 2018, and May 2019. During all sampling periods, forest floor roots at were also collected from the 12 trees.

The objectives of this study were to: 1) compare the percent of fungal colonization in adventitious canopy roots and forest floor soil roots; 2) compare root-associated fungal community diversity in canopy soils and forest floor soils at ambient conditions over seasonal sampling periods between the two sites; and 3) implement a rainfall experiment and compare root-associated fungal community diversity in canopy soils at ambient conditions to canopy soils manipulated to receive increased or decreased rainfall amounts.

It was hypothesized that: (H1) canopy soils have a lower percent of fungi colonizing the fine-roots because canopy soils are physically separated from the forest floor soils, which may limit dispersal (see Looby *et al.* 2017); (H2) the root-associated fungal communities in canopy soils at ambient conditions are distinct from those associating with forest floor roots because they are two different soil environments that experience varying microclimates (Dangerfield *et al.* 2017) and nutrient fluxes (see Ch. 4); (H3) root-associated fungal community composition in canopy soil environments will shift in response to the different rainfall treatments reflecting their sensitivity to drought and extreme rainfall (Barnes *et al.* 2018, Lagueux *et al.* 2021); (H4) there will be seasonal changes in root-associated fungal community composition in ambient soil environments and across canopy soil treatments because these communities are partially shaped by edaphic properties (Sikes *et al.* 2014), and some of these properties are dependent on seasonal climate; and (H5) root-associated fungal community composition in ambient soil environments and across canopy soil treatments will be different between the two sampling sites because although they have similar species composition, there are other factors such as soil moisture and

nutrient gradients that can shape these communities (Trudell and Edmonds 2004, McBurney *et al.* 2017).

3.2 MATERIALS AND METHODS

3.2.1 Study area

This study was conducted from April 2018 to May 2019. The study sites are located in two old-growth riparian forests. One stand is located in the Queets River Watershed (47.608 N, 124.051 W) on the westward side of the Olympic National Park and has been described as one of the most structurally complex forests on the Pacific Northwest Coast (Van Pelt *et al.* 2006) [Fig. 3.2a]. The other site is located ~29 km north in the Hoh River Watershed (47.822 N, 124.205 W) on land owned by The Nature Conservancy and is categorized as a highly productive old-growth forest (Tuo 2020, unpublished data) [Fig. 3.2b]. These areas are known for their cool, wet winters, and warm dry summers (Rohrig and Ulrich, 1991). Annual rainfall averages between 240-300 cm/yr, with a majority of rainfall happening between October and June (O'Keefe and Naiman 2006).

The dominant old-growth conifer and hardwood species are Sitka spruce and bigleaf maple. Red alder (*Alnus rubra* Bong.), Douglas-fir, and western hemlock also occur in these stands. The dominant understory species, accompanied by large woody debris, are salmonberry, redwood sorrel (*Oxalis oregana* Nutt.), sword fern, and vine maple. The dominant bryophytes established in the canopy include hanging moss (*Antitrichia curtispindula* [Hedw.] Brid.) and tree moss (*Isoetecium* spp.), which co-occur with the dominant vascular epiphytes including Oregon spikemoss (*Selaginella oregana* D.C. Eaton) and licorice fern (*Polypodium glycyrrhiza*). Lichens (e.g., N-fixing *Lobaria* spp.) are also common in the canopy environment.

3.2.2 Tree selection, rigging, and canopy access

Due to the hazards that are involved with tree climbing, it was impossible to have a completely randomized design when selecting the trees to climb for this study. In order to randomize selection as much as possible, all bigleaf maples within the old-growth study sites were assessed for structural integrity. Out of all the trees that passed safety criteria (e.g., no major leaning or slanted codominant splits), six were randomly selected at each site. Each tree was rigged and accessed using single-rope climbing techniques (Perry 1978), and a line of nylon cord remained in the trees for the duration of the experiment to allow for repeated access.

3.2.3 Root sampling prior to experimental implementation

In April 2018, fine roots were sampled from both canopy and forest floor soils to determine the percent of fungi colonizing the roots. To sample roots from canopy soils, a small saw and trowel were used to gently loosen the live epiphytic mats to access the canopy soil underneath. The presence of adventitious roots was confirmed, and three mats of canopy soil (20 cm x 20cm x 25 cm) were collected from a random location on each branch of the 12 trees, respectively.

To confirm that we were sampling forest floor roots from the same host tree while avoiding the collection of roots from neighboring tree species, large lateral roots of the host tree were located and manually explored with a trowel to locate fine roots. Once located, a surface area with dimension 20 cm x 20 cm was delineated and soil with roots intact were sampled to a depth of 25 cm. This was repeated three times at each tree. Canopy soils are organic, and due to their fibrous and matted nature, taking a soil core is not feasible. While fixed volume soil cores are typically used to sample bulk soil and roots, to confirm that we were sampling bigleaf maple

roots and maintain a consistent sampling technique between soil types, we opted to sample forest floor soil fine roots in a similar manner. Triplicate samples were combined in the same bag, labelled, placed in a cooler, and transported back to the University of Washington campus. Soils were stored at 4°C for no more than two days prior to separating out the fine roots for clearing and staining. Root separation techniques are further discussed in Section 3.2.5.

3.2.4 Experimental manipulations

In order to manipulate rainfall inputs to canopy soils, four trees had canopy plots used as ambient controls, four trees had a custom designed roof fixed to a branch to create a roof - interception structure (Fig. 3.3a), and four had a custom designed gravitational irrigation system (Fig 3.3b). Experimental structures were designed to manipulate a fixed surface area (1 m²) on each selected branch. The structures either (i) diverted roughly one-half of the throughfall from the branch, i.e., decreasing rainfall input by half; or (ii) increased rainfall input to twice what would have normally fallen on a branch. The experimental structures were attached to the branch in April 2018 and were allowed to equilibrate for one month before root sampling. The structures were left in place for the entire duration of the experiment (May 2018-June 2019).

3.2.5 Root separation/collection

Due to the matted nature of canopy soils, standard sieving and washing methods are not applicable to separate and collect fine roots. Fine roots were obtained by soaking each sample in DI H₂O to loosen the mats of canopy soil, and then forceps and a dissecting scope (Leica) were used to tease apart and separate epiphytic rhizoids, rhizomes, and decomposed organic litter from the adventitious bigleaf maple roots. Adventitious roots were then carefully removed from the

soil mats and placed in a sterilized petri dish with 50% ethanol solution for further cleaning. Forest floor fine roots were collected by rinsing each combined triplicate sample in a 50-mm sieve with DI H₂O. Fine roots were then placed in a sterilized petri dish in 50% ethanol solution for further cleaning. To remove the remainder of adhered debris with minimal damage to any extraradical hyphae or the roots, each sample was further rinsed and cleaned in 50% ethanol solution under a dissecting scope. During the root dissection and cleaning process, random subsamples of adventitious and forest floor roots were set aside for confocal microscopy.

3.2.6 Staining and clearing

All roots were cleared using a modified method based on the KOH approach (Philips and Hayman 1970). Once roots were separated from canopy and forest floor soils and rinsed thoroughly, they were placed in tissue cassettes. The cassettes were placed in labelled jars of 8.5% KOH and were heated at 60°C for up to 30 minutes. Following the heating step, the original KOH was discarded and replaced with fresh 8.5% KOH. Roots were left in the KOH solution for up to 3 days, and KOH was periodically changed when the solution turned deep red, orange, or yellow. Once the roots were cleared, the cassettes were rinsed thoroughly and placed in 1.5% NaOCl for up to 1 hour to remove any remaining color. Following the bleach, roots were rinsed thoroughly and soaked in a 2.5% HCl solution for 10 minutes to facilitate proper penetration and binding of the stains.

3.2.7 Root staining for percent colonization

Following the HCl soak, the cassettes were placed in labelled beakers respective to sample ID. Lactoglycerol was prepared from a 1:1:1 solution of DI H₂O, Lactic Acid, and Glycerol.

Trypan blue (TB) was added to create a .05% stain solution. The TB Lactoglycerol was added to the beakers, and the cassettes were soaked for 24 hours. Follow the 24-hour staining period, cassettes were rinsed in DI water and subsequently soaked in Lactoglycerol solution for up to 24 hours to remove excess TB. The stained samples were stored at 4°C until prepared for grid-intersect counting.

3.2.8 Root staining for confocal microscopy

Following the clearing protocol, the roots that were set aside were prepared for confocal microscopy. These roots were mounted in 5% agarose, sectioned out with a razor blade, and 150µm cross- and lateral sections were cut using a vibratome. The sections were subsequently soaked for 2-3 hours in a 1:200 dilution (PBS, pH 7.4) of Alexa Fluor Wheat Germ Agglutinin conjugate. Sections were located under a dissecting scope and mounted in PBS on a glass slide. Samples were then viewed on a Leica confocal microscope using a 488-nm argon laser set for excitation and fluorescence detection between 490 and 620 nm (similar to techniques used in Rath *et al.* 2014). Scans were saved for further observation.

3.2.9 Percent fungal colonization

Percent fungal colonization was determined using a modified version of the grid-line intersect method (Giovannetti and Mosse 1980). This modified approach tallied both the stained fungal structures and also any pigmented fungal structures (e.g., dark septate endophytes [DSEs]). In brief, a 9cm petri dish with 1 cm² gridlines was used for counting stained or pigmented structures. To perform these counts, roots from each sample were cut into ~1cm segments. A very minimal amount of lactoglycerol was added to keep the roots from drying out,

but not enough to allow them to move freely during counting. Then 50 adventitious canopy and forest floor roots from each sample were randomly selected, totaling 100 roots per tree. A ‘fungal structure present’ count was taken each time a stained fungal structure or pigmented fungal structure was encountered, and a general count was taken for each root-grid intersection whether a fungal structure was present or not. Each plate was rearranged and examined 3 times to calculate an average. A total of 1,200 roots were observed. For each sample, colonization was calculated by dividing the number of stained roots by the total number of root-grid intersections.

3.2.10 DNA extraction

Following the initial observation of fungal structures and determination of % fungal colonization, adventitious canopy roots and forest floor roots were sampled seasonally from the 12 trees in May 2018, August 2018, November 2018, and again in May 2019. Roots from canopy soils under experimental rainfall conditions were sampled from within the manipulated area following the same root sampling procedure discussed in Section 3.2.3. Soils with roots intact were stored at 4°C for no longer than two days prior to root dissection following methods discussed in Section 3.2.5. Once roots were cleaned, they were immediately frozen and subsequently lyophilized for DNA extraction. All DNA was extracted using an optimized protocol (see Appendix A) for the Synergy™ 2.0 Plant DNA Extraction kit (OPS Diagnostics).

3.2.11 High throughput sequencing on two platforms

Following extractions, fungal DNA was amplified and subsequently barcoded and sequenced on two high-throughput platforms. First, the MinION Nanopore sequencer was used following the methods thoroughly discussed in Ch. 2 (Mafune *et al.* 2019). Briefly, Fungal DNA

was amplified using the forward primer ITS1F-Kyo2 (5' TAG AGG AAG TAA AAG TCG TAA 3' [Toju *et al.*, 2012], and the reverse primer LR3-i (5' CCG TGT TTC AAG ACG GG 3') with MinION tags attached. Polymerase Chain Reaction (PCR) was performed on each sample of extracted DNA in triplicate form in 20 μ l reactions using PrimeStar GXL Taq Polymerase Master Mix (TaKaRa Bio Inc). The conditions for PCR were: 5 cycles of denaturation at 98°C for 10 secs, annealing at 55°C for 30 secs, ramping up at 2°C/sec to 68°C for 2-minute extension, followed by 28 cycles at 98°C for 10 seconds, 55°C for 20 seconds, and 68°C for 90 seconds. All PCRs included a negative control.

Once completed, triplicate reactions were pooled together respective to sample and purified with AMPure magnetic beads (Beckman Coulter) and resuspended in 20 μ l molecular-grade H₂O. Concentrations were determined using the Qubit Fluorimeter 2.0 (Thermo-Fischer Scientific). Using the barcode expansion pack (EXP-PBC-096), each sample was assigned a respective barcode which was attached to the tagged primers using LongAmp Taq 2x MasterMix (New England Biolabs), per ONT's PCR barcoding protocol. The Ligation Sequencing Kit (SQK-LSK109) was used to complete the library preparation and samples were pooled together in equal amounts to total 1 μ g, as specified in ONT's protocol. For the MinION approach, samples were sequenced on separate flowcells respective to the season they were collected. In other words, 24 barcoded samples from May 2018 were sequenced on one flowcell, the 24 samples from August 2018 were sequenced on another flowcell, etc. All sequence runs included a negative control and the even mock community control used in Bakker (2018).

To supplement the sequence data from the MinION sequence runs, all gDNA samples and the mock community were sent to the University of Oregon's Genomics and Cell Characterization Core Facility (GCF3) for Illumina sequencing. Briefly, the sequences were

amplified using the modified Smith & Peay (2014) ITS1F and ITS2 primer pair (White et al 1990, Gardes and Bruns 1993). The libraries were prepared using the V3 Reagent Kit (Illumina), and sequenced on an Illumina MiSeq system (2 x 300 bp). Negative controls and the Bakker (2018) mock community were included.

3.2.12 Downstream processing

The MinION sequence data were processed using methods discussed in Mafune *et al.* (2019) with a few pipeline modifications. First, the raw sequence data were demultiplexed and sorted by respective barcode using Guppy (v. 3.4.4). NanoFilt (De Coster *et al.*, 2018) was used to remove sequences with a Q-score <10. Sequences were then aligned using the -Gins1 model in MAFFT (Kato & Standley, 2013), and distance matrices were calculated for each alignment in PHYLIP (Felsenstein, 2005). Mothur (Schloss *et al.*, 2009) was used for *de novo* OTU clustering at 95% sequence similarity using OptiClust (Wescott & Schloss, 2017). The rest of the downstream analysis was identical to the methods used in Mafune *et al.* (2019). Briefly, a python script sorted sequences by respective OTU, sequences in each OTU were aligned in MAFFT, and another python script calculated a consensus sequence from the alignment. Consensus sequences were then manually assigned taxonomic identity using the National Center for Biotechnology Information's (NCBI) and the UNITE (Nilsson *et al.* 2019) databases.

The Illumina data were demultiplexed, sorted by barcode, and processed through the Usearch/UNOISE pipeline [v. 11.0.667 (Edgar 2010)]. The *-fastq_mergepairs* command was used to assemble forward and reverse paired-end sequences. The *-fastq_filter* command was used to quality filter reads at a max error of 1, which is the expected number of errors in a read (Edgar and Flyvbjerg 2015). Subsequently, the *-fastx-uniques* command was used to de-replicate quality

filtered reads and the sequences were clustered into OTUs at 97% similarity using the -*cluster_otus* command. Sequences were denoised, chimeras were filtered out, and zero-radius OTUs (zOTUs) were generated. Any zOTUs with <5 sequences were removed. Identities were assigned taxonomic identity using the UNITE (Nilsson *et al.* 2019) database, and taxonomic assignments were further inspected using the NCBI database.

The sequences generated from both platforms were identified at the kingdom, phylum, class, order, family, genus, and species level at 88%, 90%, 92%, 93%, 94%, 95%, and 97%, respectively. If information in the databases was unavailable for any of these taxonomic levels, 'Unknown' was assigned. Non-fungal zOTUs and OTUs were filtered out during assignment.

3.2.13 Statistics

The fungal colonization, MinION, and Illumina data tables were all imported into Rstudio, and all subsequent analyses were conducted using R software (R Core Team, 2018). The significance level was set at $\alpha = 0.05$ for all statistical tests. For the fungal colonization dataset, paired t-tests were used to test if there was any significant difference between percent fungal colonization in adventitious canopy roots *vs.* forest floor roots between the two research sites.

The mock community data from both platforms were imported into Rstudio to visualize and determine if the MinION surpassed the Illumina MiSeq's ability to recover and identify mock community members to genus and species level. Subsequently, taxonomy, OTU, and metadata for the MinION and Illumina datasets were then imported into Rstudio and two Phyloseq objects (Mcmurdie and Homes 2014) were generated from the Illumina and MinION sequence data. Data from the Illumina phyloseq object were subsetted for further analyses. The first subset of data was fungal communities identified in the canopy and forest floor at ambient conditions only.

The second subset was of the fungal communities identified from all canopy plots, including root-associated communities from ambient and manipulated canopy branches.

Observed OTU richness and alpha diversity (Shannon's and Simpson's diversity indices) were estimated. An Analysis of Variance (ANOVA) was executed on the diversity measures from the Illumina dataset to further test if: (1) richness and alpha diversity measures were significantly different between season and treatment at the canopy level; and (2) richness and alpha diversity measures were significantly different in canopy and forest floor environments at ambient conditions. Prior to running the ANOVA, the data were confirmed to fit normality and variance assumptions with Shapiro's test for normality and Levene's test for equal variances. After the ANOVAs were executed, Tukey's HSD posthoc tests were run to determine differences among groups occurred.

Illumina species counts were then transformed to relative abundance and Bray-Curtis dissimilarity matrices were calculated for each subset of data using the 'vegdist' function in the vegan package (Oksanen *et al.* 2018). Permutational analysis of variance (PERMANOVA) using the 'adonis2' function from the vegan package (Oksanen *et al.* 2018) was executed on the ambient data to test if season (summer, fall 2018, spring 2018/2019 combined), type of soil (canopy vs. forest floor), site (Hoh vs. Queets), and the interactions of these factors had any significant effect on fungal community composition. Another PERMANOVA was carried out on the canopy soil data to test if seasonal sampling period, treatment (2x, 1/2x, and ambient amounts of rainfall), site, and the interactions of these factors had any significant effect on fungal community composition. PERMANOVA's were performed with 9,999 permutations. A principal coordinate analysis (PCoA) based on Bray-Curtis distance matrices was also performed on both subsets of data.

MinION data were further explored at the genus and species level to determine what fungal species were more abundant between soil environments and across canopy treatments. An indicator species analysis (Dufrene and Legendre 1997) using the 'multipatt' function from the indicpecies package (De Cáceres 2013) was further implemented on observed species richness from the MinION dataset to determine if there were certain fungal species associating with canopy and forest floor soils and across canopy soil treatments. Species with indicator values > 0.50 and $p\text{-value} \leq 0.05$ were considered significant to the respective grouping variable.

3.3 RESULTS

3.3.1 Fungal Colonization

Roots that were examined for counts and under the confocal microscope demonstrated that there was an abundant amount of hyphal structures occurring in both canopy (Fig. 3.4) and forest floor roots (Fig 3.5). Further, the confocal scans revealed that adventitious canopy roots had extensive intraradical hyphae. These hyphal structures included intracellular and intercellular hyphae within and among the cortical cells (Fig. 3.6a), which included arbuscules and intracellular dense coils (Fig 3.6b, c). Intraradical hyphae were also observed within epidermal cells of canopy roots (Fig 3.4, 3.6b) and in root hairs, though root hairs were not always present on randomly sampled roots (Fig. 3.7).

Percent fungal colonization in adventitious canopy roots and forest floor roots of the 12 old-growth maple trees ranged from 24-95% and 50-91%, respectively. Fungal colonization was slightly lower in canopy adventitious and forest floor roots sampled from the Hoh Rainforest ($52.3\% \pm 7.1$ and $64.7\% \pm 2.8$, respectively) when compared to canopy adventitious and forest floor roots sampled from the Queets Rainforest ($60.7\% \pm 8.4$ and $65.5\% \pm 7.1$, respectively).

However, there were no significant differences between the percent of fungi colonizing adventitious canopy ($p = 0.47$) and forest floor ($p = 0.92$) roots between the two sites (Fig 3.8). When data from both sites were combined, fungal colonization in canopy adventitious roots ($56.5\% \pm 5.4$) was slightly lower than forest floor roots ($65.1\% \pm 3.6$); but overall, there was no significant difference between bigleaf maple canopy soil adventitious roots and forest floor roots ($p = 0.20$).

3.3.2 Sequence output

MinION sequence runs were performed on four separate flowcells respective to sampling period (i.e., DNA from 24 barcoded root tips from one sampling period, a mock community, and a negative control were sequenced on one flowcell). The sequence run for samples collected in spring 2018, summer, fall 2018, and spring 2019 generated 19,035,742; 12,694,123; 8,762,323; and 17,244,232 raw reads. After basecalling and demultiplexing, 15,008,617; 7,294,305; 1,821,358; and 7,426,353 raw reads from spring 2018, summer, fall 2018, and spring 2019 were filtered into the Guppy 'pass' folder respective to their barcode ID. After quality filtering and chimera removal, a majority of samples retained enough sequences to randomly subset 6800 sequences for the alignment step. The exceptions were two of the negative controls and one sample from spring 2018, which only had 2411 sequences remaining post quality filtering.

For the Illumina MiSeq run, all samples were sequenced on one flowcell. The sequencing run produced 24,556,242 raw reads. After demultiplexing, merging, chimera removal, and quality filtering 15,654,843 reads remained.

3.3.3 Mock community recovery

The MinION outperformed Illumina on mock community recovery at the genus level, with the exception of the MinION mock community sequenced during the fall sampling season (Fig. 3.9). This particular sequence run recovered one less mock community member than Illumina. Both sequencing technologies failed to recover *Ustilago maydis* and *Rhizomucor miehei*. The MinION consistently recovered the mock community genus *Neosartorya* and Illumina did not. In contrast, Illumina successfully recovered *Rhizophagus* from the mock community and MinION did not. Nevertheless, the MinION did successfully recover *Rhizophagus* and other members of Glomeromycota from the root samples. MinION also did not consistently recover *Alternaria alternata*.

At the species level, MinION outperformed Illumina. The mock community consisted of 19 genera. Each genus was represented by one species except *Fusarium*, which was represented by three species. The MinION resolved 17 of the 19 genera to the correct species level, including all three *Fusarium* species, and did not misidentify species. Illumina resolved 14 of the 19 genera, and only ten of those were consistently identified to the correct species level. It also did not successfully separate the three *Fusarium* species. Therefore, the MinION sequence data were used for genus and species-level inquiries.

Both platforms demonstrated comparable biases when sequencing genera from the evenly mixed mock community. On both platforms, the relative abundances of *Naganishia* and *Fusarium* were consistently over-estimated. The MinION recovered *Chytriomycetes*, *Penicillium*, *Saiotella*, and *Saccharomyces* at higher abundances than Illumina MiSeq. Illumina did successfully recover *Asperilligus*, *Chytriomycetes*, *Penicillium*, *Rhizophagus*, *Saccharomyces*, and

Saiotella from the even mock community, but the representation was <1%, whereas the MinION recovered these species (excluding *Rhizophagus*) at higher relative abundances.

3.3.4 Root-associated fungal community diversity in canopy and forest floor soils at ambient conditions and between sites

Observed OTU richness of root-associated fungal communities was significantly different between adventitious canopy roots and forest floor roots ($p < 0.05$). Seasonality also had an effect on observed OTU richness (Table 3.1), and the posthoc analysis demonstrated the significant differences occurred between spring and fall and summer and fall ($p < 0.05$). Both Shannon and Simpson diversity measures were also significantly different between canopy adventitious and forest floor root-associated communities. Observed richness and both diversity indices were higher in forest floor rooting systems ($p < 0.05$, Fig. 3.10).

When exploring beta diversity among samples (Table 3.2), both soil type and site had a significant effect on fungal community composition ($p < 0.01$). The interaction among soil type and site was also significant ($p < 0.01$), demonstrating that the composition of root associated fungal communities was not solely dependent on the two individual factors, but on the combined effects of soil type and season. The PCoA analyses on Bray-Curtis distances (Fig. 3.11) demonstrated clear clustering of root-associated fungal communities between the canopy and forest floor soil environment. Further, there was more distinct clustering in the canopy soil environment that differentiated the two sites. Seasonality did not impact root-associated fungal community diversity in either soil environment at ambient conditions.

Different indicator species were identified associating with roots from either the canopy soil or the forest floor soil environment at ambient conditions (Table 3.3). Root associates indicative

of the canopy soil environment were *Cryptosporiopsis ericae*, *Pezicula radicicola*, *Athelopsis* sp., *Helotiales* sp., *Hyphodiscus* sp., *Mycena* sp., *Neonectria* sp., and *Rhizophagus* sp. ($p \leq 0.05$). At the forest floor level, an unidentified species from Basidiomycota and *Agrocybe erebia* were indicators ($p \leq 0.05$). Genus and species-level diversity in canopy soils and forest floor soils at ambient conditions is depicted in Table 3.4, supplemented by Fig. 3.12.

3.3.5 Root-associated fungal communities in canopy soils under experimental manipulation and between sites

When testing for differences in observed OTU richness and alpha diversity across canopy soil experimental manipulations, treatment had no significant effect ($p > 0.05$). However, seasonality did have an effect on observed richness ($p \leq 0.01$, Table 3.5). The biggest differences in richness occurred between the spring and fall and summer and fall sampling periods ($p \leq 0.01$, Fig. 3.13).

When exploring beta diversity under canopy soil experimental manipulations; treatment, season, and site all had a significant effect on root-associated fungal communities ($p \leq 0.01$, Table 3.6). When exploring the interactions between the above factors, there was a significant interaction between treatment and site ($p < 0.01$), demonstrating that the effect that treatment had on fungal communities depended on site. The PCoA analyses on Bray-Curtis distances (Fig. 3.14) did demonstrate some clustering of root-associated fungal communities among the two treatments and ambient conditions, as well as between sites.

Indicator species were identified across the experimental treatments in the canopy soil environment. The genus *Basidiodendron* and an unidentified species from the family *Hyaloscyphaceae* were indicative of the irrigation treatment ($p \leq 0.01$). *Basidiodendron* was also

only identified to be an associate with adventitious canopy roots in the Queets rainforest. *Pezicula radiciala* was an indicator for both ambient canopy soils and canopy soils under the irrigation treatment ($p \leq 0.01$). *Rhizophagus intraradices* was an indicator for the roof-interception treatment ($p \leq 0.01$). Genus and species-level diversity in canopy soils at ambient conditions and across treatments is depicted in Table 3.7, supplemented by Fig. 3.12.

3.3.6 Obligate mutualists

Both canopy and forest floor rooting systems were found to associate with a diversity of arbuscular mycorrhiza. The MinION identified seven genera of arbuscular mycorrhiza, which matched most closely to *Archaeospora*, *Diversispora*, *Dominikia*, *Entrophosphora*, *Glomus*, *Rhizoglomus*, and *Rhizophagus*. In canopy soil adventitious rooting systems, species from *Rhizophagus* and *Glomus* had the highest relative abundances, followed by *Rhizoglomus* and *Dominikia*. In forest floor rooting systems *Dominikia* and *Rhizophagus* were most common, followed by *Glomus* and *Rhizoglomus*. *Archaeospora*, *Diversispora*, and *Entrophosphora* were identified, but at negligible relative abundances and only *Diversispora* was identified in both canopy and forest floor rooting systems (Figure 3.12).

When exploring species-level resolution, the MinION identified *Rhizophagus irregularis* and *R. intraradices* to species level in both canopy and forest floor soils. Though the genus *Glomus* was identified in both rooting systems, species level was only resolved in forest floor rooting networks. These identities matched most closely to *Glomus macrocarpum* and *G. tetrastratosum*.

3.3.7 Dark septate endophytes and other potential symbionts

There was a diversity of dark septate endophytes associating with roots in both canopy and forest floor environments. Notably, the genus *Cryptosporiopsis* was found in canopy soils at higher relative abundances in the fall sampling season and under the roof-interception treatments (Table 3.7). It was also found associating with forest floor roots, particularly in the summer sampling season (Table 3.4). The MinION was able to resolve some of the *Cryptosporiopsis* identities to the species level of *C. ericae*. *Meliniomyces* was found in both soil environments, but at higher relative abundances across canopy soil samples. Due to suspiciously long sequence reads in the MinION dataset, *Meliniomyces* inferences were pulled from the Illumina dataset. It was present in adventitious canopy roots across all experimental manipulations during at least one of the sampling periods, with the highest relative abundance under the roof-interception treatment.

Hyphodiscus, *Oidiodendron*, *Mycena*, *Neonectria*, *Spirosphaera*, and *Tricladium* were commonly recovered genera (Table 3.4 and Table 3.7). *Hyphodiscus* was recovered from all canopy soil treatments; however, it rarely occurred during the summer season with the exception of the roof treatment at the Hoh site. It was also present in forest floor soil environments, but was not recovered during the fall sampling season. *Neonectria* was recovered from all treatments, except at ambient conditions in the summer at the Hoh site. It was also common in forest floor roots and was recovered at higher abundances. *Oidiodendron* was recovered at higher relative abundances from adventitious canopy roots under the roof treatments during the spring 2018, fall 2018, and spring 2019 sampling periods. It was also found associating with some of the forest floor rooting systems, but only in the spring 2018 and summer sampling periods. Some of the forest floor associates were resolved to most closely match *Oidiodendron maius*.

The genus *Mycena* was also recovered at high relative abundances from both canopy and forest floor roots across all sampling periods, with the lowest relative abundances in summer. It was identified across all three canopy soil treatments, and was more common under the roof-interception treatment and the irrigation treatment. Some of the *Mycena* identities were resolved to the species level, matching most closely to *M. leptcephala* and *M. arcangeliana*.

The genus *Spirosphaera* was identified from both canopy adventitious and forest floor roots during all sampling periods, with the smallest recovery during the fall 2018 sampling period. At ambient conditions, it was found at higher relative abundances in canopy adventitious rooting systems, with the exception of the fall 2018 sampling period when relative abundances were comparable. The highest relative abundance was recorded from an adventitious root sample at ambient conditions during the spring 2019 sampling period, and in general relative abundances were higher at ambient conditions.

Tricladium was also found associating with both rooting systems and was most abundant in forest soils at ambient conditions, with the lowest relative abundance during the summer sampling period. In the canopy soil environment, it was found at higher relative abundances at ambient conditions during the spring 2018, summer, and spring 2019 sampling periods; and under both experimental conditions in summer.

3.4 DISCUSSION

3.4.1 Canopy soils – a separate, yet interconnected soil system

Canopy soils are unique environments that contribute to the structural and functional components in the ecosystems where they prevail (Nadkarni 1986, Tejo et al 2014, Looby *et al.*

2020). The pathways in which canopy soils contribute to ecosystem productivity, host tree health, and biogeochemical cycling are complex and remain under-studied. These organic soils may be confined to tree branches, but studies have demonstrated that they are not completely decoupled from the forest floor environment. For example, nutrients are leached from canopy soils to forest floor soils (see Ch. 4 and Nadkarni 1986). It was also reported that N cycling in canopy soils of a tropical montane cloud forest was sensitive to slight changes in forest floor nutrient availability (Matson *et al.* 2014).

Though canopy soils have been emphasized to hold important ecological roles, understanding how they benefit their host tree and surrounding ecosystem is challenging, especially because certain host trees grow adventitious roots into their canopy soil environment. Past research has found that host tree root biomass in canopy soils is negligible compared to the forest floor and that adventitious roots likely have reduced capabilities to uptake nutrients (Hertel *et al.* 2011). However, these conclusions were based on the lack of mycorrhizal associations in the root system being studied.

In contrast, other studies have emphasized the ecological importance of adventitious roots in providing an alternative path for host tree nutrient uptake (Nadkarni 1981, Perez *et al.* 2005, Kennedy *et al.* 2010); including the presence of mycorrhizal communities (Orlovich *et al.* 2013, Nilsen *et al.* 2020). However, there are very few studies that have explored fungal communities in canopy soils (Pittl *et al.* 2010, Looby *et al.* 2020, Nilsen *et al.* 2020), and even fewer that have directly explored the root-associated community diversity (Orlovich *et al.* 2013). To our knowledge, there were no previous studies that explored root-associated fungal community diversity in canopy soils for trees that associate with arbuscular mycorrhiza. Furthermore, there

have been no studies that tested how these communities change over time and under environmental stressors.

This study first determined the amount of fungi colonizing adventitious canopy and forest floor roots of 12 old-growth bigleaf maples located in two temperate old-growth rainforests sites in Washington State. It then used high-throughput sequencing to temporally explore root-associated fungal communities in canopy and forest floor soils from the same host trees. Root samples were collected in May 2018 (spring), August 2018 (summer), November 2018 (fall), and May 2019 (spring). During this study, canopy soils were subject to an experimental manipulation that increased rainfall by two-fold or decreased it by half the ambient amount. Four trees remained at ambient conditions for comparisons.

The experimental manipulations were implemented to go beyond fundamental information regarding root-associated fungal community diversity between canopy soils and forest floor soils, and explored how decreased and increased rainfall conditions may impact root-associated fungal diversity in canopy soils. This is particularly crucial because these soils are less buffered from environmental extremes. This is especially relevant because these old-growth rainforests are predicted to face wetter winters and drier summers, and the associated extreme rainfall and drought events could cause changes in root-associated fungal communities (Barnes *et al.* 2018, Lagueux *et al.* 2021); which in return could alter the structure and function of these ecosystems (Bennet and Classen 2020).

Surprisingly, canopy soils under both experimental treatments had higher soil moisture content when compared to ambient conditions. Higher moisture contents were expected from the irrigation-enhanced manipulation, but it was expected that the roof rainfall-interception treatment would decrease rainfall interception and as a result decrease canopy-soil moisture content.

Though half the amount of rainfall of a fixed area of the branch was diverted, there appeared to be other physical processes that actually caused an *increase* in moisture content relative to the ambient conditions. Nevertheless, both manipulations *did impact* soil moisture levels, providing new insights on the complexity of these canopy-soil environments, as well as their root-fungal interactions.

3.4.2 Fungal structures in adventitious and forest floor roots

This study found that there was no significant difference between percent fungal colonization in canopy and forest floor soils between the two sites and overall. Therefore, the first hypothesis that fungal colonization would be lower in adventitious roots was rejected. Both rooting systems had fungal colonization >55%. Dark septate endophytes and arbuscular mycorrhizal structures were observed in both systems. When observing the types of structures, adventitious canopy roots often showed intracellular intermediate or *Paris*-type arbuscular mycorrhizal structures (see Smith and Smith 1997, Dickson 2004), whereas *Arum*-type structures and vesicles were more commonly observed in forest floor roots. There are no published studies comparing the physiology and morphology of bigleaf maple adventitious roots to their counterpart in the forest floor, but a study found that the associated morphology and physiology of adventitious roots belonging to *Salix syringiana* were similar to forest floor roots (Nadkarni and Primack 1989). This suggests that structures formed by arbuscular mycorrhiza in bigleaf maple roots may be somewhat dependent on the type of root (adventitious vs. forest floor), soil environment (canopy vs. forest floor), and seasonal growth patterns.

It has been determined that one type of fungus can form vesicles and *Arum*- and *Paris*-type structures, often dependent on the host plant (Gerdemann 1965, Bedini et al 2000). It is also

known that one host plant can have a diversity of arbuscular associates, though the mechanisms underlying niche differentiation and co-occurrence are poorly understood (McBurney *et al.* 2017, Powell and Rillig 2018). Additionally, both *Paris*-type, *Arum*-type, and intermediate arbuscular structures can occur in the same rooting system (Ahulu *et al.* 2005, Kubota *et al.* 2005). The fact that both structures are occurring in two separate rooting systems belonging to the same host tree provides a unique opportunity to study the *Arum-Paris* continuum further.

3.4.3 Root-associated fungal community diversity in canopy and forest floor soils

Fungal community alpha and beta diversity (e.g., OTU richness, Shannon and Simpson indices, and community composition among samples) were different between canopy and forest floor rooting systems at ambient conditions, but only richness differed across seasons. Both observed richness and Shannon and Simpson indices were lower in canopy soil environments. The same was reported for fungal community richness, Shannon diversity, and beta diversity in a tropical montane cloud forest (Looby *et al.* 2020) and also from a temperate rainforest in New Zealand (Nilsen *et al.* 2020). Further, differences in community composition among these samples was not only dependent on the soil environment and site, but the interaction of these two factors. This allowed for the acceptance of the second hypothesis that the root-associated fungal communities in canopy soils at ambient conditions are distinct from those associating with forest floor roots. This also allowed for the acceptance of the Hypothesis 5a that these communities would also be dependent on the sample location. Seasonality did not have an impact on beta-diversity in forest floor soils and canopy soils at ambient conditions, therefore Hypothesis 4a was rejected.

In general, canopy soils experience extreme cycles of drying and rewetting (Coxson and Nadkarni 1995). In the temperate rainforests of western Washington, they have also been reported to have higher cation exchange capacity, acidity, and fluctuations in temperature (Aubrey *et al.* 2013, Tejo *et al.* 2014). Trudell and Edmonds (2004) also found that the diversity of epigeous macrofungi was distinct in two old-growth stands with similar species composition, and found that diversity was dependent on soil moisture and N gradients. These edaphic properties can all be partial determinants in shaping root-associated fungal communities, and some of these properties can be spatially dependent on site; which may be the reason that the interaction of site and soil type is significant. Another factor that may shape these communities is limitations on fungal dispersal.

3.4.4 Root-associated fungal communities differ across canopy soil rainfall treatments

When looking at alpha diversity across the canopy soil rainfall treatment levels (2x, 1/2x, and ambient rainfall), there was no notable effect of treatment on observed richness or diversity measures. However, there was a difference in observed richness between summer and fall and fall and spring, respectively. The differences in observed richness between these two sampling periods could be dependent on several factors, including transitioning from the summer drought season to the wet season and from the wet dormant season to the spring growing season, respectively.

The difference between canopy soil root-associated fungal community beta diversity was dependent on the interaction of treatment and site. This suggests that fungal communities associating with adventitious roots will be sensitive to extended periods of drought and increased rainfall, which will also be influenced by the spatial location of canopy soils. *Beta*-diversity also

differed among seasons under the experimental manipulation. These findings allowed for the acceptance of the hypotheses (Hypothesis 3, 4b, and 5b) that root-associated fungal community composition across canopy soil treatments will be different among treatments, among seasons, and between the two sampling sites. This indicates that root-associated fungal communities in canopy soil environments seasonally respond to the magnitude of rainfall and likely other microclimatic factors; suggesting the root-associated fungi in canopy soils may be acting as adaptive facilitators to these seasonal extremes. The microclimatic-induced shifts that these root-associated communities are experiencing can impact host-tree resiliency to climatic changes, as well as forest-level biogeochemical cycles (see Ch. 5, Barnes et al. 2018). Further, root-associated fungal communities in canopy soils were spatially different, which may be influenced by interactions among functional groups of fungi and also interactions among the epiphytic canopy communities.

There are no comparable studies that have implemented an experiment at the canopy soil level to explore the response of root-associated fungal communities. However, a study in this same region found that canopy soil bacterial communities shifted when a canopy branch was severed from the host tree, even though it remained hanging in the canopy environment (Dangerfield *et al.* 2017). This shows that bacterial communities in canopy soils are shaped not only by the canopy environment, but by their host tree. These observed shifts could potentially be related to how bacterial communities interact with the fungi in the adventitious mycorrhizosphere (see Artursson *et al.* 2005).

3.4.5 Obligate mutualists and ambiguous associates

Arbuscular mycorrhizal-associated fungi were common in both rooting systems, with higher diversity in forest floor soils. *Rhizophagus* spp. and *Glomus* spp. were most abundant in canopy soil environments, and *R. intraradices* was an indicator for the roof-interception (1/2x) treatment. Arbuscular species are obligate mutualists and their associations suggest that adventitious roots of old-growth bigleaf maple are exploiting canopy soils for their nutrients, which has been controversial (see Hertel et al 2011).

Adventitious roots were also found commonly associating with dark septate endophytes (DSE), and some were observed to have loose pigmented ‘sheaths’ surrounding the fine roots. One of the indicator species for the canopy soil environment was the DSE *C. ericae*, which is known to form ericoid mycorrhiza (Yang *et al.* 2018). *Meliniomyces bicolor* was also identified, and is known to form both ericoid and ectomycorrhizal associations (Villa-Ruiz *et al.* 2004, Grelet *et al.* 2009).

The genus *Oidiodendron* was also identified from a majority of adventitious roots. These most closely matched to *O. maius*, but they fell below the 97% species ID threshold. However, species-level identification was resolved in the forest floor. *Oidiodendron maius* is a well-studied ericoid mycorrhiza, and species from the *Oidiodendron* genus are facultative in nature (Rice and Currah 2005, Fadaei *et al.* 2020). *Mycena* spp. were also relatively abundant in both the root-associated communities, and a *Mycena* sp. was indicative of canopy soil environments. *Mycena* is a diverse genus and their ecological role has widely been regarded as obligate saprotrophs. However, they have been found to have plant growth promoting effects (Grelet *et al.* 2017) and colonized plant roots *in vitro*, suggesting their role in the environment is versatile and understudied (Thoen et al 2020).

Species from the *Acer* genus associate with arbuscular mycorrhiza, but some of the species within the root-associated fungal communities warrant further functional studies to determine where these fungi fall within the mutualism-antagonism continuum. To our knowledge, this is the first study to use high-throughput sequencing to characterize the fungal communities associating with old-growth bigleaf maple trees, and therefore it is not surprising that this study has several first reports of root-fungal interactions.

It is unlikely that bigleaf maples are forming ectomycorrhizal associations, and they are not in the Ericaceae family. However, the presence of these organisms and their facultative nature with other rooting systems suggests that there may be previously unreported biotrophic interactions, especially in the canopy soil environment where some of these root-associates may be helping increase the stress tolerance of the host tree to environmental extremes that might occur during periods of drought or intense rainfall.

3.4.6 Considerations

It is acknowledged that the rainfall experimental manipulation was only implemented at the canopy soil level. This was justified because: 1) canopy soils are confined to their branches and are more easily manipulated than forest floor soils; 2) the elk activity in these old-growth ecosystems would continuously threaten the integrity of experimental structures at the ground level; and 3) understanding root-associated fungal dynamics in canopy soils under rainfall extremes was a prioritized inquiry. Therefore, this study did not address how forest floor root-associated communities would respond to periods of increased dryness or wetness. However, these forests do experience a summer drought period and there was no significant shift in forest floor community diversity measures during the summer sampling period. Longer temporal

studies, including inter-annual studies, would be needed to gain further insights, as it remains difficult to isolate and experimentally manipulate a tree's rooting system at the forest floor level. This is why canopy soils offer an excellent testbed for *in situ* soil studies.

Canopy soils have been increasingly recognized as important habitats for epiphytic species, insects, birds, and mammals; and the forest canopy has been considered one of the last biotic frontiers (Erwin 1983). However, our understanding of the importance that adventitious roots hold in host tree nutrient acquisition and host tree resiliency has only begun to develop over the last 40 years. This study not only demonstrated that canopy roots had comparable amounts of fungal structures to their forest floor counterparts, but it also proved that they were associating with obligate mutualists and a wide array of facultative species. Further, canopy soils have higher concentrations of plant available N and P (see Ch. 4) when compared to the forest floor for their host trees to exploit.

3.5 CONCLUSION

Canopy soils are prevalent in ecosystems across the globe, providing habitat to a diversity of species while contributing to the cycling of nutrients. They appear to be important ecological compartments that are capable of providing resilience at the ecosystem level, for example during extended periods of rainfall or prolonged droughts. This becomes especially relevant when host trees have adventitious rooting systems that associate with a diversity of fungi. However, no research has explored how root-associated fungi in canopy soils may contribute to host tree nutrient acquisition and resiliency during certain times of the year and under climate-change scenarios.

This study compared root-associated fungal communities in canopy and forest floor soils of bigleaf maple at ambient conditions. It also implemented an experiment in the canopy soil environment to explore how root-associated fungal diversity responded to increased and

decreased rainfall levels. It is one of the first to investigate the temporal dynamics of root-associated fungal community diversity in canopy soils and forest floor soils of an old-growth temperate rainforest. Additionally, it is the only study that explores how the root-associated fungal diversity in canopy soil environments respond to changes in rainfall levels.

Observed OTU richness and both Shannon and Simpson diversity indices of root-associated fungal communities were significantly lower in canopy soils of old-growth bigleaf maple trees. Further, beta diversity of fungal communities associating with adventitious canopy roots were distinct from forest floor roots. In canopy soils, root-associated fungal community diversity also appeared to be impacted by increased and decreased rainfall levels throughout the year, which was also influenced by the spatial scale of sampling location. These results suggest that root-associated fungi in canopy soils environments are acting as adaptive facilitators to environmental extremes at a spatial and temporal scale. For example, canopy soils are subject to more stressful conditions, including extreme shifts in soil microclimate, but they also have pools of plant available nutrients throughout the year. Therefore, the host trees adventitious rooting system may favor a different suite of mycorrhizal and endophytic fungi during certain seasons; reflecting the different mechanisms in which root-associated fungi can benefit the plant host.

Further, this study provides first reports of several species of fungi, whose guilds are ambiguous, associating with adventitious roots. Many of these species are facultative in nature, and may be forming previously unreported mutualisms in the canopy soil environment. More research is required to fully understand the functional roles of these root-associated fungi, and it is emphasized that the canopy soil environment provides a unique soil system for studying the functional responses of root-associated fungi *in situ*. This is because they are confined to tree branches and can be subjected to experiments that elucidate functional processes that may be obscured by the complex interactions in the forest floor soil environment.

This research contributed fundamental information needed to build a more comprehensive understanding of the biotic processes in canopy soils and the role they may hold for these productive, temporally stable old-growth forests in the face of a changing climate. It has been

just over 40 years since pioneering studies have emphasized how canopy soils provide unique structural and functional components in the ecosystems they prevail. This study demonstrates that adventitious rooting systems associate with a different diversity of fungi, and that these communities shift in response to seasonal changes and varying rainfall levels. This builds upon the theory that canopy soils provide an essential compartment that contributes to host tree resource acquisition, inherently contributing to the resiliency of these forests in a changing world.

Figures



(a) (b)
Figure 3.1: An old-growth *Acer macrophyllum* branch engulfed in living epiphytes with thick mats of canopy soil below (a), and an exposed section of canopy soils and adventitious roots (b).



Figure 3.2: Images from the Queets research site (a) and the Hoh research site (b) in the Olympic National Forest in Washington, USA..



Figure 3.3: The custom designed roof structure that was fixed to branches to intercept and divert 1/2x the rainfall off the branch (a), and the custom designed irrigation structures that collected a fixed surface area of rainfall and redistributed to the experimental branch.

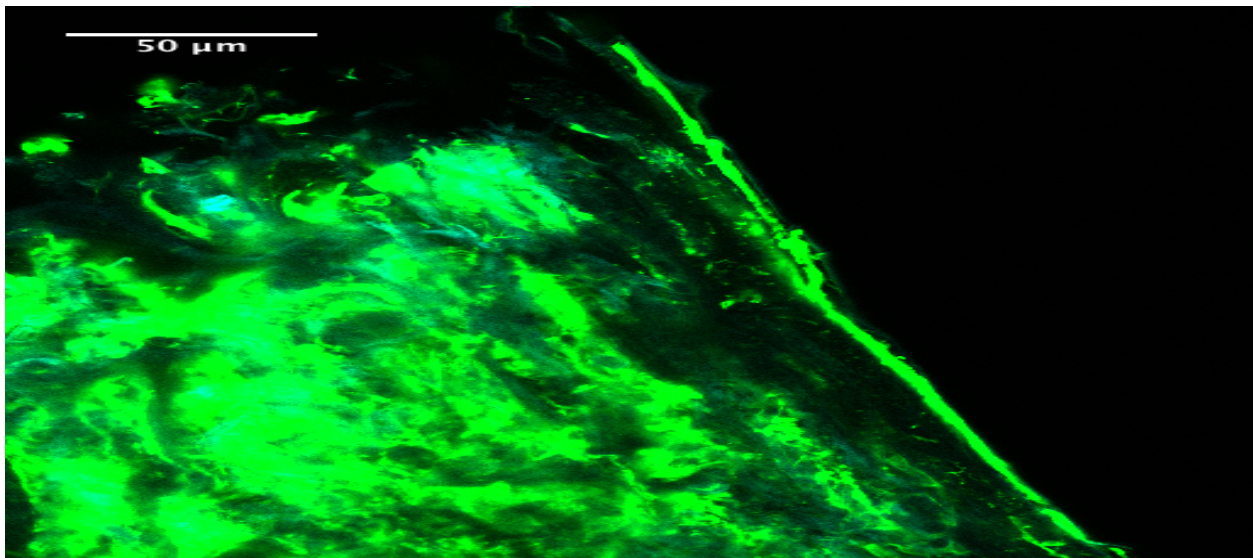


Figure 3.4: Extensive fungal hyphae in both cortical and epidermal cells of an adventitious root from canopy soils. Fungal hyphae were stained with Alexa Fluor 488 Wheat Germ Agglutinin and stained structures emitted a green fluorescence under the confocal laser.

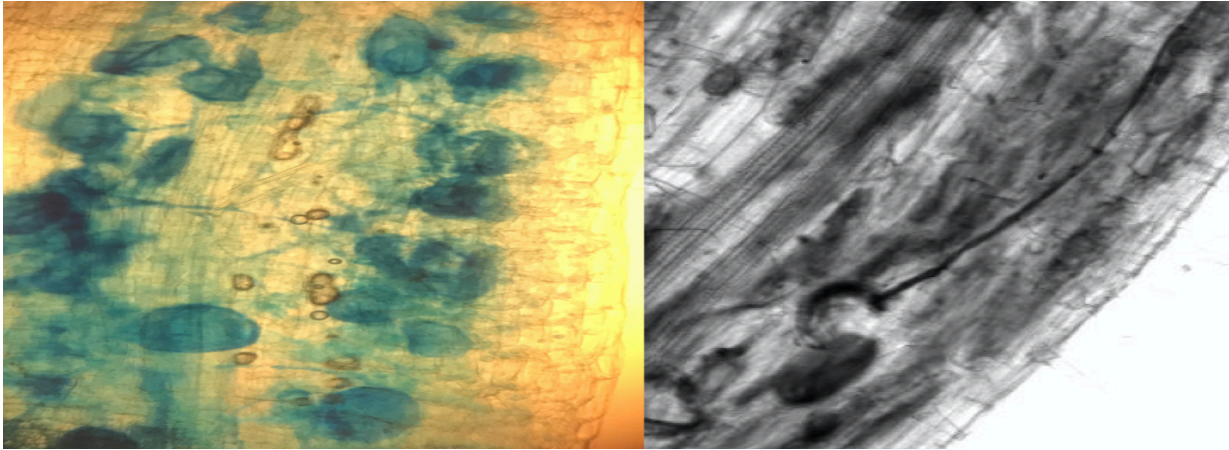


Figure 3.5: Arbuscular mycorrhizal vesicles stained with Trypan Blue (left). Arbuscular mycorrhizal vesicles, arbuscules, and intraradical hyphae stained with Trypan Blue under the confocal microscope (right). Both images are from forest floor roots of bigleaf maple.

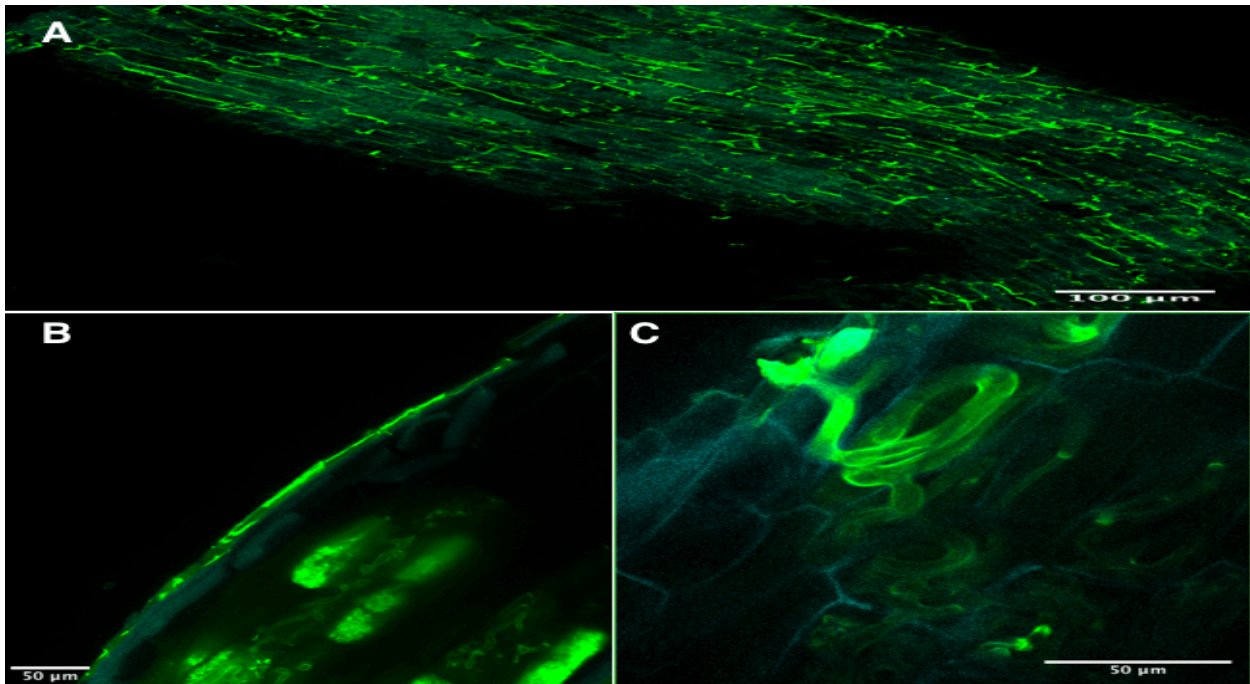


Figure 3.6: Intercellular runner hyphae (a); hyphae in the epidermal cells, and intracellular arbuscules and coils (b); and a close-up of some intracellular coils (c) of bigleaf maple canopy adventitious roots.

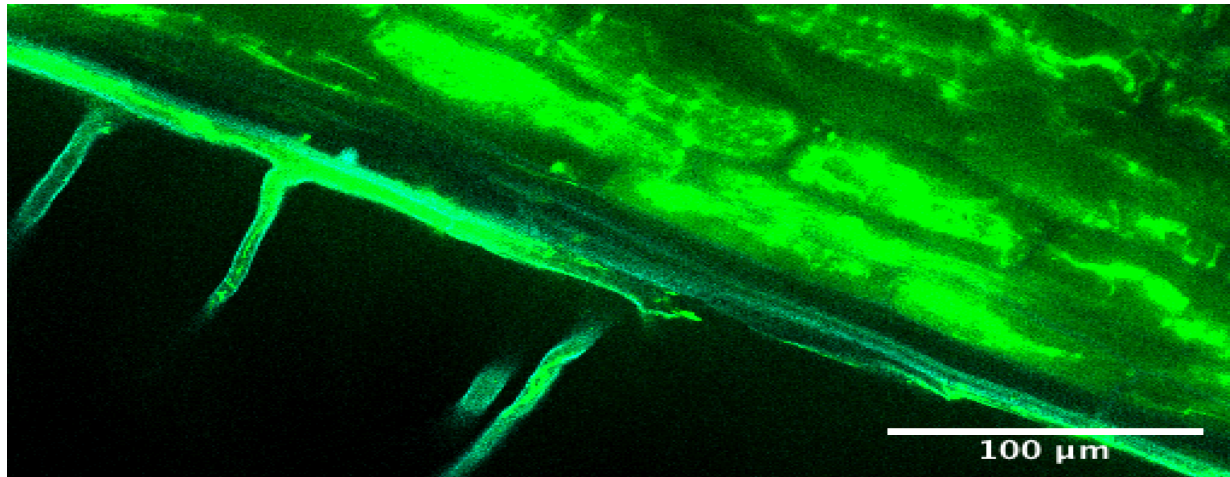


Figure 3.7: Fungal hyphae present in/around epidermal cells and root-hairs.

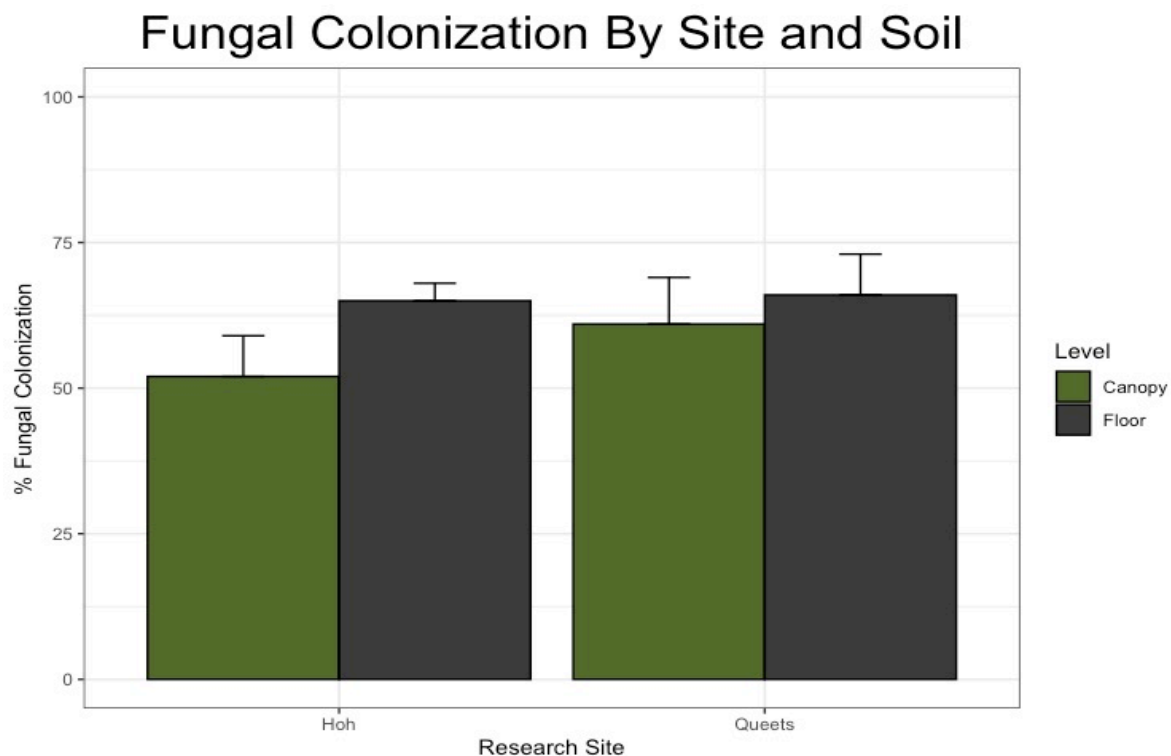


Figure 3.8: Fungal colonization in old-growth bigleaf maple roots collected from canopy and forest floor soils. Averages were slightly lower in canopy adventitious and forest floor roots sampled from the Hoh Rainforest ($52.3\% \pm 7.1$ and $64.7\% \pm 2.8$, respectively) when compared to the Queets Rainforest ($60.7\% \pm 8.4$ and $65.5\% \pm 7.1$, respectively).

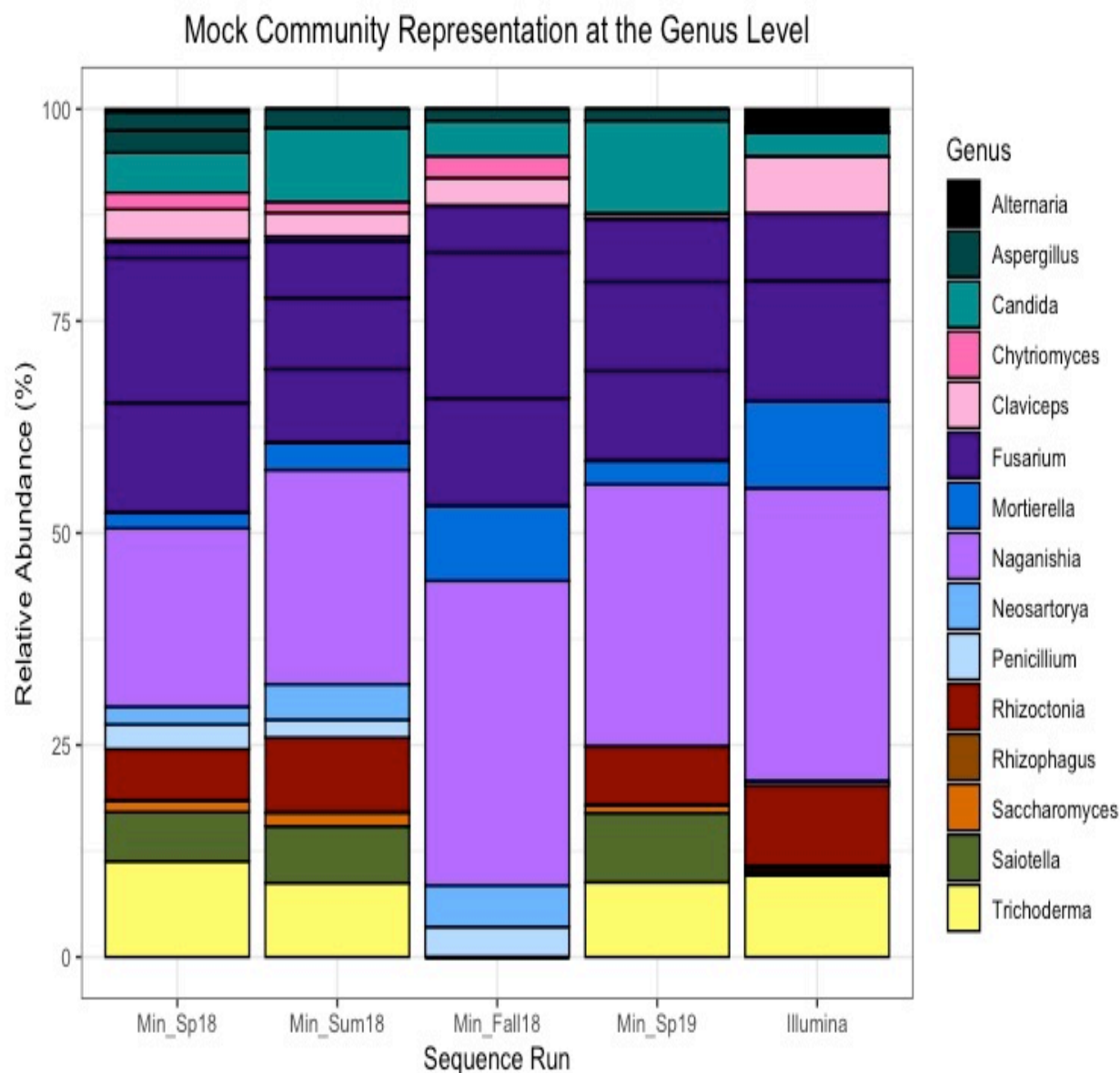


Figure 3.9: Genus-level identities and relative abundances of the mock community used as positive controls for sequencing. A mock community was sequenced on each MinION flowcell (1 per sampling period), and also on the Illumina MiSeq run.

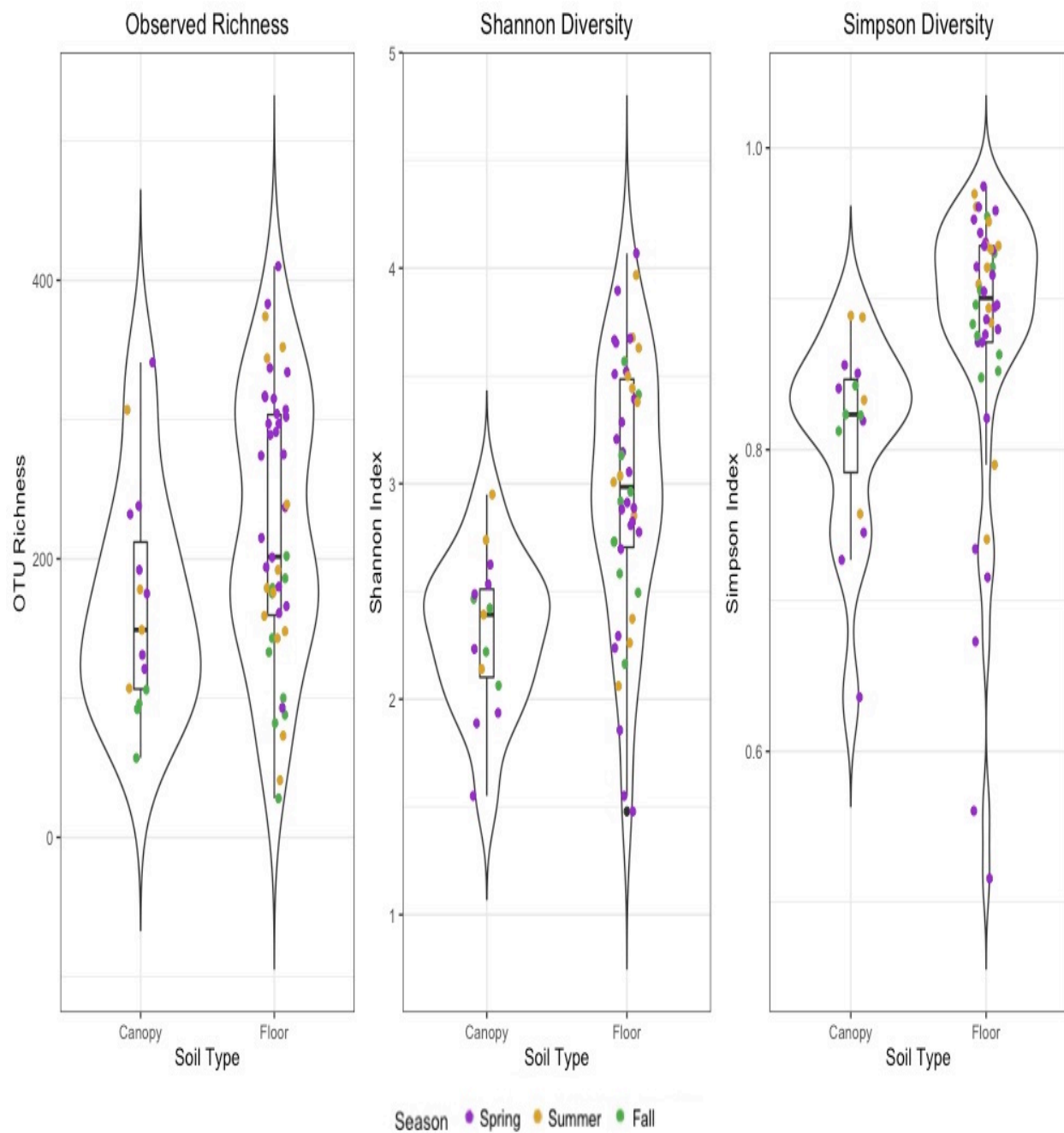


Figure 3.10: Violin plots with data points and boxplot showing observed OTU richness and Shannon and Simpson Diversity indices of root-associated fungal communities in canopy and forest floor soils at ambient conditions.

PCoA of Root-associated Fungal Communities Between Canopy and Forest Floor Soils

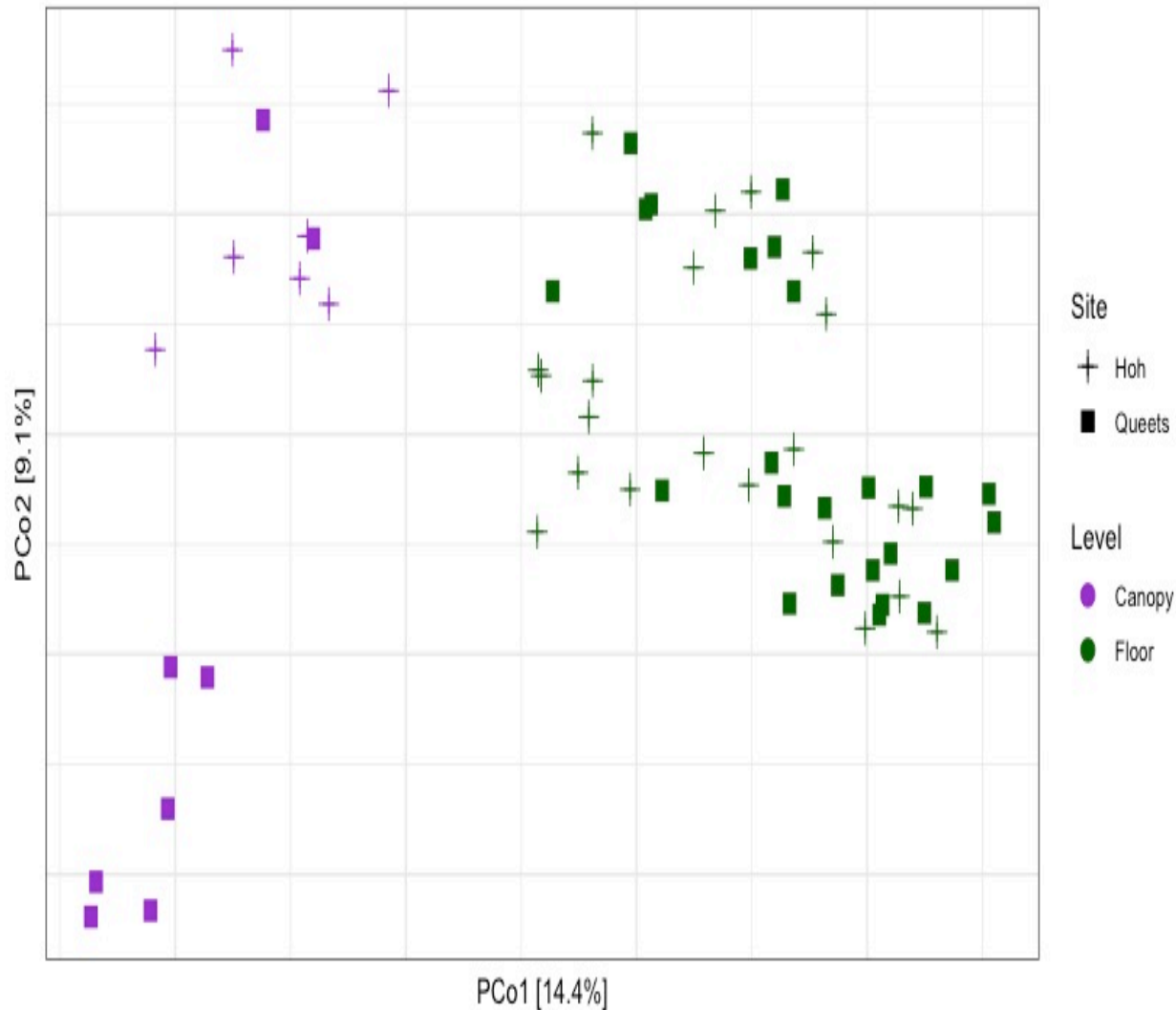


Figure 3.11: Principal coordinates analysis (PCoA) plot of root-associated fungal communities based on Bray-Curtis distances of OTU relative abundances between canopy and forest floor soils at ambient conditions across sampling periods.

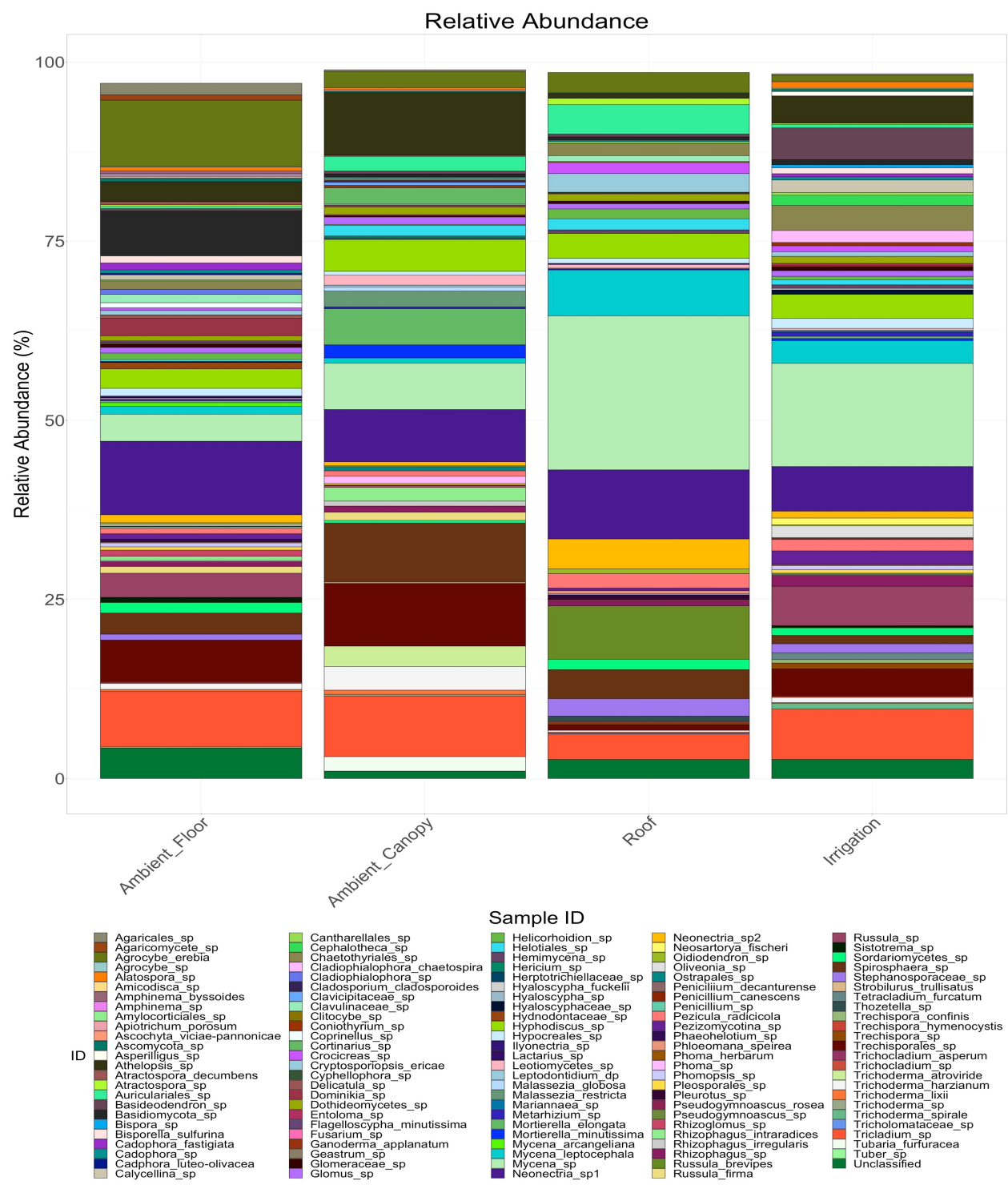


Figure 3.12 Community relative abundances ($\geq 0.1\%$) from the MinION sequence data between ambient canopy and forest floor soils, canopy soil treatments (irrigation and roof-interception).

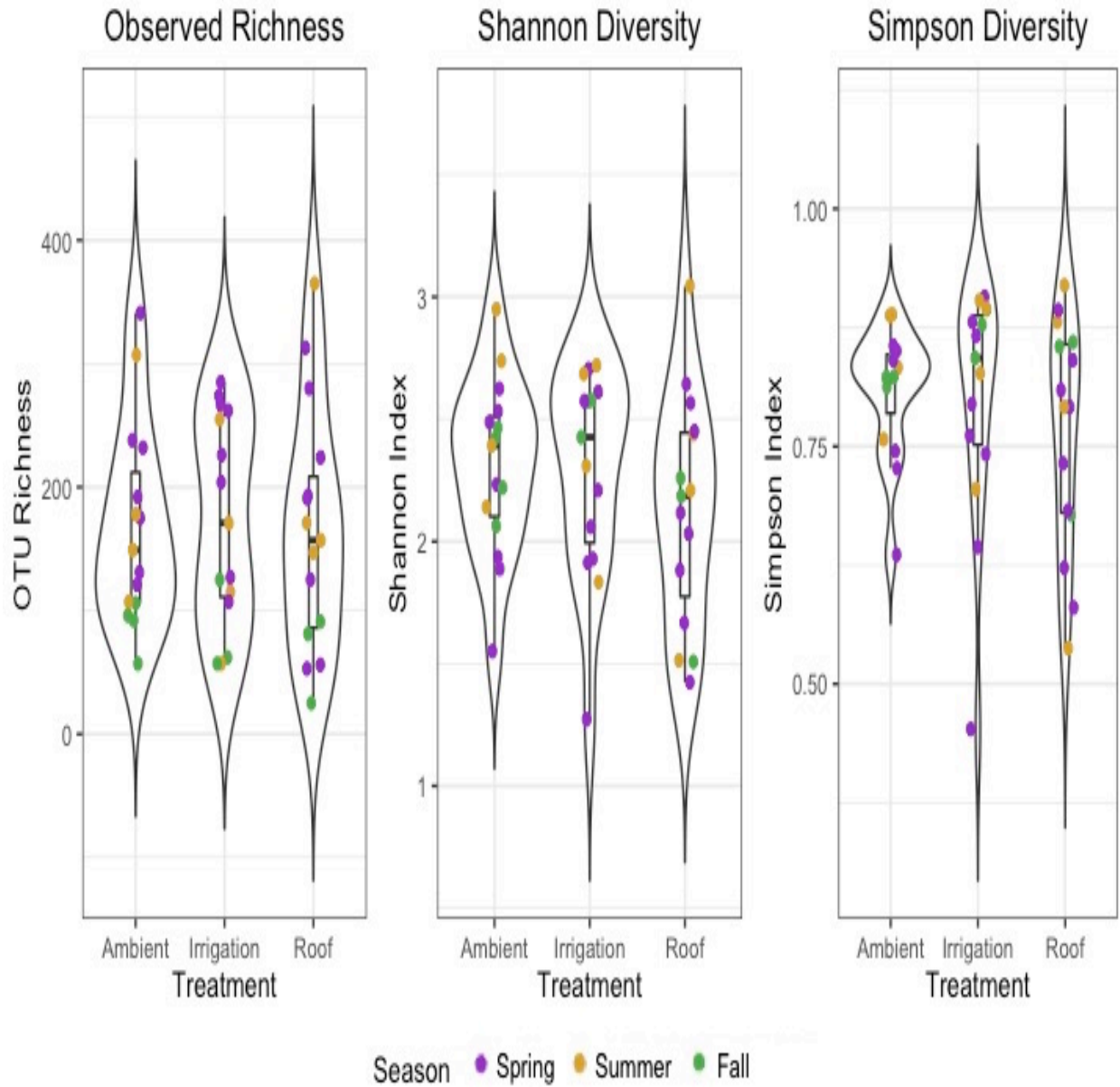


Figure 3.13: Violin plots with data points and boxplot showing observed OTU richness and Shannon and Simpson Diversity indices of root-associated fungal communities across canopy soil treatments and sampling period.

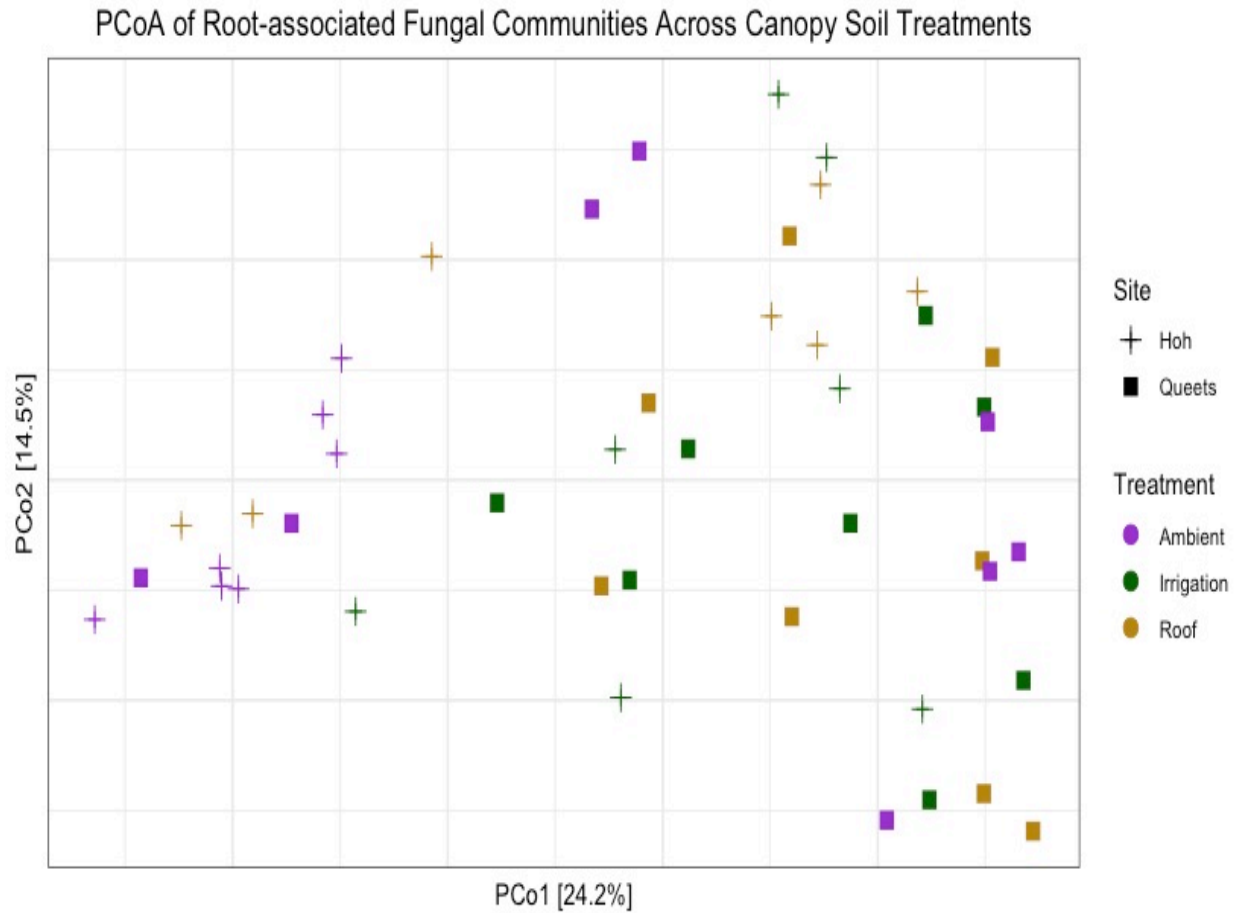


Figure 3.14: Principal coordinates analysis (PCoA) plot of root-associated fungal communities based on Bray-Curtis distances of OTU relative abundances across canopy soil treatments and site.

Tables

Table 3.1: ANOVA tables showing statistical results for differences in alpha-diversity measures, including observed OTU richness, Shannon diversity, and Simpson diversity between canopy and forest floor root-associated fungal communities (Soil Type), season, and site.

	DF	Sum Sq	Mean Sq	F-value	p-value
Canopy and Forest Floor Alpha-Diversity ANOVAs					
<i>Observed Richness</i>					
Soil Type	1	33,327	33,327	5.44	<0.05*
Site	2	1,769	1,796	0.29	0.59
Season	2	176,398	88,199	14.40	<0.01**
Residuals	56	342,930	6,124		
<i>Shannon Diversity</i>					
Soil Type	1	5.11	5.11	16.10	<0.01**
Site	1	0.65	0.65	2.05	0.16
Season	2	0.55	0.28	0.87	0.43
Residuals	56	12.22	0.31		
<i>Simpson Diversity</i>					
Soil Type	1	0.05	0.05	5.88	<0.05*
Site	1	0.01	0.01	0.40	0.53
Season	2	0.03	0.02	1.83	0.17
Residuals	56	0.47	0.01		

Table 3.2: PERMANOVA table showing statistical results for differences in beta-diversity of root-associated fungal communities between canopy and forest floor soils (Soil Type), sampling period (Season), and the interaction (Soil:Season). Canopy and Forest Floor Beta-Diversity PERMANOVA

Canopy and Forest Floor Beta-Diversity PERMANOVA					
	DF	Sum Sq	R²	F-value	p-value
Soil	1	2.70	0.12	8.69	<0.01**
Season	3	0.71	0.03	1.14	0.21
Site	1	0.85	0.04	2.74	<0.01**
Soil:Season	2	0.50	0.02	0.80	0.86
Soil:Site	1	0.97	0.04	3.12	<0.01**
Season:Site	2	0.50	0.02	0.80	0.86
Residuals	51	15.88	0.72		

Table 3.3: Indicator root-associates between the canopy and forest floor soils at ambient conditions and across canopy soil treatments.

Indicator Species	Indicator Value	p-value
Between Canopy and Forest Floor Soils at Ambient Conditions		
Canopy		
<i>Neonectria sp.</i>	0.85	<0.01**
<i>Mycena sp.</i>	0.82	<0.01**
<i>Pezicula radicicola</i>	0.80	<0.01**
<i>Hyphodiscus sp.</i>	0.72	<0.01**
<i>Helotiales sp.</i>	0.68	0.02*
<i>Rhizophagus sp.</i>	0.59	<0.01**
<i>Cryptosporiopsis ericae</i>	0.53	0.01**
<i>Athelopsis sp.</i>	0.52	<0.01**
Floor		
<i>Basidiomycota sp.</i>	0.83	<0.01**
<i>Agrocybe erebia</i>	0.78	<0.01**
Across Canopy Soil Treatments		
Irrigation		
<i>Basideodendron sp.</i>	0.61	0.01**
<i>Hyaloscyphaceae sp.</i>	0.60	<0.01**
Roof-interception		
<i>Rhizophagus intraradices</i>	0.61	<0.01**
Ambient + irrigation		
<i>Pezicula radicicola</i>	0.73	0.04*

Table 3.4: Relative abundances that represented >0.5% of community composition in forest floor and canopy adventitious roots of bigleaf maple trees at ambient conditions. Indicator species (Table 3.3) for the two soil environments are in bold. (Sp=spring, Su=summer, Fa=fall)

Relative Abundance Between Canopy and Forest Floor at Ambient Conditions												
	HOH						QUEETS					
	Canopy			Floor			Canopy			Floor		
Taxa	Sp	Su	Fa	Sp	Su	Fa	Sp	Su	Fa	Sp	Su	Fa
<i>Agaricales sp.</i>	-	-	1.4	-	-	8.3	-	-	-	-	-	1.4
<i>Agaricomycetes sp.</i>	-	-	-	-	-	-	-	-	-	3.7	-	-
<i>Agrocybe erebia</i>	-	3.0	-	14.9	-	3.2	-	10.6	-	10.6	-	26.7
<i>Agrocybe sp.</i>	-	-	-	-	-	0.5	-	-	-	-	-	-
<i>Alatospora sp.</i>	-	-	-	-	0.6	1.8	-	0.9	-	-	-	-
<i>Amphinema byssoides</i>	-	-	-	0.6	-	1.8	-	-	-	-	-	-
<i>Amylocorticiales sp.</i>	-	-	-	-	-	-	-	-	-	-	-	0.9
<i>Apiotrichum porosom</i>	-	-	-	-	-	-	-	-	-	-	0.9	-
<i>Archaeorhizomyces sp.</i>	-	-	-	-	-	0.5	-	-	-	-	-	-
<i>Ascochyta viciae-pannonicae</i>	-	-	-	-	-	-	1.3	-	-	-	-	-
<i>Ascomycota sp.</i>	-	-	1.1	-	1.7	-	-	-	-	-	1.0	-
<i>Athelopsis sp.</i>	14.1	17.1	17.6	-	7.0	-	3.7	-	-	1.5	8.2	-
<i>Atractospora decumbens</i>	-	-	0.9	-	-	2.3	-	-	-	-	-	-
<i>Atractospora sp.</i>	-	-	-	-	1.1	-	-	-	-	-	-	-
<i>Auriculariales sp.</i>	-	-	-	-	-	-	11.6	-	0.7	-	0.5	1.2
<i>Basideodendron sp.</i>	-	-	-	-	-	-	-	-	2.3	-	1.3	0.6
<i>Basidiomycota sp.</i>	-	1.9	-	12.7	-	7.0	0.5	-	-	9.5	-	7.9
<i>Bisporella sulfurina</i>	-	-	-	-	-	-	-	-	-	-	-	5.9
<i>Cadphora luteo-olivacea</i>	-	-	-	-	-	1.2	-	-	-	-	-	-
<i>Cadophora fastigiata</i>	-	-	-	1.6	0.5	3.4	-	-	-	-	-	-
<i>Cadophora sp.</i>	1.0	-	-	-	-	1.1	-	-	-	1.4	-	-
<i>Calycellina sp.</i>	-	-	-	0.6	2.0	-	0.5	-	-	-	1.4	-
<i>Cantherellales sp.</i>	-	-	-	-	0.5	-	-	-	-	-	-	-
<i>Cephalotheca sp.</i>	-	-	-	-	-	-	-	-	-	-	-	-
<i>Chaetothyriales sp.</i>	0.7	-	-	0.8	0.5	2.3	-	-	-	-	2.0	1.9

<i>Cladiophyalophora sp.</i>	-	-	-	-	-	-	-	-	-	-	-	3.6
<i>Cladosporium cladosporoides</i>	-	-	-	-	-	-	0.6	1.9	-	-	0.5	-
<i>Clavicipitaceae sp.</i>	2.8	-	-	-	-	-	-	-	-	-	-	-
<i>Clavulinaceae sp.</i>	-	-	-	3.3	-	-	-	-	-	3.3	-	0.6
<i>Coprinellus sp.</i>	-	-	-	-	-	4.3	-	-	-	-	-	-
<i>Cortinarius sp.</i>	-	13.8	-	-	-	-	-	-	-	-	-	-
<i>Crocicreas sp.</i>	-	-	-	-	1.2	-	-	-	-	-	0.9	-
<i>Cryptosporiopsis ericae</i>	-	-	-	-	1.1	-	0.6	-	-	1.1	1.4	-
<i>Delicatula sp.</i>	-	-	-	-	-	-	-	-	-	-	-	2.9
<i>Dominikia sp.</i>	1.4	-	-	1.9	0.9	5.5	-	-	-	1.5	-	4.8
<i>Dothideomycetes sp.</i>	1.5	-	2.8	-	-	2.7	-	-	1.9	0.7	-	-
<i>Flagelloscypha minutissima</i>	-	-	-	-	-	1.2	-	-	-	-	-	-
<i>Geastrum sp.</i>	-	-	-	-	-	-	-	-	-	-	-	0.9
<i>Glomeraceae sp.</i>	-	-	-	-	-	1.3	-	-	0.6	-	1.1	-
<i>Glomus macrocarpum</i>	-	-	-	-	-	-	-	-	-	-	-	0.5
<i>Glomus sp.</i>	-	-	5.6	-	0.5	0.7	-	-	-	0.9	1.5	1.0
<i>Helicorhoidion sp.</i>	-	-	-	-	3.9	-	-	-	-	-	1.5	-
<i>Helotiales sp.</i>	4.4	-	0.6	-	-	-	1.3	-	2.5	1.1	-	-
<i>Hemimycena sp.</i>	-	-	-	-	-	-	-	-	-	-	0.5	-
<i>Hericeum sp.</i>	-	-	1.7	-	-	-	-	-	-	-	-	-
<i>Herpotrichiellaceae sp.</i>	-	-	-	-	-	-	-	-	0.8	-	-	-
<i>Hyaloscypha sp.</i>	-	-	-	-	-	-	-	-	0.5	-	-	-
<i>Hyaloscypha fuckelii</i>	-	-	-	-	-	-	-	-	-	-	0.6	-
<i>Hydnodontaceae sp.</i>	-	-	-	-	-	5.5	-	-	-	-	-	-
<i>Hygrocybe pseudoconica</i>	-	-	-	-	-	0.5	-	-	-	-	-	-
<i>Hyphodiscus sp.</i>	2.5	-	4.7	1.0	6.3	-	10	-	9.4	1.1	7.7	-
<i>Hypocreales sp.</i>	-	-	-	2.3	3.0	-	0.9	-	2.2	-	0.7	-
<i>Lactarius sp.</i>	-	-	-	-	-	0.7	-	-	-	-	-	-
<i>Leotiomyces sp.</i>	-	-	8.0	-	-	-	-	-	-	-	-	-
<i>Leptodontidium sp.</i>	-	-	-	-	-	-	-	1.1	-	-	-	-
<i>Leptosphaericeae Sp.</i>	-	-	-	-	-	-	0.5	-	-	-	-	-
<i>Malassezia globosa</i>	-	-	3.5	-	-	-	-	-	-	-	-	-
<i>Malassezia restricta</i>	-	-	13.3	-	-	-	-	-	-	-	-	-
<i>Metarhizium sp.</i>	-	-	-	-	0.7	-	-	1.5	-	-	-	-
<i>Mortierella minutissima</i>	-	-	-	-	-	-	-	10.7	-	-	-	-

<i>Trechisporales sp.</i>	7.4	–	–	2.7	9.4	4.8	3.1	12	29.7	5.8	1.1	11.4
<i>Trichocladium asperum</i>	–	–	–	–	–	–	–	–	–	–	0.6	–
<i>Trichocladium sp.</i>	–	–	–	0.8	–	–	–	–	–	–	–	–
<i>Trichoderma atroviride</i>	–	17.1	–	–	–	–	–	–	–	–	–	–
<i>Trichoderma harzianum</i>	–	19.2	0.7	–	–	–	–	–	–	–	5.0	–
<i>Trichoderma sp.</i>	–	–	1.1	–	–	–	–	–	–	–	–	–
<i>Trichoderma spirale</i>	–	–	–	–	–	–	–	–	–	–	–	–
<i>Trichoderma lixii</i>	0.8	–	–	0.9	–	–	–	2.7	–	0.7	–	–
<i>Tricholomataceae sp.</i>	–	–	–	–	–	–	–	–	–	–	–	–
<i>Tricladium sp.</i>	16.4	6.7	4.0	11.5	3.7	6.6	–	22.1	1.4	10.8	1.0	12.8
<i>Tubaria furfuracea</i>	–	–	12.1	–	–	–	–	–	–	–	–	–
<i>Tuber sp.</i>	–	–	–	–	–	0.7	–	–	–	–	–	–

Table 3.5: ANOVA tables showing statistical results for differences in alpha-diversity measures, including observed richness, Shannon diversity, and Simpson diversity of root-associated fungal communities across canopy soil treatments (Treatment), sampling period (Season), sampling point (Sample), and the interaction between treatment and season (Treatment:Season).

Canopy Treatments Alpha-Diversity ANOVAs					
	DF	Sum Sq	Mean Sq	F-value	<i>p</i>-value
<i>Observed Richness</i>					
Treatment	2	502	251	0.04	0.96
Site	1	856	856	0.15	0.70
Season	2	105,441	52,720	9.04	<0.01**
Residuals	39	227,482	5,833		
<i>Shannon Diversity</i>					
Treatment	2	0.30	0.15	0.86	0.43
Site	1	0.03	0.03	0.18	0.63
Season	2	0.57	0.29	1.66	0.20
Residuals	39	6.73	0.17		
<i>Simpson Diversity</i>					
Treatment	2	0.02	0.01	0.73	0.49
Site	1	0.00	0.00	0.00	0.98
Season	2	0.05	0.02	2.02	0.15
Residuals	39	0.44	0.01		

Table 3.6: PERMANOVA table showing statistical results for differences in beta-diversity of root-associated fungal communities across canopy soil experimental treatments (Treatment), sampling period (Season), and the interaction (Treatment:Season).

Canopy Treatments Beta-Diversity PERMANOVA					
	DF	Sum Sq	R²	F-value	<i>p</i>-value
Treatment	2	1.42	0.11	2.74	<0.01**
Season	3	1.03	0.08	1.99	<0.01**
Site	1	1.01	0.07	3.90	<0.01**
Treatment:Season	4	0.65	0.05	0.63	0.97
Treatment:Site	2	1.03	0.08	1.99	<0.01**
Season:Site	2	0.32	0.02	0.62	0.93
Residuals	31	8.02	0.59		

Table 3.7: Relative abundances that represented >0.5% of community composition in canopy adventitious roots of bigleaf maple trees across treatments (ambient, irrigation, roof-interception), season (Sp=spring, Su=summer, Fa=fall), and site (Hoh and Queets). Indicator species (Table 3.3) for treatments are in bold.

Taxa	Relative Abundance across Canopy Soil Treatments																	
	HOH									QUEETS								
	Ambient			Roof			Irrigation			Ambient			Roof			Irrigation		
	Sp	Su	Fa	Sp	Su	Fa	Sp	Su	Fa	Sp	Su	Fa	Sp	Su	Fa	Sp	Su	Fa
<i>Agaricales sp.</i>	-	-	1.4	-	-	1.6	2.1	1.1	-	-	-	-	-	-	-	-	-	-
<i>Agrocybe erebia</i>	-	3.0	-	-	-	-	-	0.8	-	10.6	-	16.4	-	-	-	-	4.5	-
<i>Alatospora sp.</i>	-	-	-	-	-	-	-	-	-	0.9	-	-	-	-	-	-	4.5	-
<i>Ascochyta viciae-pannonicae</i>	-	-	-	-	-	-	-	-	1.3	-	-	-	-	-	-	-	-	-
<i>Ascomycota sp.</i>	-	-	1.1	-	-	-	-	-	-	-	-	-	-	-	-	1.3	0.8	-
<i>Asperilligis sp.</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.4	-	-
<i>Athelopsis sp.</i>	14.1	17.1	17.6	-	2.3	-	-	4.0	3.7	-	-	-	-	-	-	12.1	-	4.6
<i>Atractospora decumbens</i>	-	-	0.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Atractospora sp.</i>	-	-	-	5.0	-	-	-	-	-	-	-	-	-	-	-	-	0.5	-
<i>Auriculariales sp.</i>	-	-	-	16.2	-	6.9	-	-	11.6	-	0.7	-	1.1	2.8	-	-	-	-
<i>Basideoendron sp.</i>	-	-	-	-	-	-	-	-	-	-	2.3	-	2.3	8.1	-	18.4	-	-
<i>Basidiomycota sp.</i>	-	1.9	-	0.9	-	-	0.8	1.2	0.5	-	-	1.6	-	-	-	1.5	-	-
<i>Bispora sp.</i>	-	-	-	-	-	-	-	2.5	-	-	-	-	-	-	-	-	-	-
<i>Bisporella sulfurina</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.0
<i>Cadophora fastigiata</i>	-	-	-	-	-	-	-	0.8	-	-	-	-	-	-	-	1.8	-	-
<i>Cadophora sp.</i>	1.0	-	-	-	1.0	-	-	1.4	-	-	-	-	-	-	-	1.1	-	-
<i>Calycellina sp.</i>	-	-	-	-	-	-	1.7	0.9	7.7	0.5	-	-	-	-	-	-	-	-
<i>Cantherellales sp.</i>	-	-	-	-	-	-	-	-	-	-	-	1.4	-	-	-	2.4	-	-

Chapter 4: Nutrient Mineralization and Micro-climatic Interconnections in Canopy Soils of Old-growth *Acer macrophyllum*, in Temperate Rainforests, Washington, USA

4.1 INTRODUCTION

The temperate rainforests in western Washington are recognized for their high rainfall regimes and extensive areas of old-growth forests where evergreen trees live to ≥ 300 years and deciduous trees are older than 200 years of age (Van Pelt *et al.* 2006, Fonda 1974). These rainforests are known to be some of the most productive forests in the terrestrial biosphere (Grier 1979). Many of these old-growth forests are located in alluvial floodplains where they form dynamic riparian forest habitats (Naiman and Bilby 1998). They are characterized by their high gross primary productivity levels, high C sequestration rates, and branches supporting abundant biomasses of epiphytic species (Franklin *et al.* 1981, Pérez *et al.* 2005, Enloe *et al.* 2006, Tejo *et al.* 2014). Globally, they represent one of the most carbon-dense forest types (Keith *et al.* 2009) and provide habitat for a diversity of species (Franklin 1988).

The wet climates of these forests allows thick mats of epiphytic plant communities to survive on tree branches and they continuously increase in size as they intercept litterfall that decays and forms canopy soils. As these layers of epiphytic and organic material increase in thickness each year, the bottom layers decompose and form what is described as canopy soils or arboreal Histosols (Nadkarni *et al.* 2002, Nadkarni 1984, Enloe *et al.* 2006, O’Keefe and Naiman 2006). It has been estimated that a single tree can hold ≥ 500 kg of dry epiphytic biomass (e.g., Naiman *et al.* 2010; Van Pelt, unpublished data), and canopy soil depths greater than 35 cm have been reported (Tejo *et al.* 2015).

Even though these organic mats are mostly confined to the canopy environment, they are

key structural and functional components of these forests. For example, they provide habitat to a diversity of flora, fauna, fungi, and prokaryotes, they intercept throughfall rainfall and atmospherically derived nutrients (Nadkarni 1986, Clark *et al.* 2000, Bidwell *et al.* 2019), and function as an additional pool of carbon and nutrients high above the forest floor (Tejo *et al.* 2014). Extensive adventitious rooting systems grow from aboveground tree branches into these canopy soils, which are especially prominent in bigleaf maple (*Acer macrophyllum* Pursh) - the focus tree species of this research (Fig. 4.1 [Geiser *et al.* 2020]). Canopy soils can also impact soil dynamics occurring at the forest floor level; e.g., from rainfall percolating through canopy soils and leaching to the forest floor or when a tree branch or canopy soil mat falls to the ground (Nadkarni 1986, Nadkarni and Matelson 1992, Tejo *et al.* 2014).

Research over the last four decades has demonstrated the importance of canopy soils in resource conservation for host trees, epiphytes, and the ecosystem as a whole, where they prevail (e.g., tropical montane cloud forests and temperate rainforests). Yet, studies have been limited due to the difficulty of accessing the tree canopy and lack of established sampling methods; additionally, a majority of existing canopy soil research has been conducted in tropical and subtropical forests. It becomes important to illuminate the role canopy soils hold regarding the nutrient dynamics (e.g., N and P cycling) in these ecosystems because, per unit area, old-growth temperate rainforests represent the longest living and largest stores of carbon on earth (Urrutia-Jalabert *et al.* 2015, Smithwick *et al.* 2002). Interactions among the terrestrial N, P, and C cycles can potentially shape how these ecosystems respond to global change (Zaehle 2013, Luo *et al.* 2020); for example, the ability to sequester C can depend on the available supply of N and P in soils (Falkowski *et al.* 2000).

A few existing studies have shown that canopy soils have greater or similar rates of N_{\min} (per gram of soil) than forest floor soils in tropical forests (Matson *et al.* 2014, Cardelús *et al.* 2009, Wanek *et al.* 2002) and in a Chilean temperate forest (Pérez *et al.* 2005). All these studies focused on N cycling and the nutrient status of canopy soils, but only the latter discussed potential host tree nutrient acquisition through canopy adventitious roots. In Washington's old-growth forest stands, Tejo *et al.* (2014) reported that canopy soils enhance the total nitrogen (N) and carbon (C) pools by 20% and 25%, respectively. Obligate fungal mutualists (arbuscular mycorrhiza [Nadkarni 1981]), as well as a unique suite of fungal taxa (see Ch. 3) have also been identified associating with adventitious rooting systems in these temperate rainforests. The presence of nutrients, adventitious roots, and mycorrhiza suggests that there may be less competition for nutrients in the canopy soil compartment, consequently becoming more cost efficient for the host tree to grow adventitious roots. However, research describing seasonal fluxes of plant available nutrients in the canopy soil environments in forests, compared to the forest floor, are scarce.

What remains unknown is how canopy soils may be enhancing the mineralized nutrient pools accessible by the host tree's adventitious rooting system, as well as which and how much of these nutrients are being leached to the forest floor directly from the canopy soil compartment (e.g., during a rainfall event). It is also of timely importance to understand how canopy soil nutrient dynamics may be impacted by increased and decreased rainfall conditions, as these ecosystems in western Washington are predicted to experience future wetter winters and prolonged summer drought (Mote and Salathé 2010, Bachelet *et al.* 2011).

These seasonal extremes, especially prolonged drought, could increase the mortality risk of these old-growth tree species, consequently reducing forest productivity (Desoto *et al.* 2020) and

result in a loss of habitat. The extent to which canopy soils act as a buffer for host tree resiliency in the face of environmental change remains unknown and becomes important to consider. This is especially relevant today as bigleaf maples have experienced increased mortality across Washington State. The old-growth maples on the western side of Washington's Olympic peninsula, where this study took place, have not shown evidence of decline (Betzen 2018).

To investigate if nutrient dynamics in bigleaf maple canopy soils of western Washington's riparian old-growth forests may be contributing to host tree resiliency and ecosystem-level nutrient cycling, research needs to explore how canopy and forest floor soil environments are impacted by changes in climatic variables. This is only possible by experimentally manipulating these environments to understand the seasonal changes in nutrient cycles of these isolated canopy soil systems and to compare to the changes occurring in the forest floor. This research was designed to explore seasonal and annual changes in mineralization rates of inorganic nitrogen (i.e., ammonium $[\text{NH}_4\text{-N}]$ + nitrate $[\text{NO}_3\text{-N}]$) and inorganic phosphorus (i.e., phosphate $[\text{PO}_4\text{-P}]$).

To do this, an experiment was implemented to simulate increased and decreased rainfall conditions and test their impacts on mineralization rates in the canopy soil environments. These experimental manipulations could not be set up at the forest floor level because of the high population of elk in these forests and also due to National Park rules and restrictions specific to one of the research sites. Ambient measurements were taken during each sampling period at the forest floor at the same time as the canopy soils were experimentally manipulated. Lastly, water was applied to canopy soils to simulate a rainfall event and to measure the magnitude of nutrients leaching from canopy soils to the forest floor soils during the spring growing season.

The objectives of this research were to: (1) explore seasonal variations in nitrogen and phosphorus mineralization rates (N_{\min} and P_{\min}) in canopy and forest floor soils; (2) test if increased and decreased rainfall levels affect rates of N_{\min} and P_{\min} in the canopy soil environment; (3) determine if canopy soils enhance mineralized nutrient pools on a per mass and *areal basis*; and (4) determine the concentration of dissolved organic C (DOC), $\text{NO}_3\text{-N}$, $\text{NH}_4\text{-N}$, total nitrogen (TN) and $\text{PO}_4\text{-P}$ leaching from canopy soils to the forest floor during a rainfall event. Based on the scope of this research and current literature, several hypotheses were postulated.

The **first hypothesis** was that increased rainfall amounts in canopy soils would increase rates of N_{\min} and P_{\min} . It has been shown that N_{\min} and plant available P will increase during periods of wetting or under increased rainfall intensity events (Venterink *et al.* 2002, Matzner and Borken 2008, Chen *et al.* 2017). In contrast, drought has been shown to decrease plant available N and P (Sardans and Peñuelas 2004, He and Dijkstra 2014), as well as decrease the rates of N_{\min} (Deng *et al.* 2021).

The **second hypothesis** was that seasonal rates of N_{\min} and P_{\min} at ambient conditions would be higher in canopy soils when compared to forest floor soils on a *per mass basis*. One piece of evidence that drove this hypothesis is that studies of canopy soils in this study region have shown high inorganic N concentrations (Tejo *et al.* 2014). Also, a majority of intercepted litterfall is dominated by maple leaves from the host tree, and studies have demonstrated that high rates of N_{\min} in forest floor soils have been linked to high N concentrations of maple leaf litter (Fried *et al.* 1990, Turk *et al.* 2008). Another piece of evidence that drove this hypothesis was that rates of N_{\min} in canopy soils of a tropical montane forest were higher on a *per mass basis* when compared to the forest floor soils (Matson *et al.* 2014). This linked directly to our **third hypothesis**, which

was that rates of N_{\min} and P_{\min} in canopy soils would contribute to higher total nutrient pools and fluxes in these forest stands; preliminary analyses by Tejo *et al.* (2014) suggested that canopy soils did increase forest N pools by 20%, and detectable extractable P was measured in canopy soils (Nadkarni 1984).

The **fourth** and final **hypothesis** was that canopy soils are sites of dynamic nutrient pools and fluxes which will leach plant available N and P, as well as TN and DOC, to the forest floor soils during high rainfall events. This hypothesis was driven by a past study that proved that the presence of epiphytic mats on bigleaf maple branches altered rainfall chemistry and increased certain mineral nutrient concentrations (Nadkarni 1986). Another study that contributed to formulating this hypothesis reported higher concentrations of $PO_4\text{-P}$ and $NH_4\text{-N}$ in stemflow from a common conifer in these ecosystems than measured in rainfall (Edmonds *et al.* 1991).

To execute this project, two riparian old-growth sites were selected in the Hoh and Queets temperate rainforests located in Washington state. Six trees in each stand were rigged for climbing access to the canopy ($n = 12$ trees). At each tree, a sampling plot was designated at the forest floor level near the base of the tree and another on a canopy branch. In the canopy soil plots, four trees were left at ambient conditions, four had a custom-designed partial rainfall-interception roof fixed to a branch, and four had a custom-designed gravitational irrigation system (Fig. 4.2). The experimental structures allowed for *in situ* manipulation of rainfall amounts at the canopy soil level to determine if experimentally manipulated increased or decreased rainfall conditions would change the rates of N_{\min} and P_{\min} compared to ambient conditions.

4.2 MATERIALS AND METHODS

4.2.1 Study area

Research was conducted from May 2018 through June 2019 in two old-growth temperate rainforests in western Washington State. One site is located in the Queets River Watershed (47.608°N, 124.051°W) on the western side of the Olympic National Park which has been described as one of the most structurally complex forests on the Pacific Northwest Coast (Van Pelt *et al.* 2006). The other site is located in the Hoh River Watershed (47.822°N, 124.205°W) on land owned by The Nature Conservancy that has been categorized as a highly productive old-growth forest (Tuo 2019).

The dominant conifer in both study areas is Sitka spruce [*Picea sitchensis* (Bong.) Carrière]. Bigleaf maple is the dominant deciduous species and can account for up to 34% of total forest cover (Canfield 1941). Western hemlock [*Tsuga heterophylla* (Raf) Sarg.], red alder (*Alnus rubra* Bong.), and Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] are scattered amongst the dominant trees. The understory is dominated by vine maple (*Acer circinatum* Pursh), redwood sorrel (*Oxalis oregana* Nutt.), salmonberry (*Rubus spectabilis*) and sword fern (*Polystichum munitum* [Kaulf.] C. Presl). Some of the dominant bryophytes established the canopy include hanging moss (*Antitrichia curtispindula* [Hedw.] Brid.) and tree moss (*Isoetes* spp.). The dominant vascular epiphytes include Oregon spikemoss (*Selaginella oregana* D.C. Eaton) and licorice fern D.C. Eaton (*Polypodium glycyrrhiza*).

Both sites experience cool, wet winters and warm, dry summers. The mean annual rainfall is ca. 3000 mm (though higher rainfall inputs were recorded in some sampling plots). The majority of rainfall occurs between October and June (O'Keefe and Naiman 2006). The soil parent material at both the Hoh and Queets sites is mixed alluvium. The forest floor soils in the Queets

stand are composed of the Huel soil series (a fluvial Entisol soil order) and the Tealwhit soil series (an aquic Inceptisol soil order) (Bechtolf and Naiman 2009), whereas the soils in the Hoh stand are composed of the Huel soil series and the Hoh soil series (an udic Inceptisol soil order). The soil drainage in both forest watersheds ranges from poor to well drained depending on the area. The canopy soils have been described as an arboreal Histosol (Nadkarni *et al.* 2002, Enloe *et al.* 2006), and specifically the bigleaf maple canopy soils have been classified as a Typic Haplohemist (Tejo *et al.* 2014). Both the forest floor and canopy soils are typically classified as acidic soils, even though the forest floor soils are less acidic (pH values ranging from 5.4-6.3; Edmonds 1991) compared to canopy soils where the pH ranges from 4.6-5.0 (Tejo *et al.* 2014).

4.2.2 Canopy access and experimental plots

The research was conducted in the two old-growth forests located in the Hoh and Queets watershed. Six trees were selected ($n = 12$) at each site. Each tree was assigned two plots for soil sampling (canopy soil and forest floor soil). For this study, it was not possible to have a complete randomized design for selecting trees because the structural integrity of trees must be assessed for safety measures. Based on 10 years of climbing experience, the team assessed and selected all bigleaf maple trees in the stand that were suited for safe climbing (e.g., no major leaning) and that had large branches capable of holding the experimental rainfall structures. Out of the identified trees, six trees in each watershed were randomly selected for climbing and were subsequently rigged for access to the canopy. To access the canopy for experimental set-up and soil sampling, single-rope climbing techniques were used (Perry 1978).

4.2.3 Throughfall rainfall experiments

In order to manipulate rainfall input at the canopy soil level, four canopy plots were used as ambient controls, four had a custom designed roof fixed to a branch to create a rain-interception structure, and four had a custom designed gravitational irrigation system (Fig. 4.2). Experimental structures were designed to manipulate a fixed surface area (1 m^2) on each selected branch. The structures either diverted roughly 1/2x of the rainfall from the branch (roof), i.e., decreasing rainfall input by half, or an irrigation system increased rainfall input to twice what would have normally fallen on a branch (irrigation). For the 1/2x throughfall treatment, the spacing and size of the gutters were designed to reroute half the throughfall rainfall off the covered area holding the canopy soils. The structures were built out of transparent acrylic and polyethylene plastics and had an open design to allow for light penetration and air flow.

For the 2x throughfall treatment, custom built gravitational irrigation collection buckets with a fixed surface area of 1 m^2 were hung indirectly above the branch (i.e., natural rainfall interception was not compromised) and irrigation tubing was cut to cover the 1 m^2 experimental surface area of the branch located below. This allowed the branch to intercept normal throughfall during a rain event while the irrigation system redistributed roughly the same amount to the designated area. The experimental structures were attached to the branches in April 2018 and allowed to equilibrate one month before soil sampling. The structures were left in place for the entire duration of the experiment (May 2018-June 2019).

4.2.4 Soil temperature, local throughfall, bulk density, and estimated canopy surface area

To measure local microclimatic variables, a HOBO weather station (Onset LLC) equipped with temperature sensors was deployed on each of the 12 trees. The weather station was secured

in the canopy and extension cables were used to insert the sensors in both canopy soils and forest floor soils. At each site, two rain gauges were also calibrated, connected to a weather station (Onset LLC), and randomly placed under the forest canopy to determine the average local throughfall rainfall. Weather stations were programmed to log climate data every 30 minutes during the one-year duration of the project. Data were offloaded, imported, and saved to a computer during each sampling period.

To calculate mineralization rates of the canopy soils on a hectare basis, soil mass (mg/kg) was converted to an areal unit (kg/ha) using bulk density (BD) measurements from each respective plot. To determine canopy soil BD, a volume of soil ranging from 103-271 cm³ was collected three times from each canopy branch. To determine forest floor BD, a soil corer with fixed volume liners was used to collect three BD samples around the base of each tree. Each BD sample was placed in a labelled bag. Once back at the lab, all soils were placed in a drying oven for 48 hours at 65°C to determine dry weights. Average BD was calculated on a per plot basis; i.e., each canopy branch plot and each forest floor plot was assigned the average BD calculated from the triplicate soil samples collected from their respective plots.

An estimation of canopy soil surface area was required to convert the canopy soil N_{\min} and P_{\min} values into contents (kg ha⁻¹ yr⁻¹). This was done by randomly selecting and delineating a circular plot with an area of 1/3 ha (radius = 106.9 m) within each site. All old growth bigleaf maple trees within the plot were carefully observed from the ground by the same individual for consistency (K. Mafune). Due to many of the trees being unsafe to climb, measuring tapes and reference measurements from trees that were safe to be assessed were used to conservatively estimate length and width of each canopy soil branch on all individual trees within the circular plots. Surface area for each old-growth stand was estimated by calculating total surface area for

each branch on each tree, and summing those values to approximate canopy soil surface area per hectare for the bigleaf maple trees.

4.2.5 Net nitrogen and phosphorous mineralization

Rates of N_{\min} and P_{\min} were determined seasonally *in situ* using the tube incubation method (Raison *et al.* 1987). This method is commonly used for determining rates of N_{\min} and has been adapted to determine rates of P_{\min} (e.g., Noe 2011, Noe *et al.* 2013). For consistency, the tube method was used to determine seasonal rates of N_{\min} and P_{\min} . In each canopy soil plot, six polyvinyl chloride (PVC) tubes with a diameter of 3.8 cm were inserted within the experimental area on each manipulated branch. For forest floor soils, six tubes were randomly inserted within a 3 m radius of the host tree's trunk at each tree plot. All cores were inserted at a depth of 15 cm in the organic canopy soil and the A horizon at the forest floor level. Sampling from the A horizon at the ground forest floor level is justified because the pure organic (O) horizon in these forests was observed to be very shallow and most of the O material was a fibric Oi horizon (leaves, twigs, needles with little decomposition). The A horizon (topsoil) was observed to be deep enough at each site and represented a mix of organic matter and mineral soil. Therefore, if an O layer was present it was carefully removed prior to inserting the tubes into the A horizon.

Rates of N_{\min} and P_{\min} were determined from samples collected in May-June 2018 (spring 2018), August-September 2018 (summer), October-November 2018 (fall 2018), and again in May-June 2019 (spring 2019). Due to high wind, high rainfall, and snow on branches, it was not safe to sample during the winter dormant season. At the start of each sampling period, six cores were inserted in each sampling plot. Three of those tubes with soil cores intact were immediately removed, labeled, bagged, placed in a cooler, and transported back to a cold room (4°C) located

at the University of Washington's campus. The other three tubes were left in place and the top of the tube was sealed with breathable plastic bags and rubber bands to avoid throughfall inputs while allowing for gas exchange. The soils were then incubated *in situ* for 30 days. Upon collection, tubes with soil cores intact were labelled, bagged, placed in a cooler, and transported back the cold room.

Once samples were back in the lab, $\text{NH}_4\text{-N}$, $\text{NO}_3\text{-N}$, and $\text{PO}_4\text{-P}$ were extracted within three days of sampling. The KCl method (Keeney and Nelson 1982, Van Miegroet 1995) was used to extract $\text{NH}_4\text{-N}$ and $\text{NO}_3\text{-N}$. Briefly, 2g from each triplicate plot sample (6 g total) was combined in a sterile 50 ml centrifuge tube and 30 ml of 2M KCl was added. The tubes were shaken for one hour on a table shaker, and then the solution was filtered into a sterile 50 ml centrifuge tube free of debris using Whatman 1440-090 ashless filter papers placed on acid-washed funnels. For $\text{PO}_4\text{-P}$, the Bray-P1 method (Bray and Kurtz 1945) was selected because both canopy and forest floor soils in these ecosystems are acidic. Briefly, 2.22 g of ammonium fluoride was dissolved in deionized water, 5 ml of hydrochloric acid was added, and the solution was diluted to 2 L. Subsequently, 1 g of each triplicate plot sample (3 g total) was combined in a sterile 50 ml centrifuge tube and 21 ml of Bray-P1 solution was added. The tubes were shaken on a table shaker for 5 minutes, and the solution was filtered using the same procedure noted above. Samples were stored at 4°C for no more than three days prior to being analyzed for $\text{NH}_4\text{-N}$, $\text{NO}_3\text{-N}$, and $\text{PO}_4\text{-P}$ at the School of Environmental and Forest Sciences Soil Analytic Lab (SEFS SAL). Concentrations of $\text{NH}_4\text{-N}$ and $\text{NO}_3\text{-N}$ were measured using an auto-analyzer (Perstorp Analytical 500 Series Flow-injection. Silver Spring, MD), and $\text{PO}_4\text{-P}$ was measured using ion chromatography (Dionex DX-120, Thermo Fisher Scientific, Sunnyvale, CA).

When triplicate soil samples were being pooled for extraction, a sub-sample was taken from each individual sample to determine wet weight for all field moist soil samples. Subsequently, the weighed soils were placed in soil tins and put in a drying oven at 65°C for 36 hours to determine dry weight and calculate percent gravimetric water content (going forward referred to as soil moisture content). It was confirmed that 36-hours of drying was a long enough duration by periodically weighing samples and observing if there was any more weight loss. Wet and dry weights were also used to calculate the average dry:wet ratio for each pooled sample and convert concentrations from mg/L to mg/kg dry soil. Further, canopy and forest floor soil bulk densities (BD) were used to convert concentration (mg/kg dry soil) to content (kg/ha), and the surface area correction factor was applied to canopy soil values to produce kg/ha for canopy soils in a hectare of the watershed.

To determine seasonal rates of Net N_{\min} and P_{\min} , mineralization rates were determined over 30 days during spring 2018, summer 2018, fall 2018, and spring 2019. Those rates were divided by the number of days they had been incubated in the tubes to determine a daily rate of Net N_{\min} and P_{\min} . Those daily rates for the seasonal periods were then multiplied by 91.25 days to estimate seasonal Net N_{\min} and P_{\min} rates. Then annual Net N_{\min} and P_{\min} rates were calculated by summing the seasonal mineralization rates.

4.2.6 Canopy soil leachates under ambient conditions

To quantify what resources may be leached from canopy soils to the forest floor during a short period of heavy rainfall, custom-designed leachate collection gutters (Fig. 4.3) were fastened underneath a branch in each of the 12 trees in spring 2019. All leachates were collected at ambient conditions. Briefly, these structures were designed by cutting vinyl open-top roof gutters into 1 m segments and capping the ends. Subsequently, a sterilized collection container

was glued to the bottom side of the gutter, and four small holes were drilled into the bottom to allow water to drain while filtering out most debris. After the gutters were attached under each branch perpendicular to the linear axis of the branch, 1 L of deionized water was slowly poured onto a 0.2 m² section of canopy soil above the point of attachment. The water then percolated through the canopy soil and the leachates accumulated in the collection container. The gutters remained in place for 60-minutes prior to carefully removing, capping, and labelling each leachate sample. Samples were placed in a cooler and transported back to the University of Washington and stored at 4°C for no more than three days.

To determine the concentration of NO₃-N, NH₄-N, PO₄-P, Total N, and DOC (mg/ml) in the leachates, the solutions were filtered into a sterile 50 ml centrifuge tube using Whatman 1440-090 ashless filter papers fitted in acid-washed funnels. The filtered solutions were stored at 4°C for ~24 hours prior to being analyzed at SEFS SAL. Blanks (control) were processed and analyzed with each set of extractions. DOC and TN were measured using a DOC analyzer with an added TN unit (VCSH model, Shimadzu Co., Kyoto, Japan). Concentrations of NO₃-N, NH₄-N, and PO₄-P were measured using the same equipment noted in the previous section.

4.2.7 Statistical Analyses

To calculate inorganic N and P concentrations and express them in mg/kg of dry soil, the original concentrations (mg/L) were calculated using the following equation:

$$\text{Concentration (mg/kg)} = ((E_{\text{amount}} \times (R_{\text{value}} - B)) / S_{\text{dry}})$$

Where E_{amount} is the amount of solution added to the soil sample to extract inorganic nutrients, R_{value} is the concentration in mg/L, B is the blank concentration, and S_{dry} is the weight

of dried soil extracted. To express concentrations (mg/kg dry soil) as content (kg/ha), the values were then multiplied by average bulk densities. To calculate canopy soil content, a correction factor to account for their limited surface area was applied.

To determine seasonal rates of N_{\min} and P_{\min} , concentration and content values from soil samples taken on day 1 of the 30-day incubation (T0) were subtracted from the values taken on day 30 (T1), respectively. This resulted in Net N_{\min} and P_{\min} over a 30-day period. The monthly values were then used to estimate the seasonal values by dividing the monthly value by 30 to get daily values, and then multiplying it by 91.25 to represent seasonal (quarterly) N_{\min} and P_{\min} . Annual N_{\min} and P_{\min} rates were then estimated, excluding the winter dormant season, by adding the averaged seasonal values together (spring, summer, fall, spring), respective to treatment and soil type. *Areal* rates (kg/ha) were calculated by multiplying *per gram* rates (mg/kg) by their respective BDs, and then canopy soil rates were further corrected by multiplying the calculated values by a 11.3% and 11.8% surface area correction factor for the Hoh and Queets stands.

All data were imported and further analyzed in Rstudio (RStudio Team v. 1.1.383, 2017) using the base R (R Core Team v. 3.4.3, 2017) package, unless otherwise noted. All plots were made using the *ggplot()* function (Wickham 2016). Statistical significance was based on 95% confidence ($\alpha= 0.05$) for all tests. First, data were subsetted by soil level (canopy and forest floor) and further organized by treatment groups to test if there were any correlations between P_{\min} and soil moisture, P_{\min} and soil temperature, N_{\min} and soil moisture, and N_{\min} and soil temperature using Spearman's rank correlation and the '*cor.test*' function in R.

Data were then grouped by each sampling period and summarized using the '*dplyr*' package (Wickham *et al.* 2021). Following this step, the Wilcoxon-Mann-Whitney test was used to test if soil moisture contents and temperatures were significantly different in canopy soils and forest

floor soils at ambient conditions. Then the Kruskal-Wallis test was used to determine if there was any significant effect of treatment on moisture contents and temperature across all seasons. To further explore moisture and temperature differences on a seasonal basis, data were subsetted by season and the Kruskal-Wallis test was implemented.

Then, the N_{\min} and P_{\min} were briefly explored as a multivariate dataset using the *vegan* package (Oksanen *et al.* 2013). First, the *'decostand'* function was used to standardize the N_{\min} and P_{\min} by range. Subsequently, the *betadisper()* function was used to test homogeneity of the multivariate dispersion. Then a non-metric multi-dimensional scaling (nmds) analysis was implemented using the *'global'* model, Gower distances, and four dimensions. To view any patterns, centroids for treatment and sampling period were calculated, and were used to cluster the data in ordination space. Additionally, the *envfit()* function was used to overlay moisture contour lines.

Following these steps, linear mixed models (LMM) were used to examine differences on rates of N_{\min} and P_{\min} between canopy soils and forest floor soils. This was done using the *nlme* package in R (Pinheiro *et al.* 2006). For the model, the two spring seasons were combined to account for the effect of seasonality instead of sampling period; fixed effects included temperature and moisture, and random effects included season and tree nested within site. LMMs were also used to determine the effect of treatment and season on temperature, moisture conditions, and N_{\min} and P_{\min} rates. For fixed effects, the treatments and their interactions with season were included, as well as temperature and moisture, and a random effect of tree within site. Both N_{\min} and P_{\min} rates were 'square power' transformed in order to meet the normality and homoscedasticity assumptions of the linear mixed model. Predictor variables were not highly

correlated (variance inflation factor < 2.5). Coefficients with 95% confidence intervals for the slope parameter (β) that did not overlap zero were considered to be important predictors.

LMMs were chosen using backward selection, initiating with the model containing all hypothesized fixed effects and their interactions (Zuur *et al.* 2009). Information-theoretic approach for model selection with Akaike Information Criterion, corrected for small sample sizes (AICc) as a measure of model parsimony (Burnham and Anderson 2002), was relied upon to determine the quality of the model's results. The optimal random effects-structure was determined with the likelihood ratio test of restricted maximum likelihood (REML) estimates for each model (Zuur *et al.* 2009). In addition, the significance of random effects in the LMMs were evaluated by using a parametric bootstrapping approach (1000 simulations). Models containing no random effects were fit as general linear models using the *stats* package in R, and models with random effects were fit as linear mixed-effect models using the *lme4* package (Bates *et al.* 2015) in R. For all models, model diagnostics were run to ensure that residuals were normally distributed, that residuals had a constant variance or homoscedasticity, and residuals' error terms were independent.

4.3 RESULTS

4.3.1 Soil microclimate conditions

Under ambient conditions, the canopy and forest floor soil mean annual moisture contents were significantly different ($p \leq 0.01$, Figure 4.4b). The mean annual soil moisture content in canopy soil environments was 47%, which was significantly higher than the 31% measured at the forest floor soil level ($p \leq 0.01$); the highest significant differences between the canopy and forest floor soils were recorded in spring 2018 ($p \leq 0.01$) and fall 2018 ($p \leq 0.01$). Site had a

significant effect on mean annual moisture contents at the forest floor level ($p \leq 0.01$, Fig. 4.4b). The Queets had an average annual moisture content of 28% in the forest floor soils compared to 35% at the Hoh site.

At ambient conditions, there were no significant differences in mean annual temperatures between the canopy and forest floor soils. Canopy soils had higher, but not significant, average temperatures compared to the forest floor soils during the spring and summer of 2018 and spring of 2019, and lower average temperatures in fall of 2018 (Fig. 4.4a). Site did have an effect on forest floor soil temperatures at ambient conditions. At the canopy soil level, neither treatment or site had a significant effect on soil temperatures.

When exploring moisture patterns across canopy soil treatment levels, site did not have a significant effect on canopy soil moisture contents under any of the treatments ($p > 0.05$). Treatment also did not have a significant effect on mean annual canopy soil moisture contents ($p > 0.05$). Annual soil moisture content was highest under the irrigation treatment, followed by the roof-interception treatment, and then ambient conditions; demonstrating the although rainfall was diverted off that fixed-area on the branch, soil moisture content remained higher than those measured at ambient conditions. Though moisture content was slightly higher under both treatments, there were no significant differences. The annual average soil moisture content was 58% under the irrigation treatment, 55% under the roof-interception treatment, and 47% under the ambient treatment. Seasonality did have an effect on canopy soil moisture contents, therefore the data were subsetted and the effect of treatment was tested on an individual seasonal basis.

Annual throughfall inputs to the forest floor averaged 3789 mm (± 454 mm). The highest amount of throughfall rainfall input to the forest floor occurred during fall [November-

December] 2018 and early winter [December-January], with the lowest levels of throughfall input recorded in the summer [June – August] of 2018 (Fig. 4.5).

4.3.2 Seasonal N_{\min} and P_{\min} at ambient conditions in canopy and forest floor soils

There was no significant effect of site on N_{\min} and P_{\min} at ambient conditions or across canopy soil treatments, therefore it is not discussed further. Throughout the duration of the project, mineralization and immobilization rates were highly variable during the sampling periods (Table 4.1). When comparing net N_{\min} between canopy soils (at ambient conditions) and forest floor soils (Fig. 4.6a), forest floor soils had significantly lower rates of N_{\min} ($\beta = -38331$, $p \leq 0.01$). When examining this trend by season, forest floor soils had significantly lower rates of N_{\min} than canopy soils in the fall ($\beta = 24021$, $p \leq 0.05$) and spring ($\beta = 34452$, $p = 0.01$), but not during the summer season, which is when one might expect microbial processes to slow down in this drier time of the year. In the spring sampling season of 2019, net N immobilization ($-19.8 \pm 14.1 \text{ mg kg}^{-1} \text{ season}^{-1}$) was observed at the forest floor level, due to a large immobilization of $\text{NH}_4\text{-N}$ ($-21.9 \pm 13.0 \text{ mg kg}^{-1} \text{ season}^{-1}$). During the fall 2018 season, rates of N_{\min} in the canopy soils at ambient conditions were $159.9 \pm 70.5 \text{ mg/kg}$, compared to $33.8 \pm 9.1 \text{ mg/kg}$ at the forest floor level. In the summer sampling season, rates of N_{\min} were higher in canopy soils left at ambient condition when compared to N_{\min} in the forest floor soils, but these differences were not statistically significant.

At ambient conditions, rates of P_{\min} were significantly different between canopy soils and forest floor soils, with significantly lower rates measured in the forest floor soils ($\beta = -139.4$, $p \leq 0.05$). When looking at seasonal trends (Fig. 4.6b), P_{\min} was significantly higher in the spring sampling seasons ($\beta = 174.5$, $p = 0.01$). In canopy soils, P_{\min} was observed during spring 2018

(466.4 ± 97.2 mg/kg), fall 2018 (160.0 ± 37.0 mg/kg), and spring 2019 (354.7 ± 188.5 mg/kg). However, there was immobilization during summer of 2018 (-359.9 ± 51.7 mg/kg). When comparing P_{\min} in canopy soils and forest floor soils at ambient conditions, there was more mineralized $PO_4\text{-P}$ in canopy soils during all seasons except summer, when immobilization of $PO_4\text{-P}$ was observed in the canopy whereas mineralization of $PO_4\text{-P}$ was observed in forest floor soils (13.6 ± 22.7 mg/kg).

4.3.3 Canopy soil and forest floor P_{\min} , N_{\min} , moisture, and temperature

Prior to subsetting data by treatment group, the canopy data were explored for general correlations. There was a moderate positive correlation between soil moisture and rates of N_{\min} ($R = 0.47$, $p < 0.01$, $p = 0.05$), as well as a moderate positive correlation between soil moisture and rates of P_{\min} ($R = 0.28$, $p = 0.05$). There was also moderate negative correlation between N_{\min} and soil temperature ($R = -0.48$, $p < 0.01$), but no clear pattern between P_{\min} and soil temperature. To account for patterns within each treatment, canopy soil P_{\min} and N_{\min} data were organized by treatment to test for potential relationships between N_{\min} and both soil moisture and soil temperature, followed by P_{\min} and both soil moisture and soil temperature. Under all three manipulated throughfall treatments in the canopy soils, there was a weak to moderate positive correlation between both N_{\min} and soil moisture (Fig. 4.7a) and P_{\min} and soil moisture (Fig. 4.7c). In contrast, all three treatments had a weak to moderate negative correlation when observing the relationship between soil temperature and N_{\min} (Fig. 4.7b). The correlations between P_{\min} and soil temperature were also negative for all three treatments. Though Spearman's correlation coefficient demonstrated that there was little to no correlation among P_{\min} and soil temperature

under the 1/2x and 2x throughfall treatments, there was a weak negative correlation under ambient throughfall conditions (Fig. 4.7d).

4.3.4 Seasonal N_{\min} and P_{\min} under canopy soil rainfall experiments

In canopy soils, N_{\min} was observed under all three treatments during spring 2018, fall 2018, and spring 2019 sampling periods (Fig. 4.9, Table 4.2). Immobilization of $\text{NH}_4\text{-N}$ ($-12.1 \pm 26.8 \text{ mg kg}^{-1} \text{ season}^{-1}$) was observed under ambient conditions in spring 2018, however the amount of mineralized $\text{NO}_3\text{-N}$ ($58.1 \pm 19.5 \text{ mg kg}^{-1} \text{ season}^{-1}$) resulted in net N_{\min} for that sampling period. In summer, N mineralization was observed under the ambient ($28.8 \pm 48.9 \text{ mg kg}^{-1} \text{ season}^{-1}$) and 1/2x throughfall ($68.3 \pm 37.7 \text{ mg kg}^{-1} \text{ season}^{-1}$) treatment, but N immobilization was observed under the 2x throughfall treatment ($-22.6 \pm 18.3 \text{ mg kg}^{-1} \text{ season}^{-1}$). This resulted from seasonal immobilization of $\text{NH}_4\text{-N}$ ($-32.9 \pm 16.4 \text{ mg kg}^{-1} \text{ season}^{-1}$).

The homogeneity of dispersions among treatment and season were significantly different ($p < 0.05$), demonstrating that the group dispersions were heterogeneous. The NMDS analysis (stress = 0.03) displayed clear grouping by sampling period (Fig. 4.8a) and some general patterns across treatments (Fig. 4.8b). In both ordination plots, there was evident heterogeneity among samples and patterns related to moisture content.

Following these multivariate analyses, the data were subsetted into seasonal groups and the two spring seasons were combined to further model how the rainfall treatments and season affected rates of N_{\min} and P_{\min} . For rates of N_{\min} , treatment had a significant effect with N_{\min} rates recorded as significantly higher with the roof treatment ($\beta = 193.5$, $p = 0.01$), and even higher with the irrigation treatment ($\beta = 212.5$, $p \leq 0.01$), which reflects the higher moisture contents that both these treatments induced.

Season also influenced the effect of treatment on rates of N_{\min} . For the fall and spring seasons, the irrigation treatment increased N_{\min} , but during summer, the irrigation treatment decreased rates of N_{\min} . During the fall and spring the roof treatment increased N_{\min} rates, and the effect of the roof treatment was negligible during the summer months. In regards to rates of P_{\min} , both the roof and irrigation treatment had a significant effect on P_{\min} rates during the spring months ($\beta = 682.76, p \leq 0.01$ and $\beta = 474.96, p = 0.05$, respectively). The roof treatment had a greater effect on P_{\min} rates than did the irrigation treatment.

4.3.5 Annual rates of N_{\min} and P_{\min}

When comparing annual N_{\min} and P_{\min} rates (mg/kg/yr) on a soil *mass basis* in canopy soils and forest floor soils under ambient conditions (Table 4.2), N_{\min} and P_{\min} rates were higher in canopy soils (Fig 4.10a). The canopy soils had $143.7 (\pm 21.5)$ mg $\text{NO}_3\text{-N kg}^{-1} \text{ yr}^{-1}$ and $211.7 (\pm 46.2)$ mg $\text{NH}_4\text{-N kg}^{-1} \text{ yr}^{-1}$ mineralized on an annual basis, which totaled a rate of N mineralization of $355.3 (\pm 54.7)$ mg Net N $\text{kg}^{-1} \text{ yr}^{-1}$. The annual N mineralization rates in the forest floor A horizon were $58.2 (\pm 3.9)$ mg $\text{NO}_3\text{-N kg}^{-1} \text{ yr}^{-1}$ and $9.4 (\pm 8.6)$ mg $\text{NH}_4\text{-N kg}^{-1} \text{ yr}^{-1}$, totaling $67.6 (\pm 9.5)$ $\text{kg}^{-1} \text{ yr}^{-1}$. In canopy soils, annual P_{\min} was $945.2 (\pm 93.6)$ mg $\text{kg}^{-1} \text{ yr}^{-1}$. In the forest floor A horizon, annual P_{\min} was $387.6 (\pm 34.5)$ mg $\text{PO}_4\text{-P kg}^{-1} \text{ yr}^{-1}$. This suggested that annually canopy soils have 5.3x more mineralized Net N and 2.4x more mineralized $\text{PO}_4\text{-P}$ on a *per mass basis* compared to mineralization rates measured in the forest floor A horizon.

Mean annual rates of N_{\min} and P_{\min} among the canopy soil rainfall treatments (Table 4. 3) had the highest rates of N_{\min} and P_{\min} under the 2x rainfall conditions, followed by 1/2x rainfall conditions, and then the lowest rates at ambient conditions, which was consistent with the increase-decrease patterns of moisture content. Under 2x conditions, there was $110.8 (\pm 8.6)$ mg

$\text{NO}_3\text{-N}$ $\text{kg}^{-1} \text{yr}^{-1}$ and $611.7 (\pm 61.7) \text{ mg NH}_4\text{-N kg}^{-1} \text{yr}^{-1}$ mineralized, totaling an annual N_{min} rate of $722.5 (\pm 66.0) \text{ mg kg}^{-1} \text{yr}^{-1}$; annual P_{min} was $1951.9 (\pm 148.4) \text{ mg kg}^{-1} \text{yr}^{-1}$. Under 1/2x conditions, there was $237.1 (\pm 9.9) \text{ mg NO}_3\text{-N kg}^{-1} \text{yr}^{-1}$ and $442.6 (\pm 85.1) \text{ mg NH}_4\text{-N kg}^{-1} \text{yr}^{-1}$ mineralized, totaling an annual N_{min} rate of $679.7 (\pm 90.4) \text{ mg kg}^{-1} \text{yr}^{-1}$; annual P_{min} was $1812.5 (\pm 169.2) \text{ mg kg}^{-1} \text{yr}^{-1}$. When comparing to canopy soils at ambient conditions, annual N_{min} and P_{min} increased 2-fold and 1.9-fold and 2-fold and 1.8-fold under the irrigation and roof-interception treatment, respectively.

Mean annual mineralization rates *per mass* ($\text{mg kg}^{-1} \text{yr}^{-1}$) for N and P were converted to an *areal basis* ($\text{kg ha}^{-1} \text{yr}^{-1}$) for both canopy soils and the forest floor soil A horizon, as shown in Table 4.3. Although canopy soils have higher annual mineralization rates when expressed in units of *soil mass*, this is not observed when canopy soil annual mineralization rates are converted to an *areal basis* (Fig. 4.10b). This is a result of the surface area correction factor that was applied to account for the limited surface area of bigleaf maple canopy soils in a hectare. As a result, annual N_{min} in canopy soils at ambient conditions is $5.3 (\pm 1.3) \text{ kg ha}^{-1} \text{yr}^{-1}$ and P_{min} is $13.7 (\pm 1.4) \text{ kg ha}^{-1} \text{yr}^{-1}$. Canopy soils under increased and decreased rainfall conditions had an annual N_{min} rate of $13.3 (\pm 1.0) \text{ kg ha}^{-1} \text{yr}^{-1}$ and P_{min} rate of $33.8 (\pm 3.0) \text{ kg ha}^{-1} \text{yr}^{-1}$, and N_{min} rate of $10.1 (\pm 2.5) \text{ kg ha}^{-1} \text{yr}^{-1}$ and P_{min} rate of $36.0 (\pm 5.2) \text{ kg ha}^{-1} \text{yr}^{-1}$, respectively. Annual N_{min} in the forest floor A horizon was $56.7 (\pm 10.9) \text{ kg ha}^{-1} \text{yr}^{-1}$ and P_{min} was $372.4 (\pm 37.9) \text{ kg ha}^{-1} \text{yr}^{-1}$.

4.3.6 Canopy soil leachates at ambient conditions

Nitrogen and P leachates ($\text{NO}_3\text{-N}$, $\text{NH}_4\text{-N}$, $\text{PO}_4\text{-P}$, and TN) and DOC were measured from ambient canopy soil branches ($N = 12$) during a simulated rainfall event (Table 4.4). Nitrate-N and $\text{NH}_4\text{-N}$ were leached at the lowest levels from canopy soils (Figure 4.11), with an average of

0.10 (\pm 0.05) mg/ml of NO₃-N and 0.48 (\pm 0.14) mg/ml of NH₄-N leachates. The average amount of total N leached was 2.02 (\pm 0.35) mg/ml. The average amount of PO₄-P leached was 0.66 (\pm 0.15) mg/ml. Total organic carbon was the most concentrated canopy soil leachate at 32.42 (\pm 4.41) mg/ml.

4.4 DISCUSSION

4.4.1 *Canopy soils as an experimental system*

Canopy soils are thick organic fibrous mats (Tejo *et al.* 2014) that are porous, consequently possessing similar attributes of a sponge and increasing moisture retention in the canopy environment when present (Nadkarni *et al.* 2002), influencing forest hydrological processes (Hargis *et al.* 2019). The majority of canopy soil volume is confined to the surface of tree branches, creating a separate soil system with host tree adventitious roots and other epiphytic roots, rhizoids, and rhizomes. The biotic and abiotic processes in canopy soils may not be completely decoupled from those in the forest floor soil environment, but they do offer a limited area of organic soil that can be experimentally manipulated to explore the mechanistic links between abiotic and biotic interactions that may be rendered obscure within the forest floor soil system.

4.4.2 *Seasonal patterns of canopy soil and forest floor soil microclimate at ambient conditions*

Canopy soils occur on tree branches and are not as insulated as forest floor soils from microclimatic fluctuations. As a result, they are less buffered from changes occurring in the surrounding environment. In past studies, they have been shown to have more extreme cycles of drying and rewetting (Coxson and Nadkarni 1995) during drought and high rainfall events, and greater fluctuations in soil temperatures (Aubrey *et al.* 2013). Until this study, how the

magnitude of micro-climatic shifts and changes in rainfall input impact ecosystem-level N and P dynamics in canopy soils remained unknown. Therefore, canopy soil moisture and temperature were recorded throughout the duration of this study to better understand how they may be linked to N and P dynamics.

In this study under ambient conditions, the canopy soils demonstrated higher moisture content during all sampling seasons, differing significantly from the forest floor A horizon in the spring 2018 and fall 2018 wet seasons ($p \leq 0.05$). When focusing in at the canopy soil level, the roof-interception and irrigation treatments also impacted moisture content, but in an unexpected direction. It was predicted that increasing precipitation amounts would through an irrigation system would increase soil moisture, and diverting precipitation off the branch would decrease soil moisture. Surprisingly, both treatments increased soil moisture when compared to ambient conditions.

This demonstrates that perhaps other physical processes that influence soil moisture content may be involved in these canopy soil systems when there is less precipitation to a fixed area relative to higher precipitation inputs in adjacent areas. For example, canopy soils are less buffered than forest floor soil environments; it is possible that they may have experienced a soil moisture content deficit when half the natural precipitation was initially diverted off the branch, causing them to reach a threshold that influenced the initiation of other physical processes that can also affect soil moisture content. Such as with less rainfall reaching a fixed area, there is the potential for more open canopy-soil pore space for fog-interception. Some other possibilities are that there was hydraulic redistribution either by diffusion of water from higher areas of moisture to lower areas of moisture, and/or maybe by the adventitious rooting system to the manipulated area. This has created questions that require more research. For example, there are canopy soils

at ambient conditions on each side of the specific areas of each treatment (i.e., 1/2x and 2x). So perhaps one needs to measure the moisture content in those adjacent ambient canopy-soil areas to see if they also changed because of being adjacent to different moisture levels (i.e., next to the 1/2x and 2x).

At the canopy soil level, the treatments had no impact on soil temperature. At ambient conditions, temperature was significantly different between canopy and forest floor soils during fall 2018 ($p \leq 0.05$). Though these differences in temperature were not significantly different during other seasons, a general pattern was observed regarding canopy soil temperature thresholds. During warm periods, canopy soil temperature was consistently warmer than forest floor soil temperature. However, during cold periods canopy soil temperature was consistently lower than that of forest floor soils. This supports the concept that canopy soils are less buffered environments. Further, the magnitude of fluctuation in canopy soils can cause shifts in fungal interactions (see Ch. 3), as these communities are pushed towards microclimatic thresholds that require an adaptive response.

Similar seasonal patterns for canopy soil moisture and temperature were reported in a neighboring old-growth temperate rainforest in a temporal study that took place from April 2010-March 2011 (Aubrey *et al.* 2013). A majority of our soil microclimatic measurements fit within the seasonal ranges of the previous study. However, when comparing temperatures in spring 2010 to spring 2018 and spring 2019, the canopy soil and forest floor soil averages increased by at least 2°C and 1°C, respectively. The average soil moisture in canopy soils decreased by 0.2% and 0.3% in spring 2018 and spring 2019 when compared to spring 2010; forest floor soil averages were comparable.

These canopy soil microclimatic patterns fit predictions that soil temperature is increasing over time because of global warming (Bradford *et al.* 2019, Zhang *et al.* 2019); consequently, causing a decrease in soil moisture (e.g., Lakshmi *et al.* 2003, Vogel *et al.* 2018). Both microclimatic shifts can impact mineralized pools of N (Cassman and Munns 1980, Knoepp and Swank 2002) and P (Thompson and Black 1948, Arenberg and Arai 2019). Although it becomes complicated to predict how increased temperature and decreased moisture may impact canopy soil mineralization rates. A few studies reported that mineralization rates were highest when temperatures increased (Son and Lee 1997, Knoepp and Swank 2002), whereas other studies have found it to be lower during warmer periods when soil moisture is low (Wong and Nortcliff 1999). However, it remains unknown if the observed changes fall within an expected range for temporal shifts in canopy soil environments. Temporal studies on changes in canopy soil microclimate remain limited, and more studies are needed to better understand if these systems are being pushed towards a threshold that may be threatening their functional integrity.

It has been reported that canopy soils can substantially increase nutrient pools found in these forest ecosystems (Nadkarni 1984, Nadkarni *et al.* 2004, Enloe *et al.* 2006, Cardelús *et al.* 2014). A research study conducted in this study's research location suggested that bigleaf maple canopy soils have a high amount of extractable N and low C/N ratio, suggesting a potential for high N_{\min} rates (Tejo *et al.* 2014). The aforementioned study conducted a onetime assessment of changes in mineralizable nutrients but did not research and study the temporal and seasonal changes in N_{\min} and P_{\min} in these particular forests, or how mineralized pools may be impacted by seasonal extremes. This temporal study built upon the concept that canopy soils can contribute significantly to ecosystem-level nutrient fluxes and that climate change may impact these forest processes. This suggest that canopy soils are more sensitive to temperature and moisture changes

over shorter periods of time (e.g., 5-10 years) which would then impact nutrient mineralization rates in this ecosystem compartment.

4.4.3 Mineralization rates of forest floor soils and canopy soils at ambient conditions

It was hypothesized that canopy soils would have higher N_{\min} and P_{\min} rates on a *per mass basis* because the thick organic mats are acting as nutrient reservoirs high above the forest floor where the organic soil layer is not well developed. When comparing seasonal N_{\min} and P_{\min} (*per mass basis*) in canopy soils and forest floor soils at ambient conditions, rates of N_{\min} and P_{\min} were greater in canopy soils in all instances except when there was a summer period of immobilization of $PO_4\text{-P}$. During that time, there was also immobilized $NO_3\text{-N}$ in canopy soils. However, the amount of mineralized $NH_4\text{-N}$ was roughly 3x greater in canopy soils than forest floor soils, which resulted in a net mineralization that was 1.6x greater than that of forest floor soils. In spring 2019, there was also immobilization of $NH_4\text{-N}$ in forest floor soils, resulting in net N immobilization for the season. This study also observed that $PO_4\text{-P}$ was immobilized in the forest floor soils during the same sampling period. Though there was immobilization at the forest floor level, 120.7 (\pm 65.5) mg/kg of $NH_4\text{-N}$ and 354.7 (\pm 188.5) mg/kg of $PO_4\text{-P}$ was mineralized in canopy soils. The fact that there was immobilization on the forest floor during the growing season suggests that old-growth bigleaf maples may be depending on their canopy soils for nutrient acquisition during periods of limited P availability.

A few other studies have compared N_{\min} in canopy soil environments to forest floor soils (Wanek *et al.* 2002, Pérez *et al.* 2005, Cardelús *et al.* 2009, Matson *et al.* 2014), and all concluded that canopy soils had higher or similar N_{\min} rates compared to forest floor soil cycles on a mass basis. From these studies, only one explored N_{\min} occurring in an old-growth temperate rainforest (Pérez *et al.* 2005). When comparing rates of seasonal N_{\min} to this study,

this study had higher rates of N_{\min} during all seasons. Especially in spring and summer seasons, this study showed net mineralization, whereas Pérez *et al.* (2005) demonstrated immobilization. It is not surprising that seasonal N_{\min} rates were different, because the other study was in the Southern Hemisphere at a higher elevation (600 m) and focused on canopy soils of an old-growth conifer. It is noteworthy that Pérez *et al.* (2005) found that immobilization prevailed over mineralization, and our study found the opposite in the old-growth temperate rainforests of western Washington.

The rates of forest floor N_{\min} in these temperate rainforests are much higher when compared to another temperate rainforest study in the Northern Hemisphere. Bisbing and D'Amore (2019) reported N immobilization in the temperate rainforests of Alaska over a 30-day study period during the growing season. To our knowledge, there are no studies that have determined seasonal rates of N_{\min} in the riparian old-growth temperate rainforests of western WA in the forest floor soil or the canopy soils.

4.4.4 Seasonal N_{\min} and P_{\min} under canopy soil experimental treatments

Seasonality had a strong effect on $\text{NO}_3\text{-N}$ and $\text{NH}_4\text{-N}$ mineralization processes. Rates of N_{\min} in canopy soils appeared to be heavily linked to soil moisture, which is consistent with studies that have explored N_{\min} in forest floor soils (e.g., Zhang *et al.* 2008, Contosta *et al.* 2011, Guntiñas *et al.* 2012) and canopy soils (Cardelús *et al.* 2009). Higher rates of N_{\min} in temperate forests during the summer season does occur (e.g., Powers 1990, Groffman *et al.* 2009, Contosta *et al.* 2011), however this study found the opposite patterns for all experimental treatments. In this study, N_{\min} was lowest during the summer season, with immobilized $\text{NO}_3\text{-N}$ at ambient conditions and net immobilization occurring under increased rainfall conditions due to immobilized $\text{NH}_4\text{-N}$.

An explanation for this may reflect the impact of increasing temperatures and summer drought on the coastal regions of the Pacific Northwest (Mote *et al.* 2014, Joyce *et al.* 2015), and the fact that canopy soils are likely more vulnerable to an increase in temperature and decrease in moisture levels. These increasing temperatures and periods of drought can cause shifts in how microbial communities function, since they are strongly influenced by these micro-climatic changes. For example, drought can increase fungal functional genes related to N-acquisition (Treseder *et al.* 2018) and can cause shifts in bacterial functional genes and community processes (Manzoni *et al.* 2012, Hammerl *et al.* 2019), which could consequently slow down processes involved in N transformations. Another explanation reflects the rainfall and fog regimes in these areas; rainfall is a crucial factor in processes that drive N_{\min} , including substrate diffusion, leaching, microbial transformations, and plant uptake (Jongen *et al.* 2013, Cregger *et al.* 2014, Chen *et al.* 2019, Li *et al.* 2019). Therefore, seasons with high amounts of rainfall could potentially influence higher rates of N_{\min} .

In general, pools of $\text{NH}_4\text{-N}$ accounted for a large percentage of N_{\min} rates. However, immobilization of $\text{NH}_4\text{-N}$ (i.e., a disappearance of $\text{NH}_4\text{-N}$ during the incubation period attributed to microbial assimilation) was observed at ambient conditions in spring 2018. Although moisture content was lower at ambient conditions compared to the other two treatments during this sampling period, there was no clear explanation for the immobilization of $\text{NH}_4\text{-N}$ at the canopy soil level because soil moisture content was lower in spring 2019 compared to spring 2018, and mineralization of $\text{NH}_4\text{-N}$ occurred at ambient conditions during that sampling period.

Immobilization of $\text{NH}_4\text{-N}$ was also observed under the 2x rainfall conditions in summer. This was the warmest sampling season, and the 2x rainfall conditions had the highest soil moisture

content of the three treatments. This indicated that there may be a moisture threshold for mineralization when temperatures are high.

Studies on P_{\min} in temperate forests are much more limited relative to studies on N_{\min} . However, it has been demonstrated that warming showed negligible changes in P_{\min} (Grierson *et al.* 1999, Menge and Field 2007, Rinnan *et al.* 2007, Zhang *et al.* 2013). In this study, seasonality had a strong effect on rates of P_{\min} (Fig. 4.9). Though not significant, P_{\min} was more closely correlated with soil moisture than soil temperature. Mineralization was observed during all seasons under all treatments, excluding summer where there was immobilization in canopy soils under all experimental conditions. An explanation for this may be that drought can increase the amount of inorganic P bound to secondary minerals and alter mechanisms that replenish soil inorganic P (Zhang *et al.* 2020). Another explanation is that higher temperatures can influence microbial metabolisms, which will increase nutrient sequestration (Jonassen *et al.* 1993), reducing the amount of available nutrients, such as $PO_4\text{-P}$.

One of the main hypotheses of this research was that the manipulated rainfall conditions would impact rates of N_{\min} and P_{\min} in canopy soil. When modeling rates of N_{\min} and P_{\min} , treatment did have a significant effect on rates of N_{\min} and P_{\min} during the spring and fall seasons and fall season, respectively (Fig. 4.9c, d). One of the theories behind this hypothesis was that when half the rainfall was diverted off a fixed area of canopy soil, that the soil moisture levels would decrease; especially because experiments that implement rainfall exclusion structures with somewhat similar designs were proven to decrease soil moisture (Sardans and Peñuelas 2004, Kundell *et al.* 2018). However, the opposite was observed in this study's canopy soils that were manipulated with the roof treatment. Canopy soils that had the roof-interception structure had higher soil moisture content than those at ambient conditions.

It was clear that soil moisture was a driver of both N_{\min} and P_{\min} , however the underlying mechanisms of why canopy soil moisture content was not lower under decreased levels of rainfall remain unknown. One possible explanation for higher moisture content under the roof treatment could be that, when rainfall is diverted, there is more exposed surface area for fog water to condense on and be distributed to the pore spaces. Another potential explanation could be that the adjacent non-manipulated areas of canopy soil are providing a source of water that could be diffused to those canopy soils under the roof-interception structures by hydraulic redistribution and water movement through the epiphytes and adventitious rooting system. Also, there is a potential that the roof initially decreased soil moisture immediately after a rainfall event, but due to evaporation and the porous nature of canopy soils, capillary action may have occurred increasing soil moisture content during the time measurements were taken. However, more research is required to better understand this phenomenon. Nevertheless, the treatment did manipulate soil moisture, albeit in an unexpected direction, and therefore remains informative about the system's response to altered moisture conditions

There was high variability observed within and among treatments in this study. One explanation for high variability may reflect spatial heterogeneity among soil N and P (Robertson *et al.* 1988, Lovett and Rueth 1999, Wang *et al.* 2007, Zhou *et al.* 2018). Another explanation could reflect the different types of litter inputs to canopy soil environments. Though litterfall returns are dominated by senesced leaves of bigleaf maple (Tejo *et al.* 2015), canopy soil chemistry could be influenced by various other litterfall inputs that are unique to each canopy soil environment. For example, some maples may be closer to red alder stands, whereas some may be closely surrounded by Sitka spruce. High variability of inorganic N and P in canopy soils may also be attributed to nutrient hotspots. Hotspots can result from varying abundances of

microbial feeding fauna (Griffiths 1994), shifts in microbial community activity (Kuzyakov and Blagodatskaya 2015), an increase in fungal decomposition, and interactions among these organisms. However, there is very little research from canopy soil environments that may help further explain this, therefore the emphasis of more studies on canopy soil nutrient dynamics are warranted.

The vast changes in N_{\min} and P_{\min} between spring 2018 to spring 2019 sampling periods also suggested that treatment likely impacted processes involved in N and P nutrient transformations after one year of the experimental manipulation. This is based on the observation that the pool of mineralized N increased by a magnitude of 100 after a year of rainfall levels were doubled (spring 2018 vs. spring 2019), whereas rates only increased by a magnitude of 2.6 at ambient conditions and under the roof-interception treatment. It is acknowledged that rates of N_{\min} were relatively low under the irrigation treatment in spring 2018, but if rates of N_{\min} in spring 2019 are compared to ambient conditions in spring 2018, the mineralized N pool was greater by a magnitude of 10.7 after enduring increased rainfall conditions for one year. Soil moisture content was observed to be a driver of these rates. Moisture was comparable for both the irrigation and roof treatment in spring 2019, but the irrigation treatment had higher rates compared to the roof treatment. This suggested that there are other underlying processes that may have been impacted differently by the two treatments.

When comparing mineralized pools of $PO_4\text{-P}$ between the spring 2018 and spring 2019, there was a widespread decrease in P_{\min} rates. At ambient conditions, there were no significant changes in P_{\min} (1.3-fold decrease in P_{\min} after one year). However, under increased rainfall conditions there was a 2.4-fold decrease in rates of P_{\min} after one year, and a large 12.3-fold decrease under decreased rainfall conditions. During the time of these measurements, soil

moisture contents under the two treatments were comparable; but P_{\min} at ambient conditions showed only a negligible change and had lower soil moisture content compared to the other two treatments. Therefore, it seems that the processes involved in P transformations may reach a moisture threshold that impact P dynamics.

4.4.5 Annual N_{\min} and P_{\min} on a soil mass vs. areal basis

Although it was hypothesized that canopy soils would have higher N_{\min} and P_{\min} rates on a *per mass basis*, the opposite was hypothesized for estimates based on an *areal basis*. This is because canopy soils have a limited surface area per hectare when compared to forest floor soils. When assessing canopy soil annual N_{\min} and P_{\min} at ambient conditions on a *per mass basis*, canopy soils contributed significantly higher rates of N_{\min} and P_{\min} compared to the forest floor soils. However, due to canopy soils being limited to branches and crotches of trees, they contribute much less when converted to an *areal basis*.

To convert canopy soils to an *areal basis*, bulk densities were measured and correction factor was established by estimating the total surface area of canopy soils in the two forest stands. It was further confirmed that the surface area estimation of the canopy soils in this study was within the range found in another unpublished case study of a single bigleaf maple (Van Pelt, unpublished data). This comparison was accomplished by scaling-up that single tree estimation to this study's stand level that was based on canopy soil surface area per tree and trees per area. Also another concurrent study independently estimated canopy soil surface area/ha (Stone 2021) and found a similar percent of canopy soil surface area per hectare.

On a *per mass basis*, both increased and decreased rainfall conditions had higher annual rates of N_{\min} and P_{\min} than ambient conditions; this is likely because both these treatments

increased soil moisture content. All three treatment conditions had higher annual rates than forest floor soils, as well. Under increased rainfall conditions, annual N_{\min} and P_{\min} were both 2-fold higher than those measured at ambient canopy conditions. Under decreased rainfall conditions, annual N_{\min} and P_{\min} were both 1.9-fold higher than ambient canopy conditions.

When comparing ambient canopy soils to forest floor soils, annual N_{\min} and P_{\min} were greater in canopy soils by a magnitude of 5.3 and 2.5, respectively. Based on these observations, the hypothesis that canopy soils mineralize more N and P *per mass* of soil was accepted, which is consistent with N_{\min} in other canopy soil studies (e.g., Cardelús *et al.* 2009, Matson *et al.* 2014). To our knowledge there are no existing studies on P_{\min} rates in canopy soils that can be used for comparative purposes, but P_{\min} was much greater in canopy soils when compared to P_{\min} in dead organic matter from a maritime pine forest (Achat *et al.* 2010) and P_{\min} from the organic horizon in temperate forest soils (Pistocchi *et al.* 2018).

Annual mineralization rates in canopy soils are much lower when converted to an *areal basis*, however bigleaf maple canopy soils are still enhancing annual $\text{NO}_3\text{-N}$, $\text{NH}_4\text{-N}$, Net N, and $\text{PO}_4\text{-P}$ nutrient pools by 5.2%, 48.4%, 8.7%, and 3.7%. Therefore, the hypothesis that canopy soils are enhancing forest ecosystem-level rates of N_{\min} (especially enhancing the pools of $\text{NH}_4\text{-N}$) and P_{\min} on an *areal basis*, was accepted. This demonstrates that canopy soils are providing a nutrient reserve for epiphytes and host tree adventitious roots, while increasing forest ecosystem-level mineralized nutrient pools. Aside from $\text{NH}_4\text{-N}$, canopy soils are not enhancing these pools substantially, but when modeling ecosystem processes and predicting future impacts of climate change, the role of canopy soils may be underestimated in these old-growth forests. Further, the seasonal timing of available nutrients in canopy soils remains an important attribute when fluxes

in the forest floor soils are lower or immobilization is occurring which leads to decreasing the availability of nutrients.

Another important consideration related to how much canopy soils enhance forest ecosystem-level nutrient pools is that this study only measured N_{\min} and P_{\min} in the dominant broadleaf species (bigleaf maple), but not in the dominant conifer (Sitka spruce). The rate of P_{\min} and N_{\min} in Sitka spruce canopy soils still remains unknown. Bigleaf maple canopy soils were shown to have more extractable $\text{NO}_3\text{-N}$ and $\text{NH}_4\text{-N}$ than Sitka spruce canopy soils (Tejo *et al.* 2014), however there was still extractable inorganic N in spruce canopy soils. Sitka spruce has also been predicted to hold much more canopy soil mass (Van Pelt unpublished data, 2012) due to the size and age. This suggests that N_{\min} and P_{\min} in spruce canopy soils enhances forest ecosystem-level nutrient pools even more on an *areal basis*, and therefore the pursuit of further studying nutrient dynamics in Sitka spruce canopy soils is encouraged.

4.4.6 Plant available nutrients and carbon leachates from canopy soils to the forest floor

Part of this study aimed to determine canopy soil leachate potential of nutrients that are limiting to plant growth ($\text{NH}_4\text{-N}$, $\text{NO}_3\text{-N}$, TN, $\text{PO}_4\text{-P}$, and DOC) at ambient conditions. To conduct this research in a safe and timely manner, deionized water was applied during the experimental manipulation instead of using natural rainfall. Concentrations of canopy soil leachates increased in the order $\text{NO}_3\text{-N} < \text{NH}_4\text{-N} < \text{PO}_4\text{-P} < \text{TN} < \text{DOC}$ (Table 4, Fig. 11). Edmonds *et al.* (1991) reported the same pattern of increase for $\text{NO}_3\text{-N} < \text{NH}_4\text{-N} < \text{PO}_4\text{-P}$ when measuring the stemflow chemistry from conifers in the Hoh River Valley, near one of the sample plots used in this study. Nadkarni (1986) also reported that the presence of epiphytes on branches

altered element concentrations in rainfall chemistry when compared to branches without epiphytes, though no inorganic nutrients were measured in that study.

Canopy soils had much more available $\text{NO}_3\text{-N}$ when compared to forest floor soils during the same time that leachate samples were collected (spring 2019). Nitrate is a mobile anion in a cation exchange rich environment and is highly susceptible to leaching, for example during heavy rainfall events. However, the trace amounts in leachates from canopy soils suggest that a majority of available $\text{NO}_3\text{-N}$ is being retained, potentially remaining accessible to the adventitious rooting networks, microbes, and other epiphytic species. There remains a possibility for plant available $\text{NO}_3\text{-N}$ to be lost through denitrification, which was not explored during this study.

As a cation, NH_4^+ binds better to anionic soil particles and can resist being leached from the soil by water infiltration and percolation. Canopy soils have been reported to have higher cation exchange capacity (CEC) than forest floor soils (Nadkarni *et al.* 2002, Enloe *et al.* 2006), and bigleaf maple canopy soils in this region were found to have an average CEC of $52 (\pm 10) \text{ cmol}_c \text{ kg}^{-1}$ (Tejo *et al.* 2014). There was slightly more $\text{NH}_4\text{-N}$ leaching out of canopy soils, but the concentrations of $\text{NH}_4\text{-N}$ in the canopy soil environment were much higher than $\text{NO}_3\text{-N}$ concentrations; accounting for 78% of the N_{min} at ambient conditions during the period that the leachate samples were collected. There is potential that $\text{NH}_4\text{-N}$ could be lost through volatilization which was not explored in this study, but generally occurs with higher pH values unlike the more acidic conditions found in canopy soils of this study.

The leaching losses of $\text{PO}_4\text{-P}$ are typically considered to be lower since $\text{PO}_4\text{-P}$ is an anion that tends to have a slow diffusion rate and often is strongly sorbed by soil particles (Addiscott and Thomas 2000, Shen *et al.* 2011), decreasing its susceptibility to loss by leaching. When these

samples were collected, rates of P_{\min} were much higher than rates of N_{\min} . Though the average amount of $PO_4\text{-P}$ in the leachate solution was greater than the inorganic forms of N, there was more total N that was leached from the canopy. This shows that although leaching of inorganic N is minimal, canopy soils may often contribute sources of N to the forest floor soil environment in what is typically considered an N-limited ecosystem (Johnson *et al.* 1982, Binkley 1986, Fenn *et al.* 1998).

Though we did not determine carbon mineralization rates during the timeframe of this study, dissolved organic carbon (DOC) was also analyzed in the leachate solution. This additional quantitative inquiry was considered important because it would provide insights on how canopy soils contribute to the forest ecosystem-level C cycle through canopy soil leachate pathways, and also because DOC interacts with soil P and N cycles (Qualls *et al.* 1991, Campbell *et al.* 2000). DOC was leached in the highest amounts. The concentrations are comparable to other studies that have explored DOC leachates from organic soils (e.g., Hentschel *et al.* 2007), from mor humus (Andersson *et al.* 2000), and from silt loams and sandy loams (Teifenbacher *et al.* 2020).

4.5 CONCLUSION

Canopy soils have been deemed to have important structural and functional roles in the forests where they prevail for ≥ 40 years, so it is important to research how much they contribute to the nutrient pools and fluxes, especially in ecosystems with high rainfall. This will help to inform the continued ability of these rainforests to be part of the compartmentalized resilience (Gilarranz *et al.* 2017) if rainfall levels decrease. In many tropical forests, nutrient fluxes occur in pulses (Lodge *et al.* 1994). It is therefore important to determine whether these temperate rainforests also have canopy soils providing pulses of nutrients at times when forests are

experiencing decreased rainfall conditions and summer drought. Especially because it was shown that soil moisture content increased when half the rainfall was diverted off of a fixed area of canopy soil, which influenced an increase in N_{\min} and decrease in P_{\min} . This also further supports that N and P cycles should be researched in parallel, because many studies explore them independently (Guignard *et al.* 2017).

This research supports the role of bigleaf maple canopy soils as a reserve for inorganic N and P production during time periods when there is high competition at the forest floor level resulting in nutrient limitations. It is also important to understand how both pools function during the early spring period when root growth starts (even the soil canopy adventitious roots) and when there is a greater demand for N and P for growth, as well as for maintenance. At the forest ecosystem-level, individual trees can graft their rooting systems together and transfer limiting growth resources among all the grafted trees (Fraser *et al.* 2006, Lev-Yadun and Sprugel 2011). The possibility for tree-to-tree root grafting between roots in canopy soils is highly unlikely, therefore adventitious roots may hold a major role in host tree resiliency at the ecosystem level when there is nutrient immobilization, nutrient transfer, or competition in the forest floor soils.

The experimental manipulations implemented during this study showed that there were significant changes in the annual rates of N_{\min} and P_{\min} under both decreased and increased rainfall conditions compared to canopy soils at ambient conditions and forest floor fluxes. Surprisingly, the roof-interception treatment increased soil moisture, which may have been influenced by fog-water interception, pore space, capillary action, and hydraulic redistribution. This suggested that canopy soils may be increasing the resilience of these forests to drought. When exploring the vulnerability of these highly productive forests to drought, the nutrient cycles in canopy soils should not be ignored. Especially because bigleaf maple is a deciduous

tree which can be negatively impacted by summer and early fall periods of drought, and canopy soils could then provide an ecological buffer to those seasonal disturbances.

Further, even though canopy soil's nutrient cycles may be physically separated from forest floor soils, they still are contributing to forest floor nutrient cycles through leachates (or when a branch, or mat, falls to the forest floor). There is also potential that adventitious roots may couple processes in canopy soils to forest floor soils and vice versa. The fact that a unique suite of fungal taxa, including mycorrhiza, (see Ch. 3) was found to associate with canopy adventitious rooting systems suggested that those associations are also playing a role in host tree resiliency. Especially in these forests that experience pulses of nutrient availability which are driven by seasonal changes in rainfall. It will be important to understand how different fungi influence the N and P cycling rates and whether they may provide the resilience for these forests during a drought or periods of increased rainfall.

Overall, this study demonstrated that canopy soils should not be overlooked when considering how these forests function in the face of a disturbance, such as changing climate. They also provide a very unique opportunity to study soil systems, because they provide a simplified, yet a relatively natural soil system that is ideally suited to isolate the effects of environmental factors, such as climatic parameters, on changes in the soil environment; making canopy soils excellent testbeds for *in situ* soil studies.

Figures**a****b**

Figure 4.1: (a) Thick lateral adventitious roots in canopy soils running parallel to the tree branch, and (b) lateral and fine severed roots after a sample was taken. Photos taken from canopy soils of *Acer macrophyllum* in Washington's old-growth forests. Photos are also featured in Geiser *et al.* 2020.

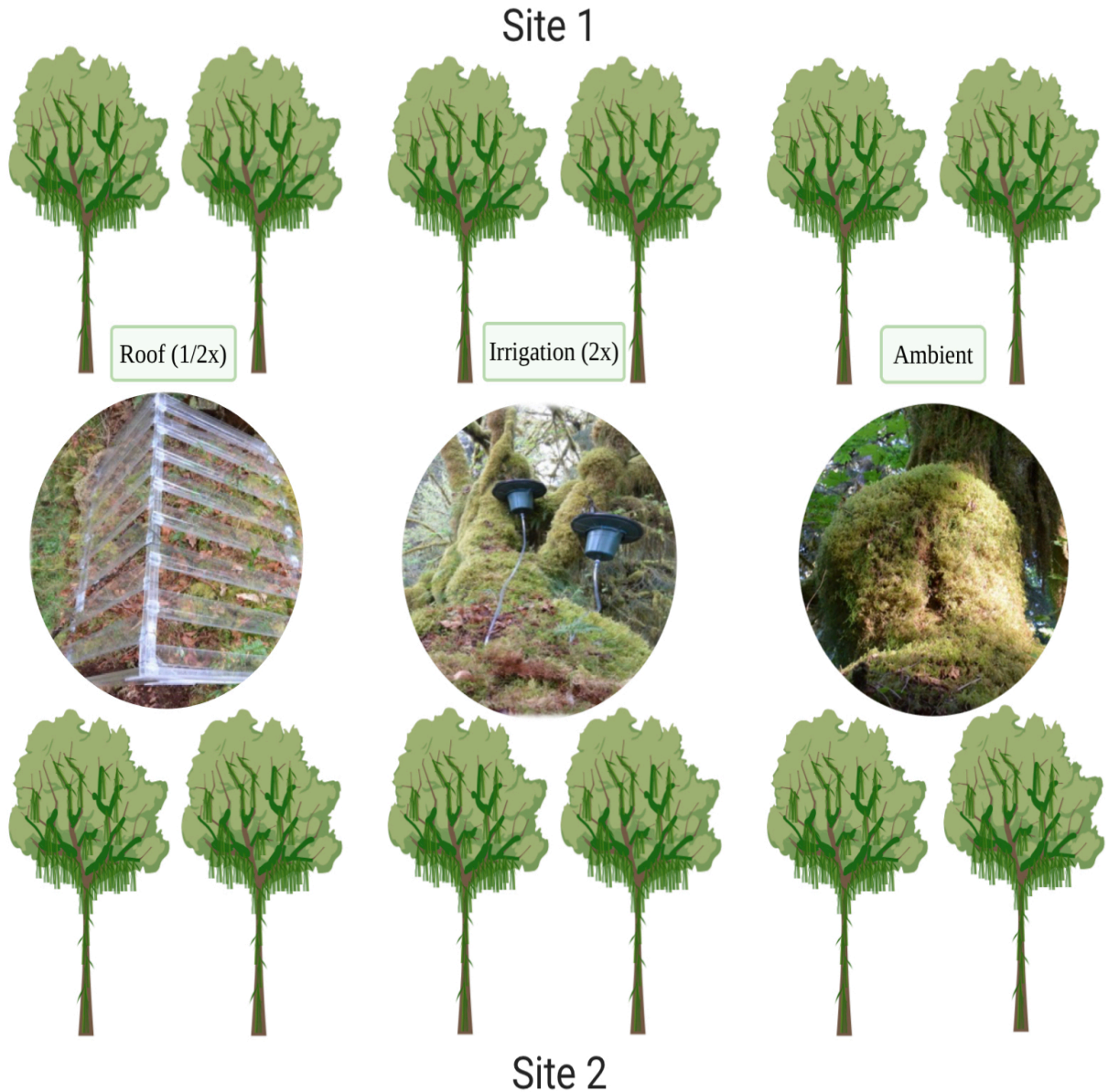


Figure 4.2: Depiction of the experimental design of this study in the Hoh and Queets watersheds in the Olympic rainforest in Washington state. Six trees at each site were selected, where two were equipped with a roof-interception structure (1/2x), two were equipped with a gravitational irrigation system (2x), and two were left at ambient conditions. The structures manipulated a controlled area of canopy soil to experience either increased, decreased, or normal rainfall conditions. In total there were twelve trees, and four of each treatment condition.

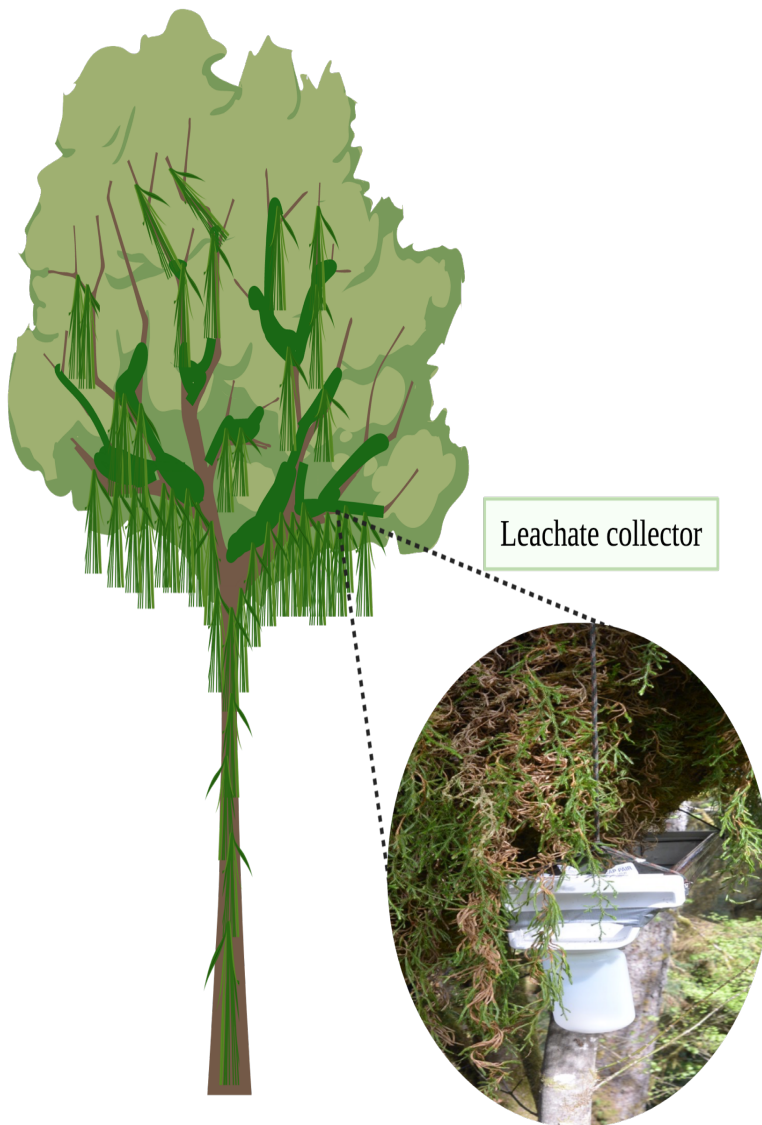


Figure 4.3: One of the 12 leachate collectors used in this study is presented here. These collectors were fastened under the branch and left to collect leachates from the simulated rainfall event on canopy soils on branches of bigleaf maple trees in the Olympic Rainforest of Washington state.

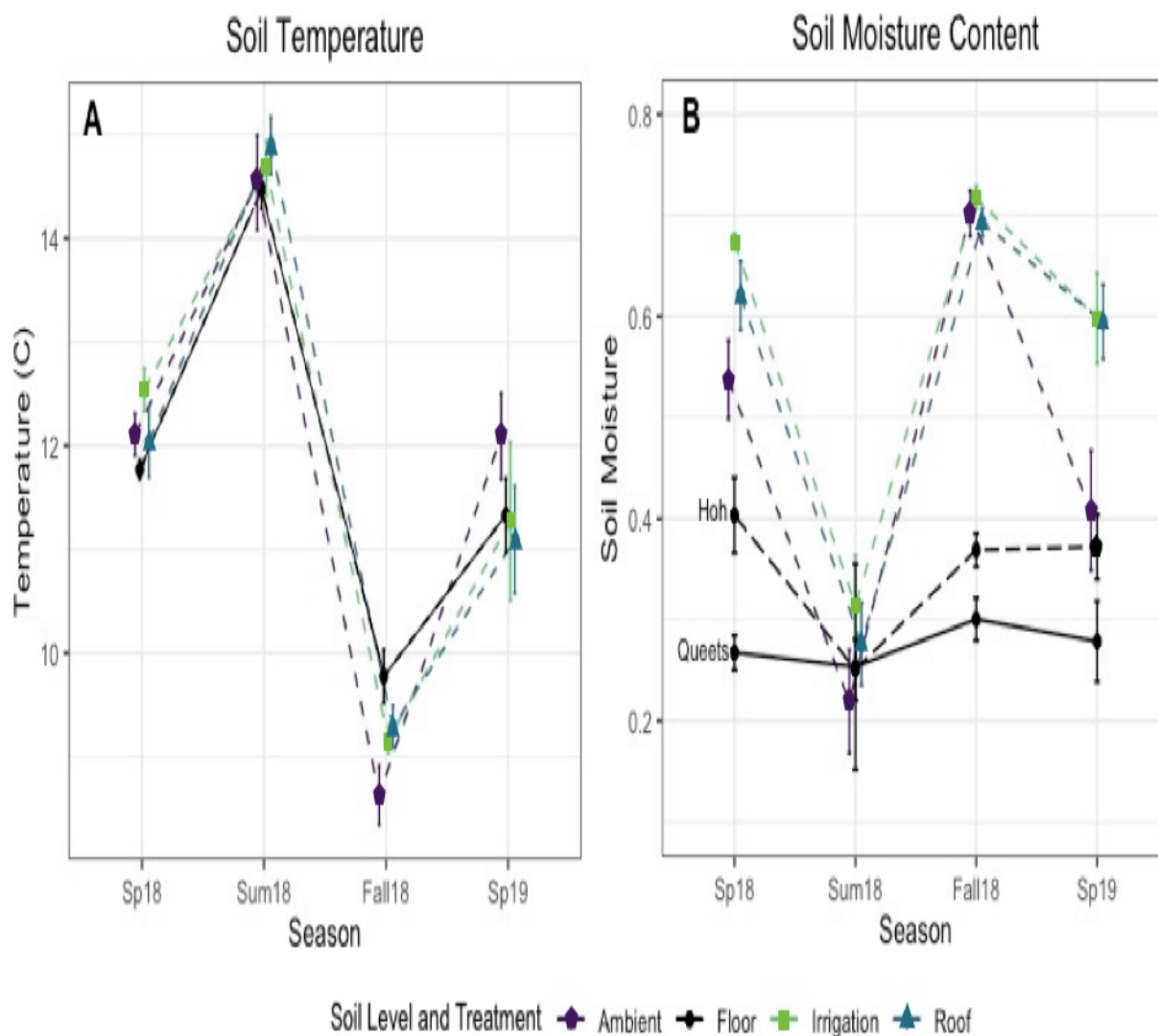


Figure 4.4: Seasonal average temperatures for the forest floor soils and in the treatments of the canopy soils (A). Seasonal average moisture contents for the forest floor soils in the Queets and Hoh watersheds and in the treatments of the canopy soils (B). These canopy and forest floor soils are in old-growth bigleaf maple stands and measured during the spring, summer and fall of 2018 and spring of 2019 in the Olympic Rainforest of Washington state. Error bars are ± 1 SE. At the forest floor level, the Queets and Hoh moisture contents are plotted separately due to being significantly different. In plot A, small-dashed lines represent temperature or moisture in the canopy soils of the precipitation treatments of ambient conditions, 1/2x interception and 2x irrigated, whereas the solid black line represent average temperature in forest floor soils. In plot B, moisture for both the Hoh and Queets site are plotted separately due to significant differences in soil moisture content between the two sites. The solid black line represents Queets forest floor moisture content and the large-dashed black line represents Hoh forest floor averages.

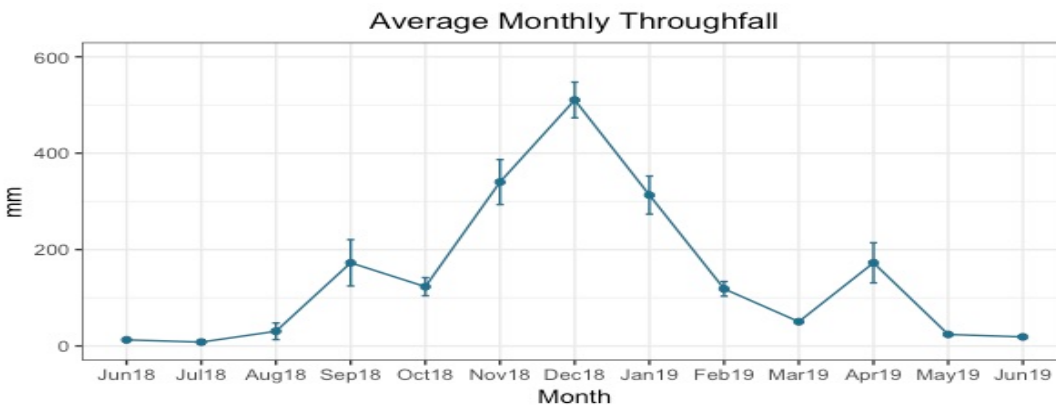


Figure 4.5: Average monthly throughfall rainfall in the old-growth temperate rainforests stands old-growth bigleaf maple canopy soils and forest floor soils for the spring, summer and fall of 2018 and spring of 2019 in the Olympic Rainforest of Washington state. Error bars represent ± 1 SE.

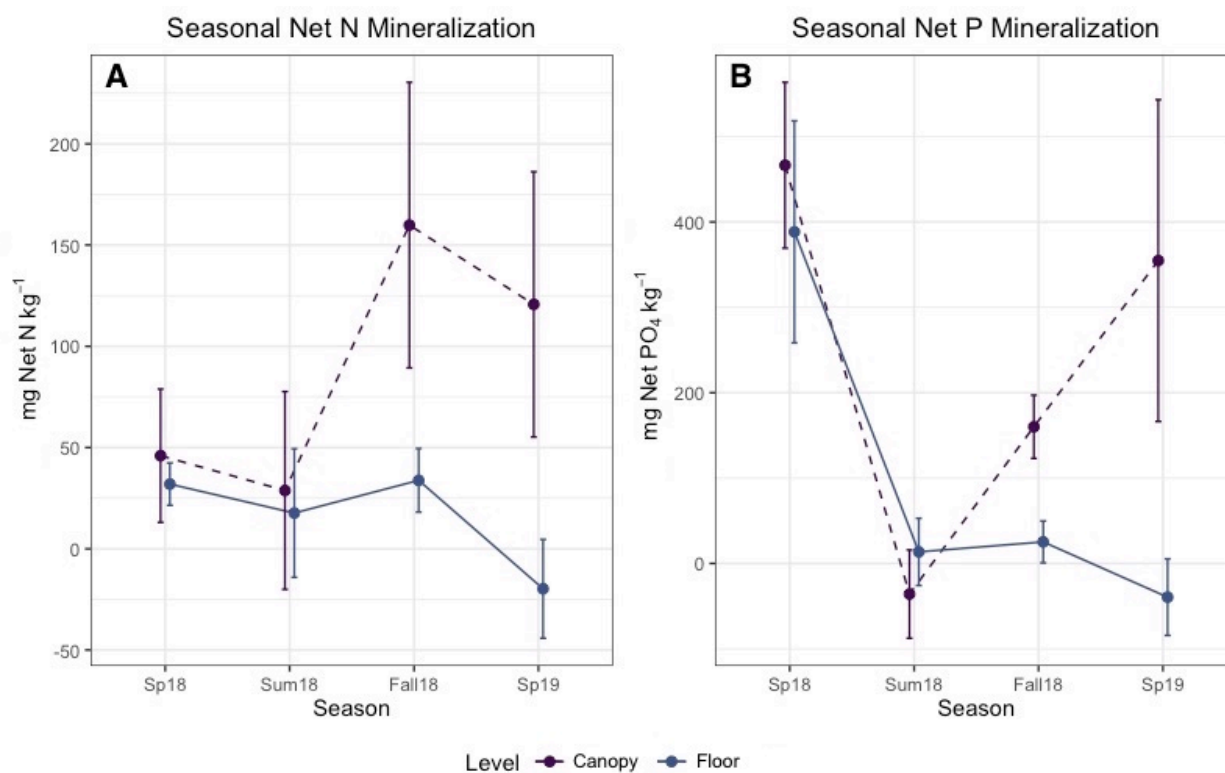


Figure 4.6: (A) Average seasonal rates of N_{\min} and (B) P_{\min} between canopy and forest floor soils at ambient conditions on a soil weight (per mass) basis. Bars denote ± 1 SE.

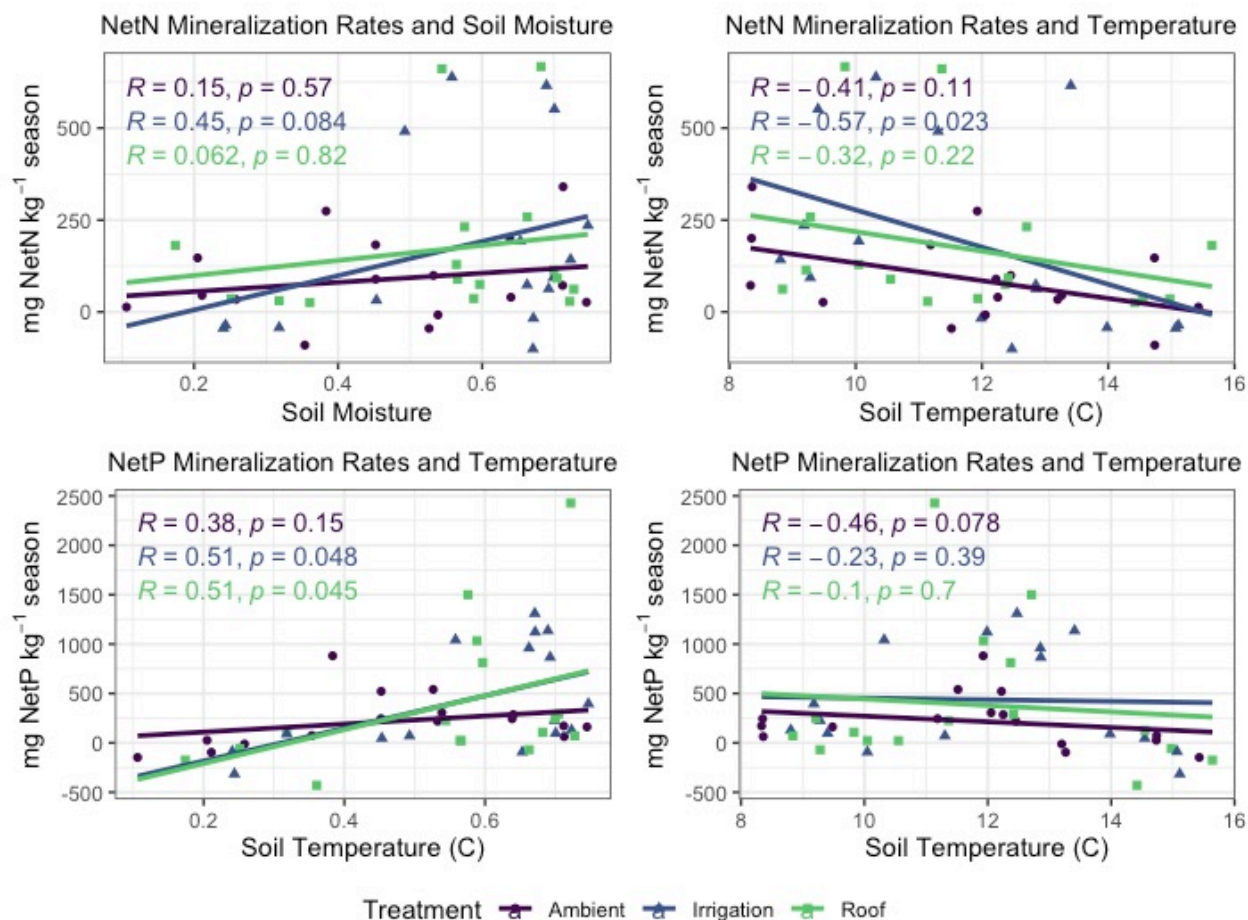


Figure 4.7: The relationship between soil moisture and N_{\min} rates (a), soil temperature and N_{\min} rates (b), soil moisture and P_{\min} rates (c), and soil temperature and P_{\min} rates on a soil weight basis (d). Groupings were based on treatment group and regression lines are based on Spearman's ranked correlation.

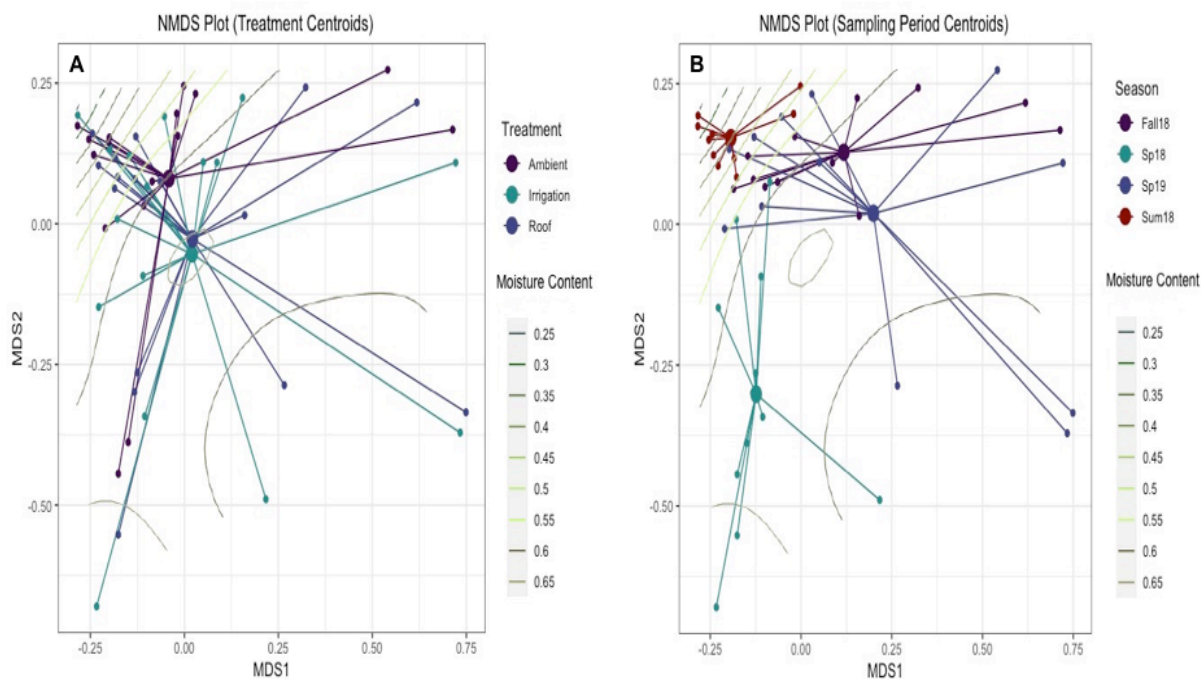


Figure 4.8: Non-metric multidimensional scaling (NMDS) ordination in canopy soil environments using Gower distances among normalized values of N_{\min} and P_{\min} grouped by: (A) treatment, and (B) sampling periods. Stress value was 0.03, indicating a good fit.

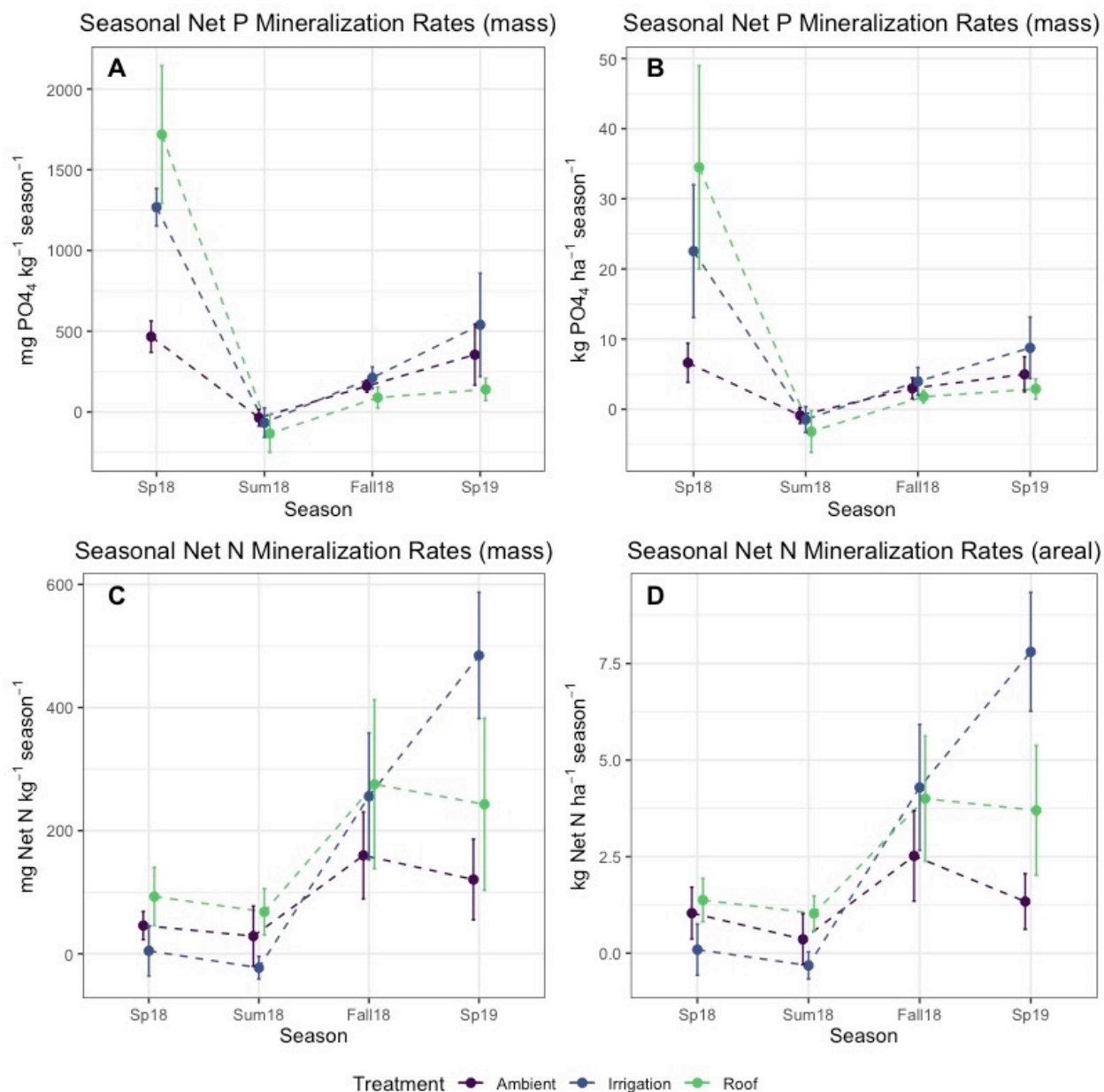


Figure 4.9: Seasonal mineralization rates of PO₄-P and Net N expressed on a *per mass basis* (A, C) and an *areal basis* (B, D) in the spring, summer and fall of 2018 and spring 2019 in the canopy and forest floor soils in the Olympic rainforests of Washington state. Error bars are ± 1 SE.

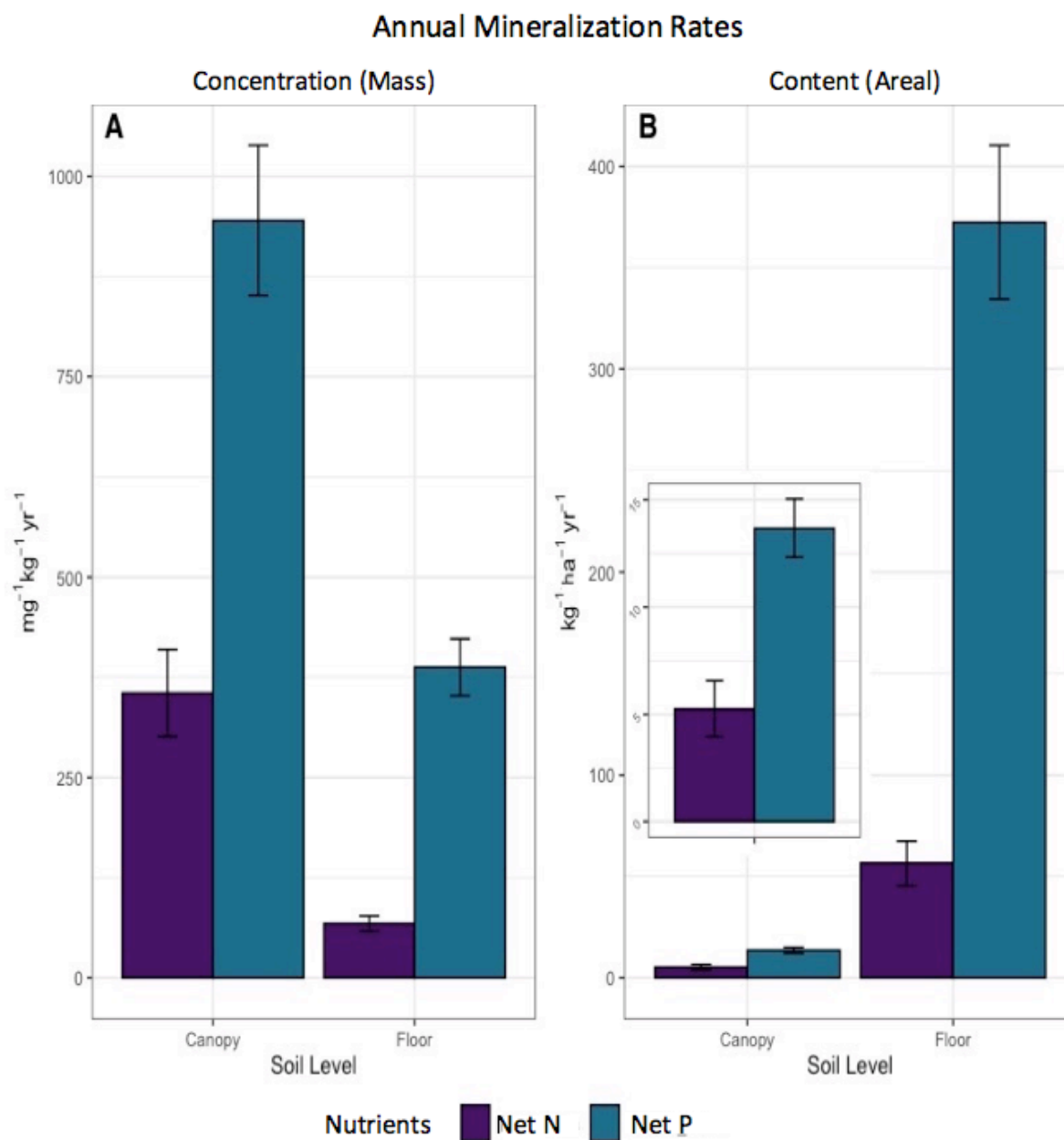


Figure 4.10: Annual N_{\min} and P_{\min} in canopy soils and forest floor soils demonstrating that canopy soils have much larger mineralized pools of N and P on a *per mass basis* (A), but much less on an *areal basis* (B) due to their limited surface area in these ecosystems. Error bars are ± 1 SE.

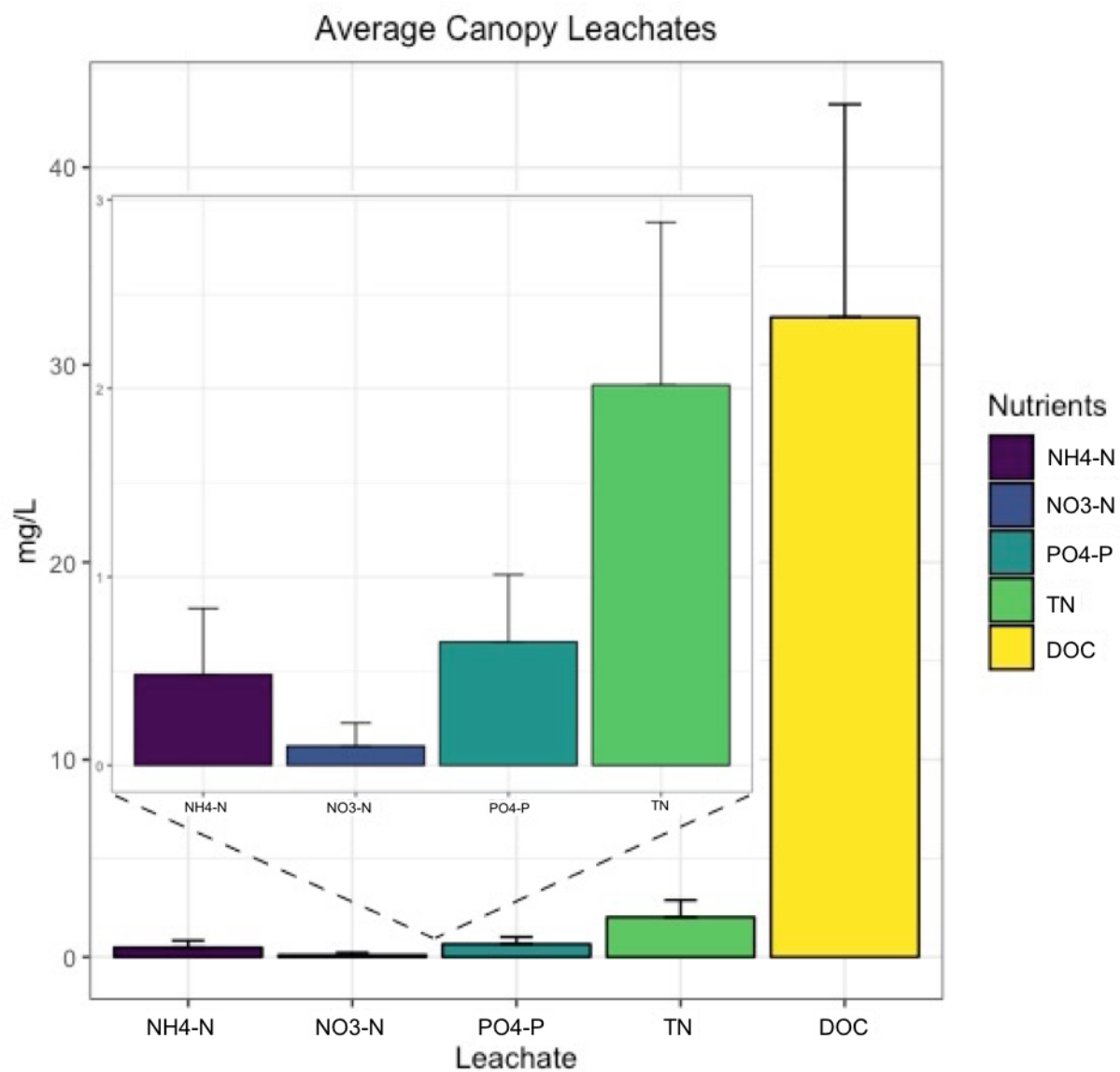


Figure 4.11: Leachate chemistry of ammonium ($\text{NH}_4\text{-N}$), nitrate ($\text{NO}_3\text{-N}$), phosphate ($\text{PO}_4\text{-P}$), total nitrogen (N), and dissolved organic carbon (DOC) collected from the 12 trees in this study. Error bars are ± 1 standard error.

Tables

Table 4.1: Seasonal means of P_{\min} and N_{\min} rates ($\text{mg kg}^{-1} \text{ season}^{-1}$) at ambient rainfall conditions. ($\text{Net N} = \Delta \text{TIN}_{(\text{T1-T0})}$; and $\text{TIN} = \text{NH}_4\text{-N} + \text{NO}_3\text{-N}$ at both T1 and T0 time periods representing seasonal periods. Negative mean values of P- PO_4 or N represent immobilization by microbes of those nutrients whereas positive mean values represent mineralization (i.e., available for uptake) of those nutrients.)

Season	Mineralized Nutrient pool	Forest Floor Soil Mean (n = 12)	Canopy Soil Mean (n = 4)
Sp18	Net P	388.3 (130.0)	466.4 (97.2)
Sum18	Net P	13.6 (39.4)	-35.9 (51.7)
Fall18	Net P	25.2 (24.4)	160.0 (37.0)
Sp19	Net P	-39.5 (44.8)	354.7 (188.5)
Sp18	Net N	32.0 (10.5)	46.0 (32.9)
Sum18	Net N	17.7 (31.8)	28.8 (48.9)
Fall18	Net N	33.8 (9.1)	159.9 (70.5)
Sp19	Net N	-19.8 (24.4)	120.7 (65.5)

Table 4.2: Seasonal rates of N_{\min} and P_{\min} ($\text{mg kg}^{-1} \text{ season}^{-1}$) under all treatments (2x=rainfall irrigation, 1/2x=roof rainfall-interception, Amb=ambient rainfall) in canopy soils and in forest floor soils. Values in parenthesis represent ± 1 SE. Shaded values represent immobilization, i.e., negative values mean a decrease in that nutrient over the time period and implying being potentially taken up (i.e., immobilized) by microbes.

		Spring 2018				Summer 2018			
Treatment		Net $\text{NO}_3\text{-N}$	Net $\text{NH}_4\text{-N}$	Net N	Net $\text{PO}_4\text{-P}$	Net $\text{NO}_3\text{-N}$	Net $\text{NH}_4\text{-N}$	Net N	Net $\text{PO}_4\text{-P}$
Canopy Soil	2x	2.01 (1.61)	2.83 (39.87)	4.84 (40.46)	1267.71 (115.85)	10.35 (5.25)	-32.93 (16.36)	-22.58 (18.28)	-66.82 (90.99)
	1/2x	70.34 (35.17)	22.72 (26.57)	93.05 (47.33)	1718.04 (426.18)	21.48 (16.32)	46.82 (37.03)	68.30 (37.66)	-134.06 (116.44)
	Amb	58.11 (19.53)	-12.14 (26.84)	45.97 (32.92)	466.36 (97.17)	-6.48 (13.74)	35.27 (35.83)	28.80 (48.85)	-35.88 (51.66)
Floor Soil	Amb	29.35 (7.61)	2.60 (3.34)	31.95 (6.04)	388.34 (75.06)	6.33 (2.11)	11.33 (18.12)	17.66 (18.34)	13.56 (22.73)

		Fall 2018				Spring 2019			
Treatment		Net $\text{NO}_3\text{-N}$	Net $\text{NH}_4\text{-N}$	Net N	Net $\text{PO}_4\text{-P}$	Net $\text{NO}_3\text{-N}$	Net $\text{NH}_4\text{-N}$	Net N	Net $\text{PO}_4\text{-P}$
Canopy Soil	2x	21.26 (12.19)	234.45 (101.92)	255.71 (102.85)	211.80 (66.89)	77.17 (15.15)	407.35 (88.50)	484.53 (102.54)	539.28 (319.88)
	1/2x	98.57 (41.33)	176.85 (143.58)	275.42 (137.00)	88.75 (64.98)	46.67 (9.93)	196.25 (133.36)	242.92 (139.74)	139.75 (69.34)
	Amb	65.53 (33.44)	94.33 (72.70)	159.86 (70.53)	160.04 (36.99)	26.50 (19.30)	94.21 (49.46)	120.71 (65.52)	354.70 (188.48)
Floor Soil	Amb	17.30 (5.52)	16.51 (8.71)	33.81 (9.07)	25.23 (14.24)	2.17 (4.38)	-21.94 (12.97)	-19.77 (14.07)	-39.50 (25.87)

Table 4.3: Annual mineralization rates on a soil weight ($\text{mg kg}^{-1} \text{ yr}^{-1}$) and areal ($\text{kg ha}^{-1} \text{ yr}^{-1}$) basis for $\text{NO}_3\text{-N}$, $\text{NH}_4\text{-N}$, Net N ($\text{NO}_3\text{-N} + \text{NH}_4\text{-N}$), and $\text{PO}_4\text{-P}$ in canopy soils at all three treatment levels (2x=rainfall irrigation, 1/2x=roof rainfall-interception, Amb=ambient rainfall) and in forest floor soils at ambient conditions.

		Annual Concentrations				Annual Contents			
Treatment		Net $\text{NO}_3\text{-N}$	Net $\text{NH}_4\text{-N}$	Net N	Net $\text{PO}_4\text{-P}$	Net $\text{NO}_3\text{-N}$	Net $\text{NH}_4\text{-N}$	Net N	Net $\text{PO}_4\text{-P}$
Canopy Soil	2x	110.76 (8.55)	611.69 (61.66)	722.49 (66.04)	1951.97 (148.40)	3.31 (.29)	9.98 (.76)	13.29 (.99)	33.77 (3.02)
	1/2x	237.06 (9.93)	442.63 (85.13)	679.69 (90.43)	1812.49 (169.23)	5.85 (.81)	6.19 (1.13)	10.10 (2.53)	35.96 (5.19)
	Amb	143.67 (21.49)	211.67 (46.21)	355.33 (54.46)	945.2 (93.57)	2.67 (.16)	2.55 (.39)	5.25 (1.31)	13.68 (1.36)
Floor Soil	Amb	58.22 (3.92)	9.40 (8.63)	67.62 (9.50)	387.63 (34.45)	51.39 (4.88)	5.27 (9.59)	56.66 (10.88)	372.43 (37.88)

Table 4.4: Average canopy soil leachate concentrations (mg/ml) (n = 12). NH₄-N = ammonium nitrogen; NO₃-N = nitrate nitrogen; PO₄-P = phosphate phosphorus; TOC = Total Organic Carbon; TN = Total Nitrogen; DON = Dissolved Organic Nitrogen.

NH ₄ -N	NO ₃ -N	PO ₄ -P	TOC	TN	DON
0.48 (0.04)	0.10 (0.01)	0.66 (0.04)	32.42 (1.21)	2.02 (0.10)	1.43 (0.10)

Chapter 5: Overall summary and future perspectives

5.1 COLLECTIVE CONCLUSION

This research has shed light on some fundamental processes in the canopy soil environment; including how N and P fluxes and root-associated fungal communities differ from the forest floor soil environment. Additionally, it provided evidence that canopy soil biotic and abiotic processes are sensitive to increased and decreased rainfall amounts, which drive soil moisture levels, in an ecosystem that is predicted to continue experiencing increased periods of rainfall and drought.

In Chapter 2, the ability of the MinION nanopore sequencer in identifying fungi to species level was tested and confirmed, providing a new methodology to identify root-associated fungi to species level. Resolving fungi to species level from shorter sequence reads provided by Illumina MiSeq is not suggested, and the results from Chapter 3 found that species-level identities were not always reliable with Illumina, although Illumina MiSeq did recover slightly higher diversity. In contrast, MinION did well with species-level resolution and also covered a breadth of diversity (especially from *Glomeromycota*), resulting in first-reports of certain fungi associating with both canopy and adventitious roots. The two sequence platforms have pros and cons, but they complement each other well, depending on the question being asked. As MinION technology continues to advance, it may provide a worthy alternative for detecting community shifts when species-level resolution is of interest.

In Chapter 3, it was determined that the suite of fungal taxa associating with adventitious roots of bigleaf maple are distinct from those associating with forest floor roots. These fungal communities were also impacted by the experimental manipulation in canopy soil environments, suggesting that they are adaptive facilitators providing a resilience component to the host tree during extended periods of rainfall or drought.

This directly links to findings in Chapter 4, which demonstrated that canopy soils have higher concentrations and mineralization rates of N and P available for plant uptake. It was also determined that on an annual basis, mineralized pools of N and P in the canopy soil environment are enhancing forest ecosystem-level pools by 5.2% and 3.7% on an *areal basis*.

5.2 FUTURE PERSPECTIVES

Evidence demonstrates that the biotic and abiotic processes happening in soils are not limited to the forest floor in ecosystems where canopy soils prevail. When present, these organic mats contribute to ecosystem-scale biogeochemical cycles, provide habitat to a diversity of species, and create an adventitious-root niche for host trees. Canopy soils of old-growth bigleaf maple have higher concentrations of inorganic N and P and a distinct suite of root-associated fungi suggesting that they are providing a resilience component for their host trees, which may be inter-connected to the temporal stability of these forests. At the ecosystem scale, they also enhance C, N, and P pools. Although the surface area of canopy soils is limited to tree branches, it is apparent that they hold a role in the structural and functional dynamics of these ecosystems. Therefore, they should be consistently considered as an integral part of these forests; especially when considering how these ecosystems will respond to a changing climate.

There are further potentials for linking the important role of canopy soils to forest ecosystem-level nutrient cycles. For example, in these riparian forests it is not uncommon to see some mature red alder trees within, or bordering, old-growth stands. Mature red alder also harbors canopy soils and develops patches of adventitious roots that are associating with N-fixing *Frankia* spp. (Kennedy *et al.* 2009), though to our knowledge there is nothing known about the mineralized nutrient pools in these canopy soils. Tejo *et al.* (2014) also reported that

Sitka spruce canopy soils contribute to C and N pools (Tejo *et al.* 2014) in these forests, and it is estimated that they harbor a much higher biomass of canopy soil due to tree size (Van Pelt, unpublished data).

Researching the biotic and abiotic processes in canopy soils of different tree species will better allow these old-growth forests with mixed tree dominance to be managed – so as to retain them in the landscape. Another study in a tropical cloud forests demonstrated that type of tree species had an effect on canopy soil nutrients and related processes (Cardelús *et al.* 2008). Pairing information from other studies with our findings on bigleaf maple canopy soils strongly suggests that there are many unknowns that still need to be answered when it comes to understanding the importance canopy soils hold in forest ecosystem-level nutrient cycling, and that these soils should not be overlooked.

This study provided fundamental information on the biotic and abiotic processes happening in bigleaf maple canopy soils. These results alone demonstrate that canopy soils are important components of the ecosystem. However, it is emphasized that the importance of studying canopy soils compared to the forest floor can help to better understand the role of canopy soils in compartmental resilience. This is especially possible because canopy soils, under decreased rainfall, showed an increase in soil moisture content. This also could be combined with other studies of microbial communities to understand how they facilitate forests to be resilient during extreme climatic events. This research suggested that if trees have roots in the canopy (where there is less uptake competition), as well as at the forest floor level; they will potentially be more nutrient-use efficient, especially in those environments that have a diversity of nutrient pulses.

Neglecting canopy soil processes and further excluding them from climate models foster an incomplete understanding of how these ecosystems function and how they will respond to

climate-related disturbances. To our knowledge, no models to date have ever included canopy soil processes or the canopy soil compartment. It would be of interest to see how their inclusion would affect model sensitivity and predictions. For example, bigleaf maple canopy soils were found to enhance the inorganic nitrogen pools of these forests by 5.2% and that could affect model sensitivity. Therefore, it becomes not only important to include them in existing models, but they also offer a unique system for model parameterization at unresolved scales – for example, including fungal processes. Fungal processes are often ignored in models, which reflects their complexity in a given environment. Many *in vitro* model systems allow these processes to be understood, but without the plethora of complex processes in natural soil systems. However, the canopy soil environment offers an experimental soil system that may provide one the opportunity to bridge this disconnect in modeling. For example, because they not only have natural biotic and abiotic processes to study but those processes are relatively disconnected from the more complex forest floor soil, lending them to be experimentally manipulated for studies.

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Appendix A

Method: Optimized protocol for fungal DNA extraction from hardwood root-tips (or other plant material) using OPS Diagnostics Synergy™ 2.0 Plant DNA Extraction Kit. Any text in blue was adjusted or standardized from the original protocol. Any text in red was an added step not included in the standard protocol. Centrifuge speeds and times can be found in the protocol supplied with the kit. For tough samples, we choose to use the zirconium oxides satellites, but the steel balls have also worked efficiently.

1. Weigh out 25-30 milligrams (mg) of dry plant tissue and place in Synergy™ tube*.
 - Fine roots/root-tips were lyophilized after field collection, and extractions were completed within one week.
2. Place cap on tubes, place on bead beater and perform a 30-60 sec dry grind.
 - Including this dry grind step results in better homogenization of tough tissues.
3. Remove from bead beater, and add 750 microliters (µl) of homogenization buffer*⁺ to each tube.
4. Place cap on tubes, place on bead beater and perform a 1 min wet grind.
5. Incubate 'milkshake' at 37°C for 15 min.

6. Transfer tubes to a centrifuge and extract supernatant.
7. Add 7-10 μ l RNase A solution*⁺ and incubate at 37°C for 15 min.
8. Add 7/10 volume isopropanol and incubate at -20°C for 10 min.
9. Transfer solution to spin column* and centrifuge.
10. Wash column with 70% ethanol and centrifuge.
11. Repeat step 10.
12. Elute from spin column by adding molecular grade water or TE, then centrifuge.
 - For this step, we chose to do a two-step elution with 60ml TE each time. Then the solution was further purified by a Polyethylene Glycol (PEG) rainfall. Based on personal observations, PCR reactions had higher success with an extra purification step.

* Included in kit.

⁺ Included in kit, but more than the standard amount is used. Extra solution can be requested from OPS Diagnostics. We use more RNase A solution, and purchase an extra bottle of the CTAB extraction buffer.

Vita



Korena Mafune was born and raised outside of Seattle, WA and has always been passionate about Washington's beautiful and diverse ecosystems. She is a proud Japanese-American and the first in her family to receive a college degree. She received her B.S. in Restoration Ecology and Environmental Horticulture from the University of Washington in 2013, where she completed an independent capstone project with Dr. Jon Bakker. The project focused on restoring native prairies, and this is really when she started 'digging' deep into soils and their below-ground processes. She knew she wanted to continue to research the 'hidden half', so directly upon completion of her B.S., she applied and was accepted to UW's School of Environmental and Forest Sciences (SEFS) graduate program. She completed her M.S. in 2015 with Drs. Daniel Vogt, Kristiina Vogt, Bruce Godfrey, and Darlene Zabowski; which was a pilot study exploring adventitious rooting systems in canopy soils. The results from this study resulted in many local and non-profit organizations funding the entirety of her PhD research, which she completed under the guidance of Drs. Daniel Vogt, Kristiina Vogt, Erica Cline, Bruce Godfrey, Joe Ammirati, and Terry Swanson (GSR). You can also find her collaborating with organizations outside the university setting with the goal of broadening participation of under-represented communities in STEM. As her PhD research comes to completion, she will transition into a new position as a Washington Research Foundation Postdoctoral Fellow in the Winkler Lab at UW. This research will explore the role microbial communities (specifically fungi and bacteria) hold in agricultural systems and further test how these organisms can be harnessed as a sustainable biotechnology. On her free time, you can find her cooking, training kung fu, hanging with her family and pets, and walking through various ecosystems foraging and luring you to come check out the mushroom(s) she just found. Photo credit: Kevin Hense.