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Diversity Analysis of Soil Fungus Communities in Disturbed, Nursery, and Mature Forest Conditions

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DIVERSITY ANALYSIS OF SOIL FUNGUS COMMUNITIES IN DISTURBED, NURSERY, AND MATURE FOREST CONDITIONS

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by

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ABSTRACT

DIVERSITY ANALYSIS OF SOIL FUNGUS COMMUNITIES IN DISTURBED, NURSERY, AND MATURE FOREST CONDITIONS

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Populations of soil fungi were examined in Derby Canyon Natives, Coeur d'Alene Forest Service nursery, Swamp Lake, and the Keechelus Lake wildlife overcrossing soils. All sampling sites were connected by their relation to the revegetation and native soil plug inoculation of the wildlife overpass. This study was an effort to describe soil fungi communities present on the overpass before plant introduction, those that plants would be bringing in their pots, and the fungi that could be introduced via soil plug transplantation. DNA was extracted from soil samples, then sequenced using next-generation sequencing methods, allowing for the analysis of species richness and evenness, diversity, and functional diversity. Both nurseries had relatively higher amounts of plant pathogens and saprotrophs, and so it was determined that the plants would not be bringing many beneficial soil fungi when introduced to the overcrossing. The mature forest area had a diverse community of fungi that included beneficial root fungi, and was deemed a suitable site to draw soil plugs. The wildlife overpass had high diversity and species richness but low functional diversity, providing evidence for the necessity of establishing more functionally diverse communities of fungi with soil plugs.

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CHAPTER I

INTRODUCTION

Research Problem

The I-90 Snoqualmie Pass East Project in Washington state's Cascade Range will introduce vegetation to the Keechelus Lake wildlife overpass in the fall of 2020. This crossing structure is part of a multimillion-dollar venture conducted by the Washington State Department of Transportation (WS-DOT) in conjunction with many other agencies including the United States Forest Service (FS), Conservation Northwest, and the Federal Highway Administration (FWHA) among others. The restoration aspect of this project aims to connect the North and South Cascade mountain range, as Interstate 90 currently presents a nigh-impassible barrier that fragments the habitat. Scores of undercrossings are planned, and many completed, in addition to three overpasses (one completed as of 2020, two planned for later phases of the project). These crossing structures will be created along a stretch of I-90 between Easton and Hyak (Figure 1).

Providing a place for animals to cross, from large ungulates to small rodents and amphibians, will potentially unify the divided populations with the added benefit of reducing animal-vehicle collisions on the interstate. The currently completed overpass rests near the south end of Lake Keechelus, rising 11 m above the interstate with two arches for northbound and southbound traffic. Kilometers of exclusionary fencing at both ends of the bridge will funnel animals towards the crossing structure, keeping them off the interstate. Atop the bridge, the walls stand 3 m high to help deter any animal from going over the sides.

General construction fill was laid over the wildlife overpass, then topped with a mulch made from the trees that had been cut down to make room for the crossing structure. The soil is not much more than a meter thick across the bridge, and the mulch provides a couple inches of a rough O_i horizon. Before being spread across the bridge, the soil sat in piles exposed to the elements for over a year. Extended exposure to this condition likely sterilized the soil of most fungi, as there were no hosts to support mycorrhizae and temperatures of the piles likely reached extremes beyond the tolerance of decomposer fungi. In the fall of 2020, plants will be introduced in order to create a more natural environment to entice animals to utilize the overpass, or even live atop the structure. An added benefit of revegetation would be the connection of soil microbes across the bridge and eventual recruitment of native flora. Vast amounts of resources have been poured into this experiment of bandaging a broken habitat, so it is in the interests of the WS-DOT and the Forest Service to maximize the survivorship of the established plants and connectivity of the communities across the interstate.

Figure 1: I-90 - Snoqualmie Pass East - Hyak to Keechelus Dam (Phase 1) - Project map

However, unless mitigation measures are taken to increase the survivorship of introduced flora, revegetation success is often variable. One review found an average survival rate of 0-60% after four years in these restoration areas (Godefroid et al. 2012). This can be attributed to a number of factors, some of which are specific to the preparation and choice in introduced plants and some that relate to the quality of the soil in the restoration area. It is not uncommon for restoration projects to take place in highly compacted, eroded, low nutrient, or otherwise poor-quality soils, so action must be taken to amend soil and lower the mortality rate that commonly plagues revegetation projects.

One potential avenue of mitigation to improve revegetation success would be the establishment of a diverse soil fungi community. Soil fungi can increase plant productivity (van der Heijden et al. 1998), alter soil physical structure (Rillig and Mummey 2006; Violi et al. 2008), and increase the amount of nutrients available to plants (van der Heijden et al. 2008). The activity of soil fungi would provide a long-term solution to the issue of soil amendment, more so than short-term methods like application of fertilizer (Ramlow et al. 2018). Taking this into consideration, the Forest Service intends to foster the development of soil fungi by taking plugs of soil drawn from local sources and transplanting them into the soil across the wildlife crossing structure. Ideally, these plugs would transfer the much-needed microbes into what is assumed to be soil that is populated with non-beneficial fungi, thus inoculating the bridge with native fungi and creating a community more like the nearby mature forest.

The plants will be planted on the bridge along with the soil in their pots, which may increase the survival rate considering the evidence of healthier and more extensive root systems in plants that were transplanted as plugs (Kokalis-Burelle 2003). Soil from

the containers transferred fungi that were colonizing the soil while the plants were being raised in the nursery. However, these fungi of nursery origin may not do much to aid the growth and survivorship of the plants introduced to the bridge – previous research has demonstrated that commercial mycorrhizae mixed in with nursery soils do not colonize plants as well as native mycorrhizae and do not survive for long after transplantation (Maltz and Treseder 2015). If native soil fungi can be established on the wildlife overpass, they should be better adapted to ensure long-term success of the newly established plants.

In order to determine whether the transplantation of the native soil plugs effectively inoculated the bridge with soil fungus communities, several questions must first be answered:

1. What is the community composition of fungi in the soil on the overpass before revegetation and soil plug inoculation?
2. What fungal communities inhabit the soil in the nursery pots where the plants are being raised?
3. What is the structure of fungal communities in the area where soil plugs will be drawn?
4. How does each community differ in terms of species richness, evenness, and overall diversity?

Research Purpose

The purpose of this research is to: 1) provide a thorough description of the fungal communities colonizing the soil in the containers at the two nurseries (where plants were being prepared for the overpass), in the soil around Swamp Lake (which has been

proposed as an area where the native soil plugs would be drawn), and in the soil atop the I-90 wildlife crossing structure; 2) define and compare species richness, species evenness, and the Simpson's diversity index for each site; and 3) provide recommendations on the procurement and placement of soil plugs.

Research Significance

Soil plays host to the most diverse community of organisms and is home to millions of species from all kingdoms (Barrios 2007). To humans, soil may seem a fixed, constant thing, but in truth it is always changing. The degradation of soil happens naturally over time with precipitation, wind, and natural disturbance, but human degradation of soil is an increasingly serious issue that threatens vast swaths of soil across the globe (Baumhardt et al. 2015). Resource management specialists and conservation ecologists are now seeking ways to conserve soil that has not been radically altered or to restore soil that has experienced severe disturbance.

Often soil conservation and restoration is approached from an agricultural standpoint. The focus here is usually improving or repairing the fertility of the soil in an effort to increase crop yields. Erosion as a result of over-working the soil is a problem that plagues farmers across the globe, as is the issue of compaction in agricultural soil after years of cultivation (Frielinghaus et al. 2001). Reclaiming mine spoils or oil shale is another focal point of soil restoration efforts, with the goal of reducing heavy metals and salinity, making the soil suitable to host plant and animal life again. In a way, these are all symptoms of a common ailment: human disturbance of the soil without any rectification of the damage done.

Anthropogenic disruption of soil negatively affects communities of soil microbes, particularly fungi (Jasper et al. 1989; Schnoor et al. 2011). Yet, the success of many restoration projects, particularly those that are introducing vegetation to a disturbed area, relies upon the soil being capable of sustaining plant life. Treatment or preparation of the soil can be a deciding factor of the efficacy of revegetation projects (Godefroid et al. 2011). Utilizing soil biota, including fungi, to provide soil treatment means taking advantage of the myriad ecosystem services supplied by these organisms, putting the flora and fauna to work in restoring soils damaged by human activities (Barrios 2007). Precedent already exists of using fungi to ameliorate disturbed soils (Anastasi et al. 2013).

While the concept of using fungi to make soils livable is not novel, the Forest Service's method of inoculating the wildlife crossing structure with native fungi via the transplantation of soil plugs drawn from local areas is new and untested. As a whole, wildlife bridges are experiments and have shown mixed success, while methods of monitoring and evaluating these structures have yet to be standardized (Clevenger and Waltho 2003). However, if it is feasible to create conditions on the bridge that mirror the forest around it, animals will perceive it as less foreign and be more likely to use it, thus justifying the construction of the overpass. A large part of naturalizing the bridge will be the installment and persistence of native flora. To aid this effort, it is critical to understand and create beneficial soil fungi communities on the wildlife overpass, as these soil fungi will increase the productivity of the introduced plants, protect the plants from pathogens, and increase the amount of nutrients available for plant uptake (van der

Heijden et al. 2008). Establishment of a diverse population of soil fungi will be of great benefit to the plants on the overpass, increasing the efficacy of revegetation efforts.

Since using soil plugs specifically to transfer soil fungi between sites has yet to be thoroughly tested for efficacy, it is crucial to establish a baseline now and give meaning to future studies conducted on the bridge. Two more wildlife overpasses are planned in the I-90 Snoqualmie Pass East Project, so any strategies deemed to be successful on the first overpass may influence decisions made around the construction and preparation of the bridges to come. Looking beyond the limited scope of the I-90 project, there are many revegetation projects all across the globe and most will face similar problems as the introduction of flora to the wildlife crossing. If the soil plug transfer successfully establishes diverse, functional communities of fungi and the survivorship of the introduced plants increases, then this method could become commonplace in restoration projects worldwide.

This study describes the baseline fungal communities, thereby allowing for future comparisons after the plants and soil plugs have been introduced. Evaluating the fungi populations before and after soil plug inoculation will illustrate the efficacy of the plug transplants and set the stage for implementation of the soil plug method in projects-to-come.

CHAPTER II

LITERATURE REVIEW

Soil Fungi

Soil is home to a truly vast number of fungi species that use many different life strategies. A study conducted in 2001 suggested that as few as 150,000 fungal species can be found in soil (Bridge and Spooner), but a review from 2007 increased that number to as many as 1.5 million species of fungi that can be found in terrestrial soils around the globe (Barrios). Species from all five major fungal phyla – Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota, and the polyphyletic Zygomycota – can be found in soil. Most soil fungi can be separated into the beneficial and the pathogenic, with saprotrophs and mycorrhizae as the beneficial groups and plant, animal, and fungal parasites as the pathogenic groups. Yeasts can be either pathogenic or beneficial, as common saprotrophs and parasites. Yeasts have played an especially important role in improving soil structure and the breakdown of soil pollutants (Botha 2011). Chytrids can also be beneficial or pathogenic, depending on the species, and have been found in wetter soils (Freeman et al. 2009). This variation in functional group is an added layer of diversity beyond the different species present in an ecosystem.

Beneficial soil fungi generally have two ecological roles: the decomposers and the mycorrhizal symbionts. Many fungal families have both mycorrhizal and decomposer species, and it is suspected that some mycorrhizal species had saprotrophic origins but it was more advantageous to associate with plants (Strullu-Derrien et al. 2018). Fungi classified as decomposers, saprophytes, or saprotrophs break down dead organic material for the purposes of consumption via the secretion of enzymes or other solvents onto a

medium and subsequent absorption of the released nutrients. Common mediums of decomposition include leaf litter, woody debris, and animal feces (particularly herbivore feces). Some fungi such as *Gymnopus perforans* specialize in decomposition of one particular medium (needle-leaf litter), whereas other species are more generalist in their choice of organic matter (Malloch 2017). Saprophytes have also been categorized into three groups: pioneer, primary component, and secondary compound decomposers (Deacon 2006). This classification is based on when they appear on a substrate and what part of the substrate they can digest, where pioneer fungi usually arrive first and decompose only cellulose and the secondary compound fungi colonize last, with the ability to digest lignin.

Decomposition of the litter layer on a forest floor requires an especially diverse community of decomposing fungi, as there are many niches to fill in the process of decomposing cellulose, hemi-cellulose, and lignin (Hättenschwiler et al. 2005). The broad array of substrates available for decomposition required an equally diverse population of fungi. Fungi are essential to fill these niches since most bacteria lack the capability of efficiently decomposing lignin (De Boer et al. 2005). Without saprotrophic fungi, many nutrients would remain in undecomposed forms, unavailable for uptake by plants. The role fungi play in decomposition is especially crucial in temperate forest environments with a predominance of needle-leaf conifers (Read and Perez-Moreno 2003). The needle-leaf litter and acidic soils characteristic of temperate forests create conditions that are unfavorable to bacteria but perfect for fungi.

Many fungi live a mycorrhizal lifestyle or have mycorrhizal forms. Mycorrhizae are fungi that associate with plant roots, trading nutrients foraged from the soil for

nutrients provided by the plants (Deacon 2006). This mutualist relationship with plants can be observed in the fossil record as early as 407 million years ago, beginning as paramycorrhizal associations with non-vascular plants like hornworts and liverworts (Strullu-Derrien et al. 2018). Now, nearly 85% of all vascular plant species are colonized by some sort of mycorrhizal fungi. Generally, mycorrhizal fungi can be classified into three overarching categories: endomycorrhizae (EM), ericoid mycorrhizae (ERM), and ectomycorrhizae (ECM) (van der Heijden et al. 2008). There are subtypes within these categories, for instance vesicular-arbuscular and coil-forming endomycorrhizae, but the primary distinction lies in whether the hyphae penetrate the root cortex (EM and ERM) or not (ECM). Endomycorrhizae can utilize branching structures called arbuscules to conduct nutrient exchange and/or have vesicles to store these nutrients, whereas ectomycorrhizae form a net of hyphae on the outside of the plant root and have hyphae penetrating the root epidermis to exchange nitrogen and phosphorous for carbon from the plant host (Deacon 2006).

Mycorrhizae provide direct benefits to plant productivity. One study observed a significant increase in biomass in eight of the eleven studied plant species (van der Heijden et al. 1998) and another discovered a correlation in plant biomass according to colonization across several geographic regions (Pánková et al. 2014). Although not all mycorrhizae affect productivity in the same manner or to the same degree – as there are several different groups of mycorrhizae and some host specificity among mycorrhizal species (Jonsson et al. 2001) – the common theme within this group is a partnership that benefits both plant and fungus. Having a mycorrhizal partner will significantly increase the amount of nitrogen, phosphorous, and water taken up by a plant, greatly augmenting

the individual's productivity (van der Heijden et al. 2008). Added surface area provided by the foraging hyphae will remain even if the fungus perishes, so a plant can continue to benefit from mycorrhizal colonization regardless of whether its fungal partner is alive or not (Read and Perez-Moreno 2003). Additionally, the net of hyphae that surrounds the host plant's roots can ward off potential soil pathogens, providing a physical barrier (van der Heijden et al. 2008). The presence of soil pathogens gives advantages to plants that are colonized by mycorrhizae that have the bulk of their biomass on the outside of the root, such as species in *Gigasporaceae* (Maherali and Klironomos 2007) or ectomycorrhizal species.

Both saprotrophs and mycorrhizae can convert pools of otherwise inaccessible macro- and micronutrients into forms that nearby plants can use (van der Heijden et al. 2008). For instance, inorganic phosphorous is often tightly bound to other soil elements, such as calcium, but the weathering action of saprotrophic fungi can release this phosphorous and allow plant uptake of the mineral (Hinsinger et al. 2011). Mycorrhizae can perform similar services via the foraging hyphae extending beyond the root of the plant. Plants are not the only organisms to benefit, as bacterial growth has been observed to increase when fungi are present to decompose lignin and hemi-cellulose (Rousk and Bååth 2011). All soil fungi can also be a food source to the many mycophages that exist in soil (Hättenschwiler et al. 2005), and when these mycophages die, fungi can recycle the nutrients held in their bodies. Even in death, fungi increase the surrounding soil's quality, as the chitin in fungal cell walls contributes nitrogen once the chitin is decomposed.

Physical structure of the soil can be altered by both groups of fungi as well. Certain vesicular-arbuscular mycorrhizal (VAM) species secrete glomalin that increases the formation of soil aggregations (i.e., peds), increasing the stability of soil (Violi et al. 2008), while hyphae both entangle soil and increase the plant root's ability to enmesh soil, further augmenting the creation of soil peds (Rillig and Mummey 2006). Increasing soil stability means less erosion over time – a particularly important service for high-precipitation or high disturbance areas. It has also been suggested that soil aggregation creates nutrient reservoirs where organic matter is shielded by mineral components (Bethenfalvey et al. 1992). Creation of peds can also lead to the formation or augmentation of pores in the soil. Hyphae can also weather mineral components of the soil to create pores, which remain intact even after the hyphae that made them has rotted away (Pointing and Belnap 2012). This improves water infiltration and percolation into the soil as long as the soil remains undisturbed.

The benefits provided by soil fungi extend beyond the direct advantage provided to plants. Soil can act as a reservoir of animal pathogens. Insect and nematode pathogens are especially abundant in undisturbed soil, whether a mature forest or a no-till agricultural field, and these fungi can either be isolated from the insect hosts themselves or from the soil if the species is an opportunistic pathogen (Sun and Liu 2008). Entomopathogens can be used as natural shields to protect plants, simply by using their innate mechanisms to attack common pests (Zhang and Feng 2018). For revegetation purposes, the presence of insect pathogens means an extra layer of protection for the introduced plants.

Disturbance does not unilaterally decrease fungal pathogen populations in soil – human additions to soil may increase its capability to harbor human pathogens. For instance, Ajello (1956) found that chicken farming increased the presence of *Histoplasma capsilatum* as a result of lowered pH and heightened organic matter. The presence of fungi normally considered pathogenic to humans is particularly interesting in soil, because it implies that these organisms are only opportunistic parasites, and do not need a living animal host to grow and reproduce (Ajello 1956).

While animal pathogens are undoubtedly relevant to human health or even natural insect control, soil fungi that are pathogenic to plants have an even greater impact by decreasing food security and crop value around the world (Strange and Scott 2005). If the aboveground plant community is not diverse, such as in a monoculture cropping system, plant pathogens will build up in the soil over time, eventually leading to reduction in biomass and productivity of the host plants (Maron et al. 2011). This effect tends to be density-dependent; only noticeable when the genetically similar plant population has become large enough that pathogens are easily shared. It has been proposed that a diverse plant pathogen community could, in turn, promote diversity in their host plants, as pathogens affect different hosts to varying degrees and overall prevent any one plant species from becoming dominant (Maron et al. 2011; Hersch et al. 2012). This is perhaps not entirely surprising, as the pathogen-host dynamic is another form of competition and competition often leads to an increase in diversity.

Environmental Effects on Community Composition

When soil is disturbed by human activity, fungi communities suffer a loss of biomass, diversity, and ability to proliferate. Studies examining this phenomenon have

been usually centered around vesicular-arbuscular mycorrhizae (VAM) in grassland-type ecosystems (Stahl et al. 1988; Jasper et al. 1991; McGonigle and Miller 2000; Schnoor et al. 2011), however the mechanisms by which disturbance affects VAM fungi can also be applied to saprotrophic and ectomycorrhizal fungi, and indeed similar results were found (Setälä and McLean 2004; Tedersoo et al. 2012). First, there is the matter of physical destruction of hyphae which can result in loss of infectivity, or colonization ability, in VAM fungi (Jasper et al. 1989). Removal of roots and stump-root complexes had similar effects on ectomycorrhizae – when the plant host is removed, so are all of the potentially infectious hyphae. Second, disruption of the soil has been demonstrated to reduce spore density, further decreasing the ability of resident fungi to proliferate (Stahl et al. 1988; McGonigle and Miller 2000). However, reduction of spores was not seen across all species and does not affect all fungi equally. For example, VAM taxa did not experience loss of colonization after perturbation, particularly in grasslands (Jasper et al. 1991).

Early colonizing species of fungi were also less affected by disruption, and so communities of soil fungi often become unevenly weighted towards these early-successional species once the inoculum of less disturbance-tolerant species is reduced (Schnoor et al. 2011). Plant nurseries can be an especially clear model of this, as the soil in nurseries often experiences low-level disturbance in the form of repotting, consistent watering, and application of herbicides, pesticides, or fungicides. For this reason, certain species or taxa tend to crop up in nursery soil more often than in natural environments. *Thelephora, Cenococcum, Rhizopgon,* and E-strain fungi have been demonstrated to be mycorrhizal symbionts particularly abundant in nursery soils (Jones et al. 2011). This created reduced evenness in the community, where a population not dominated by any

one species, genus, or other taxon was considered an even community. Trocha et al. (2006) found numerous Ascomycota mycorrhizal species in a spruce nursery, where the species composition reflected the amount of non-organic fertilizer added to the soil, demonstrating both a species bias in nursery soils and the effect of disturbance. However, these pioneer species' colonization of roots tended to decrease over time once the plant was transferred to an outdoor, non-container environment, often replaced by native mycorrhizae (Dahlberg and Stenström 1991). Soil experiencing less perturbation will see a shift in the community of fungi from the quick, early colonizers to the late colonizing, long term fungi.

Although anthropogenic disruption of soil has a generally negative effect on fungal communities through reduction of diversity and colonization, not all disturbance types have equal effect. For instance, low-intensity fires or conversion of an area to grassland or pasture will not negatively affect VAM communities (Violi et al. 2008). Soils that are particularly sparse in nutrients or have high amounts of toxic compounds will not always reduce the species richness and proliferation ability of resident soil fungi either. Particularly diverse colonies of ectomycorrhizae have been found in cinder soils that experience high amounts of grazing (Gehring and Whitham 1994) and in serpentine soils, where heavy metals are often highly concentrated, diversity of mycorrhizal fungi has been found to actually increase compared to the less-harsh soils (Branco 2010). Sizable amounts of heavy metals, such as what can potentially be found in sewage sludge, can hinder the diversity and abundance of mycorrhizae (Del Val et al. 1999) but this would be another example of anthropogenic disturbance, as these levels of toxic materials are most commonly caused by humans. Low pH in soils can actually foster

fungal growth, especially compared to bacteria growth (Rousk and Bååth 2011), and temperatures do not have much of an effect on community composition either, unless the soil experiences extreme temperatures (Linderman and Davis 2001).

Revegetation Efforts

One of the final steps in many a restoration project is the reintroduction of plants to the newly restored area. This is often a costly process, with money involved in every step. Collecting seeds or stock, raising the plants, transportation of the seedlings to the restoration area, preparation of the planting location (possibly including fencing of the area), labor costs to put the plants in the ground, and then upkeep of the established individuals all contribute to the hefty price tag of one revegetation project (Schirmer and Field 2000). These expenditures are necessary for the complete rehabilitation of an area, however, as introduction of plants can increase the water retention, porosity, bioactivity, and organic carbon and nitrogen pools while decreasing the electroconductivity and erosion of the soil (Izquierdo et al. 2005)

The ultimate fate of these efforts can vary greatly – a review of many revegetation projects found survival rates of introduced plants ranging from 0-60% after four years (Godefroid et al. 2011). It is important to note that not all revegetation projects face the same issues. For instance, reclamation of mining spoils that have had no soil amendment will have much higher rates of plant mortality, compared to a project like wetland revegetation in managed lands (Caledonia 2002). Caledonia (2002), in a study designed to create developmental benchmarks in wetland mitigation sites, reported 80% cover of native woody plants after six years and considerable amounts of volunteer recruitment from surrounding vegetation.

Numerous variables contribute to the range in reported outcomes of revegetation projects. Low number of individuals, minimal genetic diversity, and lack of soil amendment was found to result in reduced survivorship (Godefroid et al. 2011). If the introduced population is too small and has low genetic diversity, it opens the possibility of genetic drift over time and will leave the population unable to handle future disturbance. The difference between using seeds vs. seedlings or saplings also had an impact, with seedlings having the highest number of survivors over time and seeds having the least (Menges 2008). Finally, soil conditions have a marked impact on the establishment of the introduced flora. In a study of soil amendment in road reclamation , compaction, low soil moisture, scarcity of nutrients, and poor structure decreased volunteer recruitment and ability for introduced plants to establish (Elseroad et al. 2003). Most, if not all, of these aspects can be anticipated and controlled by the individuals who design these planting projects, and with careful planning a high mortality rate of introduced plants can be avoided.

Soil Fungi as a Solution

Reintroduction of plants to an area, or installation of plants into a newly created area, has a hefty price tag attached and it is essential to everyone involved in these projects to maximize the chance of success. Soil quality has been demonstrated to be a key influencing factor in this process and, with thoughtful planning and preparation, the soil in a restoration area can be changed to best fit the needs of the plants to be introduced there. Given the plentiful benefits of having a diverse community of fungi in the soil, it follows that establishment of soil fungi in a restoration zone would help ameliorate the soil and increase the survivorship of the newly added plants. Unlike adding fertilizer or

mulch, the presence or addition of fungi would provide a slow, long-lasting source of nutrients in the soil, and long-lasting amendments are more likely to increase the efficacy of revegetation (Ramlow et al. 2018).

Several aspects must be considered in the approach of using soil fungi to improve soil quality and, in turn, plant productivity. First, many mycorrhizal species, both endomycorrhizal and ectomycorrhizal, demonstrate some host specificity (Jonsson et al. 2001; Kennedy et al. 2012; Tedersoo et al. 2012; Pánková et al. 2014). Host plants can also affect the number of spores produced by their mycorrhizal symbionts, in addition to the interspecies variance in spore production (Hetrick and Bloom 1986). In terms of restoration, this means that certain fungi species will most effectively colonize the roots of introduced plants and, in return, some plant host species may foster higher chances for fungal proliferation. Equally important as the particular host plants is the diversity of the fungal community in the soil. This must not be neglected, as a diverse collection of decomposers is necessary for the most efficient decomposition of leaf litter and wood mulch (Gessner et al. 2010) while a diverse group of mycorrhizae is more likely to contain the most fitting partners and will generally contribute to higher plant productivity and, ultimately, survival (Maherali and Klironomos 2007). Host plants can influence the diversity of the community, as diversity of the fungi will influence the success of the hosts.

Given an assortment of plant species intended to be established within a restoration area, the first question must be whether the soil fungi are diverse enough to fill all decomposition niches, and the second is if there are either the key mycorrhizae or consistent colonizers that are resistant to disturbance. Fortunately, the idea of utilizing the

natural benefits of soil fungi to improve the survivorship of revegetation projects is not new. Mine spoils reclamation and oil shale revegetation efforts have a long history of using mycorrhizae inoculation to better the plants' survival (Call and McKell 1985). However, much of this research has centered around the inoculation of mining soils, which are inherently harsh and limiting to plant growth.

In reality, the potential of using fungi to improve soil health is sizable, and mostly untapped. Anastasi et al. (2013) describe how different groups of fungi – lignin decomposers, litter decomposers, and soil fungi – may be used in varying ways to break down soil pollutants. Extracellular digestion means that more than just the intended substrate may be broken down by fungi (Anastasi et al. 2013), hinting at the possibility of using fungi to decompose pollutants in the soil. For instance, lignin decomposers may destroy pollutants with aromatic rings in the act of breaking down wood mulch. For now, use of fungi to amend soils in managed public lands or recent construction areas is more conjecture than actual practice. Inoculating soil with fungi via soil plug transplantation is no exception. This practice carries the added risk of introducing invasive fungi to the restoration area. The use of commercially available mycorrhizal inoculants would introduce exotic species that could either dominate and permanently alter local fungal communities or provide plants with mycorrhizae that are not adapted to the native soil environment (Hart et al. 2018). In the mature forests around the wildlife crossing structure, ectomycorrhizae and saprotrophs that specialize in decomposing needle-leaf litter should be the most prominent groups of native fungi. Use of locally-drawn soil plugs to establish fungal communities would provide adapted fungi that carry no risk of invasion while still providing benefits to the newly introduced plants.

CHAPTER III

METHODS

Study Areas

Soil was collected at two nurseries, one mature forest, and one restoration area. The two nurseries were providing the bulk of the plants to be introduced to the wildlife overcrossing. Since soil in the plant pots will be added to the bridge along with the plants, I determined that the fungi communities present in the pots should be described. Swamp Lake was selected as the mature forest proxy, and because the Forest Service was previously considering the area as a source of soil plugs. Finally, soil was sampled on the wildlife overpass to catalogue the fungi present there before the introduction of plants.

Derby Canyon Natives (DCN) is a nursery dedicated to raising plants that are native to central Washington State, where the main complex exists at 47.570840 latitude, -120.595615 longitude, and 322 m elevation on the outskirts of the town of Peshastin, Washington, about 61.5 km northeast of the wildlife overcrossing. Climate in the Peshastin area is typical of a mid-latitude continental, sub-humid regime with average low and high annual temperatures of 1.7 ℃ and 16.3 ℃, and an average annual precipitation of 635 mm (Rossow et al. 2016) (Table 1). Although open to retail sales two days a week, DCN principally operates to raise plants for the conservation projects of many government agencies. As suggested by the name, this nursery grows only plants that are native to Washington, specializing in plants native to shrub steppe and riparian environments. Plants are cultivated primarily in containers, with some bare root stock or seed stock available for purchase. Acclimation to the central Washington climate occurs with plants' constant exposure to the environment, both being raised outdoors and in

greenhouses that are occasionally open to outside air. The greenhouse complexes are 61.5 km from the revegetation area.

Table 1: Climate and soil composition at each sampling location

Site	Annual High/Low Temps (℃)	Annual Precip. (mm)	Soil
DCN	16.3/1.7	635	65-75% peat, 25-35% perlite, limestone, mycorrhizal mix
CDA	14.75/3.58	53	80% peat, 20% mulch
SL	11.4/5.1	263.4	Kachess gravelly ashy sandy loam
BR	11.4/5.1	263.4	Construction fill

Around 22,685 plants of seven species are being raised at this nursery to be utilized for vegetation of the crossing structure (Table 2). All plants are being raised in 230 cm^3 conical pots. The specimens were potted in Pro-Mix HP Mycorrhizae soil, which is peat-based with up to 35% perlite and limestone. This soil comes pre-inoculated with a proprietary blend of mycorrhizae suited for growing flowers and vegetables. At the nursery, this soil is mixed with compost made on site and perlite is added to each pot to increase water infiltration. I was informed by Ted Alway, the nursery owner, that a 15-5-15 fertilizer with extra magnesium and calcium was applied once a week, with average 200 ppm of nitrogen.

The USDA Forest Service nursery (CDA) based out of Coeur d'Alene, Idaho is located at 47.714985° latitude, -116.825661° longitude, ~ 685 m altitude, and rests 1.9 km north of the Spokane River, and 4.8 km northwest of Coeur d'Alene proper. Climate of the area is best described as temperate with warm, dry summers. Average annual temperature is 14.75 ℃/3.58 ℃ (high/low) and mean annual precipitation is approximately 640 mm (Rossow et al. 2016). The nursery consists of 25 greenhouses and 130 acres of irrigated seedbeds, producing over 20 million seedlings per year. These

plants are for the use of both private sector and governmental agencies, to be used in projects from replanting post-logging operation to restoration of national forests. Eighty percent of the production was bare-root stock from the seedbeds, with 4 million plants produced in containers. In addition to providing plants, this nursery also cleans seeds and tempers seeds with cold storage to improve hardiness of the future seedlings. Four greenhouses made up the complex housing the plants being prepared for the wildlife crossing structure. This complex lies approximately 340 km away from the revegetation area.

Approximately 57,430 plants from 17 species are being grown here in preparation for transportation and introduction to the Lake Keechelus Wildlife Bridge. The plants were all grown from seeds, berries, or cuttings, with one batch started in 2018 and a second batch started in 2019. All specimens except the Douglas fir, Western hemlock, and Pacific silver fir are grown in 40 in^3 pots. Soil in the pots is a mix of 80% peat moss and 20% Douglas fir mulch, with vermiculite atop the soil for improved water infiltration. The plants were watered daily with an overhead sprinkler system. Integrated pest management strategies were implemented at this nursery to prevent soil pathogens from taking hold.

The first completed I-90 wildlife overpass lies at 47.322290° latitude, -121.324561° longitude, and 765 m elevation. Lake Keechelus is 1.1 km away from the bridge, and Swamp Lake rests around 2.07 km apart from the bridge. Average climate for the area is xeric and mesic, with average annual high and low temperatures of 11.4 ℃ and 5.1 ℃, and an annual precipitation of 263.4 mm (Rossow et al. 2016). The structure consists of concrete panels held together with rebar and spans both the eastbound and

westbound lanes of I-90. It is approximately 21 m from wall to wall, and the walls are 2.5 m high from the base of the arches. Construction of the bridge was completed in 2018, and around 88,000 m^3 of soil was added to the structure in the summer of 2019. Fill on the bridge was highly compacted, particularly after elk had traversed the overpass. The soil lacked structure, appearing granular in nature.

Table 2 - Plant species currently being cultivated for the I-90 wildlife overpass

Plant Species	Common Name	Nursery	Sampled? (Yes/No)
Abies amabilis	Pacific silver fir	CDA	N
Acer circinatum	Vine maple	DCN	Y
Amelanchier alnifolia	Serviceberry	CDA	Y
Aruncus dioicus	Goatsbeard	DCN	Y
Berberis aquifolium	Tall Oregon grape	CDA	N
Berberis nervosa	Cascade Oregon grape	CDA	Y
Cornus stolonifera	Red-osier dogwood	DCN	Y
Holodiscus discolor	Ocean spray	CDA	Y
Lonicera ciliosa	Orange honeysuckle	CDA	N
Lonicera involucrata	Black twinberry	DCN	N
Paxistima myrsinites	Oregon boxwood	CDA	Y
Populus trichocarpa	Black cottonwood	CDA	N
Pseudotsuga menziesii	Douglas fir	CDA	N
Ribes bracteosum	Stink currant	CDA	Y
Ribes sanguieum	Red-flowered currant	DCN	N
Rosa gymnocarpa	Baldhip rose	CDA	Y
Rosa nutkana	Nootka rose	CDA	N
Rubus parviflorus	Thimbleberry	DCN	Y
Rubus spectabilis	Salmonberry	DCN	N
Salix sitchensis	Sitka willow	CDA	N
Sambuca cerulea	Blue elderberry	CDA	N
Sambuca racemosa	Red elderberry	CDA	Y
Sorbus scopulina	Cascade mountain ash	DCN	Y
Spiraea douglasii var. menziesii	Hardhack	DCN	N
Symphoricarpos albus	Common snowberry	CDA	Y
Symphoricarpos hesperis	Creeping snowberry	CDA	N
Tsuga heterophylla	Western hemlock	CDA	N
Xerophyllum tenax	Bear grass	CDA	Y

Revegetation is planned for the fall of 2020, however there are already a few volunteer plants on the bridge, species including largehead clover (*Trifolium macrocephalum*) and some invasive weeds like prickly lettuce (*Lactuca serriola*). Only woody and herbaceous shrubs, with a few graminoid species, are planned to be added to the center of the bridge, with larger tree species being planted towards the ends of the bridge. This is largely due to safety concerns of trees falling onto the highway, as the soil on the structure is no more than 2 m thick and cannot support large root systems.

The Swamp Lake area lies between Lakes Kachess and Keechelus, around 2.3 km west of Lake Kachess and 3 km southeast of Lake Keechelus. Average elevation for the area is 740 m. Kachess Lake Road runs along the extent of Swamp Lake, providing access to research sites. The terrain is much more swampy than lake-like, despite the name, as there is very little open water. The soils are a low-slope haplosaprists, meaning an O horizon up to 109 cm thick that is comprised of highly degraded organic material (muck). This is typical of a swamp or peat bog (less so of an open-water lake) and is characterized by reduced drainage, increased fines/clays, and high cation exchange capacity (NRCS Web Soil Survey, Soil Survey Staff). The average air temperature and precipitation is identical to the bridge, as the two sampling locations were 2.25 km apart. Precipitation primarily occurs from November to February.

The sampling transect start was at 47.313675° latitude, -121.298333° longitude, and 739 m elevation (Figure 2). The soils in this area are Kachess gravelly ashy sandy loam – a course-textured soil that is well-drained with little clay and a relatively low CEC, compared to the Haplosaprists in the lake area. Dominant tree species in the area were *Thuja plicata*, *Abies grandis,* and *Pseudotsuga menziesii*. Understory vegetation

largely consisted of *Acer circinatum*, *Achlys triphylla*, *Rubus ursinus*, *Symphoricarpos albus*, and *Polystichum munitum*. The vine maple was particularly dense near the road and on the borders of the swamp but gave way to other understory flora when shaded by cedars and firs. One large nurse log bisected the transect, and woody debris was abundant in the area.

Soil Collection

Fifteen soil samples were collected at all sites, with the exception of the Coeur d'Alene nursery (CDA) where seventeen soil cores were collected. Collection methods varied between outdoor sites and the nurseries, due to the soil being contained in pots at the nurseries. Soil from pots was collected with a 7 cm plastic cylinder and transferred to a plastic sample bag. This method resulted in collection of 2.5 – 5 g of soil from each pot. Soil was collected at Derby Canyon Natives (DCN) on June 25th, 2019, and from CDA on August 9th, 2019.

The five plant species sampled at DCN were selected according to amount of representation in revegetation projects. In other words, vine maple, red-osier dogwood, and thimbleberry had the highest number of individuals that were planted on the overpass, while goatsbeard and mountain ash were being grown *en masse* for similar revegetation projects. I used a random number generator to pick three pots for each species, where the numbers generated were used to select a certain flat and then a particular pot within the flat. Plant species were selected at random from a list of species being raised at CDA. For species that had two different batches started in 2018 and 2019, such as stink currant, I collected a soil sample from one pot in each cohort. Overall, two pots from each species were sampled, with the exception of common snowberry where

one pot was sampled from the 2019 cohort. The 2018 snowberry cohort was being actively treated with insecticide, and consequently was not sampled.

Soil at the Swamp Lake (SL) site was collected on October 11th, 2019. I laid out three 15.25 m reel measuring tapes to delineate a 36.6 m transect. Every 7.32 m, beginning at zero, 1.5 m of measuring tape was used to make a 1.5 m x 1.5 m matrix, laying the tape across the transect tape to create an x-y axis (Figure 2). I chose three points along either axis for sample collection. At matrix 1, soil was collected at +/- 0.15 m from the center along the x axis and -0.15 m from the center along the y axis. Soil was drawn from points 0, 0.75, and 1.5 m along the x axis at matrix 2. At matrix 3, soil was gathered +/- 0.3 m from the center along the x axis and +0.3 m from the center along the y axis. Soil at matrix 4 was collected similarly to matrix 1, and matrix 5 had soil extracted at points +/- 0.07 m from the center along the x axis and +0.07 m from the center along the y axis. Leaf litter was cleared before inserting a trowel into the top 10 cm of soil. I inserted a trowel three times in a triangle pattern, then the loosened soil was extracted and stored in a sealable plastic bag, drawing 500-800 g of soil from each sampling point.

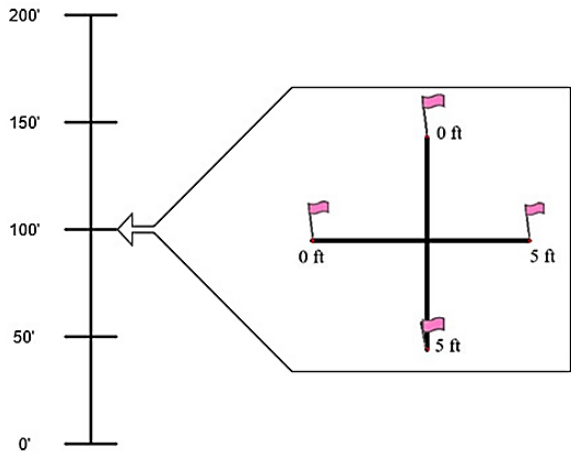

Figure 2: Simplified diagram of typical sampling transect, with example of a 1.5 m x 1.5 m sampling matrix with ends marked by flags. Original transect measured in English ft (as in diagram), but units were converted here to metric.

Soil collection atop the Lake Keechelus wildlife overpass (BR) proceeded on October 29th, 2020, and methods were similar to those performed at Swamp Lake. At this location I laid out two 30.5 m reel measuring tapes staked them into the soil to delineate a 61 m transect. Every 12.2 m, beginning at 0, a third reel measuring tape was utilized to create a 1.5 m x 1.5 m matrix. Within each matrix, I chose three points from three different quadrats to draw soil cores. At each point of sampling, wood mulch was cleared from the surface of the soil, then a metal soil probe was inserted to a depth of 10 cm. This method drew 300-500 g of soil from each point, which was deposited into a sealable plastic bag for transportation back to the laboratory.

In addition to soil collection, measurements of the soil's volumetric water content (VWC), electroconductivity (EC), and temperature were obtained by inserting a FieldScout probe into the soil at the center of each matrix. This was performed at both non-nursery sampling sites to determine whether fungi transported from the Swamp Lake area to the bridge would face significant changes in temperature, moisture, and osmotic potential (with EC as indicator). I collected VWC, EC, and temperature measurements

three times at each location: once in the fall (October 11th, 2019 for Swamp Lake and October 29th for the wildlife bridge), once mid-spring (both on May 20th 2020), and once in late spring (both on June 17th 2020). Volumetric water capacity (VWC) was measured as (water volume/soil volume) × 100, electroconductivity (EC) was measured as milliSiemens per cubic centimeter, and temperature was measured in Celsius.

I used R to create linear models to compare VWC, EC, and temperature values between sampling sites, including sampling date and time as crossed factors. An ANOVA was conducted for each physical variable to determine if matrices within the same site were significantly different. For all variables that did not conform to a normal distribution, I attempted a $\log_{10}$ transformation of the variable. If this transformation was unsuccessful, I constructed generalized linear model in lieu of the general linear model. These models were created using the core R package "nlme" (Pinheiro et al. 2020).

DNA Extraction and Sequencing

All soil samples were held at -4 °C until DNA was extracted. The cores obtained from DCN were processed the same day of collection, the sets of samples gathered at CDA and Swamp Lake were processed 24 hours after collection, and soil from the wildlife crossing structure was processed two weeks after collection. I processed all soil cores in the CWU Mycology lab. Each soil core was manually mixed in an effort to homogenize the sample and eliminate boundaries between layers in the soil. After homogenization, I separated two 250 mg subsamples from each core. For many of the nursery soil cores, these two subsamples comprised the entirety of the core and no soil was left. However, if anything remained after subsampling (always the case for the cores obtained from Swamp Lake and the overpass) this soil was archived at -4 °C. Three

subsamples were contaminated during processing, and subsequently discarded. A total of 121 subsamples were collected from 63 cores.

DNA was extracted from all subsamples by use of Qiagen DNeasy Powersoil Pro kits, following instructions provided by the manufacturer. Microbead tubes were vertically vortexed atop a VWR Scientific Products Mini Vortexer, as compared to the recommended horizontal method, but this was my only deviation from standard procedure. Seventy-five milliliters of DNA extract in aqueous solution was obtained from each subsample. Thirty microliter aliquots of each subsample were shipped on ice to the MrDNA sequencing lab in Shallowater, Texas.

Technicians at the MrDNA labs combined ITS1-F and ITS4 primers and HotStarTaq Plus Master Mix with each subsample. PCR protocol was an initial denaturing period of 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 53 °C for 40 s and 72 °C for 1 min, followed by a final elongation step at 72 °C for 5 min. Resulting products were checked for success in a 2% agarose gel, after which samples were pooled in equal amounts according to molecular weight and/or DNA concentrations. The pooled samples were then purified using Ampure XP beads. Nextera DNA sample prep kits were used to provide barcode indices for 50 ng of DNA from each sample, thus making the library. Insert size of the library was determined by use of an Experion Automated Electrophoresis Station (Bio-Rad). Prepared samples were then loaded on 600 Cycles v3 Reagent cartridges and sequenced via MiSeq 2x250 bp sequencing. Paired ends were read over 300 cycles (2x300). Each nucleotide receives a fluorescent marker that is read within the glass flow cell. Images of all cells are analyzed, and sequences within were clustered and assigned quality scores.

The MrDNA labs provided some taxonomic analysis of the resulting sequences. Barcodes, primers, sequences with less than 150 base pairs, and sequences where bases had low calling accuracy were removed from all runs. After removing these sequences, chimeras and other noise were removed. OTUs were formed by clustering sequences with 97% similarity, then named by using a BLASTn search on a database put together from NCBI and RDPII databases. Reads >250 bp and <1000 bp were joined by using Usearch, then 3'-5' reads were reoriented into 5'-3' direction, all eventually converted to FASTA and QUAL format.

Both raw and taxonomically analyzed data were shared online through Illumina BaseSpace. R package "Rqc" was used to analyze read quality (de Souza et al. 2018). Analysis by the ITS Metagenomics v1.1.0 application provided by Illumina BaseSpace was conducted to ascertain taxonomic composition of all sequences found from the forward and reverse reads of the three sequencing batches. This application searched the UNITE v7.2 database to find matches for each sequence, then returned a taxonomy report.

Diversity Analyses

I subjected all OTUs provided by MrDNA to two additional filters to remove any low-quality sequences or OTU assignments. E-value and percent homology of the associated taxa were limited to 8.14×10^{-100} and >85% respectively. The R package "vegan" provided the functions for diversity analyses (Oksanen et al. 2019). The diversity function was used to calculate the Simpson's index for each subsample, then all subsamples indices were analyzed in a mixed effects linear model that included the subsample number, soil core number, and matrix (or species, for the nursery sites) as

random effects. I created similar models for species richness (specnumber function) and species evenness (Simpson's diversity index/ln(species richness)) of each subsample.

I determined the functional group of each species by searching MycoBank databases for specimen records. Small and highly-specialized groups that were not the focus of this study, e.g. rumen associated fungi, were grouped with the saprotrophs. Yeasts and chytrids were retained as distinct categories because they were relatively large and many species lacked sufficient literature to determine an appropriate functional group. Once each species had been assigned a group, faction totals were calculated and evaluated as percentages of the whole sampled population. Group totals per subsample were also calculated. Subsample totals were compared between sites by performing an ANOVA for each group that had normal distributions within each site, or by creation of least squares models for groups that were not normally distributed at one or more sites.

CHAPTER IV

RESULTS

Soil Physical Characteristics

Average volumetric water capacity (VWC) along the Swamp Lake (SL) transect was 54.15%, and 49.57% along the wildlife bridge (BR) transect (Figure 3a). Soil VWC values were not normal, and thus were $\log_{10}$ transformed before analysis. No significant difference was found between the two outdoor locations ($F = 1.1929$, $p = 0.2856$), however VWC was significantly different according to the sampling date ($F = 4.4798$, $p = 0.0124$). VWC was not significantly different between matrices at SL ($F = 2.55$, $p = 0.105$) or at BR ($F = 0.831$, $p = 0.535$). Average electroconductivity (EC) at the five Swamp Lake matrices was 0.241 mS/cm^3, compared to an average of 0.168 mS/cm^3 in the bridge soils (Figure 3b). A significant difference between sites was discovered ($F = 4.5359$, $p = 0.04364$), but sampling date did not affect EC in the soil. Swamp Lake soils had higher EC than the bridge soils. EC was not significantly different between matrices on the bridge ($F = 1.499$, $p = 0.274$), but was different between matrices at Swamp Lake ($F = 3.558$, $p = 0.0471$) as a result of EC being highest at the second matrix and significantly lower at the fifth matrix, which was closest to the lake.

Swamp Lake soils had a mean temperature of 16.72 °C, whereas mean temperature of soil atop the wildlife bridge was 16.64 °C (Figure 3c). These data were not normally distributed, and transformations ($\log_{10}$, square root, x^2) did not successfully change the data enough to fit a normal distribution and so a generalized linear model was created to examine the relationship between location and temperature. Soil temperature did not differ significantly between the two sites ($F = 0.0318$, $p = 0.8599$), however,

temperature was significantly different between sampling dates ($F = 374.8056$, $p << 0.001$). Soil temperature did not significantly differ between sampling matrices at either Swamp Lake ($F = 0.03$, $p = 0.998$) or the wildlife crossing structure ($F = 0.002$, $p = 1$).

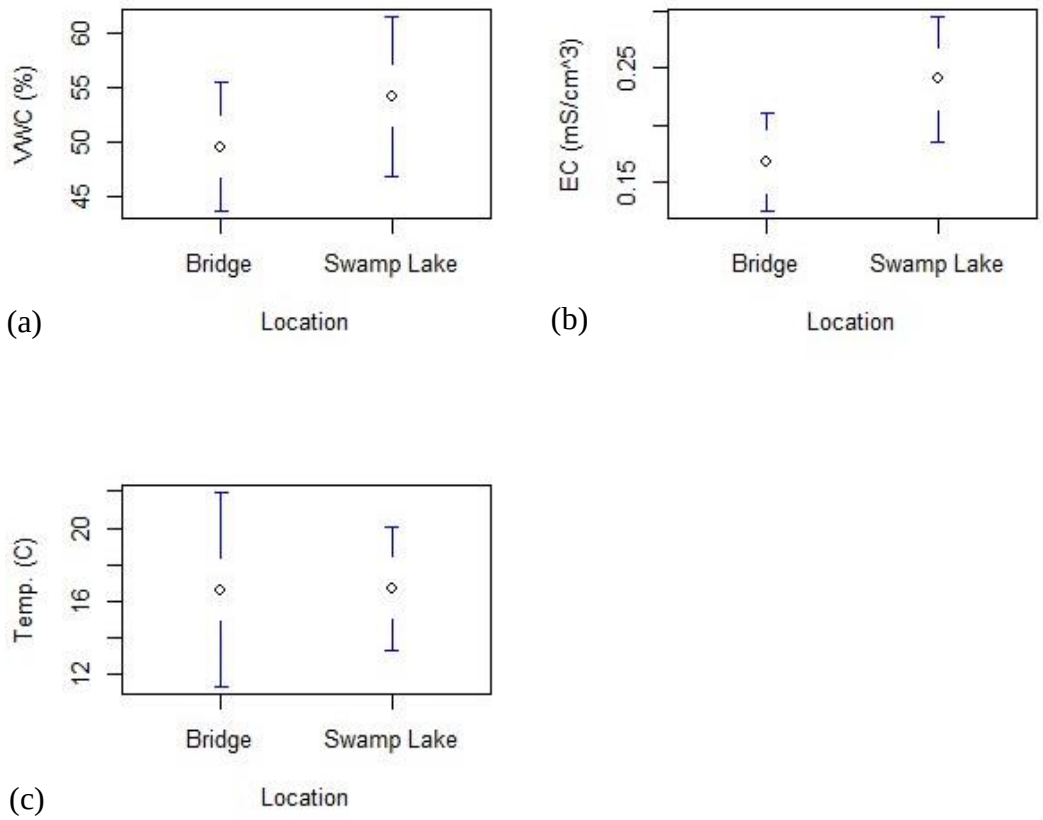

Figure 3: a) Average volumetric water capacity (VWC), as % capacity, according to site, with 95% confidence intervals. b) Mean electroconductivity (EC), in mS/cm3, according to site, with 95% confidence intervals. c) Average soil temperature in ℃ for each site, with 95% confidence intervals

Sequencing Quality

Three sequencing runs were performed to process all 121 subsamples. The first was a test batch of sixteen subsamples – four from each site – and the second and third ran 53 and 52 subsamples, respectively. The test run on the sixteen samples was conducted to determine whether the sequence data returned by the MrDNA lab would be usable in this study. The technicians at the MrDNA lab made the decision to sequence the

remaining 105 subsamples in two separate runs. Quality scores were assigned according to a PHRED algorithm. Any quality score over 30 was considered acceptable, as this would imply a base call accuracy of 99.9%. Average quality score of combined forwards and backwards reads was 32.81 for the 16-sample run, 32.72 for the 53-sample run, and 32.65 for the final 52-sample run (Figure 4). Quality scores declined as sequencing cycles progressed. The max quality score was 37.24667 within the 16-sample run, 37.5275 within the 53-.sample run, and 37.531 within the 52-sample run. Forwards reads had consistently higher quality scores than reverse reads.

For all six sequencing runs, most sequences were found once (Figure 5). Very few were isolated between 10 and 10,000 times. Few sequences were found upwards of 10,000 times. The majority of sequences found in all six runs were between 264-265 base pairs (Figure 6). 93.21% of sequences found in samples 1-16 had 264-265 base pairs and similarly in the reverse read of the first batch of samples, 93.09% of all sequences had 264-265 base pairs. In the second batch of 105 samples, the forward read of samples 1-52 had 90.98% of sequences with 264-265 base pairs. In the forward read of the next 53 samples, 93.59% of isolated sequences had between 264-265 base pairs. The reverse reads of samples 1-52 and 53-105 revealed 94.26% and 94.35%, respectively, of all sequences to have lengths of 264-265 base pairs.

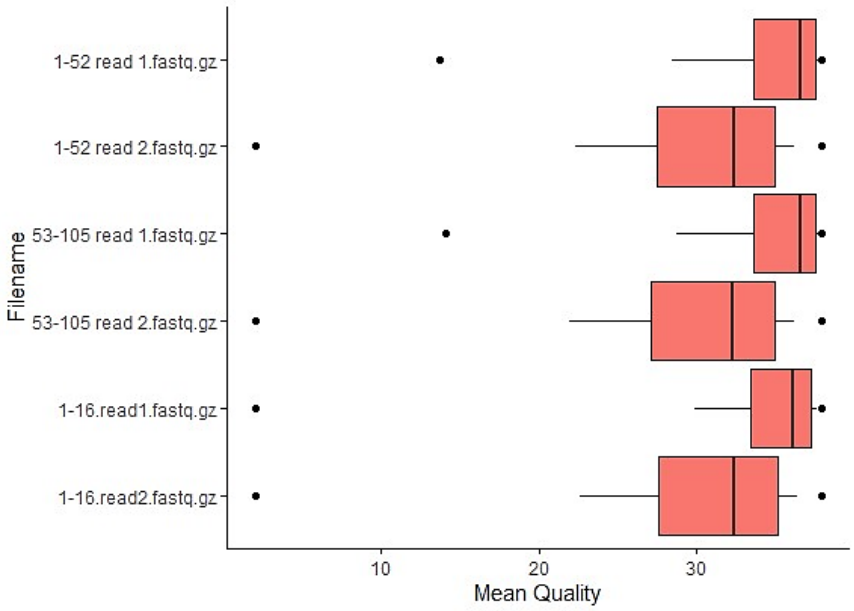

Figure 4: Mean sequence quality for forward (read1) and backwards reads (read2). File names correspond to each sequencing run.

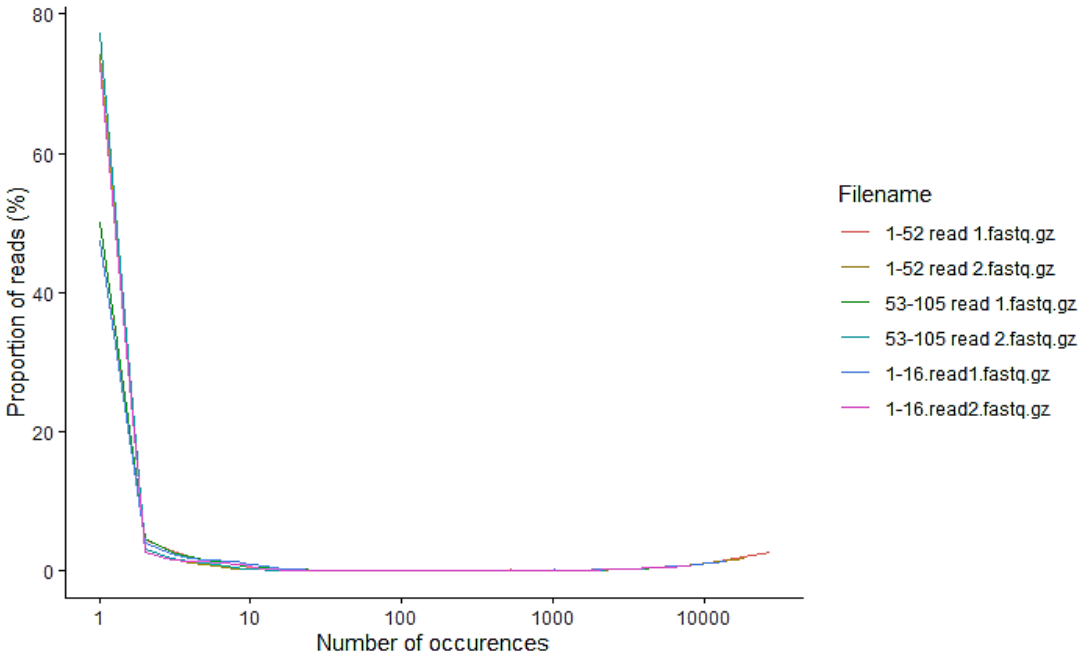

Figure 5: Proportion of reads according to the number of occurrences. Each line corresponds to a forwards or backwards read for each of the three sequencing runs.

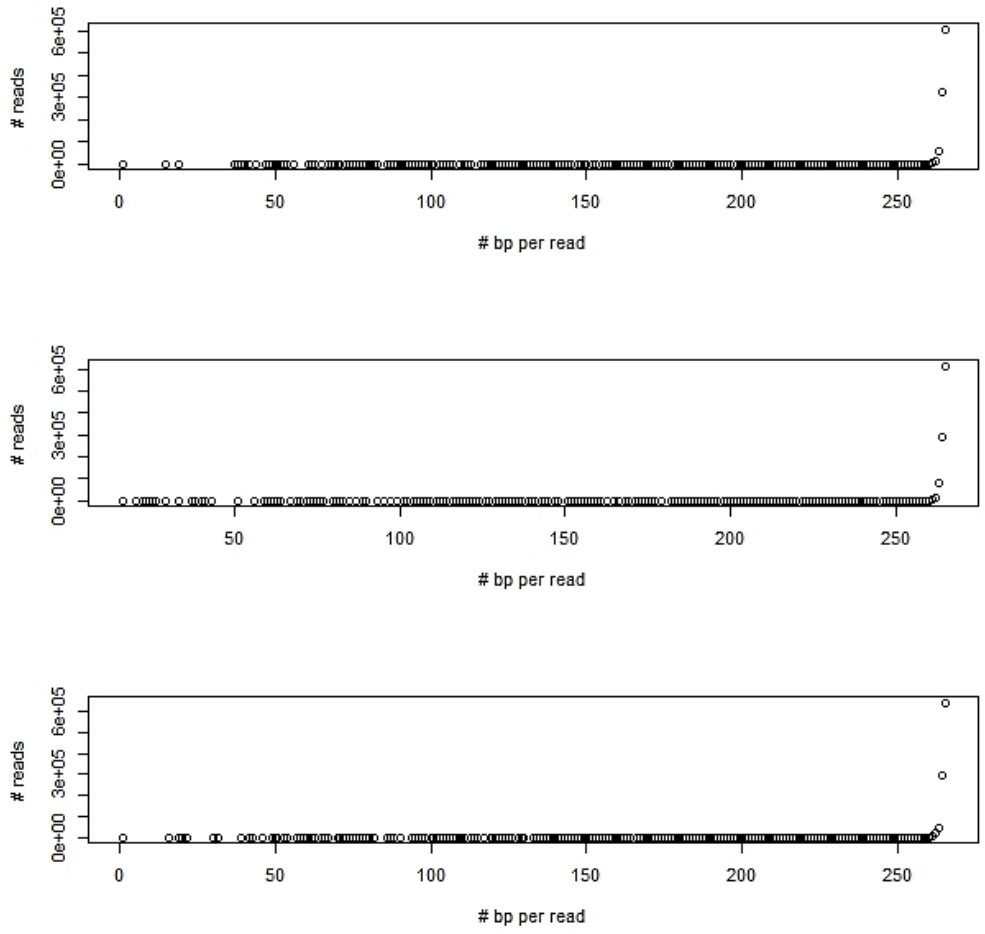

Figure 6: Number of reads according to the length of the read, as number of base pairs per sequence, for the forward read of samples 1-16 (top), the forward read of samples 17-69 (mid), and the forward read of samples 70-121 (bottom).

The use of ITS1-F and ITS4 primers resulted in the amplification of primarily fungal DNA, with 86.47% of the identified sequences falling into the Kingdom Fungi (Figure 7, left). Around 13.42% of isolated sequences were unclassified, which means that they did not have a match within the UNITE database used by the ITS Metagenomics application. Of the fungal sequences, 58.48% of all sequences were identified as Ascomycota and 12.52% were labelled as Basidiomycota by the ITS Metagenomics app (Figure 7, right). The application was unable to identify 22.17% of reads to phylum. In the first batch of samples, 70.36% of all sequences were identified to genus, whereas the

105-sample batch of samples had an average of 78.41% of reads identified to genus by the Metagenomics app (Table 2). The taxonomy analysis conducted by the MrDNA labs used a different database and placed an average of 96.43% of all sequences in the Kingdom Fungi. This analysis determined that an average of 61.55% of sequences belonged to the Ascomycota phylum and 19.97% were identified as members of the Basidiomycota.

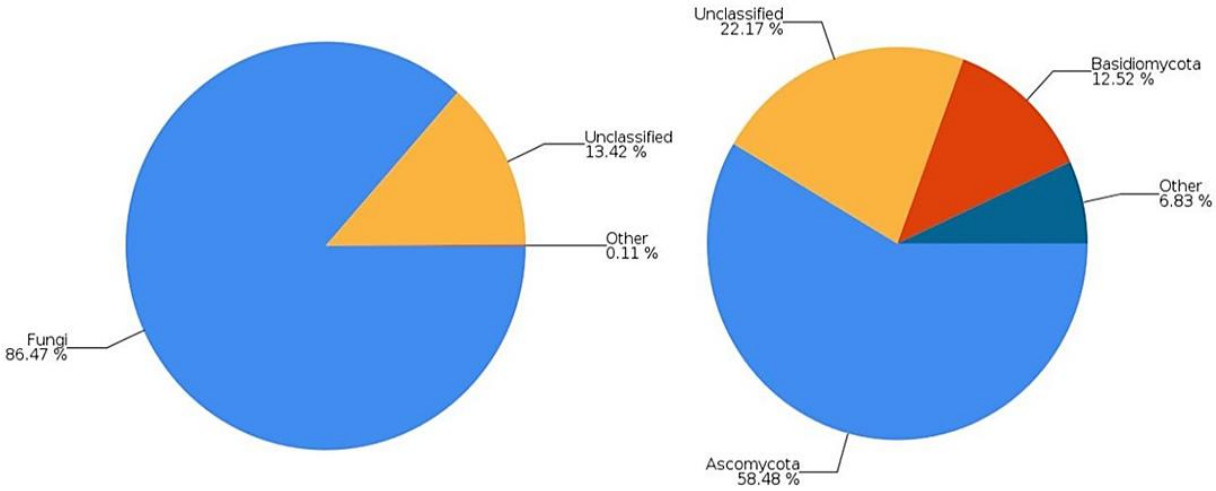

Figure 7: Classification of all reads according to kingdom (left). The "Unclassified" category may include fungal sequences that were incomplete or otherwise Classification of all reads according to phylum (right). "Unclassified" and "Other" categories include both fungal and non-fungal phyla.

A total of 2,650 OTUs were created from the sequences found in the 16-subsample batch, and 2,563 OTUs were produced from the sequences in the second batch of subsamples. Of these, 1,158 OTUs from the first batch and 1,193 OTUs from the second batch made it past the E-value and homology filters. The lowest score for sequence identity was 84.4% for OTU 2201 from the first batch of sequenced subsamples, where the closest species match was *Ophiostoma nigrocarpum*, found in one soil sample collected from an Oregon Grape plant pot in the CDA nursery. This OTU was

discarded in spite of passing the filter of having an E-value below 8.14×10^{-100}. After removing OTU2201, average percent homology was 96.67% for the 16-subsample batch and 97.39% for the OTUs in the collection of 105 subsamples, for an overall mean of 97.03% homology between the OTU sequence and the associated taxa.

Six OTUs had the cutoff E-value (8.14×10^{-100}) in the 16-subsample batch – 608, 653, 985, 1552, 2095, and 2806, or *Geoglossum glabrum*, *Sphaerostilbella aureonitens, Venturia hystrioides, Claussenomyces spp.*, *Tremella giraffa*, and *Leptodontidium elatius* respectively. From the 105-subsample batch, another six OTUs had E-values right at the cutoff – 1037, 1046, 1072, 1507, 2094, 2402, and 2715, or *Sphaerosporella spp., Taifanglania inflata, Sphaerosporella spp., Scutellinia scutellate, Rhizophlyctis rosea, Sphaerostilbella aureonitens*, and *Hygrocybe colemanniana* respectively. From the collective 2,351 OTUs from both sequencing batches, 392 OTUs from DCN soils, 462 from CDA soils, 531 from SL, and 507 from BR were unique taxa (genus or species).

Community Composition

The community of fungi in the soils at the Derby Canyon Natives nursery (DCN) was primarily composed of saprotrophs, plant pathogens, and yeast species. These groups made up respectively 35.95%, 24.51%, and 15.64% of the sampled population. Minimal amounts of endomycorrhizae, coprophilic fungi, and mycoparasites were isolated, representing 0.11%, 0.11%, and 0.55% of the whole sampled community (Figure 8). Similar to DCN, the Coeur d'Alene nursery (CDA) soils were predominately populated by plant pathogens, saprotrophs, and yeasts, with the exception of plant pathogen numbers exceeding those of saprotrophs. These three groups made up 55.68%, 22.69%, and 16.89% of the community. Endomycorrhizae, mycoparasites, and chytrids were the

least represented in the sampled population, making up 0.01%, 0.04%, and 0.24% of all the fungi (Figure 9).

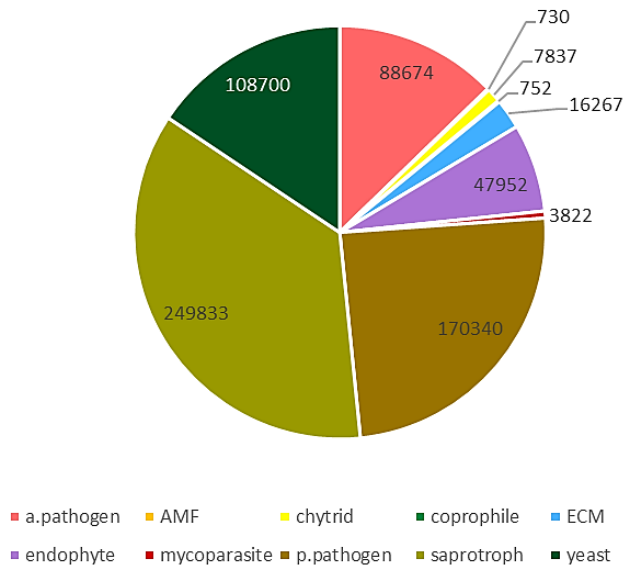

Figure 8: Composition of functional groups in Derby Canyon Native nursery soils, with total counts of each group.

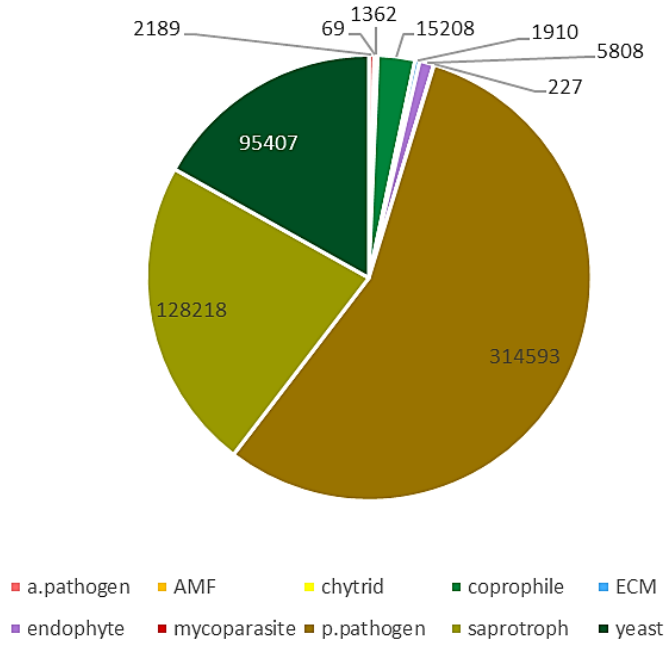

Figure 9: Functional group composition of fungi in the Coeur d'Alene nursery soils, including total counts of each group.

The fungal community isolated from soil collected at Swamp Lake were markedly different from the two nurseries (Figure 10). Ectomycorrhizae and endophytes were dominant groups, making up 29.02% and 21.63% of the population. The group encompassing saprotrophs was again in the top three, covering 33.03% of all isolated fungi. Chytrid presence was nearly non-existent – 0.004% of the community were chytrids. Endomycorrhizae again were rarely isolated, making up 0.13% of all Swamp Lake fungi. Animal pathogens were the third rarest fungi, as 0.39% of the population.

Saprotrophs were the most dominant group of fungi in the soils from the wildlife overpass, making up 63.27% of the community. Plant pathogens and coprophiles followed in abundance, as 17.25% and 7.22% of the population, respectively. The least abundant fungi groups were the endomycorrhizae, chytrids, and mycoparasites, representing 0.04%, 0.11%, and 0.15% of all the isolated fungi species that made it through the quality filters (Figure 11).

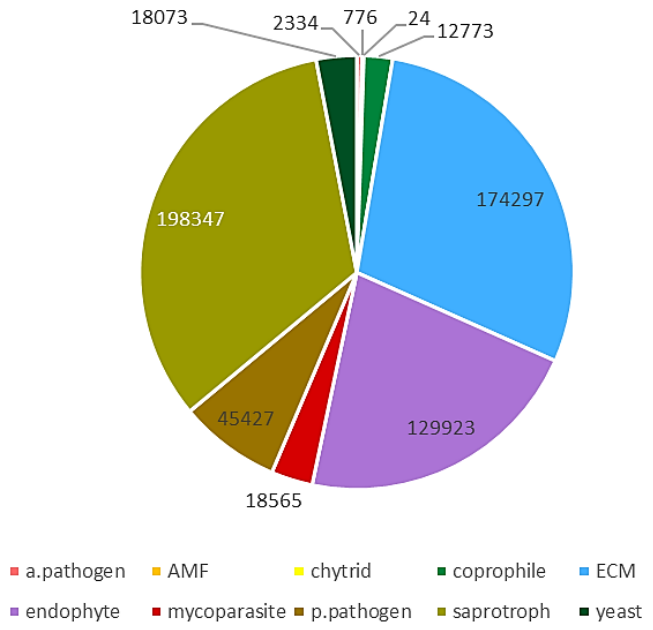

Figure 10: Distribution of functional groups in the soil collected near Swamp lake, with total counts of each group.

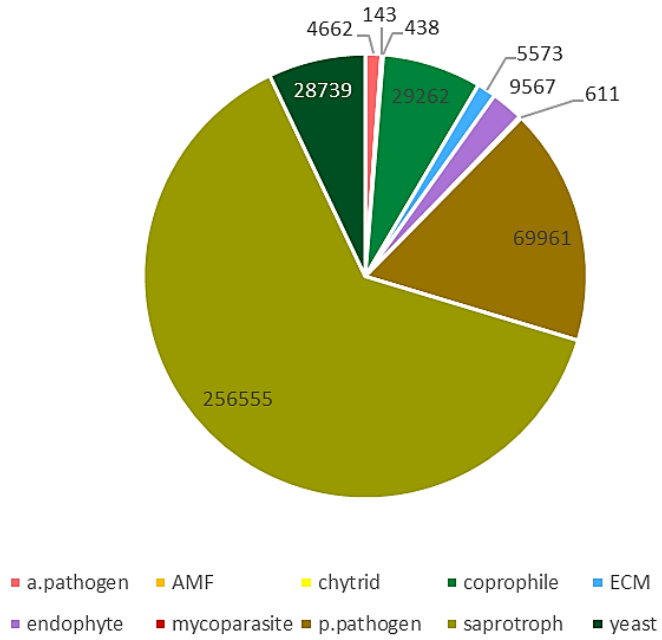

Figure 11: Functional group proportions within the fungi population in the wildlife crossing structure's soils.

All functional groups of fungi were significantly different between sampling locations (Table 3). Most groups were not normally distributed within a site, violating one assumption of the ANOVA. Log_{10} transformations were sufficient to shape the data to a normal distribution for animal pathogens, ectomycorrhizae, endophytes, mycoparasites, plant pathogens, saprotrophs, and yeasts. Chytrids, coprophilic fungi, and endomycorrhizae could not be successfully transformed to fit a normal distribution.

Fungi that primarily live as animal pathogens were significantly different between the nurseries, between the bridge and each nursery, and between Swamp Lake soils and DCN. Chytrids were significantly more abundant at DCN, but were not significantly different when comparing Swamp Lake, the bridge, and CDA to each other. Coprophilic fungi had higher numbers in the wildlife crossing soils and lower counts in DCN soils, thus these two sites were significantly different from each other. Swamp Lake and CDA were not significantly different from each other or from DCN and the bridge.

Ectomycorrhizae numbers were significantly different in pairwise comparisons of each site. The population of endomycorrhizae was significantly higher at DCN and Swamp Lake. Endophyte counts were significantly different in pairwise comparisons of all sites except in the comparison between Swamp Lake and DCN.

The bridge did not have significantly different amounts of mycoparasites from the two nurseries, however, all other pairwise comparisons demonstrated significant difference. Plant pathogen abundance was not significantly different between the two nurseries, but the nurseries were significantly different from both non-nursery locations. SL and BR were significantly different from each other. Populations of saprotrophs at CDA differed significantly from Swamp Lake soils and the wildlife crossing soils, but not from DCN soils. Saprotroph communities were also significantly different between DCN soils and the wildlife crossing structure's soil. Yeast abundance was significantly different at DCN from all other sampling sites, but all other sites were not significantly different from each other.

Table 3: F and p statistics for linear models comparing functional group to sampling site

Functional Group	**F statistic**	**p value**
Animal pathogens	31.78	<0.0001 ***
Chytrids	4.08	0.009 **
Coprophilic fungi	9.35	<0.0001 ***
Ectomycorrhizae	85.65	<0.0001 ***
Endomycorrhizae	8.05	0.0001 ***
Endophytes	47.01	<0.0001 ***
Mycoparasites	45.80	<0.0001 ***
Plant pathogens	18.24	<0.0001 ***
Saprotrophs	9.22	<0.0001 ***
Yeasts	15.82	<0.0001 ***

Diversity

Species richness of fungi was significantly different between sites ($F = 3.93$, $p = 0.0194$). This was largely due to the significant difference between the low species richness at the two nurseries compared to the relatively higher species richness in the soils collected at Swamp Lake and on the wildlife crossing structure (Figure 12). Average species richness was 120.6 at Derby Canyon Natives nursery, 131.5 at Coeur d'Alene Forest service nursery, 186.0 in Swamp Lake soils, and 191.8 in soils on the wildlife overpass. When CDA was removed from the model, sites remained significantly different ($p<0.001$).

Richness was not significantly different between sampled plant species at DCN ($F = 1.697$, $p = 0.1902$), nor between plant species at CDA ($F = 1.426$, $p = 0.2457$), nor between sampling matrices atop the wildlife bridge ($F = 1.78$, $p = 0.172$). Species richness was significantly different between matrices along the sampling transect at Swamp Lake ($F = 3.006$, $p = 0.0444$) as a result of low richness at the first sampling matrix (the closest to the road) and a relatively increased species richness at the last sampling matrix (the closest to the lake). All species richness distributions across species/matrices sampled did not initially conform to normality standards, but a $\log_{10}$ transformation was sufficient to make species richness values fit a normal distribution at each site.

Species evenness differed significantly between the four sampling sites ($F = 3.1405$, $p = 0.0423$). The low species evenness in soils from CDA contributed to this,

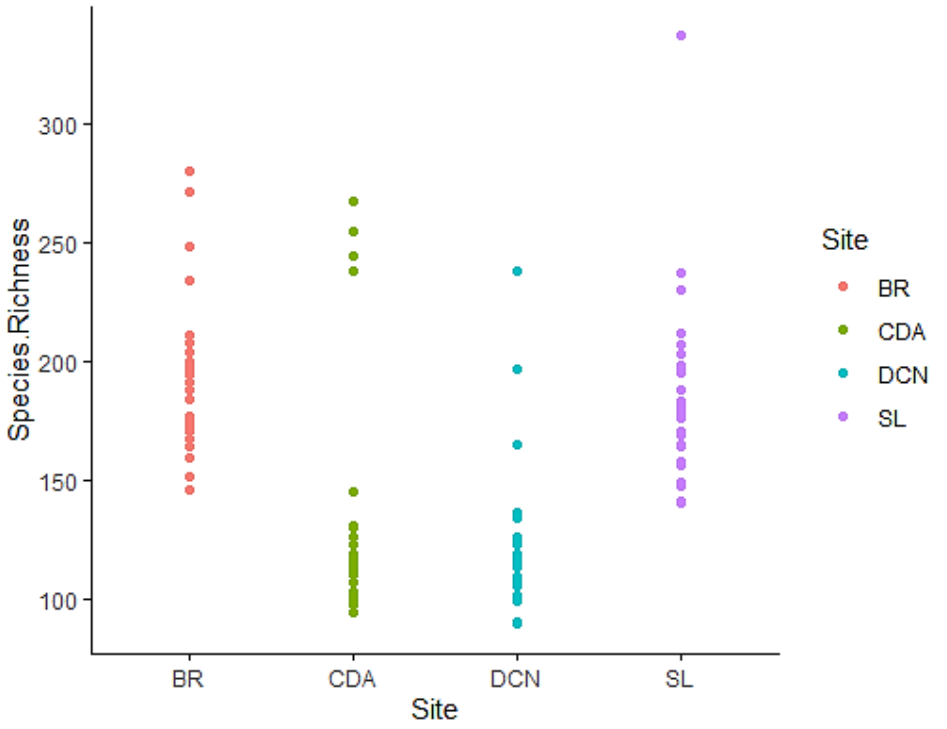

Figure 12: Species richness, as number of species present, according to site. Each point represents one subsample.

compared to the relatively higher species evenness in Swamp Lake and wildlife overpass soils (Figure 13). Mean species evenness was 0.172 in DCN soil, 0.145 at CDA, 0.162 in SL soil, and 0.162 on the wildlife bridge. When CDA was removed from the model, sites were no longer had significantly different species evenness ($p = 0.2703$).

Evenness was not significantly different between sampled plant species at DCN ($F = 0.9972$, $p = 0.4321$), between sampling matrices at Swamp Lake ($F = 1.9253$, $p = 0.1476$), nor between the matrices along the sampling transect on the wildlife crossing structure ($F = 1.7266$, $p = 0.1837$). Species evenness was significantly different between plant species sampled at CDA ($F = 2.6372$, $p = 0.0375$), as common bear grass soils had significantly reduced evenness.

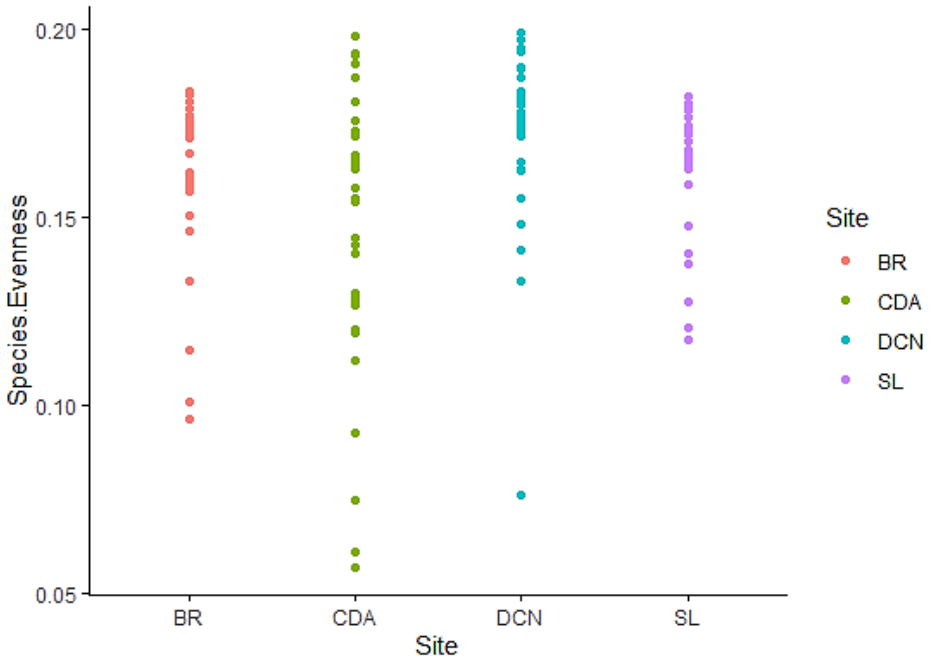

Figure 13: Species evenness according to site, with each point representing one subsample.

The four sites were significantly different in terms of diversity index scores ($F = 3.9307$, $p = 0.0194$), largely due to CDA soils having significantly less diverse fungi communities than the other four sites (Figure 14). The average diversity index for DCN was 0.822 and 0.704 for CDA soils. SL soils had a mean diversity index of 0.845 and BR soil had a mean of 0.849. When CDA was removed from the model, the remaining sites were not significantly different in terms of their diversity index ($p = 0.7004$).

Simpson's diversity index scores were not significantly different between sampled plant species at DCN ($F = 1.2598$, $p = 0.3185$), nor between sampling matrices on the wildlife crossing structure ($F = 1.4706$, $p = 0.2484$). Diversity scores were significantly different between plant species at CDA ($F = 2.8237$, $p = 0.0285$). Soil collected from common bear grass pots had significantly less diverse fungi populations than the other sampled plant species at CDA. Sampling matrices at Swamp Lake also

proved to be significantly different (F = 2.9284, p = 0.0482), as a result of low diversity at the first matrix and higher diversity at the fifth sampling matrix.

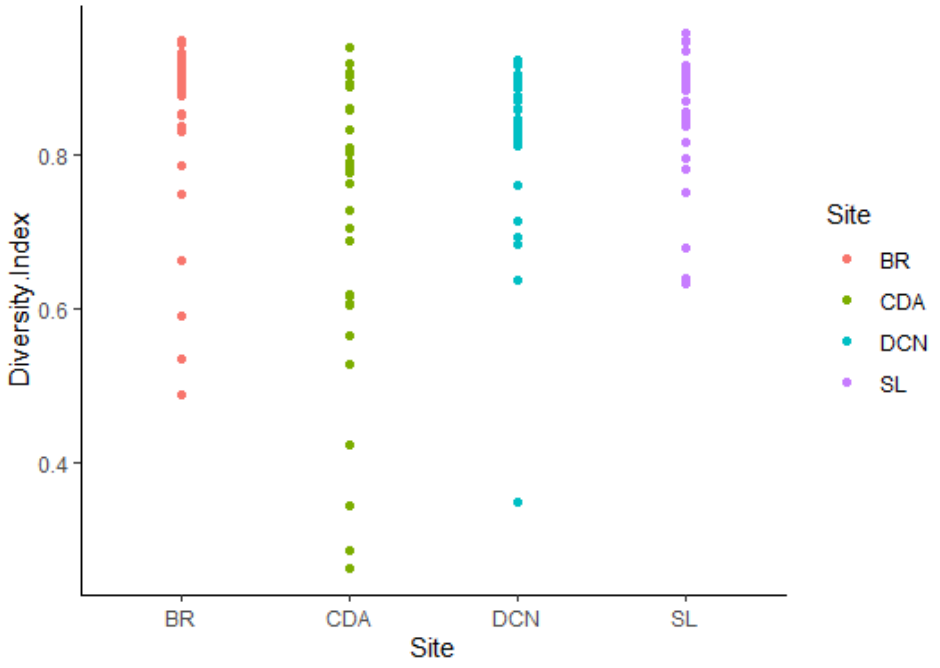

Figure 14: Simpson's Diversity Index score for each subsample, color-coded and separated by site.

CHAPTER V

DISCUSSION

Summary

The primary goals of this study were to provide a thorough description of the fungal communities at the four sampling locations and to compare the four populations' diversity characteristics. Although the results were not exactly what I was expecting, these objectives have been accomplished. Each site had unique populations of fungi, or at least distinctive proportions of all species found. Even though saprotrophs were the dominant functional group for every site except the Coeur d'Alene nursery, the genera and species that composed the saprotroph population was markedly different site to site. With the second highest mean diversity index and species richness, the wildlife crossing structure was host to a fungus population that was far more extensive than hypothesized.

Although each community was uniquely proportioned or populated, some genera were more common than others. Ubiquitous saprotroph genera such as *Penicillium* and *Cladosporium* were found in high abundance at every site and nearly every subsample, and *Mortierella* was abundant everywhere but the wildlife overcrossing. Common plant pathogen genera among the four sites were *Phoma, Lecythophora, Acremonium,* and *Alternaria*. Only Derby Canyon Natives had an abundant animal pathogen genus (*Phialemonium*), but *Ophiocordyceps* species were also present at every site. Yeast populations were prevalent only in the nursery soils, with *Cryptococcus* and *Candida* being the most consistently isolated genera across all four sites. *Rhizoscyphus ericae* was the most abundant ectomycorrhizal species everywhere but Swamp Lake, where *Cortinarius* species dominated the ectomycorrhizal community.

Aside from these frequently found fungi, many fungal species were singletons found only once within an entire transect or nursery. Fourteen and a half percent of all species isolated from Derby Canyon Natives soils were found once among the thirty subsamples. The number of singletons was higher for the Coeur d'Alene Forest Service nursery (16.9%) but lower for the two non-nursery locations (9.6% of Swamp Lake species and 10.8% of species found on the wildlife bridge). The sample-based species accumulation curves created with jackknife estimates of species richness appear to become asymptotic by the final subsamples, but the uppermost confidence interval indicates some species went undetected in the soil cores or that more soil cores needed to be drawn to catch all species (Figure 15). This is not a surprise, as previous literature has demonstrated the incredible diversity of soil fungi and the immense sampling effort required to isolate every species present in the soil microbiome (Taylor 2002; O'Brien et al. 2005). For every singleton isolated in this study's samples, hundreds more may have been present at each site, especially given the relatively small sampling area and removal of the O horizon before collecting soil.

Studying fungi comes with a degree of bias. There does not seem to be one perfect way to accurately capture all species within an area of interest, as collecting macroscopic fungi specimens or growing microscopic fungi in culture can also bias results because not all fungi make macroscopic mushrooms and not all fungi can be cultured on laboratory media (Barrios 2007). Collection of fruiting bodies has been shown to be strongly influenced by temporal factors and, even when the study spanned many seasons, the fungi growing atop the soil were not completely representative of what lies below the surface (Cuadros-Orellana et al. 2014). Furthermore, there is evidence that

gathering sporocarps or root tips to find mycorrhizae was misleading because one organism made multiple mushrooms and/or colonized multiple roots (Bridge and Spooner 2001; Taylor 2002). This was one problem avoided by using environmental DNA (eDNA) to identify the fungi at each site. Fungal DNA would be present regardless of season, as any species present would leave behind traces of DNA, whether extracellular or bound within a cell. For this reason, I did not have to include temporal bias as a potential source of error.

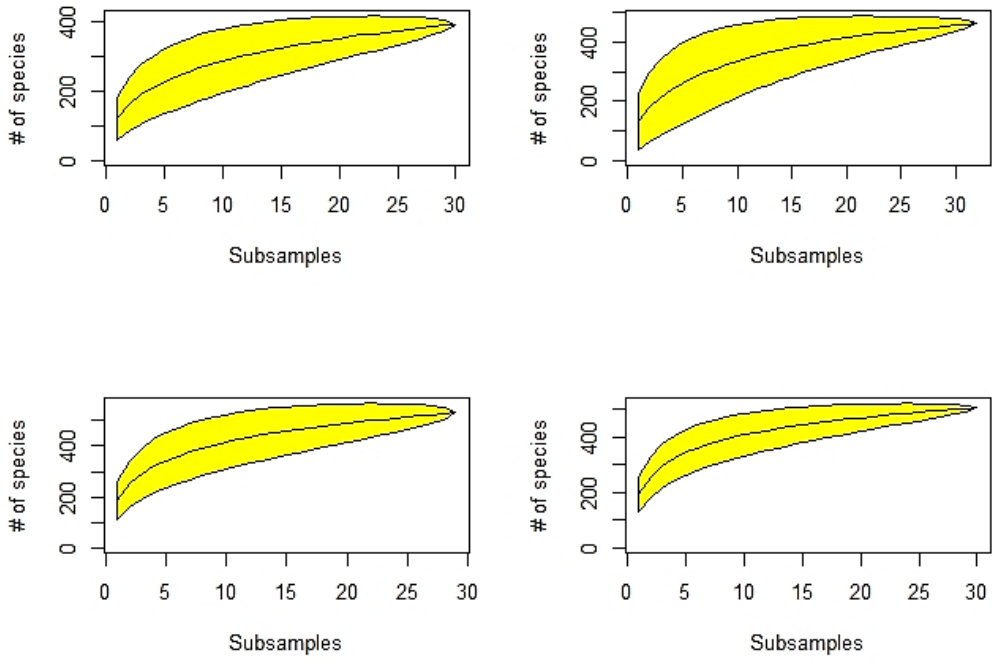

Figure 15: Sample-based species accumulation curves for DCN (top left), CDA (top right), SL (bottom left), and BR (bottom right). Yellow polygons indicate 95% confidence intervals.

While eDNA extraction may catch more cryptic species and avoid temporal issues, it is not without potential issues. Depending on soil characteristics such as aridity and temperature, DNA can remain in soil for weeks, years, or even centuries (Thomsen and Willerslev 2015). This leads to a chance of finding DNA fragments belonging to an

organism that is no longer present in the soil, creating false positives and inflating species richness. Contamination could also increase species richness erroneously, which could come from any fungi outside of the study area. Endophytes isolation would require the extraction of fungal DNA from the plant host, which is less likely than if the DNA were free in the soil. Endomycorrhizal DNA and spores can be found in the soil, however detection of these organisms can be difficult due their resistance to being cultured (Krüger et al. 2009).

eDNA must first be amplified before sequencing which means PCR must be conducted, introducing another avenue for bias. Different primers will selectively amplify certain taxa. In this study, ITS1-F and ITS4 primers were used. Previous literature has demonstrated a tendency for ITS1-F to amplify Basidiomycota over other phyla and ITS4 to amplify more Ascomycota than other phyla, and neither amplify chytrids particularly well (Manter and Vivanco 2007; Bellemain et al. 2010). Amplification of endomycorrhizal DNA is most effective when primers are used that are specifically designed for Glomeromycota DNA, which is not true for ITS1-F and ITS4 (Krüger et al. 2009). Finally, sequence identities are only as good as the database of their origin, and databases of fungal sequences can be rife with inaccuracies, such as misattributed sequences or obsolete names (Bellemain et al. 2010).

However, in spite of these limitations, this study has provided enough detail on the fungi communities at all four sites to provide a baseline for future studies of the wildlife crossing structure. Although possibly underrepresented, species outside of Ascomycota and Basidiomycota were isolated successfully, allowing us to have a general idea of the community. Species of endomycorrhizal and endophytic fungi were also

captured in my samples. Even if their abundance may be underestimated, their presence or absences affects the species richness and diversity of their site of origin, and increases our understanding of the communities.

Intrasite vs. Intersite Variation

The significant difference between some species and matrices in terms of species richness, evenness, and diversity needs to be explained. At Swamp Lake (SL), species richness increased along the transect, lowest at the first sampling matrix close to the road and highest at the last sampling matrix that was on the edge of the swamp. The root of this phenomenon is most likely the shift in plant communities from primarily vine maple and dogwood, to cedar and fir, to skunk cabbage and rushes. The change in available hosts could increase species richness, as both vine maple and dogwood are endomycorrhizal while the fir and cedar trees are ectomycorrhizal, and the ectomycorrhizae had a higher chance of being isolated from the soil samples. Ectomycorrhizae are also an especially diverse group, which would further increase the species richness in soil under ectomycorrhizal host plants (Tedersoo et al. 2010). Variance in vegetation also means a change in the litter, and more complex litter (as conifer needles would provide) requires a richer community of fungi to decompose (Hättenschwiler et al. 2005). Variation across the SL transect is an excellent example of beta diversity within what appears to be the same ecosystem, but is in truth an aggregation of patches, each experiencing change at different rates (Mori et al. 2018).

Fungi communities within the plant pots sampled at Coeur d'Alene nursery (CDA) were clearly different from species to species. Soil collected from pots bearing common bear grass, *Xerophyllum tenax*, had relatively low diversity and species

evenness, significantly reducing these indices for the entire sampling location. In natural environments, *X. tenax* is a hardy plant that can survive arid soils and can be a quick colonizer of disturbed areas (Charnley and Hummel 2011). It is therefore likely that the bear grass will survive the increased number of pathogens and reduced diversity of soil.

Diversity Index vs. Functional Diversity

A subtle distinction lies between the overall, or alpha, diversity of an ecosystem and the functional diversity of the organisms within. In alpha diversity calculations, the identity of the species does not matter. A species' mere presence will add to the diversity of the studied ecosystem by increasing the richness (Stirling and Wilsey 2001). Simpson's diversity index is centered around the proportion of species within an ecosystem, giving more weight to the abundance of a species rather than incidence (Stirling and Wilsey 2001). However, the rare species is still increasing the overall richness of the area, regardless of whether it is in the dominant functional group, and loss of these rare species will decrease the alpha diversity. A completely homogenous community could still have high species richness if many species are present that all belong to the same niche (Mori et al. 2018).

Functional diversity accounts for both identity and life strategy of all the species in question, measuring how the species affects ecosystem processes (Tilman 2001). It is not enough for the species to be present – it must fall into a specialized ecological niche to increase this type of diversity. An ecosystem with high functional diversity would have not only many species, but many different niches. Though it is a subset of total diversity, functional diversity does not completely correspond with high alpha diversity, as there could be low species richness, but many different niches filled (Tilman 2001).

An ideal, healthy ecosystem would score well in terms of both alpha diversity and functional diversity. A homogenous community with high alpha diversity is still liable to fail when severely disturbed, since all of the species would be consuming the same (or very similar) resource(s) (Mori et al. 2018). High functional and alpha diversity in a population also allows for higher species turnover, as it is less of a loss to lose one species when there are many others within the same ecological niche that can take its place (Mori et al. 2018). In this line of thinking, having high functional diversity but low species richness could also lead to ecosystem failure, because if there is only a handful of species in each niche the loss of one species could have more devastating of an effect.

For fungi specifically, both types of diversity are crucial in determining how the population operates within an ecosystem. Effect of increased alpha diversity will vary according to the lifestyle of the fungi in question. Disparate saprotrophs means better litter decomposition capability (Setälä and MacLean 2004) and diverse mycorrhizae communities leads to improved productivity of the aboveground plant hosts (van der Heijden et al. 2008), while having a variety of pathogens – plant, animal, and mycoparasitic – will create diversity in their intended targets by preventing dominance by any one species (Maron et al. 2011). Collectively having all these niches present in an ecosystem also influences overall function. Reduction or removal of any one niche would impact the populations of all other niches, fungal and otherwise, and reduce the operative capability of the entire ecosystem.

The fungi communities at Swamp Lake and the wildlife overpass demonstrate both sides of the diversity coin. On one hand is a community that has high alpha diversity and moderate functional diversity – Swamp Lake – and on the other hand is a population

with elevated alpha diversity but is dominated by saprotrophs – the wildlife bridge. Soil fungi at Swamp Lake would thus be more resilient to change, small scale disruption, and species loss than the fungi on the wildlife overpass. A more diverse assemblage of plant species would be supported by the Swamp Lake fungi as well, a fact that was obvious even qualitatively. While the alpha diversity on the bridge may be high, the functional diversity is lacking.

The reduced functional diversity of the overcrossing may be due to the homogenous nature of human disturbance. Natural disturbances tend to be patchier and less severe, barring natural disasters like hurricanes and catastrophic wildfires, than anthropogenic disruption of an ecosystem (Mori et al. 2018). Human disturbance tends to be equal across the landscape, creating more homogenized succession. This is applicable to the wildlife overcrossing since the same soil and mulch was spread across the entire structure in a relatively short time span, effectively acting as one severe disturbance event. Succession on the bridge would all begin at once. Saprotrophs usually dominate early successional populations, particularly species that can colonize quickly and compete effectively with bacteria (van der Heijden et al. 2008). Lack of a mycorrhizal presence may be the result of this disturbance too, compounded by the absence of living plant hosts. Wood mulch spread over the bridge soils provides a complex and lignin-rich litter that requires a diverse community of fungi to decompose, which may be a key factor in the relatively high diversity index of the site, as well as addition of elk feces across the bridge that would draw in coprophilic fungi.

In the soils from the two nurseries sampled in this study, we see further contrast in functional diversity. Though both had relatively lower diversity index scores than the two

outdoor sampling locations, soils from Derby Canyon Natives (DCN) had more representation among the various functional groups than what could be found in the Coeur d'Alene nursery (CDA) soils. Four factors may be at the heart of this difference in community structure. First, the soil used at DCN comes pre-inoculated with a blend of mycorrhizae (this blend appears to be proprietary, as species are not listed by the manufacturer) whereas CDA soils had no similar additions. This could explain the greater number of ectomycorrhizae in DCN pots. Second, fungicide was regularly applied at CDA but only applied if a problem arose at DCN. Unfortunately, just as overuse of antibiotics can lead to antibiotic-resistant strains of bacteria, overapplication of fungicide can precipitate a rise in resistant strains of fungi especially if the fungicide has a broad spectrum of targets (Deising et al., 2008). The dominance of plant pathogens in CDA soils could be explained by this. Third, at DCN the greenhouses were populated by many different plant species, compared to CDA where greater numbers of few species were housed in one building. Growing plants in monoculture can result in increased virulence of fungal plant pathogens (Maron et al., 2011), providing another potential explanation to the relatively higher amounts of pathogens in CDA soils. Finally, plant pathogen numbers at CDA may be an artifact of sampling more species than at DCN. However, this seems less likely considering the diverse population of plants present at Swamp Lake and the relatively smaller number of plant pathogens isolated from Swamp Lake soils.

Suggestions for Future Restoration

At present, the wildlife overcrossing is missing three groups of fungi that would be important for the success of the upcoming revegetation project: endomycorrhizae, ectomycorrhizae, and insect pathogens. Both types of mycorrhizae would aid in plant

establishment on the bridge and native mycorrhizal species could increase the competitive ability of native plants, reducing the risk of invasive species takeover (van der Heijden et al. 2008). Insect pathogens could aid in preventing herbivory that would otherwise threaten the introduced flora. The abundance of saprotrophs on the bridge, both in number of species and number of individuals, suggests that nutrient availability may be less of an issue than previously supposed.

One possible way to make up for this lack in functional diversity is to draw plugs from native soil and transplant them to the bridge. Obtaining the plugs from a local area is imperative for three reasons. First, it carries less risk of introducing exotic species to the soil, compared to inoculating with conventional store-bought mycorrhizal mixes (Hart et al. 2018). Second, the fungi would be adapted to local conditions and would be more likely to colonize the plants and soil. Third, the bridge has negligible mycorrhizae populations, so mycorrhizae transplanted via soil plug would not face much competition, thus increasing the success of inoculation. Bender et al. tested whether it was possible to inoculate corn fields with endomycorrhizae using a liquid solution of spores and met mixed results, partly due to the presence of native arbuscular fungi competitors (2019). However, this would not be an issue on the wildlife crossing, as there are few native endomycorrhizal competitors.

The area around Swamp Lake appears to be an adequate site to draw soil plugs. Ectomycorrhizae were the second most abundant functional group of fungi, so a deep soil plug taken near an ectomycorrhizal plant host, such as the plentiful grand fir, would likely capture hyphal fragments or spores that could successfully inoculate the overpass. Although endomycorrhizae were underrepresented in the samples, plants were present

around Swamp Lake that are known to be endomycorrhizal, such as *Acer circinatum*. A well-placed soil core could capture roots of such host species and spores. Implanting these plugs near another suitable host could transfer the endomycorrhizae between locations. Soil temperature and moisture content was not significantly different between Swamp Lake and the bridge, so the fungi brought from soils around the lake would not be shocked by the overpass's soils. Though electroconductivity was different, this just indicates that there would be less negative osmotic potential in the bridge's soils. Fungi can survive a broad range of osmotic potentials, so this would not be a limiting factor for inoculation success (Chowdhury et al. 2011).

Plants transported from Derby Canyon Natives have the most potential to carry insect pathogens to the wildlife crossing structure, as soils from this nursery had the highest population of animal pathogens. *Cordyceps* species were also isolated from Swamp Lake soils, which could be transported by soil plugs. Although the plant pathogen populations were sizable at the Coeur d'Alene Forest Service nursery, particularly within the bear grass soils, many species matched those occurring around Swamp Lake. Neither well-known plant pathogens like members of *Pythium, Phytophthora, Rhizophlyctis* nor species like *Phellinus weirii, Armillaria ostoyae, Heterobasidion annosum* that are known to be especially destructive in the Pacific Northwest were isolated at any of the sampling locations. It may even be a positive thing if the plants are exposed to common plant pathogens while they are being raised in the nurseries, as it could induce resistance to infection after transplantation (Cohen 2002).

Physical conditions of the soil along the Swamp Lake and wildlife overpass transects were similar, so this would not be a barrier to effective inoculation of the bridge

with native fungi. Once plants are introduced in 2020, sufficient hosts will be present that can act as hosts for transplanted ecto- and endomycorrhizae. Similar saprotroph species were found between the bridge and Swamp Lake transects, and there were unique species at each site that could complement and increase the diversity of the already robust community in the overcrossing's soil.

The soil brought to the bridge in the plant pots from the nurseries will not be adding much beneficial fungi, thus inoculation with native soil plugs would be advantageous. Ideally, the plugs will be drawn and transferred to the bridge twice – once in the spring and once in the fall – so as to avoid seasonal taxonomic bias (Cuadros-Orellana et al. 2014). Inoculant cores should also be taken in areas with diverse litter and both kinds of mycorrhizal hosts, so as to improve the chances of capturing all beneficial fungi. Each sampled site had a unique community of fungi that, when brought together, could add up to a high-functioning population that would improve soil health over time and increase the survivorship of introduced vegetation. Although a more conventional approach to soil amendment would be the application of fertilizer, consistent watering during the summer months, and frequent removal of invasive plant species, all of these goals could be accomplished or aided by establishing a diverse population of fungi atop the overcrossing.

As the baseline communities have now been described and analyzed, future studies may now examine how fungi populations on the bridge changes over time and quantify effects on the introduced vegetation. If establishment of functionally diverse fungi populations coincides with above average survival of the plants, soil plug

inoculations may become common practice to improve soil health and efficacy of restoration projects.

REFERENCES

Ajello L. 1956. Soil as natural reservoir for human pathogenic fungi. Sci. 123(3203): 876-879.

Anastasi A, Tigini V, Varese GC. 2013. The bioremediation potential of different ecophysiological groups of fungi. In: Goltapeh EM, et al., editors. Fungi as bioremediators. Berlin, Heidelberg (Germany): Springer. p. 29-49.

Barrios E. 2007. Soil biota, ecosystem services and land productivity. Ecol Econ. 64(2): 269-285.

Baumhardt RL, Stewart BA, Sinju UM. 2015. North American soil degradation: processes, practices, and mitigating strategies. Sust Switz. 7(3): 2936-60.

Bellemain E, Carlsen T, Brochmann C, Coissac E, Taberlet P, Kauserud H. 2010. ITS as an environmental DNA barcode for fungi: an *in silico* approach reveals potential PCR biases. BMC Microbio. 10(189): 1-9.

Bender SF, Schlaeppi K, Held A, van der Heijden MGA. 2019. Establishment success and crop growth effects of an arbuscular mycorrhizal fungus inoculated into Swiss corn fields. Ag Ecos and Env. 273(1): 13-24.

Bending GD, Read DJ. 1995. The structure and function of the vegetative mycelium of ectomycorrhizal plants V. Foraging behaviour and translocation of nutrients from exploited litter. New Phytol. 130(3): 401-409.

Bethlenfalvey GJ, Linderman RG, Miller RM, Jastrow JD. 1992. The role of mycorrhizal fungi in soil conservation. Myco in Sust Agri. 54(1): 29-44.

Botha A. 2011. The importance and ecology of yeasts in soil. Soil Bio and Biochem. 43(1): 1-8.

Branco S. 2010. Serpentine soils promote ectomycorrhizal diversity. Mol Ecol. 19(24): 5566-76.

Bridge P, Spooner B. 2001. Soil fungi: diversity and detection. Plant and Soil. 232(1-2): 147-154.

Caledonia MT. 2002. Establishing appropriate benchmarks for site development by documenting successional characteristics. Olympia (WA): Washington State Department of Transportation, Roadside and Site Development Unit (US). 30-39.

Call CA, McKell CM. 1985. Endomycorrhizae enhance growth of shrub species in processed oil shale and disturbed native soil. J of Range Manag. 38(3): 258.

Charnley S, Hummel S. 2011. People, plants, and pollinators: the conservation of beargrass ecosystem diversity in the Western United States. In: Dr. Jordi Lãpez-Pujol, editor. The Importance of Biological Interactions in the Study of Biodiversity. Rijeka (Croatia): Intech. p. 136-142.

Chowdhury N, Marschner P, Burns RG. 2011. Soil microbial activity and community composition: impact of changes in matric and osmotic potential. Soil Bio and Biochem. 43(1): 1229-36.

Clevenger AP, Waltho N. 2003. Long-term, year-round monitoring of wildlife crossing structures and the importance of temporal and spatial variability in performance studies. ICOET 2003. Proceedings of the International Conference on Ecology and Transportation; New York. UC Davis: Road Ecology Center. p. 293-302.

Cohen YR. 20-02. β-aminobutyric acid-induced resistance against plant pathogens. Plant Dis. 86(5): 448-455.

Cuadros-Orellana S, Leite LR, Smith A, Medeiros JD, Badotti F, Fonseca PLC, Vaz ABM, Oliveira G, Góes-Neto A. 2014. Assessment of fungal diversity in the environment using metagenomics: a decade in review. Fung Gen and Bio. 3(2): 1-13.

Dahlberg A, Stenström E. 1991. Dynamic changes in nursery and indigenous mycorrhiza of *Pinus sylvestris* seedlings planted out in forest and clearcuts. Plant and Soil. 136(1): 73-86.

Deacon J. 2006. Fungal Biology. Oxford (United Kingdom): Blackwell Publishing. p. 213-222; 256-266

De Boer W, Folman LB, Summerbell RC, Boddy L. 2005. Living in a fungal world: impact of fungi on soil bacterial niche development. FEMS Microbio Rev. 29(4): 795-811.

Deising HB, Reimann S, Pascholati SF. 2008. Mechanisms and significance of fungicide resistance. Braz J of Microbio. 39(2): 286-295.

de Souza W, Carvalho BS, Lopes-Cendes I. 2018. Rqc: a bioconductor package for quality control of high-throughput sequencing data. J of Stat Soft, Code Snip. 87(2): 1-14.

Del Val C, Barea JM, Azcón-Aguilar C. 1999. Diversity of arbuscular mycorrhizal fungus populations in heavy-metal-contaminated soils. App and Env Micro. 65(2): 718-723.

Elseroad AC, Fulé PZ, Covington WW. 2003. Forest road revegetation: effects of seeding and soil amendment. Ecol Rest 21(3): 180-185.

Freeman KR, Martin AP, Karki D, Lynch RC, Mitter MS, Meyer AF, Longcore JE, Simmons DR, Schmidt SK. 2009. Evidence that chytrids dominate fungal communities in high-elevation soil. PNAS. 106(43): 18315-20.

Frielinghaus M, Petelkau H, Schäfer H, Seidel K, Müller L. 2001. Soil indicator systems - the basis for soil conservation decisions to control soil erosion and soil compaction. Arch of Agro and Soil Sci. 47(1-2): 19-35.

Gehring CA, Whitham TG. 1994. Comparisons of ectomycorrhizae on pinyon pines (*Pinus edulis*; Pinaceae) across extremes of soil type and herbivory. Amer J of Bot. 81(12): 1509-16

Gessner MO, Swan CM, Dang CK, McKie BG, Bardgett RD, Wall DH, Hättenschwiler S. 2010. Diversity meets decomposition. Trends in Ecol and Evol. 25(6): 372-80.

Godefroid S, Piazza C, Rossi G, Buord S, Stevens AD, Aguriuja R, Cowell C, Weekley CW, Vogg G, Iriondo JM, *et al*.. 2011. How successful are plant reintroductions? Bio Cons. 144(2): 672-682.

Hart MM, Antunes PM, Chaudhary VB, Abbott LK. 2018. Fungal inoculants in the field: is the reward greater than the risk?. Func Ecol. 32(1): 126-135.

Hättenschwiler S, Tiunov AV, Scheu S. 2005. Biodiversity and litter decomposition in terrestrial ecosystems. Ann Rev of Ecol, Evo, and Syst. 36(1): 191-218

Hersch MH, Vilgalys R, Clark JS. 2012. Evaluating the impacts of multiple generalist fungal pathogens on temperate tree seedling survival. Ecol. 93(3): 511-520.

Hetrick BAD, Bloom J. 1986. The influence of host plant on production and colonization ability of vesicular-arbuscular mycorrhizal spores. The New York Bot. Garden. 78(1): 32-36.

Hinsinger P, Betencourt E, Bernard L, Brauman A, Plassard C, Shen J, Tang X, Zhang F. 2011. P for two, sharing a scarce resource: soil phosphorus acquisition in the rhizosphere of intercropped species. Plant Phys. 156(3): 1078-86.

Izquierdo I, Caravaca F, Alguacil MM, Hernández A, Roldán G. 2005. Use of microbiological indicators for evaluating success in soil restoration after revegetation of a mining area under subtropical conditions. App Soil Ecol. 30(1): 3-10.

Jasper DA, Abbott LK, Robson AD. 1989. Soil disturbance reduces the infectivity of external hyphae of vesicular—arbuscular mycorrhizal fungi. New Phytol. 112(1): 93-99.

Jasper DA, Abbott LK, Robson AD. 1991. The effect of soil disturbance on vesicular-arbuscular mycorrhizal fungi in soils from different vegetation types. New Phytol. 118(3): 471-476.

Jenkins A. 2005. Soil fungi. New South Wales (Australia): Department of Primary Industries. [Updated 2005, accessed 2020 July 6]. https://www.dpi.nsw.gov.au/agriculture/soils/biology/soil-biology-basics

Jones MD, Durall DM, Harniman SMK, Classen DC, Simard SW. 2011. Ectomycorrhizal diversity on *Betula papyrifera* and *Pseudotsuga menziesii* seedlings grown in the greenhouse or outplanted in single-species and mixed plots in southern British Columbia. Can J of Forest Res. 27(11): 1872-89.

Jonsson LM, Nilsson MC, Wardle DA, Zackrisson O. 2001. Context dependent effects of ectomycorrhizal species richness on tree seedling productivity. Oikos. 93(3): 353-364.

Kennedy PG, Matheny PB, Ryberg KM, Henkel TW, Uehling JK, Smith ME. 2012. Scaling up: examining the macroecology of ectomycorrhizal fungi. Mol Ecol. 21(17): 4151-54.

Kokalis-Burelle N. 2003. Effects of transplant type, plant growth-promoting rhizobacteria, and soil treatment on growth and yield of strawberry in Florida. Plant and Soil. 256(2): 273-280.

Krüger M, Stockinger H, Krüger C, Schüßler A. 2009. DNA-based species level detection of Glomeromycota: one PCR primer set for all arbuscular mycorrhizal fungi. New Phytol. 183(1):

Linderman RG, Davis EA. 2001. Vesicular-arbuscular mycorrhiza and plant growth response to soil amendment with composted grape pomace or its water extract. Hort Tech. 11(2): 446-450.

Maherali H, Klironomos JN. 2007. Influence of phylogeny on fungal community assembly and ecosystem functioning. Sci. 316(5832): 1756-48.

Malloch D. 2017. The saprotrophs. In: Natural History of Fungi. New Brunswick (Canada): Mycology Web Pages. [updated 2019 June 10; accessed 2020 June 7]. http://website.nbm-mnb.ca/mycologywebpages/NaturalHistoryOfFungi/Saprotrophs.html

Maltz MR, Treseder KK. 2015. Sources of inocula influence mycorrhizal colonization of plants in restoration projects: a meta-analysis. Res Ecol. 23(5): 625-634.

Manter DK, Vivanco JM. 2007. Use of the ITS primers, ITS1F and ITS4, to characterize fungal abundance and diversity in mixed-template samples by qPCR and length heterogeneity analysis. J of Microbio Methods. 71(1): 7-14.

Maron JL, Marler M, Klironomos JN, Cleveland CC. 2011. Soil fungal pathogens and the relationship between plant diversity and productivity. Ecol Letters 14(1): 36-41.

McGonigle TP, Miller MH. 2000. The inconsistent effect of soil disturbance on colonization of roots by arbuscular mycorrhizal fungi: a test of the inoculum density hypothesis. App Soil Ecol. 14(2): 147-155.

Menges ES. 2008. Restoration demography and genetics of plants: when is a translocation successful? Aus J of Bot. 56(3): 187-196.

Mori AS, Isbell F, Seidl R. 2018. β-Diversity, community assembly, and ecosystem functioning. Trends in Ecol and Eco Func. 33(7): 549-564.

O'Brien HE, Parrent JL, Jackson JA, Moncalvo JM, Vilgalys R. 2005. Fungal community analysis by large-scale sequencing of environmental samples. App and Env Micro. 71(9): 5544-50.

Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P, *et al.*. 2019. Vegan: community ecology package. R package version 2.5-6. [accessed 2020 May]. https://CRAN.R-project.org/package=vegan

Pánková H, Raabová J, Münzbergová Z. 2014. Mycorrhizal symbiosis and local adaptation in *Aster amellus*: a field transplant experiment. PLoS ONE. 9(4): 1-7.

Pinheiro J, Bates D, DebRoy S, Sarkar D. 2020. Nlme: linear and non-linear mixed effects models. R package version 3.1-148. [accessed 2020 May]. https://CRAN.R-project.org/package=nlme.

Pointing SB, Belnap J. 2012. Microbial colonization and controls in dryland systems. Nat Rev Microbio. 10(8): 551-562.

Ramlow M, Rhoades CC, Cotrufo MF. 2018. Promoting revegetation and soil carbon sequestration on decommissioned forest roads in Colorado, USA: a comparative assessment of organic soil amendments. Forest Ecol and Man. 427(1): 230-241

Read DJ, Perez-Moreno J. 2003. Mycorrhizas and nutrient cycling in ecosystems - a journey towards relevance?. New Phytol. 157(3): 475-492.

Rillig MC, Mummey DL. 2006. Mycorrhizas and soil structure. New Phytol. 171(1): 41-53.

Rossow WB, Walker A, Golea V, Knapp KR, Young A, Inamdar A, Hankins B, NOAA's Climate Data Record Program. 2016. International Satellite Cloud Climatology Project Climate Data Record, H-Series [v01r00]. NOAA Nat. Centers for Env. Info. [accessed 07/18/2020].

Rousk J, Bååth E. 2011. Growth of saprotrophic fungi and bacteria in soil. FEMS Microbio Ecol. 78(1): 17-30.

Schirmer J, Field J. 2000. The costs of revegetation. ANU Forestry and Green. Aust. Lim. 99-104.

Schnoor TK, Lekberg Y, Rosendahl S, Olsson PA. 2011. Mechanical soil disturbance as a determinant of arbuscular mycorrhizal fungal communities in semi-natural grassland. Mycor. 21(3): 211-220.

Setälä H, McLean MA. 2004. Decomposition rate of organic substrates in relation to the species diversity of soil saprophytic fungi. Oecol. 139(1): 98-107.

Stahl PD, Williams SE, Christensen M. 1988. Efficacy of native vesicular-arbuscular mycorrhizal fungi after severe soil disturbance. New Phytol. 110(3): 347-353.

Stirling G, Wilsey B. 2001. Empirical relationships between species richness, evenness, and proportional diversity. The Amer Nat. 158(3): 286-299.

Strange RN, Scott PR. 2005. Plant disease: a threat to global food security. Ann Rev Phytopath. 43(1): 83-116.

Strullu-Derrien C, Selosse MA, Kenrick P, Martin FM. The origin and evolution of mycorrhizal symbioses: from palaeomycology to phylogenomics. New Phytol. 220(4): 1012-30.

Sun B, Liu X. 2008. Occurrence and diversity of insect-associated fungi in natural soils in China. App Soil Ecol. 39(1): 100-108.

Taylor AFS. 2002. Fungal diversity in ectomycorrhizal communities: sampling effort and species detection. Plant and Soil 244(1-2): 19-28.

Tedersoo L, May TW, Smith ME. 2010. Ectomycorrhizal lifestyle in fungi: global diversity, distribution, and evolution of phylogenetic lineages. Mycor. 20(1): 217-253.

Tedersoo L, Bahram M, Toots M, Diédhiou AG, Henkel TW, Kjøller R, Morris MH, Nara K, Nouhra E, Peay KG, *et al.*. 2012. Towards global patterns in the diversity and community structure of ectomycorrhizal fungi. Mol Ecol. 21(17): 4160-70.

Tilman D. 2001. Functional diversity. In: Levin SA, editor. Encyclopedia of Diversity, vol. 3. St. Paul (MI): University of Minnesota (US). p. 109-120.

Thomsen PF, Willerslev E. 2015. Environmental DNA – an emerging tool in conservation for monitoring past and present biodiversity. Bio Cons. 183(1): 4-18.

Trocha LK, Rudawska M, Leski T, Dabert M. 2006. Genetic diversity of naturally established ectomycorrhizal fungi on Norway spruce seedlings under nursery conditions. Micro Ecol. 52(3): 418-425.

United States Department of Agriculture. Web Soil Survey. Natural Resources Conservation Service, [accessed 2020 August 3] http://websoilsurvey.sc.egov.usda.gov/.

van der Heijden MGA., Klironomos JN, Ursic M, Moutoglis P, Streitwolf-Engel R, Boller T, Wiemken A, Sanders IR. 1998. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature. 396(6706): 69-72.

van der Heijden MGA, Bardgett RD, Van Straalen NM. 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecol Letters 11(6): 296-307.

Violi HA, Barrientos-Priego AF, Wright SF, Escamilla-Prado E, Morton JB, Menge JA, Lovatt CJ. 2008. Disturbance changes arbuscular mycorrhizal fungal phenology and soil glomalin concentrations but not fungal spore composition in montane rainforests in Veracruz and Chiapas, Mexico. Forest Eco and Man. 254(2): 276-290.

Washington State Department of Transportation. I-90 - Snoqualmie Pass East - Hyak to Keechelus Dam (Phase 1) - Project map. [accessed 2020 August 2]. https://www.wsdot.wa.gov/projects/i90/snoqualmiepasseast/hyaktokeechelusdam/project-map

Zhang LB, Feng MG. 2018. Antioxidant enzymes and their contributions to biological control potential of fungal insect pathogens. App Micro and Biotech. 102(12): 4995-5004.