AN ABSTRACT OF THE THESIS OF

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Title: The Role of Plant-soil Feedback in the Invasion of *Brachypodium sylvaticum* in Douglas-fir Forests

Abstract approved: _____

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Invasive plants have the capacity to transform landscapes and alter ecosystem function, causing significant economic and ecological damage. These effects include displacement and reduction of native flora and fauna, altered fire regimes, modification of biotic and abiotic soil properties, as well as local, regional, and global economic impacts. With such large impacts it is important that we better understand invasion dynamics to help with prevention, control and mitigation of invasive species.

One process that has been associated with plant invasion is plant-soil feedback (PSF). A PSF occurs when plants alter biotic and abiotic soil properties through a variety of root exudates and litter decomposition such that subsequent plant growth is either positively or negatively affected. Positive conspecific and negative heterospecific responses have been theorized to be invasive species traits that promote invasion. Once an invasive species is removed from a system, there is a chance that PSFs generated by that species will persist in the soil, which is often referred to as ‘plant legacies’ or ‘legacy effects’ and may negatively influence restoration efforts.

In the U.S. Pacific Northwest (PNW), *Brachypodium sylvaticum* (slender false brome), a perennial bunch grass native to Eurasia, is listed as a quarantined invasive

species in California, Oregon, and Washington. Currently, *B. sylvaticum* is in the midst of rapid population growth and range expansion with populations in New York, Virginia, and Ontario, Canada. With a quickly expanding range research is critical for successful efforts to reduce the spread of *B. sylvaticum*. We developed two experiments to determine if PSF is a contributing factor to *B. sylvaticum* invasion in PNW forests. We hypothesized that 1) *B. sylvaticum* has positive conspecific and negative heterospecific PSF, 2) native species PSF has no effect on *B. sylvaticum,* and 3) PSF generated by *B. sylvaticum* will persist in the soil once removed, but over time, response of native species, soil nutrients and bacterial community composition will change from the invaded conditioned.

To test our first two hypotheses, *B. sylvaticum* and five common native plants from the Oregon Coastal range, including the economically important tree, *Pseudotsuga menziesii* (Douglas-fir), were grown in a greenhouse on wild forest soils that had either been sterilized or kept live to condition the soil biotic community to the invader and the native species. *Brachypodium sylvaticum* was then grown on soil conditioned by itself and soil conditioned by natives; each of the five native species was grown on soil conditioned by *B. sylvaticum* and on their own conditioned soils. Plant biomass along with species specific measurements (number of leaves, stems, tillers, stem diameter and height) were recorded and a relative response (RR) index was used to determine the direction of PSF for the invader and native species.

To test our third hypothesis, in March, 2015, ten plots were established in the McDonald-Dunn Research Forest located in Corvallis, OR where *B. sylvaticum* had at

least 75% cover. Herbicide was applied to half of each plot to make two soil treatments: soil with *B. sylvaticum* and soil without *B. sylvaticum*. Over a nine-month period three soil collections took place where soil was collected from all plots and treatments. Plant response was evaluated by growing four native species and *B. sylvaticum* on both soil treatments and evaluating total biomass with a RR index; plant response (via growth), soil nutrients and bacterial communities were measured for each collection period. Bacterial communities were measured with phospholipid fatty acid (PLFA) analysis and high throughput 16s rRNA amplicon sequencing.

Contrary to our hypotheses, the RR to PSF generated by *B. sylvaticum* was negative for the invader and *P. menziesii* and neutral for all other natives. Soils conditioned by *Bromus vulgaris* inhibited *B. sylvaticum* growth whereas soils conditioned by *Prunella vulgaris* and *P. menziesii* promoted *B. sylvaticum* growth. When testing for legacy effects, the RR of *P. menziesii* was negative when grown on soils where *B. sylvaticum* had been removed for six-months (six-month soils) but when grown on soils where *B. sylvaticum* had been removed for nine-months (nine-month soil) the RR of *P. menziesii* was neutral. The RR of *P. vulgaris* to six-month soils was positive while its RR to nine-month soils was negative. Nutrient and bacterial communities did not change in response to *B. sylvaticum* removal suggesting that the biotic and abiotic legacy requires longer than nine-months to be observed or *B. sylvaticum* does not affect the response variables measured.

Overall, our data suggest that PSF generated by *B. sylvaticum* does not facilitate the invasion process but does differentially affect native species growth over time. PSF

generated by native grasses may be a useful restoration tool to help prevent *B. sylvaticum* invasion and we suggest planting native species at least nine-months after *B. sylvaticum* removal.

The Role of Plant-soil Feedback in the Invasion of *Brachypodium sylvaticum* in Douglas-fir Forests

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Andrew Esterson, Author

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CONTRIBUITION OF AUTHORS

Chris Gaulke assisted with soil microbial DNA extraction, amplification, sequencing and statistical analyses for all data presented in the second and third chapters of this thesis, *The role of plant-soil feedback in the invasion of* Brachypodium sylvaticum *in Douglas-fir forests* and *The legacy of* Brachypodium sylvaticum *in an Oregon Coastal Range forest: nutrient, microbial and plant response to plant-soil feedback.*

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Introduction

Invasive Species Impacts

Invasive plant species can have strong negative impacts on native ecosystems (Vitousek et al. 1996). These effects include displacement and reduction of native flora (Hejda, Pyšek, and Jarošík 2009), altered fire regimes (Brooks et al. 2004), modification of abiotic (Grove, Parker, and Haubensak 2015) and biotic soil properties (Mangla and Callaway 2007), as well as local, regional, and global economic impacts (Kolar and Lodge 2001). It has been estimated that invasive species cause approximately 120 billion dollars in annual damages and mitigation costs in the United States (Pimentel et al. 2005). Understanding patterns of invasion and the underlying mechanisms is critical for preventing, controlling and mitigating damage by invasive species.

Invasion Hypotheses

Hypotheses explaining plant invasion are numerous and invasion ecology has been studied for well over a century (Darwin 1859; Mitchell et al. 2006). However, there is diminutive and contradicting empirical data to support any one mechanism. For example, the Enemy Release Hypothesis (ERH) posits that once a species is introduced to a new range it is released from co-evolved specialist pathogens and herbivores from the native range that restrict population growth (Keane and Crawley 2002), which subsequently allows unregulated population growth in the new range. When the ERH was tested on

populations of *Brachypodium sylvaticum*, an invasive grass in the United States, it was found that population growth rates were lower in the native range compared to invaded locales, thus supporting the ERH (Roy et al. 2010). In contrast, in a common garden experiment with 61 plant species (native, non-native and non-native invasive), Schultheis et al. (2015) found the invasive species were grazed significantly more than natives and grazing on invasive species increased with time from introduction, thus, not supporting the ERH. Following the same logic as the ERH are the novel weapons (Callaway and Aschehoug 2000) and empty niche (Hierro, Maron, and Callaway 2005) hypotheses. The novel weapons hypothesis suggest that invasive species have evolved weapons (e.g. allelopathic compounds) which in their home range do not give them a competitive advantage because of co-evolution with neighboring plants; however, in the invaded range, natives do not share a long evolutionary history with the invasive species, and therefore are not equipped with the mechanisms to deal with the weapons, thus increasing invasion success. The empty niche hypothesis posits that invasive species are released from resource competition from their native habitat and therefore can utilize resources (or empty niche space) not used by natives in the invaded land. Although invasion hypotheses differ in mechanism, most require plants to have certain functional traits to validate the hypothesis.

Invasive Species Functional Traits

Identifying patterns of functional traits that are prevalent among invasive species is a challenging task. Individuals within a species vary genetically and morphologically across time and space, which has the potential to alter the effect of a given trait with respect to invasion success. Nonetheless, many analyses of invasive species functional traits have been undertaken and have identified traits which include photosynthesis and transportation rates, nitrogen and water use efficiency, leaf area allocation, root:shoot ratio, growth rate and overall size and fitness as important factors for invasion success (Kolar and Lodge 2001; Daehler 2003; Pyšek and Richardson 2008; Van Kleunen, Weber, and Fischer 2010). These traits are not mutually exclusive and are constrained by environmental and genetic factors (Lee 2002), which raises the question, if invasive traits covary in predictable ways (i.e. leaf area linked to photosynthesis rate, size linked to fitness, etc.) is there an underlying factor that is contributing to these traits? For example, water is an essential component in photosynthesis, nutrients such as magnesium allow chlorophyll to absorb photons and nitrogen and phosphorus are ubiquitous throughout the plant as they are critical components of DNA. Perhaps the ability to acquire water and nutrients in a greater capacity compared to neighboring native species is one factor limiting or driving invasion success. Moreover, mechanisms that inhibit surrounding plants (e.g. increased soil pathogens that reduce plant growth and/or allelopathic chemicals) may also play a role in invasion dynamics.

Plant soil-feedback

One trait that has the potential to directly impact a plant's growth and development is its sensitivity to plant-soil feedback (PSF). A PSF occurs when plants affect local neighborhood biotic and/or abiotic soil properties (via root exudates and litter decomposition), such that subsequent plant growth is either positively or negatively affected, which in turn alters community composition, and therefore invasion success (Brinkman et al. 2010). PSF theory suggests that positive biotic feedback is associated with an accumulation of soil mutualists while negative feedback is associated with an accumulation of pathogens and/or pests (Klironomos 2002). Soil mutualists such as arbuscular mycorrhizae fungi (AMF) and nitrogen fixing bacteria deliver nutrients and water to plants which in turn provide the necessary resources for the aforementioned traits to become important in respect to invasion success. Conversely, soil pathogens and pests can inhibit plant performance by reducing plant vigor and/or competing for soil resources which has the potential to reduce invasion success. Additionally, PSF alterations have been shown to persist in the soil (i.e. legacy effects) long after a plant is removed (Elgersma et al. 2011; Grove, Parker, and Haubensak 2015) which in turn affects community composition and structure.

Species of Interest

Brachypodium sylvaticum (Huds.) Beauv (slender false brome), a perennial bunch grass native to Eurasia, is listed as a quarantined invasive species in California, Oregon, and Washington in the United States (Holmes et al. 2010). Currently, *B. sylvaticum* is in the midst of rapid population growth and range expansion with populations found in New York, Virginia, and Ontario, Canada (Miller et al. 2011). Invasion by *B. sylvaticum*

threatens native plant diversity, endangered species, pollinators, fire regimes, and timber production. Restoration of habitats invaded by *B. sylvaticum* is vital for the persistence of native flora and fauna. However, its strong competitive ability (Naylor-Murphy 2012) and broad extent of invasion (Halbritter et al. 2012) make restoration efforts difficult. Understanding the mechanisms behind *B. sylvaticum* invasion is critical for the prevention of its expansion and restoration of invaded environments.

Research Goals and Objectives

We conducted two experiments to determine the extent PSF plays in the role of *B. sylvaticum* invasion. Specifically, we asked the following questions related to PSF:

1. Does *B. sylvaticum* have positive conspecific PSF?
2. Does PSF generated by *B. sylvaticum* negatively influence native plant growth?
3. What effect does PSF produced by native plants have on *B. sylvaticum* growth*?*

Related to soil legacy effects, we asked:

1. Does *B. sylvaticum* produce a soil legacy effect on native plants and how long does the effect last?
2. How do native plants and the invader respond to soils that have been previously occupied by *B. sylvaticum?*
3. How do soil chemical properties and bacterial community composition change once *B. sylvaticum* is removed from the soil?

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Chapter 2:

The role of plant-soil feedback in the invasion of *Brachypodium sylvaticum* in Douglas-fir forests

Abstract

Brachypodium sylvaticum (slender false brome), a perennial bunch grass, is listed as a quarantined invasive species in the Pacific Northwest of the United States and is currently in the midst of rapid population growth and range expansion. *Brachypodium sylvaticum* invasion threatens native plant diversity, endangered species, pollinators, fire regimes and timber production. Understanding the mechanisms and effects of *B. sylvaticum* invasion is critical for the prevention of its expansion and the restoration of invaded environments. One explanation for the invasive ability of *B. sylvaticum* is plant-soil feedback (PSF). A PSF occurs when plants change biotic and/or abiotic soil properties such that plant growth is increased or decreased, which in turn alters community composition. The objective of this study was to determine the role PSF plays in *B. sylvaticum* invasion. We tested the hypotheses that 1) *B. sylvaticum* produces positive conspecific and negative heterospecific PSF on native species in a Douglas-fir forest in the Oregon coastal range and 2) that PSF generated by native species have no effect on *B. sylvaticum* growth. To test our hypotheses, *B. sylvaticum* and five common native plants, including the economically important tree, *Pseudotsuga menziesii* (Douglas-fir), were grown in the greenhouse on wild forest soils that had either been sterilized or kept live to condition the soil biotic community to the invader and the native species. *Brachypodium sylvaticum* was then grown on soil conditioned by itself and soil conditioned by natives; each of the five native species was grown on soil conditioned by *B. sylvaticum* and on their own conditioned soils. A relative response (RR) index was used to analyze biomass data to determine PSF effects. Contrary to our hypotheses, results indicate that *B. sylvaticum* has negative conspecific PSF feedback and variable

heterospecific PSF depending on the species examined. *Pseudotsuga menziesii* was the only native species studied to be negatively affected by soils conditioned by *B. sylvaticum.* Soil conditioned by *Bromus vulgaris* reduced *B. sylvaticum* growth whereas soil conditioned by *Prunella vulgaris* and *P. menziesii* promoted *B. sylvaticum* growth. In addition, *B. sylvaticum* performed better in all sterilized treatments compared to live treatments and there was a significant difference in bacterial communities between the two treatments. Overall, data suggest that PSF generated by *B. sylvaticum* does not facilitate invasion but differentially affects native species. In addition, PSFs produced by native grasses may be good restoration tools to help prevent *B. sylvaticum* invasion.

Introduction

Invasive plants have the capacity to transform landscapes and alter ecosystem function, causing significant economic and ecological damage (Vitousek et al. 1996). Invasive species effects include displacement and reduction of native flora (Hejda, Pyšek, and Jarošík 2009), altered fire regimes (Brooks et al. 2004; Poulos and Roy 2015), modification of abiotic (Grove, Parker, and Haubensak 2015) and biotic (Mangla and Callaway 2007) soil properties, as well as local, regional, and global economic impacts. Better understanding of the mechanisms behind invasion can help prevent, control, and mitigate for invasive species.

Over the past twenty years there have been a growing number of ecologists that have investigated the role of plant-microbial interactions in community assembly, especially as they relate to plant invasion (Reinhart and Callaway 2006; Eppinga et al. 2006; Mangla and Callaway 2007; Jordan, Larson, and Huerd 2008; Suding et al. 2013; Rutten et al.

2015). However, the fundamental knowledge pertaining to plant-microbial interactions (i.e., mutualistic symbioses and pathogenic responses) was first discovered in the agriculture and horticulture sector (Philippot et al. 2013) with a strong emphasis on plant health, not community diversity and maintenance. It was not until the latter part of the 20th century that ecologists began developing theories describing the roles of microorganisms in community structure (Janzen 1970; Mills and Bever 1998; Chesson 2000; Van Der Heijden, Bardgett, and Van Straalen 2008). For example, Janzen-Connell effects, which occur when specialist pathogens reduce dense populations of conspecific progeny (Janzen 1970; Connell 1971), can play a role in maintaining species diversity in both tropical (Harms et al. 2000) and grassland (Petermann et al. 2008) communities by reducing the recruitment of conspecifics in a density dependent way near reproductive plants, subsequently providing space for the recruitment of heterospecifics. However, a number of studies have shown that microorganisms play a role in plant invasions by providing biologic conditions that promote invasive plants over natives (Klironomos 2002; Mangla and Callaway 2007; Perkins and Nowak 2013).

The Enemy Release Hypothesis (ERH) (Keane and Crawley 2002; Mitchell and Power 2003; Roy et al. 2010) states that plants escape from co-evolved, specialist enemies (such as herbivores and pathogens) when they invade new landscapes and are released from negative effects of enemies on population growth and regulation (Keane and Crawley 2002). In an analysis of 473 species of naturalized plants in the United States that were native to Europe, Mitchell and Power (2003) reported a significant reduction in fungal and virus infection in naturalized plants compared to plants in their home ranges. In addition, species that showed the greatest release from pathogens tended

to be the most invasive. Although several studies provide evidence supporting the ERH, (Wolfe 2002; Mitchell and Power 2003) other studies have shown that plants in both the native and invaded ranges are equally affected by enemies (Agrawal and Kotanen 2003), which suggests that other factors such as genetics (Lee 2002), competitive ability (Callaway and Aschehoug 2000), and the capacity to alter soil properties (Rout et al. 2013) also may play a role in invasion processes.

Plant-soil feedback (PSF) (Klironomos 2002; Bever, Platt, and Morton 2012) has also been hypothesized to explain plant invasions. A PSF occurs when plants manipulate biotic and/or abiotic soil properties such that subsequent plant growth is either positively or negatively affected. This in turn can alter community composition, and therefore invasion success (Bever, Westover, and Antonovics 1997; Reinhart and Callaway 2006; Brinkman et al. 2010).

There are six possible PSF responses which can be grouped into two categories: conspecific and heterospecific feedback. Conspecific feedback refers to the effect a conditioned soil has on future generations of the species that provided the conditioning, whereas heterospecific feedback is the effect a conditioned soil has on a species that did not condition the soil. Each of these feedbacks can either be positive, negative or neutral. Conspecific positive and heterospecific negative feedbacks by invasive species provide the proper conditions for an alien plant to be a successful invader. Invasion PSF theory suggests that conspecific positive feedback is associated with but not limited to an accumulation of soil mutualists and/or becoming infected by pathogens at a slower rate compared to neighboring plants. Likewise, negative heterospecific feedback is associated

with but not limited to an accumulation of pathogens and an increased rate of infection compared to the species conditioning the soil (Klironomos 2002; van Der Putten et al. 2013). For example, when the relative response of rare native species and invasive species was measured on their own conditioned soil the native species had a more negative RR which was associated with a higher rate of pathogen accumulation compared to the invasive species (Klironomos 2002). Additionally, soils conditioned by *Chromolaena odorata*, a tropical invasive shrub, were shown to accumulate 25 times more spores of the generalist fungi, *Fusarium semitectum*, than soils conditioned without *C. odorata*, subsequently leading to a decrease in native plant performance (Mangla and Callaway 2007). However, PSF effects do not hold true for all systems because some invasive plants have been shown to have negative to neutral conspecific PSFs (Kulmatiski et al. 2008; Perkins and Nowak 2013; Del Fabbro and Prati 2015).

Due to the complexity of soil and the difficulty in studying belowground plant-microbe interactions, the exact mechanism of PSF is not completely understood. However, root exudates such as flavonoids and phytoalexins (Walker et al. 2003), sugars and amino acids (Philippot et al. 2013), along with leaf decomposition and mineralization (Aponte, García, and Marañón 2013) can alter microbial community composition. Beneficial microbes, in turn, can impact biogeochemical processes such as the nitrogen and carbon cycles (Philippot et al. 2013), and provide plants with water, nutrients and can suppress disease by secreting antimicrobial compounds (Avis et al. 2008). Conversely, deleterious microbes cause disease and compete for nutrients with plants (Van Der Heijden, Bardgett, and Van Straalen 2008).

To gain further insight into the role PSF has on invasion processes we designed a multi-phased PSF experiment with the invasive grass *Brachypodium sylvaticum* (Huds). P. Beauv. (slender false brome). The goal was to determine if PSF contributes to the invasive behavior of *B. sylvaticum.* If PSF directly promotes invasion, we would expect to see *B. sylvaticum* exhibit a positive conspecific and/or negative heterospecific PSF. If PSF does not play a significant role in the invasion of *B. sylvaticum*, then we would expect to see conspecific neutral to negative and/or heterospecific neutral to positive PSF. Additionally, we tested for reciprocal effects of PSF by native plants on *B. sylvaticum* to determine if native plants have the capacity to create soil environments that promote or reduce *B. sylvaticum* growth. If native PSF decreases *B. sylvaticum* growth, then it may be possible to plant natives to reduce soil quality for *B. sylvaticum* and protect sites from invasion. Moreover, the Pacific Northwest, which is the epicenter of *B. sylvaticum* invasion (Roy et al 2010), is home to a large sector of the timber industry that produces *Pseudotsuga menziesii* (Mirbel) Franco or Douglas-fir. After tree harvest, *B. sylvaticum* has been observed to colonize disturbed areas (Taylor et al. 2015). However, little is known about the exact impact *B. sylvaticum* has on *P. menziesii* growth. Therefore, we included *P. menziesii* in the experiment to determine if PSF by *B. sylvaticum* impacts *P. menziesii* growth. We hypothesized that *B. sylvaticum* would have positive conspecific and negative heterospecific PSF as predicted by PSF theory. We also expected native plants to have no significant impact on *B. sylvaticum* growth.

Materials and Methods

To ascertain the role PSF has on *B. sylvaticum* invasion, we conducted a two-phased PSF experiment in a greenhouse at Oregon State University (OSU) in 2015. Our experiment aimed to identify PSF effects of *B. sylvaticum* and PSF effects by native plants on *B. sylvaticum.*

Study Species

Brachypodium sylvaticum is a perennial bunch grass and is currently listed as a quarantined invasive species in California, Oregon, and Washington (Holmes et al. 2010). Originally native to North Africa and Eurasia, it is capable of rapid population growth in diverse environments including shaded forest understories, riparian zones, and full sun grasslands (Kaye and Blakelely-Smith 2006). It can persist at elevations ranging from sea level to 1,200m (Rosenthal, Ramakrishnan, and Cruzan 2008), in mesic to semi-arid moisture regimes, and at varying soil nutrient concentrations (Holmes et al. 2010). *Brachypodium sylvaticum* reproduces sexually via seeds and asexually via tiller production (Roy et al. 2010). The exact time of *B. sylvaticum* introduction to North America is unknown, but the earliest herbarium specimen is from Eugene, OR in 1939 (Chambers 1966). Genetic analyses have shown that *B. sylvaticum* was likely introduced at least twice in Oregon from native populations in western Europe (Rosenthal, Ramakrishnan, and Cruzan 2008).

Brachypodium sylvaticum may have a competitive advantage in Douglas-fir stands over a variety of plants including ferns, woody perennial shrubs, and two grass species (Naylor-Murphy 2012). However, why *B. sylvaticum* has this apparent competitive advantage in a Douglas-fir forest is not understood. Plants common in Oregon's Coastal

Range were used to test PSF effects to gain a better understanding of the competitive ability of *B. sylvaticum* in Douglas-fir forest understories. Coastal Range native species included two grasses, *Bromus vulgaris* (Hook.) Shear and *Elymus glaucus* Buckley, two forbs *Osmorhiza berteroi* DC and *Prunella vulgaris* L. and the economically important native tree, *P. menziesii.*

Seed and Pot Preparation

All plants used in the experiment were grown from seed along with *P. menziesii* saplings. *Brachypodium sylvaticum* and *O. berteroi* seed was collected in August 2014 from OSU's McDonald-Dunn Research Forest in Corvallis, OR. *Bromus vulgaris, E. glaucus* and *P. vulgaris* seed was acquired from a commercial source that produced seeds from local provenance wild populations. *Pseudotsuga menziesii* seed was collected from the Oregon central coast in a stand of trees operated by Weyerhaeuser. Ten-month and eighteen-month old (bare root) saplings were from Starker forests (Philomath, OR) and IFA Nurseries (Wilsonville, OR) respectively. Eighteen-month old saplings were stored at 3°C for three months prior to use.

For germination, all seeds were placed in sterilized plastic containers on a moist paper towel. *Osmorhiza berteroi*, *B. sylvaticum*, and *P. menziesii* seed were placed in cold stratification at 5°C for 116, 41, 44 days respectively. Seeds were removed from cold stratification and placed in a greenhouse operated by OSU to germinate. *Elymus glaucus*, *B. vulgaris* and *P. vulgaris* seeds were also placed in the greenhouse to germinate.

For the first phase, all species except *P. menziesii* were grown in sterilized 656 mL deepots while ten-month old *P. menziesii* saplings were grown in sterilized 2.31L

treepots. For the second phase, all species except *P. menziesii* were grown in 444 mL pots while eighteen-month old *P. menziesii* saplings were grown in 656mL deepots and *P. menziesii* seedlings were grown in 66mL Ray Leech Cone-tainers.

Soil Preparation

Field soil was collected in November 2014 from the top 20cm of the soil at randomly selected locations in the McDonald-Dunn Research Forest in the Oak Creek section. The McDonald-Dunn forest was chosen for the soil collection site because it is currently invaded by *B. sylvaticum* and all of our study species are common there. Soil was collected from unoccupied spaces between plants to avoid effects of present soil conditioning by living plants. Half of the collected soil was sterilized using a Consolidated SR 24-F autoclave (Consolidated Sterilizer Systems, Boston, MA) at 132°C for two hours to create two treatments: sterile and live soil. If plant response (via growth) was different between treatments than it represented a biological effect. To improve drainage in pots, sterilized potting soil (Metro-Mix Professional Growing Mix) was added to both soil types (sterilized and live) at a 4:1 ratio (potting soil:field soil). Additionally, to reduce impacts of fungus gnats (Diptera) a 10-15mm layer of sterilized sand was placed on top of the soil in all pots.

Experimental Design

To determine if PSF gives *B. sylvaticum* a competitive advantage over forest plants, we conducted a two-phased PSF experiment (Kardol, Martijn Bezemer, and van der Putten 2006; Reinhart and Callaway 2006; Brinkman et al. 2010; Perkins and Hatfield 2015) in greenhouses at OSU (Figure 2.1). Average greenhouse temperature was 21°C

and supplemental light was used to achieve 14 hours of daylight when needed. During phase one, the soil conditioning phase, species specific soil microbial communities were generated by each of the study species. Each species was planted in both soil treatments (sterilized and live) and replicated 60 times (*B. sylvaticum* was replicated 200 times to generate the larger volume of conditioned soil needed in phase two) and grown for six months. A subset of live soil was mixed at a 4:1 ratio with sterilized potting mix and placed in 656 mL deepots and left unplanted for an unconditioned soil treatment. A solution (20mL fertilizer/7.5L H_2O) of Miracle Gro All Purpose Plant Food (12-4-8) was applied to all plants twice during the conditioning phase to avoid stress from insufficient nutrients. After six months, aboveground biomass was clipped and soil from all pots from each treatment was pooled and used to create inoculum for phase two. Prior to pooling soils, 10 samples from both *B. sylvaticum* treatments (sterile and live) were randomly selected and used for genetic analysis to determine effects of soil sterilization on microbial community composition (see *Molecular Analysis*).

Phase two, the feedback stage, tested for PSF by planting *B. sylvaticum* in soil conditioned by itself, the five native species, and the unconditioned soil. Native species were planted in their own conditioned soils, soils conditioned by *B. sylvaticum,* and the unconditioned soil. We chose to use *P. menziesii* seedlings and saplings during the feedback stage to mimic naturally occurring *P. menziesii* recruitment and silvicultural planting practices. Initially, 20 replicates were planned for each treatment, however, low seed germination and seedling mortality reduced the number of replicates for some treatments (Table 2.1). All treatments were grown for a minimum of 80 days. Greenhouse pests thrips (Thysanoptera) and fungus gnats (Diptera)) were controlled after 60 days

with DECATHLON 20 WP and ENSTAR AQ pesticides that are tolerated by soil biota and have negligible non target effects (Casjens 2008; United States Environmental Protection Agency 1996). At the end of the growing period soil was carefully washed off roots and above ground and below ground biomass was collected and dried at 60°C for at least 72 hours. Height, number of stems, tillers (grasses only) and leaves were measured or counted on all plants where applicable and stem diameter was measured on *P. menziesii*.

Molecular Analysis

High throughput Illumina sequencing was used to determine differences in microbial communities at the end of the conditioning phase between sterilized and non-sterilized soils that had been conditioned by *B. sylvaticum*. Sterilized soil should have no living microorganisms, however, because the soil was not in a sterile environment (the greenhouse) recolonization likely occurred over the six-month conditioning phase.

At the termination of the conditioning phase ten samples were randomly chosen from the aforementioned treatments and frozen at -22 °C until use. For DNA extraction, samples were thawed to room temperature and using the MO BIO PowerSoil® DNA Isolation Kit (MOBIO, Carlsbad, CA USA) 0.25 g of soil from each sample was used following the manufacture's protocol with the addition of a ten-minute incubation period prior to bead beating. In two separate PCR reactions, 2ul of purified DNA were used to amplify the 16s rRNA and ITS genes of bacteria and fungi, respectively. For the 16s rRNA gene, PCR was done in triplicate using previously described primers targeting the V4 region of bacteria (Caporaso et al. 2011; Caporaso et al. 2012). PCR for the ITS gene

was completed in one reaction and targeted the ITS1-F and ITS2 regions of fungi (Bellemain et al. 2010). All amplicons were then quantified using the Qubit® HS kit (Life Technologies, Carlsbad, CA USA) according to the manufacturer's instructions and amplicons were visualized using gel electrophoresis to confirm there was ~350bp and ~450bp bands for the 16s rRNA and ITS genes, respectively. All 16s rRNA and ITS samples were pooled individually and cleaned using the UltraClean® PCR clean-up kit (MOBIO) and diluted to a concentration of 10nM. Equal molar concentrations of 16s rRNA and ITS libraries were pooled and underwent cluster generation and sequencing on an Illumina MiSeq instrument generating 250bp end reads. Only 16S rRNA libraries produced adequate numbers of reads for analysis, possibly due to preferential sequencing of short fragments, therefore, only bacteria data is presented. Sequences were analyzed using QIIME and Operational taxonomic units (OTUs) were assigned using the UCLUST algorithm against the Greengenes (version 13_8) database (DeSantis et al. 2006).

Data Analysis

Analysis of variance (ANOVA) was used to test for differences among treatments on all response variables (total mass, height, number of tillers, leaves, stems, stem diameter). For natives, data was not collected for heterospecific feedback effects between natives, therefore, the analysis only used data from native conditioned soil, soil conditioned by *B. sylvaticum,* and the unconditioned soil. All analyses were performed in R version 3.1.2 (R Core Team 2014).

A relative response (RR) index (Brinkman et al. 2010; Perkins and Nowak 2013) was used to determine the overall effect of PSF for both *B. sylvaticum* and the natives (only

total mass was evaluated for the RR index). The RR index is able to evaluate both conspecific and heterospecific feedback and is statistically robust (Perkins and Nowak 2013). Conspecific and heterospecific feedback were calculated using the following equations:

$\mathbf{RR_{conspecific}}$= ((biomass grown in own soil – mean biomass grown in own sterilized soil)/(biomass grown in own soil + mean biomass grown in own sterilized soil))

$\mathbf{RR_{heterospecific}}$= ((biomass grown in foreign soil – mean biomass grown in own soil)/(biomass grown in foreign soil + mean biomass grown in own soil))

A positive $RR_{conspecific}$ value indicates that a species performs better in soil conditioned by its own species relative to sterilized soil conditioned by its own species while a negative $RR_{conspecific}$ value indicates a species performs better in the sterilized soil compared to soil conditioned by its own species. A positive $RR_{heterospecific}$ value indicates that a species performs better in foreign soil compared to their own soil while a negative $RR_{heterospecific}$ value indicates a species perform better in their own soil compared to foreign soil. A one sample t-test was used to determine if RR values were different from zero.

To evaluate differences in microbial community composition between sterilized and live soils conditioned by *B. sylvaticum* a permutational multivariate analysis of variance (PERMANOVA) was completed using the VEGAN package (Oksanen et al. 2016) in R. Species richness (S) was calculated using open reference OTUs determined by QIIME (Caporaso et al. 2010). Differences in OTUs between treatments were calculated using a

Wilcoxon rank-sum test and false discovery rate was controlled using a BH procedure (Benjamini and Hochberg 1995).

Results

Effect of PSF on Brachypodium sylvaticum

For the live treatments, we found evidence that *B. sylvaticum* total mass was differentially affected based on which species conditioned the soil ($F=23.75$, $p<0.001$; Figure 2.2). When grown on soils conditioned by *B. vulgaris*, *B. sylvaticum* total mass was significantly reduced compared to its biomass when grown on its own live soil ($t=-2.99$, $p=0.003$), indicative of negative heterospecific PSF, but significantly increased when grown on soils conditioned by *O. berteroi* ($t=3.205$, $p<0.01$), *P. vulgaris* ($t=4.523$, $p<0.001$) and *P. menziesii* ($t=4.342$, $p<0.001$), indicative of positive heterospecific PSF. Soils conditioned by *E. glaucus* and the unconditioned treatment had no effect on *B. sylvaticum* total mass. The same trend was observed when evaluating root:shoot ratio ($F=35.61$, $p<0.001$), height ($F= 51.46$, $p<0.001$), number of tillers ($F=9.15$, $p<0.001$), and number of leaves ($F=9.68$, $p<0.001$) with the exception that *E. glaucus* had a significant negative impact on *B. sylvaticum* height ($t=-0.38$, $p<0.001$), *B. vulgaris* had no effect on root:shoot ratio, and the unconditioned soil had a positive effect on number of leaves produced by *B. sylvaticum* ($t=3.08$, $p=0.002$).

For the sterilized soils, all treatments increased total mass of *B. sylvaticum* ($F=18.57$, $p<0.001$; Fig. 2.2) compared to when grown on its own live soil. The same trend was observed when evaluating root:shoot ratio ($F=18.90$, $p<0.001$), height ($F= 4.26$, $p<0.001$), number of tillers ($F=4.74$, $p<0.001$), and number of leaves ($F=8.30$, $p<0.001$)

with the exception that *B. vulgaris* had no effect on the number leaves produced by *B. sylvaticum*.

Effect of PSF on natives

Soils conditioned by *B. sylvaticum* reduced *P. menziesii* sapling height and seedling stem diameter, but had no impact on the other native species response variables (Table 2.2). Sterilized, conspecific, and unconditioned soils had varying effects on native species' growth (Table 2.2). When grown on sterilized soil conditioned by *B. sylvaticum, E. glaucus* and *P. vulgaris* total mass and *O. berteroi* root:shoot ratio was increased compared to when grown on conspecific soils. Conversely, *P. menziesii* saplings were shorter when grown on sterilized *B. sylvaticum* conditioned soil compared to its own soil. When *E. glaucus* was grown on sterilized conspecific soil, its total mass and number of leaves, along with *O. berteroi* height, were significantly increased compared to when grown on live conspecific soil. When grown on the unconditioned soil, *E. glaucus* root:shoot ratio, the number of leaves and stems, all *P. vulgaris* response variables, *O. berteroi* root:shoot ratio and *P. menziesii* seedling root:shoot ratio and height were significantly increased compared to when grown on conspecific soil, while *E. glaucus* height and *P. menziesii* seedling stem diameter was significantly reduced compared to when grown on conspecific soil.

PSF relative response

The RR index shows that *B. sylvaticum* (one sample t-test, $p<0.001$; Figure 2.3) and *E. glaucus* (one sample t-test, $p<0.001$) have negative conspecific PSF and *O. berteroi, P. vulgaris* and *P. menziesii* seedlings have neutral conspecific PSF (Figure 2.4). The RR of

B. sylvaticum to soil conditioned by *B. vulgaris* was negative (one sample t-test, $p<0.001$) while its RR to soil conditioned by *P. vulgaris* (one sample t-test, $p<0.001$) and *P. menziesii* (one sample t-test, $p<0.001$) was positive. The RR of *P. menziesii* seedlings to soils conditioned by *B. sylvaticum* was negative (one-sample t-test, $p=0.004$).

Effect of soil sterilization on microbial community composition

After six-months of soil conditioning, there was significant evidence that sterilized and live soils conditioned by *B. sylvaticum* had different bacterial communities ($F=15.18$, $p<0.001$; Figure 2.5). 5,241 OTUs were putatively identified between both treatments. Of those, 2,562 (49%) OTUs had statistically significant different abundances between treatments and were associated with 29 unique phyla and 20 phyla that were undefined. When evaluating phyla between treatments that were statistically different, five phyla including Proteobacteria, Acidiobacteria, Bacteroidetes, Verrucomicrobia, Actinobacteria comprised 83 percent of the community composition while all other phyla represented 17 percent of the community (Figure 2.6).

Discussion

Brachypodium sylvaticum PSF

Contrary to our hypothesis, we found that *B. sylvaticum* has conspecific negative PSF which supports other studies that have shown invasive species have negative to neutral conspecific PSF (Kulmatiski et al. 2008; Perkins and Nowak 2013; Suding et al. 2013). One explanation for the conspecific negative response may be that *B. sylvaticum* accumulates and is adversely affected by soil pathogens in the invaded range. When comparing *B. sylvaticum* total mass in sterilized and live conditioned soils using the

relative response (RR) index, a negative value suggests that soil sterilization removed deleterious organisms which might be responsible for the negative RR.

The Enemy Release Hypothesis (ERH) posits that in the invaded range exotic species are free from enemies that limit population growth. Our results, along with Roy et al. (2010), who found that generalist insects in the invaded range damaged *B. sylvaticum* more compared to insects in the home range suggests that enemy release may not be responsible for *B. sylvaticum* invasion. This may be due to the amount of time since *B. sylvaticum* was introduced in the PNW, which is close to a century. Studies have shown that PSFs can become more negative with time since introduction (Diez et al 2010; Dostál et al. 2013), possibly because organism with short generations (microbes and insects) may rapidly evolve mechanism that impact *B. sylvaticum* growth.

Reducing growth rates of native species (e.g. negative heterospecific PSF produced by *B. sylvaticum*) is a second PSF that is hypothesized to benefit invasive species. In our study, *P. menziesii* was the only species with direct negative effects from *B. sylvaticum* PSF. Interestingly, inhibiting *P. menziesii* growth may indirectly reduce *B. sylvaticum* range expansion over time rather than facilitate range expansion. For example, *B. sylvaticum* has been shown to grow larger on coniferous litter rather than deciduous litter (Taylor et al. 2015). By inhibiting *P. menziesii* seedling growth, there is a chance that seedlings will not survive, thus leaving niche space available for trees from different taxa which may not provide optimal growing conditions for *B. sylvaticum*.

The absence of direct negative effects resulting from *B. sylvaticum* PSF on native grasses and forbs supports a previous study of PSF from 48 species. Of the 23 invasive

species there was no negative PSF effects to natives (Del Fabbro and Prati 2015). Unexpectedly, our data shows that *E. glaucus* and *P. vulgaris* produce more biomass on soils conditioned by *B. sylvaticum* rather than their own conditioned soil. One explanation is a localized version of enemy release. For example, soil conditioned by *B. sylvaticum* may have a different, less harmful microbial community than soils conditioned by *E. glaucus* and *P. vulgaris*, which subsequently provides an opportunity for the native species to produce more biomass.

Native PSF

We found that *B. sylvaticum* total mass was reduced in soil conditioned by the native grass *B. vulgaris*. One possible explanation for this is the biotic resistance hypothesis which posits that strongly competitive native species (plants, pathogens and/or herbivores) may control populations of exotic plants (Elton 1958). Soils conditioned by *B. vulgaris* potentially accumulated highly active native pathogens or groups of organisms that influenced soil properties such that *B. sylvaticum* was negatively impacted. An alternative explanation is that species from similar functional groups have comparable nutrient requirements, and therefore, when *B. sylvaticum* grew on soils conditioned by the native grass soil nutrients were depleted, which subsequently led to reduced *B. sylvaticum* growth. The inhibitory effects to *B. sylvaticum* growth provide a potential for site specific conditioning via *B. vulgaris* PSF to help prevent and control *B. sylvaticum* spread. To our knowledge, only one other study has documented native species PSF as having inhibitory effects on invasive species growth (Knevel et al. 2004), but other studies have shown that similar functional groups (e.g. grasses, forbs, trees) can

have negative PSF responses between species in the same functional group (Bezemer et al. 2006).

Two native forbs, *O. berteroi* and *P. vulgaris*, promoted *B. sylvaticum* growth. Native forbs have previously been shown to have positive (or less negative) PSF community effects compared to grasses, which may be explained by differences in microbiota associations and/or nutrient requirements (Bezemer et al. 2006). Similarly to the forbs, soil conditioned by *P. menziesii* promoted *B. sylvaticum* growth. This is not surprising as large portions of the invaded range are in coniferous forests, suggesting that *B. sylvaticum* has more mutualistic associations and/or is effected less by pathogens in coniferous forests compared to other systems. While studying potential sites for *B. sylvaticum* invasion, Taylor et al. (2015) found that the *B. sylvaticum* established and grew better in coniferous tree litter than litter from deciduous trees, which is quite interesting because *B. sylvaticum* has previously been described as belonging to the *Quercus-Fagetea* class (Haeggström and Skytén 1996) in its home range. Our data, coupled with Taylor et al. (2015) provides the initial framework to show that *B. sylvaticum* may be shifting ranges and that it could be associated with release from deciduous forest enemies. Future research might test *B. sylvaticum* growth responses in native deciduous forests and invaded coniferous forests. Additionally, by identifying environments that are more suitable for *B. sylvaticum* invasion (e.g. a forest understory dominated with forbs rather than grasses) land managers can establish an invasion risk scale to help focus efforts on *B. sylvaticum* establishment prevention.

Microbial Community: Sterilized vs Live Soils

In our experiment we observed differences in microbial communities between sterilized and live soils conditioned by *B. sylvaticum.* How these differences affected *B. sylvaticum* growth is unclear. Over 5000 OTUs were putatively identified, but with limited data on the ecological and physiological properties of these organisms and no culturing of specific taxa, making inferences on what impact they had on *B. sylvaticum* growth is not warranted. Therefore, our evaluation focuses on community differences between treatments at the phyla level rather than the OTU. We took this approach because groups within microbiotic phyla tend to share similar physiological and ecological properties (Fierer, Bradford, and Jackson 2007; Philippot et al. 2010).

One way to classify microorganisms is by their growth rate at varying carbon (C) concentrations. For example, Copiotrophs tend to have high nutrient requirements and elevated growth rates when C concentrations are high, while oligotrophs have lower nutrient requirements and perform better in C depleted environments (Fierer, Bradford, and Jackson 2007). In the current study, Bacteroidetes and Proteobacteria were more abundant in the sterilized treatment. Bacteroidetes and beta-Proteobacteria (a class within the Proteobacteria phyla) have been previously classified as copiotrophs (Fierer, Bradford, and Jackson 2007). One explanation for their high abundance in the sterilized treatment comes from the sterilization process itself. Sterilized soil has been shown to increase nutrient availability (Powlson and Jenkinson 1976; Troelstra et al. 2001), presumably from the dead organisms. As a result, when recolonization began (during the six-month conditioning phase), the sterilized soil could have provided a better environment for the copiotrophic taxa compared to the unsterilized soil. Furthermore,

because the sterilized soil was devoid of living organisms, the initial recolonization may have lacked strong competitive pressures between microbes compared to the live soil, ultimately leading to the high abundance of Bacteroidetes and Proteobacteria in the sterilized treatment.

An important finding in our microbial analysis is that sterilized soils do not stay free of biota in greenhouse conditions. This finding can have implications when making inferences about PSF effects. For example, it is unclear if *B. sylvaticum* performed better in sterilized soils because deleterious soil microbes from the initial field collected soil were killed during sterilization or if mutualistic microbes found in the greenhouse colonized the sterilized soil during the conditioning phase. PSF experiments that incorporate sterilization treatments should consider how recolonization of microbial communities affects nutrient availability and substrate composition which ultimately affect plant performance. Moreover, the timing of sterilization may impact results. We sterilized soil directly after the initial field collection following methods provided by Jordan et al. (2008), whereas others (Brinkman et al. 2010; Del Fabbro and Prati 2015) sterilized soil after the conditioning phase. The benefit of the latter is that there is less time for recolonization during the feedback stage. Nonetheless, we advise caution when comparing experiments using different sterilization procedures.

Conclusions

Our findings suggest that PSF generated by *B. sylvaticum* does not play a direct role in the invasion of *B. sylvaticum*. Instead, PSF produced by native forbs and *P. menziesii* may help facilitate *B. sylvaticum* invasion. The negative effect of *B. sylvaticum* on *P.*

menziesii, a forest tree of economic significance in the Pacific Northwest, suggests that silviculturalists and land managers may need to address current invasions of this nonnative grass, and take steps to limit its future spread in the Pacific Northwest. Future studies should evaluate if PSF generated by *B. sylvaticum* persists in the soil once the species is removed and determine if native plants are adversely affected by long term PSF. In addition, we suggest that *B. vulgari*s be incorporated into native seed mixes at a higher rate and forbs such as *P*. vulgaris and *O. berteroi* be removed from native seed mixes used for restoration.

Table 2.1 Phase two plant-soil feedback planting data**.** Soil type was either sterilized or live. Soil conditioning species refers to what phase one species conditioned the soil. Number of growing days varied between species based on plant performance at the end of the growing period. Traits measured varied between species based on plant morphology. *Pseudotsuga menziesii* sapling height was the difference in height from pre and post planting.

Species Grown	Soil Type	Soil Conditioning Species	Number Planted	Number Survived	Days Grown	Traits Measured
B. sylvaticum	Live	*B. sylvaticum*	19	17	89	Height, number of leaves, tillers and total biomass
B. sylvaticum	Live	*E. glaucus*	19	14	89	
B. sylvaticum	Live	*B. vulgaris*	19	16	89	
B. sylvaticum	Live	*P. vulgaris*	19	18	89	
B. sylvaticum	Live	*O. berteroi*	19	17	89	
B. sylvaticum	Live	*P. menziesii*	19	18	89	
B. sylvaticum	Live	*Control*	19	19	89	
B. sylvaticum	Sterilized	*B. sylvaticum*	19	16	90	
B. sylvaticum	Sterilized	*E. glaucus*	19	15	90	
B. sylvaticum	Sterilized	*B. vulgaris*	19	14	90	
B. sylvaticum	Sterilized	*P. vulgaris*	19	16	90	
B. sylvaticum	Sterilized	*O. berteroi*	19	16	90	
B. sylvaticum	Sterilized	*P. menziesii*	19	17	90	
E. glaucus	Live	*E. glaucus*	20	15	77	Height, number of leaves, stems and total biomass
E. glaucus	Live	*B. sylvaticum*	20	8	77	
E. glaucus	Live	*Control*	17	14	77	

Species Grown	Soil Type	Soil Conditioning Species	Number Planted	Number Survived	Days Grown	Traits Measured
E. glaucus	Sterilized	*E. glaucus*	20	12	77	
E. glaucus	Sterilized	*B. sylvaticum*	20	14	77	
B. vulgaris	Live	*B. vulgaris*	11	1	76	Height, number of leaves, stems and total biomass
B. vulgaris	Live	*B. sylvaticum*	11	4	76	
B. vulgaris	Live	*Control*	0*	0	76	
B. vulgaris	Sterilized	*B. vulgaris*	11	4	76	
B. vulgaris	Sterilized	*B. sylvaticum*	11	9	76	
O. berteroi	Live	*O. berteroi*	20	5	74	
O. berteroi	Live	*B. sylvaticum*	20	11	74	
O. berteroi	Live	*Control*	20	9	74	
O. berteroi	Sterilized	*O. berteroi*	20	9	74	
O. berteroi	Sterilized	*B. sylvaticum*	20	10	74	
P. vulgaris	Live	*P. vulgaris*	20	20	89	Number of leaves and total biomass
P. vulgaris	Live	*B. sylvaticum*	20	20	89	
P. vulgaris	Live	*Control*	20	19	89	
P. vulgaris	Sterilized	*P. vulgaris*	20	20	89	
P. vulgaris	Sterilized	*B. sylvaticum*	20	20	89	
P. menziesii (seed)	Live	*P. menziesii*	30	12	104	Height, shoot diameter and total biomass
P. menziesii (seed)	Live	*B. sylvaticum*	30	14	104	

Species Grown	Soil Type	Soil Conditioning Species	Number Planted	Number Survived	Days Grown	Traits Measured
P. menziesii (seed)	Live	*Control*	30	22	104	
P. menziesii (seed)	Sterilized	*P. menziesii*	30	14	104	
P. menziesii (seed)	Sterilized	*B. sylvaticum*	30	16	104	
P. menziesii (sapling)	Live	*P. menziesii*	20	15	111	Relative height change
P. menziesii (sapling)	Live	*B. sylvaticum*	20	19	111	
P. menziesii (sapling)	Live	*Control*	20	20	111	
P. menziesii (sapling)	Sterilized	*P. menziesii*	20	18	111	
P. menziesii (sapling)	Sterilized	*B. sylvaticum*	20	18	111	

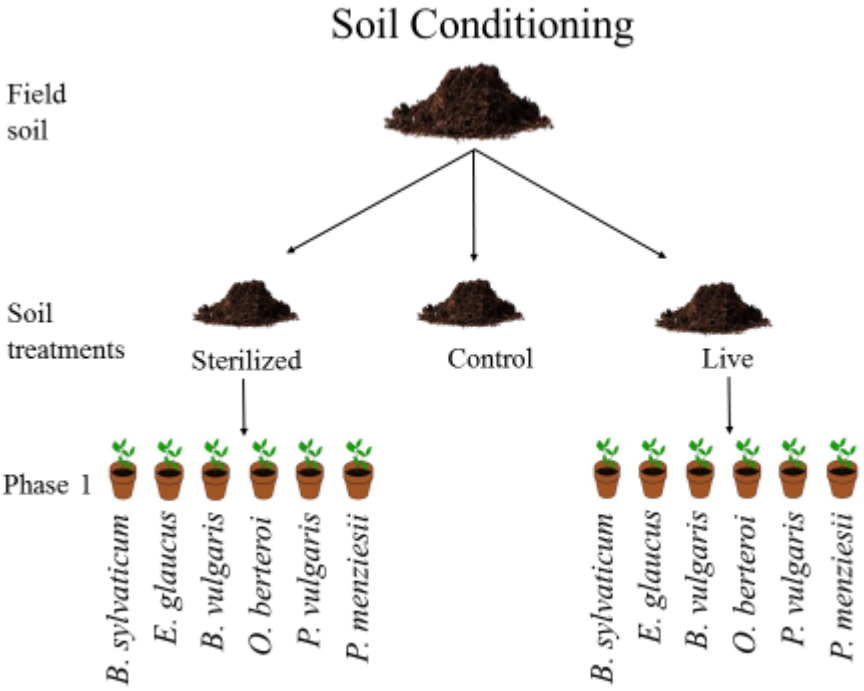
*No seedlings planted due to insufficient germination rates

Table 2.2 ANOVA results from native species response to PSF. *Bromus vulgaris* is not included in the analysis as a result from low germination rates and high mortality. Values in bold represent a statistically significant difference between a species grown in its own soil and grown in foreign soil.

Species Planted	**Trait(s) Measured**	**Soil Treatments**											
		B. sylvaticum			**Sterile *B. sylvaticum***			**Sterile *E. glaucus***			**Unconditioned**		
		t	**p**	**n**	**t**	**p**	**n**	**t**	**p**	**n**	**t**	**p**	**n**
E. glaucus	Total mass	1.524	0.129	8	2.729	**0.007**	14	4.445	**<0.001**	12	0.805	0.422	14
n=15	Root:shoot	-0.021	0.984		0.425	0.672		0.602	0.548		1.999	**0.047**	
	Number of leaves	0.708	0.480		0.834	0.406		2.068	**0.041**		3.379	**<0.001**	
	Number of stems	0.086	0.931		0.968	0.335		1.916	0.056		2.757	**<0.01**	
	Height	1.366	0.1747		1.671	0.097		1.708	0.090		-6.36	**<0.001**	
		B. sylvaticum			**Sterile *B. sylvaticum***			**Sterile *P. vulgaris***			**Unconditioned**		
		t	**p**	**n**	**t**	**p**	**n**	**t**	**p**	**n**	**t**	**p**	**n**
P. vulgaris	Total mass	1.760	0.081	20	3.039	**0.003**	20	-1.256	0.212	20	5.591	**<0.001**	19
n=20	Root:shoot	1.088	0.279		0.948	0.345		1.556	0.123		3.07	**<0.010**	
	Number of leaves	2.158	**0.03**		1.419	0.159		-0.7	0.485		5.673	**<0.001**	

Species Planted	**Trait(s) Measured**	**Soil Treatments**											
		B. sylvaticum			**Sterile *B. sylvaticum***			**Sterile *O. berteroi***			**Unconditioned**		
		t	**p**	**n**	**t**	**p**	**n**	**t**	**p**	**n**	**t**	**p**	**n**
O. berteroi	Total mass	-0.512	0.611	11	0.630	0.532	10	1.193	0.240	9	0.477	0.636	9
n=5	Root:shoot	1.602	0.117		2.813	**<0.01**		1.078	0.287		2.76	**<0.01**	
		t	**p**	**n**	**t**	**p**	**n**	**t**	**p**	**n**	**t**	**p**	**n**
	Number of stems	0.66	0.513		-0.223	0.824		0.439	0.663		-0.78	0.44	
	Height	0.698	0.489		1.895	0.065		2.18	**0.035**		1.722	0.093	
		B. sylvaticum			**Sterile *B. sylvaticum***			**Sterile *P. menziesii***			**Unconditioned**		
		t	**p**	**n**	**t**	**p**	**n**	**t**	**p**	**n**	**t**	**p**	**n**
P. menziesii (seedling)	Total mass	0.954	0.343	14	-0.480	0.632	16	-1.751	0.084	14	-0.023	0.981	22
	Root:shoot	0.633	0.529		1.025	0.308		0.093	0.926		2.045	**0.04**	
	Height	1.938	0.060		1.81	0.074		0.837	0.405		2.229	**<0.05**	
	Stem diameter	-3.208	**<0.010**		-1.364	0.177		0.836	0.406		-3.117	**<0.01**	
		B. sylvaticum			**Sterile *B. sylvaticum***			**Sterile *P. menziesii***			**Unconditioned**		
		t	**p**	**n**	**t**	**p**	**n**	**t**	**p**	**n**	**t**	**p**	**n**
P. menziesii (saplings)	Relative height difference*	-1.996	**<0.040**	19	-1.194	0.06	18	-0.502	0.617	18	1.237	0.220	20

* Relative height difference was used rather than absolute height difference based on differences in initial sapling size.



Feedback Phase

	Live Conditioned Soil						Sterilized Conditioned Soil						
	B. sylvaticum	*E. glaucus*	*B. vulgaris*	*O. berteroi*	*P. vulgaris*	*P. menziesii*	*B. sylvaticum*	*E. glaucus*	*B. vulgaris*	*O. berteroi*	*P. vulgaris*	*P. menziesii*	Control
B. sylvaticum	X	X	X	X	X	X	X	X	X	X	X	X	X
E. glaucus	X	X					X	X					X
B. vulgaris	X		X				X		X				X
O. berteroi	X			X			X			X			X
P. vulgaris	X				X		X				X		X
*P. menziesii**	X					X	X					X	X
*P. menziesii***	X					X	X					X	X

Figure 2.1 Two phase plant-soil feedback methodology. In the soil conditioning phase each species was grown on sterilized and live soils to generate species specific microbial communities. For the feedback phase, *B. sylvaticum* was grown on all soil types while native plants were only grown on their own conditioned soil, soil conditioned by *B. sylvaticum* and the control. * *Pseudotsuga menziesii* grown from seed. ***Pseudotsuga menziesii* grown from sapling.

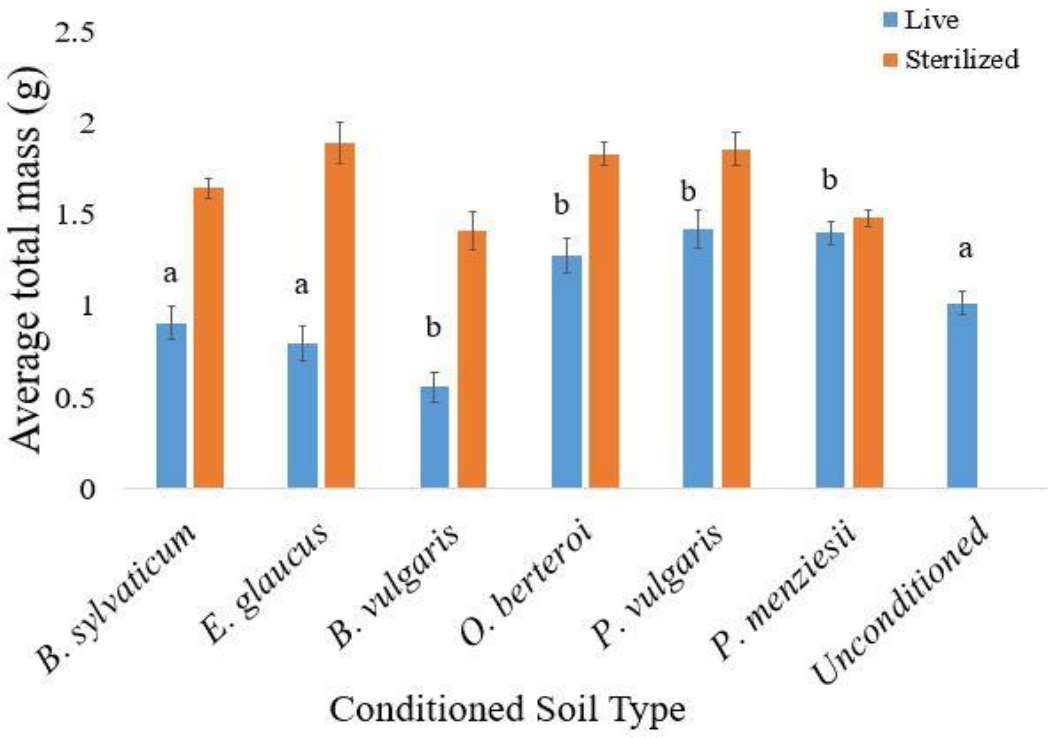


Figure 2.2 Average total mass of *B. sylvaticum* in response to species specific PSF. Each pair of bars represents the live (blue) and sterilized (red) soils which were conditioned by a specific species. *Brachypodium sylvaticum* was grown in each soil treatment and total mass was measured. Similar letters above blue bars indicate the treatment was no different than the *B. sylvaticum* treatment and different letters indicate the treatment was significantly different than the *B. sylvaticum* treatment. All sterilized treatments are statistically different than the live *B. sylvaticum* treatment. Error bars denote +/- one standard error.

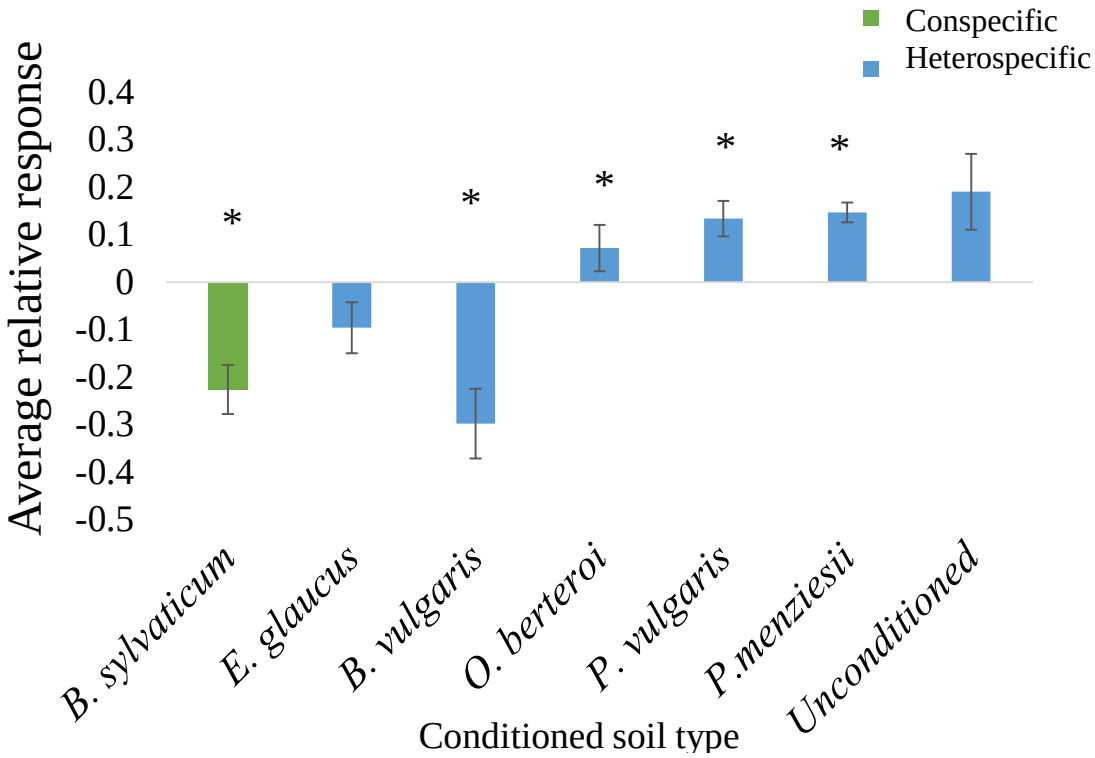


Figure 2.3 The average RR of *B. sylvaticum* to conspecific and native heterospecific PSF. Conspecific feedback (green) is the response of *B. sylvaticum* from being grown on its own conditioned soil compared to the when grown on its own sterilized soil. Native heterospecific feedback is the response of *B. sylvaticum* from being grown on soils conditioned by natives compared to when grown on soils conditioned by itself. Conditioned soil type indicates what species conditioned the soil during phase one. An asterisk above a bar indicates the average is statistically different from zero. Error bars represent +/- one standard error.

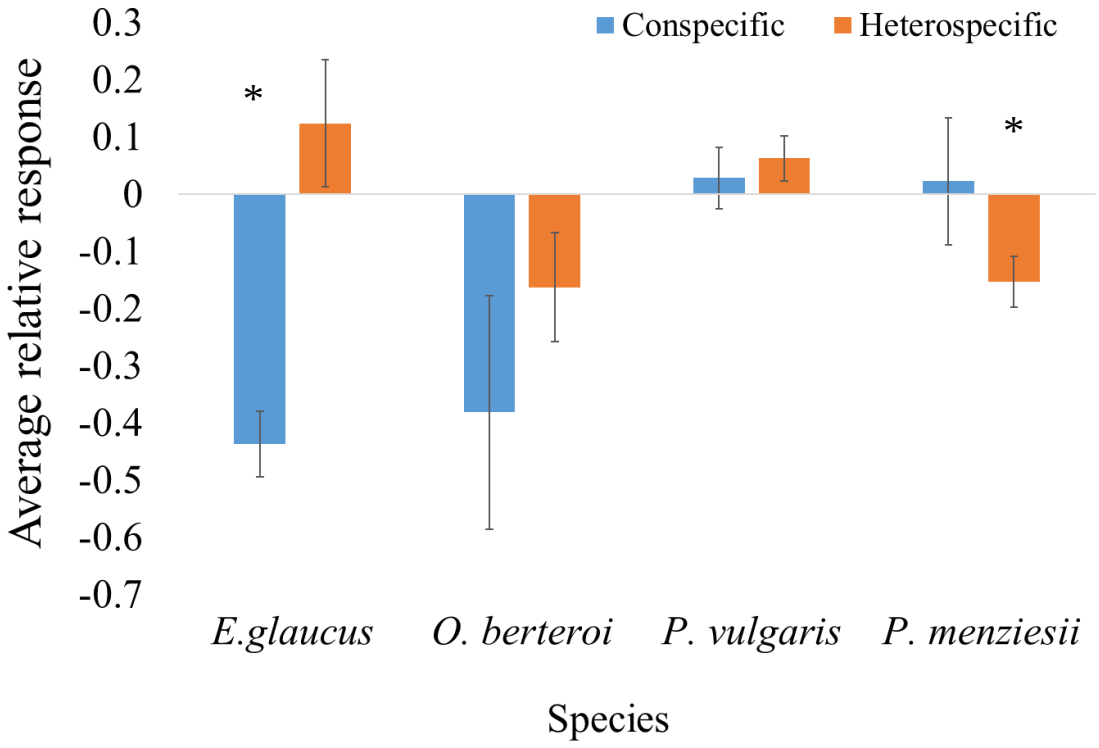


Figure 2.4 The RR of native plants to conspecific and *B. sylvaticum* heterospecific PSF. Conspecific treatments (blue) are the response of a species grown on its own conditioned soil relative to being grown on its own sterilized soil. Heterospecific responses (red) refer to how a species performs on soil conditioned by *B. sylvaticum* relative to soils conditioned by itself. An asterisk above a bar indicates the average RR is different than zero. Error bars represent +/- one standard error.

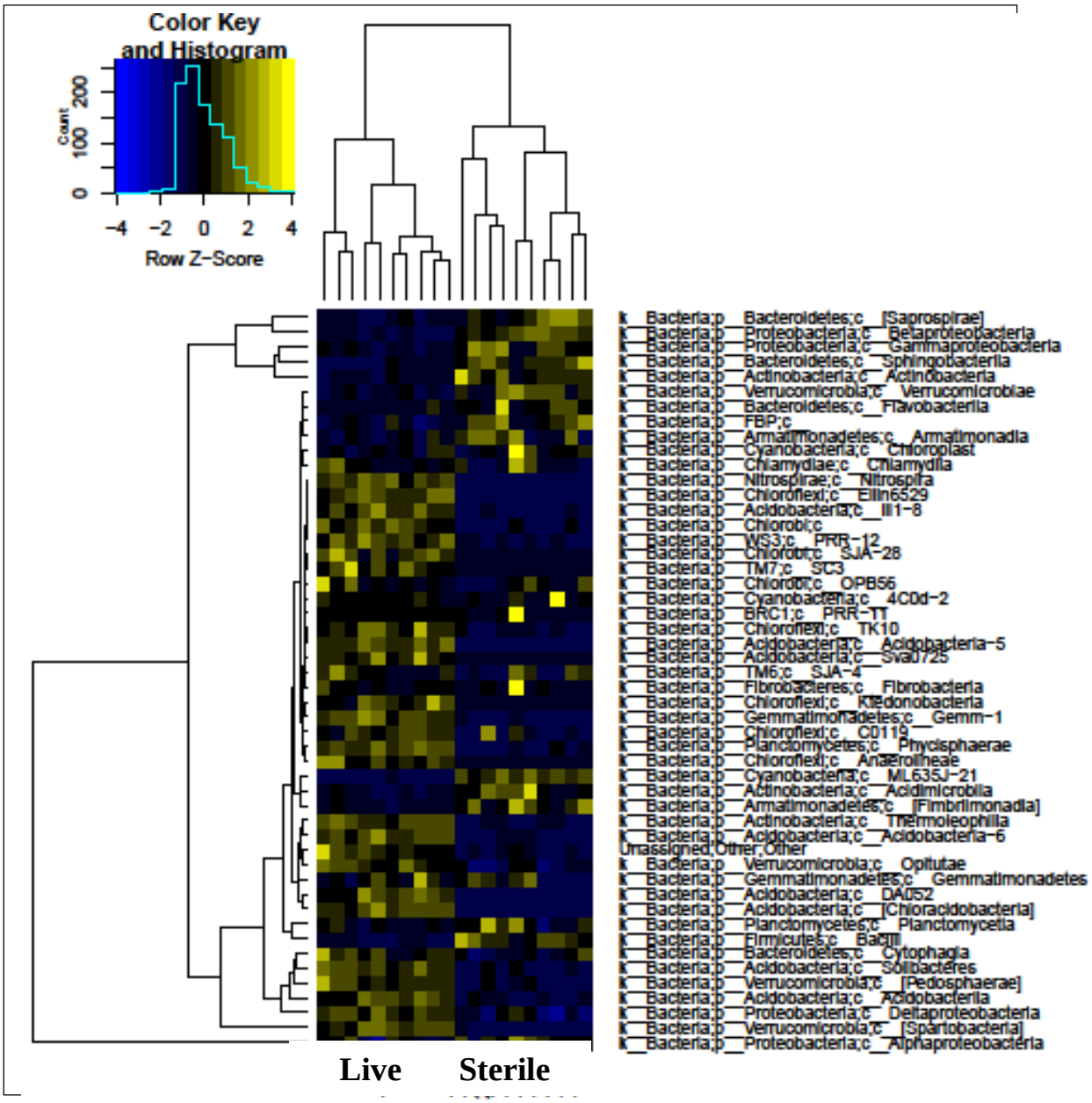


Figure 2.5 Heat map of bacteria found in live and sterilized soils after six-months of soil conditioning by *B. sylvaticum*.

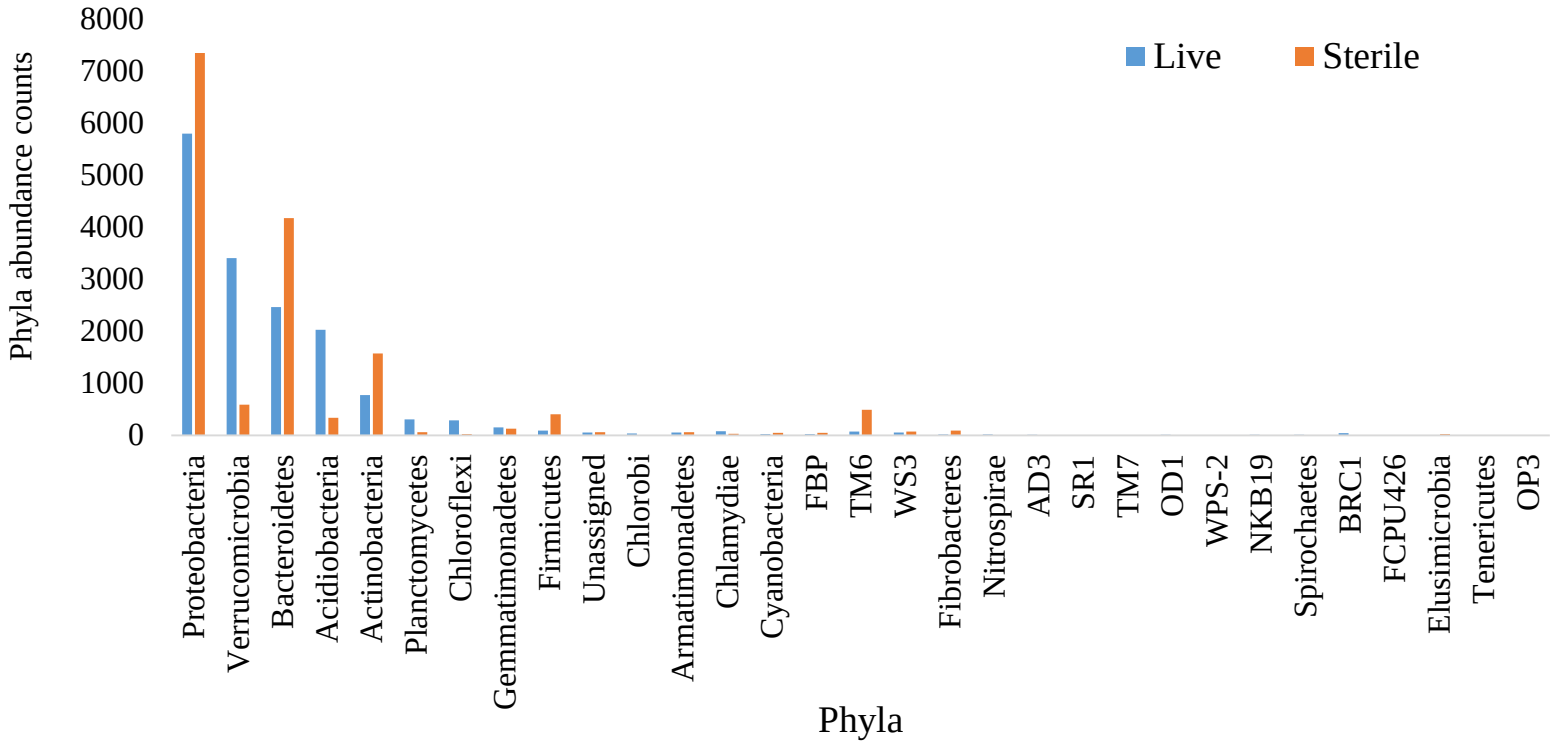


Figure 2.6 Abundance of OTUs per phyla observed in the live (blue) and sterilized (red) soils conditioned by *B. sylvaticum*. All OTUs have significantly different abundances between treatments ($F=15.18$, $p<0.001$).

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Chapter 3:

The legacy of *Brachypodium sylvaticum* in an Oregon Coastal Range forest: plant, nutrient and bacterial community response to plant-soil feedback

Abstract

Invasive species are becoming more widespread and the need to remove them is critical in order to maintain species diversity and ecological processes. However, even after invasive species are removed, there is a chance that chemical and biological soil conditions generated by the invasive species will persist in the soil, thwarting restoration efforts. This is often referred to as 'plant legacy' or 'legacy effect' and previously has been shown to have a negative impact on native species recruitment and to have facilitative effects on invasive plant establishment. The goal of our study was to determine if the invasive grass *Brachypodium sylvaticum* has either a biological and/or chemical legacy effect and if so, to what extent the legacy effect impacts *B. sylvaticum* and species native to the Oregon Coastal Range. We established ten plots in the McDonald-Dunn research forest in Corvallis, OR in areas where *B. sylvaticum* had at least 75% cover. In half of each plot *B. sylvaticum* was removed to create two soil treatments: plots with *B. sylvaticum* and without *B sylvaticum.* Over a nine-month period three soil collections took place to measure treatment effects on plant response (via total mass), soil chemical properties including pH, organic matter and nutrient concentration and bacterial community composition. Overall, our data suggest that legacy effects generated by *B. sylvaticum* differentially effect plant species. *Pseudotsuga menziesii* total mass was inhibited when growing on soils after *B. sylvaticum* had been removed for six-months, however, after nine-months of *B. sylvaticum* removal negative responses dissipated. Conversely, *Prunella vulgaris* grew better on soils after six-months from *B. sylvaticum* removal but after nine-months from *B. sylvaticum* removal soils inhibited *P. vulgar*is growth. Soil chemical properties and bacterial communities did not respond to *B.*

sylvaticum removal indicating that the legacy takes longer than nine-months to observe or *B. sylvaticum* does not alter soil chemical properties and bacterial community composition. These data suggest that overcoming legacy effects by the previous occupant may be a relatively long term rather that a short term process. Restoration efforts that involve invasive species removal should take into consideration legacy effects and may need to amend the soil to overcome harmful legacies.

Introduction

Over the past two centuries exotic species have increasingly invaded native plant communities in the United States causing billions of dollars in damages (Pimentel, Zuniga, and Morrison 2005). Once established, invasive species have the potential to alter community composition (Hejda, Pyšek, and Jarošík 2009), ecosystem functions (Sakai et al. 2001) and biotic (Mangla and Callaway 2007; Batten, Scow, and Espeland 2008) and abiotic (Aponte, García, and Marañón 2013; Grove, Parker, and Haubensak 2015) soil properties. Restoration of invaded landscapes typically focuses on removing invasive species, increasing diversity, and promoting ecological processes (Ruiz-Jaen and Aide 2005). However, long-lasting soil alterations generated by invasive species may inhibit restoration efforts, even once the invasive species are removed.

The terms ‘plant legacy’ or ‘legacy effect’ are used in ecological literature to describe changes to soil properties that persist in the soil once the species causing the change is removed (Cuddington 2011). Plants actively alter biotic and abiotic soil properties through a variety of root exudates (Walker et al. 2003; Bais et al. 2006) and biomass decomposition (Aponte, García, and Marañón 2013), which subsequently promote or

reduce growth for themselves and/or adjacent species. These processes are collectively referred to as plant-soil feedbacks (PSFs). The literature is rich with PSF examples (Klironomos 2002; Kardol, Martijn Bezemer, and van der Putten 2006; Bever, Platt, and Morton 2012; Aponte, García, and Marañón 2013; Perkins and Nowak 2013; Larios and Suding 2015; Rutten et al. 2015; Morris et al. 2016), reviews (Brinkman et al. 2010; van Der Putten et al. 2013; van der Putten et al. 2016) and a meta-analysis evaluating over 300 experiments (Kulmatiski et al. 2008). In contrast, there are few reported experiments that explore how PSF legacy effects generated by invasive species impact species, either the native or the invading species. One reason for this paucity is that evaluating PSF legacy effects requires adequate knowledge of the site history to determine what types of PSFs are present (e.g., biological and/or chemical alterations), how long they persist, and to what extent the legacy effects impact natives over time. Unfortunately, acquiring this data is often expensive, time consuming and/or impossible (e.g. site history is unknown).

Several studies provide insight into impacts of invasive species legacy effects on native plants and community composition. *Carpobrotus edulis* (L.) N. E. Br. or iceplant, an invasive succulent growing along the California coast has been shown to negatively impact germination, growth and reproduction rates of *Gilia millefoliata* Fisch. & C.A. Mey., a rare dune species, by altering soils chemical properties which persist in the soil for up to 18 months once *C. edulis* is removed (Conser and Connor 2008; Corbin and D'Antonio 2012). *Cytisus scoparius* (L.) Link or Scot's broom, an invasive nitrogen-fixing shrub, was shown to have facilitative effects on exotic species cover while having no effect on native cover over a 22-month period after removal (Grove, Parker, and Haubensak 2015), presumably from vacillating nitrogen levels. In addition, Grman and

Sudding (2010) found that shoot mass of a California native species mix was significantly reduced when growing in soils conditioned by a mix of exotic species when compared to growing in soils conditioned by the natives. The inhibitory effects of the exotic conditioned soil was suggested to be a result of either allelopathic chemical deposition, changes to nutrient availability and/or biologic community composition from previous plant communities (Grman and Suding 2010).

Experiments have shown that microbial communities have influential legacy effects as well. For example, *Pseudoroegneria spicata*, a native bunchgrass, and *Centaurea diffusa*, a common weed, did not alter microbial composition in a two-month period when growing on soils previously occupied by other species, suggesting that modifications to historical microbial community composition is a long term process (Kulmatiski and Beard 2011).

The Coast Range in Oregon provides a unique opportunity to evaluate legacy effects of the invasive grass, *Brachypodium sylvaticum* (Huds.) Beauv. (slender false brome). *Brachypodium sylvaticum* invades forests and prairies making restoration efforts both urgent and challenging. Previous work has shown that PSF generated by *B. sylvaticum* negatively effects itself and the economically important tree, *Pseudotsuga menziesii* (Mirb.) Franco or Douglas-fir, and differentially effects native species (Esterson 2016). Once commercial forests are harvested *B. sylvaticum* often invades rapidly and is typically sprayed with herbicide prior to tree plantings (Mark Gourley, Starker Forests). However, it is unclear if trees, specifically *P. menziesii*, respond differently to soil conditioning by *B. sylvaticum* once the invasive grass is removed from the system. Additionally, after *B.*

sylvaticum removal in prairie or forest settings it is not known how native plants respond to soils previously occupied by *B. sylvaticum.* The goals of this project were 1) to evaluate the response (via total mass) of the invader and native plants to soils which are currently invaded by *B. sylvaticum* and soils where *B. sylvaticum* has been removed over a nine-month period (legacy soils) and 2) to determine if legacy effects generated by *B. sylvaticum* PSF (nutrient concentration and bacteria community composition) persist in the soil once the invader is removed. We hypothesized that 1) *B. sylvaticum* and native plant response to *B. sylvaticum* removal would change over time and 2) nutrient concentration and bacteria community composition would differ between the two treatments over time.

Methods

Study Species

Brachypodium sylvaticum, a perennial bunch grass, is currently listed as a quarantined invasive species in California, Oregon, and Washington (Holmes et al. 2010). It is native to Europe and Asia and parts of North Africa, and is capable of rapid population growth in diverse environments including shaded forest understories, riparian zones, and full sun grasslands (Kaye and Blakelely-Smith 2006). It can persist at elevations ranging from sea level to 1,200 m (Rosenthal, Ramakrishnan, and Cruzan 2008), in mesic to semi-arid moisture regimes, and at varying nutrient concentrations (Holmes et al. 2010). *Brachypodium sylvaticum* reproduces sexually via seeds and asexually via tiller production (Roy et al. 2011). The exact time frame of *B. sylvaticum* introduction in the Pacific Northwest is unknown; however, the earliest documented herbarium specimen is

from Eugene, OR in 1939 (Chambers 1966). Genetic analysis has shown that *B. sylvaticum* was likely introduced at least twice in Oregon from native sites in western Europe (Rosenthal, Ramakrishnan, and Cruzan 2008).

In addition to *B. sylvaticum*, native understory plants common to Douglas-fir forests in Oregon's coastal range were used to test effects of *B. sylvaticum* PSF legacy effects. These species included two grasses, *Bromus vulgaris* (Hook.) Shear and *Elymus glaucus* Buckley, one forb, *Prunella vulgaris* L. and the economically important tree *Pseudotsuga menziesii*.

Experimental Design

This experiment utilized both field and greenhouse components to determine *B. sylvaticum* legacy effects. The field component was conducted in 2015 in the Oak Creek section of the McDonald-Dunn Research Forest, located near Corvallis, OR. *Brachypodium sylvaticum* has been present in the study site for at least 25 years and was estimated to cover 51 percent of the site in 1991 (Hubbard 1991). To our knowledge the site has not been burned or logged for at least 65 years. Ten 2.4 x 1.8 m plots were randomly established in the understory of the forest where *B. sylvaticum* abundance was greater than 75% (Figure 3.1). Each plot was divided in half along the 2.4 m edge; one side was left untreated and the other was sprayed with a 5% solution of Accord® XRT II (glyphosate; hereafter referred to as treated plots) to kill *B. sylvaticum.* We implemented our 'removal' of *B. sylvaticum* with glyphosate because it is an effective method of control and research has shown that glyphosate does not have adverse effects on microbial communities (Haney et al. 2000; Haney, Senseman, and Hons 2002).

At the time of inception (prior to herbicide treatment), and six and nine-months after *B. sylvaticum* removal, soil samples were collected from all treatments. All samples were from the top 10 cm of soil and were randomly collected from each treatment at least 0.5m inside the edge of each plot to avoid edge effects. One sample from each plot was divided in half, with one half used for nutrient analysis and the other used for microbial analysis. All of these samples were stored at -22 °C until analyses were performed. A second soil sample from each plot was used as inoculum for the feedback experiment; samples from the same treatments and collection times were pooled then stored in cool, dark conditions until used which was less than one week for all collection periods.

The feedback portion of the experiment was conducted in a greenhouse operated by Oregon State University in 2015-2016. Average greenhouse temperature was 21°C (except from June-August, 2015 when extended periods of 24-30°C temperatures occurred) and supplemental light was used to achieve 14 hours of daylight when needed. All plants used in the experiment were grown from seed. *Brachypodium sylvaticum* seed was collected in August 2014 and 2015 from the McDonald-Dunn Forest. *Bromus vulgaris, E. glaucus* and *P. vulgaris* seed was acquired from a commercial source that produced seeds from local provenance wild populations. *Pseudotsuga menziesii* seed was collected from the Oregon central coast in a stand of trees operated by Weyerhaeuser. For germination all seeds were placed in sterilized plastic containers on a moist towel. *Brachypodium sylvaticum* and *P. menziesii* seeds were placed in cold stratification at 5°C for 41 and 44 days respectively. Seeds were removed from cold stratification and placed in a greenhouse to allow germination to be completed. *Elymus glaucus, B. vulgaris* and *P. vulgaris* seeds were placed in the greenhouse when the other species' seeds were

removed from cold stratification. All species were grown in 444 mL pots except *P. menziesii* seedlings, which were grown in 66 mL Ray Leech Cone-tainers. Field soils from similar treatments were mixed 3:2 with sterilized potting soil (Metro-Mix Professional Growing Mix). Initially, 25 replicates were planned for each treatment, however, low seed germination and seedling mortality reduced the number of replicates for some treatments (Table 3.1). All treatments were grown for a minimum of 11 weeks. At the end of the growing period soil was carefully washed off roots and above ground and below ground biomass was collected and dried at 60 °C for a minimum of 72 hours. The entire feedback procedure was repeated for each of the three soil collection periods.

Nutrient Analyses

After each soil collection period, all samples collected for nutrient analysis were sent to Ward Laboratories (Kearney, NE). Analyses followed previously described protocols and tested for pH (Watson and Brown 1998), organic matter (Combs and Nathan 1998), nitrate (Gelderman and Beegle 1998), inorganic phosphorus (Mehlich 1984), potassium, calcium, magnesium, sodium (Warncke and Brown 1998), sulfate (Combs, Denning, and Frank 1998), zinc, iron, manganese, and copper (Whitney 1998).

Microbial Analyses

In order to determine microbial community composition, we chose to use two techniques: phospholipid fatty acid analysis (PLFA) and high throughput Illumina amplicon sequencing. PLFA analysis was used for the first soil collection and Illumina amplicon sequencing was used for the second and third collections. The first soil collection took place prior to herbicide treatment to get baseline soil community data,

therefore, we expected to see no difference in microbial communities. For the second and third collections we expected to see changes in microbial community composition, and therefore, chose to use Illumina sequencing because it has the capability of detecting taxa at a higher taxonomic resolution, the operational taxonomic unit (OTU).

Phospholipid fatty acid analysis

PLFAs are found in cell membranes in all living organisms. Once an organism dies PLFAs degrade rapidly (Zelles 1999) , therefore, PLFA analysis is an indicator of living soil organisms at the time of soil collection. Groups of organisms have unique PLFA biomarkers which provides PLFA analysis the opportunity to identify broad functional groups of soil organisms such as rhizobia, actinomycetes, gram positive and negative bacteria, arbuscular mycorrhizal fungi, saprophytic fungi, and protozoans. Frozen soil samples were sent to Ward Laboratories (Kearney, NE) where PLFA analysis was conducted following methodology previously described by Bligh and Dyer (1959). Fatty acids assigned to functional groups followed fatty peaks described by Bossio et al. (1998).

Molecular Techniques

For DNA extraction, samples were thawed to room temperature and using the MO BIO PowerSoil® DNA Isolation Kit (MOBIO, Carlsbad, CA USA). 0.25 g of soil from each sample was used following the manufacture's protocol with the addition of a ten-minute incubation period prior to bead beating. In two separate PCR reactions, 2 ul of purified DNA were used to amplify the 16s rRNA and ITS genes of bacteria and fungi, respectively. For the 16s rRNA gene, PCR was done in triplicate using previously

described primers targeting the V4 region of bacteria (Caporaso et al. 2011; Caporaso et al. 2012). PCR for the ITS gene was completed in one reaction and targeted the ITS1-F and ITS2 regions of fungi (Bellemain et al. 2010). All amplicons were then quantified using the Qubit® HS kit (Life Technologies, Carlsbad, CA USA) according to the manufacturer's instructions and amplicons were visualized using gel electrophoresis to confirm there was ~350 bp and ~450 bp bands for the 16s rRNA and ITS genes, respectively. All 16s rRNA and ITS samples were pooled individually and cleaned using the UltraClean® PCR clean-up kit (MOBIO) and diluted to a concentration of 10 nM. Equal molar concentrations of 16s rRNA and ITS libraries were pooled and underwent cluster generation and sequencing on an Illumina MiSeq instrument generating 250 bp end reads. Fungal samples experienced unexplained sequencing errors; therefore, only bacterial sequences were analyzed. Sequences were analyzed using QIIME and Operational taxonomic units (OTUs) were assigned using the UCLUST algorithm against the Greengenes (version 13_8) database (DeSantis et al. 2006).

Data Analysis

To determine treatment effect on plant response (total mass) a two-sample t-test was used in combination with a relative response (RR) index. RR indices are used in PSF experiments (Brinkman et al. 2010) to determine how a particular species responds to one treatment relative to another treatment. We used the following RR index to measure plant response to soils previously occupied by *B. sylvaticum*:

$RR_{BRSY\_removal}$ = ((total mass grown in treated soil – mean total mass grown in untreated soil)/(total mass grown in treated soil + mean total mass grown in untreated soil))

A one sample t-test was used to determine if the average RR was different from zero. A RR different from zero indicates *B. sylvaticum* does generate a legacy effect that impacts plant growth. To determine soil nutrient differences between treatments and collection times a univariate type three repeated measures analysis of variance (ANOVA) was used in combination with a Maulchy test for sphericity using the CAR package (Fox and Weisberg 2015) in R. When the assumption of sphericity was violated (nitrate, phosphorus, magnesium, and sodium) a Greenhouse-Geisser correction was used. When significant values were observed a post-hoc pairwise Tukey's honest significance tests was performed. A two sample t-test was used to determine differences in PLFAs for the first soil collection. For the second and third soil collections, a permutational multivariate analysis of variance (PERMANOVA) was used to analyze bacterial differences between treatment, collection time and the interaction between treatment and collection time. Beta-diversity was measured using Bray-Curtis dissimilarity, and non-metric multidimensional scaling (NMDS) was used to quantify and visualize compositional similarity of communities. Significant differences in overall beta-diversity were calculated using analysis of similarity (ANOSIM) and Kruskal-Wallis tests were used to determine differences within and among treatments. All microbial analyses were completed using the VEGAN package (Oksanen et al. 2016). All analyses were performed in R version 3.1.2 (R Core Team 2014).

Results

Plant response

When grown on soils from the first soil collection (before *B. sylvaticum* removal), a significant treatment effect was observed for *B. sylvaticum*, *P. vulgaris* and *P. menziesii* (Table 3.2). Each of these species' total mass was higher in untreated soils compared to treated soils. Six-months after *B. sylvaticum* removal no treatment effect was observed. Nine-months after *B. sylvaticum* removal, total mass of *P. vulgaris* was significantly increased when grown on the untreated soil (Table 3.2).

The average RR for *P. vulgaris* and *P. menziesii* six-months after *B. sylvaticum* removal were 0.042 and -0.126, respectively, and both values were statistically different from zero (*P. vulgaris* one sample t-test, p= 0.06; *P. menziesii* one sample t-test, p<0.001). Nine-months after *B. sylvaticum* removal the RR of *P. vulgari*s and *P. menziesii* were -0.140 and -0.041, respectively, but only the RR of *P. vulgaris* was different from zero (one sample t-test, p=0.006). The RR for each of the remaining species was no different from zero six and nine-months after *B. sylvaticum* removal.

Nutrients

When evaluating main effects, we found that collection time had a significant effect on all response variables except for organic matter and zinc, and treatment had a significant effect on iron and sulfur. There was a significant interaction between collection time and treatment for pH, manganese and calcium (Table 3.3). The general trend observed by response variables impacted by collection time was an increase in concentration (parts per million) from the first to last soil collection except for phosphorus which decreased between the first and second collection and then increased from the second to third collections (Figure 3.4).

Microbial Community

When evaluating microbial community using PLFA analysis for the first soil collection (pre-treatment) we found no differences between treated and untreated plots (Figure 3.5). The mean total PLFA biomass was 5,768 and 5,850 ng/g in the treated and untreated plots, respectively. Bacteria was approximately four times more abundant than fungi in both treatments with an average fungi:bacteria ratio of 0.22. Undifferentiated PLFAs comprised 36 and 39 percent of the total biomass for treated and untreated plots, respectively. Gram (-) and (+) bacteria equally contributed to the overall bacteria biomass for both treatments. Saprophytic fungi biomass was eight times greater than arbuscular mycorrhizae fungi (AMF) in each of the treatments.

Using DNA sequences to analyze bacterial communities from the second and third soil collections we found no evidence that collection time, treatment and the interaction between collection time and treatment had a significant effect on community composition (Figure 3.6) or beta-diversity. However, NMDS from the second soil collection suggests that some data structuring related to treatment is present (Figure 3.7). Altogether, 45 phyla were identified with Acidobacteria, Proteobacteria and Verrucomicrobia contributing to 78 percent of the observed phyla abundance. 850 OTUs were putatively identified and the most abundant across all treatments and collection times was *Chthoniobacteraceae* DA101, with an average abundance of 20 percent. For the remaining identified OTUs, 16 and 834 individual OTUs corresponded to 44 and 36 percent of the average total OTU abundance, respectively.

Discussion

Plant response

Some native species were affected by soils previously occupied by *B. sylvaticum,* which was consistent with our hypothesis. Interestingly, three opposing trends were observed in our data. First, the RR of *P. menziesii* increased (less negative) when growing on soils when time since *B. sylvaticum* removal was longest, suggesting that the legacy effect of *B. sylvaticum* on soils lasts between six and nine-months. One explanation for this legacy may be that *B. sylvaticum* disrupts ectomycorrhizal (EM) networks that play a role in *P. menziesii* seedling establishment (Bingham and Simard 2011). The direct mechanism of this process is unclear, but one possibility is that *B. sylvaticum* exudes anti-fungal compounds that deter EM fungi. Over a long occupancy in a particular site *B. sylvaticum* may devastate EM fungi populations. Once *B. sylvaticum* is removed from the system EM fungi may begin to recolonize which subsequently promotes *P. menziesii* growth. An example of this process has been observed with the invasive herb *Alliaria petiolata* (Stinson et al. 2006). In this study, Stinson et al. (2006) demonstrated that soils previously occupied by *A. petiolata* were practically void of arbuscular mycorrhizae (AM), which they suggest is from phytochemical inhibition. Also, seedlings from common tree species grew better in soils inoculated with AM fungi compared to the previously invaded soils without inoculation. Successful sequencing of our fungi DNA samples may provide evidence that supports this claim if EM fungi abundance increases between the six and nine-month soil collection.

The RR of *P. vulgaris* was adversely affected (more negative RR) by time since *B. sylvaticum* removal. Our data does not provide insight into why *P. vulgaris* performs

better in *B. sylvaticum* conditioned soils. Both species have previously been identified as having arbuscular mycorrhizae (AM) fungi associations (Abeyakoon and Pigott 1975; Moora and Zobel 1996) and it is plausible that they share generalist AM fungi that persist in the soil once *B. sylvaticum* is removed. Between six and nine-months after *B. sylvaticum* removal resources may have been depleted causing AM fungi populations to decrease which led to the negative RR in the third soil collection.

None of the grasses in the study were affected by *B. sylvaticum* removal (e.g. no RR was different from zero). This may be explained by similar resource requirements between functionally alike species. An alternative explanation may be that *B. sylvaticum* PSF does not affect the native grasses or the legacy takes longer than nine-months to dissipate.

When evaluating plant total mass between soil collections there was a significant larger amount of biomass produced in the pre-treatment soils compared to the second and third soil collections (Figure 3.2). This was likely a direct result from different greenhouse conditions between grow cycles. The first grow cycle took place between April and August in 2015, when average day length was 15 hours and there were many days when greenhouse temperatures reached 30°C. Conversely, during the second and third grow cycles, day length was fourteen hours (supplemental lights were used to achieve this) and temperatures rarely exceeded 24°C. Photoperiod has been shown to have a direct effect on photosynthetic capacity in a variety of tree species (Bauerle et al. 2012) and a positive (increased biomass) effect on grass species (Hay 1990). In addition, high temperatures can increase metabolic processes (enzyme activity) that are related to

growth and development which leads to increasing plant biomass. When environmental greenhouse conditions (temperature and daylight) were held constant between the second and third grow cycles there was no difference in biomass between collection time for each of the species.

Soil chemical properties

Contrary to our hypothesis, soil chemical properties did not respond to *B. sylvaticum* removal. Either responding to the treatment takes longer than nine-months or *B. sylvaticum* does not alter soil properties. Soil collection time had a significant effect on some nutrients. From the first soil collection to the last collection nitrate, iron and sulfur concentrations increased, with the greatest change between the second and third collections. Seasonal changes in microbial activity may explain these trends and/or nutrient requirements of coastal range species. During the growing season (March through September for species used in our study) plants require more nutrients for growth and development, thus, soil nutrient levels are depleted. After the growing season when plants go dormant and above ground biomass falls as litter, nutrient requirements are low and litter begins to decompose, thus, soil nutrients increase. Interestingly, the treated plots had no vegetation after herbicide application and yet soil nutrient fluctuations were the same between treatments and collection time. This may reflect minimal nutrient requirements of *B. sylvaticum* since it was the only species in the untreated plots.

The temporal change in phosphorus concentration was markedly different than all other nutrients. The decrease in concentration from the first to the second collection may reflect immobilization processes in which microbes converted inorganic phosphorus to

non-usable organic phosphorus. Between the first to the second collections mineralization by microbes may have led to the increase in phosphorus. Previous work has shown similar temporal phosphorus fluctuations in riparian forest (Fabre, Pinay, and Ruffinoni 1996) and range soils (Vaughn, Center, and Jones 1986) and linked phosphorus vacillations to microbial processes, plant uptake and weather. As previously mentioned, there was no difference in phosphorus concentration between treated and untreated plots, therefore plant uptake does not explain temporal changes to phosphorus for our study.

Microbial Communities

Contrary to our hypothesis, there was no difference in bacterial community composition between treatments and collection time. This suggests that changes to the bacterial community take longer than nine-months or *B. sylvaticum* does not alter the bacterial community. Other experiments have found similar patterns to microbial community structure after two-months (Kulmatiski and Beard 2011) two-years (Elgersma et al. 2011) and twelve-years (Yarwood et al. 2013) of removal of soil conditioning species. These data suggest that microbial communities are buffered against disturbance (removal of plants) and that their composition may not be solely based on aboveground plant populations, but rather soil properties such as pH and/or moisture content.

Interestingly, in an experiment by Yarwood et al. (2013), which examined the effect of litter removal on microbial community composition over a twelve-year period in a coniferous forest on the west slope of the Cascade mountain range in Oregon, the six most abundant bacteria phyla (Verrucomicrobia, Acidobacteria, Proteobacteria, Actinobacteria, Bacteroidetes and Planctomycetes) they observed were the same as in our

study, and three of the six most abundant OTUs they observed (*Chthoniobacterales* Da101, *Acidobacteria* Da052 and *Rhizobales*) were part of the top four most abundant OTUs in our study. This similarity suggests that these taxa are widespread and may play important roles in ecological processes such as regulating nutrient cycles in coniferous forests.

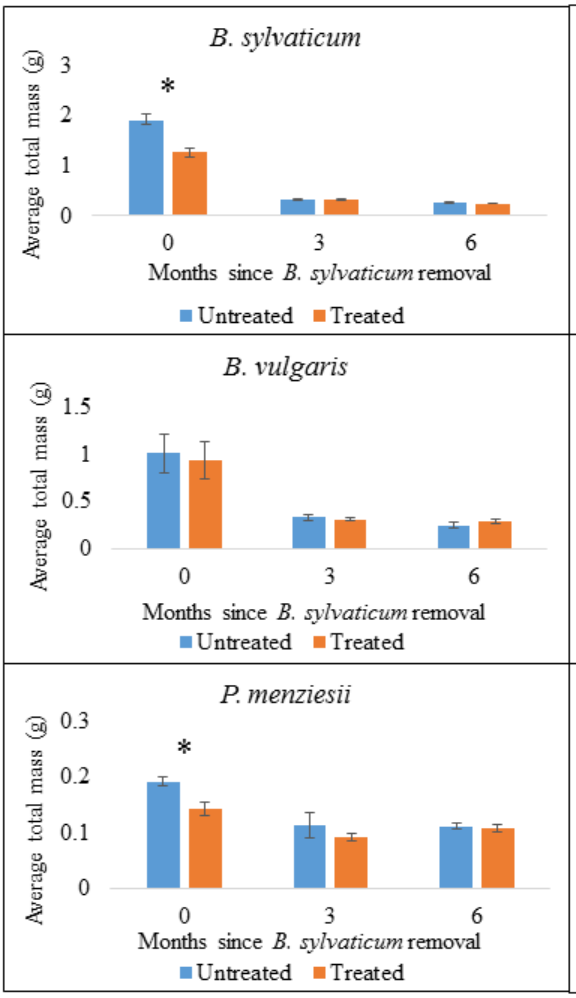
The NMDS analysis of the bacterial soil community from the second soil collection indicated some differences between treatments (Figure3.7). The structuring of the data may be reflective of low counts from differing OTUs from each treatment, but large counts between similar OTUs is heavily weighted in the statistical analysis (Adonis; vegan package) and therefore no statistical difference was observed. We recommend additional sampling to make the analysis more robust to OTUs that are present at low frequencies and/or using analyses that add more weight to taxa with low abundances.

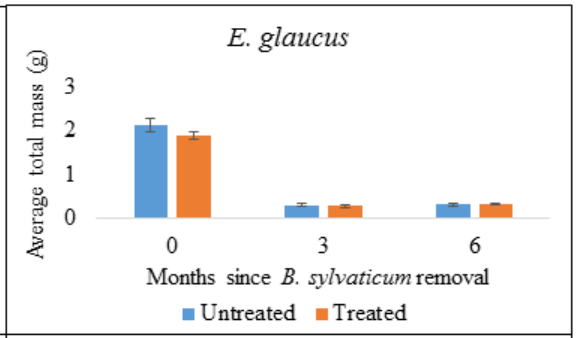
Conclusion

Legacy effects generated by *B. sylvaticum* differentially affected the relative response of native species. *Pseudotsuga menziesii* produced more biomass while *P. vulgaris* produced less biomass as the time from *B. sylvaticum* removal increased. From our data it is unclear whether the legacy is biotic, abiotic or a combination of the two, but there is an indication that between six and nine-months after *B. sylvaticum* removal plant response is affected by a diminishing legacy effect. Therefore, we recommend that land managers take into consideration the timing of revegetating a landscape after *B. sylvaticum* removal and suggest waiting at least nine-months to allow for optimal growing conditions. Future research should examine soil fungi as they may be the portion of the microbial

community that is controlling the legacy effect since we did not observe changes to bacterial communities.

Figure 3.1 Plot locations in the McDonald-Dunn experimental research forest located in Corvallis, OR. Each plot was established on existing populations of *B. sylvaticum* and is 2.4 x 1.8m. Plots were divided at 1.2m along the long side to create two treatments: untreated and treated which were sprayed with Accord XRT II (glyphosate) to remove all *B. sylvaticum.*





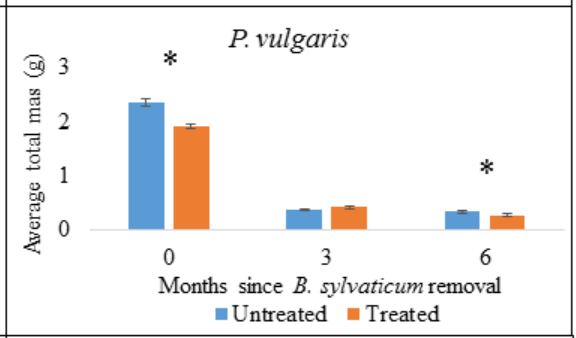


Figure 3.2 Average total mass of species grown on untreated (blue) and treated (red) soils from three soil collections (pre-treatment, six and nine-months after *B. sylvaticum* removal). An asterisk above a pair of bars indicates there is a statistical difference between the treatments. Error bars represent one standard error of the mean.

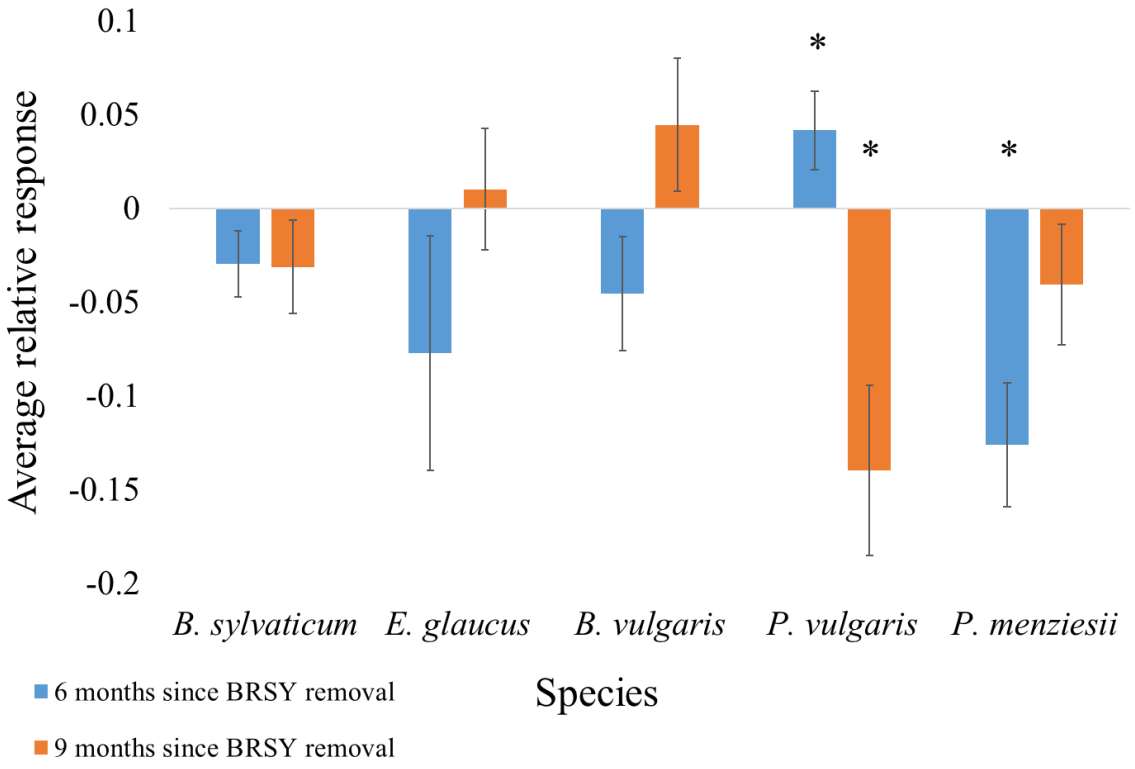


Figure 3.3 Average relative response to *B. sylvaticum* removal. Blue and red bars represent soils where *B. sylvaticum* had been removed for six and nine-months, respectively. An asterisk above a bar indicates the average RR is different from zero. Error bares represent +/- one standard error.

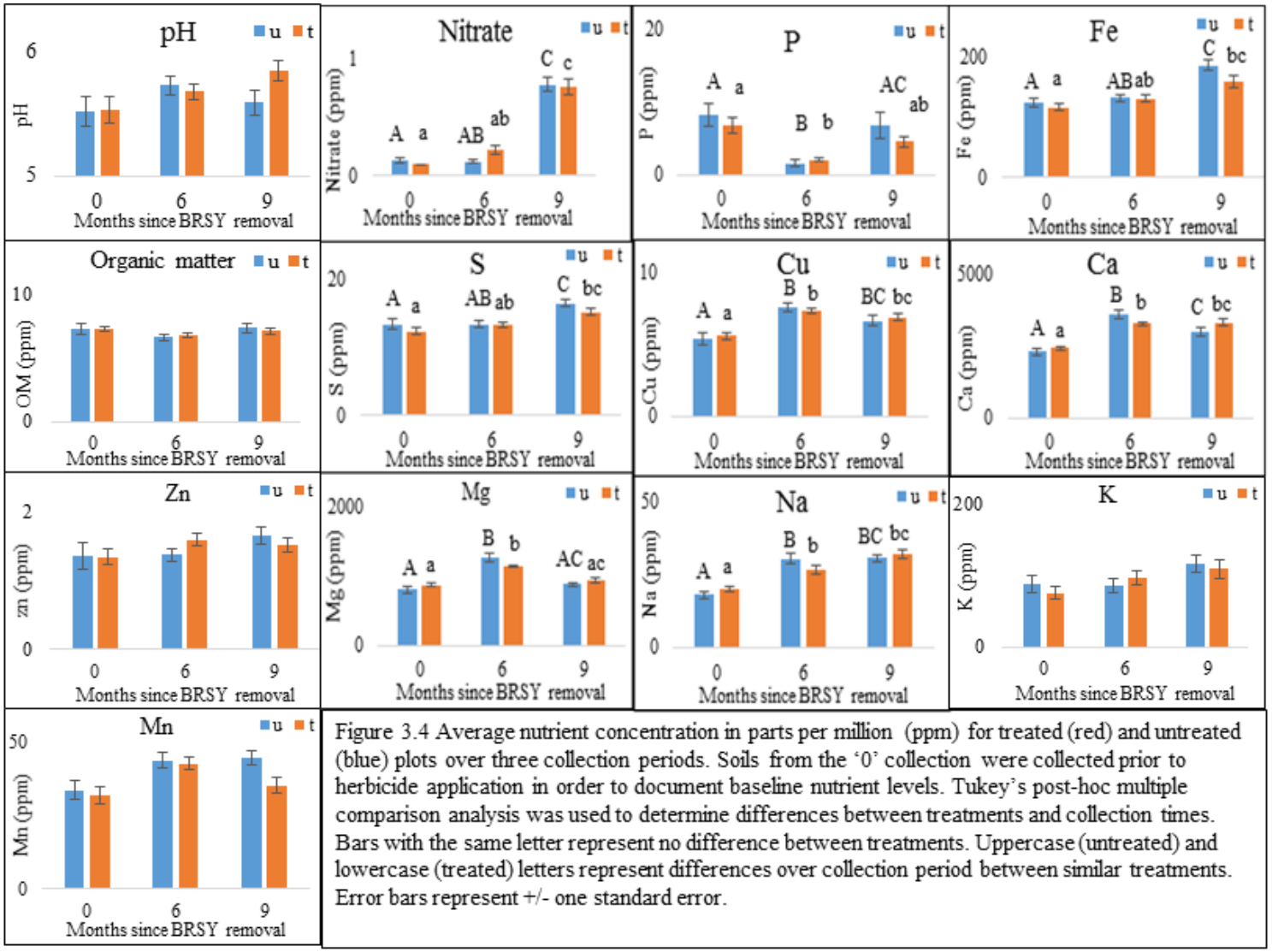


Figure 3.4 Average nutrient concentration in parts per million (ppm) for treated (red) and untreated (blue) plots over three collection periods. Soils from the '0' collection were collected prior to herbicide application in order to document baseline nutrient levels. Tukey's post-hoc multiple comparison analysis was used to determine differences between treatments and collection times. Bars with the same letter represent no difference between treatments. Uppercase (untreated) and lowercase (treated) letters represent differences over collection period between similar treatments. Error bars represent +/- one standard error.

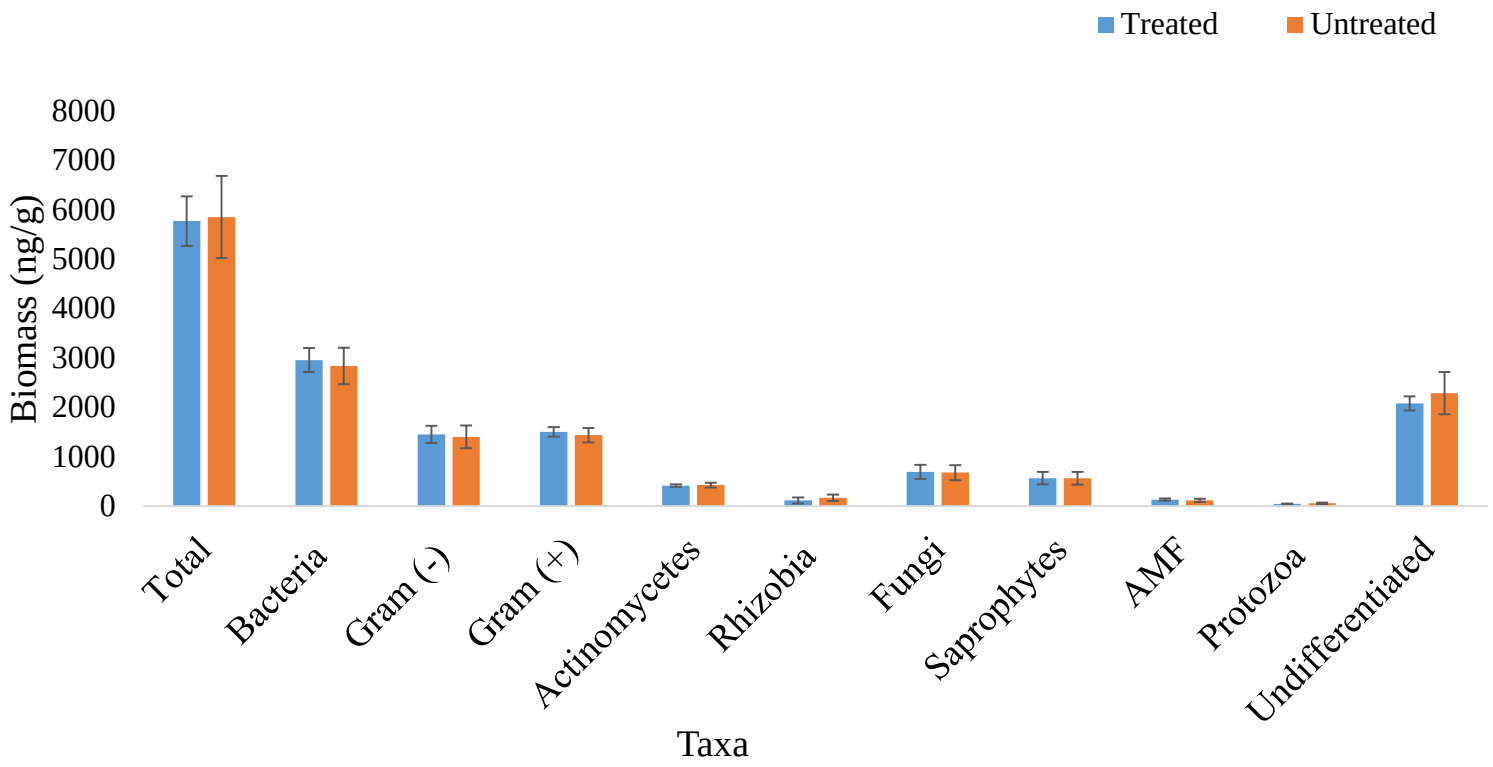


Figure 3.5 PLFA analysis biomass results from the first soil collection. Soil was collected prior to herbicide application to provide baseline microbial community data. Error bars represent +/- one standard error.

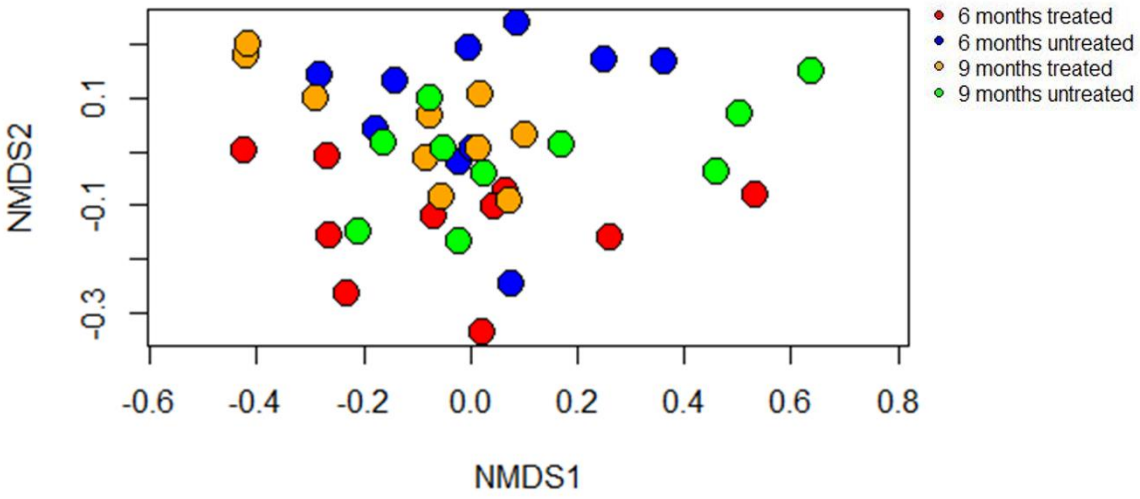


Figure 3.6 Non-metric multidimensional scaling (NMDS) visualization of bacterial communities in treated and untreated plots six and nine-months after *B. sylvaticum* removal.

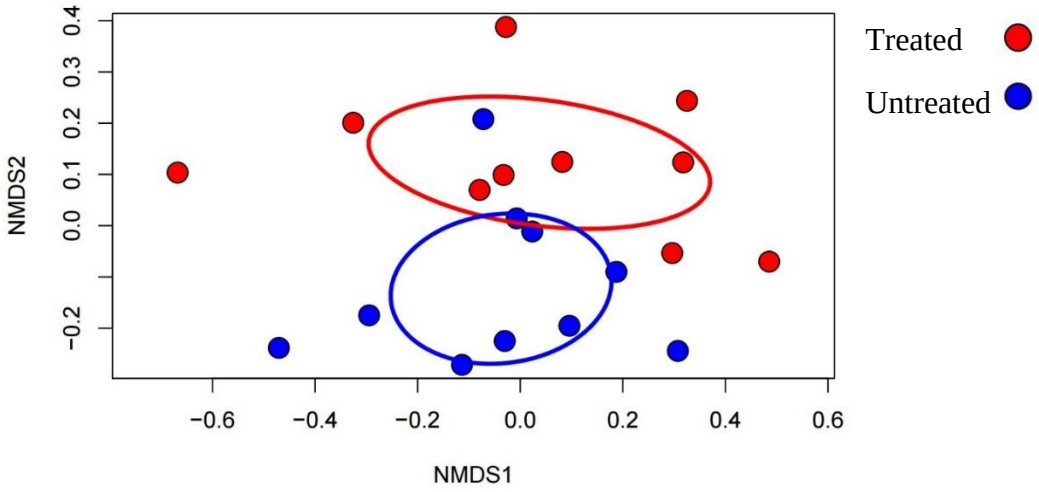


Figure 3.7 Non-metric multidimensional scaling (NMDS) visualization of bacterial communities in treated and untreated plots six-months after removal of *B. sylvaticum*.

Table 3.1 Number of replicates for each species per treatment and planting. Replicates vary between plantings due to differing germination success rate.

	Planting 1 Soil collected on 3/27/205			Planting 2 Soil collected on 9/27/2015			Planting 3 Soil collected on 12/9/2015		
	Untreated	**Treated**	**Days grown**	**Untreated**	**Treated**	**Days grown**	**Untreated**	**Treated**	**Days grown**
B. sylvaticum	25	25	86	19	20	85	20	19	84
E.glaucus	25	25	77	19	17	84	15	17	84
B. vulgaris	10	10	80	18	18	84	18	20	84
P. vulgaris	25	25	81	20	20	84	20	20	84
P. menziesii	15	15	94	23	23	92	23	24	85

Table 3.2 Two sample t-test for repsone of species to *B. sylvaticum* removal. Bold values represent signifcant differences between total mass of a species between treatments.

	Soil collection 1 Total Mass		Soil collection 2 Total Mass		Soil collection 3 Total Mass	
Species	t value	p value	t value	p value	t value	p value
B. sylvaticum	-4.236	**<0.001**	-0.719	0.476	0.463	0.645
E. glaucus	-1.487	0.143	-0.327	0.745	0.424	0.673
B. vulgaris	0.261	0.796	-0.563	0.576	0.929	0.359
P. vulgaris	-4.716	**<0.001**	1.462	0.151	-2.109	**0.041**
P. menziesii	-3.316	**0.002**	-0.906	0.369	-0.516	0.608

Table 3.3 Results from univariate type three repeated measures ANOVA for soil chemical properties. 'ct' indicates soil collection time, 'treatment' refers to plots with *B. sylvaticum* or plots without *B. sylvaticum* and 'ct:treatment' is the interaction between the two main effects. When the assumption of sphericity was not met the Greenhouse-Geisser correction p-value was used. Bold numbers represent statistically significant values.

Variable	**Treatment**	**DF**	**F**	**P**	**Corrected P**
	ct	2,18	3.297	0.06	
pH	treatment	1,9	2.001	0.19	
	ct:treatment	2,18	3.884	**0.039**	
	ct	2,18	2.091	0.152	
Organic matter	treatment	1,9	0.325	0.582	
	ct:treatment	2,18	0.327	0.725	
	ct	2,18	125.516	**<0.001**	
Nitrate	treatment	1,9	0.489	0.501	
	ct:treatment	2,18	1.964	0.169	0.192
	ct	2,18	18.578	**<0.001**	
Phosphorus	treatment	1,9	1.405	0.266	
	ct:treatment	2,18	1.374	0.278	0.273
	ct	2,18	30.561	**<0.001**	
Iron	treatment	1,9	5.594	**0.042**	
	ct:treatment	2,18	3.497	**0.052**	
	ct	2,18	1.766	0.199	
Zinc	treatment	1,9	0.108	0.749	
	ct:treatment	2,18	1.259	0.307	
	ct	2,18	19.117	**<0.001**	
Potassium	treatment	1,9	0.093	0.766	
	ct:treatment	2,18	1.931	0.173	
	ct	2,18	8.247	**<0.01**	
Manganese	treatment	1,9	3.946	0.078	
	ct:treatment	2,18	3.824	**0.041**	
	ct	2,18	2.633	**<0.001**	
Sulfur	treatment	1,9	6	**0.036**	
	ct:treatment	2,18	1.221	0.318	
	ct	2,18	49.803	**<0.001**	
Copper	treatment	1,9	0.089	0.772	
	ct:treatment	2,18	1.95	0.171	
	ct	2,18	34.69	<0.001	
Calcium	treatment	1,9	0.314	0.588	
	ct:treatment	2,18	5.683	**0.012**	
	ct	2,18	86.109	**<0.001**	
Sodium	treatment	1,9	0.029	0.868	
	ct:treatment	2,18	2.545	0.106	0.133
	ct	2,18	48.82	**<0.001**	**<0.001**

Variable	Treatment	DF	F	P	Corrected P
Magnesium	treatment	1,9	0.107	0.75	
	ct:treatment	2,18	6.802	0.006	

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Chapter 4:

Conclusions

Plant-soil feedback

The experiments presented in this work provide data that suggest plant-soil feedback (PSF) may play a role in the invasion success of *Brachypodium sylvaticum* in Oregon Coastal Range Douglas-fir forests. The conspecific relative response (RR) of *B. sylvaticum* was negative which does not support invasion theory. However, *B. sylvaticum* benefited from growing on soils conditioned by native forbs and *Pseudotsuga menziesii* which may be one reason why *B. sylvaticum* is good at establishing itself in *P. menziesii* forests. Once established and native species soil conditioning dissipates factors such as cespitose growth, release from native enemies (Roy et al. 2010) and/or using energy for competition rather than defense (Vandegrift et al. 2014) may promote positive population growth.

When we grew *B. sylvaticum* on sterilized and unsterilized conditioned soils *B. sylvaticum* grew better in sterilized soils. Our data does not provide insight into why this happened but two opposing scenarios are possible. One is there are soil organisms (bacteria, fungi, nematodes, etc.) in the invaded range that inhibit *B. sylvaticum* growth and the sterilization process removed those organisms from the soil. Alternatively, mutualistic microbes present in the greenhouse may have colonized the sterilized soil at a greater rate compared to the live soils because there was more open niche space available. Genetic analysis of the bacterial communities identified different communities between the treatments, but how the different bacterial communities impacted *B. sylvaticum* growth is not known.

When evaluating native species RR to soils conditioned by *B. sylvaticum* the only species negatively impacted was *P. menziesii*. It has been speculated (Vandegrift et al. 2014) that shading by *B. sylvaticum* reduces *P. menziesii* establishment, however, there is no available data to support this claim. Here, we provide data that shows *P. menziesii* seedling growth is directly reduced by *B. sylvaticum* PSF, but the mechanism remains allusive. Data from the legacy experiment suggest that the effects of *B. sylvaticum* soil conditioning diminishes after nine-months from *B. sylvaticum* removal. One explanation for this may be that *B. sylvaticum* disrupts ectomycorrhizal (EM) networks that play a role in *P. menziesii* seedling establishment. The direct mechanism of this process is unclear, however, one possibility is that *B. sylvaticum* exudes anti-fungal compounds that deter EM fungi.

Legacy Effects

Soils previously occupied by *B. sylvaticum* differentially affected the RR of native species. *Pseudotsuga menziesii* benefited from long term *B. sylvaticum* removal while *Prunella vulgaris* growth was reduced as time since removal increased and native grasses were not impacted. This underscores the complexity of soil conditioning and the interactions among and between plants and microbes. Our data did not provide evidence for a mechanism to explain the RR of plants because soil chemical properties and bacterial communities did not respond to the treatment over the course of the experiment. Either more time is needed to visualize the legacy, or *B. sylvaticum* does not impact soil chemical properties and bacterial communities. *Pseudotsuga menziesii* (Bingham and Simard 2011) and *P. vulgaris* (Moora and Zobel 1996) have been associated with

mutualistic ectomycorrhizal and arbuscular mycorrhizal fungi, respectively, therefore, plant response may have been attributed to alterations in fungal community composition. Future analysis of the fungal communities using DNA samples extracted in the legacy experiment may provide evidence to support this clam.

PSF Methodology

Methods used to measure PSF vary between investigators and experiments and should be taken into consideration when evaluating data. One commonly used method is to compare plants that have been grown on sterilized and non-sterilized conditioned soil (Brinkman et al. 2010). Presumably, sterilizing soil results in a medium free of living organisms and allows the investigator to determine if the PSF is of a biotic origin. However, as our data showed, after six-months of being in a greenhouse, the sterilized soil had numerous bacteria phyla present and some were more abundant in sterilized soils than unsterilized soils. Thus, 'sterilized' soils are not actually void of living organism and microbial communities found in them may not represent the communities from where the soil originated, but rather microbial communities that are present in the greenhouse which may lead to inaccurate analyses. To mitigate the chance of microbial community colonization from greenhouse organisms we suggest sterilizing the soil directly after the conditioning phase (Del Fabbro and Prati 2015) instead of before the conditioning phase (Jordan, Larson, and Huerd 2008). This directly reduces the length of time microbial recolonization could occur.

Alternatively, the entire experiment could take place in the field which would eliminate any undesired microbial colonization. Field experiments also benefit from

using soils that are conditioned by the entire plant community rather than one species, which is common in greenhouse experiments. Heinze et al. (2016) demonstrated that PSF responses do differ between greenhouse and field studies which can result in misleading interpretations of greenhouse data. Both types of experiments (field and greenhouse) provide benefits and challenges to investigators, therefore, if feasible, a combination of the two methods may provide the most insight when studying PSFs of a given system.

Management Implications

Restoring sites invaded by *B. sylvaticum* is a challenging task. In a simplistic model, *B. sylvaticum* needs to be removed and kept from re-establishing itself. Research has shown that herbicide application coupled with high severity fires are good methods of removing the grass (Fjeran 2014; Poulos and Roy 2015) and can be effective at reducing *B. sylvaticum* percent cover in subsequent years. However, if fire severity is low, the fire will promote *B. sylvaticum* cover the following year (Poulos and Roy 2015). An additional reason to have a high severity fire is to overcome long term biotic and abiotic legacy effects generated by *B. sylvaticum*. Hebel et al. (2009) showed that when exposed to high severity fire, microbial community composition and soil chemical properties were significantly different then in soils exposed to low severity fires. In addition, they observed non-native plants to have a reduction in biomass when grown on soils exposed to high severity fires.

Once *B. sylvaticum* is removed, planting native grasses may be the best way to reduce chance of a subsequent invasion. Our data indicates that soils conditioned by *Bromus vulgaris* reduced *B. sylvaticum* growth. Species from the same functional group may have

comparable nutrient requirements and/or microbial associations. In theory, native grasses will use similar nutrients as *B. sylvaticum* and potentially promote biotic interactions with soil pathogens that may also infect *B. sylvaticum*. When *B. sylvaticum* is re-introduced into the restored area biotic and abiotic soil conditions generated by native grasses will reduce *B. sylvaticum* growth, giving established grasses a better chance to compete with the invader.

The timing of revegetating a site after *B. sylvaticum* removal is also important. Our data shows that *P. menziesii* should not be planted for at least nine-months after *B. sylvaticum* removal. Although not statistically significant, there is an indication that the two native grasses produce more biomass in soils nine-months after *B. sylvaticum* removal (Figure 3.3). Therefore, we recommend waiting at least nine-months after removing *B. sylvaticum* before planting native species.

Closing Remarks

Understanding the processes associated with biological invasions will help improve methods used to control and mitigate invasive species establishment and distribution. PSF is one process that contributes to invasion but should not be the only process considered when evaluating a site. Other factors such as site history, disturbance and human activity all play a role in invasion processes. Collaboration between scientists, land managers and public officials (via policy) is required if negative impacts by invasive species are going to be reduced. Ultimately, continued research is needed to better understand invasion processes and associated mitigating techniques.

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