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Identifying and Remediating the Microbial Legacy Effects of Invasive Grass for the Purpose of Improved Restoration

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Identifying and Remediating the Microbial Legacy Effects of Invasive Grass for the Purpose of Improved Restoration

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by

Brooke E. Pickett

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ABSTRACT OF THE DISSERTATION

Identifying and Remediating the Microbial Legacy Effects of Invasive Grass for the Purpose of Improved Restoration

by

Brooke E. Pickett

Doctor of Philosophy,
Graduate Program in Evolution, Ecology, and Organismal Biology
University of California, Riverside, March 2019
Dr. Emma Aronson, Chairperson

Biological plant invasions impact the function and biodiversity of ecosystems across the globe by displacing native plant species and altering the physical and chemical soil environment. In California, invasive grasses have displaced native plants, transforming much of the endemic coastal sage scrub (CSS) to nonnative grasslands. This has occurred for several reasons including increased competitive ability of invasive grasses and long-term alterations to the soil environment, called legacy effects. Despite the magnitude of this problem, however, it is not well understood how these legacy effects have altered the soil microbial community and, indirectly, native plant restoration.

Chapter one explores how invasive plants change the abundance and diversity of three important fungal symbionts (arbuscular mycorrhizal fungi, ectomycorrhizal fungi, and fungal pathogens), as well as the implications these changes may have for ecosystem health. I finish off the chapter by discussing restoration efforts designed to ameliorate fungal legacy effects of invasive plants.

Chapter two assesses the microbial composition of soils collected from an uninvaded coastal sage scrub (CSS) community (uninvaded soil) and a nearby 10ha site from which the invasive grass, Harding grass (*Phalaris aquatica* L.), was removed after 11 years of growth (post-invasive soil) in order to better understand the long-term impact of invasive grasses on soil microbes. Our findings indicate that Harding grass may create microbial legacy effects in the soil that likely cause soil conditions inhibitory to the germination rate, biomass, and length of California sagebrush, but not the other two native plant species.

Chapter three seeks to understand if differences in native plant growth can be explained by biotic legacy effects and if these legacy effects can be reversed. We measure the growth of three CSS species inoculated with either uninvaded soil or sterilized uninvaded soil and planted into a site with known microbial legacy effects. Our findings indicate that differences in native plant growth can be explained by changes in the soil microbial community and that remediation of the soil microbial community through inoculation can improve restoration in post-invasive sites.

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CHAPTER 1

Impacts of Invasive Plants on Soil Fungi and Implications for Restoration

Abstract

Biological plant invasions impact the function and biodiversity of ecosystems across the globe by displacing native plant species and altering the physical and chemical soil environment. While much is known about direct competition between invasive and native plants, ecologists have just begun to uncover the less obvious impact of plant invasion: changes to the soil fungal community. Fungi are important to the survival of many plant species and an integral part of a healthy soil system. Arbuscular mycorrhizal fungi are plant mutualistic symbionts that associate with many species and provide necessary services, such as increasing surface area for root water absorption and resistance to pathogens, while ectomycorrhizal fungi play an equally important role and are critical for plant nutrient acquisition in boreal and temperate forests. Invasive plants are altering the soil fungal community in ways that indirectly impact the structure of native plant communities, sometimes for years after the invasive plant has been removed from an area (i.e., legacy effects). These changes make restoration especially difficult in areas from which long-term plant invasions have been eradicated; in some cases these changes can be so severe that even with active management, they take months or decades to reverse.

Keywords: mycorrhizal, fungi, roots, legacy effects, restoration, microbial, invasion

Introduction

The global scale of plant invasion means we need to understand it better at all levels in order to prevent further damage. While much research has been conducted about the ecosystem impact of invasive plants, ecologists have recently begun to uncover a less obvious, but important, consequence of plant invasion: changes to the soil fungal community.

Fungi are ubiquitous and the principal decomposers of organic debris in ecosystems all around the world (Desprez-Loustau et al., 2007). They are essential to decomposition and nutrient cycling in most intact environments, ranging from unicellular aquatic chytrids to large mushroom fruitbodies with extensive mycelial networks. They acquire their food by exuding enzymes into their environment, breaking apart the bond structures in complex compounds, and subsequently absorbing the dissolved nutrients and molecular components. Some fungi exist as symbionts of plants and animals while others exist as free-living cells. Symbionts can interact with their host as mutualists, parasites, or in a way that does not affect the host (commensalism) (Desprez-Loustau et al., 2007). Commensalism, in this context, not only includes symbionts but also free-living microorganisms performing nutrient transformations critical to plant growth, such as nitrification and denitrification (Ehrenfeld et al., 2005).

Fungal mutualists interact with plants through mycorrhizal symbiosis, a symbiotic association between fungal hyphae and the roots of a vascular plant that can be characterized as either arbuscular mycorrhizal, ectomycorrhizal, or ericoid (Allen 1991). These mycorrhizal fungi grow in the rhizosphere of the plant and can be either intracellular (arbuscular mycorrhizal fungi; AMF) or extracellular (ectomycorrhizal; ECM). Plants and their symbionts communicate through molecular and genetic feedback during which fungi provide growth-limiting nutrients, such as nitrate and phosphate (Allen 1991), and even facilitate plant-to-plant exchange of nutrients and carbohydrates (Simard et al., 1997). These plant-fungal associations are extremely important to the survival of a majority (~ 90%) of all plant species (Hayward et al., 2015).

When invasive plants are introduced to a healthy ecosystem, they can disrupt fungal mutualistic associations with native plants. Moreover, plant invasions may even prevent these mutualistic associations from occurring by altering soil nutrient dynamics, changing soil food webs, or introducing plant pathogens (Allen 1991; Ehrenfeld et al., 2005). Although not always negatively impacting mutualisms with native plants, these changes brought on by plant invasion can last for years (Belnap et al., 2005; Cuddington 2012; Ehrenfeld et al., 2005; Eviner et al., 2010; Hamman and Hawkes 2013; Jordan et al., 2008; Pringle et al., 2009; Simard et al., 1997) after the invasive plant has been removed and are termed “legacy effects” (Kulmatiski and Beard 2011). These legacy effects are normally defined as the abiotic and biotic impact of a species that persist long after the invasive species has been eradicated or extirpated from an area (Cuddington 2012).

Studies focused on understanding the legacy effects of invasive plant growth on native plants can have either similar or conflicting results, largely dependent upon the native and invasive species studied (Bozzolo and Lipson 2013). As a result, many suggestions for improving soils after invasive species removal have been anecdotal, and are context-dependent.

In this chapter, we will discuss how invasive plants may change the abundance or diversity of three important fungal symbionts (arbuscular mycorrhizal fungi, ectomycorrhizal fungi, and fungal pathogens), as well as the implications these changes may have for ecosystem health. We will finish off the chapter by discussing restoration efforts designed to ameliorate fungal legacy effects of invasive plants.

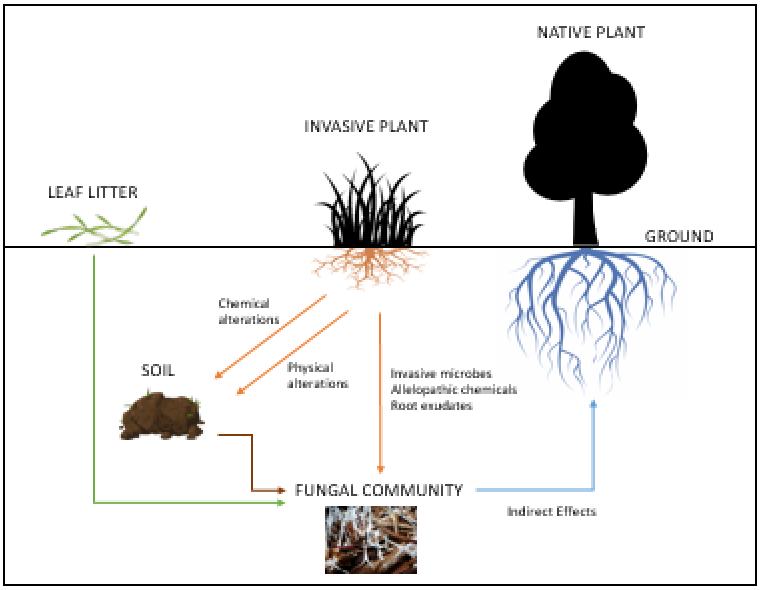
Biotic Impact of Invasive Plants

Plant-soil interactions can be abiotic or biotic, meaning that plant composition can alter the chemical composition of the soil or the microbial composition of the soil and vice versa. Not until 1985, however, did papers linking the words plant and soil begin to appear in the BIOSIS database. Since then, papers about plant-soil interactions have appeared at a rate of 3,500 per year (Ehrenfeld et al., 2005). So while the field is relatively new, it is growing quickly and becoming more diverse.

Early investigations of plant-soil feedbacks focused on physical properties of the soil, such as texture, water content, and temperature. Researchers then began investigating the chemical and biogeochemical components of plant-soil feedbacks, such as the pH, carbon, and nitrogen content of soils (Ehrenfeld et al., 2005). Currently, there is more focus on the role of microbes in regulating and responding to plants and the larger environment. This increased focus on microbes is due to their critical importance to the ecology of all macro-organisms: they are major decomposers in all ecosystems, important to the survival of most plant species, and an integral part of both carbon and nitrogen cycles.

Many studies have demonstrated shifts in microbial communities due to invasive plant growth (Bozzolo and Lipson, 2013; Callaway et al., 2004; Elgersma and Ehrenfeld 2011; Lankau and Lankau, 2014; Lekberg et al., 2013; Kulmatiski and Beard 2011; Simard et al., 1997). However, the phenomenon of *fungal* shifts in response to invasive plants is less understood, and potentially has many implications for maintaining biodiversity and function of invaded ecosystems. Throughout this section we will explore the ways in which invasive plants alter the fungal community (Figure 1).

Figure 1. Diagram showing the biotic impacts of invasive plants. Orange arrows: the invasive plant alters the chemical and physical soil components, which has an indirect effect on the fungal community composition. The invasive plant directly affects the fungal community through introduction of invasive microbes, allelopathic chemicals, and root exudates. Green arrow: leaf litter can alter the fungal community composition if the invasive plant leaf litter has a different quality (C:N) than that of the native plant leaf litter. Blue arrow: all of these alterations to the soil fungal community have indirect effects on the growth of native plants.



Arbuscular and Ectomycorrhizal Fungi

Fungal hyphae, or collectively the mycelium or mycelial network, are filamentous strands of fungal cells which compose the main body of the fungus, and the fungal vegetative structure that is often branching and filamentous (Parniske 2008). In soils, fungal hyphae grow throughout the soil matrix, with the direction of apical growth (from the apex to the hyphal tip) often dependent on an environmental stimulus. These hyphae exhibit a variety of morphological structures and functional modifications. Arbuscular mycorrhizal fungi (AMF) form arbuscules, small branching structures within cortical root cells, which are the sites of the bi-directional exchange of carbon and nutrients, such as phosphorus, between the plant and fungi (Parniske 2008). Ectomycorrhizal fungi similarly exchange nutrients with plants, but they form a dense hyphal sheath that surrounds the root surface, rather than penetrating the root cells (Hock 2012) (Figure 2) This mutualism provides a fungus with carbohydrates and the plant with an increased surface area for water and mineral absorption.

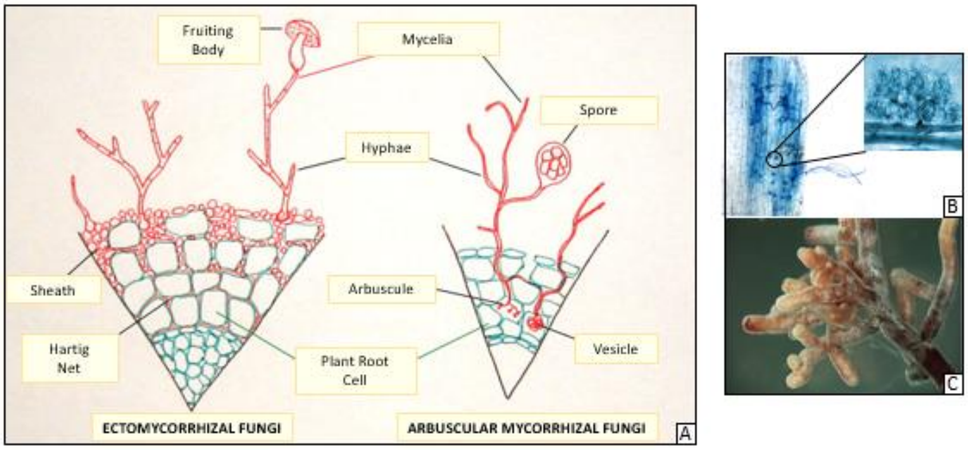


Figure 2. A) Diagram depicting the similarities and differences between ectomycorrhizal and arbuscular mycorrhizal fungi. B) An arbuscule inside of a plant root. C) Ectomycorrhizal fungal hyphae growing on a plant root.

Arbuscular mycorrhizal fungi are obligate plant symbionts. These AMF are arguably the most common plant mutualistic symbionts, consisting of at least 145 groups (Bever et al., 2001). They associate with most plant species and are especially important for the uptake of phosphorus (Batten et al., 2006; Lankau and Lankau 2014), an integral nutrient for plant growth. Over the years, researchers have discovered that AMF not only increases plant access to phosphorus, but they also provide resistance to pathogens (Batten et al., 2006; van Grunsven et al., 2010), stabilize soil aggregates (Miller and Jastrow 2000), alter plant communities (Bever et al., 2001), and even ameliorate the allelopathic effect of some invasive plants (Barto et al., 2010). Most AMF are generalists, meaning they associate with many plant taxa, while others are specialists, and associate with only one or merely a few plant taxa.

The widespread distribution and low host-specificity of most AMF suggests that when plants invade a healthy soil system, they can readily form associations with AMF. Since most invasive plants can probably form arbuscular mycorrhizas (Richardson et al., 2000), it isn’t surprising to find that numerous opportunistic invasive plants also associate with AMF to their own advantage (Smith et al., 2008). When associating with fast-growing, small-spored fungal taxa, such as *Glomus*, which can colonize via mycelia fragments, an invasive plant may be even more likely to thrive (Bongard 2013). These associations with generalist AMF may allow invasive plants to outcompete and displace native plants which are either non-mycorrhizal (such as *Brassica* spp.), weakly mycorrhizal, or do not form associations with generalist AMF, in contrast to the generalist invader. One recent example of such an invader is *Vincetoxicum rossicum*, a forb that displaces native plants and was found to associate with four different AMF subgroups (*Glomus intraradices*, *G. caledonium*, *G. fasciculatum*, and *G. mosseae*), which are highly infective and remarkably efficient at phosphorus uptake. These same subgroups, however, were absent from the rhizosphere of each native plant growing within the invasive plant patches (Bongard 2013). This finding suggests that the invasive plant’s ability to associate with fungal generalists allows it to thrive and may improve its ability to displace native plants.

Some invasive plants have the ability to degrade local mycorrhizal fungi, a finding termed the "Mycorrhizal Degradation Hypothesis" (Vogelsang et al., 2004) (Figure 3). Degradation of local AMF can change the soil in ways that hinder native plants and help invasive plants. Examples of this include *Alliaria petiolata*, a non-mycorrhizal plant, which has been known to produce glucosinolates which are potentially toxic to AMF, and *Myrica faya*, a plant which carries nitrogen fixing microbes, from the genus *Frankia* along with it to the invaded range (van der Putten et al., 2007).

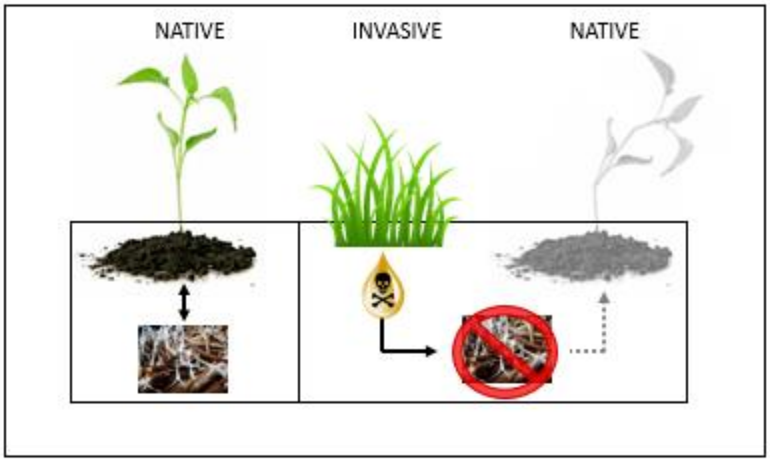


Figure 3. Diagram illustrating the Mycorrhizal Degradation Hypothesis. In the left panel, we see a healthy native plant in a mutualistic relationship with AMF. In the right panel, the invasive plant is producing a chemical exudate that eliminates beneficial fungi, thereby preventing fungal association with the native plant and eventually native plant death.

While researchers have only just begun exploring the impact of invasive plant species on AMF abundance (Bongard 2013; Richardson et al., 2000; Smith et al., 2008), it is evident that invasive plants can have the potential to either increase (Bozzolo and Lipson 2013; van der Putten et al., 2007; Vogelsang et al., 2004) or decrease (van der Putten et al., 2007; Zhang et al., 2009) the abundance and diversity of AMF. Increased AMF abundance with invasion may happen when the native intact plant community naturally associates with fewer AMF taxa than the invading mycotrophic (mycorrhizal) plants (Barto et al., 2010; Bozzolo and Lipson 2013; Simard et al., 1997; Tanner and Gange 2013). In fact, if the invader is mycotrophic, a monoculture of the invasive plant may still harbor a more species-rich AMF community than a diverse community of native plant species (Lekberg et al., 2013). This increased abundance of AMF by the invader may actually feedback to increase invasion (Paudel et al., 2014). However, if the invading plant is non-mycorrhizal, then AMF abundance and diversity will decrease relative to pre-invasion soil (Zubek et al., 2016). A recent comprehensive field study (Greipsson and Ditommaso 2006) compared AMF abundance in soils invaded by one non-mycorrhizal and two mycorrhizal plant species. All three invaders reduced AMF abundance and richness, but the non-mycorrhizal plant reduced AMF abundance and richness to a greater extent. However, this pattern is not always so evident: if an invader is mycotrophic, but not a good host for AMF, then it may actually decrease the AMF abundance (Mummey and Rillig 2006; Shah et al., 2010). Certain invasive plants may associate with particular groups of AMF, which may be different than those hosted by local native plants (Busby 2011). In these cases, invasion could subsequently bolster the abundance of some AMF groups, while decreasing the diversity or abundance of others.

In the presence of invasive plants, some studies show shifts in either AMF diversity (Hawkes et al., 2006) or from fungal specialists to generalists (Allen 1991; Bunn et al., 2015), as well as differences in the prevalence of fungal versus bacterial utilization of leaf litter (Hawkes et al., 2006). However, the identity and functional group status of both the native and invasive plant may dictate their effects on AM fungal symbionts. For instance, a recent meta-analysis (Tanner and Gange 2013) reported that invasions may not necessarily cause a shift in AMF associations, unless the native and invasive plant are in different functional groups. If an invader decreases AMF abundance or only increases the abundance of the particular AM fungal associate, then this could negatively impact native plant communities which are dependent on AMF for survival (Tanner and Gange 2013). Changes to soil AMF abundance and diversity may not be short-lived; in fact, they could last long after the invader is gone (Shannon et al., 2014). Such biotic legacy effects can occur when plant-soil interactions are altered by invasive plants for long periods of time.

The timing of AMF response to invasion is still largely a mystery (Day et al., 2015). A recent meta-analysis (Tanner and Gange 2013) reported that AMF colonization of native plants may decrease due to legacy effects of invasive plants. However, it is unclear how quickly these legacy effects occur or attenuate after an invasive plant is removed, as well as how soon the community may return to the structure and functioning of the previously native state (Day et al., 2015). In certain instances, after an invasive is removed, any changes in AMF abundance and diversity are fleeting, because differences in abundance and richness return rapidly with the return of the native vegetation type (Bozzolo and Lipson 2013; Endresz et al., 2013). In contrast, in other studies (Zubek et al., 2016) even a highly mycorrhizal invasive plant may not rapidly alter the AMF community, even after 29 weeks. Another study shows some recovery of AMF communities 6 years after the removal of an invasive known to decrease AMF abundance (Kulmatiski and Beard 2011). In some cases invasion can lead to the development of a novel AMF community over decadal time scales (Busby 2011; Zhang et al., 2009; Zubek et al., 2016). Overall, the recovery of the AMF community could take a long time. Furthermore, shifts in AMF may be dependent on an invasive plant’s functional traits (Belnap and Phillips 2001; Busby 2011; Day et al., 2015; Shannon et al., 2014), which may ultimately be the best predictor for the extent of AMF response to invasion, and subsequent recovery.

Although AMF and other groups of mycorrhizal fungi, such as the ectomycorrhizal fungi (ECM or EM fungi), are phylogenetically distant (James et al., 2006) and functionally distinct, they both play key roles in ecosystem functioning. In boreal and temperate forests, ECM are facultative symbionts that play an important role in plant nutrient acquisition (Read and Perez-Moreno 2003). In fact, ECM take up about 80% of all plant nitrogen in boreal forests (Hobbie and Hobbie 2006). However, the impact of invasive plants on the soil composition of ECM has not been well studied (Wolfe et al., 2008). The few papers that do tackle this issue have either found inhibition of ECM in the presence of a non-mycorrhizal invader (James et al., 2006; Jansa et al., 2008; Hausmann and Hawkes 2009) or suggest that an invasive plant may elicit an allelopathic effect on EM fungi (Grove et al., 2012). Similarly as with AMF, ECM associations with native plants can be inhibited by invasive plant presence (Castellano and Gorchov 2012).

Invasive plants also introduce invasive AMF and ECM into the invaded range. Very little is known about the invasion process of AMF, but we do know that AMF propagules can be transferred long distances by wind, water, and agriculture (Sieverding and Oehl 2005). When AMF is introduced to a new area, it spreads very slowly from the point of introduction, but can persist for up to a several years in the soil without a host (Sieverding and Oehl 2005). Introduction could be problematic if the AMF are generalists and associate with invasive plants; in these cases it may not have an overall negative impact on an ecosystem that already harbors AMF. Normally ECM are beneficial to plants, but they have been shown to cause damage to invaded ecosystems by competing with native fungi, facilitating in the co-invasion of trees (Hayward et al., 2015), and changing the soil foodwebs (Dickie et al., 2016). It is still unknown, however, what effect these invaders have on native host physiology and native fungal communities.

Fungal Pathogens

Soil pathogens contribute to the spatial and temporal patterns of natural systems through negative plant-soil feedbacks (van der Putten 2003) and may influence plant diversity by suppressing dominant plants (Bever et al., 1997). Certain pathogens target either a group of related plant species or only one host plant genus.

Increase in global trade and the subsequent movement of plants has increased the number of introduced plant species and the pathogens they carry (Brasier 2003). Some invasive pathogens have been introduced intentionally as biological controls (Schwartz et al., 2006), but most may be introduced inadvertently over trade routes. The fact that fungi are small and inconspicuous may be a major factor in their success as invaders and may be why these pathogens can spread faster than the host plants that carry them.

Pathogens brought over by invaders have been shown to decimate native plant populations. The lack of host resistance to invasive pathogens has caused severe environmental and agricultural damage in invaded areas (Desprez-Loustau et al., 2007). Some examples of pathogenic microbes, often studied by plant pathologists and mycologists, are *Phytophthora cinnamomi* which infects Eucalyptus trees in Australia (Weste 1981), *Armillaria luteobubalina* which has killed off 38% of plants in coastal ecosystems (Shearer 1998), *Phytophthora ramorum* which has infected more than 70 plant species in California and causes sudden oak death (Venette and Cohen 2006), and *Phytophthora kernoviae* which is the latest of many *Phytophthoras* recently found in the UK (Brasier 2003) (Figure 4).

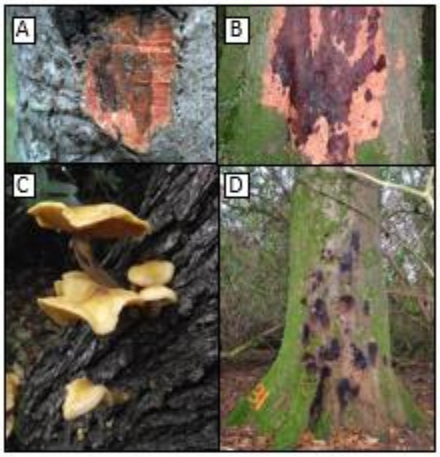


Figure 4. A) Phytophthora cinnamomi B) Phytophthora kernoviae C) Armillaria luteobubalina D) Phytophthora ramorum

Most studies concerning the spread of invasive fungal pathogens focus primarily on agricultural rather than natural systems. In a majority of papers, the invading pathogen that causes a devastating agricultural epidemic are those that coevolved with crop plants and were somehow reunited with their host (Desprez-Loustau et al., 2007). The most well-known example of this is the Irish potato famine caused by *Phytophthora infestans*. In contrast, in natural systems, most harmful invasive pathogens did not coevolve with the plants they infect so the host plants have never been exposed to the pathogen before (Desprez-Loustau et al., 2007).

The main body of research that does focus on invasive pathogens in natural systems primarily deals with invasive forest pathogens. North American forests are continually threatened by invasive pathogens and several species of trees have already been essentially eliminated by them. The best example of this is the chestnut blight which killed off most of the mature native chestnut trees in the northeast US in only 30 years (Bramble 1936), replacing them with a variety of other hardwood species. Pests and pathogens may be even more harmful to these hardwood forests than the invasive plants that carried them there (Lovett et al., 2006), with over 20 invasive pathogens infecting forests in the US and Canada (Pimentel 2002).

Invasive pathogens in forest ecosystems are currently in the process of removing several foundation tree species that control productivity, water levels, forest structure, and microclimate (Ellison et al., 2005). When an entire species of tree is wiped out or an entire life stage or size class of tree is eliminated, the forest ecosystem can change dramatically. The loss of these species can negatively impact nutrient fluxes, water movement, biodiversity, and food webs (Loo 2009). The indirect effects of these species losses are difficult to calculate and could extend for multiple forest generations. If a relatively minor tree species is lost, the impact of the invasive pathogen may actually be small, but if a keystone species is lost there could be long-lasting cascading effects (Ellison et al., 2005). It is important to note that not all introduced pathogens are harmful to these forests (Liebhold et al., 1995), but more research is needed to identify those that are harmful before they spread.

The damage wrought by invasive pathogens is clearly wide-spread. Approximately 65-85% of plant pathogens are considered invasive (Pimentel 2002). Thus, there is a critical need for invasive pathogen ecology to elucidate the extent to which invasive pathogens harm natural systems. Based on our current understanding, it is unclear whether or not invasive fungal pathogens persist in the soil for years after invasive plants have been removed or whether these pathogens interact with other microbes in the soil to the detriment of native species. In other words, more research is needed to bridge the gap between plant pathology and ecology to better understand the impact of invasive pathogens in natural systems (Desprez-Loustau et al., 2007).

Introduction of non-native pathogens is one way invasive plants influence soil pathogen composition. However, they have also been shown to influence the abundance and diversity of native fungal pathogens in invaded sites, in ways that are often either beneficial (Nijjer et al., 2007) or detrimental to their growth. Alkaloids produced by these pathogens can inhibit generalist pathogenic fungi, which inadvertently stimulates the growth of host-specific pathogens (Hol and van Veen 2002). This accumulation of pathogens specific to the invasive plant may actually allow native plants to thrive (van de Voorde et al., 2011). Some studies have shown that certain invasive grasses may produce chemicals which are said to have an inhibitory effect on competitors and may deter herbivory or either repel pathogens (Liebhold et al., 1995; Richardson et al., 2000). In contrast, some invasive plants may release chemicals known to attract pathogens (Accumulation of Local Pathogens Hypothesis) (Eppinga et al., 2006), which could act as a "pathogen reservoir," leading to reduced competition by local plants (Day et al., 2016).

Restoration Efforts to Reverse Biotic Changes

Plant communities are dependent upon soil microbial communities, therefore, native plant restorations may ultimately not be successful unless the microbial and plant communities are simultaneously restored. The idea of using microbes, either a component of the native-plant associated microbial community or an entire whole soil inoculum isolated from an intact ecosystem, as a biological control against the spread of invasive species has gained popularity in recent years.

Restoration ecologists are now applying AMF cultures (Zubek et al., 2016), whole native soil, or biological crust to their restorations in hopes of augmenting native plant establishment (Figure 5). Addition of native soil to restoration sites has been found in some studies to decrease invasive plant cover and increase the native plant cover (Rowe et al., 2009). It is important to compare the methods of these types of studies to understand what inoculation method is most successful for combatting a particular invader (Belnap et al., 2005; Stinson et al., 2006; van de Voorde et al., 2011). Some studies, for example, remove the invasive plant before applying soil inoculum to restore soil fungal abundance (Hamman and Hawkes 2013) or combine fertilizer with the inoculum. Microbial soil inoculations have been found to actually inhibit the allelochemical effects of an invasive plant on a native plant species (Mishra et al., 2012).

Other possible means of managing invasive plants at the microbial level include the addition of sugar, sawdust, or activated charcoal to soils. Sugar and sawdust can increase microbial growth and store excess soil nitrogen from invasive plants in the microbial biomass (Szili-Kovaks et al., 2007) (Figure 5). This method has been successful for some invasive sites, but not all (Eviner et al., 2010). Soil additions of activated carbon are believed to bind invasive plant allelochemicals and remove them from the soil solution (Lankau 2010). Because allelochemicals are short-lived, this technique is most useful if the invasive plant is still present in a site (Eviner et al., 2010). Studies have shown that native plant growth increases with the addition of activated charcoal under invasion by spotted knapweed (*Centaurea maculosa*), diffuse knapweed (*Centaurea diffusa*), and cheatgrass (*Bromus tectorum*) (Eviner et al., 2010). Activated carbon can have numerous other effects on the soil (binding organic substrates, changing soil nitrogen concentration, and changing the carbon-to-nitrogen ratio), (Eviner et al., 2010) so further research is needed to decouple these effects with the aforementioned binding of allelochemicals.

Restoration of Arbuscular Mycorrhizal Fungi and Ectomycorrhizal Fungi

Invasion by non-mycorrhizal plants can sometimes reduce the abundance of AMF in the soil, negatively impacting native plants that are dependent on AMF for survival. A decrease in AMF abundance can encourage further invasion by non-mycorrhizal plants, thus maintaining invasive plant dominance and inhibiting native plant growth (Reinhart and Callaway 2006). This is of special concern considering many other studies have found invasive species that are less dependent than native plants on AMF (Ehrenfeld 2011; Elgersma and Hock 2012; Mishra et al., 2012; Requena et al., 1997; Rowe et al., 2009). In situations where the invader is known to be non-mycorrhizal, restoration strategies that increase the soil AMF abundance could be especially effective combined with native plant seeding and planting AM host plants. AMF addition to soil has been useful in some restorations efforts (Koziol and Bever 2016), but not all (Aprahamian et al., 2016). In some cases, when an invaded site has sufficient AM propagule pressure, adding additional AMF may not have any effect on AM abundance or native plant performance (Lankau 2010). Furthermore, a singular increase in AMF abundance may not be sufficient for restoring native plant diversity, but rather an increase in AMF diversity along with augmenting specialist AMF propagule pressure may improve restoration outcomes (Bever et al., 2001).

Although some land managers consider the co-invasion of ectomycorrhizal fungi to be a threat to native communities, as of yet there have been minimal evidence-based management strategies documented by practitioners (Dickie et al., 2016). Removal of plants that associate with ectomycorrhizal fungi, chemical sprays, and sporocarp removal have been performed, but the success of these strategies are debatable. Picking mushroom caps has been shown to have little impact on invasive fungal populations (Egli et al., 2006), but this may be because studies have focused mainly on fungi that are neither short-lived nor reproduce sexually (Dickie et al., 2016). Fungicide is another option, but it may also damage native fungi, thereby doing more harm than good to native plants.

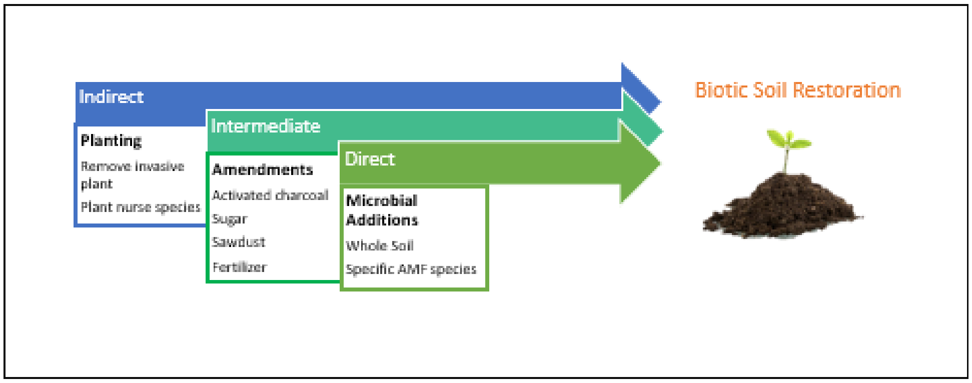


Figure 5. Diagram showing the indirect, intermediate, and direct methods of biotic soil restoration. Indirect methods include removing invasive plants and planting nurse species. Intermediate methods include soil amendments such as activated charcoal, sugar, sawdust, and fertilizer. Direct methods include the addition of whole soil or specific AMF species to the soil.

Fungal Pathogens and Implications for Restoration

Very few papers recommend restoration strategies for mitigating the effects of invasive pathogens (Dickie et al., 2016) and even less recommend strategies for preventing the accumulation of pathogens by invasive plants. Most restoration strategies for combatting pathogens are primarily focused on agriculture, not natural systems, and those that do cover natural systems focus primarily on hardwood forests. Restoration of chestnut trees has been extensively studied in the wake of the aforementioned chestnut blight fungus. Recommended strategies include planting blight resistant trees (Jacobs 2007), creating strains of blight fungus that are less virulent (Milgroom and Cortesi 2004), and crossbreeding trees (Pliura et al., 2011), such as naturally resistant Asian chestnut trees and American chestnuts. Although many papers focus on gene manipulation as a restoration strategy, others suggest more large-scale strategies such as maintaining tree stand structure, maintaining healthy and resistant tree species, and timber extraction

(Waring and Hara 2005). Some researchers recommend inoculating specific ectomycorrhizal fungi to boost the vigor of infected trees (Dulmer et al., 2014). Blight fungus and hardwood tree infections could potentially be used to guide further research about ecological restoration in other natural systems ravaged by invasive pathogens.

The most successful strategy for combatting invasive fungi is to prevent them from being introduced in the first place. This could involve either banning plants that associate with known invasive fungi or by preventing nurseries from inoculating their plants with invasive fungi (Hayward et al., 2015).

Conclusion

Arbuscular and ectomycorrhizal fungi play important roles in the nutrient acquisition and maintenance of biodiversity. Evidence concerning the impact of invasive plants on these fungal groups has been mixed, with AMF occasionally illustrating an increase in abundance (Bozzolo and Lipson 2013; van der Putten et al., 2007; Vogelsang et al., 2004), a decrease in abundance (Busby 2011), or a shift from specialist to generalist AM taxa (Allen 1991; Bunn et al., 2015). These conflicting results underscores the importance of future research on the response of AMF to invasion and invasive plant management, with an emphasis on the role of factors driving their response, such as invasive plant functional group (Bunn et al., 2015).

Although little is known about ECM, highlighting a need for future investigation, the evidence suggests that both non-mycorrhizal invasive plants (Hausmann and Hawkes 2009; James et al., 2006; Jansa et al., 2008) and allelopathic invasive plants (Grove et al., 2012) may inhibit their EM fungal growth, which may interfere with plant nutrient acquisition in both boreal and temperate forests. The introduction of harmful invasive ECM which facilitate in the co-invasion of trees may further disrupt forest symbioses (Dickie et al. 2016).

Invasive plant encroachment into ecosystems have unintended consequences for microbial pathogens, such as influencing the abundance and diversity of native fungal pathogens in ways that benefit their growth or harm native plants. Indeed, invasive plants alter soil fungal composition (Jacobs 2007; Nijjer et al., 2007; Pimental 2002). The spread of invasive pathogens by invasive species has been widely covered in agricultural research. Future research should focus on invasive pathogens that are being transported by an invasive host plant to natural systems other than hardwood forests. A majority of studies focused on natural systems, emphasize primarily hardwood forests and the loss of foundation tree species. The loss of these foundation species has impacted nutrient fluxes, water movement, biodiversity, and food webs of infected forests (Loo 2009). These sorts of large-scale changes could have cascading effects that last for many generations.

Conflicting results and a lack of microbial data has led to case-dependent, anecdotal restoration recommendations. The results of inoculation experiments are very encouraging for improving restoration efforts. However, it may be particularly useful in the future to evaluate exactly how the microbial composition changes for each invasive plant, especially at the species level or for plant functional groups.

While more restoration ecologists are making decisions based on important microbial-plant mutualisms, much more information is needed concerning the long-term impact of invasion on fungi, especially mycorrhizal fungi and fungal pathogens.

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CHAPTER 2

Legacy Effects of Invasive Grass Impact Soil Microbes and Native Shrub Growth

Abstract

In California, invasive grasses have displaced native plants, transforming much of the endemic coastal sage scrub (CSS) to nonnative grasslands. This has occurred for several reasons including increased competitive ability of invasive grasses and long-term alterations to the soil environment, called legacy effects. Despite the magnitude of this problem, however, it is not well understood how these legacy effects have altered the soil microbial community and, indirectly, native plant restoration. We assessed the microbial composition of soils collected from an uninvaded coastal sage scrub (CSS) community (uninvaded soil) and a nearby 10ha site from which the invasive grass, Harding grass (*Phalaris aquatica* L.), was removed after 11 years of growth (post-invasive soil). We also measured the germination rate, biomass, and length of three CSS species and Harding grass grown in both soil types (uninvaded and post-invasive). Our findings indicate that Harding grass may create microbial legacy effects in the soil that likely cause soil conditions inhibitory to the germination rate, biomass, and length of California sagebrush, but not the other two native plant species. Specifically, California sagebrush growth was lower in the post-invasive soil, which had more Bacteroidetes, Proteobacteria, *Agrobacterium*, *Bradyrhizobium*, *Rhizobium* (*R. leguminosarum*), *Candidatus koribacter, C. solibacter*, and Rhizophilic AMF, and less Planctomycetes, Acidobacteria, *Nitrospira*, and *Rubrobacter* compared to the uninvaded soil. Shifts in soil microbial community composition such as these can have important implications for restoration strategies in post-invasive sites.

Key Words: coastal sage scrub, invasive species, Harding grass, restoration, soil microbial community.

Management Implications

Microbial and plant communities are interdependent, therefore reassembly of one component may be limited by reestablishment of the other component. (Lankau et al. 2014). Thus, restoration success may be limited unless both the microbial and plant communities are restored. Previously, most suggestions for improved restorations have been context dependent and invasive plant removal and site preparation methods largely consisted of burning, grazing, herbicide, or manual weed pulling, without concern for the belowground communities. Furthermore, little emphasis was placed on the underlying mechanisms which may have led to invasion (Eviner et al. 2010). Now we understand that legacy effects of invasive species can last for decades or centuries (Belnap et al. 2005; Eviner et al. 2010), sometimes impacting the growth of native plants. If these legacy effects are microbial, they will require novel restoration strategies to repair the damage done to native plant communities.

Most restoration strategies to ameliorate biotic legacy effects consist of invasive removal, planting natives, soil amendments, removing topsoil, and microbial inoculation. However, these strategies have all had inconsistent results (Blumenthal et al. 2003; Walker et al. 2007), possibly because the restoration strategy was not tailored to the specific invasion. It may be important to first identify the legacy effects caused by a particular invasive plant before attempting restoration. In regard to this study, the changes in soil microbial composition brought on by Harding grass (*Phalaris aquatica* L.) indicate that it may be important to restore the soil to a microbial community composition resembling that of the uninvaded soil. This could be accomplished by simply inoculating the native plants with uninvaded soil before transplanting seedlings into the post-invasive site. Instead of just planting native species in a post-invasion site and hoping establishment will occur, alterations to soil microbial composition could ensure native plant establishment and longevity. It may also be possible to use plants such as Coyote brush (*Baccharis pilularis* DC.) and Purple sage (*Salvia leucophylla* E. Greene) as nurse species since their germination and growth were unaffected by the post-invasive soil.

Introduction

In the United States there are now over 300 rangeland invasive plants that cost about $2 billion annually to treat (Ditomaso 2000). Many of these weeds poison animals, increase the cost of raising livestock, alter sensitive habitat, decrease land value, deplete resources, and reduce plant diversity (Ditomaso 2000; Eviner et al. 2010). In California, historical ranching and agriculture, anthropogenic nitrogen deposition, and invasion have transformed much of the landscape from coastal sage scrub (CSS) to nonnative grassland (Bowler 2000), decreasing shrub cover by 90% (Westman 1981). Coastal sage scrub is characterized by low-growing shrubs in coastal and inland California and northwest coastal Baja California. This habitat type is a hotspot of endemic species, 100 of which are proposed for or under protection (Rubinoff 2001). A number of rare and endangered species, such as the California Gnatcatcher (*Polioptila californica*) (Rubinoff 2001) and Palos Verdes blue butterfly (*Glaucopsyche lygdamus palosverdesensis*) rely on CSS for survival. The cover of an important host species for the endangered California Gnatcatcher, California sagebrush (*Artemisia californica* Less.), has also decreased from 17.7% to 6.1% in the last 62 years (Antonio and Vitousek 1992). Despite the importance of CSS and threats to it, research in this system has been sparse (Lowry et al. 2013). Due to the magnitude of invasion and shrinking of CSS, it is important to better understand the extent of ecosystem impacts caused by invasive plants and particularly invasive grasses.

Invasive grasses can alter water flow, soil quality, pH (Kourtev et al. 2003), carbon storage (Eviner et al. 2010), inputs of N and other elements (Ehrenfeld 2003), and organic matter (Saggar et al. 1999) in soils. This, along with increased competitive ability, allow invasive grasses to replace native species and dominate the landscape. However, the impacts of invasive plants on soil microbial communities, and associated indirect impacts on native plant communities are less well understood.

Microbes interact with plant species as mutualists, pathogens, decomposers of organic matter, and critical facilitators of the carbon and nitrogen cycles (Ehrenfeld et al. 2005). Alterations to the soil microbial community can be induced by variations in the carbon:nitrogen ratio of plant litter, which can cause dramatic shifts between fungal and bacterial dominant communities in the soil (Dickens et al. 2013). Invasive plants can also alter microbial communities through the production of root exudates (Bais et al. 2006), or by directly introducing translocated microbes (Vellinga et al. 2009).

These changes in the microbial composition of the soil brought on by invasive plants can indirectly affect native plant growth (Bever et al. 2010; Cuddington 2011; Dickens et al. 2013; Eviner et al. 2010; Hawkes et al. 2006; Jordan et al. 2008; Mangla and Callaway 2008) sometimes in negative ways (Mangla and Callaway 2008). Alteration of important nutrient cycles (Liao et al. 2008), changing of soil food webs, or inhibition through introduction of plant pathogens (Belnap et al. 2005; Jordan et al. 2008) can cause native plant cover to decline. However, studies focused on understanding the indirect effects of invasive plant growth on native plants often have conflicting results, largely dependent upon the native and invasive species studied (Bozzolo and Lipson 2013), the length of invasion, invader cover, and site specifics (Eviner et al. 2010). For these reasons, it may be important to understand how key invasive grasses affect natives in context-dependent ways (Eviner et al. 2010).

Invasive grasses can outcompete native plants in three different ways: the accumulation of local pathogens hypothesis, the enhanced mutualism hypothesis, and soil priming. The accumulation of local pathogens hypothesis states that invasive plants recruit local pathogens, resulting in exclusion of native plant species (Eppinga et al. 2006), while the enhanced mutualism hypothesis states that invasive plants thrive by forming stronger mutualisms in the invaded range (Reinhart and Callaway 2006). Priority effects occur when one species begins growing in a location before another species (Dickson et al. 2012), sometimes leading to alternative stable states (Suding et al. 2004). Evidence for priority effects occur in the findings of several studies that have shown invasive species regularly begin growth before native species (Wainwright et al. 2012), colonize disturbances, and grow quickly in the absence of other individuals (Parendes and Jones 2000).

Thousands of acres in private and public CSS lands are invaded by Harding grass (*Phalaris aquatica* L.), a highly aggressive (Tran and Cavagnaro 2010), perennial, mycorrhizal (Asghari and Cavagnaro 2011), deep-rooted bunchgrass from the Mediterranean (Ditomaso 2000). It forms large monocultures in both riparian and upland systems of the invaded range. In the United States, it is found most commonly in the coastal valleys, foothills, and along roadsides from Oregon, to California, and grows best in highfertility conditions (Harrington and Lanini, 2000). It is a favored pasture grass because it is drought tolerant, able to persist in a wide variety of soils, and does well under heavy grazing (Langer 1990). Harding grass is known to outcompete and displace native plants and land managers have struggled with establishing native plants in Harding grass removal areas. Despite the prevalence and invasiveness of Harding grass in CSS, little is known about its impact on native CSS plants, as well as soil microbes and abiotic properties.

To better understand the long-term effects of invasive grasses on native plants and associated soils, we monitored the performance of native and invasive plants grown in soils previously invaded by Harding grass (hereafter post-invasive soil), as compared to uninvaded soil, and characterized soil microbial communities in these two soil types. We hypothesized that (1) native plants would have a slower germination rate and smaller size in the post-invasive soil relative to the uninvaded soil, (2) the microbial community composition of the uninvaded soil would differ from the post-invasive soil, due to differences in plant community assemblage, and (3) changes in soils associated with Harding grass would retain conditions inhibitory to native plant conditions: a legacy effect of invasion.

Materials and Methods

Greenhouse Experiment

In order to test our first hypothesis, we designed a greenhouse experiment where we grew three native shrub species and Harding grass in soils that had been conditioned in the field by native plants versus soils that had been conditioned in the field by the invasive grass. We collected soils from Rancho Sierra Vista (RSV), a park unit of the Santa Monica Mountains National Recreation Area. RSV is a lowland site in Southern California that was originally CSS, but has a long history of ranching and agriculture that have transformed much of the landscape to nonnative grassland. The soil series is Mipolomol consisting of loamy, mixed, superactive, thermic, shallow Entic Haploxerolls (US Department of Agriculture). The first recorded siting of Harding grass in RSV occurred in November 2002 (Calflora, 2014). Records of the site since the 1950's show that the site was open, dry field agriculture, with the Harding grass being planted for livestock fodder in the 1970's before the Santa Monica Mountains National Recreation Area was established in 1978. The invasive grass spread quickly as nothing would eat it and control efforts did not begin until the late 1990's-early 2000. Harding grass rapidly expanded to approximately 10ha in RSV (34°09'10.3"N, 118°57'08.2"W), forming a complete monoculture. The invasive grass was fully established in the 10ha site, reaching ~1.1m in height, while the native plants displaced in this site were also fully established and reached ~1.2m in height. Removal of Harding grass by park managers began in 2006, using a combination of mowing and herbicide application (glyphosate). By 2013, the Harding grass infestation was reduced to routine maintenance levels and in several areas eradicated. Although this post-treatment area of Harding grass was surrounded by intact CSS to provide ample propagules, native plant recruitment was virtually nonexistent several years later. Park managers were concerned that traditional restoration strategies, in which the native plant seedlings were out-planted in large numbers, might prove ineffective in restoring the native plant population if there were underlying unfavorable soil conditions.

The greenhouse experiment was arranged as a full factorial design using native shrubs California sagebrush (*Artemisia californica* Less.), Purple sage (*Salvia leucophylla* E. Greene), and Coyote brush (*Baccharis pilularis* DC.) and the invasive grass, Harding grass with soil source, either uninvaded or post-invasive. All four species are mycorrhizal and non-leguminous. The native species were chosen because they are dominant species in CSS that continue to grow in the uninvaded soils surrounding the post-invasive site. In October 2013, for each of the two treatments (uninvaded or post-invasive) 1000 seeds of each species were divided among 10 pots per species for a total of 40 pots per treatment. Forty pots were filled with uninvaded soil and the other 40 with post-invasive soil. We placed trays containing the plant pots in random locations throughout the ~14m^2 bench space in the back of the greenhouse. We made sure that the treatments were separated by at least 1m to prevent cross-contamination during watering.

Uninvaded soil was defined as soil in which intact CSS had historically grown uninterrupted, while post-invasive soil was defined as soil in which Harding grass had grown for 11 years (2002-2013) or more. The post-invasive soil was collected at ~10 randomly selected locations across the 10ha post-invasive site at RSV and homogenized. Uninvaded soil was collected directly around the 10ha post-invasive site where the native plants used in this study were prevalent. Approximately 19L of soil from each site was collected from the top 15cm of soil with sterilized shovels. Uninvaded soils were collected approximately 1m away from intact native plants, while post-invasive soils were collected at least 10m away from any vegetation.

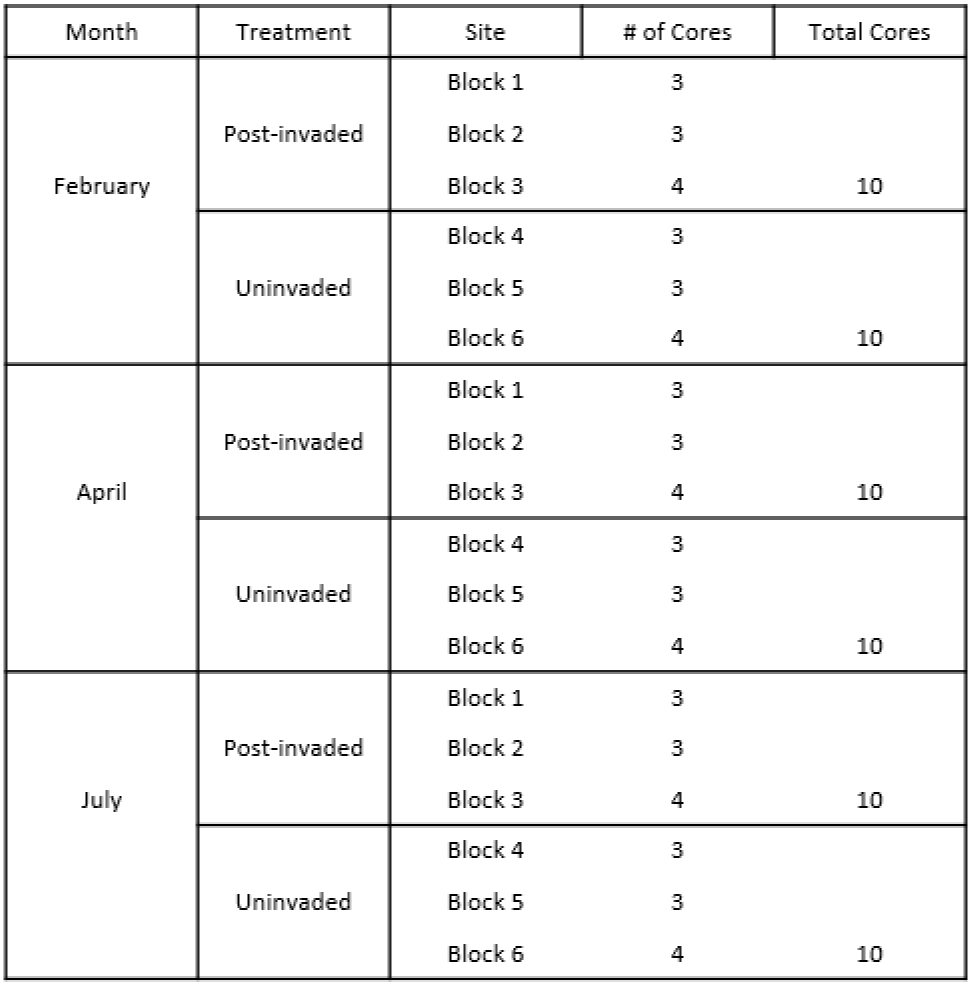
Immediately after seeding, the pots were watered three times a week to field capacity with regular tap water and stored in an open lath house structure under uniform light exposure at the park's nursery at RSV. The lath house is a large wooden construction on the National Park Service grounds in RSV. It has a corrugated metal ceiling and wooden walls that give way to netting halfway up, allowing a constant flow-through of air and natural lighting. The plants grew in the greenhouse for seven months, prior to destructive sampling at the conclusion of this experiment.

We harvested plants on May 24, 2013. All plants from each pot were harvested, for a total of 461 plants and an average number of ~ 6 plants per pot. At harvest, the entire plant was carefully and slowly excavated from the soil with soil knives and repurposed smooth wooden sticks to ensure minimal root loss, after which the shoot was separated from the root with shears. The length data was recorded, then the shoots and roots were dried, cleaned, coiled, and stored at room temperature in the laboratory at University of California, Riverside (UCR). The germination rates, number of individuals, and dried seedling biomass were recorded. Germination rate was calculated as (# of seedlings per pot/100 seeds). For all plants we recorded the length and biomass of the dried root and shoot separately.

Field Sampling for Nitrogen and Microbial Composition

In order to test our second hypothesis, to determine if Harding grass may have altered the soil microbial community, soil cores were collected in and around the 10ha post-invasive site (34°09’10.3”N, 118°57’08.2”W). Uninvaded soils were collected directly under the drip-line of native plants, while post-invasive soils were collected at least 10m away from any vegetation in the 10ha post-invasive site. Soil samples were taken as 5cm deep soil cores with a sterile corer. The site was split into three blocks as was the surrounding intact sage scrub (Figure 1). At total of 60 soil cores were collected over three months (Table 1). On February 10, 2014 and April 15, 2014, about a year after the plants were harvested in the greenhouse, a total of 20 soil cores per month were collected with 10 soil cores collected in the uninvaded blocks and 10 soil cores collected in the post-invasive blocks. For post-invasive soil, three cores were collected in Block 1, three were collected in Block 2, and four were collected in Block 3 (an extra sample was collected from Block 3 in case of error) for a total of 10 cores. For uninvaded soil, three cores were collected in Block 4, three in Block 5, and four in Block 6 (Figure 1). Soil cores were classified as either “uninvaded” or “post-invasive” soil samples. The samples were placed directly on dry ice in the field and stored at -20°C at UCR until analysis.

Table 1. Source and number of soil cores collected during February, April, and July 2014.



Month	Treatment	Site	# of Cores	Total Cores
February	Post-invaded	Block 1	3	
		Block 2	3	
		Block 3	4	10
	Uninvaded	Block 4	3	
		Block 5	3	
		Block 6	4	10
April	Post-invaded	Block 1	3	
		Block 2	3	
		Block 3	4	10
	Uninvaded	Block 4	3	
		Block 5	3	
		Block 6	4	10
July	Post-invaded	Block 1	3	
		Block 2	3	
		Block 3	4	10
	Uninvaded	Block 4	3	
		Block 5	3	
		Block 6	4	10

Figure 1. Aerial view of the 10ha site from which Harding grass (Phalaris aquatica) was removed (green rectangle). The three red sites represent blocks from which post-invasive soil was collected, while the three

blue sites represent blocks from which the uninvaded soil was collected. Sampling locations were randomly chosen within blocks.

Soil cores for nitrogen analysis were collected on July 23, 2014. A total of 20 soil cores were collected with 10 soil cores collected in the uninvaded blocks and 10 soil cores collected in the post-invasive blocks. For post-invasive soil, three cores were collected in Block 1, three were collected in Block 2, and four were collected in Block 3 (an extra sample was collected from Block 3 in case of error) for a total of 10 cores. For uninvaded soil, three cores were collected in Block 4, three in Block 5, and four in Block 6. They were placed on dry ice in the field and transported to UCR.

Soil Assays

Nitrogen extraction was performed on the same day as soil collection: 10g of soil were combined with 40ml of 2M KCl, placed on a shaker table for 1h at 200rpm, allowed to settle for 1h, gravity filtered through a filter (with a new filter for each sample), collected in a plastic vial, and frozen until analysis. Ammonium analysis followed Weatherburn (1967) and nitrate analysis followed Doane and Horwath (2003). For ammonium analysis, 80ul of sample were mixed with 60ul of salicylate solution and 60ul of bleach solution, then read on a microplate reader at 650nm. For the nitrate analysis, 100ul of sample were combined with 100ul of reagent solution (50ml vanadium chloride solution, 3.3ml sulfanilamide solution, 3.3ml *N*-(1-Naphthyl)ethylenediamine (NED) solution, and 400ml DI water) and read on a microplate reader at 540nm. Two technical replicates were run for each sample.

Five uninvaded soil samples and five post-invasive soil samples collected in July were dried at 60°C for 48h. About 70mg per sample were weighed into small tin cups and folded carefully into pellets. The samples were then run on a FLASH 2000 elemental analyzer (Genecraft Labs, Jakarta, Indonesia) to determine the total C and N concentration. Soil moisture was determined by weighing out about 8g of the same 10 samples in tins and recording the weight before and after drying at 60°C for 48h. A pH meter (Thermo Fisher Scientific, Wilmington, DE, USA) was used to take the pH of the same 10 samples.

DNA Extraction, Quantification, and Barcoded Amplicon Sequencing of 16S

The 16S ribosomal RNA gene (16S rRNA) V3 and V4 regions were analyzed to classify the diversity of bacteria in the soil. Microbial DNA was extracted using a MO BIO PowerSoil DNA Isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's instructions and using a PowerLyzer 24 bench top bead-based homogenizer (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). A NanoDrop 2000/2000c UV-Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) was used to quantify the DNA in soil extracts. PCR for bacteria and archaea was performed using primers that target the 16S V3 and V4 regions (S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21; Klindworth et al., 2012) of the 16S rRNA gene. Microbial genomic DNA (2.5ul) was combined with forward and reverse primer (5ul each), and 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, Massachusetts, USA) (12.5ul). A Bio-Rad

MJ Research PTC 200 Thermocycler was used to amplify 96 samples at a time with the following program: 95°C for 3 min, 25 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 5 min, and hold at 4°C. AMPure XP beads (Beckman Coulter Genomics, Danvers, Massachusetts, USA) were used to purify the 16S amplicon without primer and primer dimer sequences. Dual indices and Illumina sequencing adapters were attached to the amplicon using the Nextera XT Index Kit (Illumina, San Diego, California, USA). Amplicon DNA (5ul) was combined with 2x KAPA HiFi HotStart ReadyMix (25ul), Index 1 and 2 primers (5ul each), and PCR grade water (10ul). The same thermocycler was used with the following program: 95°C for 3 min, eight cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, 72°C for 5 min, and hold at 4°C. A second bead cleanup was used to purify the final library before quantification. The samples were verified with gel electrophoresis after every step. The samples were quantified in duplicate using the Quant-iT PicoGreen dsDNA assay kit (Life Technologies, Grand Island, New York, USA). All samples were pooled together in equimolar concentrations then sequenced with an Illumina MiSeq instrument at UCR.

DNA Extraction, Quantification, and Barcoded Amplicon Sequencing of AMF

The SSU rRNA gene was analyzed to classify the diversity of mycorrhizae in the soil. Microbial DNA was extracted using a MO BIO PowerSoil DNA Isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's instructions and using a PowerLyzer 24 bench top bead-based homogenizer (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). A NanoDrop 2000/2000c UV-Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) was used to quantify the DNA in soil extracts. PCR for fungi was performed using the primer pairs WANDA-AML2. Microbial genomic DNA (1 μL) was combined with forward and reverse primer (5 μL each), and Phusion DNA Polymerase (Thermo Fisher Scientific, Wilmington, DE, USA) (12.5ul). A Bio-Rad MJ Research PTC 200 Thermocycler was used to amplify 96 samples at a time with the following program: 95°C for 2 min, 40 cycles of 95°C for 30 s, 60°C for 1 min, 68°C for 1 min, and hold at 10°C. AMPure XP beads (Beckman Coulter Genomics, Danvers, Massachusetts, USA) were used to purify the extracts and amplicon. Dual indices and Illumina sequencing adapters were attached to the amplicon. Diluted (1:10) amplicon DNA (1 μL) was combined with Phusion (12.5 μL), Index 1 and 2 primers (2.5 μL each), BSA (0.1 μL) and PCR grade water (6.4 μL). The same thermocycler was used with the following program: 95°C for 2 min, 15 cycles of 95°C for 10 s, 55°C for 30 s, 72°C for 30 s, and hold at 10°C. A second bead cleanup was used to purify the final library before quantification. The samples were verified with gel electrophoresis after every step. The samples were quantified in duplicate using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Wilmington, DE, USA). All samples were pooled together in equimolar concentrations then sequenced with an Illumina MiSeq instrument.

Data Analysis

To analyze the plant length and biomass data, we used JMP13 statistical software (JMP, Version 13. SAS Institute Inc., Cary, NC, 1989-2007) to perform a 1-Way analysis of variance (ANOVA) with the factor: soil type (uninvaded or post-invasive) separated by species (California sagebrush, Coyote brush, Purple sage, and Harding grass). Plant root and shoot lengths and root and shoot biomass are not reported in separate figures because they show the same trend as the mean length and biomass graphs.

Quantitative Insights into Microbial Ecology (QIIME; Kuczynski et al., 2012) was used to quality filter the 16S sequences and determine taxonomic identity against the Greengenes reference databases using 97% similarity. Analysis of similarity (ANOSIM) was performed in QIIME using a Unifrac index to statistically compare community similarity among treatments. We performed alpha diversity analyses and generated PCoA plots using QIIME. Beta diversity analyses were performed using MicrobiomeAnalyst (Dhariwal et al. 2017). To analyze the abundance of certain taxa in the soil samples, we used JMP13 to perform a two-way ANOVA with factors soil type (uninvaded or post-invasive), month (April or February), and soil*month at the phylum, class, order, family, and genus levels. We used the vegan (Oksanen et al. 2018) and ggpubr (Kassambara 2018) packages in R to perform a PERMANOVA and generate figures, respectively.

For SSU, we used smalt (http://www.sanger.ac.uk/science/tools/smalt-0) to remove PhiX contamination and cutadapt (Martin 2011) to filter sequences. We used the forward read and checked quality with FastQC (Andrews S. 2010). Demultiplexing was performed in QIIME 1.9.1 and taxonomy was assigned using BLAST against the MaarjAM database (Öpik et al. 2010). We assigned families of Glomeromycotina to AMF functional groups: rhizophilic, edaphophilic, and ancestral using AMF resource allocation. The guild approach of classifying AMF is outlined in Weber *et al.*, (2018) and organizes AMF by biomass allocation: edaphophilic AMF have high allocation to extra-radical hyphae, rhizophilic AMF have high allocation to root colonization, and ancestral AMF have lower allocation to root colonization and soil hyphae than the other two groups (Table 2). The rhizophilic guild may protect plant roots from pathogens (Sikes et al. 2010; Treseder et al. 2018), while the edaphophilic guild improves plant nutrient uptake.

Table 2. AMF hyphal functional scheme as described in (Weber *et al.*, 2018). 1. Powell et al. 2009); 2. (Hart and Reader 2002); 3. (Varela-Cervero et al. 2015); 4. (Varela-Cervero et al. 2016a); 5. (Varela-Cervero et al. 2016b).

Functional Group	Families
Rhizophilic	Glomeraceae^{1,2,3,4,5} Claroideoglomeraceae^{1} Paraglomeraceae
Edaphophilic	Gigasporaceae^{1,2,5} Diversisporaceae^{1,2,5}
Ancestral	Archaeosporaceae Ambisporaceae Acaulosporaceae^{1,2,5}

	Pacisporaceae

Results and Discussion

We found significant differences in plant growth and the composition of microbial taxa between uninvaded soils and soils from which invasive Harding grass had been removed after thriving for many years. These changes in soils associated with Harding grass are a legacy effect of invasion.

Soil Chemical Properties

The amount of nitrate was higher in post-invasive soil (*p*<0.001), while ammonium did not differ between soil types. The uninvaded soil had an average of 1.64% C and 0.17% N, while post-invasive soil had an average of 2.58% C and 0.22% N. The total N and total C concentration was significantly higher (*p*<0.05) in the post-invasive soil, while the C:N ratio did not differ between the two soil types (*p*>0.05) (Figure 2). Both soil types had similar levels of moisture (*p*>0.05) and pH (*p*>0.05).

The higher amount of total C may be a result of Harding grass roots left in the soil after invasive plant die-off. These large root networks are decomposed by soil microbes over time, releasing carbon into the soil.

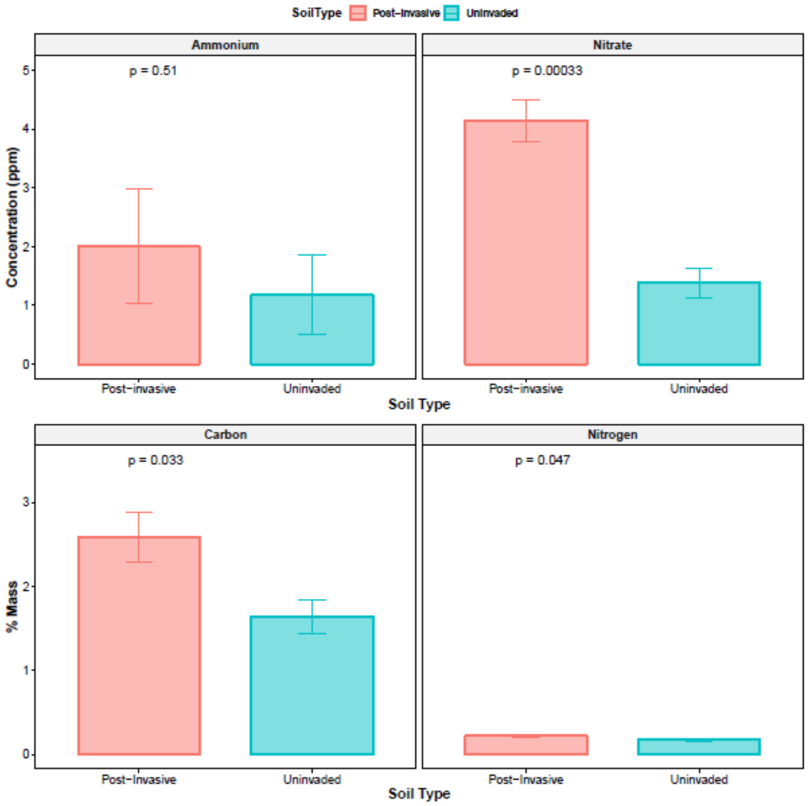


Figure 2. The ammonium concentration (ppm), nitrate concentration (ppm), total N (% mass), and total C (% mass) (clockwise) of post-invasive (red) and uninvaded (blue) soil in July 2014. Standard errors are represented in the figure by the error bars attached to each column.

Bacterial Composition

The total bacterial composition (Figure 3) and richness, as measured with alpha diversity metrics, of the uninvaded and post-invasive soils did not differ ($p > 0.05$). We did, however, find differences in relative abundance at various taxonomic levels between the soils.

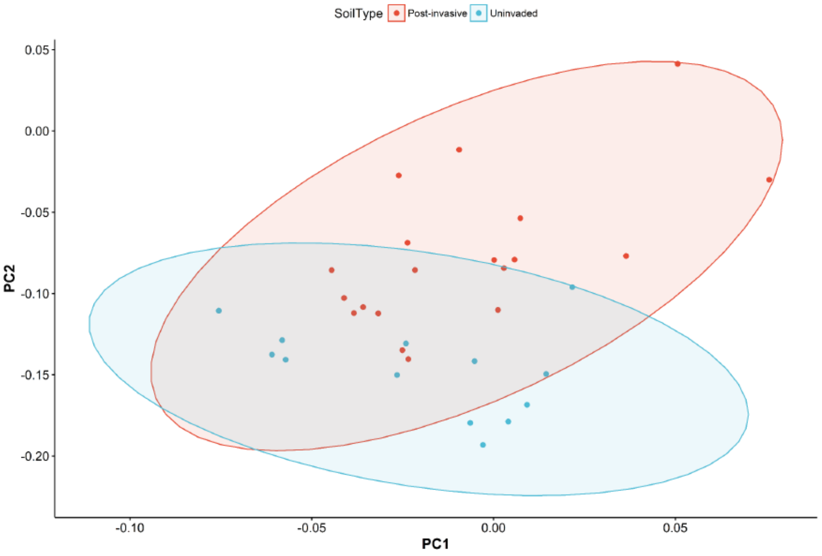


Figure 3. Principal coordinates analysis based on the weighted Unifrac distance metric for all bacterial taxa. Ellipses represent standard deviations of the weighted average of treatments at the 95% confidence level.

Seven phyla dominated all samples, with Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Planctomycetes, Proteobacteria, and Verrucomicrobia accounting for ~94% (range of 89-96%) of sequences in each sample. Thirty-three additional phyla were present but not dominant in both soils types and of those non-dominant phyla, 21 were present but not consistent across all soils. There were significant differences in the relative abundance of certain taxa between uninvaded and post-invasive soil: in post-invasive soil, there was relatively more Bacteroidetes ($p<0.0001$) and Proteobacteria ($p<0.001$) than uninvaded soil, and a lower amount of Planctomycetes ($p<0.05$) and Acidobacteria ($p<0.01$) (Figure 4).

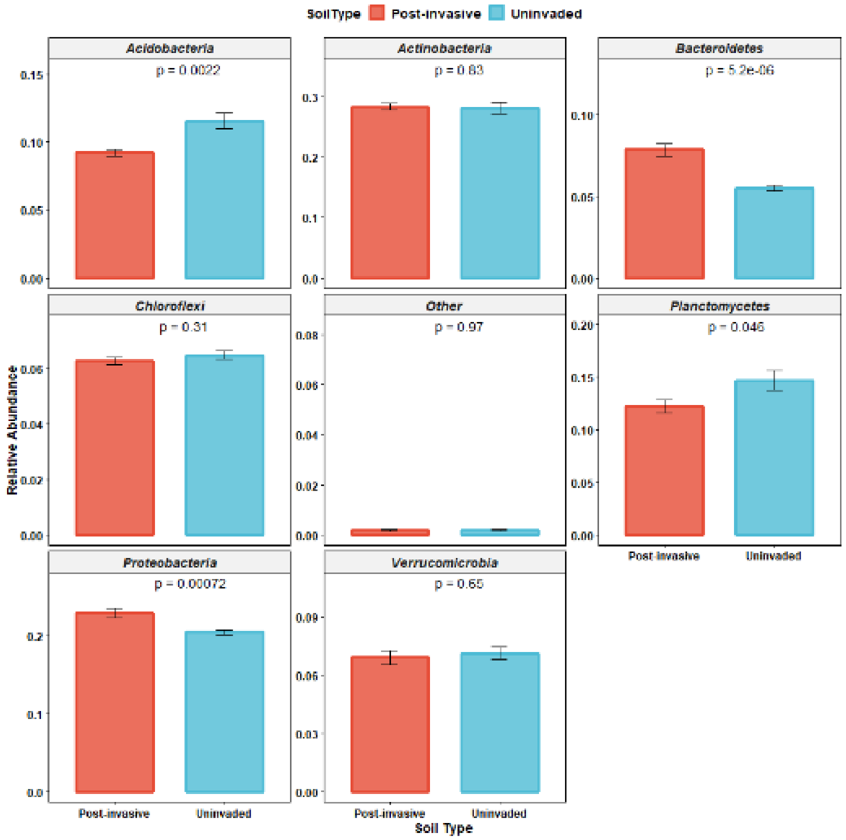


Figure 4. Relative abundances of the dominant bacterial phyla (Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Planctomycetes, Proteobacteria, Verrucomicrobia) within and across soil types. “Other” indicates the combined relative sequence abundance of the additional, rare phyla.

A total of 149 orders were present in all soils, with just 12 orders together accounting for more than half of the observed sequences in each sample: Actinomycetales, Burkholderiales, Chthoniobacterales, Gaiellales, Gemmatales, Rhizobiales, Rhodospirillales, Rubrobacterales, Saprospirales, Solibacterales, Solirubrobacterales, and Sphingomonadales accounted for ~52% (range 46-58%) of sequences in each sample. Of these, Actinomycetales (Actinobacteria), Rhizobiales (Proteobacteria), and Rubrobacterales (Actinobacteria) were most abundant and accounted for ~21% of sequences in each sample. The relative abundance of certain orders differed between uninvaded and post-invasive soil: in post-invasive soil there were more Actinomycetales (p<0.0001), Burkholderiales (p<0.0001) and Sphingomonadales (p<0.05); whereas in uninvaded soil, there was more Rubrobacterales (p<0.001).

The *Rubrobacter* genus was the most abundant genus identified, accounting for ~3.9% of sequences in each sample. This genus was most abundant in uninvaded soil compared to post-invasive soil (p<0.05). Of the remaining 114 detected low-abundance genera, six differed between soil types: *Agrobacterium* (p<0.01), *Bradyrhizobium* (p<0.01), *Rhizobium* (p<0.0001), *Candidatus koribacter* (p<0.01), and *C. solibacter* (p<0.001) were more abundant in post-invasive soil, while *Nitrospira* (p<0.05) was more abundant in uninvaded soil (Figure 5). Within *Rhizobium*, only *R. leguminosarum* (a mutualistic symbiont of legumes) was detected.

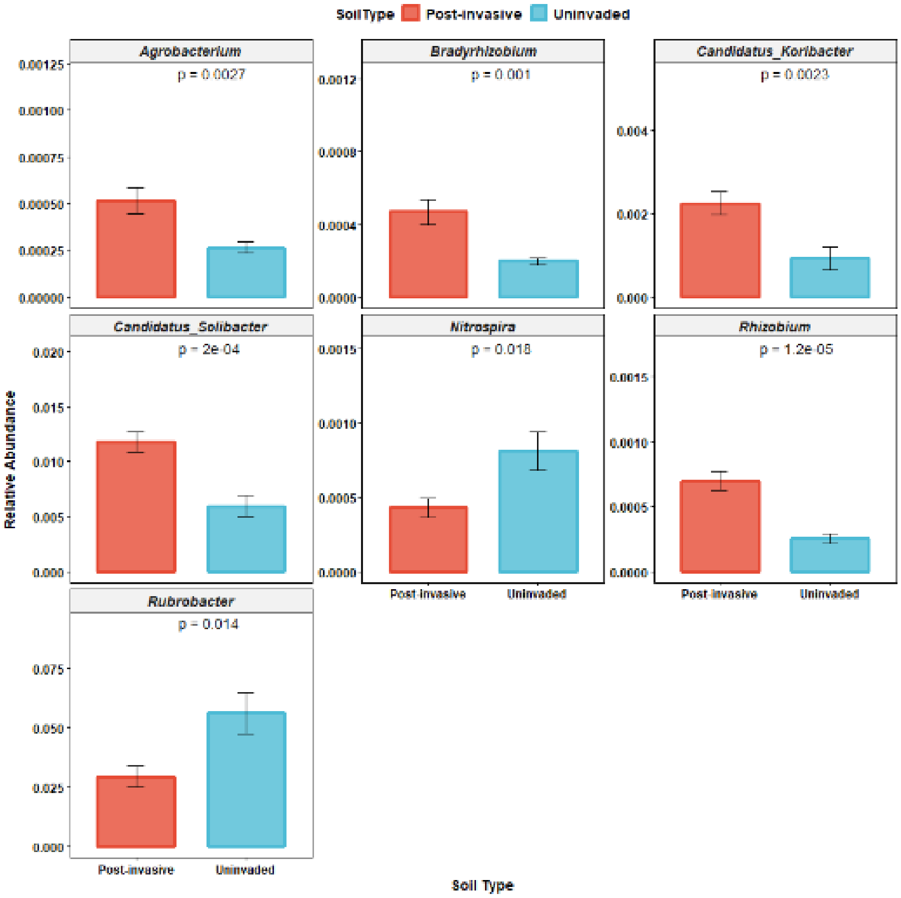


Figure 5. Relative abundances of genera (Agrobacterium, Bradyrhizobium, Candidatus Koribacter, Candidatus Solibacter, Nitrospira, and Rubrobacter) that were significantly different between soil types. Standard errors are represented in the figure by the error bars attached to each column.

Proteobacteria and Bacteroidetes are commonly found in a variety of soils, mainly those rich in nutrients, while *Bradyrhizobium* and *Rhizobium* are symbiotic nitrogen fixers that can be found in plant root nodules. *Candidatus koribacter* and *C. solibacter* (Acidobacteria) may be important for nitrate reduction (Ward et al. 2009) and *Nitrospira* is a nitrite-oxidizing bacteria that is integral to the nitrogen cycle and increasing plant-available nitrogen in soils.

The increased amount of nitrate in the post-invasive soil may be the reason nitrate-reducing bacteria are more abundant in these soils, though the decreased abundance of nitrifying bacteria, such as *Nitrospira*, has been found in other studies (Rice 1964). *Rubrobacter* is a genus well adapted for living in semi-arid, exposed soils, it may be that this genus is indicative of a healthy soil community, so its displacement by the invasive grass may be a sign of declining soil health.

The greater abundance of *Rhizobium* and *Bradyrhizobium* in the post-invasive soil is odd considering Harding grass is a non-leguminous plant. In fact, other studies show a direct negative effect of invasive plants on N-fixing microbes (Sanon et al. 2009). A few studies have shown that diazotrophic bacteria exist in grass roots and that these grasses derive a significant amount of N from them, but the bacteria involved are not Rhizobia (Umali-garcia and David 1980). Rhizobial bacteria exclusively nodulate legumes, and are found on only one non-legume taxon, *Parasponia*. This association is a recent host switch by the *Bradyrhizobia* involved and not a historical relationship (Lafay et al. 2006). It may be that Harding grass is associating with free-living N-fixers in some way that benefits its growth.

Fungal Composition

The total fungal composition (Figure 6), of the uninvaded and post-invasive soils did not differ (*p*>0.05). All OTUs belonged to 4 orders, 10 families, and 12 genera within Glomeromycotina. We found the following 11 genera: *Acaulospora*, *Ambispora*, *Archaeospora*, *Claroideoglomus*, *Diversispora*, *Entrophospora*, *Geosiphon*, *Glomus*, *Kuklospora*, *Paraglomus*, and *Scutellospora*. All samples were organized into either rhizophilic AMF, edaphophilic AMF, or ancestral AMF guilds as described earlier. Rhizophilic AMF richness was higher in post-invasive soil than uninvaded soil (*p*<0.05), while richness of edaphophilic and ancestral AMF did not differ by soil type (Figure 7). This finding points to the Enhanced Mutualism Hypothesis: the post-invasive soil has more rhizophilic AMF, which are thought to protect plant roots from pathogens, possibly allowing the Harding grass to invade and thrive. While it cannot be shown with this study, it is possible that the Harding grass brought the rhizophilic AMF with it when it invaded (Sieverding and Oehl 2005). It is also possible that rhizophilic AMF abundance increases in soils with higher nitrate concentration or that the Harding grass is more mycorrhizal than the native plants.

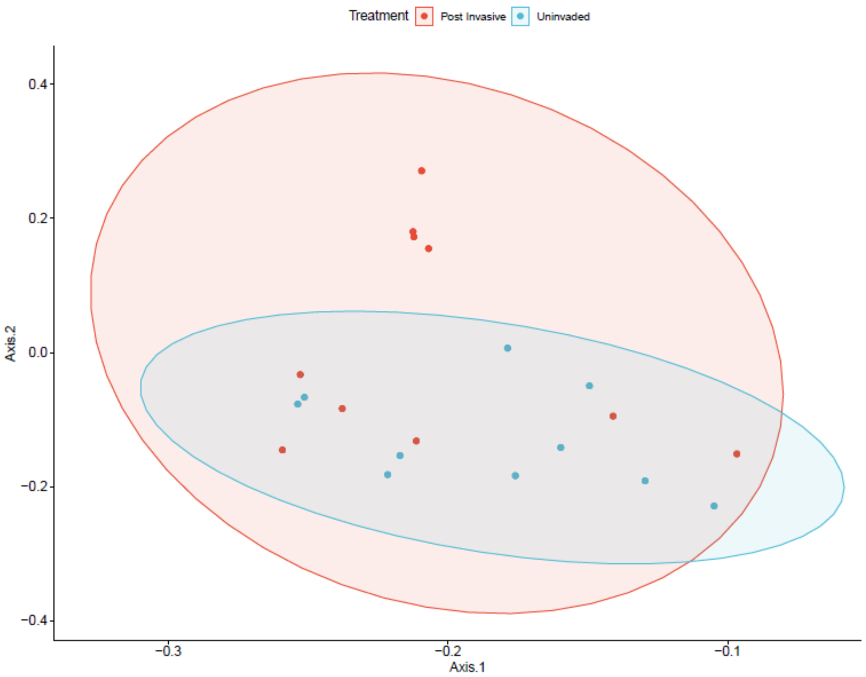


Figure 6. Principal coordinates analysis plot based on the weighted Unifrac distance metric for all arbuscular mycorrhizal fungal (AMF) taxa. Ellipses represent standard deviations of the weighted average of treatments at the 95% confidence level.

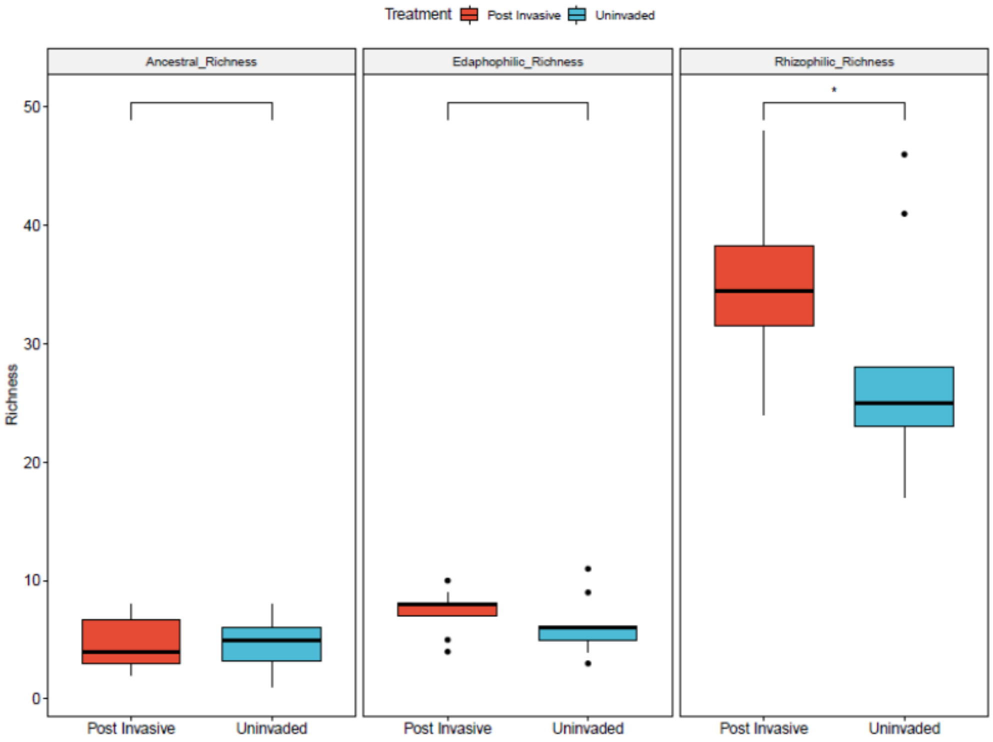


Figure 7. Arbuscular mycorrhizal fungal (AMF) soil communities by functional group using the phylogenetic scheme (Weber et al., 2018). Richness was defined as the number of times a unique taxonomic unit was discovered in each sample. * denotes significant difference by soil type at $p < 0.05$.

Plant Growth Trends

After 7 months of growth (October – May 2013) in the greenhouse, there was a significant correlation between plant growth and soil type that was species-specific. The invasive, Harding grass, had a larger biomass ($p<0.05$) and native, California sagebrush, had a larger biomass ($p<0.001$) and length ($p<0.05$) in uninvaded soil compared to post-invasive soil. Purple sage and Coyote brush growth were not affected by soil type.

The germination rate for the species ranged from 1% to 11.6%. Low germination rate can be typical of a growth experiment, such as this one, in which fertilizer was not added to the soil mix. The seed was also collected from RSV and could have been a weaker year/crop. The only significant differences in mean germination rate occurred for Harding grass (*p*<0.01) and California sagebrush (*p*<0.05) grown in uninvaded versus post-invasive soil (Figure 8). The Harding grass germination rate was 2.31 times lower in uninvaded soil (4.2% ± 1% SE) than in post-invasive soil (9.7% ± 2% SE). The California sagebrush germination rate was 2.5 times higher in uninvaded soil (2.5% ± 0% SE) than in post-invasive soil (1% ± 0% SE). The Coyote brush and Purple sage germination rates were not significantly different between soil types.

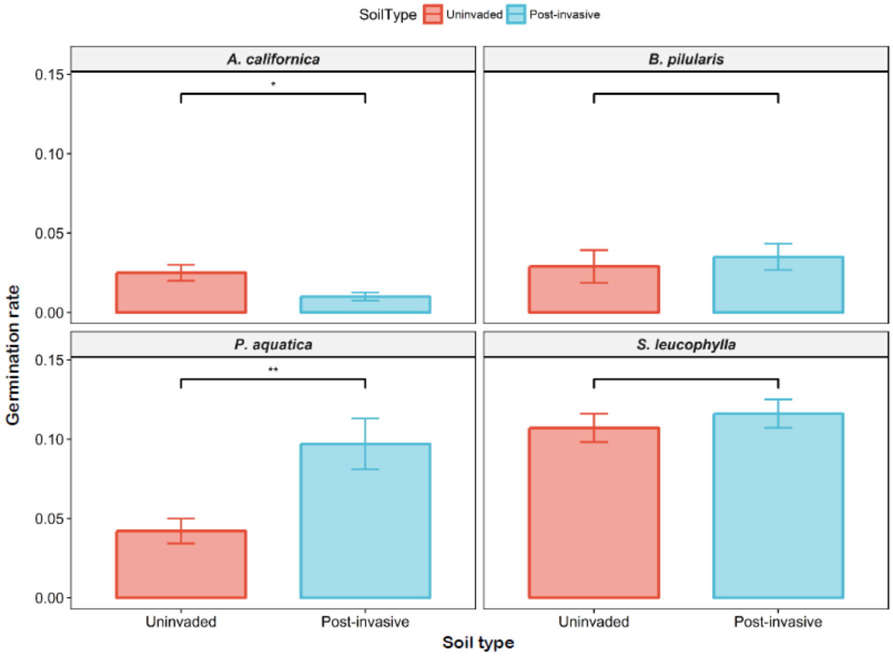


Figure 8. The germination rate of California sagebrush (Artemisia californica) (n=35), Coyote brush (Baccharis pilularis) (n=64), Purple sage (Salvia leucophylla) (n=210), and Harding grass (Phalaris aquatica) (n=139) (clockwise) in uninvaded (red) or post-invasive (blue) soils. Standard errors are represented in the figure by the error bars attached to each column. * denotes significant difference by soil type at p < 0.05 ** denotes significant difference by soil type at p < 0.01

The mean total biomass for all species ranged from 0.02g to 0.6g per pot. Again, the only significant differences occurred for the Harding grass and California sagebrush grown in uninvaded versus post-invasive soils (Figure 9). The Harding grass mean total biomass was 1.8 times higher in uninvaded soil than in post-invasive soil and California sagebrush mean total biomass was 3.5 times greater in uninvaded soil than in post invasive soil. For California sagebrush, the mean root mass ($p<0.01$) and the mean shoot mass ($p<0.001$) were also higher in the uninvaded soil. For Harding grass, the mean root mass ($p<0.05$), but not mean shoot mass ($p>0.05$) was also higher in the uninvaded soil. The mean total biomass for Coyote brush and Purple sage were not significantly different. The biomass root:shoot ratio was not significantly different between the uninvaded and post-invasive soil for all plants.

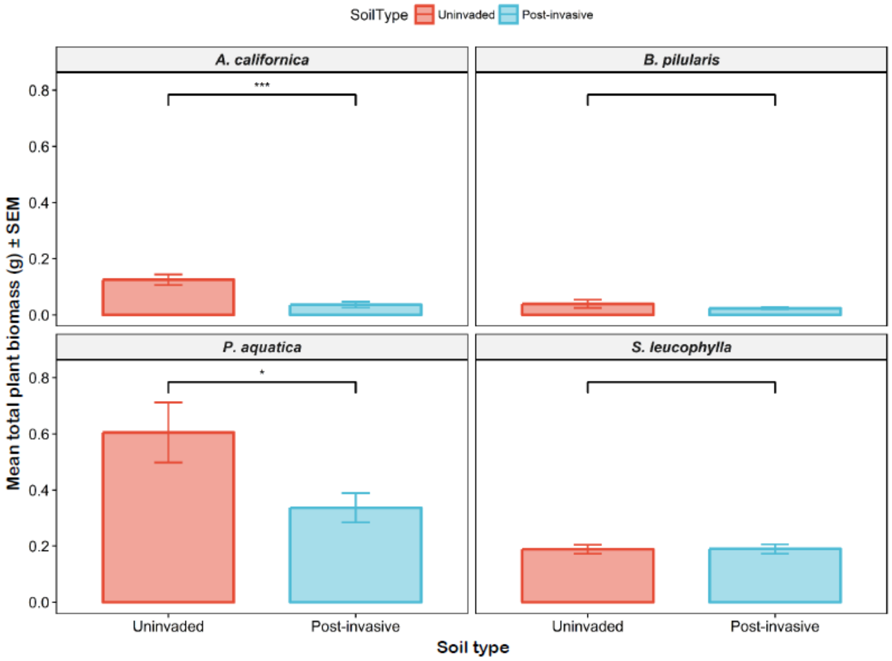


Figure 9. The mean total plant biomass of California sagebrush (Artemisia californica) (n=35), Coyote brush (Baccharis pilularis) (n=64), Purple sage (Salvia leucophylla) (n=210), and Harding grass (Phalaris aquatica) (n=139) (clockwise) in uninvaded (red) or post-invasive (blue) soils. The mean total biomass for all species ranged from 0.02g to 0.6g per pot. Standard errors are represented in the figure by the error bars attached to each column. * denotes significant different by soil type at p < 0.05 *** denotes significant different by soil type at p < 0.001

The mean total seedling length for all species ranged from 13.25mm to 308.15mm per pot. The only significant difference occurred for the California sagebrush grown in uninvaded versus post-invasive soils (Figure 10). The California sagebrush mean total length was 2.05 times larger in uninvaded soil than in post-invasive soil. The mean shoot length ($p<0.05$), but not the mean root length ($p>0.05$), was higher in the uninvaded soil. The Harding grass, Purple sage, and Coyote brush mean total lengths were not statistically different between soil types (Table S1). The length root:shoot ratio was not different between soil types for all plants except Purple sage (*p*<0.01), which had a higher root:shoot ratio in uninvaded soil.

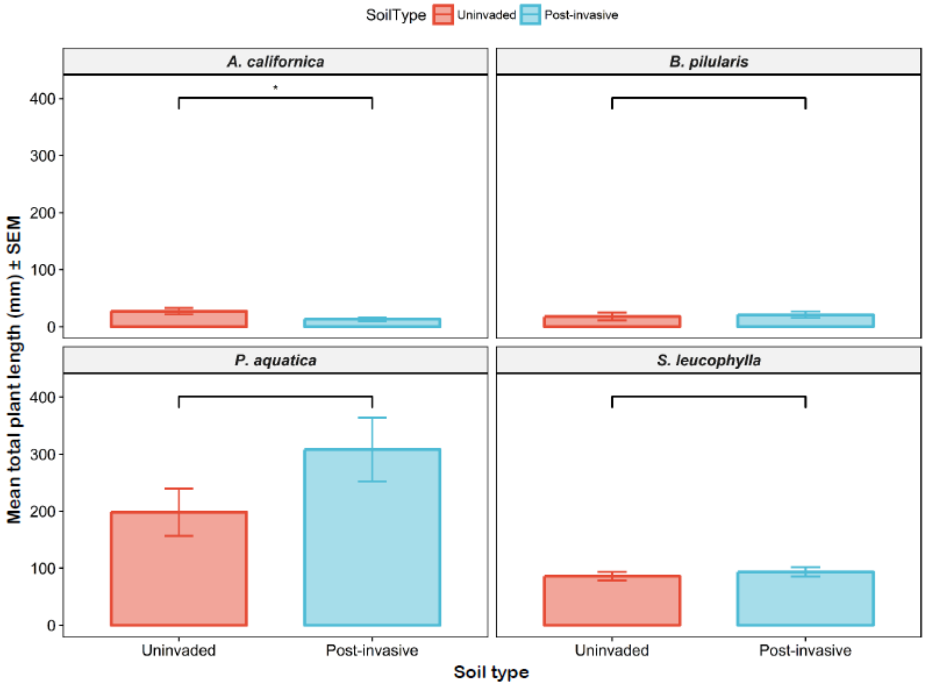


Figure 10. The mean total plant length of California sagebrush (Artemisia californica) (n=35), Coyote brush (Baccharis pilularis) (n=64), Purple sage (Salvia leucophylla) (n=210), and Harding grass (Phalaris aquatica) (n=139) (clockwise) in uninvaded (red) or post-invasive (blue) soils. The mean total seedling length for all species ranged from 13.25mm to 308.15mm per pot. Standard errors are represented in the figure by the error bars attached to each column. * denotes significant difference by soil type at $p < 0.05$.

The primary goal of this study was to determine if the growth of three native shrubs and invasive Harding grass would differ between uninvaded soil and soil from which a long-term Harding grass invasion had been removed. We found that while the growth of two native species (Purple sage and Coyote brush) did not differ by soil type, one native species, California sagebrush and the invasive, Harding grass did. The size of both California sagebrush and Harding grass was decreased in post-invasive soil, the germination rate of California sagebrush was lower in the post-invasive soil, and the germination rate for Harding grass was higher in the post-invasive soil, ostensibly due to higher soil N.

It is important to point out here that this study does not indicate whether bacteria and fungi are drivers or passengers in explaining the soil legacy effects. Rather, this study gives us a better understanding of the microbial legacy effects in post-invaded sites. We cannot directly explain the plant growth responses, but we can inform our understanding of what happens in these sites. It may be possible to explain plant growth responses if pathogenic and saprophytic fungal abundances were also quantified, as these microbial groups are very important to the healthy functioning of CSS.

Harding Grass

The accumulation of local pathogens hypothesis may help explain why Harding grass growth was lower in post-invasive soil. That over time, the Harding grass monoculture caused an increase in *Agrobacterium* spp. in the post-invasive soil. This increase in a potential plant pathogen could cause a decline in Harding grass growth relative to the uninvaded soil which has a lower abundance of *Agrobacterium*.

Under the enhanced mutualism hypothesis, it could be that Harding grass was able to form stronger mutualisms with microbes that exist in the uninvaded soil, but no longer survive in large numbers in the post-invaded soil. When Harding grass first invaded it was introduced into a highly diverse system with many species of native plants that interact with a variety of soil microbes. Invasive plants can take advantage of these interactions, resulting in significantly higher biomass when grown in previously uninvaded soil. For example, a study by Maron and Connors (1996) found that the invasive annual grass, Ripgut brome (*Bromus diandrus* Roth.), had significantly higher root and shoot biomass when grown in soil collected under native shrubs that form mutualisms with nitrogen-fixing bacteria compared to soil collected one meter away. It could be that the increase in rhizophilic AMF and increased abundance of *Rhizobium* and *Bradyrhizobium* observed in the post-invasive soil provided a growth benefit to the Harding grass.

It is also possible that Harding grass was able to prime the soil in ways that first increased, but then decreased its own growth. When a plant such as Harding grass invades, it usually increases the amount of N in the soil. A meta-analysis by Liao et al. (2008) found that invaded soils had ~20% larger N pools and ~51% faster rates of N-mineralization compared to uninvaded soils. Unsurprisingly, Harding grass increased nitrate and total N in post-invaded soil relative to uninvaded soil, most likely because of its high quality leaf litter (low C:N). Increased soil N promotes the growth of bacteria which quickly mineralize N into plant available forms (NH_3 and nitrate). The increased abundance of Proteobacteria and Bacteroidetes, which are likely copiotrophic (found in environments rich in nutrients) in post-invasive soil could be due to this increase in soil nitrate. Differences in N supply have been found to alter the relative abundance of copiotrophic taxa in other studies (e.g., Actinobacteria, Bacteroidetes, and β-Proteobacteria) (Fierer et al. 2007; Ramirez et al. 2012). This sort of soil-priming would stimulate the growth of Harding grass, which may be better adapted to high-N environments than many native shrubs (Perry et al. 2010). A monoculture of Harding grass could have been the result of this soil priming. However, after many years of maintaining this monoculture, the microbial community may have shifted far enough from the original uninvaded soil community to negatively impact the growth of Harding grass, which grew faster and larger in the naïve uninvaded soil. Transporting rhizophilic AMF with it to the invaded range could have provided Harding grass with more nutrients and pathogen protection, allowing it to establish and form a monoculture.

Native Plants

Of the three native plants studied, California sagebrush was the only one with significantly decreased mass and length in the post-invasive soil. This could be explained by an increase in soil N or an increase in pathogenic or free-living microbes. Increased soil N could promote the growth of plants, such as Harding grass, that are better adapted to high N levels and harm the growth of plants, like California sagebrush, that are not (Perry et al. 2010). The increased abundance of several potentially beneficial bacterial taxa and rhizophilic AMF with Harding grass invasion could have potentially helped California sagebrush growth, if not for the increased soil N. Alternatively, the favoring of certain microbial groups by the invasive grass in post-invasive soil may have allowed certain microbes to outcompete microbial taxa that are important to the growth of California sagebrush. It is also possible that the *Agrobacterium* accumulation in the post-invasive soil was pathogenic to California sagebrush.

It may be that the other native plants studied, Purple sage and Coyote brush, were unaffected either because the invasive did not alter the abundance of bacteria and AMF critical to their health and well-being, or they are more resilient to changes in soil N. There could be a potentially complex interaction between the soil nutrients and soil biota such that these two native plants were benefited by the increased *Rhizobium*, *Bradyrhizobium*, and Rhizophilic AMF in the post-invasive soil, despite the elevated N.

Reduced native plant colonization in the field could be a result of elevated soil nitrate or an accumulation of pathogenic bacteria. There may be some unmeasured legacy effects as well, such as an alteration to the pathogenic or saprophytic soil fungi composition or soil physical characteristics that may reduce establishment that were not measured in a greenhouse experiment. In the greenhouse, the soil is mixed before potting and the plants are provided with ideal growth conditions, if this experiment were repeated as a field experiment, we may find that Coyote brush and Purple sage are instead inhibited by unmeasured legacy effects in the soil.

It is possible that these observed trends are due to the loss of the native species or pre-invasion environmental differences, abiotic changes that were not measured, presence of glyphosate in the soil, or other biotic changes such as shifts in pathogenic or saprotrophic fungi. We can probably rule out pre-invasive conditions because of the long duration of invasion. Glyphosate is also an unlikely factor because its half-life is approximately two weeks and it does not have residual soil activity on the seedbank. While it took several years of treatment before the population was eradicated (locally), several years had elapsed after herbicide treatment had ceased. In many other areas of RSV, native recolonization from the seed bank has occurred after glyphosate application to other invasive plants. Both uninvaded and post-invasive sites have similar sun exposure, moisture ($p > 0.05$), slope aspect, soil texture (Mipolomol), pH ($p > 0.05$), and elevation. We did observe differences in the total N and C concentration of soils so it is most likely that the unique traits of Harding grass that differentiate it from native shrubs are responsible for the trends observed in this study.

Although this study shows evidence that exotic grass invasions, and their subsequent removal, have lasting impact on above and belowground ecosystems, it has some spatial limitations. For instance, we collected our samples from one site in southern California which was previously invaded by Harding grass, as well as an intact site, replete with native vegetation. Therefore, our conclusions are limited solely to this ecosystem. However, we observed reduced native plant performance after Harding grass removal over several years prior to conducting our experiment in this particular site, which motivated our study in comparing the intact and post-invasion sites within this particular study location. The justification for our experimental setup is three-fold: 1) replicating a large-scale post-invasive site would be costly and impractical: this would require another site with a long-term Harding grass monoculture and several years to remove said monoculture, 2) park restoration ecologists observed this specific Harding grass invasion site over several years and noted the lack of native plant growth after invasive removal, and 3) this is a natural laboratory, with "invasion" as the treatment, therefore, it would be unethical to "treat" a native area with invasive plants for the sake of replication.

Legacy Effects

Due to the long period of invasion and the persistence of differences between the microbial communities and nutrient composition in the uninvaded and post-invasive soils after the invasive grass was removed, these results should be considered legacy effects of Harding grass. Some long-term plant invasions are known to change soil in ways that persist for years after the invasive plant has been removed ("legacy effects"; Kulmatiski and Beard 2011), although this is not always the case (Jordan et al. 2008). Legacy effects are normally defined as the abiotic and biotic impact of a species that persist long after the species has been removed from an area (Cuddington 2011). In some cases, even with active management, legacy effects can be so severe that they take decades to reverse (Eviner et al. 2010). Several short-term studies have shown that invasive plants can alter soil biotic communities in ways that disrupt plant community composition, plant-soil interactions, and plant-plant interactions (Reinhart and Callaway 2006). Taken in concert with our findings, it appears that these effects of invasion may not always attenuate over time.

Legacy effects of invasive plants have been shown to affect native species in sometimes contradictory ways. Studies focused on understanding the effect of invasive plant growth on natives often have conflicting results, largely dependent upon the specific native and invasive species studied (Bozzolo and Lipson 2013), the length of invasion, invader percent cover, and site specifics (Eviner et al. 2010). While relatively little is known about legacy effects (Corbin and D'Antonio 2012), it does seem that legacies in soil are directly dependent on the invasive being studied (Hausmann and Hawkes 2009). It may be that the specific ways in which Harding grass alters the soil is key to understanding the observed plant growth trends.

Overall, the findings of our study indicate that Harding grass creates microbial legacy effects in the soil that likely cause soil conditions inhibitory to the growth of some native CSS plants. Future work will focus on determining if differences in native plant growth can be explained by biotic changes in the soil brought on by Harding grass and if remediation of soil microbial conditions through soil inoculations could improve restoration in these post-invasive sites. We will specifically explore if changes in the composition of bacteria, pathogenic fungi, and arbuscular mycorrhizal fungi (AMF) explain differences in native plant growth.

Inoculation experiments have shown encouraging results for restoration efforts, however, it may be useful to evaluate how certain functional groups of invasive plants alter the soil microbial composition. This would provide us with an accurate framework to inform restoration practices. Much more information is needed about the long-term impact of invasion on soil microbes in order to properly restore the biotic properties of soils.

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CHAPTER 3

Identifying and Remediating the Microbial Soil Legacy Effects of Invasive Grass for Improved Restorations

Abstract

Invasive grasses in California have transformed once healthy California sage scrub (CSS) into nonnative grasslands. This has occurred partly due to legacy effects, changes in the soil microbial composition brought on by invasive grasses that can sometimes have long-term impacts on the soil and the native vegetation. Despite the rapid shrinking of CSS, it is not well understood how legacy effects alter the microbial community or if these alterations actually impact native plant growth. In this study, we have directly tied long-term changes in the soil microbial community to native plant growth. We measured the growth of three CSS species inoculated with either uninvaded soil or sterilized uninvaded soil and planted into a site with known microbial legacy effects. Our findings indicate that differences in native plant growth can be explained by changes in the soil microbial community and that remediation of the soil microbial community through inoculation can improve restoration in post-invasive sites. Specifically, native plants *Artemisia californica* and *Baccharis pilularis* grew larger in the unsterilized uninvaded soil inoculum plots, which had a larger abundance of Gemmatimonadetes and *Glomus*, compared to the sterilized soil inoculum plots.

Introduction

There are over 300 rangeland invasive plants in the United States that alter sensitive habitat, poison animals, reduce plant diversity, and deplete resources (Ditomaso 2000). In California, shrub cover has decreased by 90% largely due to plant invasion, transforming the landscape from coastal sage scrub (CSS) to nonnative grassland (Westman, 1981). Coastal sage scrub is a hotspot of endemic species, characterized by low-growing shrubs in the inland and coastal areas of California and northwest coastal Baja California. A number of rare and endangered species rely on CSS for survival, with 100 of the endemic species proposed for or under protection (Rubinoff, 2001). The cover of California sagebrush (*Artemisia californica* Less.), a foundation species in CSS, has decreased from 17.7% to 6.1% in the last 62 years (Antonio & Vitousek, 1992). Despite the fragility and importance of CSS, research conducted in this system has been sparse (Lowry et al., 2013). It has become very important to the survival and health of CSS to better understand the ecosystem impact caused by invasive plants and especially invasive grasses.

Invasive grasses alter carbon storage in the soil (Eviner et al., 2010), inputs of nitrogen and other elements (Ehrenfeld, 2003), organic matter content (Saggar, McIntosh, Hedley, & Knicker, 1999), water flow, and soil quality. Researchers have observed how these changes in soil physical and chemical properties can increase the competitive ability of invasive grasses and allow them to dominate a landscape. However, the impact of invasive plants on soil microbial communities, and the indirect impact of these changes on native plant communities, are less well understood.

Microbes are critical facilitators of the carbon and nitrogen cycles, primary decomposers of organic matter, and important mutualists and pathogens of most plant species (Ehrenfeld, Ravit, & Elgersma, 2005). Variations in the carbon:nitrogen ratio of plant litter as a result of invasion, can alter the soil microbial community, sometimes causing dramatic shifts between fungal and bacterial dominant communities in the soil (Dickens, Allen, Santiago, & Crowley, 2012). Introduction of translocated microbes (Vellinga, Wolfe, & Pringle, 2009) and root exudates (Bais, Weir, Perry, Gilroy, & Vivanco, 2006) from invasive plants can also contribute to changes in the soil microbial community.

Soil bacteria are important decomposers and contributors to nutrient cycling in CSS. Changes to their composition brought on by invasive plants have been found in other studies (Kuske et al. 2002) and may have important implications for native plant growth.

Arbuscular mycorrhizal fungi (AMF) are the most common plant mutualistic symbionts and they associate with most plant species (Bever et al. 2001). They not only increase plant access to phosphorus (Batten et al. 2006; Lankau and Lankau 2014), but stabilize soil aggregates (Miller and Jastrow 2000), provide resistance to pathogens

(Batten et al 2006; Klironomos 2002), and ameliorate the allelopathic effect of some invasive plants (Barto et al. 2010). The generalist nature of AMF allow them to form associations with invading plant species, sometimes allowing the invader to outcompete and displace native plants that are non-mycorrhizal or weakly mycorrhizal. Other invasive plants do not associate with AMF, but instead degrade them (Mycorrhizal Degradation Hypothesis) (Vogelsang et al. 2004). It has become clear that when a plant invades, it has the potential to either increase or decrease the abundance and diversity of AMF, sometimes by introducing invasive AMF species into the invaded range (Sieverding and Oehl 2005).

All of these changes in the soil microbial community brought on by invasive plants can indirectly affect native plant growth (Bever et al., 2010; Cuddington, 2012; Dickens et al., 2012; Eviner et al., 2010; Hawkes, Belnap, D’Antonio, & Firestone, 2006; Jordan, Larson, & Huerd, 2008; Mangla, Callaway, & Callaway, 2008). Native plant cover can decline if important nutrient cycles are altered (Liao et al. 2008), food webs change, or plant pathogens are introduced to the soil (Belnap et al. 2005; Jordan et al. 2008). The indirect effects of invasive plant growth on native plants are largely dependent upon the native and invasive species studied (Bozzolo and Lipson 2013), site specifics, time of invasion, and invader cover (Eviner et al. 2010), creating conflicting results across studies. It may be important, therefore, to understand how certain important invasive grasses affect native plants in context-dependent ways (Eviner et al. 2010).

Changes in the soil microbial composition sometimes last for years after the invasive plant has been removed. These changes are termed “legacy effects” and are defined as the biotic and abiotic impact of a species that persists after the species has been removed from an area (Cuddington 2011).

A prevalent invasive grass in CSS ecosystems is *Phalaris aquatica* L. (Harding grass), a species known to outcompete and displace native plants by forming large monocultures in riparian and upland systems of the invaded range. It is highly aggressive (Tran and Cavagnaro 2010), mycorrhizal (Asghari and Cavagnaro 2011), perennial, and deep-rooted, having been transplanted as pasture grass from the Mediterranean (Ditomaso 2000) to the United States coastal valleys, foothills and roadsides from Oregon to California. Despite the invasiveness of this grass, little is known about its impact of native CSS plants or on soil microbes and abiotic properties.

We conducted a greenhouse study to better understand how long-term invasions alter soil microbial conditions and native plant growth. The performance of three native plants (*Artemisia californica* Less., *Salvia leucophylla* E. Greene, and *Baccharis pilularis* DC.) and the invasive grass, *P. aquatica*, were compared in uninvaded soil vs. soil previously invaded by *P. aquatica* (post-invasive soil). We found a species-specific correlation between plant growth and soil type along with differences in relative abundance at various microbial taxonomic levels between the soil types.

To better understand if these observed differences in plant growth were biotic in origin and reversible, we monitored the performance of the same three native plant species inoculated with either unsterilized or sterilized uninvaded soil after transplant into the post-invaded site. We also characterized the bacterial and fungal communities in the treatment sites and the surrounding intact and post-invasive sites. We hypothesized that (1) differences in native plant growth can be explained by changes in the soil microbial community and (2) remediation of the soil microbial conditions through inoculation will improve restoration in post-invasive sites.

Materials and Methods

Field Site

Rancho Sierra Vista (RSV) is a lowland site in Southern California that was originally California sage scrub (CSS), but has a long history of ranching and agriculture that have transformed much of the landscape to nonnative grassland. Since the 1950's this site was open, dry field agriculture, with soil series Mipolomol, consisting of loamy, mixed, superactive, thermic, shallow Entic Haploxerolls (US Department of Agriculture). The first recorded siting of *P. aquatica* in RSV occurred in November 2002 (Calflora, 2014). It was first planted for livestock fodder in the 1970's before the Santa Monica Mountains National Recreation Area was established in 1978. As nothing would it eat, *Phalaris aquatica* rapidly expanded to approximately 10ha (34°09'10.3"N, 118°57'08.2"W), forming a monoculture. The invasive grass was fully established, reaching ~1.1m in height. Control efforts began in the late 1990's-early 2000 and removal of *P. aquatica* by park managers over the 10ha began in 2006, using a combination of mowing and herbicide application (glyphosate). By 2013, the *P. aquatica* infestation was reduced to routine maintenance levels and in several areas eradicated. Although this post-treatment area of *P. aquatica* was surrounded by intact CSS to provide ample propagules, native plant recruitment was virtually nonexistent several years later. Park managers were concerned that traditional restoration strategies, in which the native plant seedlings were out-planted in large numbers, might prove ineffective in restoring the native plant population if there were underlying unfavorable soil conditions.

Experimental Setup

The experiment was arranged as a randomized block design with native plants California sagebrush (*Artemisia californica* Less.), Purple sage (*Salvia leucophylla* E. Greene), and Coyote brush (*Baccharis pilularis* DC.) inoculated with either unsterilized (UT) or sterilized (ST) uninvaded soil. All three species are mycorrhizal and non-leguminous and were chosen due to their dominance in CSS and their abundance in the uninvaded soils surrounding the post-invasive site. On January 22, 2014, the 10ha site was arranged into three blocks that each contained 10 plots (1 m x 1 m). Six of the 10 plots were single-species plots containing six plants each and the remaining four plots were mixed species plots containing two of each plant species (Figure 1). We created mixed species plots to investigate 1) whether there were any interspecies effects on the plant growth or microbial composition of the soil and 2) if certain species could act as nurse plants for those that grew poorly in the post-invasive soil. Each plot was replicated three times (one per block) and none of the plots mixed plants inoculated with ST or UT soil. A total of 180 plants were planted in the experiment. However, the mixed-unsterilized experimental plot in block one was lost (probably due to herbivory) so 174 plants were analyzed, instead of 180.

Soil Inoculum

Uninvaded soil was defined as soil in which intact CSS has historically grown uninterrupted. The uninvaded soil used in this study was collected from an intact stand (no history of livestock or cultivation) of CSS in RSV, California. It was collected about 1 mile West of the 10ha field site on the side of an east facing slope (34°09’16.0”N 118°57’54.7”W) where *S. leucophylla, B. pilularis,* and *A. californica* are prevalent. The soil was collected at ~10 randomly selected locations across the slope. Approximately 19 L of soil was collected using a sterilized 20 cm diameter shovel, from the top 15cm of soil. About half of the soil (9.5 L) collected was transported back to the lab for sterilization to eliminate any plant-associated microbes. The soil was first sieved through a 1 cm^2 stainless steel mesh and then steam-sterilized in a process involving a 24 hour steam-sterilization, followed by a 48 hour incubation period, and a second 24 hour sterilization period. The unsterilized and sterilized soils were never mixed together. They were each combined with Berger BM2 peat moss germinating mix in a sterilized cement mixer at a ratio of 4 peat moss: 1 soil. These mixtures are referred to as either sterilized (ST) or unsterilized (UT) soil inoculum hereafter.

Plant Growth Conditions

On January 22, 2014, 1000 seeds per species were sown in three separate plant flats (540 mm x 280 mm x 50 mm) filled with commercial potting mix and peat moss. After germination and two weeks of growth, n= 100 seedlings per species were transplanted into cone-tainers (3.8 cm diameter x 21 cm deep) with one seedling in each cone-tainer. Each cone-tainer was packed with either the unsterilized or sterilized soil inoculum, a 20 mm space between the soil surface and the top of the cone-tainer was made to allow room for irrigation. The seedlings were watered every other day and were grown in a mesh outdoor greenhouse with indirect sunlight on the SAMO property. Conditions in the greenhouse were a mean temperature of 29.4°C max and 14.4°C min. Plants were placed in random locations throughout the outdoor greenhouse and the treatments were separated by at least 1m to prevent cross-contamination during watering. After about 5 months of growth (Sep. 6, 2013-Jan. 22, 2014) under these conditions, 18 of the *A. californica* grown with sterilized soil inoculum, 7 *A. californica* grown with unsterilized soil inoculum, and 1 *B. pilularis* grown with sterilized soil inoculum died. Due to this mortality, n=60 plants per species (instead of the original 85) were transplanted into the 10ha post-invasive site (Figure 2) on January 22, 2014. The field site is a NW facing slope in full sunlight. Power augers were used to drill holes about 15 in deep x 8 in wide into which the seedlings were planted after careful removal from the conetainers and a shake to dislodge excess soil. The seedlings were then watered once a week by volunteers using a utility vehicle mounted with a water tank.

Plant Harvest

We destructively harvested plants on August 20, 2014 after about eight months of growth in the field. One plant from each plot was randomly chosen for harvesting, for a total of 30 out of 174 plants. At harvest, the entire plant was carefully and slowly excavated from the soil with soil knives to ensure minimal root loss, after which the shoot was separated from the roots with shears. The shoots were placed into clean paper bags and the roots were shaken lightly to remove loose soil and placed in sterile bags (Whirlpak by Nasco, Inc.) for transport to the laboratory at UCR. Rhizosphere soil was defined as the soil still clinging to the surface of the roots after being shaken, while bulk soil samples were collected under each harvested plant with soil knives. All soil and root samples were placed on dry ice in the field and transported to a -20°C freezer at the laboratory in UC Riverside within 24h.

Soil Core Collection

Soil cores were collected throughout the field experiment starting on February 10, 2014. Soil cores collected during every other month (150 soil cores total) were not frozen, but placed on dry ice and immediately shipped to the laboratory from the field and processed for nitrogen analysis. Soil cores collected during the other 3 months (213 soil cores total) were stored at -20°C for DNA extraction and sequencing (Table 1) at the laboratory in UC Riverside. Soil cores for sequencing were collected directly under the drip-line of the native plants at a depth of 5cm with a sterile corer. One soil core was collected in each unmixed plot under every plant and three cores were taken in each mixed plot (one per species) during each sampling session. Soil cores for nitrogen analysis were also 5cm deep, but were taken from the middle of each plot, equidistant from the plants inside.

During each sampling session, 10 additional soil cores were also collected under the canopy of the surrounding intact CSS and another 10 soil cores were collected in random locations throughout the 10ha site that was not inside an experimental plot. These last 20 soil cores were meant as uninvaded soil and post-invasive soil controls, respectively. A total of 71 soil cores were taken during every sampling event for sequencing and 50 soil cores for nitrogen analysis. These collections happened six times during the experiment for a grand total of 363 soil cores taken. Using a soil temperature probe, we also measured soil temperature and water content next to each spot from which a soil core was collected.

Leachate Analysis

Plant available nitrogen (nitrate and ammonium) was determined for each soil core. Forty mL of 2M KCl (148g KCl + 1L H_20) was added to 10g of the original soil core. The sample was placed on a shaker table for 1hr at 200rpm before settling for another 1hr. The supernatant was then gravity filtered and the final extract was collected in vials. Ammonium analysis followed Weatherburn (1967) and nitrate analysis followed Doane and Horwath (2003). For ammonium analysis, 80ul of sample were mixed with 60ul of salicylate solution and 60ul of bleach solution, then read on a microplate reader at 650nm. For the nitrate analysis, 100ul of sample were combined with 100ul of reagent solution (50ml vanadium chloride solution, 3.3ml sulfanilamide solution, 3.3ml *N*-(1-Naphthyl)ethylenediamine (NED) solution, and 400ml DI water) and read on a microplate reader at 540nm. Two technical replicates were run for each sample.

16S DNA Extraction, Quantification, and Barcoded Amplicon Sequencing

The 16S ribosomal RNA gene (16S rRNA) V3 and V4 regions were analyzed to classify the diversity of bacteria in the soil. Microbial DNA was extracted using a MO BIO PowerSoil DNA Isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's instructions and using a PowerLyzer 24 bench top bead-based homogenizer (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). A NanoDrop 2000/2000c UV-Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) was used to quantify the DNA in soil extracts. PCR for bacteria and archaea was performed using primers that target the 16S V3 and V4 regions (S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21; Klindworth et al., 2012) of the 16S rRNA gene. Microbial genomic DNA (2.5ul) was combined with forward and reverse primer (5ul each), and 2x KAPA HiFi HotStart

ReadyMix (KAPA Biosystems, Wilmington, Massachusetts, USA) (12.5ul). A Bio-Rad MJ Research PTC 200 Thermocycler was used to amplify 96 samples at a time with the following program: 95°C for 3 min, 25 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 5 min, and hold at 4°C. AMPure XP beads (Beckman Coulter Genomics, Danvers, Massachusetts, USA) were used to purify the 16S amplicon without primer and primer dimer sequences. Dual indices and Illumina sequencing adapters were attached to the amplicon using the Nextera XT Index Kit (Illumina, San Diego, California, USA). Amplicon DNA (5ul) was combined with 2x KAPA HiFi HotStart ReadyMix (25ul), Index 1 and 2 primers (5ul each), and PCR grade water (10ul). The same thermocycler was used with the following program: 95°C for 3 min, eight cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, 72°C for 5 min, and hold at 4°C. A second bead cleanup was used to purify the final library before quantification. The samples were verified with gel electrophoresis after every step. The samples were quantified in duplicate using the Quant-iT PicoGreen dsDNA assay kit (Life Technologies, Grand Island, New York, USA). All samples were pooled together in equimolar concentrations then sequenced with an Illumina MiSeq instrument at UCR.

We also performed a BLAST search to verify Cyanobacteria findings. We filtered the OTU table by taxonomy (specifically Cyanobacteria), then chose representative sequences based on this filtering. The BLAST search was performed using the NCBI BioSystems Database (Geer et al., 2010) and was limited to only Cyanobacteria.

AMF DNA Extraction, Quantification, and Barcoded Amplicon Sequencing

The SSU rRNA gene was analyzed to classify the diversity of mycorrhizae in the soil. Microbial DNA was extracted using a MO BIO PowerSoil DNA Isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's instructions and using a PowerLyzer 24 bench top bead-based homogenizer (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). A NanoDrop 2000/2000c UV-Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) was used to quantify the DNA in soil extracts. PCR for fungi was performed using the primer pairs WANDA-AML2. Microbial genomic DNA (1 μL) was combined with forward and reverse primer (5 μL each), and Phusion DNA Polymerase (Thermo Fisher Scientific, Wilmington, DE, USA) (12.5ul). A Bio-Rad MJ Research PTC 200 Thermocycler was used to amplify 96 samples at a time with the following program: 95°C for 2 min, 40 cycles of 95°C for 30 s, 60°C for 1 min, 68°C for 1 min, and hold at 10°C. AMPure XP beads (Beckman Coulter Genomics, Danvers, Massachusetts, USA) was used to purify the extracts and amplicon. Dual indices and Illumina sequencing adapters were attached to the amplicon. Diluted (1:10) amplicon DNA (1 μL) was combined with Phusion (12.5 μL), Index 1 and 2 primers (2.5 μL each), BSA (0.1 μL) and PCR grade water (6.4 μL). The same thermocycler was used with the following program: 95°C for 2 min, 15 cycles of 95°C for 10 s, 55°C for 30 s, 72°C for 30 s, and hold at 10°C. A second bead cleanup was used to purify the final library before quantification. The samples were verified with gel electrophoresis after every step. The samples were quantified in duplicate using a Qubit 2.0 Fluorometer

(Thermo Fisher Scientific, Wilmington, DE, USA). All samples were pooled together in equimolar concentrations then sequenced with an Illumina MiSeq instrument.

DNA Extraction from Roots

Sterile forceps were used to carefully remove large pieces of soil and debris from the root surface, the root ball was then split if needed, and placed into a sterile petri dish filled with 10% bleach. The root was then submerged, pressed gently, and moved back and forth in the bleach until excess soil disengaged from the root. The roots were then washed with milliQ water to clean off any remaining bleach, cut into 1cm pieces, and 0.15g were weighed out into bead tubes for extraction. Bead solution and C1 solution was added to the tubes before a 1hr incubation in a 65°C heating block. Tubes were vortexed every 15min for 5-10sec then microbial DNA extraction was carried out using a MO BIO PowerSoil DNA Isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's instructions and using a PowerLyzer 24 bench top bead-based homogenizer (Mo Bio Laboratories, Inc., Carlsbad, CA, USA).

Data Analysis

To analyze the plant length and biomass data, we used JMP13 statistical software (JMP, Version 13. SAS Institure Inc., Cary, NC, 1989-2007) to perform a linear regression between plant length in UT compared to ST soil.

Quantitative Insights into Microbial Ecology (QIIME; Kuczynski et al., 2012) was used to quality filter the sequences and determine taxonomic identity against the Greengenes reference databases using 97% similarity. Analysis of similarity (ANOSIM) was performed in QIIME using a Unifrac index to statistically compare community similarity among treatments. We performed alpha diversity analyses and generated PCoA plots using QIIME. Beta diversity analyses were performed using MicrobiomeAnalyst (Dhariwal et al. 2017). To analyze the abundance of certain taxa in the soil samples, we used JMP13 to perform a two-way ANOVA with factors soil type (uninvaded or post-invasive), month (April or February), and soil*month at the phylum, class, order, family, and genus levels. We used the vegan (Oksanen et al. 2018) and ggpubr (Kassambara 2018) packages in R to perform a PERMANOVA and generate figures, respectively.

For SSU, we used smalt (http://www.sanger.ac.uk/science/tools/smalt-0) to remove PhiX contamination and cutadapt (Martin 2011) to filter sequences. We used the forward read and checked quality with FastQC (Andrews S. 2010). Demultiplexing was performed in QIIME 1.9.1 and taxonomy was assigned using BLAST against the MaarjAM database (Öpik et al. 2010).

Results

Bacteria and Leachate

The amount of nitrate was higher in post-invasive soil ($p<0.001$), while ammonium did not differ between soil types. The total bacterial composition of the treatment and control plot soils were not different from each other ($p>0.05$) in February and April (Figure 1). The bacterial composition did not differ by block or mixed/non-mixed plots.

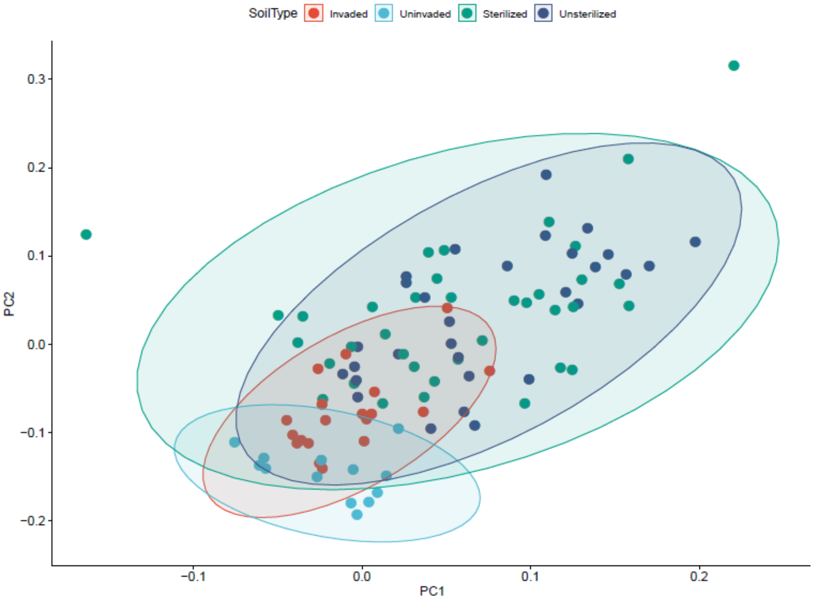

Figure 1. Principal coordinates analysis plot based on the weighted Unifrac distance metric for bacterial taxa in treatment and control plots. Ellipses represent standard deviations of the weighted average of treatments at the 95% confidence level.

For the destructively sampled plants in August, the roots and rhizosphere microbial composition were different from each other ($p<0.01$), with the rhizosphere composition differing by treatment (UT or ST) ($p<0.01$) (Figure 2). We found differences in relative abundance at various taxonomic levels between the roots and rhizosphere samples and the rhizosphere in UT compared to ST soils.

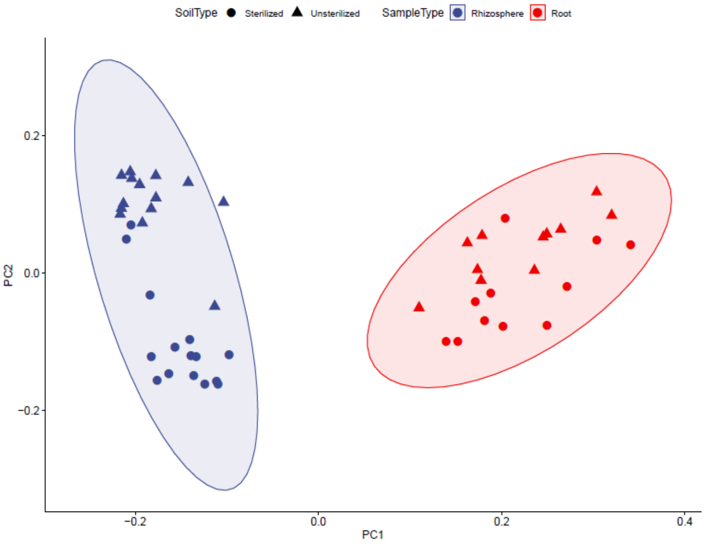

Figure 2. Principal coordinates analysis plot based on the weighted Unifrac distance metric for bacterial taxa. Ellipses represent standard deviations of the weighted average of treatments at the 95% confidence level. The rhizosphere soil is represented in blue, while the roots are represented in red.

Roots and Rhizosphere. Eleven phyla dominated all root and rhizo samples, with Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Firmicutes, Gemmatimonadetes, Nitrospirae, Planctomycetes, Proteobacteria, and Verrucomicrobia accounting for ~95% of sequences in each sample. Cyanobacteria alone made up ~41% of sequences in each root sample. Forty-two additional phyla were present but not dominant in both roots and rhizosphere. There were significant differences in the relative abundance of certain taxa between root and rhizosphere: in rhizosphere, there was relatively more Acidobacteria ($p<0.0001$), Actinobacteria ($p<0.0001$), Bacteroidetes ($p<0.0001$), Chloroflexi ($p<0.0001$), Gemmatimonadetes ($p<0.0001$), Nitrospirae ($p<0.0001$), Planctomycetes ($p<0.0001$), Verrucomicrobia ($p<0.0001$), and a lower amount of Cyanobacteria ($\mathrm{p}<0.0001$) and Firmicutes ($p<0.0001$) (Figure 3). The representative sequences in the BLAST search results matched to Cyanobacteria genera at ~85% identity on average with good E-values.

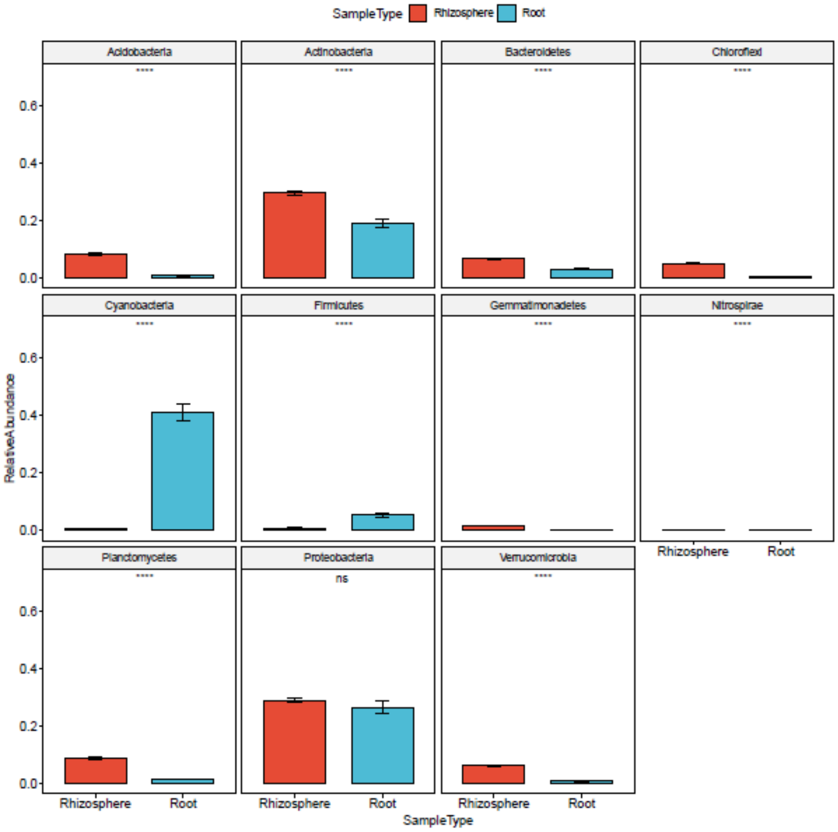

Figure 3. Relative abundances of genera (*Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Firmicutes*, *Gemmatmonadetes*, *Nitrospirae*, *Planctomycetes*, *Proteobacteria*, *and Verrucomicrobia*). Ten were significantly different between root and rhizosphere samples. Standard errors are represented in the figure by the error bars attached to each column.

The Streptomyces genus was the most abundant genus identified, but did not differ in abundance between roots and rhizosphere. Of the remaining 284 detected low-abundance genera, there were several differences between the root and rhizosphere. Here we will only mention the seven most abundant: Kaistobacter (p<0.0001), Rhodoplanes (p<0.0001), and Rubrobacter (p<0.001) were more abundant in the rhizosphere, while Agrobacterium

($p<0.0001$), Bacillus ($p<0.001$), and Rhizobium ($p<0.0001$) were more abundant in the roots.

Rhizosphere by Treatment. Nine phyla dominated the rhizosphere samples, with Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Gemmatimonadetes, Planctomycetes, Proteobacteria, Firmicutes, and Verrucomicrobia accounting for 95% of each sample. Thirty-three additional phyla were present, but not dominant in the rhizosphere of both UT and ST treatments. There were significant differences in the relative abundance of Gemmatimonadetes ($p<0.01$) with more abundance occurring in UT rhizosphere soil compared to ST rhizosphere soil.

Fungal Composition

The total fungal composition of the treatment and control plot soils were not different from each other ($p>0.05$) in February and April. All OTUs belonged to 4 orders, 9 families, and 11 genera within the phylum Glomeromycota. We found the following 11 genera: *Acaulospora*, *Ambispora, Archaeospora, Claroideoglomus, Diversispora, Entrophospora, Geosiphon, Glomus, Kuklospora*, *Paraglomus*, and *Scutellospora*. In UT treatment plots we found a larger abundance of *Glomus* ($p<0.05$), while in ST plots we found a larger abundance of *Ambispora* ($p<0.05$) and *Geosiphon* ($p<0.05$) (Figure 4).

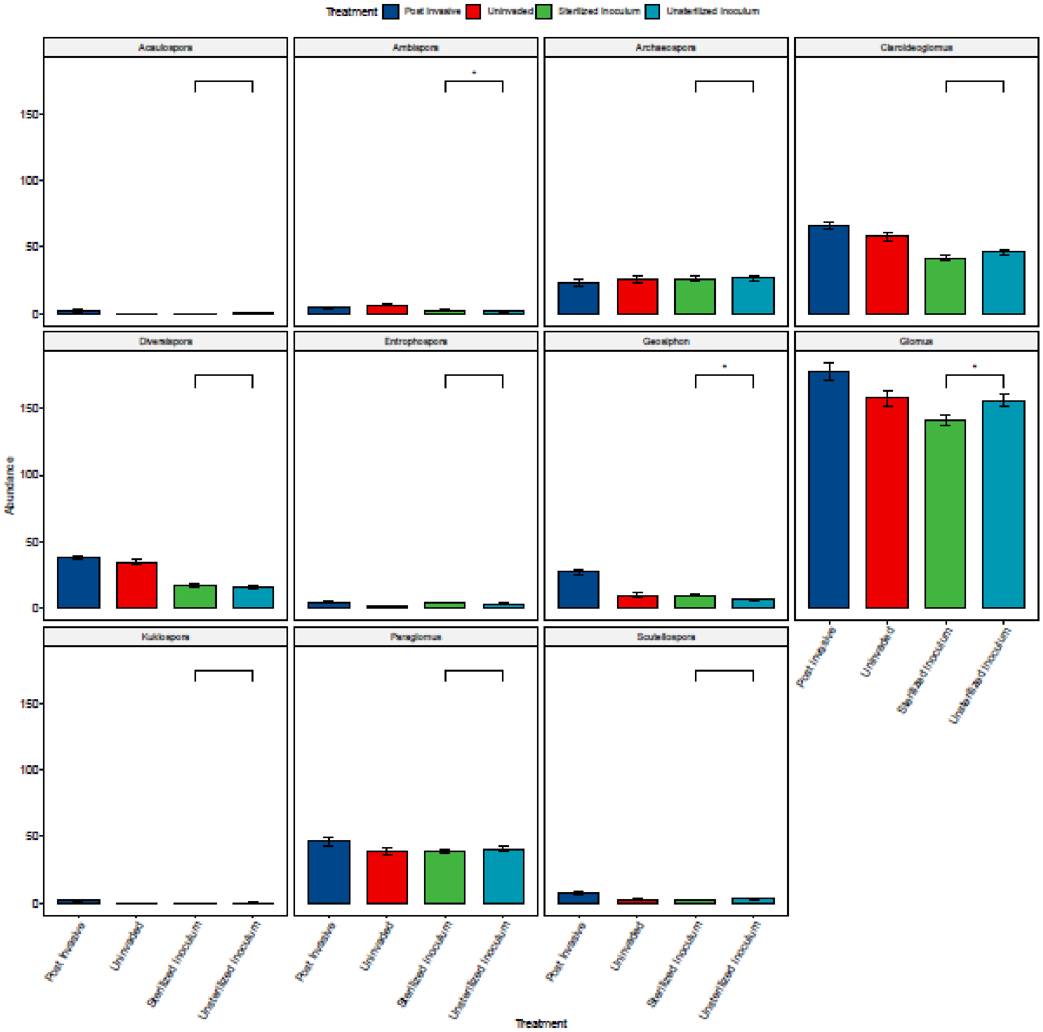

Figure 4. Relative abundances of genera (*Acaulospora*, *Ambispora*, *Archaeospora*, *Claroideoglomus*, *Diversispora*, *Entrophospora*, *Geosiphon*, *Glomus*, *Kuklospora*, *Paraglomus*, and *Scutellospora*). Three were significantly different between treatment plots. Standard errors are represented in the figure by the error bars attached to each column.

Plant Growth Trends

After about eight months of growth in the field (February-August), there was a significant correlation between plant growth and soil type that was species-specific (*p*<0.01). The total seedling length for all species ranged from 9cm-66.5cm and the total biomass ranged from 1.76g-21.99g in the last month of growth. The *A. californica* (*p*<0.05) (Figure 5) and *B. pilularis* (*p*<0.01) (Figure 6) had a larger length in the UT plots compared to the ST plots. *Salvia leucophylla* was not affected by inoculum type.

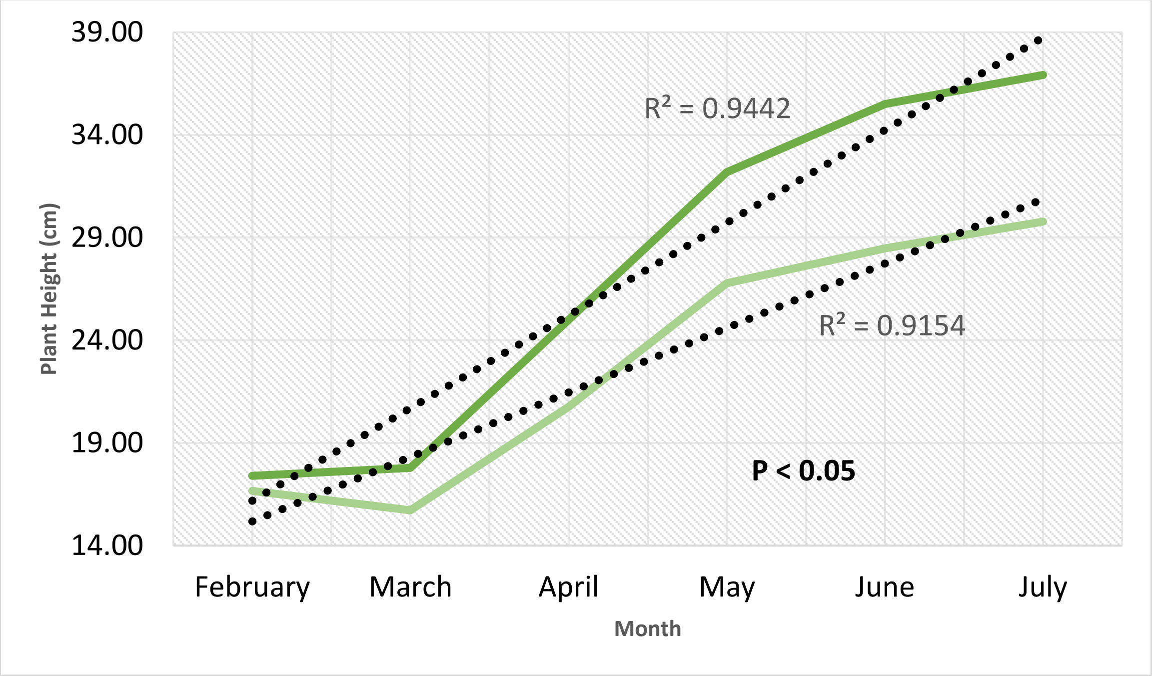

Figure 5. *Artemisia californica* plant height with unsterilized (dark green) compared to sterilized (light green) uninvaded soil inoculum.

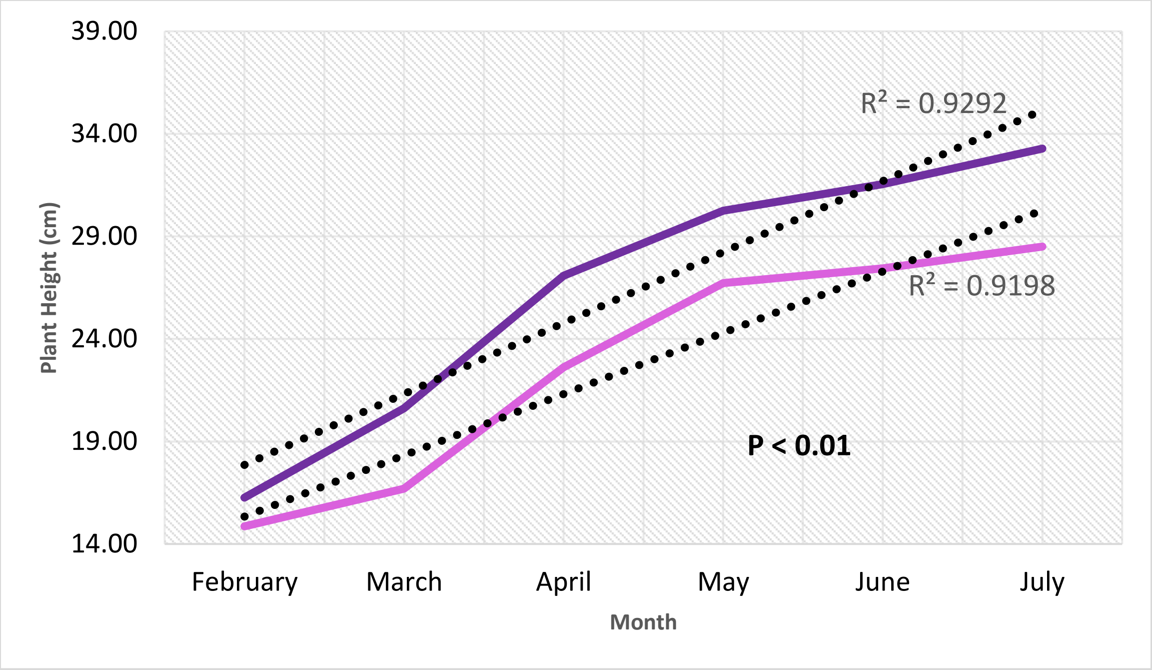

Figure 6. *Baccharis pilularis* height with unsterilized (dark purple) compared to sterilized (light purple) native soil inoculum.

After about five months of growth (Sep. 6, 2013-Jan. 22, 2014) in the cone-tainers, before transplant into the post-invasive site, 18 of the *A. californica* grown with ST, 7 *A. californica* grown with UT, and 1 *B. pilularis* grown with ST died. During growth in the field, 6 *A. californica* grown in ST plots and 1 grown in UT plots died, while 1 *S. leucophylla* grown in a ST plot and 1 in a UT plot died.

Discussion and Conclusion

We found significant differences in native plant growth and the composition of microbial taxa between UT and ST plots, indicating that the post-invasive microbial community in the ST plots is different than that of the uninvaded soil community in the UT plots in ways that impact plant growth.

Bacterial Composition

Roots and Rhizosphere. The root and rhizosphere communities of the 30 destructively sampled plants were different in their overall microbial composition. This is most likely due to the lack of selective pressure for microbial species in the rhizosphere compared to the roots. The most striking difference was the large abundance of cyanobacteria in the roots compared to the rhizosphere soils. Cyanobacteria are photosynthetic bacteria and the only prokaryotes able to produce oxygen. Some are N-fixing and live in a symbiotic relationship with plants or fungi (Dodds et al. 1995). The large amount of cyanobacteria and Firmicutes in these roots suggests that these bacteria are very important for the growth of native shrubs and gives us more insight into the healthy functioning of these systems. We must keep in mind, however, that Cyanobacteria are most commonly found in moist soils, not arid soils such as these. It could be that this finding is due to database bias and not an actual trend. In order to check this, we performed a BLAST search using the NCBI BioSystems Database. Since each of the reference sequences showed a similar high percentage match to multiple Cyanobacteria genera, this is a good indication that the OTUs genuinely represent Cyanobacteria. Such a finding is novel and deserves further investigation.

Rhizosphere by treatment. The only significant difference between soil treatments was the abundance of *Gemmatimonadetes*, a phylum well adapted for living in arid, exposed soils (DeBruyn et al. 2011). It may be that this phylum is indicative of a healthy soil community, so its lower abundance in the ST soil may be a sign of declining soil health.

Fungal Composition

Glomus is the largest genus of AMF, comprising ~85 species (Kirk et al. 2008). They are obligate symbionts that are dependent on plant roots for their survival. Not much is known about the particulars of *Ambispora,* but it is known to form mycorrhizal associations with plants. *Geosiphon*, however, does not form mycorrhizal associations with plants, but instead forms an endosymbiotic relationship with cyanobacteria (Schussler 2002), which we found in high abundance in native plant roots. This increased abundance of *Glomus* in the UT plots may be the reason we observed greater *A. californica* and *B. pilularis* plant growth. It may be that these native species depend on *Glomus* in particular for survival, therefore, the lack of *Glomus* in the post-invasive soil would decrease their growth and possibly prevent them from naturally establishing in the site.

It is possible that these observed trends are due to abiotic changes that were not measured, presence of glyphosate in the soil, or other biotic changes such as shifts in pathogenic or saprotrophic fungi. Glyphosate has a half-life of approximately two weeks and does not have residual soil activity on the seedbank. Additionally, several years had elapsed between herbicide treatment and transplant of seedlings into the site. As far as unmeasured abiotic changes, all plants were subject to the same soil additions, sun exposure, moisture, slope aspect, soil texture (Mipolomol), pH, and elevation. We did not observe differences in either plant growth or microbial composition by block. It is most likely that the microbial changes brought on by Harding grass are responsible for the trends observed in this study.

It is also possible that this study was not able to grasp the full scope of alterations to the soil microbial community. When soil core sampling occurred we took cores at the drip zone of the plant, hoping to capture both rhizosphere and bulk soil communities. However, it may be that the surrounding bulk soil overwhelmed the minute differences between rhizosphere soils in the UT compared to ST plots. This may be why we observed differences by treatment (UT or ST) only in the rhizosphere of the destructively sampled plants, but not in the soil cores taken in February and April. In the future, it may be best for studies such as this to either sample closer to the roots or sample plants throughout the experiment, rather than just at the end.

Although this study shows evidence that exotic grass invasions, and their subsequent removal, have lasting impact on above and belowground ecosystems, it has some spatial limitations. For instance, we collected our “uninvaded soil” from one site in southern California that was intact and replete with native vegetation and conducted the main body of the study in one “post-invasion” site. Therefore, our conclusions are limited solely to this ecosystem. However, our previous greenhouse experiment revealed that there were some sort of microbial legacy effects from the invasive grass in this particular site and we observed reduced native plant performance after *P. aquatica* removal over several years prior to conducting this field experiment.

Conclusions

Previously, during the greenhouse project mentioned earlier, we were able to show that there are soil legacy effects left behind by the invasive *P. aquatica* in the study site that affect the growth of some CSS native plants. Now we understand that these legacy effects have a definite microbial component. There are several short-term studies that show invasive plants can alter the soil biotic community (Reinhart and Callaway 2006) and several others that suggest there may be some long-term biotic legacy effects. Here, we show that there are definite changes in the bacterial and fungal composition of soils that persist for years after an invasive plant has been removed and that these changes can alter native plant growth.

Our findings indicate that differences in native plant growth can be explained by changes in the soil microbial community and remediation of the soil microbial community through inoculation can improve restoration in post-invasive sites. This means that we can improve native plant establishment by inoculating seedlings with intact uninvaded soil or perhaps with certain genera of AMF (such as *Glomus*) that are important to the survival of native species in the specific study site.

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