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UNIVERSITY OF CALIFORNIA  
SANTA CRUZ

**DEVELOPMENT AND PERSISTENCE OF SOIL LEGACY EFFECTS OF AN  
INVASIVE SHRUB AND IMPLICATIONS FOR REFORESTATION**

A dissertation submitted in partial satisfaction  
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Ecology and Evolutionary Biology

by

**Sara E. Grove**

June 2014

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## Abstract

### Development and persistence of soil legacy effects of an invasive shrub and implications for reforestation

Sara E. Grove

Invasive species can change the environment in ways that persist long after the species itself is gone, called a “legacy effect.” Legacy effects of invasive species include changes to the abiotic and biotic soil environment and can lead to positive feedbacks that inhibit restoration success and reinforce invasion. The objective of this research was to examine the legacy of *Cytisus scoparius*, an aggressive nitrogen-fixing shrub, on soil chemistry, ectomycorrhizal fungi, and Douglas-fir forest regeneration. My work highlights the importance of allelopathy and nitrogen enrichment in the disruption of the mutualism between Douglas-fir and ectomycorrhizal fungi.

The temporal aspects of biological invasions are important and understudied. The impacts of invasive species may change in magnitude and even direction with time following invasion. In a novel contribution, I used a combination of field and greenhouse experiments to determine how soil legacies develop, persist and change over time.

I found that soils invaded by *C. scoparius* harbor less ectomycorrhizal fungi, and that growth of Douglas-fir is linked to the abundance of these fungi. Adding *C. scoparius* mulch into uninvaded forest soils revealed that Douglas-fir grew more when mulch was added, but only in the presence of activated carbon, suggesting that allelopathy may be inhibiting Douglas-fir success.

To examine how long soil legacies persist after *C. scoparius* removal, I removed *C. scoparius* at different times over a 22-month period, then measured soil properties. One

month following *C. scoparius* removal, nitrogen availability increased, but by 10 months, dropped to less than what was found in live *C. scoparius* stands, suggesting that the legacy of nitrogen enrichment is not stable and declines with time following removal. Following *C. scoparius* removal, exotic grasses quickly established and dominated the sites, and they may contribute to the suppression of Douglas-fir seedling growth. I also used a chronosequence approach to assess how quickly soil-mediated impacts develop after invasion. I planted Douglas-fir into soils collected from *C. scoparius* invasions of different ages. Surprisingly, the seedlings grew larger in soils that had been invaded longer; in this case nitrogen fertilization seems to be more important than other effects of *C. scoparius* invasion.

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## INTRODUCTION

Invasive species can cause dramatic changes to both the physical and biotic environment, and these changes often result in the loss of native species, shifts in species dominance, and changes in resource availability (Williamson 1996, Parker et al. 1999, Ehrenfeld 2003, Lambertini et al. 2011). Vast effort and resources are spent to control invasive plants. Usually we make the assumption that once these resources are spent and the invader is successfully removed, the impact of that species on the community is also eliminated. The objective of my dissertation is to understand how the impacts of an invasive species change with time following colonization, and how long these impacts persist as soil legacy effects following invader removal. I measured how allelopathy, soil nutrient availability, and local mycorrhizal fungi change as a result of invasion, and how these factors effect restoration. Providing relevant information to invasive species managers to inform an important conservation problem was an additional aim of this research.

In some cases, the changes that result from biological invasion may represent an alternative stable state that is dominated and maintained by the invader (Paine et al. 1998, Scheffer and Carpenter 2003, Suding et al. 2004, Kulmatiski 2006). Thus changes wrought by invasive species can persist and hinder native species from reoccupying the habitat even after the invader is removed (Vitousek et al. 1987, Maron and Jefferies 2001, Corbin and D'Antonio 2004b). Because of this legacy effect, standard restoration practices may fail to recover the ecosystems to

preinvasion conditions (Bakker and Berendse 1999, Zedler 2000, Hobbs and Harris 2001).

The temporal aspects of biological invasions are important and understudied. The impacts of invasive species may change in magnitude and even direction with time following invasion (Strayer et al. 2006). In addition, while it is commonly accepted that legacy effects of invasion occur, they are often assumed rather than demonstrated. We have few definitive examples of systems where invasive species were removed, but their impacts were documented to persist, and even fewer examples where the quantitative trajectory of impacts was followed over time following removal.

The soil legacies of invaders may likely involve biotic interactions such as plant-fungal interactions, which are important drivers of plant composition and diversity (Bever 1994, van der Heijden et al. 1998, Hartnett and Wilson 1999, Klironomos 2002, 2003, Reynolds et al. 2003). Mycorrhizae are a ubiquitous coevolved mutualism between plants and fungi. Ectomycorrhizal fungi (EMF) sheath the surface of fine plant roots and extend into the soil to access water and mineral resources that are otherwise inaccessible to their hosts (Smith and Read 1997); their role in phosphorus and N acquisition is of particular importance (Wallenda and Kottke 1998, Read and Perez-Moreno 2003). Invasive plants may reduce the abundance and richness of local mycorrhizal fungi and thereby disrupt positive feedbacks between native plants and their associated fungal symbionts (Richardson et

al. 2000, Bever 2003, Levine et al. 2006, Mitchell et al. 2006, Wolfe et al. 2008, Pringle et al. 2009, Vogelsang and Bever 2009).

In addition to displacing the host species, invasive plants could disrupt the mycorrhizal mutualisms of native plants through a number of possible mechanisms, which include changes in soil chemistry and nutrient pools/fluxes. Allelopathy, in which plants produce toxic compounds that give them an advantage over competitors, has received much attention recently as part of the Novel Weapons Hypothesis of invasion success (Bais et al. 2003, Callaway and Ridenour 2004, Vivanco et al. 2004, Jarchow and Cook 2009, Lankau et al. 2009, Inderjit et al. 2011). Mycorrhizal fungi that did not evolve in the presence of these toxic compounds may also be suppressed by them (Perry and Rose 1983, Rose et al. 1983, Schreiner and Koide 1993), with possible negative effects for their plant partners. For example, the invasion of *Alliaria petiolata* reduces colonization by EMF, resulting in depressed tree seedling recruitment (Artursson et al. 2005, Stinson et al. 2006, Wolfe et al. 2008). The *A. petiolata* system is well studied and has shown definitively that invaders have the potential to suppress mycorrhizal mutualisms. However, it remains unknown how general this phenomenon may be.

Another possible mechanism for the disruption of mycorrhizal mutualisms is through changes in soil nutrient pools (Treseder 2004). Lilleskov (2008) found that communities of ectomycorrhizal fungi changed in response to atmospheric N deposition, and Egerton-Warburton et al. (2007) showed a decline in the abundance and diversity of mycorrhizal fungi after experimental N-fertilizer addition in

grasslands. Invasive species that alter nutrient pools through N-fixation have been shown to produce long-lived legacy effects on soil biogeochemistry and plant community composition (Vitousek 1990, Corbin and D'Antonio 2004a). After the removal of an N-fixing invader it can take several years for nitrogen and carbon pools to return to preinvasion conditions (Maron and Jefferies 2001, Marchante et al. 2008, Von Holle et al. 2013). Nitrogen addition by invasive plants may suppress or alter mycorrhizal communities in the same way as atmospheric deposition and artificial fertilization does. Nitrogen enrichment would be expected to reduce mycorrhizal dependence and lead to a loss of particular fungal species (Johnson 1993).

For my dissertation research I examined how quickly soil-mediated impacts develop after invasion of the ecosystem, and also whether and how quickly the system returns to its previous state after removal of the invader. Specifically, I studied the degree to which *C. scoparius* invasion affects the mycorrhizal mutualism of Douglas fir, the temporal extent of these effects, and the mechanisms involved. I quantify the decline of ectomycorrhizal fungi in *C. scoparius*-invaded clearcuts and examined the contribution of three not mutually exclusive explanations: the absence of a suitable host, soil N enrichment, and allelopathy. The overall purpose of my research was to determine if changes in the biotic soil environment caused by *C. scoparius* invasion have consequences for the regeneration of forests.

Douglas-fir (*Pseudotsuga menziesii* var. *menziesii*) is native to western North America and is an economically important timber species in the US (Fowells 1965, Arno and Hammerly 1977). Ectomycorrhizal fungi are ubiquitous in Douglas-fir



forests, and Douglas-fir seedling establishment can depend critically on the presence of ectomycorrhizae (Molina et al. 1992, Nara 2006). Though clearcutting is the most economical practice for commercial logging, it has profound ecological consequences, including the reduction of mycorrhizae in the absence of host trees (Perry et al. 1987, Hagerman et al. 1999), and the optimal habitat it creates for the invasion of exotic species (Deferrari and Naiman 1994, Davis et al. 2000).

*Cytisus scoparius* (Fabaceae, Scotch boom) is a N-fixing legume, native to Europe, and is one of the most problematic invasive weeds in the Pacific Northwest (Peterson and Prasad 1998, Prasad 1998). It was introduced to the US over 200 years ago and by the 1930s it was recognized as a major problem in the west (Mack 2003). *Cytisus scoparius* does not associate with ectomycorrhizal fungi, and (at least) in its native range it forms arbuscular mycorrhizal associations (Wang and Qiu 2006). *Cytisus scoparius* leaf and shoot tissue contains quinolizidine alkaloids (Wink et al. 1983, Wippich and Wink 1985), and as an N-fixer it is known to increase N availability (Dancer et al. 1977, Wheeler et al. 1987, Diquelou and Roze 1999, Fogarty and Facelli 1999, Haubensak and Parker 2004, Caldwell 2006). I hypothesize that the alkaloids and N-enrichment that accompany *C. scoparius* invasion alter biotic interactions, and ultimately impact Douglas-fir forest restoration.

In western Washington, *C. scoparius* invasion and control is a major problem for forest management. In both 2008 and 2009, we planted 7500 trees into 5 separate clearcuts that had been invaded by *C. scoparius*, and within six months over 90% of the trees died in both years (Parker and Haubensak 2011). The *C. scoparius* was

entirely removed before trees were planted, so direct competition for resources was essentially eliminated and cannot explain the high mortality rates of the Douglas-fir seedlings. These results led us to speculate that *C. scoparius* might have indirect effects on Douglas-fir regeneration, effects that remain even after the invader is removed. This speculation is supported by previous work of Haubensak and Parker (2004) who showed inhibitory effects of *C. scoparius* soil on biomass of a small herbaceous species in nearby prairie systems of this region.

#### *Dissertation chapters*

In chapter one, I used a factorial greenhouse experiment to test for this soil legacy effect. I planted Douglas-fir seedlings into *C. scoparius* invaded and nearby uninvaded forest soils that were collected from the vicinity of the failed plantations. Douglas fir seedlings grown in *C. scoparius* invaded soils were smaller than seedlings grown in uninvaded forest soils and had less EMF colonization. I also added *C. scoparius* litter to uninvaded forest soils and found an intriguing pattern that Douglas-fir grew more when mulch was added, but only in the presence of activated carbon. Activated carbon binds organic molecules and may mitigate allelopathy. These results indicate that *C. scoparius* invasion alters the soil so that it is less suitable for Douglas-fir growth, and this effect could be mediated by the disruption of the ectomycorrhizal mutualism.

In Chapter two, I conducted a field experiment to examine how long soil legacies persist after the removal of *C. scoparius*. I removed *C. scoparius* at different times over a 22-month period and allowed areas to be *C. scoparius*-free for 0, 1, 10, and 22 months before planting Douglas-fir seedlings and measuring soil properties. To evaluate how soil legacy effects influenced the restorability of invaded areas, I measured nutrient availability at the time of tree planting, the survival and growth of Douglas-fir across time since *C. scoparius* removal treatments, and the abundance of exotic grasses and forbs. One month following *C. scoparius* removal, as the leaf and shoot tissues decomposed, soil nitrogen availability spiked, but by 10 months after removal N availability dropped to less than what was found in untreated intact *C. scoparius* stands. Even though mineralized N decreased with time following the removal of *C. scoparius*, the concentrations in the soil at 22 months post removal were still high relative to uninvaded Douglas-fir forest soils in the region, which are considered to be highly N limited. Following *C. scoparius* removal, exotic grasses and forbs quickly established and dominated the sites. With time following *C. scoparius* removal, the abundance of these exotics continued to increase and may have also contributed to the suppression of Douglas-fir seedling growth.

In chapter 3, I used a chronosequence approach to assess how quickly soil-mediated impacts develop after *C. scoparius* invades Douglas-fir clearcuts. I collected soils from invaded and uninvaded patches within clearcuts of different ages. Sites ranged from 3-31 years since they were last harvested, and clearcut age was a rough proxy for duration of invasion. In the greenhouse, I planted Douglas-fir seeds into

each of these soils and measured seedling growth, ectomycorrhizal root colonization, and leaf nitrogen content. Surprisingly, Douglas-fir grew larger in invaded soils relative to uninvaded soils from the same clearcut, and Douglas-fir seedlings grew slightly larger as the age of clearcut increased. The positive effect of *C. scoparius* invasion and invasion duration on Douglas-fir growth appears to be a result of N-enrichment associated with *C. scoparius*. Ectomycorrhizal root colonization was suppressed by *C. scoparius* invasion, but there was no evidence that reduced colonization reduced Douglas-fir growth in invaded soils. However, the abundance of ectomycorrhizal root colonization positively correlated with seedling growth in uninvaded soils. Another surprising finding was that the duration of time in the absence of ectomycorrhizal host plants (ie Douglas-fir) did not affect the abundance of ectomycorrhizal colonization of seedlings in the uninvaded soils, indicating that ectomycorrhizal spores and propagules of at least some species are either very long lived or are readily dispersed into clearcuts. Another unexpected finding was that Douglas-fir seedlings grew better in soils from areas that had been deforested longer, suggesting that in more recently harvested soils pathogen pressure could be higher.

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## CHAPTER 1

### **Allelopathy and nitrogen enrichment contribute to soil legacy effects following an exotic plant invasion**

#### **Abstract**

Invasive species may leave behind legacies that persist even after removal, inhibiting subsequent restoration efforts. We examined the soil legacy of *Cytisus scoparius*, a nitrogen-fixing, putatively allelopathic shrub invading the western US. We tested the hypothesis that allelopathy plays a critical role in the depressive effect of *Cytisus* on the key native Douglas-fir, both directly on tree growth and indirectly via effects on its ectomycorrhizal fungi (EMF). In a greenhouse factorial experiment, we used activated carbon to inhibit *Cytisus*-produced allelochemicals and sucrose to reduce elevated nitrogen (N). We found that: 1) *Cytisus*-invaded soils depressed Douglas-fir growth compared to uninvaded forest soils, despite the N fertilization effect. The effect of adding *Cytisus* litter was positive only in the presence of activated carbon, providing evidence for a role of allelopathic compounds. Activated carbon did not increase growth in the absence of *Cytisus* litter. Sucrose addition provided only weak support for the positive effect of *Cytisus* litter. 2) Seedlings grown in *Cytisus* soils had lower EMF abundance compared to those in uninvaded forest soils. In forest soil from one site, adding *Cytisus* litter also decreased EMF abundance. Douglas-fir growth increased significantly with EMF across sites and soils, suggesting that changes in EMF were linked to tree growth. The fungal taxon *Cenococcum geophilum* was significantly depressed in *Cytisus* soils compared to

forest soils, while *Rhizopogon rogersii* abundance was similar across soil types.

These results together suggest an overall negative effect of *Cytisus* on the growth of a dominant native tree and its fungal symbionts.

## **Introduction**

Great effort and resources are spent to control invasive plants, usually under the assumption that once the invader is removed, the impact of that species on the community is also eliminated. However, abiotic and biotic changes during invasions may result in loss of native species, changes in community composition and structure, and changes in resource availability and nutrient cycling (Vitousek et al. 1987, Maron and Jefferies 2001, Rothstein et al. 2004, Suding et al. 2004, Asner et al. 2008, Rascher et al. 2011). These changes can persist and hinder native species from reoccupying these habitats even after the invader is removed, known as a ‘legacy effect’ (Maron and Jefferies 2001, Corbin and D'Antonio 2004b). Soil legacies of invasive plants can include elevated nitrogen (Vitousek et al. 1987, Evans et al. 2001, Dougherty and Reichard 2004), carbon storage (Christian and Wilson 1999), soil salinity (Vivrette and Muller 1977, D' Antonio 1993), and changes in soil chemistry via allelopathy (Inderjit et al. 2006, Pollock et al. 2008).

Allelopathy, in which plants produce toxic compounds that give them an advantage over competitors, has received much attention recently as an explanation for the invasion success of certain introduced species. (Bais et al. 2003, Callaway and Ridenour 2004, Vivanco et al. 2004, Pollock et al. 2008, Jarchow and Cook 2009,

Lankau et al. 2009). Allelochemicals released by invaders are thought to be especially detrimental to native plants in their introduced range, because they lack an evolutionary history with the invader and therefore may have fewer mechanisms to counter the toxicity of allelochemicals (Callaway and Aschehoug 2000, Bais et al. 2003, Fitter 2003, Hierro and Callaway 2003). A legacy effect driven by allelopathy can occur if allelochemicals accumulate in the soil and continue to have negative effects on the establishment and growth of native vegetation (Bais et al. 2003, Hierro and Callaway 2003, Lankau 2010). This type of legacy may also indirectly affect plant growth because allelochemicals can disrupt microbial mutualisms such as mycorrhizal associations (Perry and Rose 1983, Rose et al. 1983, Schreiner and Koide 1993). For example, abundant evidence suggests that the invader *Alliaria petiolata* (garlic mustard) causes depressive effects on the mycorrhizae of native species through its production of mustard oils (Schreiner and Koide 1993, Roberts and Anderson 2001, Kliebenstein 2004). However, to our knowledge no studies have examined the legacy of indirect impacts on native species via persistent changes in the soil biota.

*Cytisus scoparius* (hereafter *Cytisus*) is considered one of the most invasive species in western North America, with widespread distribution in California, Washington and Oregon (Peterson and Prasad 1998, Isaacson 2000). Invasion by *Cytisus* into managed forests in the Pacific Northwest has resulted in the loss of more than \$40 million annually in timber revenue and control expenses in the state of Oregon alone (Hulting et al. 2008). *Cytisus* is a nitrogen (N)-fixer and can enrich soil

N pools (Helgerson 1979, Wheeler et al. 1979, Wheeler et al. 1987). It also produces high concentrations of alkaloids, primarily sparteine (Wink et al. 1983, Gresser et al. 1996, Wink 2002). Sparteine has been demonstrated to provide defenses against herbivores (Wink et al. 1982) and to inhibit seed germination in some species (Wink 1983). However, there is surprisingly little known about the ecological effects of quinolizidine alkaloids in general, and of *Cytisus*-produced compounds specifically.

Throughout much of the Pacific Northwest, Douglas-fir (*Pseudotsuga menziesii*) is both the dominant native and the primary harvested species (Curtis and Carey 1996). In previous work, we showed that Douglas-fir seedlings planted into clearcut sites that had been long-invaded by *Cytisus* experienced drastic mortality despite the absence of *Cytisus* (Parker and Haubensak 2011). This, taken with prior work that showed inhibition of target native species grown in *Cytisus* soil (Haubensak and Parker 2004), strongly suggested a negative soil legacy of *Cytisus* invasion.

This study was designed to examine the soil-based legacy of *Cytisus* on the growth of Douglas-fir. We expected that the net impact of *Cytisus* on Douglas-fir would integrate a positive effect of nitrogen fertilization and a negative effect of allelochemistry, possibly mediated by mycorrhizae. To test for the effect of nitrogen enrichment, we added sucrose to reduce soil available N (Blumenthal et al. 2003). To test for the effect of allelopathy, we used activated carbon (AC) to adsorb organic compounds, such as the alkaloids released during the breakdown of *Cytisus* litter (Mahall and Callaway 1992, Wardle et al. 1998, Ridenour and Callaway 2001, Kulmatiski and Beard 2006).

Based on the poor performance of Douglas-fir in *Cytisus*-invaded field sites, we predicted that 1) Douglas-fir seedlings grown in *Cytisus* invaded soils would be smaller than seedlings grown in uninvaded forest soils, and 2) adding *Cytisus* litter to uninvaded forest soils would decrease Douglas-fir seedling growth. We expected that 3) adding sucrose would reduce the N fertilization effect of both *Cytisus*-invaded soils and soils amended with *Cytisus* litter. We also predicted that adding activated carbon would ameliorate allelopathic effects, and therefore 4) the effect of adding *Cytisus* litter would be positive when combined with activated carbon. We further expected that the negative effects of *Cytisus* on Douglas-fir growth would be accompanied by the suppression of ectomycorrhizal fungi (EMF), and so 5) colonization by EMF would be lower on Douglas-fir seedlings grown in *Cytisus* invaded soil or with *Cytisus* litter. If allelochemicals mediate this reduction in EMF, 6) the addition of activated carbon should ameliorate the suppression of EMF by *Cytisus* litter.

## **Methods**

We examined the effects of *Cytisus* on Douglas-fir growth and its associated EMF in a fully randomized factorial greenhouse experiment. In November 2008, we collected soils from two pairs of sites in the south Puget Sound region of western Washington. The two pairs were on opposite sides (hereafter referred to as “East” and “West”) of Joint Base Lewis-McChord (JBLM). Each pair included a Douglas-fir forest (47°03’12N, 122°40’47W and 47°02’28N, 122°29’30W) and a nearby clearcut that had been heavily invaded by *Cytisus* (47°02’51N, 122°40’01W and 47°02’01N,

122°31'51W). Organic material was scraped off the surface of the mineral soil; regular spades were used to excavate the top 30 cm of mineral soil, which was passed through a 1.61 cm<sup>2</sup> sieve. Collections were made from at least 10 holes with diameter of approximately 0.5 m, across an area of approximately 1 ha within each site. We transported the soils to the greenhouse at the University of California, Santa Cruz, where collections from each site (east forest, west forest, east *Cytisus*, and west *Cytisus*) were homogenized; we added soil amendments and homogenized again before dividing and distributing into D60 Deepots (6.4 cm x 36 cm, Stuewe and Sons, Tangent, OR).

The three soil amendments were *Cytisus* litter, activated carbon, and sucrose. Each of these amendments was added to the four soil types (west and east forest and west and east *Cytisus*) in all possible combinations for a total of 32 different soil-treatment combinations. For the *Cytisus* litter addition, we collected fragments of green branches of *Cytisus* from stands of mature plants with a hedge-trimmer at the same time as the soil collections. The plant material was then cut by hand into approximately 2-5 cm segments; diameters of fragments did not exceed 3 mm. The leaf and stem material has high concentrations of sparteine, an N-based quinolizidine alkaloid (Haubensak and Parker, unpublished data). We refer to this material as 'litter' while acknowledging that it did not senesce and fall to the ground before collection. *Cytisus* litter decomposes rapidly (Haubensak 2001) and alkaloids degrade quickly (Gross and Wink 1986), so in order to evaluate a chemistry effect of *Cytisus* we used green material.



To test for allelopathic effects of *Cytisus* litter on Douglas-fir seedlings, we added activated carbon (“AC,” Marineland Aquarium Products, Cincinnati, OH). We ground the AC into a fine dust with a mortar and pestle and added it at a rate of 1% w/v, or 10 cm<sup>3</sup> per liter of soil.

Sucrose was added to examine whether soil N enrichment by *Cytisus* increases Douglas-fir growth. Sucrose is a readily available form of carbon and increases N immobilization by microbes, thereby decreasing plant-available N (Blumenthal et al. 2003). We added 1.52 g of sucrose per L, based on a 200 g C m<sup>-2</sup> addition rate (Blumenthal et al. 2003). Sucrose was added once at the start of the experiment.

Within two weeks of soil collection, we planted 420 one-year-old Douglas-fir seedlings into the array of soil amendment treatment combinations described above (N = 15 for each treatment). Seedlings were purchased from Silvaseed (Roy, Washington) and were initially grown in potting soil from seed collected from mature trees from two distinct seed zones in Western Washington (seed zone 030-05 and 422-15). The (030-05) seed zone is near the town of Hoquiam, which is a coastal zone at a slightly lower elevation than JBLM. The (422-15) seed zone is in Ashford, where environmental conditions and elevation are more similar to those found at JBLM. For each treatment/soil combination, the 15 replicates were split between 8 (422-15) trees and 7 (030-05) trees. Seed zone was included as a random factor in all analyses. Eight seedlings were inspected and found to have no mycorrhizae at the time of planting.

To prevent mycorrhizal cross-contamination between soil treatments, we sterilized all containers and potting tools in a 10% bleach solution immediately prior to planting and the potting tools were treated again between handling a given soil type. The Douglas-fir seedlings were planted into their respective soil treatments and capped off with approximately 2 cm of 30 grit sand to reduce the splash of ectomycorrhizal spores during watering. We watered the trees from above to further reduce cross contamination between individual pots. The seedlings were left to grow outdoors in the natural light and temperature conditions for 19 months.

Beginning in December 2008 we measured seedling height every 10 weeks through the spring of 2010. On April 23, 2010 we measured the final tree heights and calculated growth rates. At that time we harvested the aboveground biomass of all surviving seedlings. We cut each tree at the root crown, placed them into paper envelopes and dried them at approximately 61° C for up to 24 days after which they were weighed on an analytical balance. Height and biomass patterns were similar therefore we only report biomass results here.

During final harvest of Douglas-fir seedlings, we collected subsamples of soils from control (unamended) forest and *Cytisus* soils for nutrient analyses. These subsamples were dried at 105°C for 24 hours, ground with mortar and pestle, then sent to A and L Western Laboratories (Modesto, CA) for the following analyses: soil organic matter, plant-available P (Olsen-Bray), percent N, nitrate pool size, cations, and pH.

### ***Ectomycorrhizal abundance and identity***

We measured the abundance of ectomycorrhizal fungi on 120 trees across both east and west soils and across four of the experimental soil treatments: 1) unamended *Cytisus* soils 2) unamended forest soils, 3) forest soils with *Cytisus* litter addition and 4) forest soils with both *Cytisus* litter and AC additions. We measured a minimum of 56 randomly selected root tips on each of 15 replicate trees. To prepare the roots for microscopy, we gently shook off the excess soil and briefly soaked the entire root mass in a bath of DI water. We divided the root mass longitudinally into approximately equal portions, 2-3 depending on the size of the root mass. We randomly selected which of the sections would be analyzed for mycorrhizal colonization. The roots selected for mycorrhizal assessments were further cleaned in a series of water baths, where the roots were agitated to remove the soil. Occasionally we very gently used our hands and/or dissecting instruments to physically loosen the remaining persistent large soil aggregates. We took great care not to remove hyphae from the root tips. The roots selected for mycorrhizal assessment were cut with scissors into 3-6 cm fragments and stored in DI water at 3° C. Within seven days of the initial processing the roots were examined for EMF. To measure the abundance of EMF across soil treatments, the Douglas-fir root tips were placed into gridded petri dishes and the roots that terminated nearest to the corner of each of the grid cells were assessed for the presence or absence of EMF and each root tip was assigned a morphotype. To calculate the proportion of root tips colonized by EMF we divided the number of roots tips with EMF by the total number of root tips observed.

We extracted the fungal DNA from representative samples of each morphotype encountered. DNA was extracted from the fine root tips with a DNeasy plant mini kit (Qiagen). We amplified the ITS region of rDNA with fungal specific primers ITS-1F and ITS-4 (Gardes and Bruns 1993) and Green GoTaq master mix (Promega). The PCR product was cleaned with ExoSAP-It (USB Products) and through a series of ethanol precipitations. The cleaned PCR product was cycle sequenced on an ABI 3100 Sanger Sequencer (Applied Biosystems, Foster City, CA, USA). To obtain species identification we used Geneious (Drummond et al. 2009) to view, manually edit, create contigs and match our nucleotide sequences with the vouchered specimens in the NCBI GenBank database. Our assessments of the presence/absence of EMF were well supported by our sequence data. However, most morphotypes did not correspond to a single taxon, while two morphotypes corresponded consistently with a particular fungal taxon. For these two taxa, *Rhizopogon rogersii* and *Cenococcum geophilum*, we used microscopy to quantify individual colonization rates.

### ***Statistical analyses***

For soil nutrients, we used 2-way ANOVA to examine the fixed effects of ‘type’ (forest versus *Cytisus*) and ‘site’ (east versus west). Only unamended soils were analyzed, so the effects of *Cytisus* litter, sucrose, and AC were not included here.

First we tested for the significance of differences among our treatments overall, with a 4 factor (soil type / litter / sucrose / AC) factorial ANOVA model with seed

source as a random effect. East and west sites showed dramatically different patterns in soil nutrient parameters (see Results), which could strongly influence the effects of various treatments; therefore we analyzed east and west sites separately. The full models were highly significant for both the east and west (Table 1).

We then tested a series of *a priori* hypotheses using planned contrasts. We compared Douglas-fir seedling growth in *Cytisus*-invaded soil versus forest soil. We tested for an N fertilization effect by comparing soils with sucrose added to those with no sucrose added for 1) *Cytisus*-invaded soil, and 2) forest soil to which *Cytisus* litter had been added. To test for allelopathy in *Cytisus*-invaded soils, we compared activated carbon addition to no activated carbon. To test for an allelopathic effect of adding *Cytisus* litter while controlling for other potential positive effects of activated carbon, we used a 2-way factorial mixed model ANOVA with litter and activated carbon as fixed effects and seed source as a random effect.

Using ANOVA, we compared colonization of EMF across the four treatments for which we assessed EMF abundance: *Cytisus*-invaded soil, forest soil, forest soil with litter, and forest soil with litter and activated carbon. We used subsequent one-tailed t-tests (assuming unequal variances) to test these specific hypotheses: forest soil > *Cytisus*-invaded soil; forest soils without *Cytisus* litter > with *Cytisus* litter added; and forest soils with activated carbon added along with *Cytisus* litter > *Cytisus* litter added without activated carbon. These comparisons were done for overall EMF abundance, the abundance of *Rhizopogon*, and the abundance of *Cenococcum*. We normalized the

data using arcsine square-root transformations, but the *Cenococcum* data could not be normalized and was analyzed using a one-tailed, non-parametric Wilcoxon test.

We explored the relationship between Douglas-fir seedling growth and EMF colonization for the subset of trees for which we had assessed EMF, analyzing east and west samples separately. In order to avoid treatment effects, we used only the control (unamended) forest and *Cytisus* soils in this analysis, and a preliminary ANCOVA found no significant effect of soil type and no significant interactions (data not shown). Therefore, the two soil types were combined in a regression of aboveground biomass on EMF colonization. All statistics were performed with JMP v. 9.0.0 (SAS Institute 2010).

## **Results**

### **Soil nutrients**

For virtually all the soil parameters measured (Table 2), there was a significant site \* soil interaction term, suggesting that the differences between *Cytisus* and forest soils were contingent on site (Table 3). *Cytisus* soils were 60% lower in organic matter compared to forest soils in the west site, but no different in the east. *Cytisus* soils had higher % N than the forest soils in the east site (0.785 v 0.454%), but lower in the west site (0.365 v 0.523%). That pattern was opposite for available P: *Cytisus* soils had half the available P as the forest soils in the east site, but over three times more in the west. *Cytisus* soils had 90% greater cation exchange capacity (CEC) than forest soils in the east site, but there was no difference between *Cytisus* and forest

CEC values in the west site. There were other such inconsistencies between east and west sites among the other nutrient cations such as Mg, Ca and S (Table 2). Based on these patterns, we determined that the east and west sites were fundamentally different and chose to analyze the east and west sites separately in all comparisons for seedling growth and ectomycorrhizal colonization.

### **Effects of *Cytisus scoparius* on Douglas-fir seedling growth**

*Cytisus* had an overall negative effect on Douglas-fir seedling growth. Despite large differences in soil nutrient status between the east and west sites, the suppressive effect of *Cytisus* soil was clear in both sites. In the east site, Douglas-fir seedlings grown in *Cytisus* invaded soil were 17% smaller than seedlings grown in soils collected from uninvaded forest soil ( $F_{1,199} = 13.6$ ,  $P = 0.0003$ , Fig. 1). In the west site, Douglas-fir seedlings were 13% smaller in *Cytisus* invaded soil ( $F_{1,209} = 11.2$ ,  $P = 0.0009$ ; Fig. 1).

Activated carbon treatments produced mixed support for allelopathy. The addition of activated carbon to *Cytisus*-invaded soil had no effect on seedling growth in the east site ( $F_{1,199} = 0.089$ ,  $P = 0.76$ ; Fig. 2), and had an unexpected negative effect in the west site ( $F_{1,209} = 4.55$ ,  $P = 0.03$ ; Fig. 2). However, with fresh *Cytisus* litter, the effect of activated carbon was positive. That is, in uninvaded forest soils, the effect of adding *Cytisus* litter depended on whether activated carbon was added to the mixture, with activated carbon boosting the impact of litter from neutral to positive in one case and from negative to positive in the other (Fig. 3). In the west, the interaction between litter and activated carbon was significant ( $F_{1,106} = 5.37$ ,  $P =$

0.02) and the interaction was marginally significant in the east ( $F_{1,108} = 3.192$ ,  $P = 0.07$ ).

Our sucrose addition treatment did not consistently reduce plant growth. Adding sucrose to *Cytisus*-invaded soil resulted in a marginally significant, 11% decrease in Douglas-fir seedling growth in the east ( $F_{1,199} = 3.6$ ,  $P=0.059$ ) but had no effect in the west ( $F_{1,209} = 0.11$ ,  $P = 0.10$ ). In uninvaded forest soils, adding sucrose along with *Cytisus* litter was not different from adding litter by itself in either the east ( $F_{1,199} = 0.193$ ,  $P = 0.66$ ) or west ( $F_{1,209} = 1.022$ ,  $P = 0.31$ ).

### **Effects of *Cytisus scoparius* on ectomycorrhizal fungi**

We found substantial evidence that *Cytisus* had a negative impact on fungal mutualists of Douglas-fir. In the east site, *Cytisus*-invaded soil had 22% lower EMF colonization than uninvaded forest soil, a significant difference (one-tailed  $T = 2.37$ ,  $DF = 22.31$ ,  $P = 0.01$ ; Fig. 5). In the west site, *Cytisus* soil had 13.8 % less EMF colonization, a marginally significant difference ( $T = 1.47$ ,  $DF=20.74$ ,  $P = 0.08$ ; Fig. 5). In forest soils, *Cytisus* litter addition decreased EMF colonization in the east site by 16% ( $T = 2.19$ ,  $DF = 25.01$ ,  $P = 0.018$ ) and had no effect on EMF in the west site ( $T = 0.41$ ,  $DF = 21.19$ ,  $P = 0.65$ ; Fig. 5). The effect of adding activated carbon to forest soil along with *Cytisus* litter counteracted the negative effects of the litter in the east site but not in the west (Fig. 5). In the east site, EMF were 15% more abundant on Douglas-fir roots when activated carbon was added with *Cytisus* litter compared to the litter addition alone ( $T = 1.94$ ,  $DF = 19.32$ ,  $P = 0.03$ ). In the west site, activated



carbon did not have a positive effect on ectomycorrhizal fungi abundance ( $T = 1.03$ ,  $DF = 22.06$ ,  $P = 0.84$ ). Degree of colonization by EMF positively predicted final Douglas-fir biomass and explained a substantial proportion of the variance in biomass in soils from both sites (east:  $R^2 = 0.42$ ,  $N = 27$ ,  $P = 0.0002$ , west:  $R^2 = 0.32$ ,  $N = 29$ ,  $P = 0.001$ ; Fig. 6).

Fungi identified by ITS included the following genera: *Amanita*, *Cadophora*, *Cenococcum*, *Hygrophorus*, *Laccaria*, *Phialocephala*, *Rhizopogon*, *Rhizoscyphus*, *Suillus*, *Thelephora* and *Wilcoxina*. Because most of these taxa did not clearly correspond to a morphotype, we could not evaluate all the individual responses to *Cytisus* soils and litter. However, two taxa, *Rhizopogon rogersii* and *Cenococcum geophilum*, were clearly identifiable using microscopy. Root colonization by *Rhizopogon rogersii* was not significantly lower in *Cytisus*-invaded soil than forest soil in the east ( $T = 1.19$ ,  $DF = 24.97$ ,  $P = 0.12$ ) or west ( $T = 1.14$ ,  $DF = 27$ ,  $P = 0.13$ ) (Fig. 7). *Cytisus* litter addition did not decrease *Rhizopogon* colonization in forest soils (east:  $T = 0.86$ ,  $DF = 25.60$ ,  $P = 0.80$ , west:  $T = 0.43$ ,  $DF = 23.77$ ,  $P = .66$ ). Additionally, activated carbon did not increase *Rhizopogon* colonization in soils amended with *Cytisus* litter (east:  $T = 0.06$ ,  $DF = 23.16$ ,  $P = 0.52$ , west:  $T = 0.52$ ,  $DF = 21.87$ ,  $P = 0.69$ ). In contrast, the abundance of *Cenococcum geophilum* on Douglas-fir roots was 55% lower in long-invaded *Cytisus* soil compared to the uninvaded forest soil in the east (one-tailed Wilcoxon,  $\chi^2 = 5.06$ ,  $P = 0.02$ ; Fig. 7). In the west, *Cenococcum* abundance decreased by 81% in *Cytisus* soils ( $\chi^2 = 8.05$ ,  $P = 0.004$ ) compared to forest soils (Fig. 7). The addition of litter to forest soils

marginally significantly decreased the abundance of *Cenococcum* in the east ( $\chi^2 = 2.38$ ,  $P = 0.12$ ) but not in the west ( $\chi^2 = 0.29$ ,  $P = 0.58$ ). Activated carbon did not increase *Cenococcum* abundance in either site (east:  $\chi^2 = 0.65$ ,  $P = 0.42$ ; west:  $\chi^2 = 0.35$ ,  $P = 0.55$ ).

## Discussion

The soil legacy of *Cytisus*, a putative allelopathic N-fixer, may be comprised of both positive and negative effects: *Cytisus* elevates soil N while also producing leaves with high concentrations of sparteine, a toxic alkaloid (Gresser et al. 1996). Our previous results suggested negative effects of *Cytisus* have the potential to outweigh positive effects on the growth of native herbaceous species (Haubensak and Parker 2004). Here we found that the growth of a dominant native tree and its fungal symbionts also demonstrated a net negative impact of the invader. However, we found complex and variable patterns in exactly how nitrogen fertilization and allelopathy interact to produce a legacy effect through both direct and indirect mechanisms.

We had two different approaches to measuring the effects of *Cytisus* on soils: 1) comparing uninvaded forests to nearby, long-invaded clearcuts, and 2) adding fresh *Cytisus* litter directly to uninvaded forest soils. The *Cytisus*-invaded soils used in our study had been invaded for several decades and so could be considered a “worst-case scenario” in terms of the legacy effects of *Cytisus* on the soils. On the other hand, other site differences between the two intact forests and the two invaded clearcuts

could strongly influence our results. Certainly the four locations varied substantially in their nutrient status and stoichiometry, which probably reflect underlying site history and soil features, as well as (and interacting with) the role of *Cytisus* invasion.

Our experimental addition of *Cytisus* litter circumvents the problem of underlying variability among the sites, and it can suggest how a recent *Cytisus* invasion might influence abiotic and biotic features of an intact forest soil environment. For example, if soil microbial communities change slowly over time in response to *Cytisus* invasion, long-invaded soils would represent an endpoint; addition of activated carbon to these soils would not show a benefit to microbial communities, because the sensitive species would be absent and therefore unable to respond. In contrast, activated carbon would have the potential to ameliorate the effects of *Cytisus* litter on an intact forest microbial community, thereby allowing for the indirect effects of allelopathy to be seen.

### **Direct effects of *Cytisus* on Douglas-fir**

As we predicted, the net effect of soil long invaded by *Cytisus* was to suppress the growth of Douglas-fir seedlings. Several others have demonstrated similar suppression effects of invasive species' soil legacies on target native species. For example, Batten et al. (2008) showed that the soil microbial community was altered in soils invaded by the exotic grass *Aegilops triuncialis*, resulting in delayed flowering, decreased biomass and increased root allocation in some native species. However, we found more examples in the literature of studies that demonstrated a

legacy effect without a specific mechanism tested (Grman and Suding 2010). Several mechanisms have been suggested for legacies, including altered resources (Corbin and D'Antonio 2004a) or accumulation of pathogens (Bever 1994, Klironomos 2002). However, comparative studies that use invader-conditioned soils and uninvaded soils, and that demonstrate reduced growth of target native species in the former relative to the latter, are surprisingly uncommon.

Allelopathy is another possible mechanism by which invasive plants may alter the soil and affect growth of subsequent colonizers. For example, catechin root exudates produced by *Centaurea maculosa* contribute both to success in its introduced range and depressed growth of native grasses (Ridenour and Callaway 2001). The production of mustard oils by the invader *Alliaria petiolata* results in depressed tree seedling recruitment (Stinson et al. 2006, Wolfe et al. 2008). In our study, the addition of activated carbon to *Cytisus*-invaded soils did not increase Douglas-fir growth. The effectiveness of activated carbon in field and lab studies is mixed, suggesting an indiscriminate nature of activated carbon's affinity for organic compounds. For example, Kulmatiski and Beard (2006) showed that some native species responded positively and others negatively to activated carbon addition following non-native removal. Other studies have shown no effect of activated carbon on target species (Barto and Cipollini 2009). In this study, the lack of effect of activated carbon on Douglas-fir growth may reflect ineffectiveness at binding sparteine, the primary alkaloid produced by *Cytisus*. An alternative explanation is that allelopathic compounds produced by *Cytisus* do not directly affect Douglas-fir

growth but instead have an indirect effect via its mycorrhizal partners. As explained above, adding activated carbon to long-invaded soils would not reveal such an indirect effect because those mutualistic partners would presumably be absent from those soils.

In support of this hypothesis, we did find a strong interaction effect in forest soils between the addition of *Cytisus* litter and the addition of activated carbon. We will explore the potential role of ectomycorrhizae in this pattern below. In both sites, activated carbon transformed the effect of litter into a strong positive effect. Interestingly, without activated carbon, litter addition depressed tree growth in soils from the east site, whereas litter appears to have a slight positive net effect in the soils from the west site. This may suggest that the net sum of positive and negative factors can vary from site to site. We expected that the net effect of litter addition would be more positive in the site with greater nitrogen limitation (i.e., lower nitrogen availability), but our nutrient analysis did not support our hypothesis.

Nitrogen fertilization likely explains the positive effect of *Cytisus* litter on Douglas-fir growth. *Cytisus*-invaded soils have elevated rates of plant-available nitrogen in California grasslands (Haubensak 2001), and glacial outwash prairies (Haubensak and Parker 2004). However, our sucrose addition results only weakly support the hypothesis that Douglas-fir growth is responding to nitrogen enrichment by *Cytisus* litter in forest soils. When added to the *Cytisus*-invaded soil, sucrose significantly reduced growth in one of the two sites, but when combined with the litter addition treatment, sucrose did not significantly reduce growth. We added a

single dose of sucrose at the onset of the experiment, and it is likely that the effect of sucrose on N availability was intensive but short lived (Torok et al. 2000). Further, slower-growing species from infertile sites, like Douglas-fir, may not respond strongly to short-term changes in N dynamics (Chapin et al. 1990).

### **Indirect effects of *Cytisus* on Douglas-fir: Ectomycorrhizal fungi**

The abundance of ectomycorrhizal fungi was lower on seedlings grown in *Cytisus* soils compared to uninvaded forest soils. Adding *Cytisus* litter to forest soil also suppressed EMF colonization, at least in soil from one site. These effects on mycorrhizal fungi are likely to have important implications for the growth and establishment of Douglas-fir seedlings. Others have found that EMF can be critical to conifer establishment because of their role in nutrient acquisition (Read et al. 2004, Nuñez et al. 2009, Teste et al. 2009). For example, some Pinaceae in nutrient limited environments show higher leaf nutrient content and faster growth when colonized by EMF (Parke et al. 1983, Abuzinadah and Read 1986, Gehring and Whitham 1994, Van Tichelen and Colpaert 2000). Consistent with those studies, we also found that Douglas-fir growth was positively and significantly related to mycorrhizal colonization across sites and soil treatments.

One possible explanation for the negative effects of *Cytisus* on ectomycorrhizae is that mycorrhizal fungi may be suppressed by allelopathic compounds. We tested this with addition of activated carbon to forest soils with *Cytisus* litter; EMF colonization increased in the presence of activated carbon relative to soils with *Cytisus* litter alone,

but only in one site. Few studies have experimentally shown that invasive species inhibit the mycorrhizal colonization of native species via the mechanism of allelochemicals. An exception is Zhang et al (2007) who examined interactions between invasive and native species grown in soil containing constructed arbuscular mycorrhizal fungi (AMF) communities. Using activated carbon and extracts from the rhizome of the invader, they showed that the depressive effect of the invader on the native was via allelochemicals contained in those extracts. We have similar experiments in progress that will more directly measure the effects of *Cytisus* allelochemicals on the growth of the fungi associated with Douglas-fir.

Until recently, allelopathy mediated by mycorrhizae has been an unexplored mechanism by which invasive plants may impact native plant communities. The invader *Alliaria petiolata*, an entirely non-mycorrhizal plant, suppresses both arbuscular (Stinson et al. 2006, Callaway et al. 2008, Vogelsang and Bever 2009) and ectomycorrhizal fungi (Wolfe et al. 2008) in invaded soils. *Alliaria* and other plants in the Brassicaceae suppress mycorrhizal fungi through root exudates of anti-fungal allelopathic mustard oils (glucosinolates) (Kliebenstein 2004). The end result is that establishment, growth and competitive ability of native plants are negatively impacted. It is still unclear whether this type of impact is a common and important feature of plant invasions.

Two other mechanisms could also contribute to the reduction we saw in EMF colonization of Douglas-fir seedlings in *Cytisus* soils and with *Cytisus* litter: nitrogen enrichment and absence of suitable plant hosts. In the presence of elevated nitrogen,

some plants will suppress mycorrhizal associations (Treseder and Allen 2002, van Diepen et al. 2007, Hoeksema et al. 2010). This could occur in our system in response to nitrogen enrichment of the soil by *Cytisus*, and we are investigating this possibility in a separate experiment. Finally, *Cytisus* itself does not associate with ectomycorrhizal fungi, and EMF generally require a plant host in order to persist in a site over the long term (Molina et al. 1992, Outerbridge and Trofymow 2004). Therefore soils that have been invaded for several decades are likely to be depauperate of ectomycorrhizal fungi suitable for Douglas-fir. All considered, the long-invaded *Cytisus* soils supported what are perhaps surprisingly high rates of EMF colonization on Douglas-fir. This could be explained either by long-distance dispersal of fungal spores or by extended spore dormancy, although little is known about the longevity of EMF spores in the absence of suitable plant hosts (Nara 2009).

In addition to overall EMF colonization, we quantified the abundance of two specific fungal taxa, *Cenococcum geophilum* and *Rhizopogon rogersii*, which are broadly distributed and commonly associated with Douglas-fir. The two taxa responded differently to *Cytisus* invasion. *Rhizopogon* was not significantly reduced in the *Cytisus*-invaded soils, which may reflect the ability of *Rhizopogon* spores to remain dormant in the absence of a host for at least a decade (Bruns et al. 2009). In contrast, we found that *Cenococcum* was strongly reduced in the *Cytisus*-invaded soil compared to uninvaded forest soil. The apparent differential sensitivity of different fungal species to *Cytisus* invasion would be expected to result in shifts in overall community structure and composition. Ectomycorrhizal fungi vary in their effects on



their plant host, providing different degrees of benefit and even being parasitic under certain conditions (Johnson et al. 1997, Jones and Smith 2004). Thus differential sensitivity of mycorrhizal fungi to the soil changes that accompany invasions may have important consequences for reforestation success.

In conclusion, *Cytisus* invasion has a net negative impact on Douglas-fir growth through its effects on soil, consistent with an earlier bioassay and with the hypothesis that *Cytisus* is an allelopathic invader. Ectomycorrhizal fungi are also suppressed in *Cytisus* soils, and the strong relationship we found between plant size and mycorrhizal colonization could reflect the potential for the suppression of mycorrhizae to feed back to tree growth and establishment. The legacy of *Cytisus* invasion integrates both positive and negative factors; and as we found here, their relative importance is likely to be highly context-specific.

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**Table 1.1** ANOVA output for full factorial mixed model with seed source as a random effect testing the effects of soil factors on final dry biomass of Douglas-fir seedlings after 19 mo of growth. Soil type = *Cytisus*-invaded soil or forest soil, Litter = with or without, Activated carbon = with or without, Sucrose = with or without. Degrees of freedom = 1, 199 for the East site, and 1, 209 for the West site.

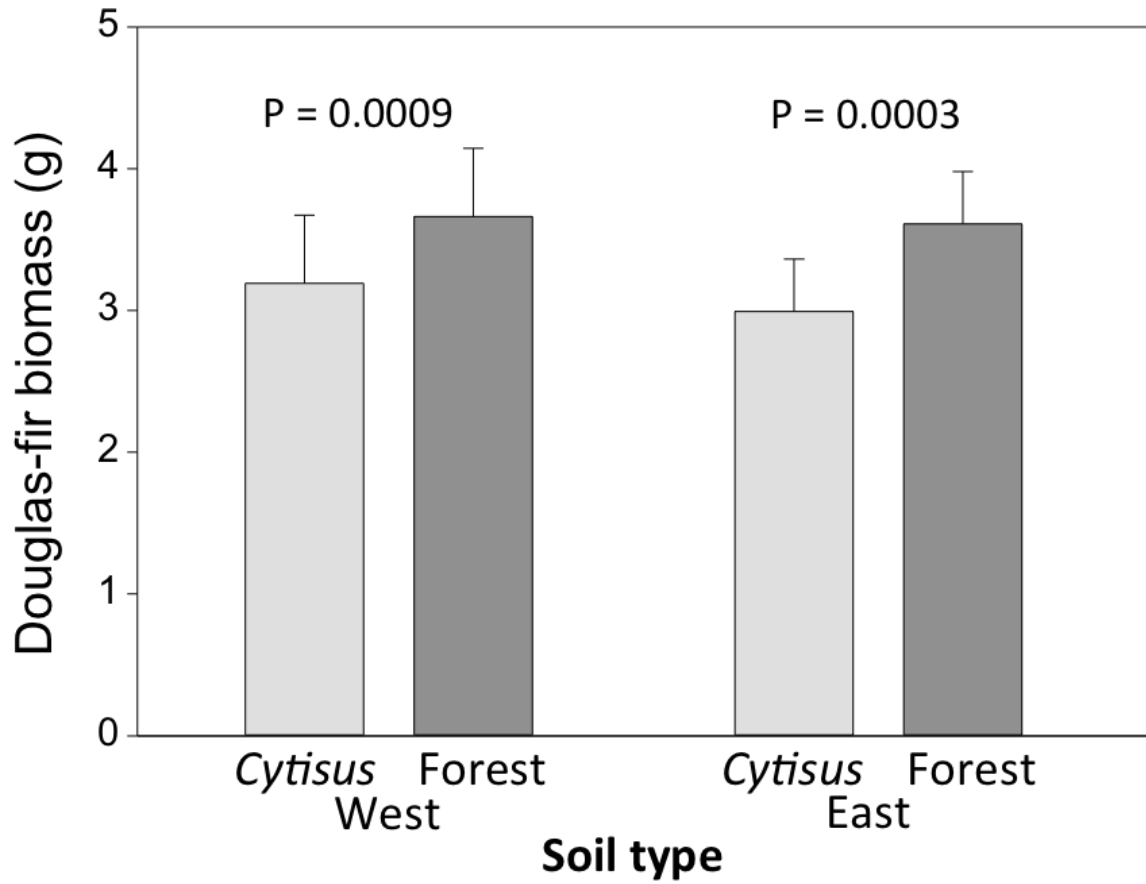
Source	West		East	
	F	P	F	P
Soil type	11.2559	0.0009*	13.6194	0.0003*
Litter	22.3300	0.0001*	0.8973	0.3447
Soil type*Litter	0.0873	0.7679	1.4179	0.2352
Activated carbon	0.1227	0.7265	1.0980	0.2960
Soil type*Activated carbon	6.9501	0.0090*	2.1950	0.1400
Litter*Activated carbon	1.8495	0.1753	1.2268	0.2694
Soil type*Litter*Activated carbon	3.3720	0.0677	1.6956	0.1944
Sucrose	0.0793	0.7786	3.3238	0.0698
Soil type*Sucrose	3.9547	0.0480*	0.8624	0.3542
Litter*Sucrose	0.1599	0.6896	0.6135	0.4344
Soil type*Litter*Sucrose	0.6452	0.4228	0.9952	0.3197
Activated carbon*Sucrose	1.8711	0.1728	0.7547	0.3860
Soil type*Activated carbon*Sucrose	0.0112	0.9158	0.0594	0.8077
Litter*Activated carbon*Sucrose	0.0017	0.9667	0.7225	0.3963
Soil type*Litter*Activated carbon*Sucrose	0.0241	0.8767	1.4010	0.2380

**Table 1.2:** Soil parameters measured in *Cytisus*-invaded (*Cytisus*) and uninvaded (Forest) mineral soils (0-15 cm). West and east soils were from two sites on JBLM property. Values are means (N = 14) with one standard error in parentheses. SOM = soil organic matter; CEC = cation exchange capacity.

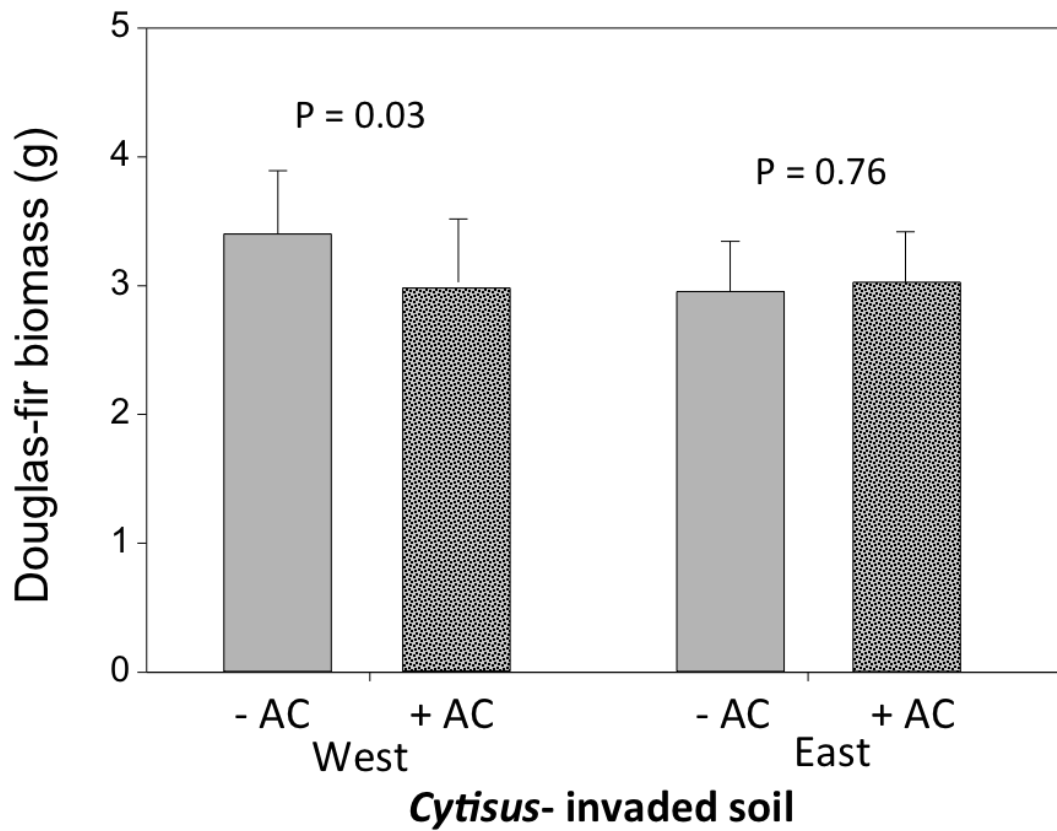
	<i>Cytisus</i>		Forest	
	East	West	East	West
C:N	16.29 (0.42)	21.57 (1.66)	29.57 (0.78)	24.42 (2.85)
Total N (%)	0.785 (0.02)	0.365 (1.66)	0.454 (0.78)	0.523 (2.85)
SOM	21.99 (0.45)	13.54 (0.99)	23.03 (0.72)	21.71 (2.46)
Available P	16.34 (1.47)	70.51 (3.60)	53.0 (7.97)	33.49 (5.31)
pH	5.49 (0.04)	5.70 (0.05)	5.61 (0.03)	5.14 (0.03)
K	92.68 (25.5)	85.30 (17.3)	98.77 (35.5)	90.73 (47.5)
Mg	40.29 (1.69)	74.96 (5.53)	73.87 (2.96)	55.63 (3.42)
Ca	316.57 (18.9)	609.81 (46.2)	702.91 (29.8)	396.53 (26.5)
Na	29.31 (1.23)	33.81 (3.18)	29.70 (1.73)	29.96 (2.55)
CEC (meq/100g)	3.11 (0.21)	5.10 (0.31)	5.86 (0.28)	4.41 (0.38)
NO <sub>3</sub> <sup>-</sup> -N (ppm)	10.29 (1.34)	7.03 (2.28)	7.14 (1.60)	11.07 (1.61)

**Table 1.3:** Results for 2-way ANOVAs for all soil variables with “Site” (East versus West) and “Soil” (*Cytisus*-invaded versus uninvaded forest). DF = 1 for each main effect and interaction term.

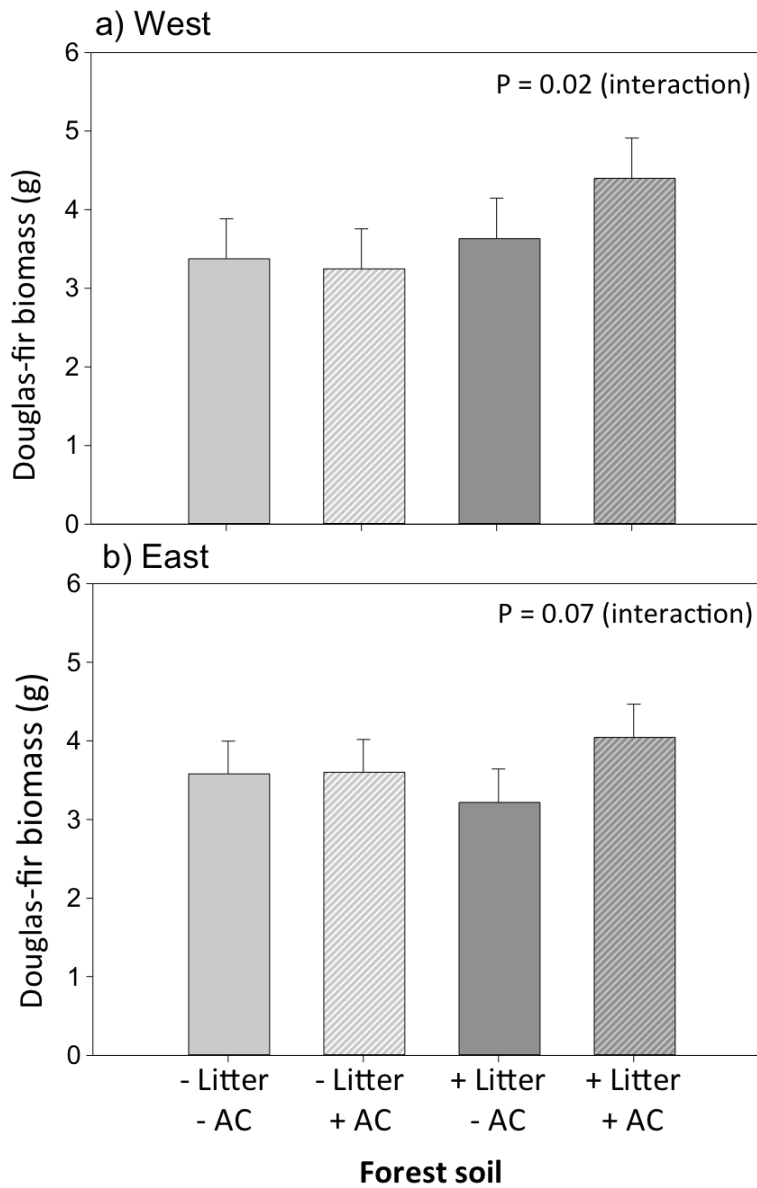
	Source	F	P
C:N	Site	0.0017	0.962
	Soil	22.31	<0.0001
	Site*Soil	9.31	0.055
Total N (%)	Site	87.09	<0.0001
	Soil	21.22	0.001
	Site*Soil	168.5	<0.0001
SOM	Site	12.34	0.002
	Soil	10.99	0.003
	Site*Soil	6.57	0.017
Available P	Site	11.25	0.003
	Soil	0.001	0.974
	Site*Soil	50.84	<0.0001
pH	Site	11.85	0.002
	Soil	32.92	<0.0001
	Site*Soil	84.29	<0.0001
K	Site	0.05	0.822
	Soil	0.02	0.864
	Site*Soil	0.001	0.991
Mg	Site	5.01	0.031
	Soil	3.77	0.061
	Site*Soil	51.95	<0.0001
Ca	Site	0.042	0.832
	Soil	7.33	0.012
	Site*Soil	88.06	<0.0001
Na	Site	1.07	0.311
	Soil	0.57	0.453
	Site*Soil	0.85	0.362
CEC	Site	0.82	0.373
	Soil	11.82	0.002
	Site*Soil	32.84	<0.0001
NO <sub>3</sub> <sup>-</sup> -N	Site	0.037	0.854
	Soil	0.066	0.792
	Site*Soil	4.248	0.051



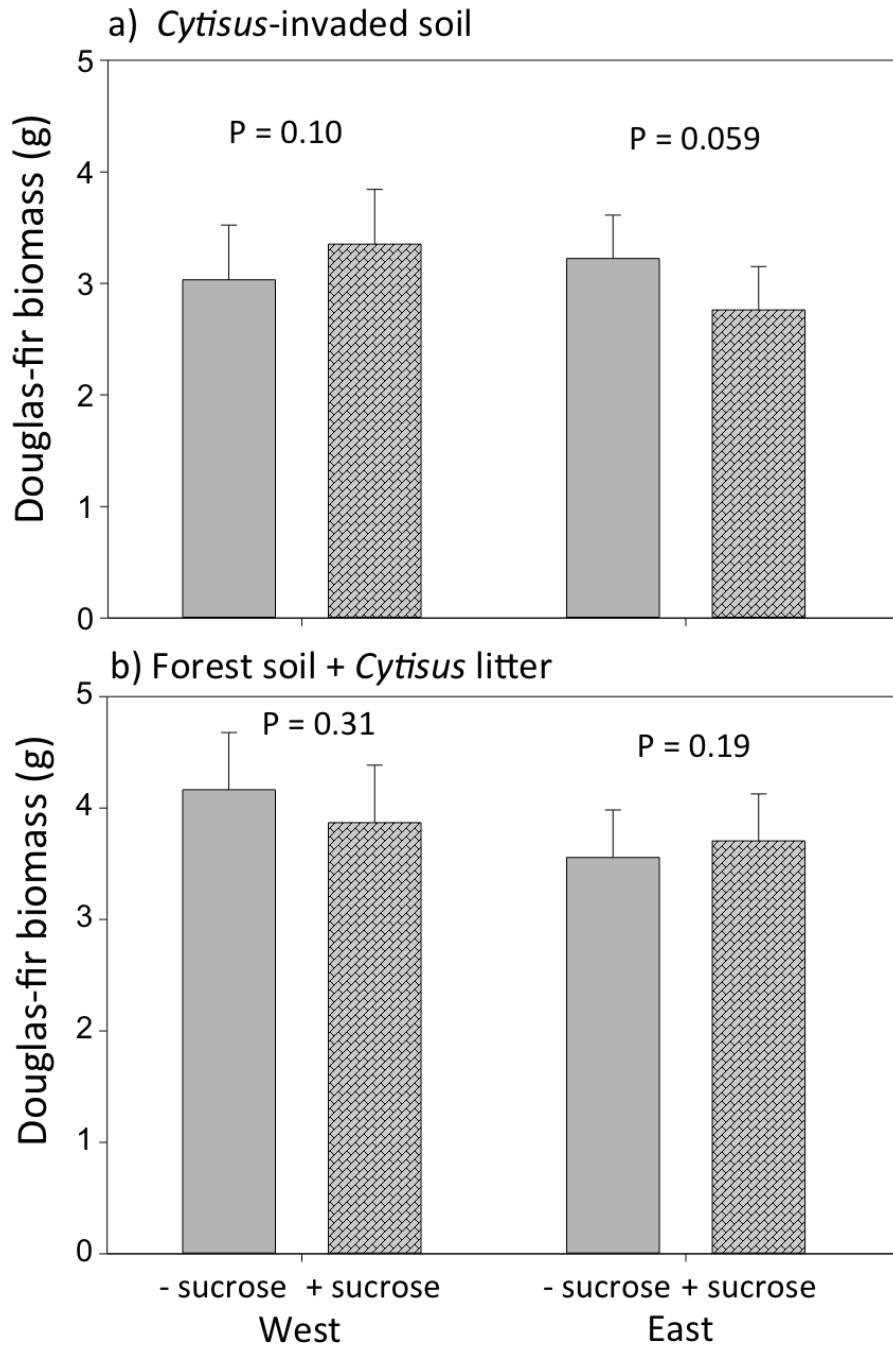
**Figure 1.1.** Aboveground dry biomass (g) of Douglas-fir seedlings after 19 months of growth in either *Cytisus*-invaded soils or uninvaded forest soils, collected from two sites (east and west) at Joint Base Lewis McChord in southwestern Washington.



**Figure 1.2.** The effect of activated carbon (AC) addition on aboveground biomass (g) of Douglas-fir seedlings after 19 months of growth in *Cytisus*-invaded soils collected from two sites (east and west) at Joint Base Lewis McChord.

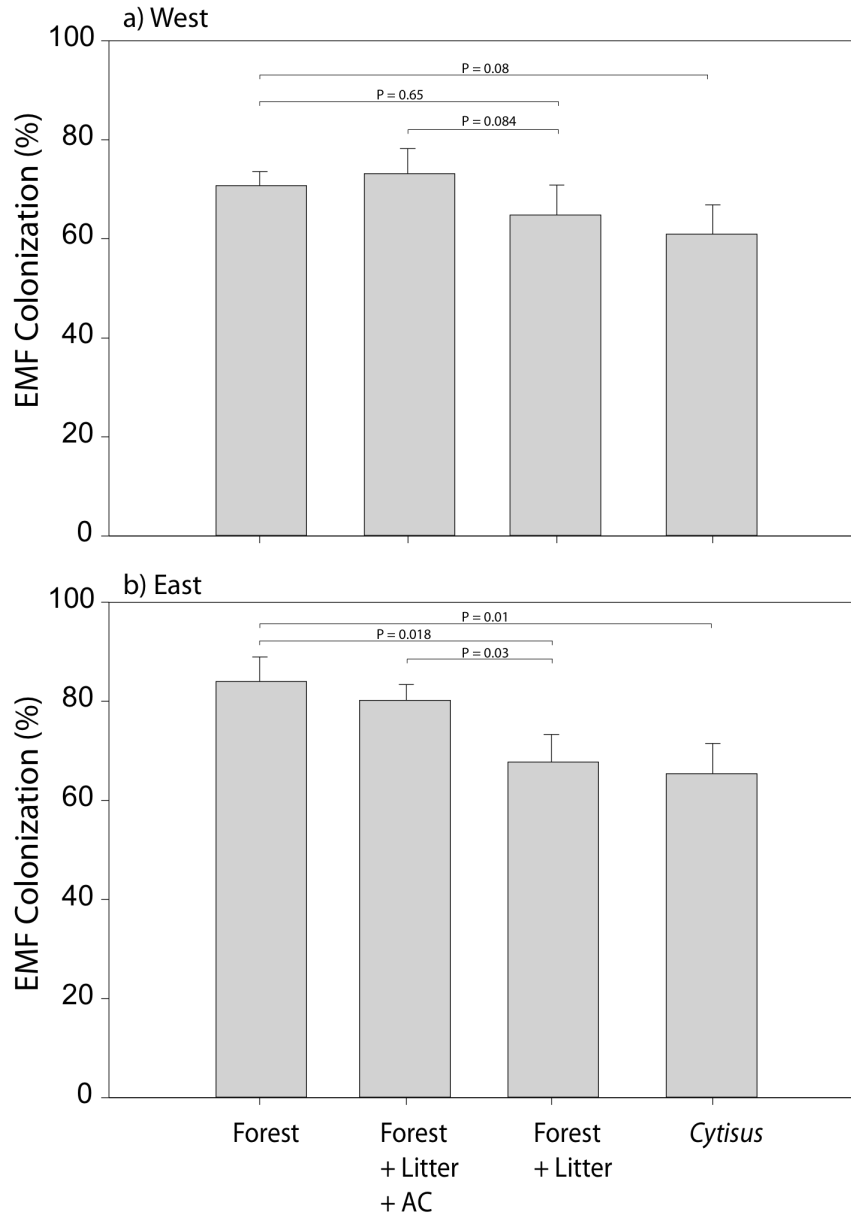


**Figure 1.3.** The joint effects of adding *Cytisus* litter (Litter) and activated carbon (AC) on aboveground biomass (g) of Douglas-fir seedlings after 19 months of growth in *Cytisus*-invaded soils collected from two sites (east and west) at Joint Base Lewis McChord.



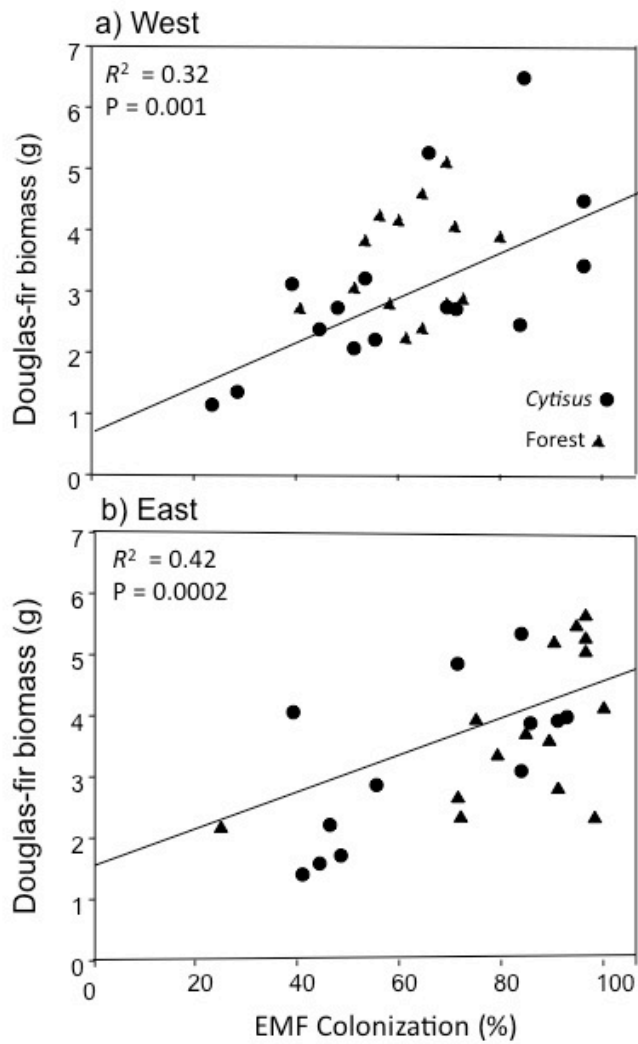
**Figure 1.4.** The effect of adding sucrose on aboveground biomass (g) of Douglas-fir seedlings grown in two types of soils: a) *Cytisus*-invaded soils, and b) uninvaded soils to which *Cytisus* litter was added. Soils were collected from two sites (east and west) at Joint Base Lewis McChord.

Fig 5.

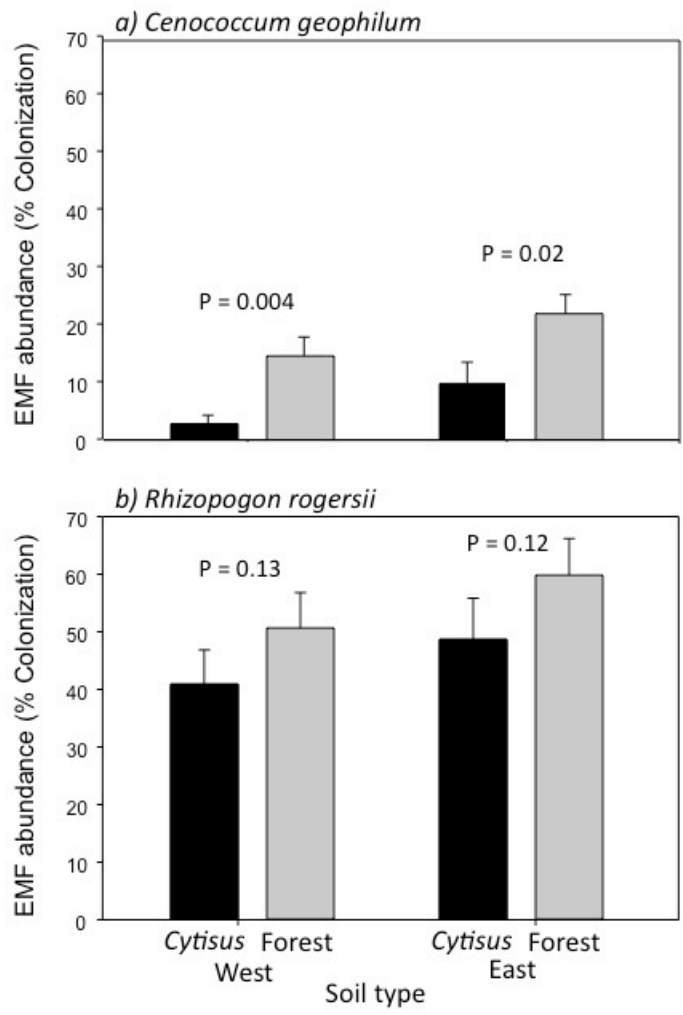


**Figure 1.5.** Colonization of roots by ectomycorrhizal fungi (EMF) for Douglas-fir seedlings grown in four types of soils: uninvaded forest soils with no amendments, forest soils to which both *Cytisus* litter and activated carbon were added (+Litter +AC), forest soils to which only *Cytisus* litter was added, and soils long invaded by *Cytisus*. Soils were collected from two sites (east and west) at Joint Base Lewis McChord.





**Figure 1.6.** Aboveground biomass (g) of Douglas-fir seedlings is significantly predicted by the percentage of roots colonized by ectomycorrhizal colonization (EMF). Data from *Cytisus*-invaded and uninvaded forest soils (with no amendments) were combined for this analysis; soils were collected from two sites (east and west) at Joint Base Lewis McChord.



**Figure 1.7.** Colonization of Douglas-fir roots by two species of ectomycorrhizal fungi (EMF), *Cenococcum geophilum* and *Rhizopogon rogersii*, for seedlings grown in uninvaded forest soils and soils long invaded by *Cytisus*. Soils were collected from two sites (east and west) at Joint Base Lewis McChord.

## CHAPTER 2

### **Persistence of a soil legacy following removal of the invader *Cytisus scoparius* and implications for reforestation**

#### **Introduction**

Vast effort and resources are spent to control invasive species. It is typically assumed that once these resources are spent and the invader is successfully removed, the impact of that species on the community is also eliminated. However, invasive species may change the environment in ways that persist long after the species itself is gone; this is called a “legacy effect” (Ehrenfeld and Scott 2001, D'Antonio and Meyerson 2002, Corbin and D'Antonio 2004a). Legacy effects have been shown to inhibit native species from reoccupying habitat after an invader is removed (Vitousek et al. 1987, Maron and Jefferies 2001, Corbin and D'Antonio 2004a, Maron et al. 2011). There are several possible mechanisms by which invasive plants can impart persistent soil legacy effects. Soil legacies can result if a species changes the abundance and composition of soil biota, such as increased soil pathogen loads (Eppinga et al. 2006), or reduced abundances of beneficial microbial mutualists (Stinson et al. 2006, Pringle et al. 2009, Vogelsang and Bever 2009). Legacy effects can also result through changes in the availability of soil nutrients. Soil nutrient availability and cycling can be altered if an invasive species takes up resources and renders them unavailable to other species, or if an invader increases the availability of nutrients through nitrogen fixation or increasing decomposition rates (Allison and Vitousek 2004, Rothstein et al. 2004). These changes in nutrient dynamics can also

result in soil legacies if nutrient availability is not restored to conditions that favor native species following invader removal (Maron and Jefferies 2001, Ehrenfeld 2003, Corbin and D'Antonio 2012).

Some legacy effects are self-reinforcing and appear to promote an 'alternative stable state' which are unable to be successfully restored to preinvasion conditions. The discussion of whether and how often invaded ecosystems may represent a "stable state" is a major debate in invasion ecology (Suding et al. 2004, Suding and Hobbs 2009, Yelenik and D'Antonio 2013). The speculations are limited, however, because we have few examples where the trajectory of impacts has been followed over time after invader removal as the soil environment returns to pre-invasion conditions. These gaps in our knowledge of legacies have limited our ability to successfully restore species and ecosystem functioning post-invasion. The legacy effects of invaders can inhibit the success of restoration by facilitating reinvasion by the same or other exotic species, which in turn, can prevent the establishment and success of native plants (Simberloff and Von Holle 1999, Richardson et al. 2000, Maron and Jefferies 2001).

Nitrogen-fixing species comprise a significant body of invaders worldwide (Daehler 1998), and a large number of studies report the N enrichment impacts associated with various widespread or locally aggressive species such as *Morella faya* (Vitousek et al. 1987), *Prosopis glandulosa* (Archer 1995), *Acacia* spp. (Le Maitre et al. 2011) and *Robinia pseudoacacia* (Von Holle et al. 2005, Castro-Diez et al. 2009).

In general, most N studies on N-fixing invaders have documented soil N enrichment with invasion, and a subset have suggested that this increased N can persist as a soil legacy (Vitousek et al. 1997, Corbin and D'Antonio 2004b, Vitousek et al. 2013). Its possible that these N additions can continue to have effects on ecosystems after invader removal. What is clear from these studies is that invaders that increase available N have the potential to produce soil legacy effects (Vitousek et al. 1997, Corbin and D'Antonio 2004b, Vitousek et al. 2013). A few studies have found that plants with nitrogen-fixing associations can alter nitrogen and carbon pools to such an extent that it can take years to return to pre-invasion levels (Maron and Jefferies 2001, Marchante et al. 2008) while other studies have found N availability returns to preinvasion levels quickly following invader removal (Malcolm et al. 2008, Hughes et al. 2012). However, with only a few studies to compare, it is unclear how general legacy effects of N-fixing invaders are, and its even less clear how long they persist and what factors control the duration of soil legacies of N-fixing invaders (Arkema et al. 2006, Strayer et al. 2006, Suding and Hobbs 2009). Following invader removal, where soil legacy effects occur, restoration may require additional efforts that aim to mitigate soil legacy effects (Corbin and D'Antonio 2012).

Nitrogen enrichment may positively affect the growth of recolonizing plant species, particularly fast growing species with the ability to capitalize on increased nutrient availability, such as exotic grasses and forbs (Milberg et al. 1999, Davis et al. 2000). Thus, legacies of N-fixers can be positive for some species (e.g., Maron and Jeffries 2001), but may also have negative effects on other species by changing plant

community dynamics. Specifically, the secondary invasion of fast growing exotic grasses following N-fixing invader removal may, in turn, inhibit the establishment and growth of native species that are less able to take advantage of increases in available N.

Here we examine the duration of time soil legacies persist after the removal of *Cytisus scoparius*, a widespread N- fixing invader. We removed *C. scoparius* at different times over a 22-month period and allowed areas to be *C. scoparius*-free for 0, 1, 10, and 22 months before planting Douglas-fir seedlings and measuring soil properties. To evaluate how soil legacy effects influence the restorability of invaded areas, we measured nutrient availability at the time of tree planting, the survival and growth of Douglas-fir across time since *C. scoparius* removal treatments, and the abundance of exotic grasses and forbs.

## **Methods**

### **Study System**

*Cytisus scoparius*, a large shrub in the Fabaceae, is considered an important weedy pest plant in the western US (Mobley 1954, Gilkey 1957, Peterson and Prasad 1998, Isaacson 2000). It is native to Europe (Hegi 1926) and has been introduced as an ornamental in many places throughout the globe (e.g. Chile, Canada, India, South Africa, New Zealand, Australia and the United States). In the United States, its range in the Pacific Northwest region is extensive (Hulting et al. 2008), and it invades open

sites extremely rapidly (Parker and Reichard 1998, Parker 2000). Much research has focused on the control and removal of *C. scoparius*, particularly from grasslands (Hoshovsky 1986, Sheppard et al. 2000, Dunn 2002).

Douglas-fir (*Pseudotsuga menziesii* var. *menziesii*) is the dominant tree of lowland forests in the Pacific Northwest. Its latitudinal distribution extends from central British Columbia to central California (Hermann and Lavender 1990), and it is an economically important timber species (Fowells 1965, Arno and Hammerly 1977). *Cytisus scoparius* invades timber harvested areas forming dense stands (Parker et al 2012), resulting in a loss of more than \$100 million annually in timber revenue and control expenses in the Pacific Northwest (Hulting et al. 2008, Matsen 2011).

To determine how long the legacy effects of *C. scoparius* invasion persist after *C. scoparius* has been removed, we conducted a field experiment in four heavily invaded, formerly forested sites in western Washington (Mason County). Each of these sites is managed by the Green Diamond Resource timber company and had been clearcut at least eight years before the start of the experiment (Sites: Research 2003, Tires 2003, Ants 1997, McEwan 1984) and has since been extensively invaded by *C. scoparius*. The soils are characterized as glacial outwash gravelly loamy sand (USDA NRCS 2012).

In May 2011, we installed four 20 x 20 m plots in four separate sites (16 total plots). In July 2011, July 2012, and April 2013, we removed *C. scoparius* from one randomly selected plot per site so that by the end of these removal treatments, *C. scoparius* had been absent from plots for 22 months, 10 months, and 1 month

respectively. In the control plots, *C. scoparius* was left in place and unmanipulated. One approach to studying legacy effects is to remove the invader and then sample the responses to invader removal at different times after initial invader removal. With this approach, time of year of sampling is confounded with time since removal. We took a different approach in order to avoid this problem; instead we varied the time of invader removal, but sampled the responses Douglas-fir survival and growth, soil nutrients, and exotic grass and forb colonization at the same time across all treatments. We know that nutrient pulses vary to an extreme degree across seasons (Powers 1990, Laverman et al. 2000), especially in the Pacific Northwest, with wet winters and dry warm summers. Our approach allowed us to minimize temporal variation of nutrient pulses and allowed us to collect soil samples and plant trees at the same time. To remove *C. scoparius* from the treatment plots we hired professional contract crews associated with the Green Diamond Resources timber company to apply Garlon 4 Ultra (5 gallons/ removal plot at 1.3% concentration) herbicide treatments. Sprayed plants were left to senesce *in situ*. Prior to each removal treatment, we used a line-intercept method to measure *C. scoparius* cover and density along a 30-m transect that ran diagonally across each plot, ensuring that each plot started with at least 75% cover of *C. scoparius*. In April 2013, after the final *C. scoparius* removal treatment, we planted 50 Douglas-fir seedlings, approximately 2.5 m apart, into each treatment plot (N = 50 seedlings/plot x 3 plots/site x 4 sites = 600 seedlings). Seedlings were donated from the Washington State Department of Natural Resources Webster's Forest Nursery (Tumwater, Wa), and were grown from



seed collected from the Kitsap seed zone. Prior to out-planting, seedlings were grown for one year in a seed bed, then harvested, root pruned, and transplanted into another nursery bed where they were grown for a second year. At the time of planting we measured initial seedling heights, which ranged from 11-63 cm. Douglas-fir seedlings were not planted into the untreated plots because *C. scoparius* was still growing in those plots, and impacts of soil changes on Douglas-fir seedling growth would be confounded with direct competition from *C. scoparius* (Zielke et al. 1992, Prasad 2000).

### ***Field Sampling***

In effort to minimize post planting issues such as drought and transplant shock we followed standard forestry practices and out-planted the Douglas-fir seedlings in April. At the time of tree planting (April 27 – 29, 2013), we collected soil cores in order to characterize the soil legacy effects of *C. scoparius* following its removal. We collected soil samples from two of the three *C. scoparius* removal treatments( 10 and 22 month removals as well as intact ‘control’ plots). We did not sample soil from the third herbicide treatment plot (‘1 month removal’) because the herbicide application had just occurred and the plants had not yet died and therefore N content within the plant tissues would not had yet been cycled into the soil. One month later, after tree planting and soil sampling (May 28 - 31 2013), we collected soil cores from the final *C. scoparius* removal plot and re-collected soils from the live intact *C. scoparius* ‘control’ plots to account for seasonal differences between the April and

May sampling points. We collected soil cores every 5-m along a 30-m transect that ran diagonally across each plot, beginning at the 5-m mark and ending at the 25-m mark to avoid sampling in the edge of plots. At each 5-m sampling interval we used a 5-cm diameter coring device, driven 20 cm into the mineral soil (N = 5 soil cores/plot x 4 plots/site x 4 sites = 80 soil cores). All soil cores were collected within 20 cm of the nearest *C. scoparius* stem (dead stems in the treated plots and live stems in the control). Large rocks and root material were removed by hand, then the samples were stored on ice, after which they were returned to a 3 ° C refrigerator until shipment to Northern Arizona University for N and P analysis.

Soil samples were analyzed for available nitrogen (N), phosphorus (P), and pH. To measure available N, a 7-day laboratory aerobic incubation was conducted (Binkley and Hart 1993) using field-moist soils. Two ~10-g subsamples were weighed into specimen cups; one subsample was immediately extracted with 50 mL 2M KCl and the other subsample was capped and incubated in a dark box at room temperature for 7 days. At the end of the 7-day period the incubated subsample was extracted with 50 mL of 2M KCl. All KCl extracts were shaken for 30 minutes then refrigerated for 24 hours to allow samples to settle. After 24 h, extracts were filtered through Whatman #1 filters; filtrates were then frozen until analysis. Available P was assayed with a NaHCO<sub>3</sub> extraction on air-dried soils (Olsen 1954; Indiaty et al 2004). Both P and N samples were analyzed on a Lachat (QuikChem Method 10-115-01-1-A, Orthophosphate in Waters for P: Method 12-107-06-2-A for NH<sub>4</sub><sup>+</sup> and 12-107-04-

1-B for  $\text{NO}_3^-$ ) at the Colorado Plateau Analytical Laboratory at Northern Arizona University. Soil pH was measured on all samples using a 1:1 soil slurry.

In November 2013, after one growing season, we assessed Douglas-fir seedling survivorship and measured height of all surviving seedlings. We also recorded if the seedlings had experienced herbivory. At the same time, we used a standard point intercept method to measure the abundance (% cover) of all other individual species besides Douglas-fir and *C. scoparius* in each plot. We recorded each species encountered every 0.5 m along a 30-m transect that ran diagonally across each plot. We then classified each species observed as native or exotic, and recorded its growth form (grass, forb, tree, shrub, or fern).

### ***Statistical analyses***

We compared the cover of *C. scoparius* at the time of the removal across treatments with a mixed model ANOVA with time since removal included as a fixed ordinal effect and site as a random effect. We determined if pH or the availability of N and P change with time following the removal of *C. scoparius* using a mixed model ANOVA with time since removal included as a fixed ordinal effect and site as a random effect.

To evaluate the effect of time since *C. scoparius* removal on Douglas-fir seedling survival we used a logistic regression model with time since removal and site as fixed effects. Seedling growth was analyzed as change in height with an

ordinal mixed model ANOVA with time since removal as a fixed effect and site included as a random effect. In the field, at the time of the final growth measurements we frequently observed seedlings that had been damaged by herbivores, presumably by deer, and often the current years growth was absent. To determine if the degree of herbivory varied among time since removal treatments we used a logistic regression model with time since removal as a fixed effect and site as a random effect.

In order to evaluate the effect of time since *C. scoparius* removal on percent cover of exotic grasses, forbs and of native plants, we used a mixed model ordinal ANOVA with time since removal as a fixed effect and site included as a random effect to evaluate the effect of time since *C. scoparius* removal on the percent cover of exotic grasses and forbs and of native plants.

All analyses were done in JMP v 10 (SAS Institute). For all ordinal ANOVA mixed models we used Tukey's post hoc tests to compare means among the time since *C. scoparius* removal treatment groups.

## **Results**

Immediately prior to removal, *C. scoparius* cover was greater than 75% in all treatments. However, because the individual plants continued to grow throughout the duration of the experiment, percent cover did vary across time since removal

treatments ( $F = 7.79$ ,  $DF = 3,9$ ,  $P = 0.007$ ; Fig 1) and treatments imposed later had higher *C. scoparius* cover at the time of removal.

We found no difference in N availability (net  $\text{NO}_3^- + \text{NH}_4^+$  produced in 7-d incubations) between the April and May field sampling dates in the plots where *C. scoparius* was left intact. Therefore April and May data were combined to provide one pre-removal estimate of nitrogen availability. Soil N availability spiked one month following *C. scoparius* removal; in those plots, there was 35% more available N than in intact *C. scoparius* plots. Ten months following *C. scoparius* removal, however, N availability was 30% less than areas where *C. scoparius* remained intact, and by 22 months post removal N availability decreased another 6% ( $F = 22.23$ ,  $DF = 3$ ,  $80.75$ ,  $P = 0.0001$ ; Fig 2).

Unlike N, P availability was significantly greater in intact *C. scoparius* plots in April compared to May ( $F = 26.63$ ,  $DF = 1, 32$ ,  $P = 0.0001$ ; Fig 3), limiting our ability to compare P availability across all treatments. Instead we compared P availability in soils that were collected during the same sampling point. That is, for the April sampling point we compared the 10 and 22 mo *C. scoparius* removal treatments to the intact plots sampled in April. We found no difference in P availability 10 and 22 mo following *C. scoparius* removal compared to the soils in intact *C. scoparius* plots ( $F = 1.85$ ,  $DF = 2, 50$ ,  $F = 0.167$ ). For the May sampling point we compared the 1 mo *C. scoparius* removal plots to the intact plots sampled in May. Again, we found no difference in P availability one month following *C. scoparius* removal compared

to the plots where *C. scoparius* was left intact ( $F = 0.991$ ,  $DF = 1$ ,  $P = 0.326$ ). Thus phosphorus availability varied with time but not with treatment.

Similar to P availability, soil pH was 5% higher in May compared to April in intact plots ( $F = 34.88$ ,  $DF = 1,34$ ,  $P = 0.0001$ ; Fig 4), and so comparisons with intact plots were made as above. One month following *C. scoparius* removal we found a slight (4.9%) decrease in soil pH ( $F = 22.76$ ,  $DF = 1,22.77$ ,  $P = 0.0001$ ) relative to control (intact) plots sampled in May. We found no difference in soil pH in the 10 mo or 22 mo post removal treatments versus *C. scoparius* intact plots sampled in April ( $F = 0.763$ ,  $DF = 2,54$ ,  $P = 0.47$ ; Fig 4).

Douglas-fir seedling survival was unaffected by time since removal ( $\chi^2 = 2.68$ ,  $P = 0.26$ ; Fig. 5). However, time since invader removal affected growth of surviving seedlings ( $F = 7.717$ ,  $DF = 2, 504$ ,  $P = 0.0005$ ; Fig. 6). Seedlings planted 22 months post invader removal grew 37% less than seedlings planted into 1-mo post removal plots and 29 % less than seedlings planted in 10-mo post-removal plots. There was no difference in seedling growth between seedlings planted 1 and 10 months post invader removal. Because the proportion of seedlings affected by herbivores (40-44%) did not vary between time since removal treatments ( $\chi^2 = 1.75$ ,  $P = 0.41$ ) we additionally compared seedling survival and growth excluding seedlings that had been browsed. Site was a significant factor for Douglas-fir survival ( $\chi^2 = 48.28$ ,  $P = 0.0001$ ) and herbivory ( $\chi^2 = 232.9$ ,  $P = 0.0001$ ). That is, some sites were more affected by herbivory than others.

However, the cover of exotic grasses and forbs increased markedly with time following *C. scoparius* removal ( $F = 4.09$ ,  $DF = 3, 9$ ,  $P = 0.04$ : Fig 7a), but the cover of native vegetation cover did not ( $F = 0.55$ ,  $DF = 3, 9$   $P = 0.67$ : Fig 7b). Exotic grass and forb cover more than doubled (40%) one month following removal, compared to plots where *C. scoparius* was left intact (18%). With time following *C. scoparius* removal, cover of exotics continued to increase, and by 22 months post removal exotic grasses and forbs covered ~75% of the plots.

## **Discussion**

After *C. scoparius* removal, there was a large initial pulse of N into the soil imparting a soil legacy effect in the absence of the invader. The N enrichment associated with *C. scoparius* invasion is presumably a result of  $N_2$  fixation by *Bradyrhizobium* that associate with *C. scoparius* (Parker et al. 2006). High N content in *C. scoparius* leaf, stem and root tissues is released into the soil environment through decomposition. One month following the removal of *C. scoparius* a large pulse of inorganic nitrogen was released, resulting in 35% more available N relative to areas where *C. scoparius* remained intact and alive. After 10 months following removal, N availability was 30% less than under intact *C. scoparius*, and by 22 months following removal, N availability decreased another 6%. Even though mineralized N decreased with time following the removal of *C. scoparius*, the

concentrations in the soil at 22 months were still high relative to uninvaded Douglas-fir forest soils in the region (Perakis et al 2012). Lowland Douglas-fir forests in the Pacific Northwest are frequently N limited and foresters often add N fertilizer to forest stands to increase Douglas-fir growth rates. Peterson and Hazard (1990) compared the effect of N addition to Douglas-fir growth across Pacific Northwest forest plantations and found that in the region of our study, N additions resulted in a 21-23% increase in Douglas-fir growth, indicating that the forest in this region are N limited.

The concentrations of mineralized N found in our study are within the range other researchers have found in soils invaded by *C. scoparius* and other nitrogen fixing legumes (Von Holle 2013, Marchante et al 2009, Vitousek and Walker 1989). Several studies have found that N availability markedly increases with *C. scoparius* invasion (Dancer et al. 1977, Wheeler et al. 1987, Diquelou and Roze 1999, Fogarty and Facelli 1999, Haubensak and Parker 2004, Caldwell 2006), but see (Shaben and Myers 2010 ). The studies that directly compared soil N in invaded and uninvaded sites have found that available N increased from 26 to 714% (Fogarty and Facelli 1999 (118-714%), Caldwell 2006 (26%); Haubensak and Parker 2004 (127%). The degree to which *C. scoparius* or other N-fixing invaders change N availability likely depends on initial pre-invasion soil conditions, such as the availability of N and P, and soil texture, as well as the extent and duration of the invasion and the presence of native N-fixers (Liao et al. 2008, Castro-Díez et al. 2014).

Despite the widespread recognition that N-fixing invaders change nutrient



cycling and availability, only a few studies have tracked the persistence of N enrichment following invader removal (Maron and Jefferies 2001, Marchante et al. 2008, Hughes et al. 2012, Von Holle et al. 2013). In a chronosequence study, Hughes et al (2012) compared mineralized N across soils that were previously invaded by the legume *Falcataria moluccana* but that had been eradicated for different durations of time. Similar to our findings, immediately following the removal of *F. moluccana* inorganic N increased by 300% relative to never before invaded soils, however in areas where *F. moluccana* had been eradicated for 3 years, inorganic N had returned to concentrations found in nearby native uninvaded forest soils (Hughes et al 2012). In contrast, Von Holle et al (2013) found long-persisting N legacy effects of the invasive legume *Robinia pseudoacacia* 14 years following a hurricane that removed entire stands of the invader. They found that extractable N was greater where the *R. pseudoacacia* had been removed 14 years earlier than in nearby stands of native forest stands (which had never been invaded). In this *R. pseudoacacia* system, the pool sizes of inorganic N ranged from 3.5-4.0 µg N/g dry soil in the invaded 4.2-4.7 µg N/g dry soil in the formerly invaded soils, while in our system the N inputs of *C. scoparius* were much larger overall, in the range of 10 µg N/g dry soil in the invaded stands to 7.5 µg N/g dry soil in the 22 month post removal stands.

Due to the nature of our study system and the logistics of site selection, it was not possible to incorporate a control treatment uninvaded by *C. scoparius*. At the onset of our study, each clearcut was heavily invaded by *C. scoparius* and there were no areas within these clearcuts that were uninvaded, which is typical of many *C.*

*scoparius* invasions following deforestation. We were able to compare the mineralized N found in our study with N values of Douglas-fir soils reported in the literature. Perakis et al. (2012) reported the mean pool size of mineralized N in a Pacific Northwest Douglas-fir forest to be 0.15  $\mu\text{g/g}$  of mineral soil. These values are dramatically lower than the mineralized N pools found in all of our treatments. The mineralized N pool in the *C. scoparius* live intact treatment was 6-fold higher than Douglas-fir forest soils, and the 1 mo post removal treatments were 2 orders of magnitude higher than typical values reported for forest soils in the region (Perakis 2012, Sollins et al 1990, Fenn et al. 1998 ). The inorganic N pool sizes and mineralization rates in *C. scoparius* invaded soils are consistent and well within the range of values reported from similar soils within 70 km of the present study (Haubensak and Parker 2004). In general, spatial and temporal variation inherent in nitrogen cycling, demands caution when comparing N availability of samples collected at different times of year, on different years, and in different locales. However, nitrogen in *C. scoparius* invaded soils and uninvaded forest soils differed by 2 orders of magnitude, and differences this extreme are not likely explained by seasonal and spatial variation alone.

We did see a sudden drop in N availability between 1 mo and 10 mo following the removal of the N-fixing invader. Some of the differences we find in N availability with time following invader removal are in part likely due to  $\text{NO}_3^-$  leaching, as these glacial outwash soils are relatively coarse in texture, well drained and receive considerable rainfall. Change in N retention following the removal of a

N-fixing invader may be due to a mismatch between N availability and N uptake by the existing plant community (Yelenik and D'Antonio 2013). It is possible that in some cases mineralized soil N remains in the system when the intact vegetation takes up the added N, particularly if the species are fast growing early colonizers. In systems where N is lost more quickly, the other species may be slower growing and less adept at utilizing the N. We suspect that much of the large pulse of N that was made available within a month of killing *C. scoparius* was lost rather quickly, through leaching, because the abundance of fast growing nitrophylllic species were relatively sparse immediately following the removal of the dominant species. We do not expect that denitrification played a large role in N loss following *C. scoparius* removal because these gravelly soils are not prone to water logging and anaerobic conditions. It is also plausible that some of this lost N was not actually lost and was instead taken up by the colonizing vegetation.

The added nitrogen that results from *C. scoparius* invasion appeared to have promoted the growth of exotic forbs and grasses including *Tanacetum vulgare*, *Leucanthemum vulgare*, *Hypochaeris radicata*, *Digitalis purpurea*, *Agrostis capilaris*, *Holcus lanatus*, *Arrhenatherum elates*, and *Phalaris arundinacea*. One explanation for the success of exotic invasive species is their ability to readily capitalize on increased resources (Davis et al 2000). In the plots where *C. scoparius* was left untreated, we found only 20 % of the *C. scoparius* understory was occupied by other exotic species. By 10 months after removal of *C. scoparius*, the cover of exotic grasses and forbs more than doubled, and hit 60-70% by 22 months. Our

results suggest that an increase in N availability following the invasion and removal of *C. scoparius* may promote the establishment and growth of fast growing exotic nitrophyllic grasses and forbs.

In contrast, native vegetation cover was not promoted by the nitrogen fertilization associated with removing *C. scoparius*. The native plants that colonize lowland Douglas-fir forest after harvest are adapted to low nutrient conditions and are primarily perennial shrubs and forbs, which tend to be slower growing (Kruckeberg 1995, Swanson et al. 2011). In our sites these included *Gaultheria shallon*, *Arctostaphylos uva ursi*, *Corylus cornuta*, *Cornus nuttallii*, *Symphoricarpos albus* and *Prunus emarginata*. Rather than providing a positive fertilization effect on native vegetation, N enrichment instead appears to be creating an “invasion meltdown” that furthers the invasion of exotic species (Simberloff and Von Holle 1999, Ehrenfeld and Scott 2001, Von Holle et al. 2005, Liao et al. 2008) by changing interspecific competitive dynamics, and the structure of the plant community from woody shrub and perennial forb dominance to grass and annual forb dominance.

However, the native tree Douglas-fir did show highest growth rates in plots planted 1 month after *C. scoparius* removal, which may reflect a positive response to the initial flush of nitrogen. Over a 22 month period the benefit to Douglas-fir growth decreased as did the availability of mineralized nitrogen. The Douglas-fir growth may be tracking N availability, or it may reflect more complex ecological interactions. The rapidly growing exotic grasses and forbs may compete with Douglas fir after *C. scoparius* removal. Harrington et al (2013) found that early growth of Douglas-fir in

this region is more limited by water than nitrogen, which suggests that competition with exotic grasses and forbs for water as well as nutrients may be important. It is likely that both N loss and exotic grass competition are contributing to decreased growth of Douglas-fir seedlings 22 months post *C. scoparius* removal.

Because N fixation can create high demand for phosphorus, we expected that P availability should be very low or depleted while N fixers are present, but that following *C. scoparius* removal treatments, P should be released into the soil through decomposition. However, we found no effects on phosphorus availability with time following *C. scoparius* removal. One possible explanation is that as P was released during *C. scoparius* decomposition, it was immediately taken up by plants colonizing the plots. Because we did not see a pulse of P following removal treatments, it is also possible that the *C. scoparius* tissues were either not high in P content, or whatever P was released was quickly bound to soil clay particles and did not remain labile in the soil. It is unlikely that P was lost through leaching following *C. scoparius* removal treatments due to its high affinity to be adsorbed by small soil particles.

### **Management Implications**

Our work demonstrates that with time following *C. scoparius* removal, soil N first increases in the system and then relatively quickly is either utilized by fast growing exotic grasses and forbs or is leached out of the soil. The initial pulse of N release facilitated the invasion of other exotic grasses and forbs. From a forestry

perspective, the implications of this secondary invasion will depend on what factors are most limiting to Douglas-fir seedling growth. If grass competition inhibits the success of planted seedlings, then applying management strategies that minimize the N inputs will be important. In our study, we allowed the herbicide treated stands of *C. scoparius* to decompose in place. Therefore the nitrogen in the *C. scoparius* tissues was cycled back into the soil. If we had removed the aboveground portion of *C. scoparius* after killing it, less nitrogen could enter the system, fast growing exotic species may not be as successful and slow growing native shrubs and trees would be at less of a disadvantage. The outcome following the removal of the aboveground tissues may however depend on how much of the nitrogen released following *C. scoparius* treatments is from the decomposition of aboveground versus the belowground roots tissues.

It is also worth considering timing of the herbicide application. When *C. scoparius* was sprayed in April, a large pulse of N was released into the soil and this coincides with the active growing season of other plants in the lowland Douglas-fir community, particularly the exotic grasses and forbs. Knowing that a pulse of N is going to be added to the environment following *C. scoparius* removal, it may be advantageous to kill the *C. scoparius* during the fall and winter months while the exotic grasses and forbs are dormant. This approach could result in increased leaching of the N and possibly aid in the recovery of native species and the release of Douglas-fir seedlings from competition with fast growing nitrophylllic exotics.

Our results also provide some insights as to when the best time to plant Douglas-fir seedlings following *C. scoparius* herbicide applications. Our findings suggest that a ‘sooner the better’ planting strategy following *C. scoparius* herbicide applications may be a best approach. In our study, the seedlings planted immediately following *C. scoparius* removal, grew the largest, presumably because of both the N enrichment associated with the invader and the lag time before exotic grasses and forbs became dominant. To disentangle the relative importance of N loss and competitive exclusion following invader removal further studies are required.

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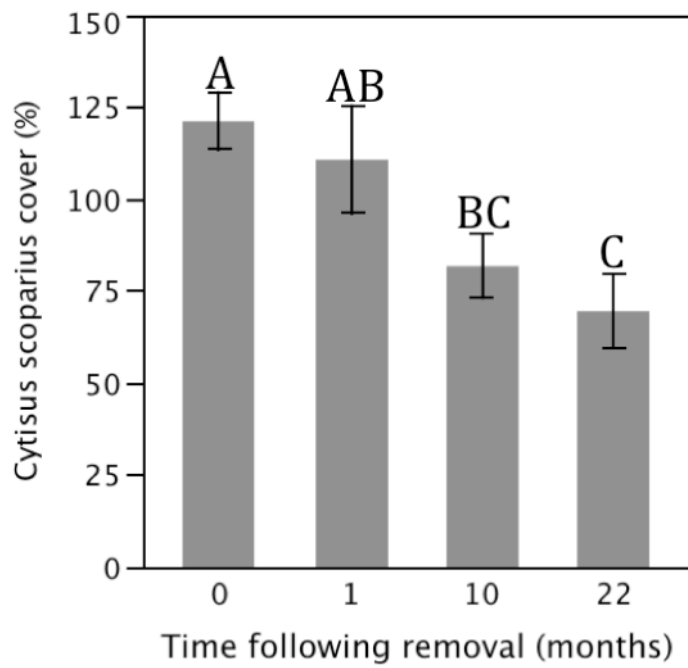
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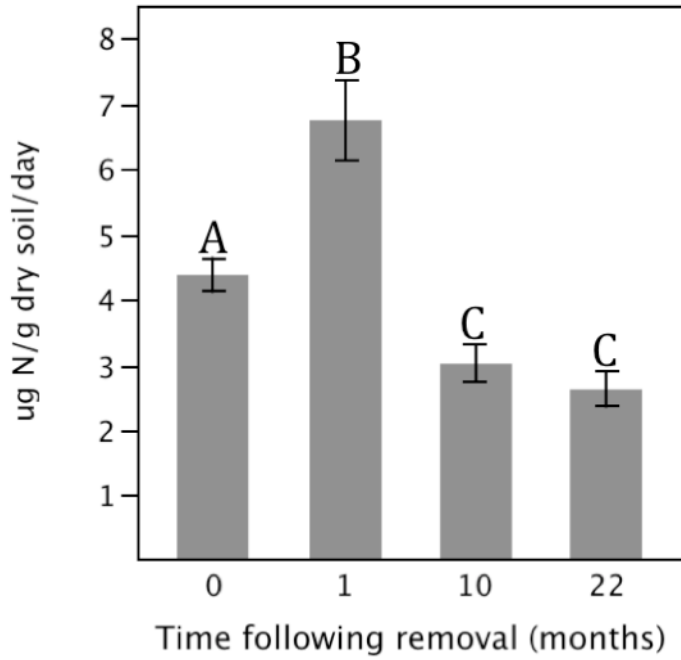
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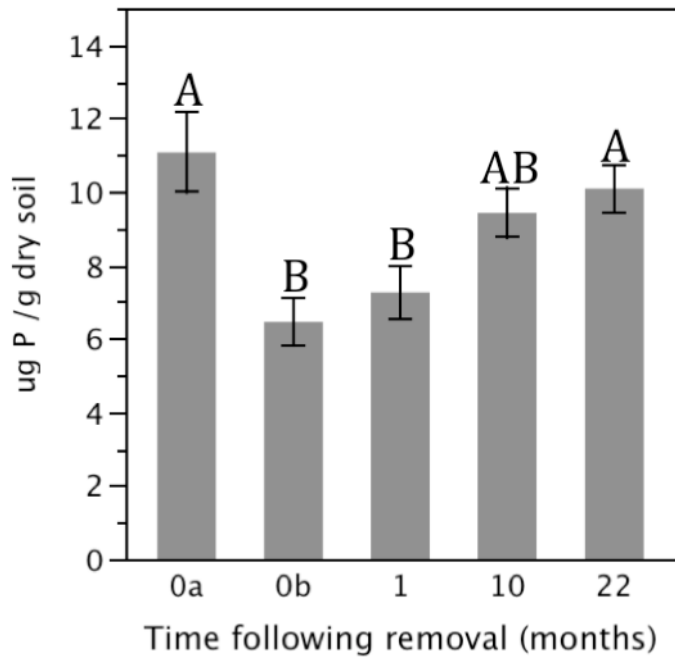
**Figure 2.1.** Percent cover of *Cytisus scoparius* prior to herbicide removal treatments.

Error bars = +/- 1 standard error.

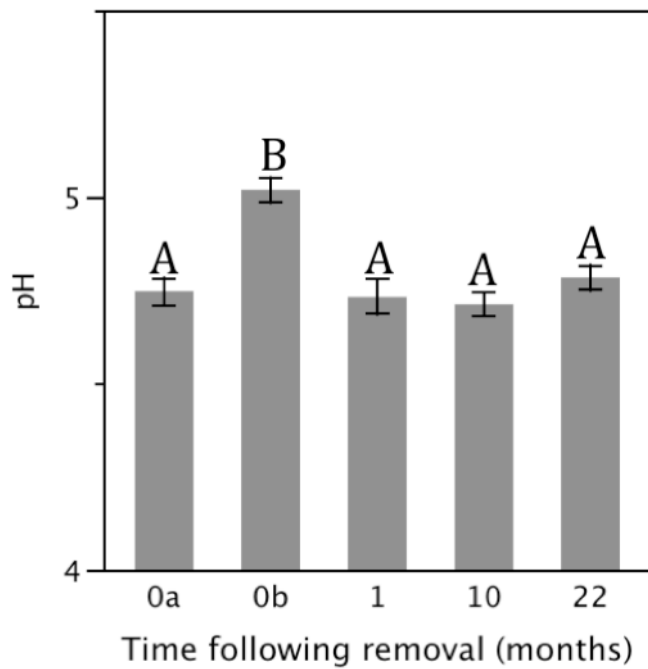


**Figure 2.2.** Mineralized nitrogen ( $\mu\text{g N03-} + \text{N04/g dry soil/day}$ ) availability 0, 1, 10 and 22 months after the removal of *Cytisus scoparius*. Error bars =  $\pm 1$  standard error.

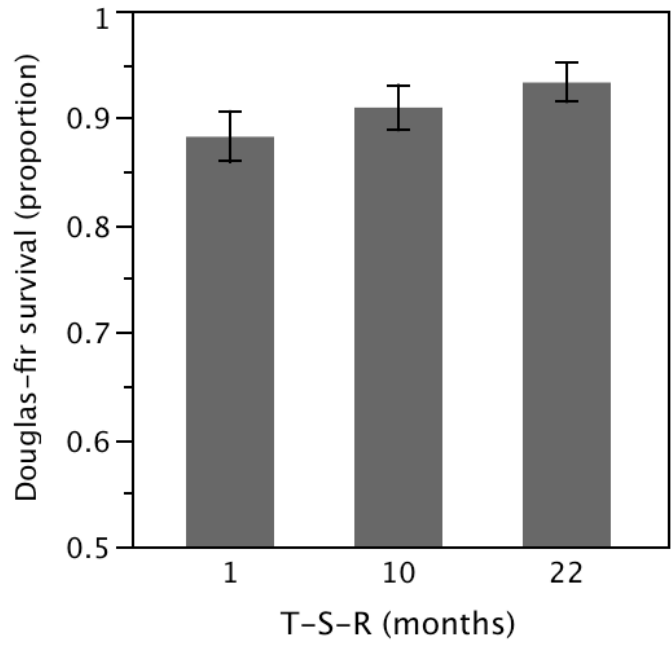




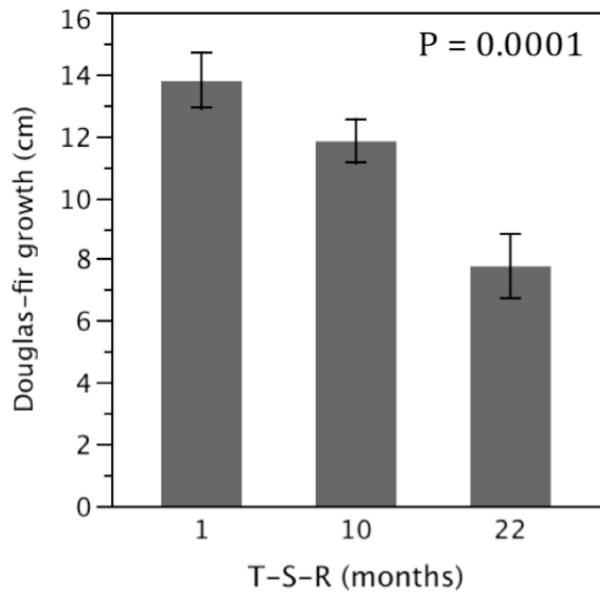
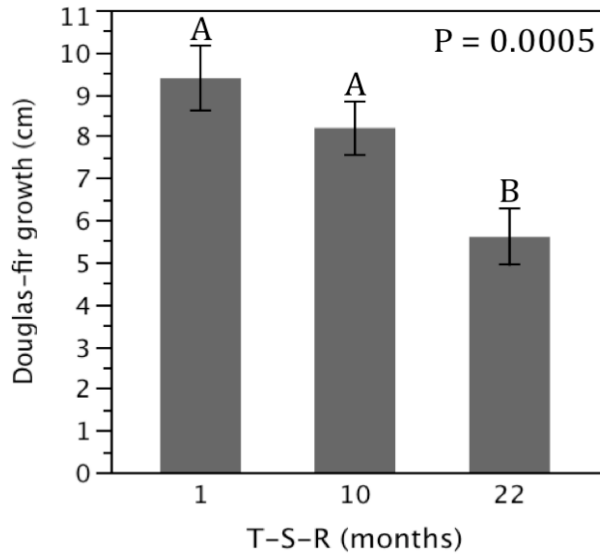
**Figure 2.3.** Phosphorus availability 0, 1, 10 and 22 months following *C. scoparius* removal, 0a were samples collected in April 2013 from the live intact *C. scoparius* ‘control plots’ and 0b were collected in the same control plots in May 2013. Error bars = +/- 1 standard error.



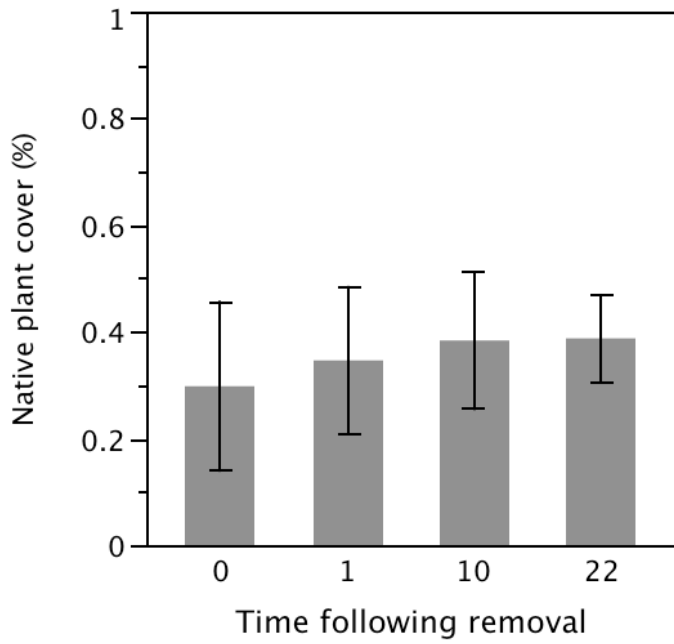
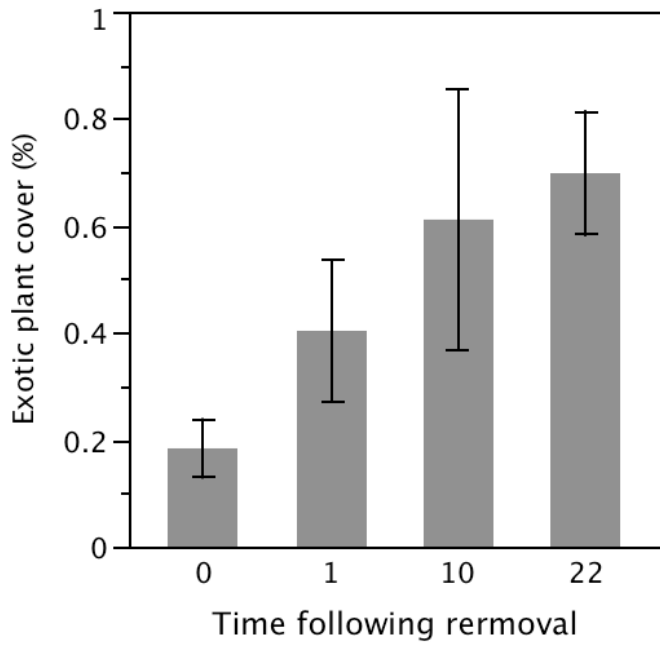
**Figure 2.4.** Soil pH 0, 1, 10 and 22 months following *C. scoparius* removal 0a were samples collected in April 2013 from the live intact *C. scoparius* 'control plots' and 0b were collected in the same control plots in May 2013. Error bars = +/- 1 standard error.



**Figure 2.5.** Douglas-fir seedling survival planted into areas that had *Cytisus scoparius* removed 1, 10 and 22 months prior to planting. Error bars = +/- 1 standard error.



**Figure 2.6.** Douglas-fir seedling growth in areas where *Cytisus scoparius* was removed 1, 10, and 22 months prior to planting including (top panel) and excluding (bottom panel) deer browsed seedlings. Error bars = +/- 1 standard error.



**Figure 2.7.** The percent cover of exotic and native plants with time following *C. scoparius* removal. Error bars = +/- 1 standard error.

## CHAPTER 3

### **Temporal dynamics of soil-mediated impacts on ectomycorrhizal abundance following *Cytisus scoparius* invasion, and implications for Douglas-fir forest restoration**

#### **Introduction**

Invasive species can cause dramatic changes to both the physical and biotic environment (Vitousek 1990, Williamson 1996, Parker et al. 1999, Ehrenfeld 2003, Lambertini et al. 2011) and are one of the most serious threats to biodiversity and causes of ecosystem change worldwide (Millennium Ecosystem Assessment 2005). As a result, management of invasive species is often a major component of conservation and restoration efforts (Simberloff and Vitule 2013). Plant species can influence soil biota, which in turn can differentially influence the success of individual species within a community. Feedbacks between biotic and abiotic factors during invasion may result in loss of native species, shifts in dominance, and changes in resource availability (Suding et al. 2004, Bever et al. 2010, Suding et al. 2013). Thus changes wrought by invasive species can persist and hinder native species from reoccupying habitat even after the invader is removed (Maron and Jefferies 2001, Corbin and D'Antonio 2004b). Because of this “legacy effect”, standard restoration practices may fail to recover ecosystems to pre-invasion conditions (Bakker and Berendse 1999, Zedler 2000, Hobbs and Harris 2001).

It has been suggested, but rarely tested, that impacts of invasive species may change in magnitude and even direction with time following invasion (Strayer et al.

2006). For example, one might expect impacts to increase as the invader population increases, individual plants grow in size, and ecological changes accumulate. On the other hand, it is also possible that ecological or evolutionary change may attenuate the impacts of an invader over time (Agrawal and Kotanen 2003, Phillips and Shine 2004, Strauss et al. 2006, Langkilde 2009). Most studies have quantified the impacts of invasive species by contrasting invaded areas to uninvaded reference areas or by comparing pre-invasion to post-invasion conditions (Parker et al. 1999, Vila et al. 2011). Because each case is a snapshot in time, these studies don't reveal the dynamics of impacts. There is a need for long-term studies that characterize invasion impacts through time. Understanding the temporal dynamics of invasion impacts can inform which populations to prioritize for management. For example, if the impacts of an invader worsen with time, prioritizing the management of new populations may result in better restoration outcomes than attempts to restore areas that have long been accumulating changes caused by the invader. Alternatively, if the impacts of a population develop quickly and duration of invasion does not exacerbate further development of legacy effects, then managing long invaded areas may be equally as successful as restoration of more recently invaded areas. Nitrogen-fixers represent a significant number of invaders worldwide and can have ecosystem level impacts (Daehler 1998). Since litter of nitrogen-fixing invaders is generally higher in nitrogen content than the litter of non-N fixing native vegetation, the tissues decompose faster and increase the availability of nitrogen in soil (Allison and Vitousek 2004, Liao et al. 2008, Castro-Diez et al. 2012). Nitrogen-fixing invaders

have been shown to create soil legacies that facilitate and maintain the dominance of species that can quickly utilize the added nitrogen and gain a competitive advantage over slower growing natives (Maron and Jefferies 2001, Corbin and D'Antonio 2004a, Von Holle et al. 2013). Invasive species that alter soil nutrient cycling dynamics seem particularly likely to create impacts that change with time following invasion. Numerous studies have documented changes in nutrient pools and nutrient cycling with invasion (Vitousek et al. 1987, Archer 1995, Liao et al. 2008, Le Maitre et al. 2011). Few have studied the temporal dynamics of these changes with time following invasion.

Soil mediated impacts of invasive species may involve biotic interactions such as the disruption of mycorrhizal mutualisms between plants and fungi (Mitchell et al. 2006, Pringle et al. 2009, Vogelsang and Bever 2009). Mycorrhizae are a ubiquitous coevolved mutualism between plants and fungi, and can be an important driver of plant community composition and diversity (Bever 1994, van der Heijden et al. 1998, Hartnett and Wilson 1999, Klironomos 2002, 2003, Reynolds et al. 2003). Ectomycorrhizal fungi (EMF) form a sheathing mantle on the surface of fine plant roots and extend into the soil to access water and mineral resources that are otherwise unavailable to their hosts (Smith and Read 1997); their role in phosphorus and nitrogen acquisition is of particular importance (Wallenda and Kottke 1998, Read and Perez-Moreno 2003). Invasive plants that reduce the abundance and richness of local mycorrhizal fungi may disrupt positive feedbacks between native plants and their associated fungal symbionts (Bever 1994, Richardson et al. 2000, Levine et al. 2006,



Mitchell et al. 2006, Wolfe et al. 2008, Pringle et al. 2009, Vogelsang and Bever 2009). In addition to displacing the host species, invasive nitrogen-fixing plants could disrupt the mycorrhizal mutualisms of native plants through a number of possible mechanisms, which include changes in soil chemistry and nutrient cycling dynamics.

Invasive species that produce compound that have toxic effects on mycorrhizal fungi has been implicated as an explanation for the success of some exotic invaders (Callaway and Ridenour 2004, Inderjit et al. 2006). For example, *Alliaria petiolata* produces a suite of secondary compounds called glucosinolates, and the invasion of *A. petiolata* reduces colonization by ectomycorrhizal fungi, resulting in depressed tree seedling recruitment (Artursson et al. 2005, Stinson et al. 2006, Wolfe et al. 2008). Nitrogen-fixing invasive plants may disrupt the mycorrhizal mutualism of native plants through the production of alkaloids. These nitrogen rich compounds can be toxic to other organisms and provide protection against pathogen and herbivore attack (Wink et al. 1982, Wippich and Wink 1985). Because alkaloids have anti pathogen effects, then it follows that they could also have inhibitory effects on beneficial microbes such as mycorrhizal fungi. However, to our knowledge no studies have explicitly investigated the relationship between alkaloid toxicity and mycorrhizal fungi.

*Cytisus scoparius* (Scotch broom) is an introduced and highly invasive woody shrub in the Pacific Northwest, where it has spread rapidly and extensively into open areas with high light availability (Parker and Reichard 1998, Parker 2000) including

areas that have been deforested for timber. *Cytisus scoparius* is a N-fixing legume that increases soil nitrogen availability (Dancer et al. 1977, Wheeler et al. 1987, Diquelou and Roze 1999, Fogarty and Facelli 1999, Haubensak and Parker 2004, Caldwell 2006). *Cytisus scoparius* does not form ectomycorrhizal associations, and its dominance in forest habitats may impact the abundance of ectomycorrhizal fungi, which could lead to a positive feedback that favors its own growth over ectomycorrhizal plants, such as Douglas-fir. In addition to not being a suitable host for ectomycorrhizal fungi, adding *C. scoparius* litter to otherwise uninvaded soil suppresses colonization of ectomycorrhizal fungi on Douglas-fir seedlings as well as seedling growth (Grove et al. 2012). This suggests that the litter of *C. scoparius* may contain compounds that have toxic effects on ectomycorrhizal fungi. We hypothesize that *C. scoparius* invasion affects mycorrhizal fungi through 1) exclusion of suitable ectomycorrhizal host plants, 2) changes in soil nitrogen availability and 3) toxicity through the breakdown of alkaloid rich stem and leaf tissues.

Commercial logging practices in the Pacific Northwest typically remove all or nearly all of the individual Douglas-fir trees. Large-scale removal of Douglas-fir is the most cost effective timber harvest method, but causes declines of forest associated species (Simberloff 1999, Thompson et al. 2003, Linden and Roloff 2013), including the abundance and diversity of ectomycorrhizal fungi (Rose et al. 1983, Pilz and Perry 1984, Perry et al. 1987, Hagerman et al. 1999), as well as the mammals, birds and insects, that disperse mycorrhizal spores (Powers 1989). In Douglas-fir forests ectomycorrhizal fungi are ubiquitous and Douglas-fir seedling establishment can

critically depend on the presence of ectomycorrhizae (Molina et al. 1992, Nara 2006). The duration of time that ectomycorrhizal spores and propagules can remain alive in the absence of a suitable host plant is still unknown for most species (Bruns et al. 2009, Nguyen et al. 2012). It is also unclear how the duration of time between deforestation and reforestation affects the survival and recruitment of ectomycorrhizal fungi or how long following deforestation does the abundance of EMF remain sufficient to facilitate Douglas-fir survival and growth.

Forests in the Pacific Northwest provide an ideal opportunity to study changes of invader impact over time following deforestation and invasion. *Cytisus scoparius* invasion begins rapidly after Douglas-fir forests are harvested for timber. Here, we used a chronosequence of many independent invasions of different ages to characterize the development of soil mediated impacts over time. Our study design disentangles the impacts of deforestation and *C. scoparius* invasion on EMF abundance, nitrogen availability, and ultimately Douglas-fir seedling growth. We additionally conducted a soil conditioning experiment in the greenhouse to similarly separate the effects of plant host loss and *C. scoparius* invasion on ectomycorrhizal fungi abundance and Douglas-fir growth. Specifically we ask: 1) What is the impact of invasion on ectomycorrhizal fungi, nitrogen availability, and Douglas-fir success? 2) After invasion, do these impacts experience rapid shifts or do changes accumulate slowly over time? 3) Above and beyond host removal that occurs with timber harvest, does *C. scoparius* invasion impact the ectomycorrhizal mutualism, and does this affect Douglas-fir growth?

## **Methods**

### **Chronosequence experiment**

*Field Sites:* In spring (March 24-April 2) 2012, we collected soils from 16 sites over a broad geographic area in western Washington in deforested areas experiencing active invasion of *C. scoparius*. Of these 16 sites, 14 are managed by the Washington State Department of Natural Resources (WA- DNR) and the remaining 2 are managed by Lewis McChord military base (Sites: Beal and Nisqually). Each site was historically Douglas-fir forests that had been clearcut or otherwise harvested for timber, and they vary from 3-31 years in the duration of time since they were last harvested (Table 1). The sites were primarily clearcuts although some harvests did leave scattered individual trees or clumps of trees in tact. All of the soils we collected were at least 15 m from any live Douglas-fir and were unaffected by them. We selected sites to minimize differences in underlying soil fertility. All sites selected for this study had gravelly sandy loam soils and were characterized as “glacial soils” by the Natural Resource Conservation Service (NRCS). Additionally, we restricted our sites to those classified by WA-DNR as “Site Class III”. The site class designation is derived from NRCS soil data and the height of the tallest dominant tree species in fully stocked stands 50 years post planting, and it is used as an indicator of site fertility so that foresters can project forest biomass for a given stand. All soils were collected in clearcuts experiencing active invasion of *C. scoparius*. The stochastic nature of *C. scoparius* invasion created invaded and uninvaded patches within each of

these clearcuts. *Cytisus scoparius* begins to invade clearcuts immediately after removal of the Douglas-fir overstory, and we used time-since-last timber harvest as a proxy for duration of invasion. Even the youngest clearcuts had robust patches of *C. scoparius*: however, there was no guarantee that our sampling in the oldest clearcuts was also from the oldest *C. scoparius* patches within those clearcuts. Therefore our test for an effect of time is conservative.

**Soil collection:** In April 2012, we collected approximately 4 gallons of soil from the uninvaded and *C. scoparius* invaded patches in each of the 16 clearcuts (N = 2 soil treatments x 16 time since harvest sites = 32 treatments). The samples were harvested from directly beneath the canopy of *C. scoparius* in the invaded patches and from beneath a locally native perennial woody shrub in the uninvaded patches. The diversity of native shrub species encountered in the uninvaded patches was low (personal observation) and generally included *Gaultheria shallon*, *Vaccinium ovatum*, *Rhododendron macrophyllum*, *Mahonia aquifolium*, and *Arctostaphylos uva-ursi*. All of these native shrubs, except *Mahonia aquifolium* (Berberidaceae), are in the Ericaceae family and form ericoid or arbutoid mycorrhizal associations (Douglas et al. 1989, Xiao and Berch 1992, Gorzelak et al. 2012), while *C. scoparius* and *M. aquifolium* form arbuscular mycorrhizal associations (Wang and Qiu 2006). Plants that form ericoid mycorrhizae do not share fungal partners with ectomycorrhizal plants like Douglas-fir. In contrast, ericaceae plants that form arbutoid mycorrhizae can share fungal symbionts with ectomycorrhizal plants (Hagerman et al. 2001, Krpata et al. 2007), but in our study sites only *Arctostaphylos uva-ursi* is arbutoid

mycorrhizal. Therefore this is the only shrub that may share fungal symbionts with Douglas-fir.

We used a shovel blade to scrape away any existing moss and litter before collecting the top 25 cm of field soil. The soils were stored uncovered in 5 gallon buckets in the shade and then transported to the greenhouse at the University of California, Santa Cruz, where they were potted up into 164 mL cone-tainers (Stuwe and Sons, Tangent OR). For each of the 32 treatments (time since harvest x invasion status) we created 54 replicates and 3 replicates of each treatment were included in each of the 18 greenhouse blocks ( $N = 32 \text{ treatments} \times 3 \text{ replicates/ treatment/ block} = 1782$ ).

To prevent mycorrhizal cross contamination between soil treatments, we sterilized all cone-tainers and potting tools in a 10% bleach solution immediately prior to potting. To reduce the splash of mycorrhizal spores from one soil treatment to another we capped each cone-tainer with ~2 cm of 30 grit sand. To further prevent mycorrhizal spores from splashing from one soil treatment to another we spaced the cone-tainers 4.5 cm apart and top-watered.

Douglas-fir seeds were obtained from Silvaseed (Roy, Washington) and were harvested from Eatonville Washington (seed zone 422). We cold stratified the seeds by soaking them for 24 hours in tap water and then refrigerating for four weeks. In May 2012, Douglas-fir seeds were planted into each replicate pot, and arranged in a randomized block design.

### ***Douglas-fir Response***

Four weeks post seed planting we measured germination success. In August 2012, three months after the initial planting we began measuring seedling heights and continued to measure seedling heights approximately every 10 weeks until the final block was taken down in November 2013. At the time of collecting height data, we also observed germination of *C. scoparius* seed in some replicates. Any replicate that was invaded was excluded from further analysis to avoid the confounding effect of competition between Douglas-fir and *C. scoparius*. In August of 2013, we began to harvest the experiment one block at a time. At the time a block was harvested the final seedling heights were measured and the aboveground portion of each surviving seedling was clipped off, oven dried at 61° C for one week, and weighed to obtain aboveground dry biomass. On November 1, 2013 we completed the takedown of the final block (block 18).

### ***Ectomycorrhizal Abundance***

We measured the proportion of roots colonized by ectomycorrhizal fungi on 190 seedlings from 4 blocks. To prepare the roots for microscopy we carefully extracted the intact root mass from the soil and removed adhered soil particles by briefly soaking the root mass in a deionized water bath. The entire root mass was cut into 0.5 cm segments. To measure the abundance of ectomycorrhizal fungi we placed all root segments into a grided petri dish and selected the 72 roots segments that terminated

nearest to the corner of each grid cell. The 72 subsampled root segments were stored at 3° C in DI water for up to 7 days. Each of the root tips was assessed for the presence or absence of EMF and assigned a morphotype. We used a molecular approach to determine the mycorrhizal status of ambiguous roots when microscopy was insufficient.

We used a DNeasy mini plant kit (Qiagen) to extract fungal DNA from the ambiguous root segments . We amplified the ITS region of rDNA with the fungal specific ITS-1F and ITS-4 primers (Gardes and Bruns 1993) and a Green GoTaq master mix (Promega). The PCR products were cleaned with ExoSap-It (USB Products). The PCR product was sequenced on an ABI 3100 Sanger Sequencer (Applied Biosystems, Foster City, CA) at the University of California Berkeley DNA sequencing facility. To determine if the fungal sequences were actually ectomycorrhizal we used Geneious (Drummond et al. 2009) to view, edit, create contigs and match our nucleotide sequences with vouchered records in the NCBI GenBank database.

### ***Leaf Nitrogen Content***

To evaluate if nitrogen enrichment associated with *C. scoparius* invasion occurred and to evaluate the relationship between N enrichment and Douglas-fir seedlings growth in *C. scoparius* invaded soils we measured the nitrogen content of Douglas-fir needles. Within each block all needles from the same treatment (age of timber harvest x invasion status) were combined and ground in a Wiley Mill, then



200 – 250 mg of each sample (N = 124) was analyzed for % N on a CNS Vario Max Analyzer (Elementar). Because a minimum of 200 mg was required for analysis, sometimes the combined seedling mass was not sufficient to be included for N analysis.

### ***Statistical analyses***

To assess the effect of timber harvest age and *C. scoparius* invasion, on Douglas-fir germination, and to incorporate the greenhouse block effect as a random factor we analyzed the germination response as a GLMM and compared nested models using AIC. The GLMM and model comparisons were performed in R with the lme4 package.

We modeled Douglas-fir seedling biomass, leaf nitrogen content, and ectomycorrhizal root colonization with ANCOVA models, including age of timber harvest and invasion status as fixed factors. Greenhouse block was included as a random factor for biomass, but not for leaf nitrogen content or ectomycorrhizal colonization, for which we were unable to maintain the block structure. The ANCOVA models were performed with JMP v. 10 (SAS Institute 2013)

### **Soil conditioning experiment**

To better understand the relative impacts of host removal and *C. scoparius* invasion on the success of Douglas-fir germination, growth, and ectomycorrhizal

fungi, we implemented a fully randomized soil conditioning greenhouse experiment with soils collected from uninvaded and recently clearcut sites in the Capitol State Forest in Western Washington (Thurston County). In December 2010, we collected 5 gallons of field soil from the 3 recently clearcut uninvaded Douglas-fir forests. The soils used in this study were collected from clearcuts that have never been invaded by *C. scoparius* and that were clearcut between 7 months- 2 years prior to the start of this study. We used soils from such recent clearcuts to capture the maximum abundance of ectomycorrhizal fungi in the soil shortly following host removal. In the field, prior to soil collection, we scraped aside any living vegetation and litter and collected approximately 3-4 gallons/site of soil 20 cm below the soil surface. Soils were brought back to the University of California, Santa Cruz greenhouse and potted up into 983 ml D60 Deepots (6.4 cm x 36 cm, Stuewe and Sons, Tangent, OR).

To condition the soils, one third of each of the soils was planted with Douglas-fir seedlings, one third with *C. scoparius* seeds, and the last third remained unplanted as a control (N = 7 replicates/ conditioning treatment x 3 soil conditioning treatments x 3 field soils = 63). In November 2011, after the soils had been conditioned for 11 months, the *C. scoparius* and Douglas-fir plants were removed and the soils with the same conditioning treatment were homogenized and repotted into 350 164 mL pots. Douglas-fir seeds were planted into all replicates. The seeds used in this experiment were from the same seed source as described above in the chronosequence experiment and were stratified in the same way.

In January 2012, eight weeks after initial planting, we measured Douglas-fir seed germination. Following germination all replicates were capped with sand to reduce mycorrhizal spore splash between replicates (as described above). All germinated seedlings were allowed to grow for 10 months after initial planting and in September 2012, final seedling height measurements were recorded. At that time, we harvested the aboveground biomass of all surviving seedlings. We cut each tree at the root crown and dried them at approximately 61° C for 7 days and then weighed. Height and biomass patterns were similar, therefore we only report biomass results here. We also measured leaf nitrogen content, as above.

We measured the abundance of ectomycorrhizal fungi on 57 seedlings (N = 19 seedlings/treatment x 3 treatments) following the methods described previously.

Belowground biomass was also measured on all the seedlings that were not destructively sampled for EMF assessment. To measure belowground biomass we gently excavated the intact root systems from the soil rinsed in a water bath, dried at 61° C for a minimum of 7 days and then weighed.

### ***Statistical analysis***

We used logistic regression to compare the proportion of seeds germinated across the three soil conditioning treatments. The full model included each of the conditioning treatments as predictor variables and germination as a nominal response variable. We modeled Douglas-fir seedling biomass, leaf nitrogen content, and ectomycorrhizal root colonization with ANOVA models and included soil

conditioning treatment as a fixed effect. For all tests that had significant effects, we used Tukey's post-hoc test to compare treatments.

## **Results**

### **Chronosequence experiment**

#### ***Douglas-fir seed germination and growth***

The comparison of the different nested models revealed that neither year or invasion status was a significant contributor to germination success ( $P = 0.56$ ). There was a positive effect of invasion and time since timber harvest on Douglas-fir seedling growth, as measured by aboveground biomass ( $F = 25.58$ ,  $DF = 1$ ,  $P = 0.0001$ ; Fig. 1). Overall seedlings in invaded soils grew 18% larger than seedlings in uninvaded soils from the same site ( $F = 20.93$ ,  $DF = 1$ ,  $P = 0.0001$ ; Fig. 1), and there was a marginally significant interaction of timber harvest age and invasion status ( $F = 3.48$ ,  $DF = 1$ ,  $P = 0.06$ ) and a significant block effect on Douglas-fir biomass ( $F = 2.81$ ,  $DF = 1$ ,  $P = 0.0001$ ). Because both main effects (timber harvest age and invasion status) were significant, we additionally used separate linear regression models to evaluate the effect of time following invasion and time following timber harvest on aboveground Douglas-fir seedling biomass. In *C. scoparius* invaded soils, the duration of invasion had a significant, but very small positive effect on Douglas-fir seedling growth ( $r^2 = 0.008$ ,  $P = 0.045$ ). In uninvaded soil, the duration of time since the last timber harvest had a significant positive effect on seedling growth ( $r^2 = 0.05$ ,  $P = 0.0001$ ), that is seedlings grew larger in soils that were harvested longer

ago.

### ***Ectomycorrhizal colonization***

The percent of Douglas-fir root tips colonized by ectomycorrhizal fungi was 31% greater in uninvaded soil compared to invaded soil ( $F = 14.89$ ,  $DF = 1$ ,  $P = 0.0002$ ; Fig. 2). Ectomycorrhizal root colonization was not affected by timber harvest age ( $F = 1.05$ ,  $DF = 1$ ,  $P = 0.31$ ; Fig. 2) and there was no interaction of time and invasion status ( $F = 0.351$ ,  $DF = 1$ ,  $P = 0.554$ ). To evaluate the relationship between the abundance of ectomycorrhizal fungi and Douglas-fir seedling growth we used separate correlation coefficients for invaded and uninvaded soil conditions. The abundance of ectomycorrhizal fungi was correlated with increased Douglas-fir seedling growth, but only for trees in uninvaded soil ( $R = 0.26$ ,  $P = 0.01$ ; Fig. 3). Seedling growth in *C. scoparius* invaded soils was unrelated to the abundance of EMF ( $R = 0.10$ ,  $P = 0.32$ ; Fig 3)

### ***Leaf Nitrogen Content***

We found no effect of invasion ( $F = 0.50$ ,  $DF = 1$ ,  $P = 0.48$ ) or time following timber harvest ( $F = 1.30$ ,  $DF = 1$ ,  $P = 0.26$ ) on nitrogen leaf content in Douglas-fir needles.

### **Soil Conditioning Experiment**

### ***Seed Germination and Douglas-fir growth***

Seed germination was 55% less in *C. scoparius* conditioned soil relative to the control and Douglas-fir conditioned soil ( $\chi^2 = 20.90$ ,  $P = 0.0001$ ; Fig 5) (N = 284). As measured by aboveground dry biomass, Douglas-fir seedlings grew 50% larger in soils conditioned by *C. scoparius* compared to seedlings in the control (F = 10.86, DF = 2, 176,  $P = 0.0001$ ; Fig 6) and there was no difference in seedling size between the seedlings grown in the control and Douglas-fir conditioned soil (Fig 6).

### ***Ectomycorrhizal colonization***

Soil conditioning treatment had no effect on the overall proportion on Douglas-fir roots colonized by ectomycorrhizal fungi (F = 0.45, DF = 2, 54,  $P = 0.64$ ; Fig 7) the mean proportion of roots colonized ranged from 72-78%. The ectomycorrhizal genus *Rhizopogon* was identifiable based on distinctive morphology. *Rhizopogon* root colonization was 85% higher in the control relative to the Douglas-fir and *C. scoparius* conditioned soils (F = 16.28, DF = 2, 54,  $P = 0.0001$ ; Fig 8). The proportion of roots colonized by *Rhizopogon* on seedlings from the *C. scoparius* and Douglas-fir conditioned soils was not different (Fig 8).

### ***Leaf Nitrogen Content***

Douglas-fir seedlings grown in soils conditioned by *C. scoparius* had 44% higher leaf nitrogen content than seedlings grown in the control, and leaf nitrogen content

was not different in the control and Douglas-fir conditioned soils ( $F = 4.93$ ,  $DF = 2$ ,  $P = 0.01$ ; Fig 9).

### ***Discussion***

We expected that with time following timber harvest the abundance of ectomycorrhizal fungi would decrease and that the lack of ectomycorrhizal fungi would have consequences for Douglas-fir seedling growth. Surprisingly we found relatively high (45 %) ectomycorrhizal colonization rates in the deforested soils, and duration of time the absence of suitable host plants did not result in declines of ectomycorrhizal fungi, suggesting that: 1) whatever ectomycorrhizal fungi are lost as a result of deforestation happens quickly and that 2) the presence of ectomycorrhizal host plants was not as important to the persistence of ectomycorrhizal fungi as we had thought.

The relatively high proportion of colonized Douglas-fir roots grown in soils that had been deforested for upwards of 30 years suggests that at least some Douglas-fir associated species of ectomycorrhizal fungi have long lived spores and/or are readily dispersed into areas that have long been deforested. Longevity of ectomycorrhizal spores is not well understood (Baar et al. 1999, Bruns et al. 2009), but we know that some pine associated species can last at least a decade (Nguyen et al 2012). We know that the seeds of many plants can persist in soil as a seed bank for many decades and then germinate when extrinsic conditions are favorable to their success. Because mycorrhizal fungi are generally not free living (aside from dormant spores),

it makes intuitive sense that the spores of mycorrhizal fungi that associate with plants that have long seed dormancy, might also have equally long dormant periods, so that they can respond in sync when environmental conditions are suitable. However, Douglas-fir seeds generally only remain viable in soil for 1-2 years and do not exhibit long dormancy periods (Isaac 1943), and in our study sites (and in the Pacific Northwest in general), the plants that abundantly colonized deforested areas were not suitable alternative hosts for ectomycorrhizal fungi.

Invasion by *C. scoparius* did result in reduced colonization of ectomycorrhizal fungi on Douglas-fir seedling roots. There are several possible mechanisms *C. scoparius* invasion may have resulted in the disruption of the ectomycorrhizal mutualism.

First, *Cytisus scoparius* may have reduced the abundance of ectomycorrhizal fungi through the production and release of toxic alkaloid compounds. We found that ectomycorrhizal fungi were suppressed in the chronosequence experiment, but not the soil conditioning experiment. This discrepancy could be due to differences in exposure/contact of aboveground *C. scoparius* leaf and stem tissues. The toxic alkaloid compounds of *C. scoparius* are found primarily in the leaf and stem tissues (Wink et al. 1982, Wink et al. 1983). The soils used in the soil conditioning experiment were from uninvaded clearcuts and had never had *C. scoparius* leaf and stem tissues incorporated, while the chronosequence soils had been experiencing aboveground *C. scoparius* tissue additions via annual senescence for 3-30 years. We therefore expect that the chronosequence soils could have contained the toxic



alkaloids while the soil conditioning soils did not.

A second possible explanation for the disruption of the mycorrhizal mutualisms is through changes in soil nitrogen pools (Treseder 2004). Nitrogen enrichment associated with *C. scoparius* invasion is well documented in this region (see chapters 1 and 2). If ectomycorrhizal fungi are involved in nitrogen acquisition for Douglas-fir, then nitrogen enrichment may have reduced the dependence of Douglas-fir on ectomycorrhizal fungi, or led to losses of particular fungal species that are particularly important in nitrogen acquisition (Johnson 1993, Hoeksema et al. 2010). Other work has demonstrated that nitrogen enrichment can have direct effects on ectomycorrhizal fungi. For example, atmospheric nitrogen deposition can change ectomycorrhizal fungi communities (Lilleskov et al. 2008), and experimental nitrogen additions in grasslands reduced the abundance and diversity of mycorrhizal fungi (Egerton-Warburton et al. 2007). If atmospheric nitrogen deposition and nitrogen fertilizers can impact ectomycorrhizal fungi, then nitrogen enrichment from nitrogen-fixing invaders is likely driving changes in mycorrhizal communities as well.

Despite the negative effects of *C. scoparius* on ectomycorrhizal fungi, the soil mediated impacts of *C. scoparius* had a net positive, rather than negative effect on Douglas-fir seedling growth in both the chronosequence and soil conditioning experiments. The positive Douglas-fir growth response reported here was likely mediated by the fertilization effect of nitrogen enrichment. Several studies have reported elevated nitrogen in soils invaded by *C. scoparius* (Dancer et al. 1977, Wheeler et al. 1987, Diquelou and Roze 1999, Fogarty and Facelli 1999, Haubensak

and Parker 2004, Caldwell 2006). No studies to our knowledge have found nitrogen enrichment to benefit native plants in invaded communities, instead the impacts of nitrogen-fixing invaders are reported as negative (Ehrenfeld 2003, Von Holle et al. 2005, Elgersma et al. 2011). One possible explanation for the switch from negative to positive effects of *C. scoparius* invasion, and nitrogen enrichment in particular, may be in part because ectomycorrhizal fungi are less important to seedling growth in greenhouse conditions compared to in the field (Brinkman et al. 2010), where conditions are less hospitable. In the field, the dry season can be stressful for Douglas-fir seedlings, while in the greenhouse the trees were continuously watered. In our greenhouse experiments, Douglas-fir seedlings were grown in isolation and free from competitors, while in the field, fast growing grasses and forbs quickly establish and dominate areas following *C. scoparius* removal (see chapter 2. These fast growing herbaceous competitors may be better suited to utilize the nitrogen enrichment than Douglas-fir, which has high nutrient use efficiency and employs a slow growing but long lived strategy (Milberg et al. 1999, Davis et al. 2000, Tateno and Takeda 2010, Urban et al. 2013).

Reforestation of invaded deforested areas continues to be an ongoing management problem even after *C. scoparius* is removed (Parker and Haubensak 2011). In a previous greenhouse study, we found that soils that had been long invaded by *C. scoparius* suppressed Douglas-fir seedling growth and ectomycorrhizal colonization, and that adding a *C. scoparius* litter treatment to otherwise uninvaded forest soils inhibited both ectomycorrhizal colonization and Douglas-fir growth

(Grove et al. 2012). It was surprising to us that in the experiments presented here, the soil legacy effects of *C. scoparius* on Douglas-fir growth are positive rather than negative. Methodological differences between these experiments and our previous work may explain the apparent switch from inhibition to facilitation of Douglas-fir growth. In our earlier work, the aboveground leaf and stem tissues were incorporated into the soil at the time of planting Douglas-fir seedlings, but they were excluded in the soil conditioning experiment. The soils used in the chronosequence were collected in late March-early April and had not received any additions of aboveground tissues since leaf senescence the previous fall (Haubensak 2001). The chronosequence soils also never had *C. scoparius* stem tissues directly incorporated. *Cytisus scoparius* stems are somewhat unique for a woody perennial, in that they remain fleshy and green and photosynthesize year round (Bossard and Rejmanek 1992) and contain sparteine and other alkaloid defense compounds that are thought to provide protection against herbivore and pathogen attack (Wippich and Wink 1985, Gresser et al. 1996). When the stem tissues are incorporated into the soil following *C. scoparius* control treatments, these alkaloids are released into the soil through decomposition, and may inhibit the growth and even survival of Douglas-fir seedlings directly, or indirectly through suppression of mycorrhizal fungi.

In the soil conditioning experiment, seed germination was suppressed by 45% in the *C. scoparius* conditioned soils relative to the control and Douglas-fir conditioned soils. This intriguing negative soil mediated effect of *C. scoparius* was not however observed in the chronosequence experiment. One possible explanation for the

inhibition of seed germination is due to differences in osmotic potential or water holding capacity caused by differences in soil texture between treatments. A large volume of *C. scoparius* root biomass was left in the *C. scoparius* conditioned soils, and as a result the soil texture was different from the Douglas-fir conditioned soil and the control (personal observation). Seed germination could have also possibly been suppressed by chemical inhibition from *C. scoparius* alkaloids, although the alkaloid rich aboveground plant tissues were not incorporated into the soil.

Our findings suggest that the effects of *C. scoparius* invasion and deforestation develop quickly, and intensify only slightly if at all with time following timber harvest and invasion. In the chronosequence study, there was a great deal of variation in seedling growth within each year following *C. scoparius* removal treatment, and overall we found a significant, but very slight positive effect of duration of invasion.

It was also surprising to us that the nitrogen content in Douglas-fir needles was higher in invaded soils in the conditioning experiment but not in the chronosequence, and that there was no relationship between invasion duration and leaf nitrogen. One possible explanation for this is that nitrogen did actually accumulate in the soil over time in *C. scoparius* invaded soils, but because the Douglas-fir was no longer nitrogen-limited, the leaf nitrogen content did not increase. It is also possible that any increase in nitrogen experienced by Douglas-fir due to invasion occurred early on in the experiment, and that after 18 months the N was either used by the seedlings during peak photosynthesis during the previous spring and summer months, or was

leached from the soil with watering. An alternative explanation is the ability of legumes to halt nitrogen fixation once they are no longer nitrogen limited (Truchet and Dazzo 1982, Streeter 1988, Carroll et al. 1990). *Cytisus scoparius* may fix enough nitrogen nearly immediately upon colonization to fulfill the plants nitrogen needs, leading to down regulation of nitrogen fixation, such that nitrogen does not accumulate in the soil over time.

### **Management Implications**

Our findings suggest that because the impacts of *C. scoparius* invasion change only minimally over time following initial establishment, Douglas-fir reforestation efforts of long invaded areas may not necessarily be more problematic to restore than recently invaded areas. For many good reasons natural resource managers often prioritize recent invasions for control over older populations. Generally speaking because younger populations are smaller in size, control is often more feasible. Invasive species that have long lived seeds, such as *C. scoparius*, will also have larger seed banks in soils that have long been invaded, and this inhibits restoration success. Feasibility and seed bank aside, areas that have long been invaded by *C. scoparius* do not necessarily have higher soil nitrogen or fewer ectomycorrhizal fungi than areas that have only recently been invaded. If these are important drivers of reforestation success, then areas that have long been invaded should be as restorable as recent invasions. This is encouraging because in the Pacific Northwest, many of the *C. scoparius* invasions have persisted for several decades. Although, it may take more

initial effort to control long established populations of *C. scoparius*, the same degree of soil amendments such as carbon or mycorrhizal inoculum additions should not increase with invasion age. If the impacts of N-enrichment and loss of ectomycorrhizal fungi that result from *C. scoparius* invasion are important factors that contribute to the restorability of Douglas-fir, than long invaded sites should still be considered for Douglas-fir reforestation.

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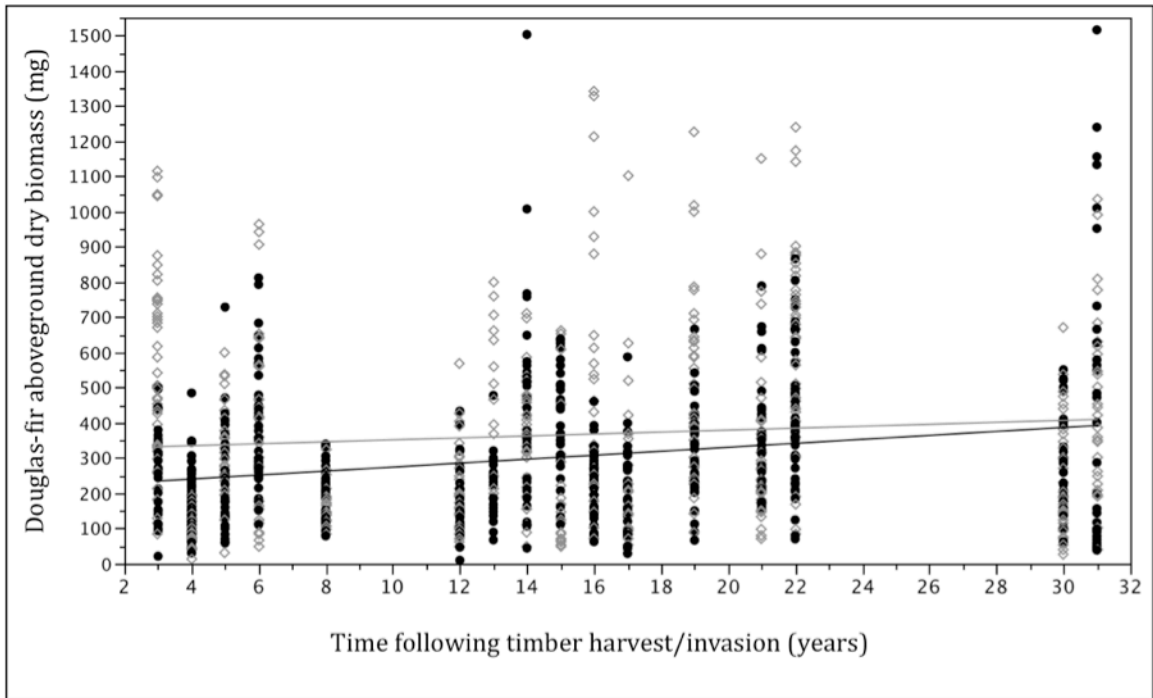
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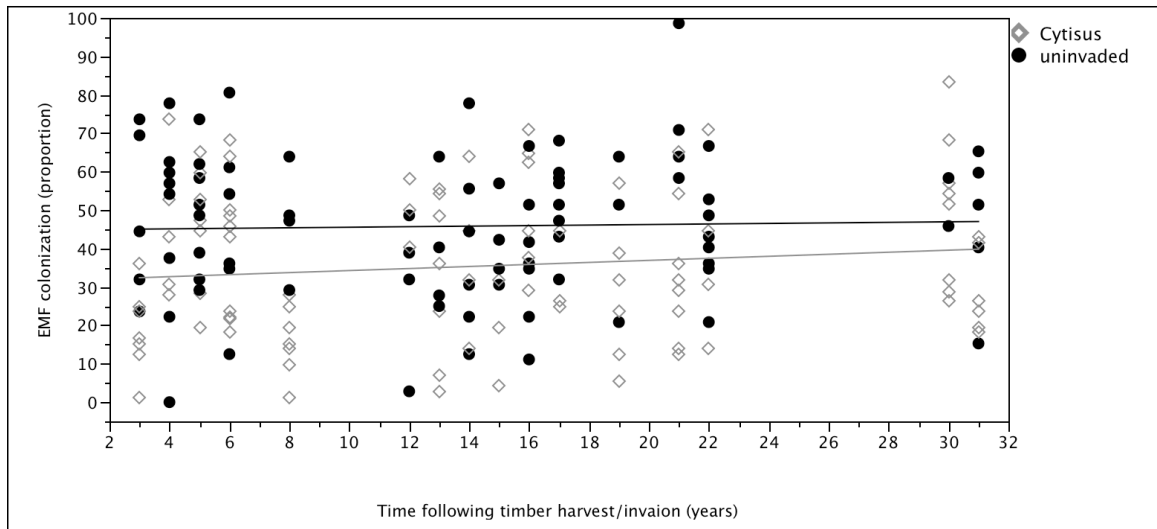


**Table 3.1.** Sixteen deforested sites of varying age (3-31 years) in western Washington used for chronosequence study.

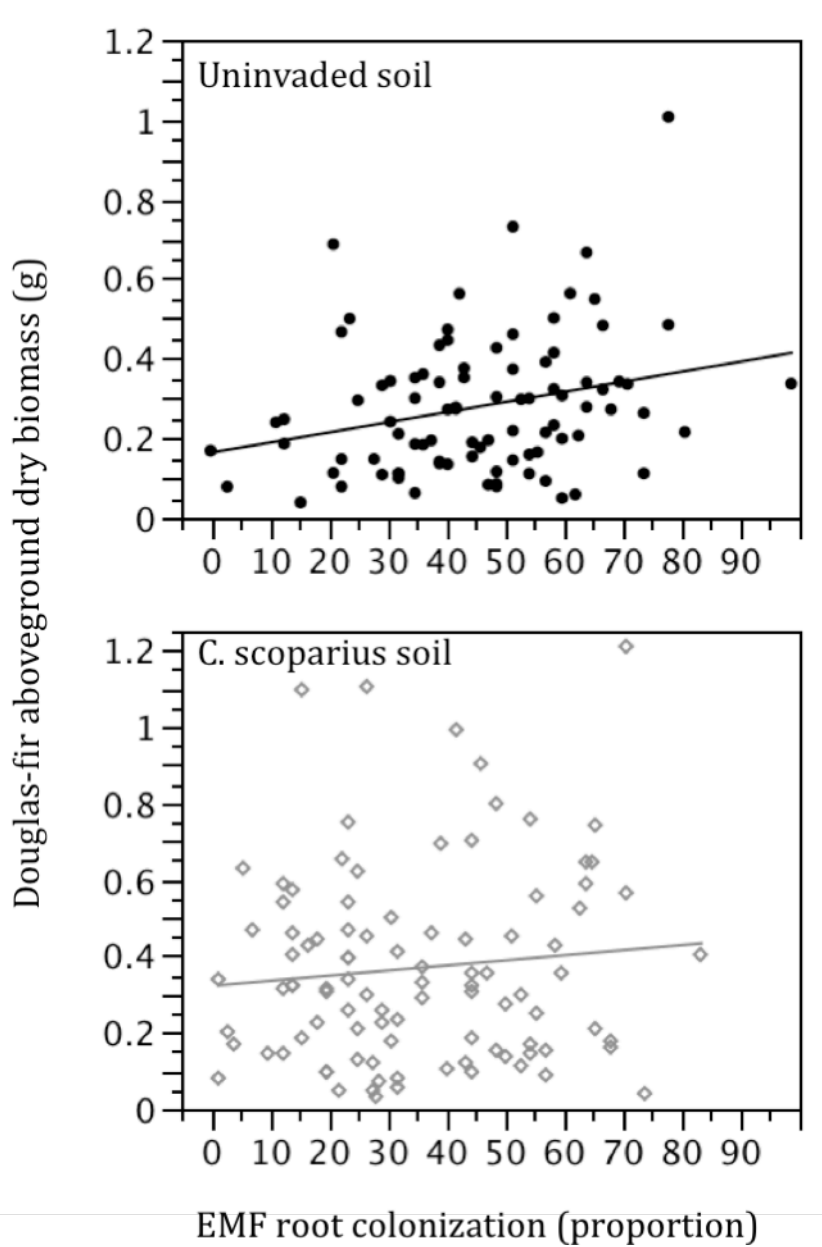
Clearcut	Harvest yr	UTMs	Size (ha)
Beal	1981	47°04'31.15"N, 122°37'10.03"W	1.76
Nisqually	1982	45°59'28.37"N, 122°38'35.32"W	4.46
Rainier	1990	46°52'43.45"N, 122°39'54.74"W	2.31
Elfendahl Pass 2	1991	47°28'14.83"N, 122°54'54.91"W	4.55
Horseshoe 1	1993	47°24'04.50"N, 122°40'30.44"W	3.29
Hurd 1	1995	47°26'15.61"N, 122°55'42.99"W	8.78
Waddel Creek	1996	37°05'52.58"N, 122°16'44.65"W	3.34
Tahuya River	1997	47°26'02.81"N, 122°57'16.91"W	14.35
Elfendahl Pass 1	1998	47°28'37.51"N, 122°54'39.98"W	5.23
Sandhill road 2	1999	47°28'22.73"N, 122°51'56.44"W	9.19
Sherwood	2000	47°20'51.21"N, 122°51'16.87"W	12.07
Haven way	2004	47°26'28.03"N, 122°57'38.82"W	8.40
Algyer	2005	46°53'02.05"N, 122°38'55.36"W	25.21
Sandhill road 1	2006	47°28'59.47"N, 122°51'21.63"W	24.68
Hurd 2	2006	47°25'14.98"N, 122°56'46.69"W	13.04
Keycenter	2007	47°21'26.90"N, 122°44'56.90"W	2.45
Mclane	2008	47°00'02.86"N, 122°59'47.40"W	2.23
Horseshoe 2	2009	47°24'07.19"N, 122°40'47.27"W	3.56



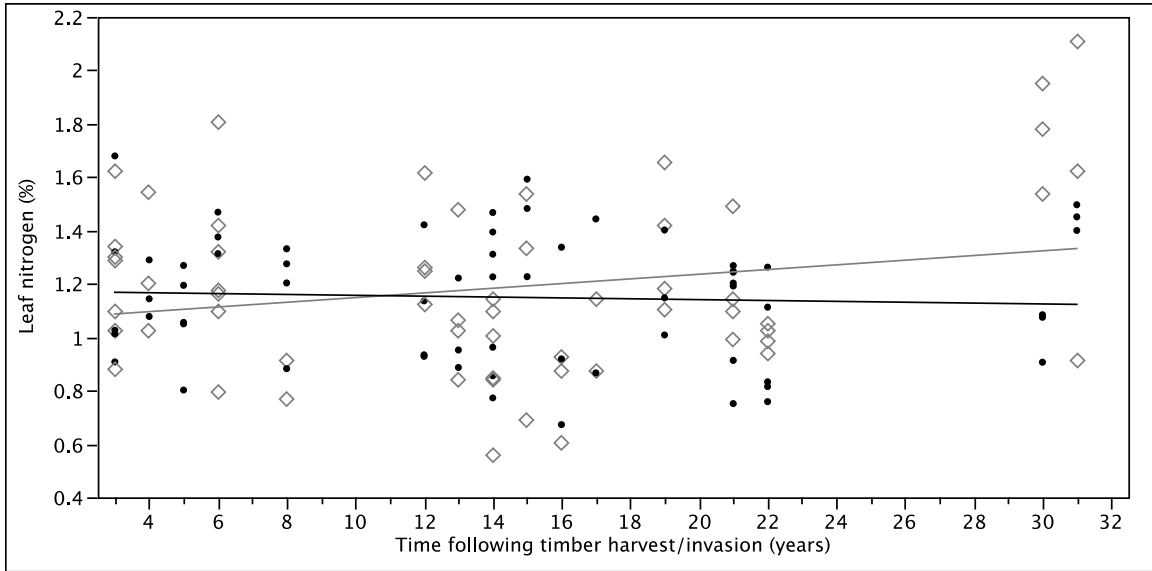
**Figure 3.1.** Douglas-fir growth as measured as aboveground dry biomass (mg) with time following timber harvest (black circles) and *C. scoparius* invasion (open grey diamonds).



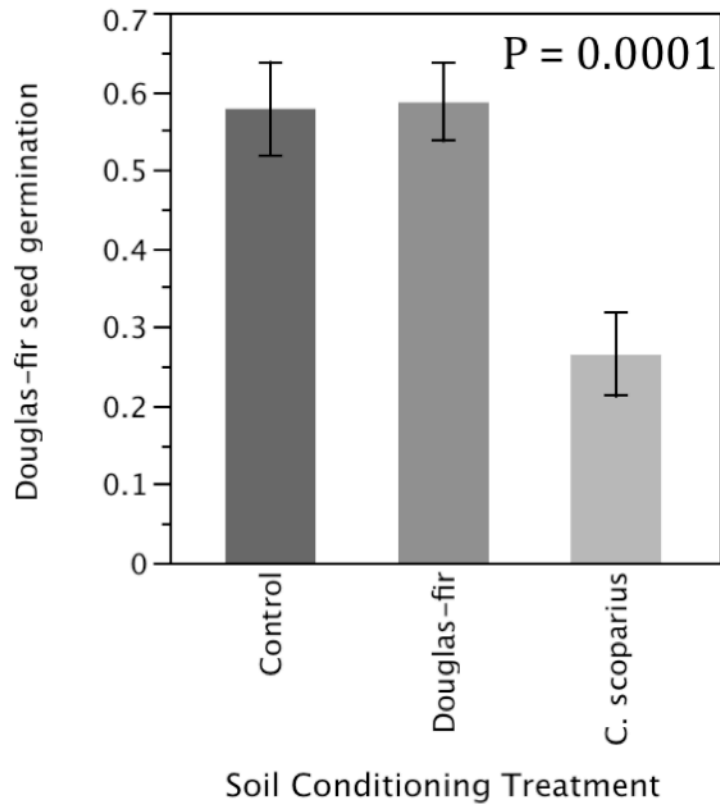
**Figure 3.2.** The proportion of Douglas fir roots colonized by ectomycorrhizal fungi with time following timber harvest (solid black circles) and *C. scoparius* invasion (open grey diamonds).



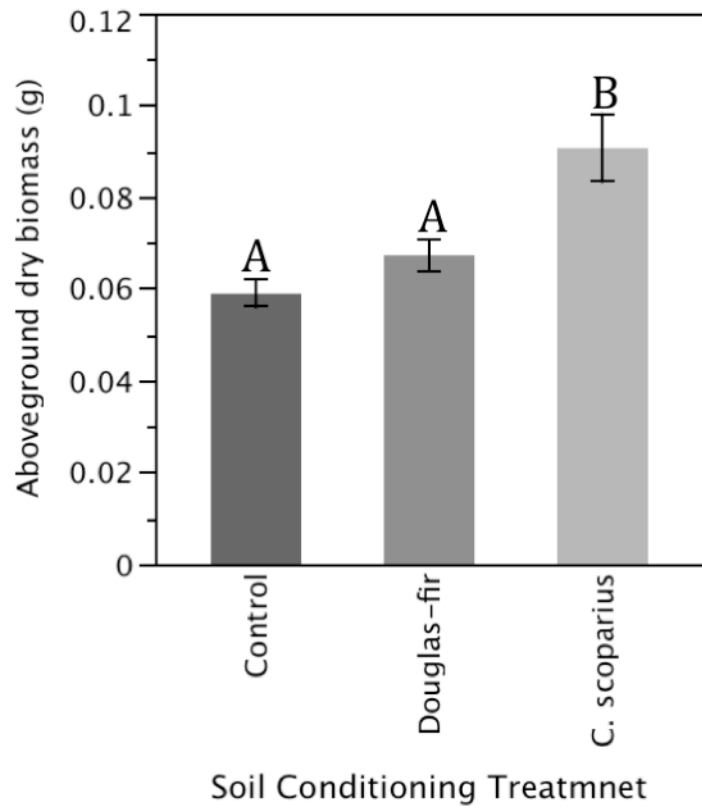
**Figure 3.3.** The relationship between ectomycorrhizal colonization of Douglas-fir seedling roots and seedling growth in uninvaded deforested soils (solid black circles) and *C. scoparius* invaded soils (open grey diamonds).



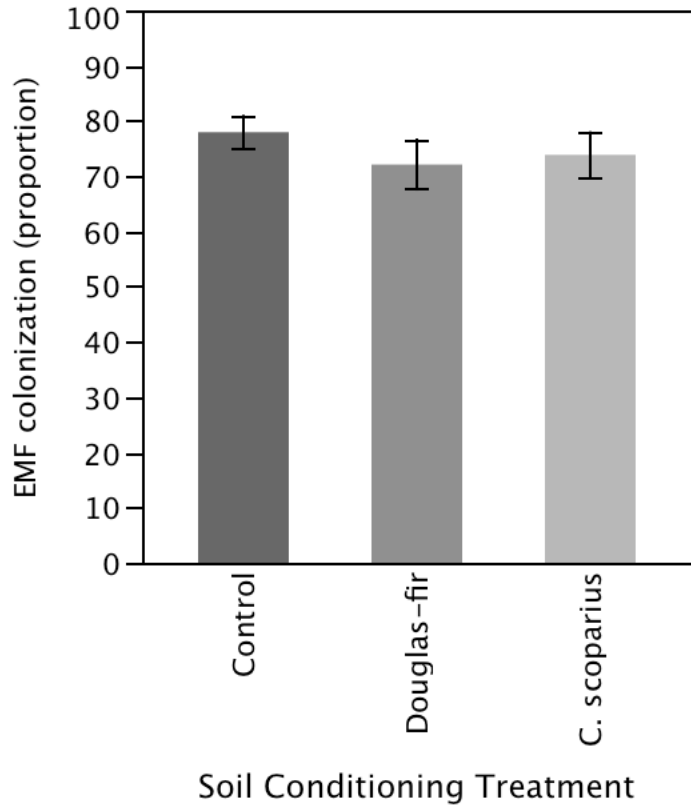
**Figure 3.4.** Leaf nitrogen content with time following timber harvest (solid black circles) and *C. scoparius* invasion (open grey diamonds).



**Figure 3.5.** Proportion of Douglas-fir seeds that germination in soils conditioned by Douglas-fir, *C. scoparius* or a control. Error bars = +/- 1 standard error

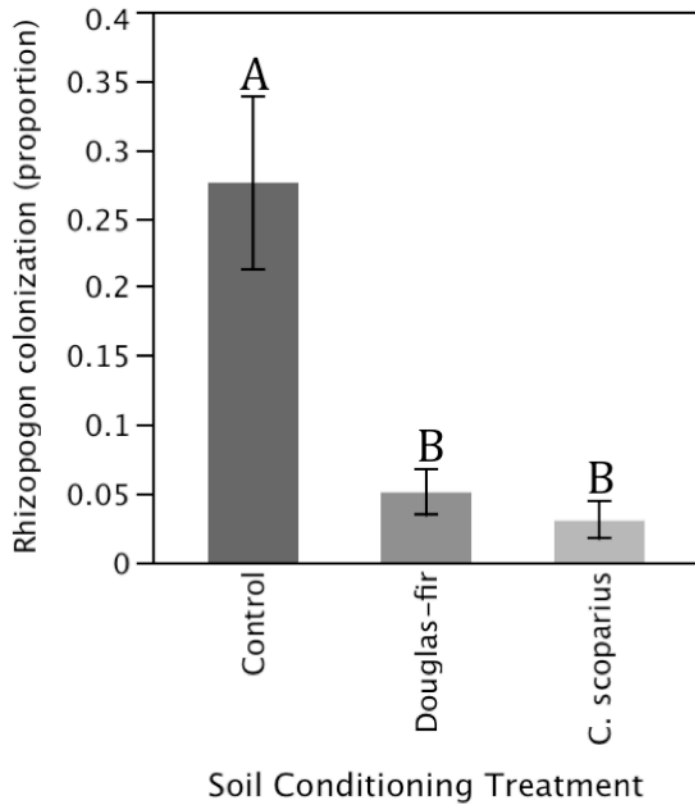


**Figure 3.6.** Douglas-fir aboveground dry biomass after growing for 10 months in soils conditioned by Douglas-fir, *C. scoparius* and a control. Error bars = +/- 1 standard error

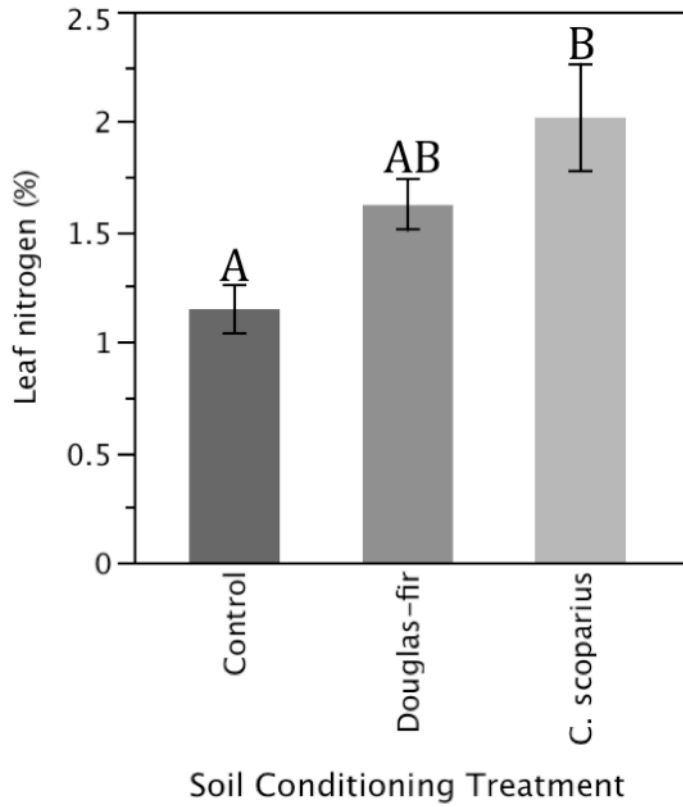


**Figure 3.7.** The proportion of Douglas-fir roots colonized by ectomycorrhizal fungi from seedlings grown in soils conditioned by Douglas-fir, *C. scoparius*, or a control. Error bars = +/- 1 standard error





**Figure 3.8.** The proportion of Douglas-fir roots colonized by *Rhizopogon* spp. on seedlings grown in soils conditioned by Douglas-fir, *C. scoparius*, or a control. Error bars = +/- 1 standard error



**Figure 3.9.** Leaf nitrogen content in Douglas-fir needles grown in soils conditioned by Douglas-fir, *C. scoparius* or a control. Error bars = +/- 1 standard error

## SYNTHESIS

In the last 50-60 years, since the publication of Charles Elton's influential book, *The Ecology of Invasions by Animals and Plants*, it has become widely and increasingly recognized by conservation practitioners and scientist that invasive species are an important problem. By homogenization of the landscape, invasive species have profound impacts on biodiversity and ecosystem functioning. Biological invasions by introduced species can also impact the aesthetic value of natural places and have significant impacts on our economy.

For my dissertation research I chose to study *Cytisus scoparius* in the Pacific Northwest, because it invades and impacts both natural and managed landscapes and has been notoriously difficult to control. In the Pacific Northwest, *C. scoparius* invasion costs 100 million dollars annually in lost timber and agriculture revenue and control expenses. Land managers throughout the region have reached out to the scientific community and asked for research that can help inform management decisions aimed at reducing the ecological and economic impacts of *C. scoparius* invasion. An overarching goal of this research was to work collaboratively with invasive species managers and contribute to the conservation of biodiversity, ecosystem functioning, as well as the aesthetics and economy of the Pacific Northwest.

The idea that biological invasions can result in alternative stable states has recently gained popularity among invasion ecologists and posits that invaded areas

may not be able to return to preinvaded conditions following the removal of the invader due to hysteresis, or a situation where there are two or more stable states can occur in a given environment, and the restoration from one state to another requires something different than what initially caused the state shift in the first place. Knowing when and if an invasion has resulted in an alternative stable state would, in theory, be useful for managers who need to make decisions about which species, and which populations of those species should be prioritized for control. In addition to addressing an applied conservation problem, my research aimed to provide an example of an alternative stable state caused and maintained by a biological invasion. Currently a number of conceptual models for identifying alternative stable states exist, but there is no practical approach that can be applied in the field to real world invasions that can determine definitively if an invaded condition is irreversible due to hysteresis. To assume that a degraded invaded ecosystem is in an alternative stable state, with little possibility for recovery, is a risky proposition and could result in unnecessary abandonment of impacted areas, leading to further invasion and degradation of our natural resources.

In order for an invasion to result in an alternative stable state, the changes made by the invader need to persist as 'legacy effects' following invader removal. Well-studied legacy effects include changes in nutrient cycling, disturbance, or fire regimes. Recent work has highlighted the importance of the indirect impacts of invasive plants through changes in soil biota, but the examples are few. Furthermore, the extent to which those shifts persist after invasion as a legacy effect has rarely been

described. Our understanding of legacies is limited by a lack of empirical evidence of how they develop, persist, and change over time. For my dissertation research I investigated both the development and persistence of impacts on abiotic and biotic soil properties over time following *C. scoparius* invasion.

*Cytisus scoparius* invades many different open habitats throughout its introduced range. I have focused my research on the invasion of Douglas-fir forest habitat following timber harvests, usually in the form of clearcutting. Following *C. scoparius* removal, reforestation efforts of these clearcuts have often failed, and within a couple of years the treated areas return to the invaded state. This phenomenon was the underlying motivation for my dissertation research. It has been suggested that adding a species with a unique functional group into a community can change the resilience of that community. Douglas-fir forests are generally nitrogen limited, and because *C. scoparius* fixes nitrogen and therefore increases soil fertility, it has the potential to create changes that could persist as soil legacies. It is also hypothesized that *C. scoparius* invasion could impart soil legacies through allelopathy. The leaf and stem tissues of *C. scoparius* contain antimicrobial defense compounds (quinolizidine alkaloids) that could affect soil biota that are beneficial to native plants. These alkaloid compounds may also build up and persist in the soil and have direct negative effects on the success of Douglas-fir and other native plants. Through N-fixation and allelopathy, *C. scoparius* could create soil legacies that hinder the resilience and recovery of forest habitat following *C. scoparius* removal. In my dissertation work I characterized the complex relationships between allelopathy and nitrogen enrichment

as mechanisms for the disruption of positive feedbacks between Douglas-fir and ectomycorrhizal fungi.

Ectomycorrhizal fungi associate with plant roots, and improve water and nutrient uptake and offer protection against root pathogens. This symbiotic relationship can be disrupted by the invasion of exotic plants and is not necessarily restored following invader removal.

In chapter 1 of my dissertation I confirmed the existence of a soil legacy effect following *C. scoparius* removal. Comparing the growth of Douglas-fir seedlings in soils collected from long invaded clearcuts and uninvaded forest, I found that Douglas-fir grown in the previously invaded soils were smaller than the seedlings grown in uninvaded forest soils. I also compared the abundance of ectomycorrhizal fungi on these seedlings and found that the seedlings grown in the *C. scoparius* invaded soils had less ectomycorrhizal root colonization than seedlings grown in the uninvaded forest soils. I additionally tested the potential role of allelopathy (the suppression of neighboring plants through the release of toxic phytochemicals) as a mechanism for the negative legacy effect. *Cytisus scoparius* mulch addition reduced both the abundance of EMF and Douglas-fir growth. These findings together led me to believe that legacy effects of *C. scoparius* are at least in part responsible for the failed restoration of invaded forest habitat, and that the mechanism could potentially be due to the allelopathic effects of *C. scoparius* litter on the ectomycorrhizal mutualism. If the soil legacy effect and failed Douglas-fir reforestation is mediated by

the loss of EMF fungi due to invasion than the reintroduction of these fungi to the soil at the time of revegetation may improve restoration success.

The prevalence of allelopathy has historically been and continues to be a contentious topic in plant ecology (Harper 1975). The methods used to detect allelopathy are often confounded by experimental artifacts (Lau et al 2007). The activated carbon addition approach used in this study is no exception. Along with binding allelochemicals, activated carbon can increase nutrient availability in soil, and this can result in increased plant success. In this study, when activated carbon was added to uninvaded soils without any *C. scoparius* litter, Douglas-fir seedling growth was not improved, indicating that the effects of *C. scoparius* litter on EMF abundance and Douglas-fir are not merely due to methodological artifacts. As a first pass at detecting allelopathy in our system, the activated carbon addition method was a reasonable place to start. To further understand the role of allelopathy in this system, we need to measure concentrations of alkaloid compounds in invaded soils, which is the focus of future work. To further understand if these putative allelochemicals contribute to soil legacy effects, it would be useful to understand how long alkaloids can persist in soil following *C. scoparius* removal.

The soils used in the allelopathy experiment were from either intact uninvaded forests or from clearcuts that have been invaded for several decades. Because I did not also include uninvaded clearcut soils in this experiment, it was impossible to determine how much the decrease in EMF abundance was due to the legacy effects of *C. scoparius* per se, and how much was a result of the clearcutting, which removes

EMF host plants. To disentangle the impacts of deforestation and invasion on EMF abundance and Douglas-fir success I used two additional greenhouse studies: a soil conditioning experiment and a chronosequence experiment (reported in chapter 3).

A major difference between the soil conditioning experiment and the allelopathy greenhouse experiment (reported in chapter 1) was that in the soil conditioning experiment, I used soils collected from recent clearcuts (1-2 years), and these soils had never been invaded by *C. scoparius*. In the greenhouse, I experimentally “invaded” the clearcut soils by planting in *C. scoparius*. By comparing the *C. scoparius* conditioned soils to unplanted control soils, I was able to determine how EMF abundance is affected by clearcutting and invasion. Results from this experiment do not corroborate the findings from the allelopathy experiment (chapter 1). Instead, I found no difference in EMF abundance on Douglas-fir seedlings grown in the *C. scoparius* conditioned and the unplanted control soils. There was remarkably high EMF colonization (75-80%) in both the control and *C. scoparius* conditioned soils. Douglas-fir seedling growth was actually improved by the *C. scoparius* conditioning treatment. These results were strikingly different from the first greenhouse experiment, where *C. scoparius* had negative impacts on both EMF and Douglas-fir growth. I suspect that the very recently clearcut soils used in the soil conditioning experiment contained a larger pool of EM fungi than the long invaded and treeless soils used in the allelopathy experiment (chapter 1). An even more important factor that may explain the differences between these experiments is that I did not incorporate the aboveground *C. scoparius* tissues into the soil in the soil



conditioning experiment, and because these field soils had never been invaded, they have never interacted with *C. scoparius* leaf and stem tissues. In a sense, the lack of a negative effect of *C. scoparius* soil conditioning on EMF and Douglas-fir growth provides some support that the leaf and stem tissues of *C. scoparius* may have allelopathic effects. It would be worth doing a field experiment to see if removing the aboveground *C. scoparius* biomass following mechanical control improves restoration success. Removing the *C. scoparius* biomass would be more labor intensive and therefore expensive, and to my knowledge, has not been attempted by *C. scoparius* managers. However, by removing the aboveground biomass, Douglas-fir may be able to benefit from the nitrogen enrichment associated with *C. scoparius* and avoid the negative effects of allelopathy.

To date there are very few studies that have tracked the development of soil legacy effects following plant invasions, and it is unknown if changes to the soil environment are exacerbated with time. The chronosequence experiment (reported in chapter 3) was designed to evaluate how *C. scoparius* impacts change with time after the initial invasion. By comparing invaded and uninvaded areas within the same clearcut, I was able to tease apart the effects of deforestation and invasion on EMF and Douglas-fir.

The results from the chronosequence study suggest that the changes that result from *C. scoparius* invasion on EMF abundance develop quickly and that the abundance of EMF was unaffected by invasion duration. It remains unclear what is actually driving the reduction in EMF colonization on the Douglas-fir seedlings

grown in the invaded soils. It could be that Douglas-fir seedlings are actually divesting resources in the fungi because the invasion actually increases soil fertility to the same degree that associating with ectomycorrhizal fungi does. It is also possible that the nitrogen enrichment that occurs with *C. scoparius* invasion is having negative effects on EMF, or that the putative allelocompounds of *C. scoparius* have reduced EMF abundance. Because Douglas-fir growth was not negatively affected by the decrease in EMF colonization it seems very likely that the Douglas-fir may have abandoned the partnership with at least some EM fungi, perhaps the fungi that are best suited for N acquisition.

In the chronosequence study, the soil legacy effects of *C. scoparius* had a positive effect on Douglas-fir growth. Similar to the soil conditioning experiment, I did not include aboveground *C. scoparius* tissues in the soil, and therefore I excluded the tissues that contain the putative allelochemicals. However, if alkaloids persist in the soil as part of a legacy effect, than I should have seen a negative effect of *C. scoparius* invasion, because presumably the invaded soils have received annual inputs of *C. scoparius* leaves, when they senesce in the fall. Since I did not find a negative effect of *C. scoparius* invasion on Douglas-fir growth in the chronosequence experiment I assume that either the alkaloids did not persist in the soil for very long following *C. scoparius* removal, or the benefit of nitrogen enrichment associated with the invasion outweighs the negative effect of allelopathy when Douglas-fir are grown in isolation in the greenhouse.

In the soil conditioning experiment and the chronosequence experiments, Douglas-fir benefited from the nitrogen enrichment that occurs with *C. scoparius* invasion and the overall impacts of invasion were positive. Yet, in actual field conditions Douglas-fir seedlings often experience high mortality. This may be because in the field Douglas-fir seedlings are not growing in isolation and have to compete with other plants. Douglas-fir and other clearcut colonizing species in the region are generally woody perennials and are adapted to low nitrogen conditions. I have found that when *C. scoparius* is removed from deforested areas, fast growing exotic grasses and forbs quickly colonize and dominate (chapter 2). These fast growing species may be better than the slow growing woody natives at acquiring the added N.

It is thought that invaded ecosystems in alternative stable states cannot be restored to preinvasion conditions because of positive feedbacks that have been created by the invasion and will ultimately result in reinvasion. In order for changes in the biotic and abiotic soil environment to impact the restorability of an invaded system following invader removal, the changes must persist in the absence of the invader. However, if following *C. scoparius* removal the invasion induced changes in soil conditions do not persist to create positive feedbacks that facilitate reinvasion, then the framework for the alternative stable state hypothesis breaks down. My dissertation research and the research of many others has documented that underlying soil conditions change following *C. scoparius* invasion, and that restoration following *C. scoparius* removal has often been unsuccessful. In chapter 2 I used a field

experiment to assess if nitrogen enrichment that occurs with *C. scoparius* invasion persisted following invader removal and to determine if the nitrogen enrichment had consequences for Douglas-fir success that could ultimately lead to an alternative stable state. I compared the nitrogen status and Douglas-fir growth in areas where *C. scoparius* was removed for 0,1,10 and 22 months. My results confirm that soil conditions change with *C. scoparius* invasion. One month following the *C. scoparius* herbicide treatment the availability of nitrogen spiked as the nitrogen content in the plant tissues were released through decomposition. However, within 10 months following invader removal, the nitrogen availability decreased to less than what was available within invaded areas. I further found that Douglas-fir seedlings grew better in the more recently treated plots where nitrogen availability was also highest. The soil conditions associated with *C. scoparius* invasion seem to be recovering towards preinvasion conditions and did not appear to persist as a long-term legacy effects following invader removal. Interestingly, exotic grasses and forbs colonized the areas and by 22 months following removal covered 70% percent of the area. These secondary invaders may have played a role in the suppression of Douglas-fir growth. Nearly two years following *C. scoparius* removal, reinvasion by *C. scoparius* was minimal and Douglas-fir survival was high. Our findings do not appear to provide direct support for *C. scoparius* invasion as a driver of an alternative stable state. However, there are some important caveats to consider. First, because there was no uninvaded control, I was not able to decipher if the nitrogen status after 22 months post removal was still elevated relative to uninvaded clearcut soils. Second, we are

also unable to quantitatively determine the relationship of exotic grass and forb invasion and reforestation success and it remains somewhat unclear how these secondary invaders impact reforestation.

It would be interesting to know whether the short lived nitrogen enrichment effect of *C. scoparius* has delayed impacts on Douglas-fir survival and growth by facilitating the establishment of competitively dominant exotic grasses and forbs. Half of the Douglas-fir seedlings planted into these experimental removal plots remain in the field, and future work could assess these long term effects. If in the long term, the secondary invasion of the grasses and forbs suppress Douglas-fir, then managers should consider applying soil amendments that alter the carbon to nitrogen ratios to mitigate the impacts of N enrichment. Future research in the *C. scoparius*-Douglas-fir system should investigate how N-enrichment facilitates the invasion of these exotics, and how these exotics further impact reforestation following *C. scoparius* removal.

My dissertation research provides insight into an emerging concept in invasion biology: that soil legacy effects can impact native species long after the removal of the invader. In a novel contribution, I characterized the development of soil legacies and how quickly a system recovers towards pre-invasion conditions following invader removal. My work has identified that the soil-mediated impacts of an N-fixing invader can have positive and negative effects. Nutrient enrichment and allelopathy that result from invasion can influence a ubiquitous mutualism between a dominant tree and ectomycorrhizal fungi. The outcome of the mutualism disruption on native

plant success is context dependant, and in some circumstances the abundance of ectomycorrhizal fungi may not be important to native plant establishment following invader removal. Globally, many important invasive plants are N-fixing shrubs, with the same presumed syndrome of both positive and negative effects as seen in this system. This work provides an example for how these invaders affect ecosystems, and can guide our understanding of how their impacts develop and can be mitigated. Findings presented in my thesis suggest that soil mediated impacts of invasion can occur immediately following invasion, and that even the large changes in soil chemistry that often result from invasion of an N-fixer, will not necessarily have lasting impacts that maintain the system in an alternative stable state.