UNIVERSITY OF CALIFORNIA,
IRVINE

The Response of Mycorrhizal Fungi to Ecological Restoration in Coastal California

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

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2023

DEDICATION

To Jonaé Varela, my greatest source of inspiration.

In Memory of Romelia Bañuelas and Maria De La Cruz Hernandez.

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There have been so many people who have supported my journey to UC Irvine. No other person has been more supportive than my fiancé Jonaé Varela. She has been at my side at every step of my academic journey from Whittier College to Cal Poly Pomona. As an undergraduate student, Jonaé inspired me to pursue my doctoral education. Without her inspiration I would have never dreamed of receiving my PhD.

To my parents, John and Maria Guadalupe Bañuelas, who have fueled my excursions and instilled a sense of adventure and dedication in my life. This dissertation is also dedicated to my Grandmothers Romelia Bañuelas and Maria De La Cruz Hernandez who are no longer with us. They have always been a source of inspiration and joy. I would also like to commemorate my Uncle Albert who also passed away during my PhD and was always supportive. To all my extended family and friends, you truly inspire me.

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ABSTRACT OF THE DISSERTATION

The Response of Mycorrhizal Fungi to Ecological Restoration in Coastal California

David C. Banuelas
Doctor of Philosophy in Biological Sciences
University of California, Irvine, 2023
Professor Kathleen K. Treseder, Chair

While 80% of plant species are associated with mycorrhizal fungi, the impacts of ecological restoration on mycorrhizal fungi are rarely explored in scientific literature. Ecological restoration is an interdisciplinary endeavor, requiring partnerships with a diverse array of stakeholders. Increasing partnerships with restoration practitioners and mycologists will fill important knowledge gaps in our understanding of restoration efforts. Since mycorrhizal fungi provide a myriad of services to plant hosts, the efficacy of restoration efforts can improve with such partnerships. For my first chapter, I collaborated with the Newport Bay Conservancy to explore the effects of invasive species removal on mycorrhizal fungi. For my second and third chapters, I partnered with Save the Redwoods League to examine the response of mycorrhizal fungi to restorative thinning and road removal. Taken together, my dissertation chapters provide evidence that fungal communities play an important role in the success of restoration efforts and can inform management decisions.

In Chapter 1, I assessed the effect of full and selective removal of the invasive Brazilian pepper tree (*Schinus terebinthifolius*) on mycorrhizal fungi. To provide a trajectory for the restoration of the mycorrhizal community, I examined the effect of selective removal on the native Arroyo willow (*Salix lasiolepis*). An important distinction between the selective and full removal was that the full approach deployed a soil amendment. The results showed a disparity between full and selective removal, where the diversity of ectomycorrhizal fungi (EMF) declined significantly after selective

removal. However, full removal led to a significant increase in the diversity of EMF. The results of our study point to the soil amendment as a significant factor that led to the increase of EMF.

For Chapter 2, I assessed the effects of logging and restorative thinning in second-growth redwood (*Sequoia sempervirens*) forests. Mycorrhizal fungi are important in these ecosystems, where redwoods use arbuscular mycorrhizal fungi (AMF) and EMF. However, following timber harvests, pine trees (Pinaceae) were aerially seeded. Since pine trees use EMF exclusively, the encroachment of pine woodland can restrict the use AMF by redwood forests. Our predictions were supported with the significantly lower abundance and diversity of AMF in second-growth forests. This trend was found in soils and a specialized root structure for AMF known as the rhizonode. When pine trees were removed, we expected the presence of AMF would change. However, the presence of AMF was still lower compared to old-growth. The results of our study provide a new trajectory for the restoration of second-growth forests, where restoration efforts should aim to establish a mycorrhizal community indicative of old-growth.

Chapter 3 explored the effect of road removal on mycorrhizal fungi and the broader fungal community. In this chapter, I continued the examination of restoration efforts in second-growth redwood forests. Since 2004, road removal has been an integral part of ecosystem restoration. I expected road removal efforts would lessen the diversity and abundance of mycorrhizal fungi. However, we found a recent road removal led to an increase in the abundance of EMF. In accordance with our predictions, pathogenic fungi that are detrimental to redwood health were lowest in old-growth forests and the oldest removal site. Moreover, saprotrophic fungi involved in decomposition were lowest in the road-center compared to the roadside. The results of this study show that restoration efforts should focus on restoring saprotrophic fungi in the road-center, which facilitate nutrient cycling and the recovery of redwood forests.

INTRODUCTION

A common misconception of biological conservation is that degraded ecosystems, once preserved, will recover without intervention (Van Meerbeek et al., 2019). However, anthropogenic forces have altered natural ecosystems at such a rate, ecological restoration is an important step in returning ecosystems to conditions of pre-disturbance. While restoration efforts have stressed the implementation of plants and wildlife, less focus has been paid to the effect of restoration on mycorrhizal fungi and the broader fungal community. The aim of this dissertation is to demonstrate that restoration efforts have varied impacts to the fungal community. Furthermore, each of these impacts will inform future management decisions with each chapter providing recommendations for future restoration efforts. By increasing the body of knowledge in this field, the efficacy of future restoration projects will increase.

For Chapter 1, I examined the effect of full and selective removal of invasive woodland on mycorrhizal fungi. In subtropical regions of North America, the introduction of the Brazilian pepper tree (*Schinus terebinthifolius*) has led to the decline of native plant species (Dawkins & Esiobu, 2016; Nickerson & Flory, 2015). For Southern California, the Brazilian pepper tree outcompetes the native arroyo willow (*Salix lasiolepis*), leading to a decline in height and abundance (Mullens & Gerry, 2006). Both the Brazilian pepper tree and the arroyo willow use dual mutualism, forming associations with arbuscular mycorrhizal fungi (AMF) and ectomycorrhizal fungi (EMF). Therefore, these tree species are model organisms to understand how restoration efforts affect dual mutualism.

Due to the resistance of the Brazilian pepper tree to control efforts, various removal efforts have been attempted. Control burns have been deployed with little success as it facilitates seedling establishment (Stevens & Beckage, 2009). Chemical control in the form of herbicides is effective,

however, are difficult to apply on a large scales (Enloe et al., 2021). Biological controls are successful but can take several decades to develop (Prade et al., 2022). Mechanical removal is the most effective method despite the costs associated with removal (Asmelash et al., 2016; Dawkins, 2016; Smith et al., 2011). For each removal effort, the effects to mycorrhizal fungi can have implications for the long-term success of the native plant communities.

Two hypotheses around the mechanized removal of the Brazilian pepper were established for Chapter 1. Initially, I expected the abundance and diversity of mycorrhizal fungi (AMF and EMF) would decrease resulting from the disturbance attributed to full and selective removal (Hypothesis 1). For the broader fungal community and mycorrhizal fungi, I expected significant shifts in the community composition for full and selective removals (Hypothesis 2). For my hypotheses around full and selective removal, DNA sequencing was used to identify fungal communities associated with soils and roots. The sampling in full and selective removal was collected prior to and following the removal of the Brazilian pepper tree in Southern California.

Chapter 2 examined the impact of logging and thinning on mycorrhizal fungi associated with redwood forests (*Sequoia sempervirens*). Within these ecosystems, dual mutualism involves the use of AMF and EMF (Willing et al., 2021), whereas pine trees use EMF exclusively. Following timber harvests, the aerial seeding of pine trees (Pinaceae) may disrupt the dual mutualism of second-growth redwood forests. The services provided by dual mutualism may change in response to the dominance of pine woodland. Although trees can shift their dependency between AMF and EMF, the plant hosts receive specific advantages when dual mutualism occurs (Teste et al., 2020). Therefore, any losses to dual mutualism can impair the recovery of redwood forests. When pine woodland is removed during restorative thinning, effects to dual mutualism should be considered. The rhizonode structure of redwood forests, where AMF associations occur, can serve as an

important indicator for the presence of AMF following the removal of pine woodland (Willing et al., 2021). Such investigations will determine if the invasion and removal of pine woodland disrupts the dual mutualism in redwood forests.

To investigate the impact of pine woodland in second-growth redwood forests, I examined mycorrhizal communities in three forest types: old-growth, second-growth, and thinned forests. First, I predicted EMF would be the most abundant and diverse in second-growth forests. For AMF, I expected the opposite, where AMF would be more diverse and abundant in old-growth forests. The different predictions for AMF and EMF were based on the notion that the aerial seeding of pine woodland would have a negative effect on the presence of AMF. For the second hypothesis, I predicted restorative thinning would have an intermediate effect on AMF (Hypothesis 2). Since the sampling of the thinned forests occurred within three months, I did not expect a strong effect on AMF. For my last hypothesis, I expected the composition of the broader fungal community would differ across forest types (Hypothesis 3).

In Chapter 3, I continued researching redwood ecosystems, where I examined the effect of road removals on mycorrhizal fungi. For decades, road removal has been an integral part of ecosystem restoration in redwood forests (Hagans et al., 1986; Madej, 2001, 2010; Maurin & Stubblefield, 2011). However, there is a limited knowledge on the implication of road removal on fungal guilds. Road removals can lead to the decline in the abundance and diversity of mycorrhizal fungi (Archuleta & Baxter, 2008; White et al., 2008; Wu et al., 2002). Furthermore the loss of mycorrhizal fungi can affect the survival of plant communities (Bermúdez-Contreras et al., 2022; House & Bever, 2018; Xiang et al., 2015). Following road removals, saprotrophic fungi are slow to recover, which can alter decomposition rates (Eaton et al., 2021; McGee et al., 2019). A lack of nutrient cycling resulting from the decline of saprotrophic fungi can impair the recovery of redwood forests

(Seney & Madej, 2015). The pathogenic guild of fungi can impair seedling establishment following road removal (Eaton et al., 2021). Although the effect of road removal on fungal guilds have been speculated in redwood forests (Seney & Madej, 2015), no prior study has examined this interaction directly.

We tested three hypotheses, each focused on the prevalence of (1) fungal guilds, and (2) fungal taxa. To quantify prevalence, we assessed richness and relative abundance. First, we hypothesized that roads and road removals would alter the prevalence of fungal guilds and taxa owing to the sensitivity of each to disturbance (Hypothesis 1; Eaton et al., 2021; McGee et al., 2019). Accordingly, we predicted that, compared to old-growth forest, the road scars (2005 roadside, 2013 roadside, and 2013 road-center) would harbor different community compositions of taxa, lower prevalence of mycorrhizal fungi and saprotrophic fungi, and higher prevalence of pathogenic fungi. Second, we hypothesized that fungal community composition in the road scars would approach that of the old-growth forest over time (Hypothesis 2), because the aboveground recovery of second-growth forest can require several decades (Burns et al., 2018; Iberle et al., 2020). Specifically, we predicted that the prevalence of each fungal guild—and the community composition of fungal taxa—in the 2005 roadside will be closer to that of the old-growth forest than the 2013 roadside. Third, we hypothesized that fungal community would be more disturbed in the road-center than the roadsides, because of the higher degree of disturbance within the road-center (Hypothesis 3). For that reason, we predicted that the prevalence of fungal guilds and the community composition of fungal taxa in the 2013 roadside will be closer to that of the old-growth forest than the 2013 road-center.

CHAPTER 1

Title: Mycorrhizal responses to selective and full removal of an invasive tree in riparian woodland

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Abstract

The Brazilian pepper tree (*Schinus terebinthifolius*) is an invasive species that requires significant disturbance to eradicate. Previous studies have identified associations between Brazilian pepper tree and arbuscular mycorrhizal fungi (AMF) and ectomycorrhizal fungi (EMF). However, limited research has explored the connection between disturbance from removal and the effect on mycorrhizal fungi. This study investigated the sensitivity of mycorrhizal fungi and the broader fungal community to the full and selective removal of Brazilian pepper tree. During the selective removal of Brazilian pepper tree, we examined the mycorrhizal community of the arroyo willow (*Salix lasiolepis*) to assess the influence of the restoration disturbance on native species. We used ITS2 sequencing to identify the mycorrhizal fungi present during the restoration. Our expectation was that both removal efforts would reduce the presence of mycorrhizal fungi. Contrary to our expectations, selective removal reduced EMF richness in the soil, but full removal increased EMF richness. Moreover, fungal community composition in soil and roots shifted significantly during selective and full removal. However, the community composition of EMF, specifically, remained constant across treatment types. During full removal efforts, the application of organic soil amendments may have contributed to the increase in the diversity of EMF. Selective removal will require additional measures such as soil amendments to curtail the loss of EMF.

Introduction

Despite the benefits of invasive species removal (Hanula & Horn, 2011; Perry et al., 2015; Zavaleta et al., 2001), disturbances associated with these efforts may disrupt native species (Davies et al., 2011; Kettenring & Adams, 2011). Mechanical removal, in particular, can lead to secondary invasions and a decline in the relative abundance of native species (Baughman et al., 2010; DiTomaso et al., 2010; González et al., 2017; Sher et al., 2018). What is less understood is the effect invasive removal has on the mycorrhizal partners of native species. *Schinus terebinthifolius* Raddi (Brazilian pepper tree) is a cosmopolitan invasive tree that is difficult to eradicate without substantial disturbance to soils (Dalrymple et al., 2003; Smith et al., 2011). In North America, the Brazilian pepper tree can harbor a higher diversity of mycorrhizal fungi than do native species (Dawkins & Esiobu, 2017; Gomes et al., 2018). In Southern California, the native species *Salix lasiolepis* Benth. (arroyo willow) competes directly with Brazilian pepper tree in riparian communities (Mullens & Gerry, 2006). There is a need for studies of mycorrhizal responses to the removal of Brazilian pepper tree, and the potential consequences for restoration of native plants like arroyo willow (Dawkins & Esiobu, 2017).

Controlling the invasive Brazilian pepper tree has proven challenging due to its resistance to removal. Numerous restoration efforts have been attempted. Controlled burns are inadvisable, since they can facilitate the spread of Brazilian pepper tree (Stevens & Beckage, 2009). Although chemical controls have been effective, they require applications to the cambium and are not always feasible (Enloe et al., 2021). In 2019, biological controls were introduced in Florida, and their long-term impact will be assessed in the coming decades (Prade et al., 2022). The most efficient method remains mechanical removal, though it is expensive and can take several decades to complete (Asmelash et al., 2016; Dawkins & Esiobu, 2016; C. S. Smith et al., 2011). Since each restoration

effort may yield different outcomes, it is crucial to conduct studies that characterize the fungal community after the removal of the Brazilian pepper tree. These studies can inform restoration strategies that are effective and sustainable.

Two common types of symbiotic fungi are arbuscular mycorrhizal fungi (AMF) and ectomycorrhizal fungi (EMF) (Tedersoo et al., 2020). AMF are the most common, forming mutualisms with 80–90% of plant species (Heijden et al., 2015). All AMF belong to the phylum Glomeromycota (Redecker et al., 2013). They penetrate the root cortex and construct arbuscules that facilitate resource exchange (Smith & Read, 2008). In comparison, EMF associate with 2–3% of plant species, and they belong to the Ascomycota and Basidiomycota phyla (Kuo et al., 2014). They form specialized structures (Hartig net) around the root cortex (Tedersoo et al., 2010). In grasslands and shrublands, AMF are the primary mutualism (Weber et al., 2019). In many forested ecosystems, trees can form associations with EMF, AMF, or both (Egerton-Warburton & Allen, 2001; Van der Heijden, 2001). A recent review found studies involving dual mutualism (AMF + EMF) are lacking despite their widespread occurrence (Teste et al., 2020). Since the Brazilian pepper tree and arroyo willow are dual mycorrhizal (Dawkins & Esiobu, 2017), they can showcase how both mycorrhizal types respond to invasive species removal.

By the 1980s, Brazilian pepper tree had severely invaded the Upper Newport Bay Ecological Reserve and adjacent habitat preserves ("Upper Newport Bay") in Southern California (OC Archives, 2023). Brazilian pepper tree was introduced to the region as an ornamental plant (Pincetl et al., 2013). In 2000, the State of California designated the Brazilian pepper tree as invasive (Cal-IPC, 2006). The invasion had prevented the establishment of willow thickets, which would support the nesting of the federally endangered *Vireo bellii pusillus* (Least Bell's Vireo). In 2020, 6.3 hectares of Brazilian Pepper tree stands were removed from Big Canyon Nature Park ("Big

Canyon") using two approaches: full and selective removal (Fig 1A). In full removal, to eliminate all Brazilian pepper tree roots, clearing and grubbing were deployed. In selective removal, clearing and grubbing was used sparingly to avoid damaging native willows. Additionally, a commercial inoculum consisting of AMF and EMF was added during full removal but not selective removal. Yet, commercial applications seldomly increase the presence of mycorrhizal fungi (Maltz & Treseder, 2015). Thus, we did not necessarily expect the inoculum to increase the mycorrhizal colonization in Big Canyon.

We tested two hypotheses addressing how mycorrhizal fungi would be affected by full and selective removal of Brazilian pepper tree in Big Canyon. We first posited that the relative abundance and diversity of mycorrhizal fungi would decrease owing to the disturbance caused by full and selective removal (Hypothesis 1). For the same reason, we anticipated significant shifts in the community composition of mycorrhizal fungi, as well as the overall fungal community, for both types of removal (Hypothesis 2). To investigate these hypotheses regarding the response of mycorrhizal fungi to full and selective removal, we performed DNA sequencing on soil and root samples before and after removal of Brazilian pepper trees in Big Canyon.

Methods

Site Description

We tested our hypotheses at Big Canyon (33° 37' 49.7" N, 117° 52' 48.6" W), which experiences a Mediterranean climate. The locale receives an average of 27.9 cm of rainfall each year, with a mean annual temperature of 19.9 °C (WRCC, 2023). The typical dry season runs from May through October, when the monthly rainfall is less than 2.0 cm. The wet season extends from November to April, when the average rainfall fluctuates between 2.3 and 4.2 cm each month. The dominant soil

type is Sorrento loam, on 2% to 9% slopes (NCRS, 2023). The main parcel of Big Canyon comprises 15.5 ha owned by the City of Newport Beach ("Newport Beach"; Fig. 1A). Additionally, Big Canyon Creek drains into the Upper Newport Bay, which is managed by California Department of Fish and Game (CDFW). The Kizh, Tongva, and Acjachemen Nations are the traditional stewards of the region, and continue to advise management decisions in the Upper Newport Bay (McCawley, 1996).

Restoration Approach

In partnership with Newport Beach and CDFW, the Newport Bay Conservancy led the Big Canyon restoration project. The work spanned October 2020 through January 2021. Two restoration approaches were employed: selective removal of the Brazilian pepper tree while preserving native willow trees, or full removal of all vegetation (Fig. 2E, 2F).

Selective Removal. To safeguard established arroyo willows, 0.7 hectares were designated for selective removal (see Fig. 1A). To minimize disturbance to the arroyo willow during the selective removal, excavator activity was limited. This approach is more costly than full removal and was therefore used on a smaller area. The selective removal also ensured that mature willows neighbored the young willows to be planted in the full removal area. Unlike the full removal, the selective approach left the topsoil beneath willows intact. Moreover, the organic soil amendment was not applied beneath the canopy of willows due to lack of access.

Full Removal. The entire 5.6 hectares of dense Brazilian pepper tree woodland was targeted for full removal (Fig. 2A, 2B). Because the Brazilian pepper tree can resprout from cut stumps, the removal process was conducted in two phases. Initially, chainsaw crews cleared all vegetation by removing the canopy and tree trunks (Fig. 2C, 2D). To remove the seedbank, the top five cm of

topsoil was disposed offsite along with all leaf litter and woody biomass. Subsequently, an excavator (backhoe) removed the root biomass (grubbing) along with the top 0.6 meters of topsoil. Following the removal of topsoil, the Big Canyon Creek was recontoured to support a restored riparian corridor for the outplanting of willow cuttings.

The remaining soil was mixed with an organic amendment on site and applied evenly through the full removal area by tilling or raking (ESA, 2020). The soil amendment included nutrients and a commercial inoculum manufactured by Fungi Perfecti LLC (MycoGrow® Soluble; ESA, 2020). The inoculum included ten AMF and ten EMF species (Table 1). Following full removal, arroyo willows were outplanted in the restored riparian corridor in January 2020 (see Fig. 1A ; (Pohl, 2016). The willow outplanting was cultivated from cuttings and completed prior to soil collections in February 2020.

Sampling Design

Before removal began, we collected 30 soil samples from the full removal area and 30 from the selective removal area (Fig. 1). In the full removal zone, the soil samples were gathered within the proposed restored riparian corridor. There, Brazilian pepper tree cover exceeded 90% before removal. To establish the sampling locations within the riparian corridor, we used the Random Point Generator Tool in ArcGIS Pro (2.1.0), identifying 30 random points (Fig. 1). Similarly, we selected 30 random points within the selective removal area, where the native cover was less than 30%. We collected 10 soil samples from each of the three existing willow groves (Fig. 1). After removal, we revisited the same locations to repeat the sampling process.

Sample Collection

Soil samples were collected in the full and selective removal areas between August 28th and September 3rd, 2020. After the removal of the Brazilian pepper tree was completed in January 2021, a second set of soil samples was collected from February 5th to 9th, 2021. The first collection coincided with the end of the arroyo willows' growing season in the fall, and second collection occurred during their flowering period, which typically begins in mid-January (Baldwin & Goldman, 2012).

We also examined roots from the soil cores, and we specifically targeted roots of Brazilian pepper tree (full removal) and arroyo willow (selective removal). Soil samples were collected approximately 0.2–0.5 meters from the dripline of the target tree nearest the random sampling point. We collected soil cores with a diameter of 10 cm and a depth of 10 cm. Each soil core was placed in a sterile Whirl-Pak® bag and kept on ice during the fieldwork. Within six hours of collection each day, the soil samples were stored at –20 °C in our laboratory at the University of California, Irvine (UCI). In total, we recovered 120 soil samples (30 samples × 2 removal treatments × 2 time points).

Root extraction

First, we extracted the roots from the soil. Each sample was passed through a 10 mm sieve. All samples were sieved and extracted on the same day and then stored at –20 °C. We collected fine roots (< 2 mm diameter) of the target species from the sieve. We used color and morphology to identify the appropriate tree species, following Ruas et al. (2011). We then rinsed the roots with deionized water three times. The roots were kept on ice until DNA extraction, which occurred on the same day. Since, by design, Brazilian pepper tree roots were not present in our post-removal samples from the full removal area, we only collected arroyo willow roots at that time point.

Consequently, we obtained 30 samples of Brazilian pepper tree roots (30 samples x 1 time point) and 60 samples of arroyo willow roots (3 groves x 10 samples x 2 time points), resulting in a total of 90 root samples.

DNA sequencing

We used the DNeasy PowerSoil Pro Kit (Qiagen, Redwood City, CA, USA) to extract DNA from 250 mg of each soil sample, following the manufacturer's instructions. For the root samples, we used the DNeasy Plant Pro Kit (Qiagen, Redwood City, CA, USA) and extracted DNA from 50 mg of roots. For the roots, we followed a modified lysing step developed in previous research (Gonzalez Mateu et al., 2020).

To target the fungal DNA in both soil and root samples, we employed a two-step PCR approach, focusing on the ITS2 region. In the first PCR, we amplified the DNA using the fITS7 (5′ GTGARTCATCGAATCTTTG) and ITS4 (TCCTCCGCTTATTGATATGC-3′) primers with Illumina Nextera Adapters. The PCR parameters established by Lekberg et al. were used (2018). Successful amplification at approximately 420 bp was confirmed through gel electrophoresis after the first PCR. Nineteen samples failed to amplify and were not included in the second PCR.

The samples that amplified correctly in the first PCR were used for the second PCR, where dual indexed barcodes were attached with an annealing temperature of 60 °C, following the method described by Lekberg et al. (2018). Gel electrophoresis was performed to verify successful amplification of the second PCR. After the final clean-up with Ampure XP beads (Beckman Coulter, Brea, CA, USA), the amplicon library was pooled based on gel intensity (1 µL, 2 µL, or 3 µL). The sequencing of the library was conducted at the Genomics Research and Technology Hub

(GRT Hub) at UCI (https://ghtf.biochem.uci.edu/; Irvine, CA) using the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) with 2 x 300 bp paired-end (PE) reads.

Bioinformatics

The demultiplexed sequences were pre-processed using AMPtk software v1.5.4 (Palmer et al., 2018). After concatenating all paired-end reads, the demultiplexed files were clustered into operational taxonomic units (OTUs) with a 97% similarity cutoff. This level of similarity allows for taxonomic resolution at approximately the species level (Kõljalg et al., 2013). The sequences were filtered using default parameters, and taxonomy was assigned using the UNITE database (Kõljalg et al., 2005). All non-fungal sequences were removed. Where possible, trophic modes were assigned to the OTUs using the FUNGuild database provided by AMPtk (Nguyen et al., 2016). To calculate the relative abundances of arbuscular mycorrhizal fungi (AMF) and ectomycorrhizal fungi (EMF), the OTU counts for each mycorrhizal type were summed and divided by the total OTU count for each sample. The result was multiplied by 100 to express the relative abundance as a percentage.

Statistical Analysis

All statistical analyses were performed using R Studio 4.1.2 (RStudio Team, 2021). Statistical analyses were performed separately for full versus selective removal, since the two approaches were not directly comparable. For all tests, effects were considered significant when $P < 0.05$ and marginally significant if $P < 0.10$. For paired T-tests, the "stats package" was used while the "vegan package" was used for PERMANOVA tests (RStudio Team, 2021).

Selective removal. First, we analyzed data from the selective removal treatment. To test Hypotheses 1, we used a series of paired T-tests to compare the richness or relative abundance of OTUs associated with AMF or EMF in either roots or soils. The statistical analysis for AMF and

EMF were conducted separately. Similarly, the tests for soils and roots were conducted individually. For Hypothesis 1, the dependent variable was the diversity or relative abundance of mycorrhiza, and the independent variable was treatment status ("before" or "after"). A significant treatment status effect, where OTU richness and relative abundance declined significantly after selective removal, would support Hypothesis 1.

For Hypotheses 2, we conducted a series of PERMANOVAs to determine if any changes occurred to community composition after selective removal. Due to a paucity of AMF OTUs, only EMF and the broader fungal community were considered in the PERMANOVA. The dependent variable was the relative abundance of EMF or fungal OTUs. The independent variable was timepoint (before versus after). Significant timepoint effects would support Hypothesis 2. NMDS plots were used to illustrate community composition. To identify ECM OTUs that disproportionately contributed to any shifts in community composition after selective removal, we used the "indicspecies package" in RStudio (Cáceres & Legendre, 2009).

Full removal. For soil samples from the full removal treatment, we tested our hypotheses by using the same statistical tests as above, with one exception. We did not test for changes in roots in the full removal treatment since no roots remained after the treatment (by design).

Results

Metagenomic Data

Paired-end sequences yielded 7,982,887 total reads, with 5,087,201 reads meeting minimum length for validation. After filtering, a total of 4,900,942 reads were assigned taxonomy, producing 4,061 OTUs. The FUNGuild database identified 700 saprotrophs, 303 pathotroph-saprotroph-symbiotrophs, 261 symbiotrophs, 138 saprotrophs-symbiotrophs, 246 pathotrophs, and 78

pathotroph-symbiotrophs. Soil samples were rarefied to 1,901 sequences per sample, and root samples 9,586 sequences per sample. The most common taxa in the soil and root samples were from the order Hypocreales with an unidentified trophic mode. The most abundant phylum in root and soil samples was Ascomycota, representing 69.3% of taxa found in soils and 65.8% in roots. In soil samples, 29.9% of fungal taxa belonged to Basidiomycota, and 33.8% in roots. Glomeromycota (AMF) represented less than 1% of the fungal taxa in soils and roots, likely because the primers have a known bias against AMF (Lekberg et al., 2018).

Selective removal

Hypothesis 1. The selective removal treatment led to a marginally significant decline in EMF richness in the arroyo willow soils, with the mean richness shifting from 7.12 to 5.06 OTUs (Fig 3A. $P = 0.051$). Likewise, the relative abundance of EMF in soils also tended to decline following selective removal, although the change was not significant (Fig 3B, $P = 0.180$). In contrast, selective removal tended to increase AMF richness and relative abundance in soils, although effects were only marginally significant for relative abundance ($P = 0.062$) and not significant for richness ($P = 0.12$) (Fig. 3C & D).

With respect to roots, EMF richness declined significantly with selective removal (Fig 4A, $P = 0.05$), mirroring the shift in EMF richness in soils. Yet, the relative abundance of EMF did not change significantly (Fig 4B, $P = 0.54$). Moreover, following the selective removal treatment, no significant changes occurred to the richness ($P = 0.12$) and relative abundance ($P = 0.21$) of AMF associated with arroyo willow roots (Fig 4C, 4D). We therefore rejected our first hypothesis for AMF, because it predicted that mycorrhizal richness and relative abundance would decline following selective removal. In contrast, Hypothesis 1 was partially supported for EMF, because EMF richness declined significantly in roots and marginally significantly in soil.

Hypothesis 2. Following selective removal, the community composition of broader fungi in arroyo willow soils shifted significantly, owing to an increase in variation among samples (Fig. 5A, P = 0.047). Yet, when we focused on the community composition of EMF specifically, we found no significant changes with selective removal (Fig. 5B, P = 0.762). Within arroyo willow roots, selective removal significantly altered the community composition of broader fungi (Fig. 5C, P = 0.001) but not EMF (Fig. 5D, P = 0.217). Our second hypothesis received partial support, as we observed a shift in the broader fungal community but not EMF following selective removal.

In soils, the EMF taxa *Pulvinula* sp. 1 (P = 0.015), *Anguillospora* sp. (P = 0.039), and *Tomentella* sp. 2 (P = 0.0004) were significant indicators of the time period before selective removal, compared to after selective removal (Fig. 6A). Similarly, roots of arroyo willow were associated with three EMF indicator taxa. *Pulvinula* sp. 1 decreased following selective removal (P = 0.015), while *Tomentella* sp. 3 (P = 0.038) and *Tomentella clavigera* sp. 2 (P = 0.039) increased (Fig 6B).

Full removal

Hypothesis 1. Following the full removal treatment, the richness of EMF in Brazilian pepper tree soils increased significantly (Fig 7A, P = 0.009). The relative abundance of EMF tended to rise as well, but non-significantly (Fig 7B, P = 0.120). The relative abundance (P = 0.380) and richness (P = 0.420) of AMF in Brazilian pepper tree soils did not change significantly (Fig 7C, 7D). We rejected our first hypothesis that relative abundance and richness of AMF and EMF would decline in the full removal treatment.

Hypothesis 2. After the full removal of Brazilian pepper tree, the composition of the broader fungal community changed significantly in soils (Fig 8A, P = 0.024). Specifically, variation among samples decreased. This shift was not reflected in the soil EMF community, though (Fig 8B, P =

0.667). The EMF taxa *Geopora* sp. 2 (P = 0.009) and *Geopora* sp. 4 (P = 0.009) were significant indicators of the time after full removal (Fig. 9). Our second hypothesis received partial support since a shift in the broader fungal community occurred after the completion of full removal.

Discussion

Our study examined the consequences of invasive species removal on the fungal community, particularly mycorrhizal fungi. We investigated whether the richness and relative abundance of mycorrhizal fungi would be affected by selective or full removal. Surprisingly, we observed an increase in the richness of EMF in soil after full removal (Fig. 7), despite completely removing the trees and the top 0.6 meters of soil (Fig. 2F). Likewise, EMF community composition did not significantly shift in either the full or selective removal treatments (Figs. 5 & 8). These results, together with an increase in relative abundance of AMF in soils after selective removal (Fig. 3D), contradicted our hypotheses. In other respects, mycorrhizal fungi responded as expected. For instance, selective removal decreased EMF richness in soils and roots (Figs. 3A & 4A). Moreover, the composition of the broader fungal community shifted in all cases (Figs. 5 & 8). These findings suggest that land managers may not need to supplement the mycorrhizal community during full and selective restoration, except for EMF under selective removal.

Although a commercial inoculum was used in Big Canyon, the inoculum species did not seem to establish, given that they were uncommon after restoration (Figs. 6 & 9). Instead, the organic soil amendments might have facilitated the increase in EMF richness after full removal. A combination of chitin, humic acid, and compost were applied to the full removal site. The limited research investigating these components comes from in-vitro and greenhouse studies. First, various EMF taxa can depolymerize chitin in the absence of plant hosts (Maillard et al., 2023). Similarly, humic acid stimulates the growth of mycelium in EMF (Hršelová et al., 2007). In a greenhouse study, the

addition of compost increased the colonization of EMF by *Quercus pubescens* (Pagliarani et al., 2023). These organic amendments may have likewise contributed to the increase in EMF richness after full removal.

The relative abundance of the EMF indicator species *Geopora* spp. increased after the complete removal of the Brazilian pepper tree. In other ecosystems, *Geopora* spp. is known to maintain a presence in soils despite the loss of plant hosts (Mueller et al., 2019; Shemesh et al., 2020). In willow habitats such as the one in our study, *Geopora* spp. is well known for its role in decomposition in degraded or contaminated landscapes (Bell et al., 2015; Querejeta et al., 2021; Taniguchi et al., 2018). Previous research has also determined that the infection of *Geopora* spp. occurs in mature willow (*Salix* spp.) stands (Hrynkiewicz et al., 2015). As arroyo willows in the restored riparian corridor mature, the likelihood of infection by *Geopora* spp. increases. Therefore, the full removal effort may benefit arroyo willows by the increase in the relative abundance of *Geopora* spp.

Our study builds on the few previous studies that have identified mycorrhizal taxa associated with native willow in Southern California (Allen et al., 2000; Querejeta et al., 2009; Taniguchi et al., 2018). The fungal taxa associated with arroyo willows in our study have also been identified in willow habitats outside of the region. In willow forests contaminated by mine tailings, *Pulvinula* spp. can reduce abiotic stress induced from heavy metals (Cao et al., 2020; Kolaříková et al., 2017); a *Pulvinula* taxon declined following selective removal in Big Canyon (Fig. 6A). Additionally, *Tomentella* spp. alleviate abiotic stress associated with high salinity (Hrynkiewicz et al., 2015). In our case, one *Tomentella* taxon increased, and two others declined with selective removal (Fig. 6). The soil at Big Canyon has elevated levels of salinity and heavy metals (ESA, 2016). If *Pulvinula*

and *Tomentella* also diminish salinity and heavy metal stress in arroyo willow, the reduction in *Pulvinula* and two *Tomentella* taxa may be disadvantageous to arroyo willow recovery.

When attempting to remove invasive species, widespread disturbance can hinder the long-term recovery of native plant species (Prior et al., 2018). Ideally, removing invasive plants before they become widely established is the most effective approach to minimize disturbance. However, many riparian communities in the Western US are extensively invaded, increasing the need for high-disturbance removal (Boland, 2016; DiPietro et al., 2002; Goodwin et al., 1997; Pendleton, 2002). In cases where significant disturbance cannot be avoided, the application of inocula should be considered to re-establish mycorrhizal fungi. Yet, commercial inocula typically are ineffective in restoration efforts (Maltz & Treseder, 2015). Instead, practitioners might consider using inoculum from a local reference ecosystem, since this approach more often improves mycorrhizal colonization and benefits to native plants (Hrynkiewicz et al., 2009; Maltz & Treseder, 2015).

Due to lack of riparian forests that are uninvaded by Brazilian pepper tree in Big Canyon, our study did not include monotypic stands of arroyo willow. Instead, we focused on willow trees retained during selective removal. However, examining EMF associated with monotypic stands of arroyo willows will provide a reference for the existing willows in selective removal and the newly established willows in full removal. While the goal of our study was to examine the immediate effects of restoration, long-term sampling can determine if EMF richness recovers in selective removal over time. In similar restoration projects, willow trees have taken one to three years to reach conditions resembling pre-invasion states (Goetz et al., 2022; Sher et al., 2018). Our study provides an important baseline for future studies. Lastly, our study only used DNA sequencing to identify mycorrhizal fungi. Future studies will benefit from the use of absolute abundance using

microscopy to gauge the colonization rates of mycorrhizal fungi on native and invasive root systems.

Although the removal of invasive plant species is an essential step in riparian forest restoration, we may improve outcomes by considering effects on mycorrhizal fungi. Our study yielded unexpected results, such as an increase in EMF richness following full removal of Brazilian pepper tree, possibly owing to organic soil amendments. In contrast, selective removal led to an overall decline in EMF richness where a soil amendment was not applied. Using local ecosystem inocula and organic soil amendments to augment mycorrhizal fungi may mitigate deleterious effects like these on the mycorrhizal community. Native plant recovery may improve as a result. These findings emphasize the importance of considering relationships between invasive species, mycorrhizal fungi, and native plants to ensure successful restoration of riparian ecosystems.

Figures

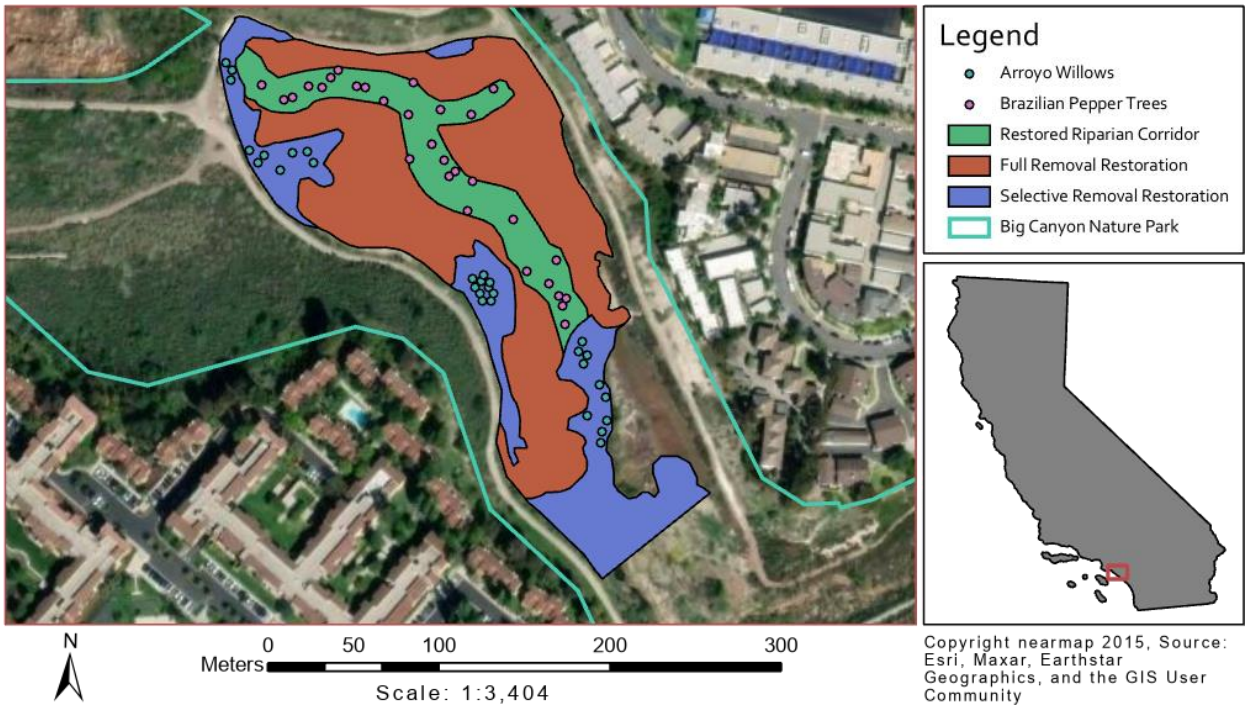


Figure 1. The boundary and project area of the Big Canyon Nature Park. The distribution of trees used in our study are represented by green or magenta circles along with red, blue, or green polygons denoting restoration types. Big Canyon is part of a large habitat reserve referred to as the Upper Newport Bay, located in Southern California.

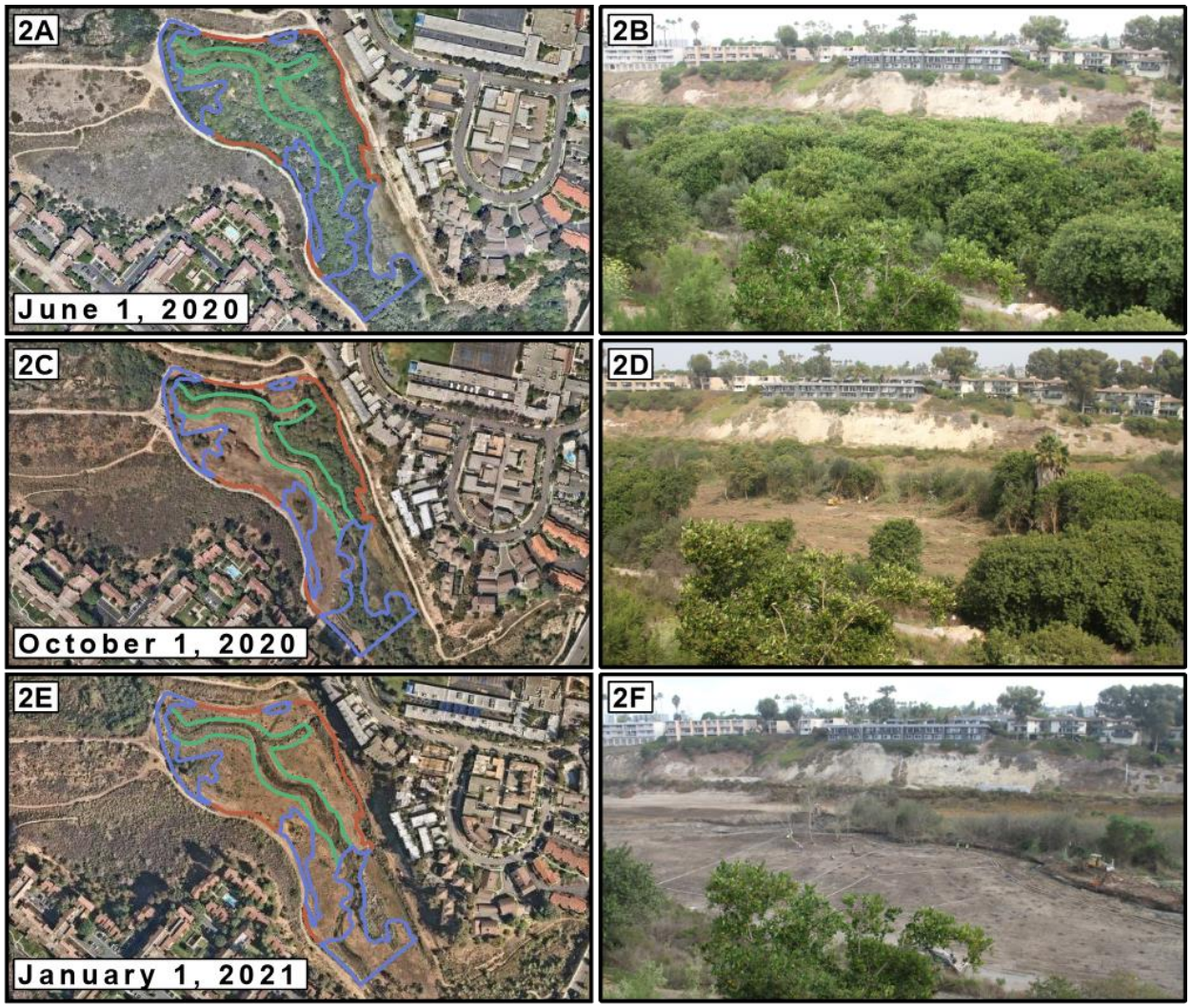


Figure 2. Progress of Brazilian pepper tree removal. Three stages are shown: Before removal (A & B), 60% completion (C & D), and after removal (E & F). Red polygon = full removal restoration. Blue polygon = selective removal restoration. Green polygon = restored riparian corridor.

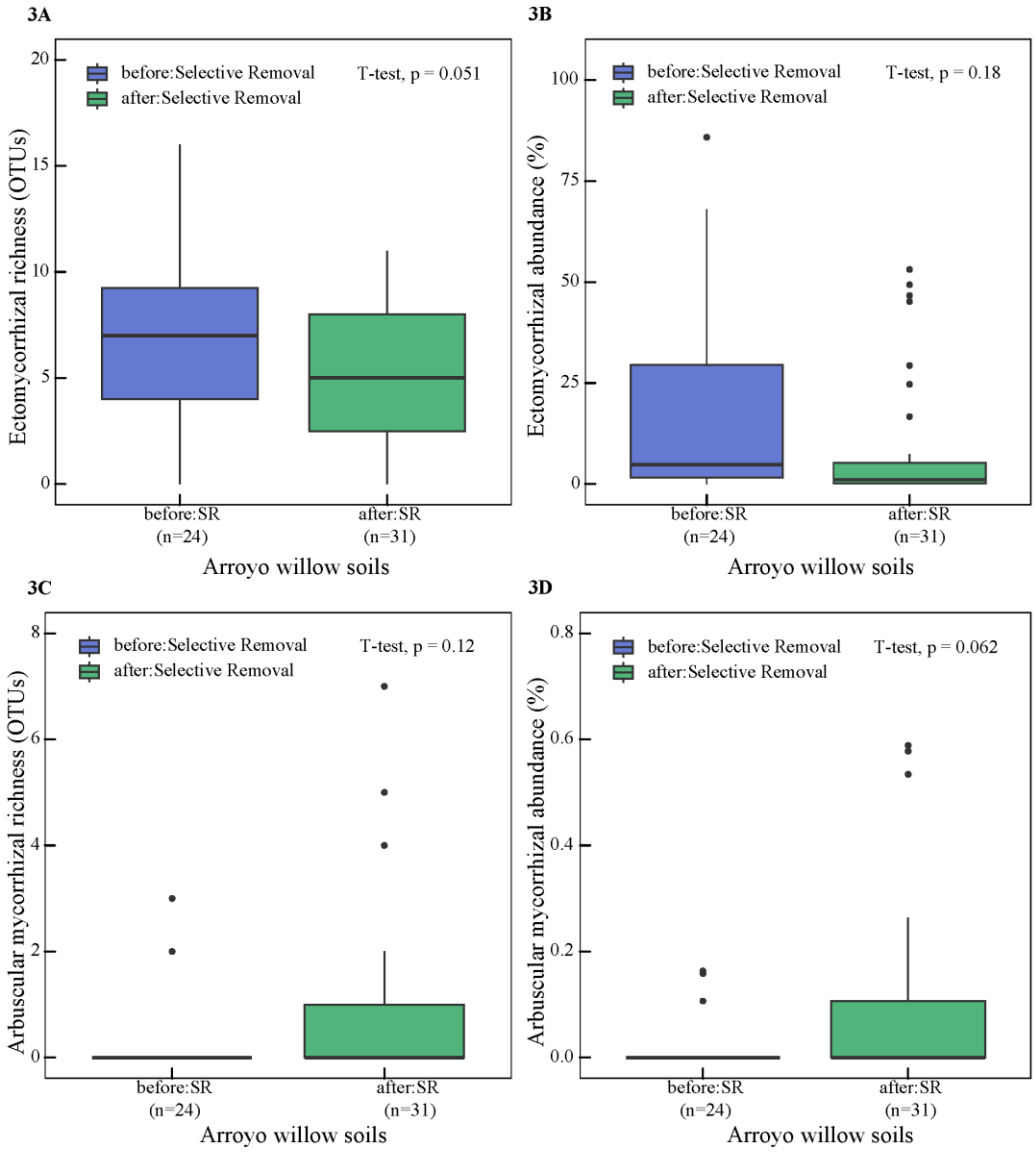


Figure 3. Richness and relative abundance of operational taxonomic units (OTUs) of mycorrhizal fungi in soils of the selective removal treatment, with ectomycorrhizal richness (A), ectomycorrhizal relative abundance (B), arbuscular mycorrhizal richness (C), and arbuscular mycorrhizal relative abundance (D). Box plots denote the interquartile range between the 25 and 75 percentiles (boxes), medians (black lines), and potential outliers (dots). Selective removal marginally significantly reduced ectomycorrhizal richness (A) but marginally significantly increased relative abundance of arbuscular mycorrhizal fungi (D).

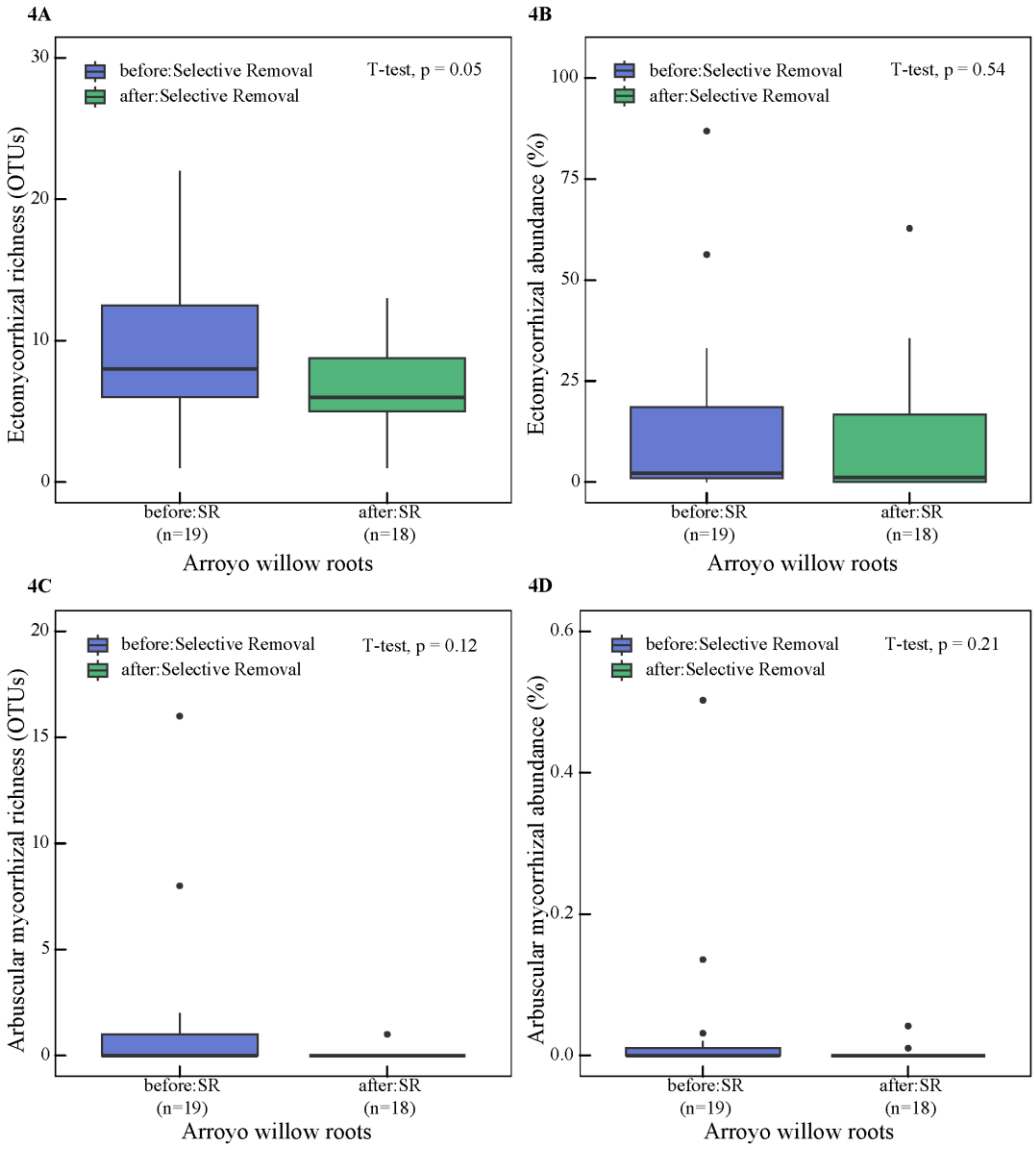


Figure 4. Richness and relative abundance of operational taxonomic units (OTUs) of mycorrhizal fungi in roots of the selective removal treatment, with ectomycorrhizal richness (A), ectomycorrhizal relative abundance (B), arbuscular mycorrhizal richness (C), and arbuscular mycorrhizal relative abundance (D). Box plots denote the interquartile range between the 25 and 75 percentiles (boxes), medians (black lines), and potential outliers (dots). Selective removal significantly reduced ectomycorrhizal richness.

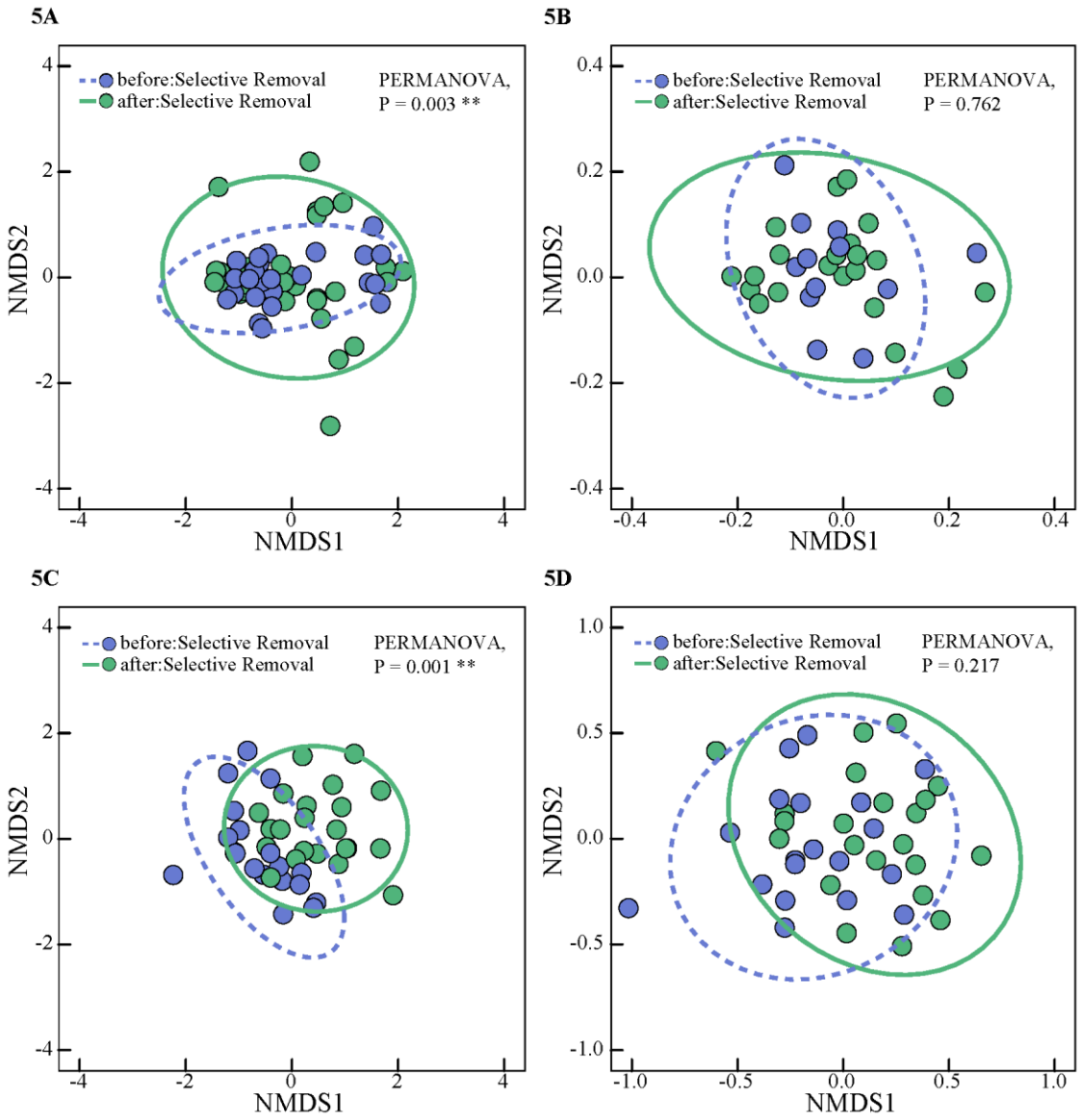
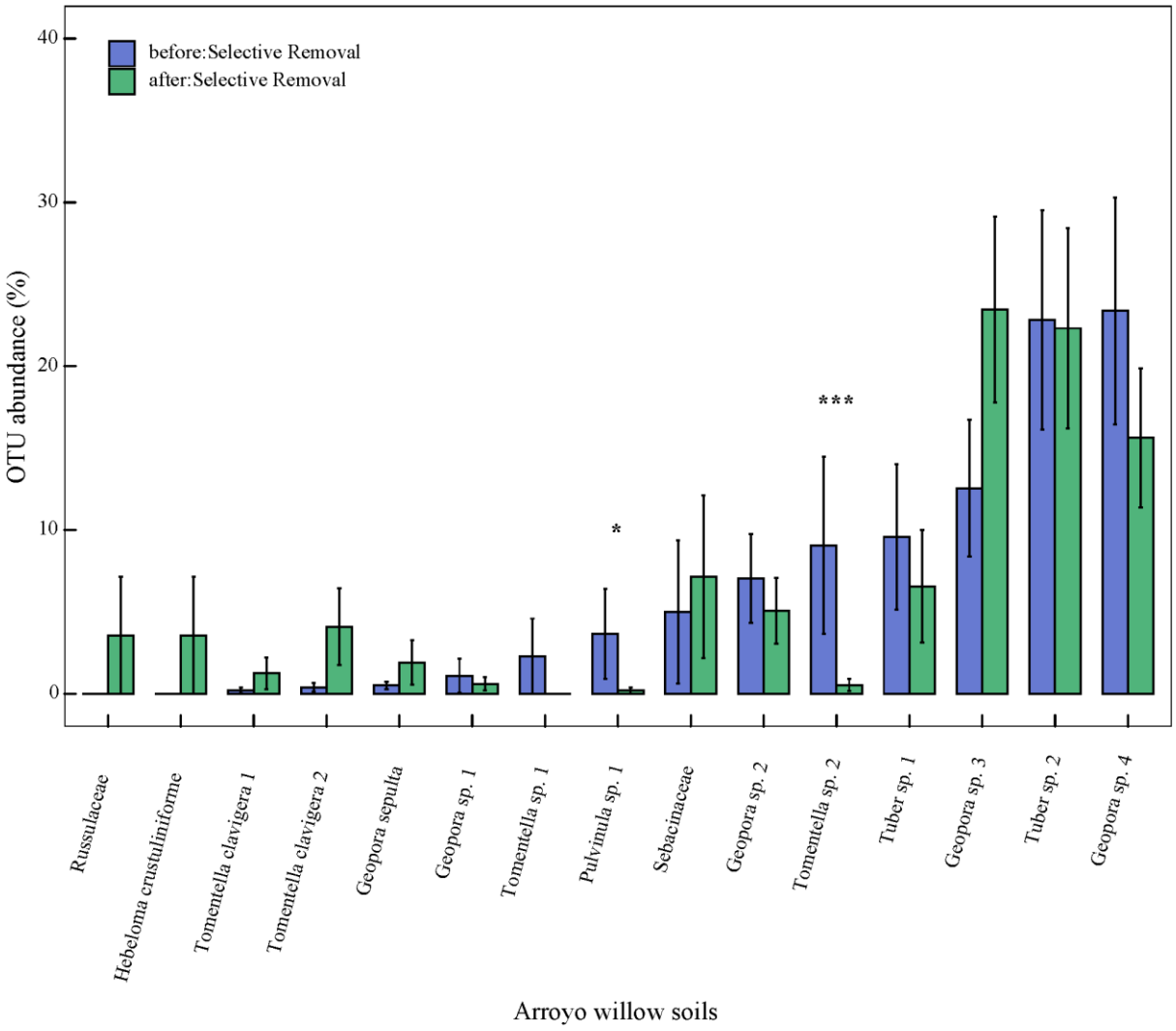


Figure 5. Non-metric multidimensional scaling (NMDS) plots of the community composition of the broader fungal community in soils (A), the ectomycorrhizal community in soils (B), the broader fungal community in roots (C), and the ectomycorrhizal community in roots (D) associated with arroyo willows in the selective removal treatment. Symbols = samples. Asterisks (*) = significant differences resulting from a PERMANOVA test. Solid or dashed lines = 97% confidence intervals. Selective removal significantly shifted the community composition of the broader fungal communities in soils (A) and roots (B).

6A


before:Selective Removal
after:Selective Removal
OTU abundance (%)
40
30
20
10
0
*
***
Russulaceae
Hebeloma crustuliniforme
Tomentella clavigera 1
Tomentella clavigera 2
Geopora sepulta
Geopora sp. 1
Tomentella sp. 1
Pulvinula sp. 1
Sebacinaceae
Geopora sp. 2
Tomentella sp. 2
Tuber sp. 1
Geopora sp. 3
Tuber sp. 2
Geopora sp. 4
Arroyo willow soils

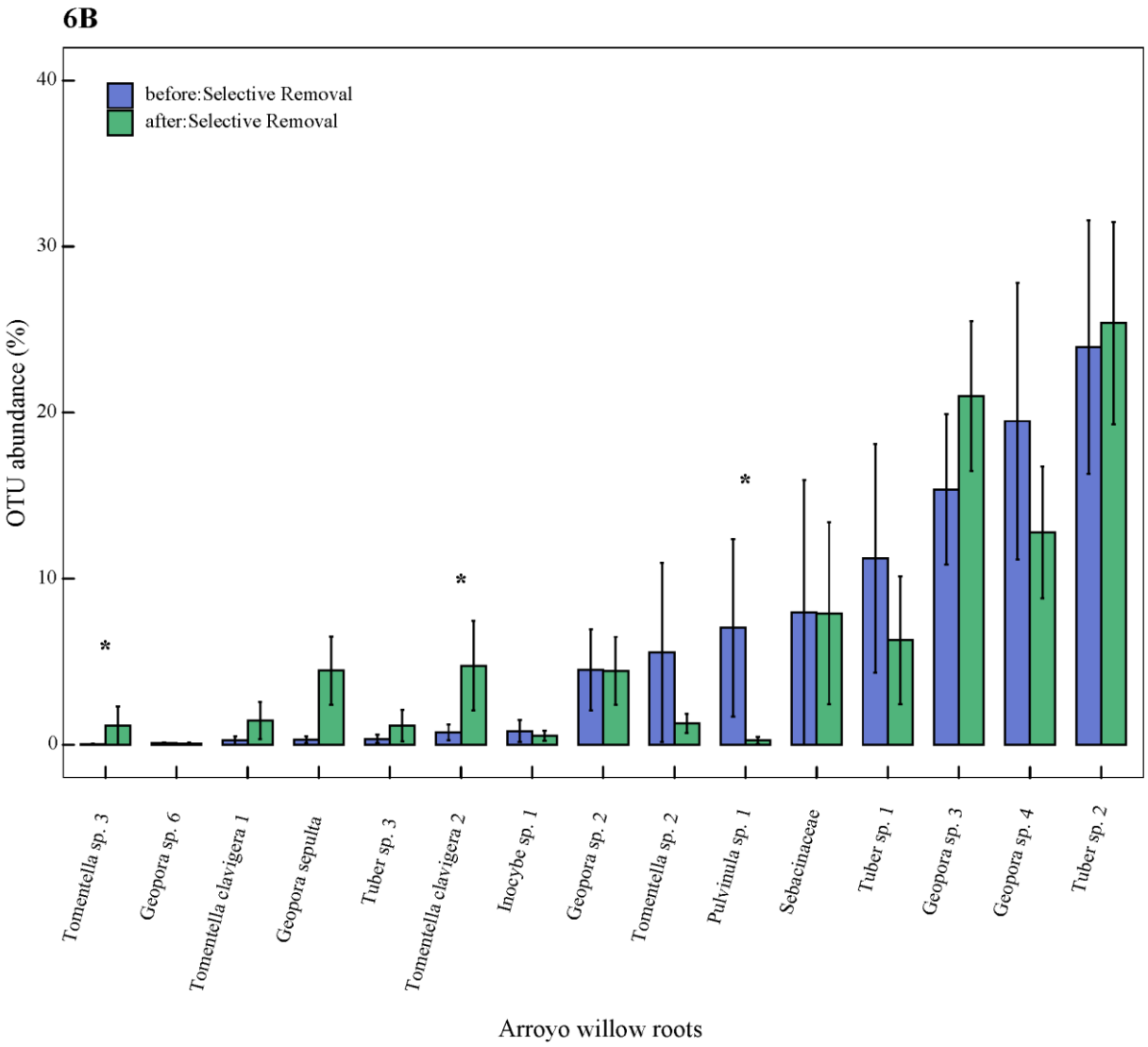


Figure 6. The relative abundance of the 15 most common operational taxonomic units (OTUs) of ectomycorrhiza in arroyo willow soils (A) and roots (B) in the selective removal treatment. Bar plots depict means ± SE. Asterisks (*) denote significant indicator species ($P < 0.05$) before versus after selective removal. *Tomentella* sp. 3 and *Tomentella clavigera* 2 were significant indicators of post-removal, and *Pulvinula* sp. 1 was a significant indicator of pre-removal.

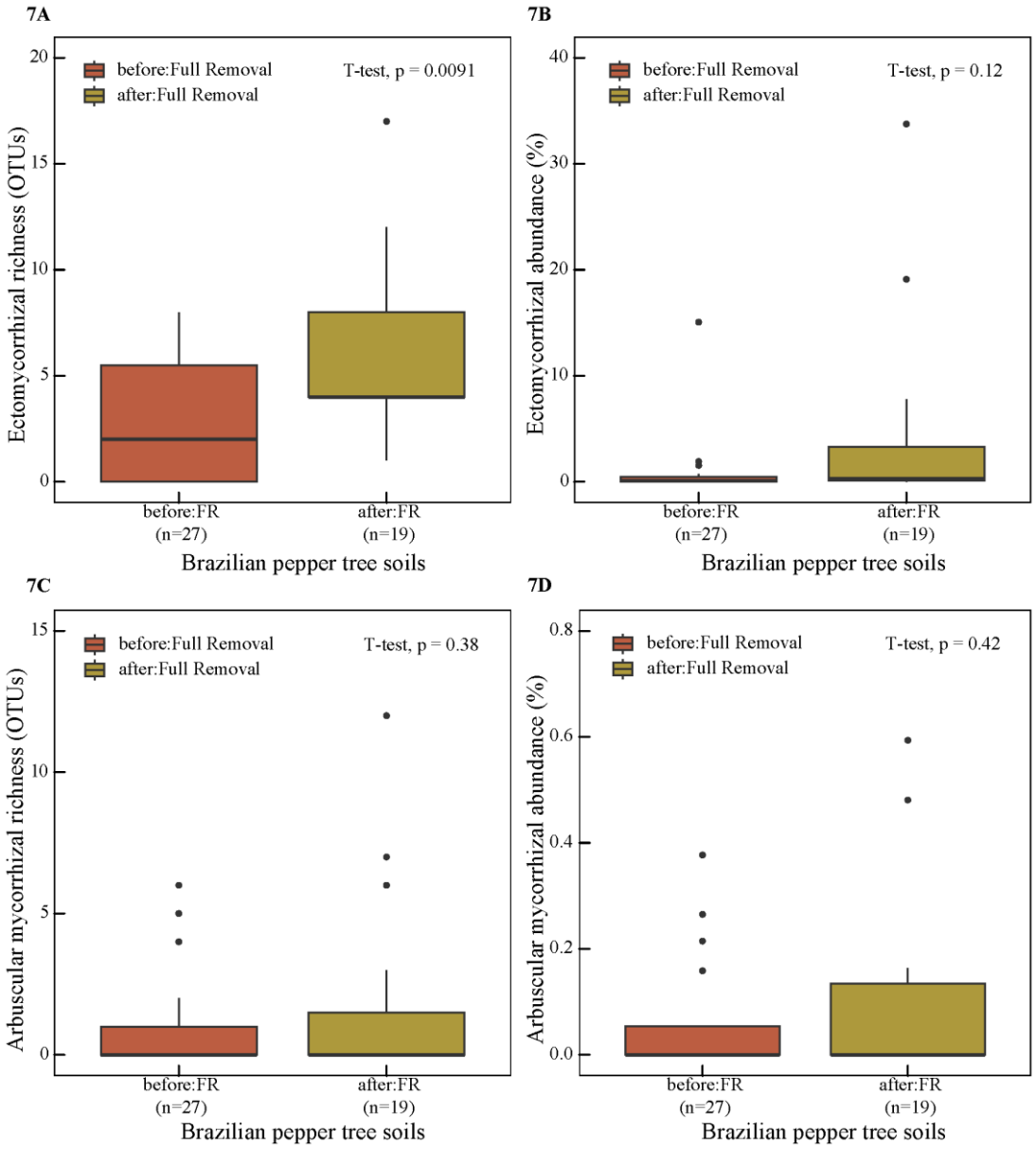


Figure 7. Richness and relative abundance of operational taxonomic units (OTUs) of mycorrhizal fungi in soils of the full removal treatment, with ectomycorrhizal richness (A), ectomycorrhizal relative abundance (B), arbuscular mycorrhizal richness (C), and arbuscular mycorrhizal relative abundance (D). Box plots denote the interquartile range between the 25 and 75 percentiles (boxes), medians (black lines), and potential outliers (dots). Full removal significantly increased ectomycorrhizal richness.

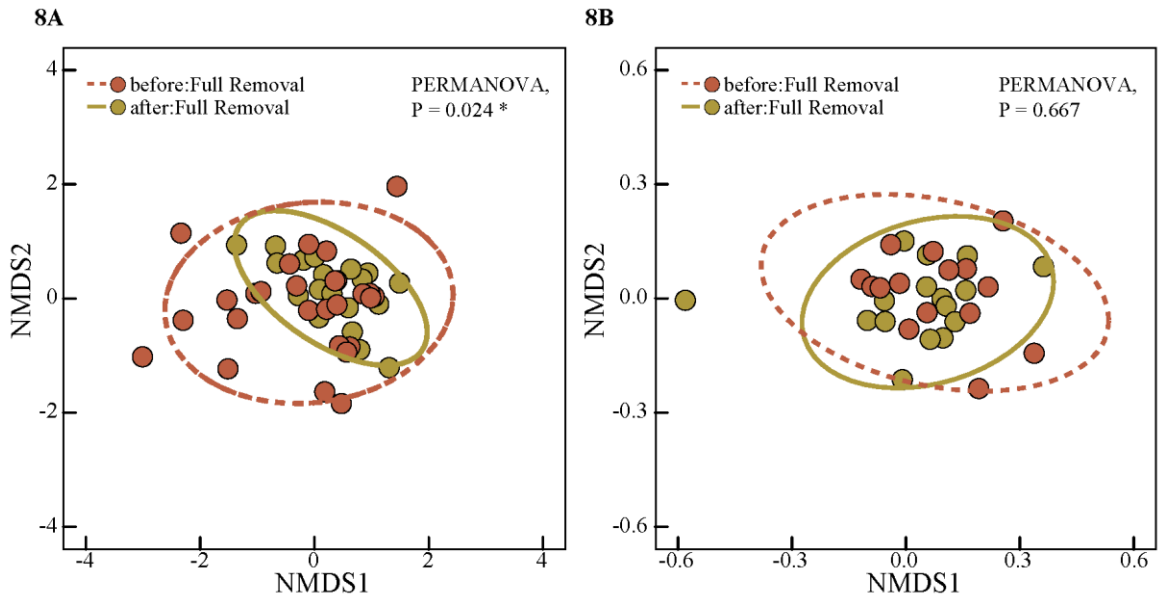


Figure 8. Non-metric multidimensional scaling (NMDS) plots of the community composition of the broader fungal community (A) and the ectomycorrhizal community in soils associated with Brazilian pepper tree in the full removal treatment. Symbols = samples. Asterisks (*) = significant differences resulting from a PERMANOVA test. Solid or dashed lines = 97% confidence intervals. Full removal significantly shifted the community composition of the broader fungal community (A).

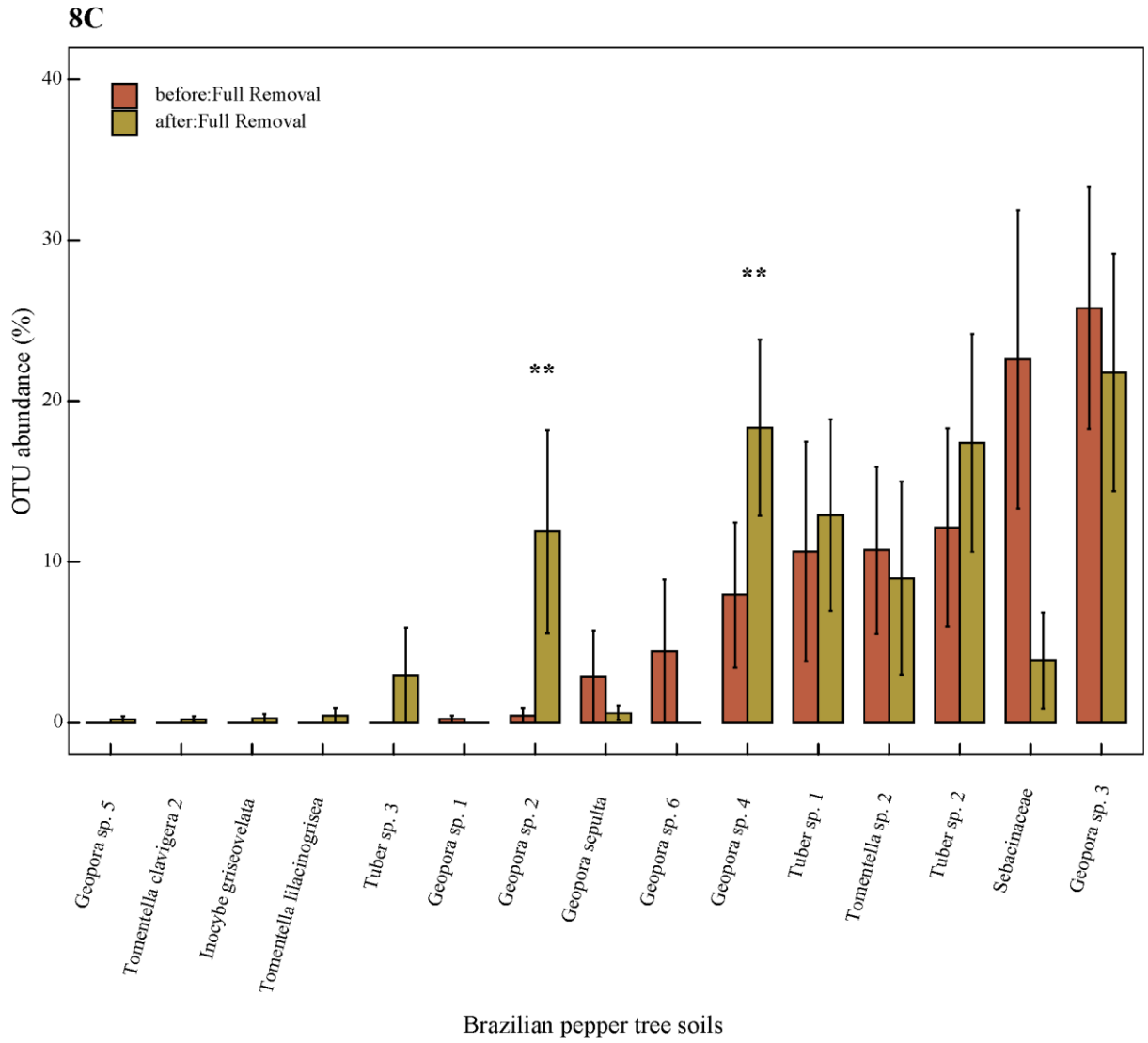


Figure 9. The relative abundance of the 15 most common operational taxonomic units (OTUs) of ectomycorrhiza in Brazilian pepper tree soils in the full removal treatment. Bar plots depict means ± SE. Asterisks (*) denote significant indicator species ($P < 0.05$) before versus after selective removal. *Geopora* species 2 and 4 were significant indicators of post-removal.

Table 1. Mycorrhizal species associated with the commercial inoculum (Fungi Perfecti LLC.) applied to full removal restoration. The product consisted of varying proportions of each species. In our sequences we did not detect any of the species at the field site.

Species	**Guild**	**Proportion**
Glomus intraradices	Arbuscular mycorrhizal fungi	34 prop/g each
Glomus mosseae	Arbuscular mycorrhizal fungi	34 prop/g each
Glomus mosseae	Arbuscular mycorrhizal fungi	34 prop/g each
Glomus etunicatum	Arbuscular mycorrhizal fungi	34 prop/g each
Glomus deserticola	Arbuscular mycorrhizal fungi	13 prop/g each
Glomus clarum	Arbuscular mycorrhizal fungi	13 prop/g each
Paraglomus brasilianum	Arbuscular mycorrhizal fungi	13 prop/g each
Gigaspora margarita	Arbuscular mycorrhizal fungi	13 prop/g each
Rhizopogon villosulus	Ectomycorrhizal fungi	208,750 prop/g each
Rhizopogon luteolus	Ectomycorrhizal fungi	208,750 prop/g each
Rhizopogon amylopogon	Ectomycorrhizal fungi	208,750 prop/g each
Rhizopogon fulvigleba	Ectomycorrhizal fungi	208,750 prop/g each
Pisolithus tinctorius	Ectomycorrhizal fungi	1,250,000 prop/g
Suillus granulatus	Ectomycorrhizal fungi	260,000 prop/g
Laccaria bicolor	Ectomycorrhizal fungi	83,500 prop/g each)
Laccaria laccata	Ectomycorrhizal fungi	83,500 prop/g each)

Scleroderma cepa	Ectomycorrhizal fungi	41,750 prop/g each
Scleroderma citrinum	Ectomycorrhizal fungi	41,750 prop/g each

CHAPTER 2

Title: The effect of logging and thinning on arbuscular and ectomycorrhizal fungi in second-growth redwood (*Sequoia sempervirens*) forests.

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Abstract

More than 90% of the redwood (*Sequoia supervirens*) range was logged extensively. Despite their resilience, management practices have impaired the recovery of second-growth forests. Prior to the establishment of the Redwood National and State Parks, Douglas-fir (*Pseudotsuga menziesii*) and Sitka spruce (*Picea sitchensis*) were aerially seeded for future timber harvests. While the deleterious effects on redwood forests are well documented, the effects on mycorrhizal fungi have yet to be considered. The aim of this study was to determine how mycorrhizal fungi associated with redwood forest are affected by the encroachment and the removal of Sitka spruce and Douglas-fir. Since redwood forests use arbuscular mycorrhizal fungi (AMF) and ectomycorrhizal fungi (EMF), we expected a decline in AMF in second-growth forests compared to old-growth forests. We had this expectation due to the presence of pine woodland that use EMF exclusively. In support of our predictions, the richness and abundance of AMF was significantly higher in the soils of old-growth. Additionally, this trend was found present in the rhizonode, a specialized root structure that harbors AMF. Where we expected EMF to be most prevalent in second-growth, there were no significant differences between old and second-growth. However, the presence of EMF was significantly higher in thinned forests compared to old-growth. Future restoration efforts should aim to restore AMF in second-growth and thinned forests to resemble below ground conditions of old-growth.

Introduction

The intrusion of trees in the Pinaceae into second-growth redwood forests, along with their exclusive reliance on ectomycorrhizal fungi (EMF), may disrupt the dual mutualism used by redwood forests. Dual mutualism occurs when trees associate with at least two mycorrhizal types, where redwood forests use arbuscular mycorrhizal fungi (AMF) and EMF (Willing et al., 2021). Both mycorrhizal types provide critical services to the plant host in the form of enhanced growth, pathogen protection, and resource acquisition (Pérez-Izquierdo et al., 2021; Smith & Read, 2008). The services provided by AMF and EMF can vary depending on abiotic and biotic conditions such as land use, soil moisture, biogeochemistry, and tree species (Dickie et al., 2013; Karst et al., 2021). While trees can shift their dependency between AMF and EMF, wide-ranging benefits occur when dual mutualism is achieved (Teste et al., 2020). Consequently, the loss of dual mutualism in second-growth redwood forest can impair recovery. When restoration involves the removal of pine woodland, further effects to dual mutualism should be considered. Additionally, special attention should be directed to the specialized root structures, known as rhizonodes, which harness specific associations with AMF (Willing et al., 2021). By determining what effect pine invasion and removal has on dual mutualism, we can address critical knowledge gaps in the restoration of redwood forests.

A previously unrecognized dimension of logging in redwood forests is the potential shift in the dependency of dual mutualism. Changes in the dependency of dual mutualism can have implications for nutrient exchange between hosts and mycorrhizal fungi. Due to their enhanced enzymatic capabilities, EMF have a greater capacity to ‘mine’ for nutrients within the soil (Plassard & Dell, 2010). Whereas, AMF ‘scavenge’ for resources, mostly assisting with N uptake without enzymatic capabilities (Lambers et al., 2008). However, EMF are expensive, requiring more assimilated C

from hosts compared to AMF (Hobbie, 2006; Lendenmann et al., 2011). The dependency of dual mutualism may shift with environmental conditions at the expense or benefit to the plant hosts (Teste et al., 2020). Furthermore, logging is known to drive shifts in the dependency of AMF and EMF in trees using dual mutualism (Boeraeve et al., 2019; Chen et al., 2019; Rodriguez-Ramos et al., 2021). When considering second-growth redwood forests, the cost-benefit of dual mutualism can change if AMF are less prevalent, and EMF is favored.

In a recent review, the dependency of dual mutualism is multifaceted, revealing three distinct categories: context-free, spatial, and temporal (Teste et al., 2020). Apart from the redwood rhizonode, the dependency of dual mutualism can vary across spatial gradients. Previously, mycorrhizal associations in old-growth redwood forests have been investigated in the southern, central, and northern ranges of California. These findings showed that the rhizonode consistently harbored a substantial presence of AMF across spatial distances (Willing et al., 2021). In contrast, there was wide-ranging variability in the presence of AMF in soil and fine root tips not associated with the rhizonode structure (Willing et al., 2021). These initial findings allude to the dependance AMF in rhizonodes as context-free, while the soils and roots were spatially dependent owing to the variability of AMF across spatial gradients. Currently, no study has determined which dependency dual mutualism exhibits in second-growth redwood forests. To mitigate effects of logging in second-growth forests, restoring dual mutualism is critical, however, studies examining these dynamics are limited.

Previous management practices that facilitated the spread of pine trees into second-growth redwood forests, can shift the dependency of dual mutualism. Much of the available research on the effects of land management comes from the Redwood National and State Parks (RNSP). Approximately 45% (16,000 hectares) of all the remaining old-growth stands in the redwood range,

occur in RNSP (Burns et al., 2018). Over 32,000 hectares of RNSP are considered second-growth, subjected to extensive clear-cutting during the mid-20th century. Prior to establishment of RNSP in 1968, trees in the Pinaceae such as Douglas-fir (*Pseudotsuga menziesii*) and Sitka spruce (*Picea sitchensis*) were aerially seeded (Teraoka & Keyes, 2011). Where pine woodland is densely populated, redwoods are no longer the dominant feature of second-growth forests. This above-ground shift in plant species composition can be problematic for the dual mutualism employed by redwood forests. Both Sitka spruce and Douglas-fir are known to primarily host EMF (Burke et al., 2008; Molina & Trappe, 1982), which may affect the dual associations of redwood forests. In other second-growth forests, the shift from dual mutualist to dense pine woodland led to the diminishment of AMF and the prevalence of EMF (Gazol et al., 2016). While the above-ground effects of pine woodland are well defined in redwood forests (Coonen & Sillett, 2015; Iberle et al., 2020; Teraoka & Keyes, 2011, 2011), below-ground aspects have received far less attention.

Despite the thinning of pine woodland in redwood forests, deleterious effects such as the loss AMF mutualist may inhibit long-term recovery. In the coming decades the Redwood Rising (RR) restoration project will thin 20,000 hectares of second-growth redwood forests in RNSP (Burns et al., 2018). Beginning in 1978, numerous thinning projects have commenced with varying degrees of success (Dagley et al., 2023; Keyes, 2011; O'Hara et al., 2020; Soland et al., 2021; Teraoka & Keyes, 2011). Historically, the Yurok Tribe managed the disturbance regime of old-growth forests with controlled burns (Lorimer et al., 2009). While second-growth redwoods could benefit from the reintroduction of prescribed fire, wet fuels and administrative difficulties make this restorative type less feasible (Berrill et al., 2013). Restorative thinning in second-growth redwood forests has proven to be the most effective tool to address the temporal shift in species composition. However, thinning alone cannot guarantee the recovery of dual mutualism. In other forested ecosystems where

restorative thinning was necessary, controlled burns and the development of a native understory led the recovery of AMF over time (Baohanta et al., 2012; Hart et al., 2018; Korb et al., 2003). Examining the immediate effects of restorative thinning in second-growth redwood forests, establishes a baseline for assessing the need for additional measures to restore AMF.

We partnered with RR to compare mycorrhizal communities in three forest types: old-growth, second-growth, and thinned forests. To shed light on the dependency of dual mutualism in these forest types, we developed three hypotheses. First, we predicted EMF would be the most abundant and diverse in second-growth forests, whereas AMF would be more abundant and diverse in the in old-growth forests. Our overarching expectation was the presence of Sitka spruce and Douglas-fir, would diminish AMF associations in second-growth and encourage EMF. Second, we hypothesized that the abundance and diversity of AMF and EMF in soils would be intermediate in the thinned stands in comparison to old- and second-growth (Hypothesis 2). We had this expectation since no additional efforts to restore AMF communities occurred following thinning treatments. Third, we anticipated the composition of the broader fungal community would differ between forest types and between sample types ("root" or "rhizonode"; Hypothesis 3). Our third hypothesis was based on previous studies where differences in the entire fungal community, including mycorrhizal fungi, are attributed to logging (Ammitzboll et al., 2021; J. Chen et al., 2019; Rähn et al., 2023; Sterkenburg et al., 2019; Tomao et al., 2020). Due to the expected prevalence of AMF in the rhizonode, we expected the roots and rhizonodes to harbor different community composition in terms of the broader fungal community.

Methods

Site Description

We tested our hypotheses within two different park units within RNSP (41° 23' 55.82 " N, 124° 02' 27.38 " W), located on the traditional territory of the Yurok (Huntsinger & McCaffrey, 1995). The old-growth locations in our study occurred in the Prairie Creek Redwoods State Park (Prairie Creek RSP), whereas the second-growth and thinning locations were in Redwood National Park (RNP). All old- and second-growth sites, including thinned forests, were no more than 2.5 km apart from each other. Both park units' border each other with Prairie Creek RSP consisting of continuous old-growth while the RNP is fragmented by second-growth (Fig. 1). Collectively both park units experience a Mediterranean climate. The mean annual minimum temperature is 6 °C followed by the maximum annual temperature at 16 °C (WRCC, 2023). Approximately 90% of the rainfall occurs between October and April, with the mean annual precipitation reaching 170.6 cm (WRCC, 2023). Coastal fog supplements the seasonal lack of rainfall in the summer months (Keyes & Teraoka, 2014). The redwood range occurs near the coast where the fog belt extends 24 km inland (Keyes & Teraoka, 2014). Within the park units, the dominant soil types consists of the Ossagon-Squashan-Goldbluffs Complex (293-294), occurring on slopes ranging from 9-50 percent (NCRS, 2023).

The thinned and second-growth forests are referred to as the Greater Prairie Creek Project Area (GPCA; Fig. 1). Using clear-cutting, the GPCPA was logged extensively between 1930 and 1978 (NPS, 2008, 2014). To restore connectivity between old-growth redwood forests, 16,688 hectares of second-growth in GPCPA have been identified for restoration (RR, 2019). Within the project area, the dominant overstory consists of Douglas-fir, Sitka spruce, and redwoods that were resprouted from cut stumps. Other trees species present in the overstory include grand-fir (*Abies grandis*), red

alder (*Alnus rubra*), and western hemlock (*Tsuga heterophylla*). Overall, the second-growth forests in the GPCPA have low species diversity and stand structure compared to old-growth (RR, 2019). Compared to the old-growth, the understory of GPCPA is also limited, consisting of sparse populations of evergreen huckleberry (*Vaccinium ovatum*), rhododendron (*Rhododendron occidentale*), salal (*Gaultheria shallon*), and sword fern (*Polystichum munitum*).

Restoration Approach

Project activities in the GPCPA and broader RR project, broke ground in 2020. In addition to the interagency management between State and Federal agencies, Save the Redwoods League was crucial in the development and coordination of the RR. The purpose of thinning in the GPCPA was to rehabilitate second-growth redwood forests that had previously been degraded by commercial timber harvests. Specifically, thinning efforts sought to reduce stand density in order to facilitate the growth of redwoods, understory vegetation, and the development of a multi-story canopy (RR, 2019). The restoration deployed variable density thinning (VDT), incorporating varying degrees of low thinning, crown thinning, gaps, skips, and conifer release to increase stand heterogeneity. The project used three restoration efforts to lower tree density: (1) Skyline Yarding/Tethered/Lop-and-Scatter Option, (2) Ground-Based Yarding/Lop-Scatter Option, and (3) Lop-and-Scatter (Fig 1). Briefly, the skyline option involves stationary cables to pull logs or whole trees off site, while the ground-based option uses tractors or skidders for removal. In contrast, the lop-and-scatter option involves felling trees and distributing them on site. In general, all treatments planned to keep a density of 100 trees per 0.4 ha (RR, 2019).

Sampling Design

We conducted our sampling during October 2020 and 2021, which coincided with the end of the active treatment season, providing safe access for field sampling. During the first field season, our

collection focused on comparing the mycorrhizal community between old-growth and second-growth forests. During the second field season, at the time of field sampling thinning treatments had been completed by September. Due to the proximity of the old-growth and second-growth, our sampling design commenced in the Streelow-Gold Bluffs management unit of the GPCPA (Fig 1.; RR, 2019). Our collection sites were also based on the access from trail and roads. To establish the sampling design, we use the random point generator in ArcGIS Pro (2.1.0), identifying 20 random points for each forest type (old-growth and second-growth) for the 2020 collection (20 soil samples × 2 forest types = 40 total samples).

The random points were split between two locations with the access points that provided the nearest distance between old-growth and second-growth (Fig. 1). At each randomly generated point, our parameters selected a point that was at least 5 m from the nearest trail and no more than 20 m away. Additionally, each point was at least 10 m from each other point. In the field, we selected the closest redwood tree from a randomly generated point and collected one soil sample approximately 1.5 m from the tree trunk. For 2021, we repeated the sampling design at the same locations, however, we collected 20 samples from an additional forest type (thinning treatment) associated with the Ground Based Yarding/Lop-and-Scatter Option (20 soil samples × 3 forest types = 60 soil samples).

Sample Collection

Upon arriving at the randomly generated point, we collected one soil sample from each tree using a soil core 5.5 cm in diameter at a depth of 10 cm (Lakago Homier 1 m Soil Sampler Probe). Approximately 950 cm^2 of soil was recovered from each sample. All soil equipment was sterilized with 70% alcohol to prevent the spread of pathogens between sites. Soil cores were collected in sterile freezer bags and kept on ice while conducting field work. The soil samples were then stored

at -20 °C at the end of each day. Following the completion of field work, the samples were transported in dry ice to the University of California, Irvine (UCI). Prior to downstream processing, the soils samples were stored at -80 °C. Due to the limitation of rhizonode tissue in the 2021 samples, we only extracted DNA from roots and rhizonodes in the 2020 collection. During the 2020 field season, access to thinned forests was not available. Therefore, mycorrhizal fungi associated with roots and rhizonodes in thinned forests were not investigated. We moved forward with extracting DNA from soils in 2021, where we included samples from the thinning treatment.

DNA Extraction

The extraction of DNA started with each soil sample being transferred to -20°C. Within one week, the soil samples were passed through a 10 mm sieve. Immediately after the sieving process, the roots were returned to storage at -20 °C for DNA extraction. For soil samples, 250 mg of soil was used for DNA extraction using the DNeasy PowerSoil Pro Kit (Qiagen, Redwood City, CA, USA) according to the manufacturer's instructions. All soil samples were sieved and extracted on the same day and then stored at -20 °C until downstream amplification. Fine root tips (less than 2 mm) and rhizonodes tissues were separated following Willing et al. except for the sample weight (2021). Instead, we extracted DNA from 50 mg of rhizonode tissue and 50 mg of fine root tips using the DNeasy Plant Pro Kit (Qiagen, Redwood City, CA, USA). The presence of the rhizonode structure verified the selection of fine root tips. We excluded fine root tips that were not directly connected to a rhizonode structure. Following the extraction of DNA from roots and rhizonodes, the samples were returned to -20 °C until downstream amplification.

DNA Sequencing

To target the fungal DNA in soil, root, and rhizonode samples we conducted a one-step PCR targeting the ITS2 region. We amplified the DNA using the 5.8SFun (5′ AACTTTYRRCA-

AYGGATCWCT) and ITS4Fun (AGCCTCCGCTTATTGATATGCTTAART-3′) primers (Taylor et al., 2016). To increase the sequencing quality, heterogeneity spacers were included with linkers pads, and dual indexed barcodes at 12 base pairs (BP; Lundberg et al., 2013; Willing et al., 2021). Prior to the PCR amplification all samples were diluted by 1/100 to avoid any PCR inhibitors (Bessetti, 2007). Each PCR reaction used 2 µL of template DNA, 2 µL of each primer diluted to 10 µM, and 12 µL of GoTaq Green Master Mix (Promega). The remaining reaction consisted of sterile water to reach a volume of 24 µL.

Thermocycler parameters started with denaturing at 95°C for 2 min, proceeded by 35 cycles of 30 s at 95 °C, 30 s at 55 °C, 30 s 72 °C, with a final extension for 10 min at 72 °C. To confirm successful amplification, a 1.5% agarose was used to confirm the presence of PCR products between 267 and 511 BP. Additional PCR attempts were used on samples that did not initially amplify on the first attempt. The final amplicon library was pooled based on gel intensity (1 µL, 2 µL, or 3 µL). The library pool was cleaned with Ampure XP beads (Beckman Coulter, Brea, CA, USA) prior to sequencing at the Genomics Research and Technology Hub (GRT Hub) at UCI (https://ghtf.biochem.uci.edu/; Irvine, CA). Sequencing was carried using the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) with 2 x 300 BP paired-end (PE) reads.

Bioinformatics

The demultiplexed sequences were pre-processed using AMPtk software v1.5.4 (Palmer et al., 2018). After concatenating all paired-end reads, the demultiplexed files were clustered into operational taxonomic units (OTUs) with a 97% similarity cutoff. This level of similarity allows for taxonomic resolution at approximately the species level (Kõljalg et al., 2013) . The sequences were filtered using default parameters, and taxonomy was assigned using the UNITE database (Kõljalg et al., 2005). To ensure that only fungal DNA remained for downstream analysis, all non-fungal DNA

was removed. When available, trophic modes were assigned to the OTUs using the FUNGuild database provided by AMPtk (Nguyen et al., 2016). To calculate the relative abundances of arbuscular mycorrhizal fungi (AMF) and ectomycorrhizal fungi (EMF), the OTU counts for each mycorrhizal type were summed and divided by the total OTU count for each sample. The results were multiplied by 100 to express the relative abundance as a percentage. Soils were rarefied at 1,454 sequences per sample while the roots and rhizonodes were rarefied together at 4,434 sequences per sample.

Statistical Analysis

All statistical analyses were performed using R Studio 4.1.2 (RStudio Team, 2021). For our hypotheses around the abundance and richness of mycorrhizal fungi, we conducted the analyses for EMF and AMF separately (Hypothesis 1, and 2). For Hypothesis 3, the composition of the broader fungal community included all assigned and unassigned fungal guilds. For all statistical tests involving abundance, diversity, and community composition we conducted our analysis for soil and root (roots and rhizonodes) associated mycorrhizal fungi individually. For all tests, effects were considered significant when $P < 0.05$ and marginal if $P < 0.10$. For ANOVA tests, the "stats package" was used while the "vegan package" was used for PERMANOVA tests (RStudio Team, 2021). To identify significant OTU's associated with forest and sample type the "indicspecies package" was deployed (Cáceres & Legendre, 2009).

Hypothesis 1 & 2

Soil Associated Mycorrhizal Fungi. To test Hypotheses 1, we used an ANOVA to assess differences in the mycorrhizal community of old- and second-growth soils. The ANOVA compared OTU richness or relative abundance of OTUs associated with either mycorrhizal type (AMF or EMF) separately. For Hypothesis 1, the dependent variable was the diversity or abundance of

mycorrhizal fungi with the independent variable being the forest type ("old-growth" or "second-growth"). Additionally, a pairwise comparison (Tukey test) was performed to assess differences between forest types. A significant effect resulting from a pairwise comparison, where second-growth soils are associated with a higher OTU richness and relative abundance of EMF compared to old-growth, would support Hypothesis 1. Oppositely, a higher AMF richness or relative abundance in old-growth forests compared to second-growth would support Hypothesis 1.

To test Hypotheses 2, we used an ANOVA to compare the OTU richness or relative abundance of OTUs in soils associated with old-growth, second-growth, and thinned forests. For the soil associated with mycorrhizal fungi of Hypothesis 2, the dependent variable was the diversity or abundance of mycorrhizal fungi with the independent variables being the forest type ("old-growth," "second-growth," or "thinned"). Additionally, a pairwise comparison (Tukey test) was performed to assess differences across forest types. Hypothesis 2 would be supported when the differences between second-growth are intermediate, indicated by the pairwise comparison. Similarly, when the pairwise comparison shows the differences between thinned and second-growth forests are insignificant would provide further support for Hypothesis 2.

Root Associated Mycorrhizal Fungi. To assess differences in root associated mycorrhizal fungi, we conducted an ANOVA test. We compared the OTU richness or relative abundance of OTUs associated with either mycorrhizal type individually. For the plant associated mycorrhizal fungi of Hypothesis 1, the dependent variable was the diversity or relative abundance of mycorrhizal fungi with the independent variables being the forest type ("old-growth" or "second-growth") and sample type ("roots" or "rhizonode"). A pairwise comparison (Tukey test) was used to assess specific differences across forest and sample types. To support Hypothesis 1, a significant effect would be considered when the richness and relative abundance of AMF in old-growth roots and rhizonodes

was higher compared to second-growth. Additional support for Hypothesis 1, would occur when the richness and relative abundance of EMF are higher in second-growth compared to old-growth.

Hypothesis 3

Broader Fungal Community. For Hypotheses 3, we conducted a series of PERMANOVA tests to assess differences in the community composition of fungi. For soil and root fungi, the dependent variable was the relative abundance of fungal OTUs. In soils the independent variable was forest type ("old-growth," "second-growth," or "thinned"), whereas the independent variables for root associated mycorrhizal fungi were forest ("old-growth" or "second-growth") and sample type ("roots" or "rhizonode"). A significant effect of forest or sample type would support Hypothesis 3. Non-metric multidimensional scaling (NMDS) plots accompanied the PERMANOVA tests, which illustrated community composition of the broader fungal community. To identify fungal OTUs that contributed overwhelmingly to differences in community composition, we used the indicspecies package in RStudio (Cáceres & Legendre, 2009). Indicator tests for soil and plant fungi were conducted separately.

Results

Metagenomic Data

Paired-end sequences yielded 10,873,437 total reads, with 5,974,955 reads meeting minimum length for validation. After filtering a total of 5,911,242 reads were assigned taxonomy, producing 6,056 OTUs. Soil samples were rarefied to 1,454 sequences per sample, whereas root samples were rarefied to 4,434 sequences per sample. The FUNGuild database identified 804 saprotrophs, 281 pathotroph-saprotroph-symbiotrophs, 765 symbiotrophs, 299 saprotrophs-symbiotrophs, 168 pathotrophs, and 93 pathotroph-symbiotrophs. After rarefying, the most common species in soil and root samples was *Lachnum* sp. (saprotrophic), accounting for 8.9% and 7.18% of the sequences

respectively. The three most abundant phyla in soil and root samples included: Basidiomycota (48.9% in soils, 52.6% in roots), Ascomycota (37.3% in soils, 36.4% in roots), and Glomeromycota (8.12% in soils, 4.89% in roots).

Hypothesis 1

Soil Associated Mycorrhizal Fungi. The ANOVA test revealed the richness of EMF was significantly different across forest types (P = 0.0322; Fig. 2A). While the richness of EMF was higher in second-growth rather than old-growth, the differences were not reflected by the pairwise comparison (P = 0.229; Fig. 2A). Across forest types, the relative abundance of EMF, the ANOVA test resulted in no significant differences (P = 0.227). While the relative abundance was highest in second-growth, the pairwise comparison to old-growth was not significant (P = 0.198; Fig. 2B).

The ANOVA test revealed significant differences in the richness of AMF across forest types ($P = 5.01 \times 10^{-7}$; Fig. 2C). In the soils of old-growth, the richness of AMF was 16.2 OTUs compared to 3.74 OTUs in second-growth, resulting in a significant difference using a pairwise comparison ($P = 2.7 \times 10^{-6}$; Fig. 2C). Similarly, the ANOVA yielded significant differences in the relative abundance across forest types (P = 0.0009; Fig. 2D). Indicated by the pairwise comparison, the relative abundance of AMF in old-growth forests was 22.9 %, whereas second-growth was 3.3 % (P = 0.003; Fig. 2D). Although the relative abundance and diversity of EMF followed the expected pattern, with a higher prevalence in second-growth, the results did not yield significant differences. However, our results demonstrated a greater prevalence of AMF in old-growth compared to second-growth forests. Taken together, we found partial support for Hypothesis 1, which predicted AMF would be most prevalent in the soils of old-growth.

Root Associated Mycorrhizal Fungi. As far as the richness of EMF for root associated mycorrhizal fungi (roots and rhizonodes), no significant difference occurred across forest type (P =

0.183), sample type (P = 0.390), and the forest:sample comparison (P = 0.198; Fig. 3A). In the fine root tips, no significant differences were observed between the richness of EMF in old- and second-growth (P = 0.999, Fig. 3A). The EMF in the rhizonode structure had a higher richness compared to second-growth, however, the differences were not indicated by the pairwise comparison (P = 0.232; 3A). As far as the pairwise comparisons for the richness of EMF between fine root tips and the rhizonode in second-growth, no significant differences occurred (P = 0.816; Fig. 3B). Similarly, no differences occurred for the same pairwise comparison for old-growth (P = 0.62; Fig. 3B).

For the relative abundance of EMF of root associated mycorrhizal fungi (roots and rhizonodes), no significant difference occurred across forest type (P = 0.128), sample type (P = 0.686), and the forest:sample comparison (P = 0.136; Fig. 3A). Following a pairwise comparison, there were no observed differences in the relative abundance of EMF in the root tips of old- and second-growth (P = 0.999, Fig. 3B). Similarly, there were no observed differences in the relative abundance of EMF in the rhizonodes of old- and second-growth (P = 0.14; Fig. 3B). As far as the pairwise comparisons for the relative abundance richness of EMF between fine root tips and the rhizonode in second-growth, no significant differences occurred (P = 0.55; Fig. 3B). Similarly, no differences were observed for the same pairwise comparison for old-growth (P = 0.83; Fig. 3B).

For the richness of AMF for root associated mycorrhizal fungi (roots and rhizonodes), significant differences were observed for the forest type (P = 0.034) and the forest:sample comparison (P = 0.0204) with no difference occurring within sample type (P = 0.4616; Fig. 3A). Following a pairwise comparison across forest type, there were no significant differences in the richness of AMF in the root tips (P = 0.993; Fig. 3C). However, the richness of AMF in the rhizonodes of old-growth was significantly higher where the richness of AMF was 15.7 compared to 6.37 in second-growth (P = 0.012; 3C). As far as the pairwise comparisons for the richness of AMF between fine root tips and

the rhizonode in second-growth, no significant differences occurred (P = 0.816; Fig. 3B). Similarly, no differences were observed for the same pairwise comparison for old-growth (P = 0.14; Fig. 3B).

For the relative abundance of AMF for root associated mycorrhizal fungi (roots and rhizonodes), significant differences were observed for the forest type (P = 0.0126) and the forest:sample comparison (P = 0.037) with no difference occurring within sample type (P = 0.268; Fig. 3A). No significant differences in the relative abundance of AMF in fine root tips occurred between old- and second-growth (P = 0.998; 3D). In contrast, a significant difference was observed in relative abundance of AMF in the rhizonodes, where the relative abundance of AMF in old-growth was 11.1 % and 0.617 % second-growth (P = 0.009; 3D). As far as the pairwise comparisons for the relative abundance of AMF between fine root tips and the rhizonode in second-growth, no significant differences occurred (P = 0.862; Fig. 3B). Similarly, no differences were observed for the same pairwise comparison for old-growth (P = 0.14; Fig. 3B).

Contrary to our predictions, the richness and abundance of EMF in roots and rhizonodes remained constant across old- and second-growth. However, the old-growth rhizonode did harbor the highest diversity and abundance of AMF but was not significantly different from the fine root tips across forest types. Overall, we only found partial support for Hypothesis 1, where we confirmed the greater prevalence of AMF in the rhizonodes of old-growth compared to second-growth.

Hypothesis 2

Across forest types, the ANOVA tests demonstrated significant differences across forest types for the richness of EMF (P = 0.0322; 2A). Between second-growth and thinned soils, the richness of EMF was similar, indicated by the pairwise comparison (P = 0.374; Fig. 2A). However, when comparing the EMF richness between thinned and old-growth, richness in thinned forest was

significantly higher (P = 0.025; Fig. 2A). For the relative abundance of EMF, there were no differences across all forest types (P = 0.227; Fig. 2B). The pairwise comparison of the relative abundance of EMF between thinned and old-growth was not significantly different (P = 0.566; Fig. 2B). Similarly, there were no significant differences in the relative abundance of EMF between second-growth and thinned forests (P = 0.809; Fig. 2B).

Following the ANOVA test, significant differences were indicated in the richness of AMF across forest types ($P = 5.01 \times 10^{-7}$; Fig. 2C). The pairwise comparison indicated the richness of AMF in old-growth soils was higher compared to thinned forests (P = 0.002; Fig. 2C). The richness of AMF in second-growth and thinned soils was similar following a pairwise comparison (P = 0.855; Fig. 2C). Similarly, the ANOVA yielded significant differences in the relative abundance across forest types (P = 0.0009; Fig. 2D). Following the pairwise comparison the relative abundance of AMF in the soils of old-growth was significantly higher compared to the thinned forests (P = 0.002; Fig. 2D). However, second-growth and thinned forests had a similar relative abundance of AMF (P = 0.905; Fig. 2D).

Our results showed that second-growth paralleled treated areas, while old-growth maintained a higher richness and abundance AMF compared to treated forests. We also observed a higher richness of EMF in thinned soils compared to old-growth. Since we found an intermediate difference in the presence of AMF thinned forests, we found partial support for Hypothesis. However, EMF was significantly higher in thinned forests compared to old-growth, contradicting Hypothesis 1.

Hypothesis 3

The community composition of fungi in old-growth, second-growth, and thinned soils yielded a marginal difference, where the old-growth community showed a slight dissimilarity from second-

growth and thinned forests (P = 0.061, Fig 4A). The community composition of fungi in rhizonode and root structures showed no significant differences when comparing forest (P = 0.267) and sample type (P = 0.925; Fig. 4). However, when considering the interaction between forest and sample type together, the community composition resulted in a significant difference (P = 0.044; 4B). Taken together we found support for Hypothesis 3 which predicted the broader fungal community of soils and root structures would have a degree of variability across forest and sample types.

Indicator taxa

Soil Indicators A total of 15 AMF OTUs, defined to phylum or order, were significant indicators of old-growth soils (Table 1). In contrast, no AMF OTU's were significant indicators of second-growth or thinned forests. Second growth soils were significantly associated with two species of EMF (*Clavulina coralloides* and *Amphinema diadema*), while thinned forest had one EMF indicator in the order of Russulaceae (Table 1).

Rhizonode Indicators The old-growth rhizonodes consisted of seven AMF OTUs identified as Glomeraceae while one AMF indicator was defined as *Dominikia* (Table 2). Additionally, old-growth rhizonodes were indicated by two EMF species (*Entoloma* sp. 1 and *Tomentella sublilacina*). Second-growth rhizonodes only had one significant association with EMF, identified as *Pezoloma ericae*.

Root Indicators In contrast to the numerous AMF OTUs associated with old-growth soils and rhizonodes, roots of old-growth were indicated by five EMF OTUs (*Pseudotomentella* sp. 1, *Rhizopogon vinicolor*, *Destuntzia fusca*, *Pseudotomentella* sp. 2, *Pseudotomentella* sp. 3, and Atheliaceae) (Table 3). Second-growth roots were significantly associated with two EMF OTU's (*Cenococcum geophilum* and Entolomataceae) and no AMF OTUs. Considering the widespread presence of AMF in old-growth soils and rhizonodes, we found support for Hypothesis 3, which

predicted differences in the community composition of fungi would be influenced by mycorrhizal fungi.

Discussion

Our study aimed to determine what effects logging and restorative thinning had on mycorrhizal fungi associated with redwood forests. Initially, we posited that aerial seeding of pine trees (Sitka spruce and Douglas-fir) would lead to the proliferation of EMF over AMF in second-growth forests. We had this inference due to the primary mutualism of *Pinus* trees being EMF, whereas redwood forests have dual mutualism with AMF and EMF. Our results demonstrated the soils and rhizonodes of old-growth harbored more abundance and diversity of AMF compared to second-growth. While EMF of second-growth followed the expected trend in soils, roots, and rhizonodes, the differences were not significant. Aside from the higher richness of EMF in the soils of thinned forests compared to old-growth, our predictions for an intermediate effect of thinning to AMF and EMF were supported. In our study, both mycorrhizal types of thinned forests mirrored second-growth, instead of the old-growth forest. No widespread differences to community composition occurred for soil and root associated fungi. Collectively, our findings indicate that soils associated with second-growth and thinned redwood forests exhibit a reduced presence of AMF compared to old-growth. Furthermore, the presence of AMF in rhizonode tissue of second-growth forests was lower compared to old-growth, thus altering the dual mutualism associated with these ecosystems.

The decline of AMF in the soils and rhizonodes of second-growth redwood forest may be attributed to lack of C allocated to mycorrhizal associations. When redwood forests are densely populated by pine woodland, C allocation to the root system is limited by light availability (Soland et al., 2021). In other forested ecosystems using dual mutualism, younger trees invest more heavily in AMF, relying more on EMF as they mature (Chen et al., 2000; Egerton-Warburton & Allen,

2001; Gange et al., 2005). This may be in part to the lower C costs associated with AMF, which allows for a greater allocation of C for tree growth (Teste et al., 2020). The benefits of dual mutualism can be important for the uptake of nutrients, where dual mutualism allows a greater flexibility to attain nutrients from a broad spectrum of soil conditions (Teste et al., 2020). The loss of AMF in second-growth redwood forests can have implications for nutrient uptake and growth.

While the literature has shown that EMF are sensitive to logging in second-growth forests (Byrd et al., 2000; Danielson et al., 2020; Marín et al., 2017; Rodriguez-Ramos et al., 2021), our study falls in line with previous studies where EMF in old- and second-growth forests were similar (Chen et al., 2019; Hui et al., 2019; Karst et al., 2021; Rodriguez-Ramos et al., 2021). As demonstrated in a previous study, the planting of Douglas-fir in forested communities shifts the mycorrhizal community away from AMF and facilitates EMF (Gazol et al., 2016). Despite the similarity in the overall EMF community between old- and second-growth in our study, the roots of old-growth were indicated by several species of EMF (*Rhizopogon vinicolor*, *Destuntzia fusca*, and *Pseudotomentella*).

Our study corroborated the findings of the Northwest Forest Plan (NWFP), where *D. fusca* was identified as a rare fungal taxa associated with old-growth forests of the US Pacific Northwest (Molina, 2008). In old-growth stands of the Canadian Pacific Northwest, the removal of large trees led to the decline *R. vinicolor* (Beiler et al., 2010). While no study has identified *Pseudotomentella* in the Pacific Northwest, *Pseudotomentella* is associated with a rare endemic pine forests (*Pinus greggii*) in Mexico's Sierra Madre Oriental (Vald et al., 2020). Therefore, these EMF taxa are associated with intact forest communities. Despite the similarity of the overall EMF community between old- and second-growth redwood forests, the loss of specific EMF taxa can be an indication of habitat degradation.

The mycorrhizal indicators of old-growth redwood forests are associated with of old-growth forests throughout the world (Beiler et al., 2010; Burke et al., 2012; Byers et al., 2020; Goodman & Trofymow, 1998; Hagenbo et al., 2018; Mafune et al., 2023; Molina, 2008, 2008; Svantesson et al., 2021; Tedersoo et al., 2008), and can be used to develop a reference inoculum for redwood forests. Specifically, the old-growth redwood forests had a higher relative abundance of *Pseudotomentella*, *Rhizopogon vincolor*, *Destunzia fusca*, *Entoloma* sp. 1, *Tomentell sublilacna*, and *Dominikia* sp. compared to second-growth and thinned forests. These mycorrhizal species have been deployed as an inoculum in other forested ecosystems (Bencherif et al., 2023; Chartier et al., 2020; Shishikura et al., 2021; Ss et al., 2010; Trappe, 2009), while *Tomentella sublilacina* is less feasible (Peay et al., 2012). Using old-growth forests as a reservoir to develop inoculums has shown to be successful in restoring the below-ground conditions of second-growth (Cortese & Horton, 2023; Policelli et al., 2020). To address the loss of mycorrhizal fungi in second-growth and thinned forests, AMF and EMF taxa can be reintroduced from inoculums developed from old-growth forests.

Our study focused on the immediate effects of thinning along with the effect of logging in young second-growth forests cut within the past 60 years. To determine the length needed for second-growth to return to conditions resembling old-growth, sampling in different age stands in second-growth are necessary. Similarly, sampling in different age stands that had been previously thinned will determine what length of time is required for the mycorrhizal community to resemble that of old-growth. We focused on roots associated with the redwood overstory; however, native understory could be a major factor in diversity of mycorrhizal fungi. Lastly, examining the absolute abundance of mycorrhizal fungi in the rhizonode and root structure will enhance future studies.

Despite their cultural, economic, and ecological importance, there is lack of knowledge around the below-ground effects of logging to redwood forests. We sought to understand how the aerial

seeding of pine woodland (Douglas-fir and Sitka spruce) affected the dual mutualism deployed by redwood forests. The results of our study demonstrated that dual mutualism in the soils and rhizonodes of second-growth redwood forest harbored less AMF compared to old-growth. While the overall EMF community was similar across old- and second-growth, there are numerous EMF taxa highly associated with old-growth. In soils of thinned forests, there was no recovery of AMF following treatment efforts, whereas the relative abundance of EMF in thinned forests was higher compared to old-growth. In addition to the above ground effects of pine woodland (Pinaceae) to second-growth redwood forests, our study demonstrates that dual mutualism is also affected. Future restoration efforts should focus on mitigating the loss of AMF to curb the lower diversity and relative abundance of AMF associated with second-growth and thinned redwood forests.

Figures

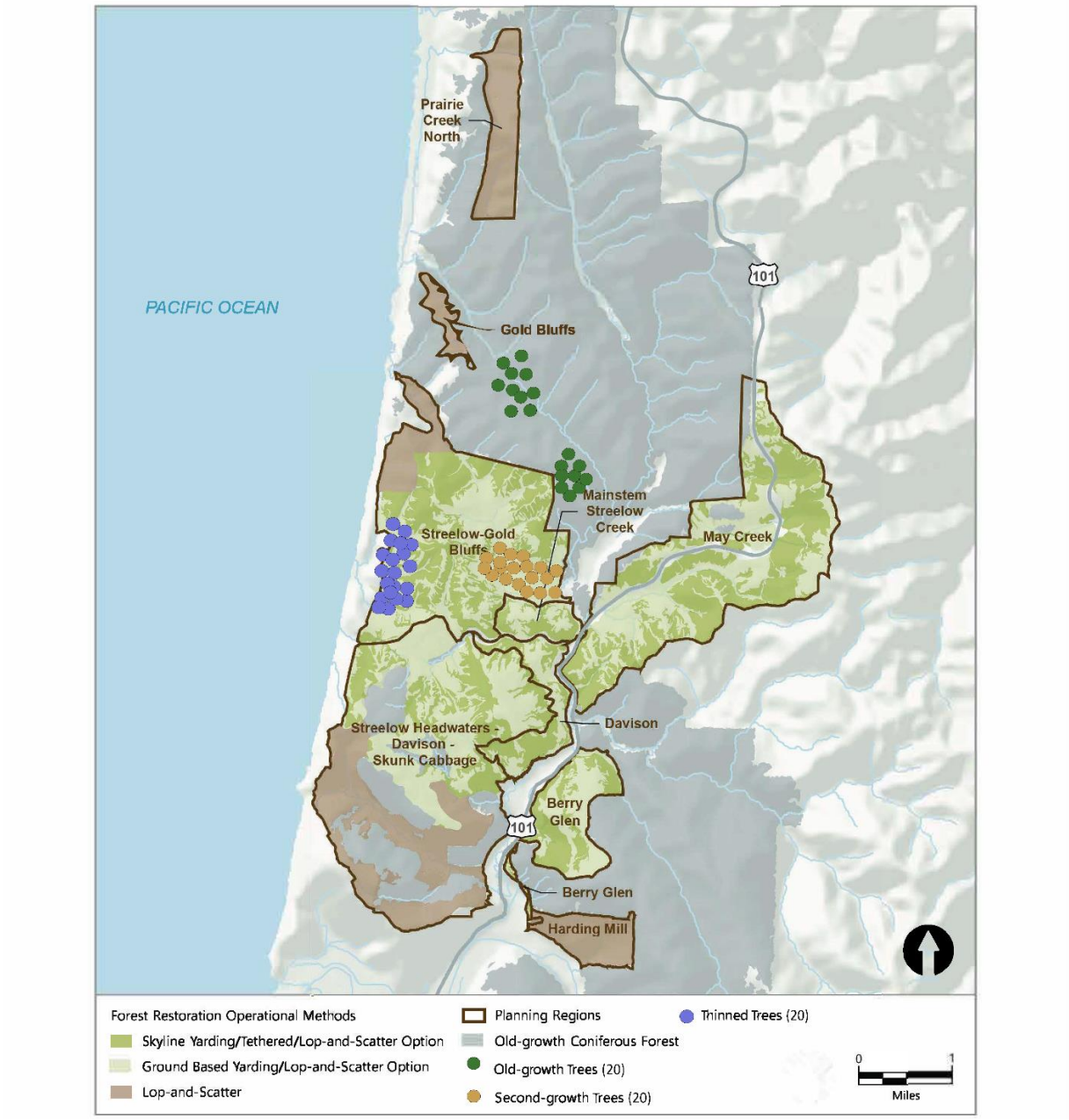

Figure 1. Description of Field Site. Trees randomly selected for our study are depicted with green (old-growth), beige (second-growth), and blue (thinned) circles. Broader restoration types of the Greater Prairie Creek Project area are shown in brown or different shades of light green. Management unit names are outlined in brown.

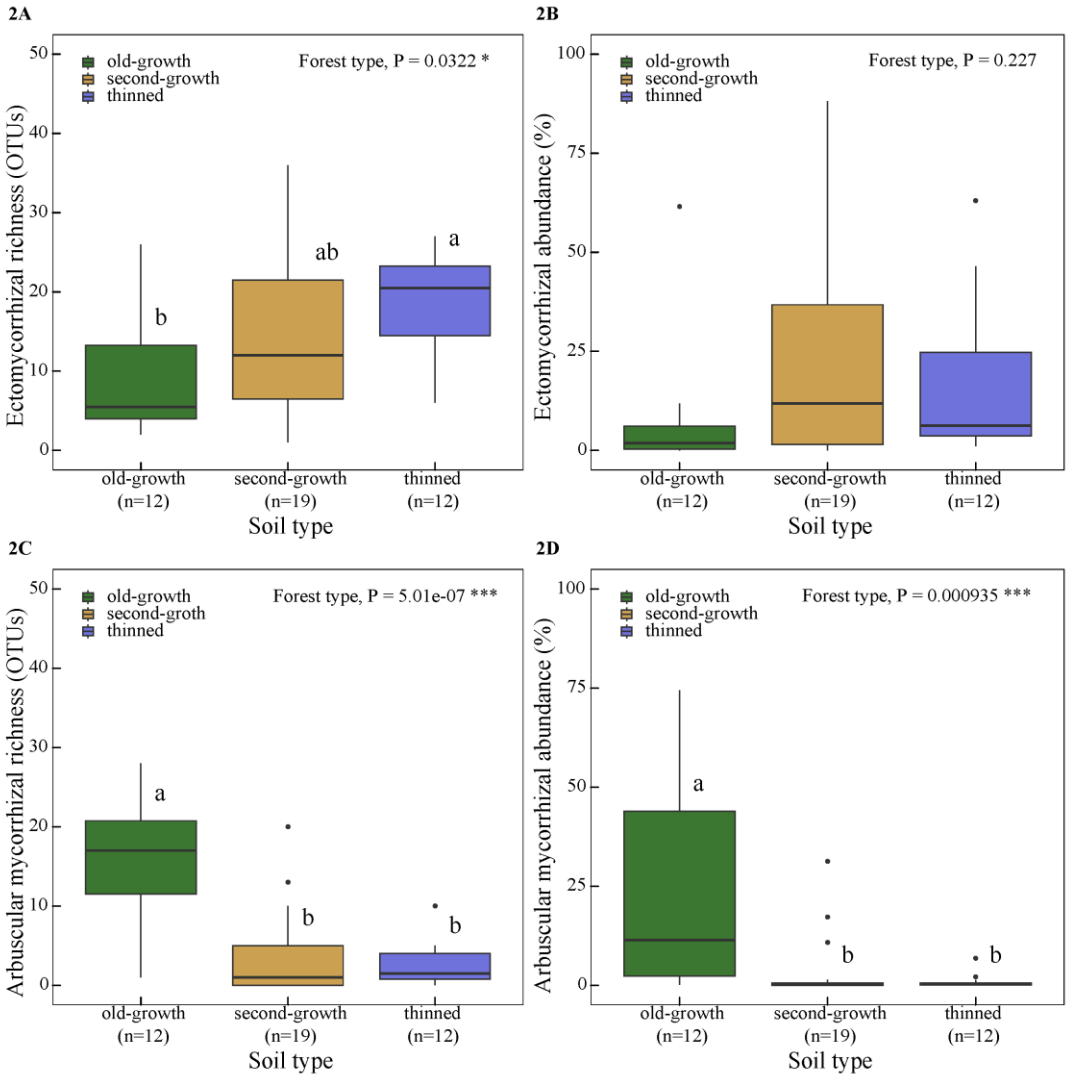

Figure 2. Richness and relative abundance of operational taxonomic units (OTUs) of soil associated mycorrhizal fungi, with ectomycorrhizal richness (A), ectomycorrhizal relative abundance (B), arbuscular mycorrhizal richness (C), and arbuscular mycorrhizal relative abundance (D). Box plots denote the interquartile range between the 25 and 75 percentiles (boxes), medians (black lines), and potential outliers (dots).

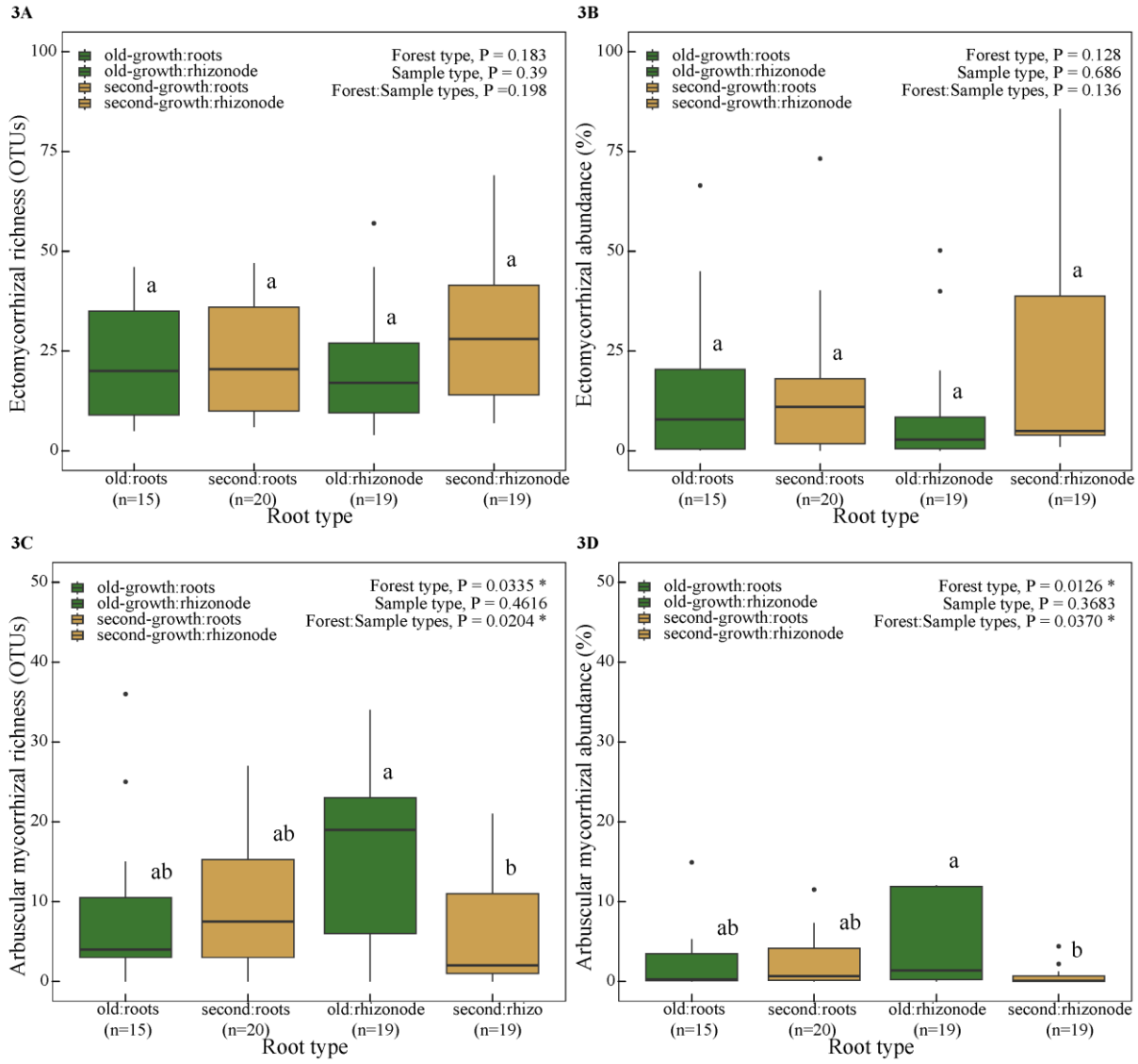

Figure 3. Richness and relative abundance of operational taxonomic units (OTUs) of plant associated mycorrhizal fungi, with ectomycorrhizal richness (A), ectomycorrhizal relative abundance (B), arbuscular mycorrhizal richness (C), and arbuscular mycorrhizal relative abundance (D). Box plots denote the interquartile range between the 25 and 75 percentiles (boxes), medians (black lines), and potential outliers (dots). Green = roots. Yellow = rhizonodes.

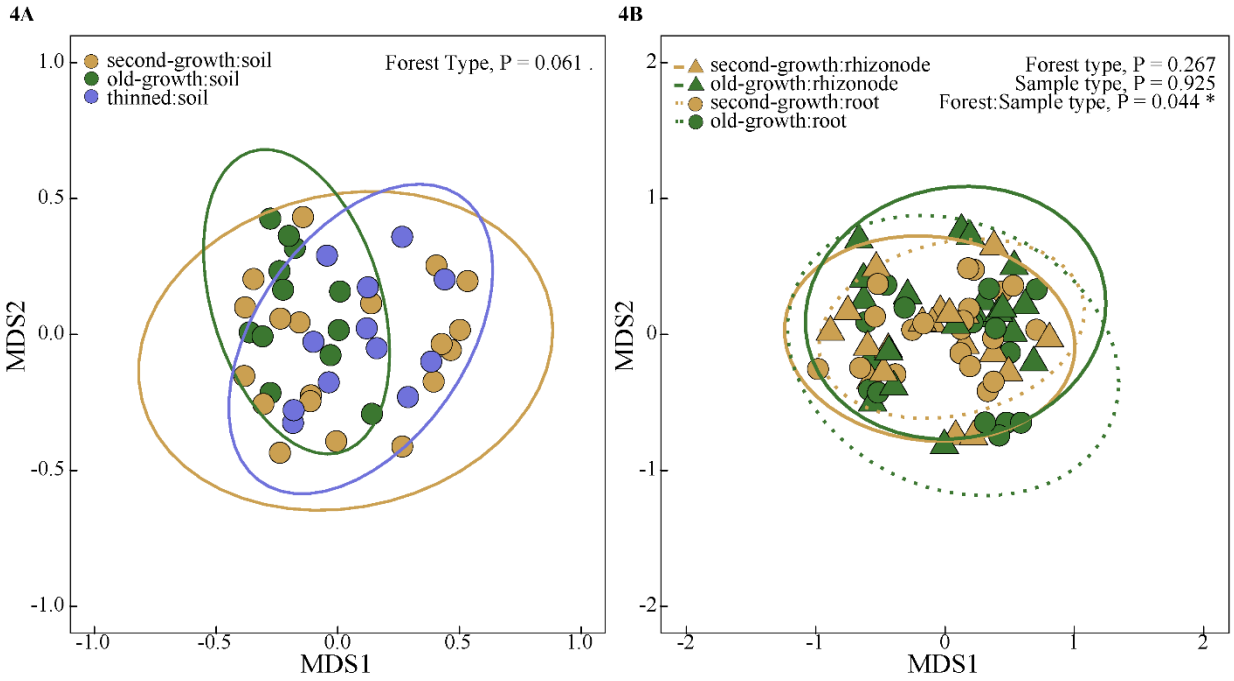

Figure 4. Non-metric multidimensional scaling (NMDS) plots of the community composition of soil fungi (A) and root associated fungi (B). Symbols = samples. Asterisks (*) = significant differences resulting from a PERMANOVA test. Solid or dashed lines = 97% confidence intervals. For soil, the forest type (squares) included old-growth (blue), second-growth (green), and thinned (blue). Root associated fungi was not included in the community composition assessment, where forest type included old-growth (green) and second-growth (yellow) with sample type consisting of the roots (circles) and rhizonodes (triangle).

Table 1. Indicator species for soil were grouped into four categories (second-growth, second-growth and thinned, old-growth and thinned). Asterisks (*) = significant differences resulting from the indicator analysis.

OTU	TAXON	ASSOCIATION	GUILD	P-VALUE	
OTU7	*Clavulina coralloides*	2nd-growth	Ectomycorrhizal	0.0495	*
OTU366	*Amphinema diadema*	2nd-growth	Ectomycorrhizal	0.0329	*
OTU23	*Solicoccozyma terricola*	2nd-growth & thinned	Undefined	0.0468	*
OTU162	Herpotrichiellaceae	old-growth	Animal Pathogen-Fungal Parasite-Undefined Saprotroph	0.0095	**
OTU715	Herpotrichiellaceae	old-growth	Animal Pathogen-Fungal Parasite-Undefined Saprotroph	0.0356	*
OTU244	Herpotrichiellaceae	old-growth	Animal Pathogen-Fungal Parasite-Undefined Saprotroph	0.0281	*
OTU133	Glomeromycota	old-growth	Arbuscular Mycorrhizal	0.0003	***
OTU159	Glomeromycota	old-growth	Arbuscular Mycorrhizal	0.0003	***
OTU130	Glomeromycota	old-growth	Arbuscular Mycorrhizal	0.0004	***
OTU2472	Glomeraceae	old-growth	Arbuscular Mycorrhizal	0.0083	**
OTU151	Glomeromycota	old-growth	Arbuscular Mycorrhizal	0.0003	***
OTU396	Glomeromycota	old-growth	Arbuscular Mycorrhizal	0.0017	**
OTU206	Glomeromycota	old-growth	Arbuscular Mycorrhizal	0.0016	**
OTU83	Glomeromycota	old-growth	Arbuscular Mycorrhizal	0.0003	***
OTU234	Glomeraceae	old-growth	Arbuscular Mycorrhizal	0.0016	**
OTU357	Glomeraceae	old-growth	Arbuscular Mycorrhizal	0.0043	**
OTU209	Glomeraceae	old-growth	Arbuscular Mycorrhizal	0.0406	*
OTU135	Glomeromycota	old-growth	Arbuscular Mycorrhizal	0.0001	***

OTU1057	Glomeraceae	old-growth	Arbuscular Mycorrhizal	0.0173	*
OTU349	Glomeraceae	old-growth	Arbuscular Mycorrhizal	0.0104	*
OTU4816	Glomeromycota	old-growth	Arbuscular Mycorrhizal	0.0083	**
OTU738	Tricholomataceae	old-growth	Ectomycorrhizal-Fungal Parasite	0.0363	*
OTU642	Myxotrichaceae	old-growth	Ectomycorrhizal-Undefined Saprotroph	0.0365	*
OTU111	*Pezicula*	old-growth	Endophyte-Plant Pathogen-Undefined Saprotroph	0.0069	**
OTU993	*Helotiales*	old-growth	Undefined	0.0135	*
OTU53	Botryosphaeriales	old-growth	Undefined	0.0031	**
OTU314	Leotiomycetes	old-growth	Undefined	0.0279	*
OTU155	Septobasidiaceae	old-growth	Undefined	0.0206	*
OTU157	*Helotiales*	old-growth	Undefined	0.0305	*
OTU501	Rhytismatales	old-growth	Undefined	0.043	*
OTU318	*Clavulinopsis*	old-growth	Undefined Saprotroph	0.0253	*
OTU2949	*Leohumicola*	old-growth	Undefined Saprotroph	0.0338	*
OTU285	*Clavulinopsis laeticolor*	old-growth	Undefined Saprotroph	0.0347	*
OTU148	*Metarhizium*	Thinned	Animal Pathogen	0.0041	**
OTU666	Herpotrichiellaceae	Thinned	Animal Pathogen-Fungal Parasite-Undefined Saprotroph	0.0061	**
OTU54	Russulaceae	Thinned	Ectomycorrhizal	0.0043	**
OTU195	Hyaloscyphaceae	Thinned	Plant Saprotroph-Wood Saprotroph	0.0451	*
OTU249	Ascomycota	Thinned	Undefined	0.0366	*
OTU976	Fungi	Thinned	Undefined	0.0372	*
OTU4521	Ascomycota	Thinned	Undefined	0.0277	*
OTU623	Sordariomycetes	Thinned	Undefined	0.0413	*
OTU8	Fungi	Thinned	Undefined	0.011	*
OTU391	Dothideomycetes	Thinned	Undefined	0.0374	*
OTU323	Hypocreales	Thinned	Undefined	0.0443	*
OTU974	Xylariales	Thinned	Undefined	0.0368	*
OTU119	Rozellomycota	Thinned	Undefined	0.046	*
OTU36	Fungi	Thinned	Undefined	0.0306	*
OTU105	Agaricomycetes	Thinned	Undefined	0.0205	*
OTU348	Leucoagaricus	Thinned	Undefined Saprotroph	0.0391	*
OTU704	*Scytalidium*	Thinned	Wood Saprotroph	0.0376	*

Table 2. Indicator species for rhizonodes were grouped into two categories (second-growth and old-growth and thinned). Guilds and OTU identification are provided for each taxon. Asterisks (*) = significant differences resulting from the indicator analysis.

OTU	TAXON	ASSOCATION	GUILD	P-VALUE	
OTU1504	*Pezoloma ericae*	2nd-growth	Bryophyte Parasite-Ectomycorrhizal-Ericoid Mycorrhizal	0.0369	*
OTU1991	*Penicillium*	2nd-growth	Dung Saprotroph-Undefined Saprotroph-Wood Saprotroph	0.0393	*
OTU429	*Leptodontidium*	2nd-growth	Endophyte	0.0353	*
OTU528	*Podosphaera xanthii*	2nd-growth	Plant Pathogen	0.0322	*
OTU1220	*Jaapia ochroleuca*	2nd-growth	Undefined	0.0018	**
OTU662	*Helotiales*	2nd-growth	Undefined	0.003	**
OTU1467	*Mucor zonatus*	2nd-growth	Undefined	0.0278	*
OTU811	Fungi	2nd-growth	Undefined	0.0247	*
OTU912	Helotiales	2nd-growth	Undefined	0.0304	*
OTU1239	Orbiliales	2nd-growth	Undefined	0.0269	*
OTU983	*Fusichalara*	2nd-growth	Undefined	0.0343	*
OTU1886	Fungi	2nd-growth	Undefined	0.0436	*
OTU967	Fungi	2nd-growth	Undefined	0.0391	*
OTU3417	Basidiomycota	2nd-growth	Undefined	0.0281	*
OTU1295	Mucorales	2nd-growth	Undefined	0.0286	*
OTU634	Ascomycota	2nd-growth	Undefined	0.0316	*
OTU117	Basidiomycota	2nd-growth	Undefined	0.0412	*
OTU5995	*Capnodiales*	2nd-growth	Undefined	0.0417	*
OTU901	*Umbelopsis ramanniana*	2nd-growth	Undefined Saprotroph	0.0077	**
OTU3161	*Ramicandelaber*	2nd-growth	Undefined Saprotroph	0.0392	*
OTU973	*Pholiota abieticola*	2nd-growth	Undefined Saprotroph	0.0323	*
OTU1129	*Geminibasidium*	2nd-growth	Undefined Saprotroph	0.0259	*
OTU864	*Neurospora pannonica*	2nd-growth	Undefined Saprotroph	0.0185	*
OTU1050	Orbiliaceae	2nd-growth	Wood Saprotroph	0.0148	*
OTU69	Herpotrichiellaceae	old-growth	Animal Pathogen-Fungal Parasite-Undefined Saprotroph	0.0448	*
OTU4935	Glomeraceae	old-growth	Arbuscular	0.001	***

			Mycorrhizal		
OTU1145	Glomeraceae	old-growth	Arbuscular Mycorrhizal	0.0065	**
OTU1094	*Dominikia*	old-growth	Arbuscular Mycorrhizal	0.0061	**
OTU495	Glomeraceae	old-growth	Arbuscular Mycorrhizal	0.0096	**
OTU5561	Glomeraceae	old-growth	Arbuscular Mycorrhizal	0.0405	*
OTU234	Glomeraceae	old-growth	Arbuscular Mycorrhizal	0.0161	*
OTU2463	Glomeraceae	old-growth	Arbuscular Mycorrhizal	0.0267	*
OTU4678	Glomeraceae	old-growth	Arbuscular Mycorrhizal	0.0456	*
OTU538	*Entoloma*	old-growth	Ectomycorrhizal	0.006	**
OTU209	*Tomentella sublilacina*	old-growth	Ectomycorrhizal	0.0019	**
OTU824	Mortierellaceae	old-growth	Endophyte-Litter Saprotroph-Soil Saprotroph-Undefined Saprotroph	0.0025	**
OTU450	Clavariaceae	old-growth	Endophyte-Litter Saprotroph-Soil Saprotroph-Undefined Saprotroph	0.0379	*
OTU1023	Zoopagomycota	old-growth	Undefined	0.0385	*
OTU1115	Fungi	old-growth	Undefined	0.0416	*
OTU613	Ascomycota	old-growth	Undefined	0.0459	*
OTU672	Cystobasidiomycetes	old-growth	Undefined	0.0429	*
OTU654	Fungi	old-growth	Undefined	0.0275	*

Table 3. Indicator species for roots were grouped into two categories (second-growth and old-growth and thinned). Guilds and OTU identification are provided for each taxon. Asterisks (*) = significant differences resulting from the indicator analysis.

OTU	TAXON	ASSOCIATION	GUILD	P-VALUE	
OTU94	*Cenococcum geophilum*	2nd-growth	Ectomycorrhizal	0.0487	*
OTU471	Entolomataceae	2nd-growth	Ectomycorrhizal-Fungal Parasite-Soil Saprotroph-Undefined Saprotroph	0.0061	**
OTU280	Helotiales	2nd-growth	Undefined	0.0453	*
OTU62	*Pseudotomentella*	old-growth	Ectomycorrhizal	0.0005	***
OTU3548	*Rhizopogon vinicolor*	old-growth	Ectomycorrhizal	0.0393	*
OTU124	*Destuntzia fusca*	old-growth	Ectomycorrhizal	0.0315	*
OTU129	*Pseudotomentella*	old-growth	Ectomycorrhizal	0.0309	*
OTU129	*Pseudotomentella*	old-growth	Ectomycorrhizal	0.0309	*
OTU1714	Atheliaceae	old-growth	Ectomycorrhizal-Lichen Parasite-Lichenized-Plant Pathogen	0.0387	*
OTU251	*Mycena*	old-growth	Leaf Saprotroph-Plant Pathogen-Undefined Saprotroph-Wood Saprotroph	0.0127	*
OTU1928	*Mycena*	old-growth	Leaf Saprotroph-Plant Pathogen-Undefined Saprotroph-Wood Saprotroph	0.0387	*
OTU2073	*Mycena cinerella*	old-growth	Leaf Saprotroph-Plant Pathogen-Undefined Saprotroph-Wood Saprotroph	0.0387	*
OTU1506	*Coniochaeta ligniaria*	old-growth	Lichen Parasite	0.0393	*
OTU4387	Clavariaceae	old-growth	Lichenized-Undefined Saprotroph	0.0378	*
OTU2113	Endogonales	old-growth	Undefined	0.043	*
OTU2135	Chytridiomycota	old-growth	Undefined	0.0372	*
OTU1577	Ascomycota	old-growth	Undefined	0.0479	*
OTU1127	Russulales	old-growth	Undefined	0.0402	*

OTU853	Agaricomycetes	old-growth	Undefined	0.0385	*
OTU1479	Agaricomycetes	old-growth	Undefined	0.0402	*
OTU1965	Basidiomycota	old-growth	Undefined	0.0418	*
OTU5287	Clavulinaceae	old-growth	Undefined	0.0402	*
OTU677	Ascomycota	old-growth	Undefined	0.0393	*
OTU789	Rozellomycota	old-growth	Undefined	0.0385	*
OTU97	Helotiales	old-growth	Undefined	0.0298	*
OTU4281	Fungi	old-growth	Undefined	0.011	*
OTU3289	Leotiomycetes	old-growth	Undefined	0.0418	*
OTU258	*Leptobacillium leptobactrum*	old-growth	Undefined	0.0321	*
OTU175	Auriculariales	old-growth	Undefined	0.0474	*
OTU258	*Leptobacillium leptobactrum*	old-growth	Undefined	0.0321	*
OTU175	Auriculariales	old-growth	Undefined	0.0474	*
OTU1414	*Helicogloea eburnea*	old-growth	Undefined Saprotroph	0.0428	*
OTU2349	*Oliveonia*	old-growth	Undefined Saprotroph	0.0385	*
OTU252	*Sistotremastrum*	old-growth	Undefined Saprotroph	0.0432	*
OTU512	*Skvortzovia furfuracea*	old-growth	Undefined Saprotroph	0.031	*
OTU1186	*Humidicutis calyptriformis*	old-growth	Undefined Saprotroph-Undefined Symbiotroph	0.0153	*

CHAPTER 3

Title: End of the road: Exploring the effects of road removal on fungal guilds associated with redwood (*Sequoia sempervirens*) forests

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Abstract

For decades road removal has been a critical part of restoration efforts in the Redwood National and State Parks. However, there is limited knowledge around the effect of road removal on fungal communities. The aim of this study was to investigate the response of fungal guilds (mycorrhizal, pathogenic, and saprotrophic fungi) to road removals in 2005 and 2013. We expected the time since removal and location (roadside or road-center) would shape the fungal community. Specifically, we hypothesized mycorrhizal and saprotrophic fungi would be less prevalent in the younger removal site, whereas pathogens would be more prevalent. Contrary to our predictions, the relative abundance of ectomycorrhizal fungi was highest in the 2013 road-center. However, only the 2005 roadside and old-growth forests were indicated by specific taxa associated with arbuscular mycorrhizal fungi. Due to disturbances associated with road removal, we expected pathogenic fungi to be most prevalent in the 2013 removal site. Contradicting our predictions, the diversity of pathogens in the 2013 roadside was significantly higher compared to the road-center. In accordance with previous studies, saprotrophic fungi in our study were least diverse and abundant in the 2013 road-center compared to the roadside. The community composition of the broader fungi was significantly different between the old-growth and the road removal sites. The results of our study provide a baseline for assessing future efforts to restore redwood forests following road removal.

Introduction

To facilitate resource extraction on natural landscapes, 885,000 kilometers of roads were established across the US (Switalski et al., 2004). When roads are no longer viable or abandoned, road removal is necessary to rehabilitate the habitat (Havlick, 2002). While the effect of road removal on abiotic soil proprieties are well defined (Grant et al., 2011; Haan et al., 2012; Larson & Rew, 2022; Robinson et al., 2010; Spooner & Smallbone, 2009; Yates et al., 2000), alterations to soil biota are less understood. Fungal communities are especially vulnerable to road removal, where functional guilds shift in response to restoration efforts (Eaton et al., 2021; McGee et al., 2019). The functional guilds include mycorrhizal, saprotrophic, and pathogenic fungi, where each guild has a particular function in an ecosystem (Albornoz et al., 2022). Despite the widespread efforts to remove roads across the globe (Coutinho et al., 2019; Fernandes et al., 2018; Zhu et al., 2018), there are limited studies on the response of fungal guilds to road removal. Since fungal guilds are tied to ecosystem function, the response of fungal guilds to road removal will indicate how effective these efforts are at restoring ecosystem function.

For decades, road removal has been an integral part of ecosystem restoration in redwood (*Sequoia sempervirens*) forests (Hagans et al., 1986; Madej, 2001, 2010; Maurin & Stubblefield, 2011). A limited body of knowledge examining the relationship between fungal guilds and roads sheds light on the potential consequences of road removal. First, road construction and abandonment can decrease the abundance and diversity of mycorrhizal fungi (Archuleta & Baxter, 2008; Coutinho et al., 2019; White et al., 2008; Wu et al., 2002). The loss of mycorrhizal fungi can affect the services provided to the plant hosts, including nutrient exchange and pathogen protection (Bermúdez-Contreras et al., 2022; House & Bever, 2018; Xiang et al., 2015). Saprotrophic fungi involved in decomposition are slow to recover following road removal (Eaton et al., 2021; McGee et

al., 2019). If saprotrophic fungi are limited, the lack of nutrient cycling can impair the recovery of vegetation (Seney & Madej, 2015). Additionally, pathogenic fungi can induce deleterious effects, impairing seedling establishment following road removal (Eaton et al., 2021). While the response of fungal guilds has been speculated in redwood forests (Seney & Madej, 2015), no prior study has examined this important interaction directly.

While old-growth redwood forests have been fragmented by road construction (Dangerfield et al., 2021), they have not experienced disturbances from road removal. They provide an important reference ecosystem for the restoration of mycorrhizal fungi in second-growth forests, exposed to road construction and removal. Primarily, redwoods in old-growth stands form mutualisms with arbuscular (AMF) and ectomycorrhizal (EMF) fungi (Willing et al., 2021). Additionally, ericoid mycorrhizal (ERM) fungi are also present, being used by understory plants in the Ericaceae (Willing et al., 2021). In other forested ecosystems, the construction or abandonment of roads can shift the community of AMF away from that of intact forests (Albornoz et al., 2023; McGee et al., 2019). Additionally, the introduction of invasive plants can harbor AMF taxa that inhibit nutrient uptake from natives (Weber et al., 2019). However, in native grasslands, the removal of invasive plants species can return the community of AMF to conditions indicative of the reference grassland (Coutinho et al., 2019; Gibson-Roy et al., 2014; Gibson-Roy & Carland, 2022; White et al., 2008). In addition, EMF introduced from disturbance can become parasitic to native plant species (Dickie et al., 2016, 2017). Currently, few studies have examined road removal on forested ecosystems involving EMF and ERM. Therefore, comparisons of mycorrhizal fungi between old-growth and second-growth redwood forests with road removal are necessary. These comparisons will identify which mycorrhizal taxa are lacking and which mycorrhizal taxa are not indicative of the old-growth forests.

Historically, redwood forests were thought to be resistant to pathogenic fungi with very few occurrences reported (Roy, 1966). Recent investigations demonstrated redwood die-back in urban forests and plantations were attributed to pathogenic fungi in the Botryosphaeriaceae family (Aćimović et al., 2018; Lee et al., 2022). Moreover, sudden oak death targets tanoak (*Notholithocarpus densiflorus*) populations, so the loss of this understory species in redwood forests can have wide ranging effects on forest structure and composition (Maloney et al., 2005). Therefore, early detection of fungal pathogens in redwood forests can inform land management strategies and mitigation efforts (Cannon et al., 2016; Munck & Bonello, 2018). Since pathogenic fungi disproportionately infect stressed trees (Lawrence et al., 2017; Slippers & Wingfield, 2007), roadside construction and removal in redwood forests may contribute to the establishment of pathogenic fungi.

In redwood forests, road scars, where roads have been removed, are associated with lower soil organic carbon (SOC) (Seney & Madej, 2015). Seney and Madej suggest the redwood forests associated with road scars lack a fungal community capable of decomposing SOC (2015). Saprotrophic fungi are critical in the development of SOC in old-growth redwood forests, since they decompose fallen trunks and branches (Sillett & Pelt, 2007). In dawn redwood forests (*Metasequoia glyptostroboides*) which are closely related to redwoods, younger dawn redwood stands have less enzymatic capacity to decompose SOC compared to older stands (Chang & Chiu, 2015). Different age stands of redwoods can yield different saprotrophic communities based on their enzymatic capacities. In other forested ecosystems, roadside versus the road-center (middle) yielded different capacities to decompose SOC with the roadsides having a greater capacity to degrade lignin (Eaton et al., 2021). Road scars associated with redwood forests offer a unique opportunity to examine the response of saprotrophic fungi to road removal in young second-growth stands.

An estimated 40% of the second-growth redwood range is fragmented by abandoned logging roads (Burns et al., 2018). Old- and second-growth redwood forests within 30 meters of highways experience greater incidents of drought stress and canopy dieback (Dangerfield et al., 2021). While major highways are permanent fixtures of redwood forests, abandoned logging roads can be removed to minimize edge effects. Much of the available knowledge around road removal comes from the Redwood National and State Parks (RNSP), where 1,046 kilometers of roads were established (Hagans et al., 1986; Madej, 2001; Maurin & Stubblefield, 2011). Three forms of road removal are used in RNSP and include: road ripping, stream crossing restoration, and full recontour (Switalski et al., 2004). RNSP introduced the concept of full recontour in 1997 (Madej, 2001). A full recountour involves returning the road scar to a natural slope indicative of pre-disturbance to prevent landslides and restore a natural stream crossing (Madej, 2001; Switalski et al., 2004). The Greater Mill Creek Project Area (GMCPA) is a long term restoration project to remove 482 kilometers of logging roads using full recountour (RR, 2019). Road removal in GMCPA began in 2004, with incremental removals occurring annually (Fig. 1). Within RNSP, the role of the fungal guilds has long been speculated, however, has not been studied directly (Seney & Madej, 2015). This project allowed us to examine the response of fungal guilds after road removal at two timepoints.

To investigate the trajectory of fungal guilds (mycorrhizal, saprotrophic, and pathogenic) following road removal, we partnered with the GMPCA. Additionally, we wanted to investigate whether the roadside or road-center yielded a different response from the fungal community. In 2020, we examined soils from two road scars: one in which removal was completed in 2005, and another in 2013. We assessed the roadsides of both scars. Additionally, we sampled the road-center

in the 2013 scar. Soils from the nearest old-growth redwood forest were used as a "control" or reference forest.

We tested three hypotheses, each focused on the prevalence of (1) fungal guilds, and (2) fungal taxa. To quantify prevalence, we assessed richness and relative abundance. First, we hypothesized that roads and road removals would alter the prevalence of fungal guilds and taxa owing to the sensitivity of each to disturbance (Hypothesis 1; Eaton et al., 2021; McGee et al., 2019). Accordingly, we predicted that, compared to old-growth forest, the road scars (2005 roadside, 2013 roadside, and 2013 road-center) would harbor different community compositions of taxa, lower prevalence of mycorrhizal fungi and saprotrophic fungi, and higher prevalence of pathogenic fungi. Second, we hypothesized that fungal community composition in the road scars would approach that of the old-growth forest over time (Hypothesis 2), because the aboveground recovery of second-growth forest can require several decades (Burns et al., 2018; Iberle et al., 2020). Specifically, we predicted that the prevalence of each fungal guild—and the community composition of fungal taxa—in the 2005 roadside will be closer to that of the old-growth forest than the 2013 roadside. Third, we hypothesized that fungal community would be more disturbed in the road-center than the roadsides, because of the higher degree of disturbance within the road-center (Hypothesis 3). For that reason, we predicted that the prevalence of fungal guilds and the community composition of fungal taxa in the 2013 roadside will be closer to that of the old-growth forest than the 2013 road-center.

Methods

Site Description

The old-growth locations of study occurred in the Jedidiah Redwoods State Park (Jedidiah Smith RSP; 41°45'57.5"N 124°06'34.4"W), with the road removal sites occurring in second-growth forests

of the Del Norte Coast Redwoods State Park (Del Norte Coast RSP; 41°44'21.5"N 124°05'09.2"W). The road removal activities occurring in the Del Norte Coast RSP are referred to as the GMCPA. Jedidiah Smith RSP and the GMCPA are located on the traditional territory of the Tolowa (Tushingham, 2013). The region experiences a Mediterranean climate, with the annual temperatures ranging from 8 to 19 °C (RR, 2019). Between 1988 and 2017, the annual precipitation ranged from 117 to 363 centimeters, occurring between October and April (RR, 2019). In summer months, coastal fog supplements the seasonal lack of rainfall (Keyes & Teraoka, 2014). The coastal fog belt in this region extends 13 kilometers inland (RR, 2019). In the Jedidiah Smith RSP and GMCPA, the most common soil types consists of the Bigtree-Mystery complex and the Coppercreek-Tectah Slidecreek complex occurring on 2% to 9% slopes (NCRS, 2023). Previous studies in the region have demonstrated the soils associated with road removal have low nutrient availability (Madej, 2010). Moreover, the roadsides are inundated with invasive plant species not indicative or old-growth forests (RR, 2019). At the time of collection there was a large presence of non-native grasses and berries.

Restoration Approach

The timber harvesting of the GMPCA began in 1908 with logging centered around the rail road networks (RR, 2019). Following timber harvesting, much of the old-growth stands were slashed and burned to make way for livestock grazing. Several strategies were deployed to remove redwood stumps that could resprout and inhibit grazing. By the 1960's, logging accelerated with clearcutting, requiring an extensive road network to accommodate larger vehicles (Arvola, 1976). Throughout the 1970's Douglas-fir was aerially seeded with second-growth forests to support future timber harvests (RR, 2019).

In 2000, lands associated with the GMPCA were sold from private timber companies to establish the Del Norte Coast RSP. Shortly thereafter, 325 miles of logging roads were targeted for removal with the first road removed in 2004 (CSP, 2010). While road removal in the GMPCA involved several methods of road removal full contour is most common. This method involves the return to topography indicative of pre-disturbance (Switalski et al., 2004). Since the inception of the road removal, locally sources redwood trees were outplanted to support restoration (CSP, 2010). In specific regions of the GMCPA, *Phytophthora* spp. has caused widespread outbreaks of sudden oak death. Therefore, many regions of the park follow a specific management plan to prevent the spread (RR, 2019). Nursery stock resistant to *Phytophthora* spp. is also necessary for seedling survival on former roads (RR, 2019).

Sampling Design

This sampling for this research coincided with the end of the active treatment season, occurring between October 26th and 30th, 2020. Since the majority of the GMCPA is closed to the public, our collection sites were near trails and roads that could be easily accessed. We selected the 2005 and 2013 removal sites due to their proximity to each other and old-growth forests (Fig. 1). All sampling on road removal sites occurred on the former roadside except for the 2013 removal, where we collected additional samples from the road-center. Due to limited access, we could not collect samples from the road-center at the 2005 removal site. To establish unbiased sampling locations, we used the random point generator in ArcGIS Pro (2.1.0). To assist the random point generator, we converted the former roads into GIS shapefiles from Google Earth Pro (Google, 2015).

We established 18 random points for each forest type (old-growth, 2005 roadside, 2013 roadside, and 2013 road middle) approximately 40-60 meters apart from each other (18 soil samples × 4 forest types = 72 total samples; Fig. 1). At each randomly generated point, our parameters selected a point

that was at least five meters from the former roadside. For all former roadsides, nine samples were collected on the eastern edge and nine on the western edge. Additionally, for the 2013 road-center, the sampling occurred in the road-center estimated from the GIS shapefile. In contrast, samples in the old-growth forests occurred between five and 10 meters from the Mill Creek Trail and were 40-60 meters apart (Fig. 1).

Sample Collection

In the field, we selected the closest redwood tree from a randomly generated point and collected one soil sample approximately 1.5 meters from the tree trunk. Upon arriving at the randomly generated point, we collected a soil core 5.5 centimeters in diameter (Lakago Homier 1 m Soil Sampler Probe). Approximately 950 centimeters ^{2} of soil was recovered from each sample to a depth of 10 centimeters. All soil equipment was sterilized with 70% alcohol to prevent the spread of pathogens between sites. Soil cores were collected in sterile freezer bags and kept on ice while conducting field work. The soil samples were then stored at -20 °C at the end of each day. Following the completion of field work, the samples were transported in dry ice to the University of California, Irvine (UCI). Prior to downstream processing, the soils samples were stored at -80 °C until downstream amplification.

DNA Sequencing

The extraction of DNA started with each soil sample being transferred to -20°C from -80 °C. Within one week, the soil samples were passed through a 10 mm sieve. Immediately after the sieving, 100 milligrams of soil were used for DNA extraction using the Quick-DNA Fecal/Soil 96 Kit (Zymo, Tusin, CA, USA). All soil samples were extracted using the manufacturer's instructions on the same day and then stored at -20 °C until downstream amplification. To target the fungal DNA in soil we conducted a one-step PCR targeting the ITS2 region. We amplified the DNA using

the 5.8SFun (5′ AACTTTYRRCAAYGGATCWCT) and ITS4Fun (AGCCTCCGCTTATTGATATGCTTAART-3′) primers (Taylor et al., 2016). To increase the sequencing quality, heterogeneity spacers were included with linkers pads, and dual indexed barcodes at 12 base pairs (BP; Lundberg et al., 2013; Willing et al., 2021).

Prior to the PCR amplification all samples were diluted by 1/100 to avoid any PCR inhibitors (Bessetti, 2007). Each PCR reaction used 2 µL of template DNA, 2 µL of each primer diluted to 10 µM, 12 µL of GoTaq Green Master Mix (Promega). The remaining reaction consisted of sterile water to reach a volume of 24 µL. Thermocycler parameters started with denaturing at 95°C for 2 min, proceeded by 35 cycles of 30 s at 95 °C, 30 s at 55 °C, 30 s 72 °C, with a final extension for 10 min at 72 °C. To confirm successful amplification, a 1.5% agarose was used to confirm the presence of PCR products between 267 and 511 BP. Additional PCR attempts were used on samples that did not initially amplify on the first attempt. The final amplicon library was pooled based on gel intensity using (1 µL, 2 µL, or 3 µL). The library pool was cleaned with Ampure XP beads (Beckman Coulter, Brea, CA, USA) prior to sequencing at the Genomics Research and Technology Hub (GRT Hub) at UCI (https://ghtf.biochem.uci.edu/; Irvine, CA). Sequencing was carried using the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) with 2 x 300 BP paired-end (PE) reads.

Bioinformatics

The demultiplexed sequences were pre-processed using AMPtk software v1.5.4 (Palmer et al., 2018). After concatenating all paired-end reads, the demultiplexed files were clustered into operational taxonomic units (OTUs or “taxa”) with a 97% similarity cutoff. This level of similarity allows for taxonomic resolution at approximately the species level (Kõljalg et al., 2013). The sequences were filtered using default parameters, and taxonomy was assigned using the UNITE

database (Kõljalg et al., 2005). To ensure that only fungal DNA remained for downstream analysis, all non-fungal DNA was removed. When available, trophic modes were assigned to the OTUs using the FUNGuild database provided by AMPtk (Nguyen et al., 2016). To calculate the relative abundances of arbuscular mycorrhizal fungi (AMF) and ectomycorrhizal fungi (EMF), the OTU counts for each mycorrhizal type were summed and divided by the total OTU count for each sample separately. The result was multiplied by 100 to express the relative abundance as a percentage.

Statistical Analysis

Each prediction was tested in two ways. First, we examined the richness and relative abundance of each fungal guild. Second, we assessed the taxonomic composition of the fungal community as a whole. We used R Studio 4.1.2 to conduct all statistical tests (RStudio Team, 2021). Effects were considered significant when $P < 0.05$ and marginally significant when $P < 0.10$.

Fungal guilds. We used a series of analyses of variance (ANOVAs) from the "vegan" package of R to examine the prevalence of each fungal guild (RStudio Team, 2021). The dependent variables were richness or relative abundance of the fungal guild. For statistical analysis we only considered fungal OTUs assigned as mycorrhizal (symbiotic), saprotrophic, or pathogenic as some OTUs have multiple guilds. The independent variable was soil type (old growth, 2005 roadside, 2013 roadside, and 2013 road-center). We then performed a Tukey posthoc test to make pairwise comparisons among the sites.

To explore which fungi contributed to any changes in the relative abundance of fungal guilds, we used the "stat" package to conduct a series of ANOVA tests on each genus within each guilds (RStudio Team, 2021). The dependent variable was relative abundance of each genus, and the independent variable was soil type.

Fungal taxa. For the composition of fungal taxa, we included all fungal OTUs whether assigned to a guild or not. We used the "vegan package" to perform a PERMANOVA test (RStudio Team, 2021). The relative abundance of each fungal taxon was used for the dependent variables, and soil type was the independent variable.

Results

Metagenomic Data

Paired-end sequences yielded 11,463,457 total reads, with 5,550,259 reads meeting minimum length for validation. After filtering a total of 5,482,185 reads were assigned taxonomy, producing 6,287 OTUs. The FUNGuild database identified 966 saprotrophs, 290 pathotroph-saprotroph-symbiotrophs, 741 symbiotrophs, 285 saprotrophs-symbiotrophs, 176 pathotrophs, and 98 pathotroph-symbiotrophs. Following the assignment of trophic mode, soil samples were rarefied to 19,000 sequences per sample. After rarefying, OTUs were assigned to seventeen phyla. The most abundant phylum in soil samples was Ascomycota, which accounted for 47.9% of taxa. Additionally, 41.8% of fungal taxa derived from Basidiomycota. Glomeromycota (AMF) represented less than 1% of the fungal taxa in soils and roots, likely because the primers have a known bias against AMF (Lekberg et al., 2018). The most common OTU in the soil samples was *Inocybe mixtilis* accounted for 4.9% of the OTUs. *Hymenogaster parksii* was also common and was present in 3.4% of the sequences.

Fungal Guilds

For AMF, richness did not differ significantly among soil types ($P = 0.498$; Fig. 2A). Their relative abundance tended to peak in the old forest, but only varied marginally significantly among soil types ($P = 0.087$; Fig. 2B). Furthermore, there were no significant pairwise differences. As a

result, we reject our hypotheses for this guild. Of the 12 AMF genera identified, only *Glomus* varied significantly among soil types ($P < 0.05$; Fig. 5; Table 1).

For EMF, there were no significant differences in richness across soil types ($P = 0.41$; Fig. 2C). In contrast, relative abundance was significantly different among soil types ($P = 0.001$; Fig. 2D). Specifically, the relative abundance of EMF was higher in the 2013 road-center than the old growth forest ($P = 0.0005$). This pairwise difference was opposite that of our predictions for Hypothesis 1. The other pairwise comparisons we predicted for Hypotheses 2 and 3 were non-significant. Thus, we reject our hypotheses for EMF. A total of 51 genera were classified as EMF, with eight genera differing significantly among soil types ($P < 0.05$; Fig. 5; Table 1).

For ERM fungi, soil types differed in richness ($P = 0.0201$; Fig. 2E), with old-growth ($P = 0.04$) and the 2013 roadside ($P = 0.03$) harboring significantly higher richness than the 2013 road-center. Nevertheless, none of the pairwise comparisons differed as predicted. Furthermore, ERM relative abundance varied among soil types ($P = 0.045$; Fig. 2F). Specifically, the 2005 roadside contained significantly higher ERM relative abundance than the 2013 road-center ($P = 0.04$), which did not support any of our hypotheses. Since no other pairwise comparison was significant, we rejected our hypotheses for ERM as well. Three genera were associated with ERM, of which *Byssoascus* varied significantly across soil types ($P < 0.05$; Fig. 6; Table 1).

For pathogenic fungi, richness varied significantly among sites ($P = 0.018$; Fig. 3A). The old growth forest had significantly lower richness than the 2013 roadside ($P = 0.02$), so this particular pairwise comparison supported Hypothesis 1. However, the other soil types did not differ significantly from that of the old growth forest, so Hypothesis 1 was only weakly supported. In addition, while we had predicted that the 2013 roadside would be closer to the old growth forest than the 2013 road-center, the opposite pattern occurred. Specifically, richness in the 2013 roadside

was significantly higher than the 2013 road-center, even though it was low in the old growth forest. In terms of relative abundance, pathogenic fungi did not differ significantly among soil types ($P = 0.725$; Fig. 3B). Thus, for pathogenic fungi, Hypothesis 1 was weakly supported at best. Hypotheses 2 and 3 were rejected. Four of the 33 pathogenic genera we identified differed significantly across soil types ($P < 0.05$; Fig. 7; Table 1).

For saprotrophic fungi, we found a significant difference in richness among soil types ($P = 0.0472$; Fig. 3C). Although the 2013 roadside yielded significantly higher richness than the 2013 road-center ($P = 0.04$), the richness of the old growth forest was closer to that of the road-center. This is the opposite of our prediction for Hypothesis 3. No other pairwise comparison was significant. Regarding relative abundance, differences among soil types were likewise significant ($P = 0.023$; Fig. 3D). As with richness, relative abundance was significantly higher in the 2013 roadside than the 2013 road-center. Nonetheless, the richness in the old growth forest was intermediate between the two, which did not support Hypothesis 3. We also rejected Hypotheses 1 and 2. We identified 303 saprotrophic genera, of which 24 genera were significantly different among soil types ($P < 0.05$; Fig. 8; Table 1).

Fungal Taxa

The community composition of fungal taxa varied significantly across soil types ($P = 0.001$; Fig. 4). Following pairwise comparisons, the community composition of fungi associated with old-growth forests was significantly different from the 2005 roadside ($P = 0.017$), 2013 roadside ($P = 0.001$), and the 2013 road-center ($P = 0.001$). The community composition of fungi associated with the 2013 road-center was significantly different from the 2005 roadside ($P = 0.04$) and the 2013 road-center ($P = 0.001$). Similarly, the community composition of the fungi in the 2005 roadside differed significantly from the 2013 roadside ($P = 0.009$). Since the community composition of the

broader fungal community still varied between the 2005 roadside and old-growth we rejected our hypotheses.

Discussion

The goal of this study was determining how road removal in redwood forests affected the fungal guilds. We predicted that the time since removal would play a role in the response of fungal guilds. Moreover, we predicted the location of fungal community on the roadside or road-center would also have a significant influence. Initially, we expected the road scars would harbor the lowest presence of mycorrhizal fungi compared to old-growth. Surprisingly, the relative abundance of EMF was highest in the 2013 road-center contradicting Hypothesis 1. We predicted pathogenic fungi would exhibit the lowest presence of pathogenic fungi in old-growth forests. In partial support of Hypothesis 1, the diversity of pathogenic fungi was highest in the 2013 roadside. Contrary to our expectations for Hypothesis 1, the presence of saprotrophic fungi in old-growth was similar to the road scars. Moreover, the community composition of the broader fungal community varied significantly, contradicting our hypothesis where we expected the 2005 roadside would mirror old-growth. Taken together the results of our study demonstrate each fungal guild has disparate responses to road removal, depending more on the location (roadside vs. road-center) than the time since removal.

The results of our study showed that mycorrhizal fungi were not affected by road scars, whereas a previous study found EMF in low abundances 33 years following the abatement of logging roads (McGee et al., 2019). This previous study pointed to the removal of topsoil from road construction for the continued decline of EMF (McGee et al., 2019). Furthermore, a previous study found AMF taxa to be more prevalent following roadside restoration in *Eucalypt* forests (Albornoz et al., 2023). In our study, we found that the overall AMF community was similar across road scars and old-

growth forests. The only disparity occurred for ERM, where our study found the 2005 roadside had a higher presence compared to the 2013 roadside. One previous study in heathlands, demonstrated ERM infection near abandon roadsides was lower than the undisturbed ecosystem (van der Bij et al., 2018). One distinction between our study and all previous studies is the degree of restoration. Where the road scars in our study were mitigated with full recountour, all previous studies examining roads occurred without intervention. Therefore, after several years, the results of our study show that mycorrhizal fungi respond positively to treatments, where EMF was more abundant in the 2013 road-center.

The management of fungal pathogens in redwood forests is a necessary task (Cannon et al., 2016), and we found that the prevalence of fungal pathogens indeed responded to road removals. In our study, the 2013 roadside had the higher diversity of fungal pathogens compared to the 2013 road-center. A higher diversity of fungal pathogens can be an indication of ongoing habitat degradation. Our study contrasted a previous study where *Fusarium* sp. was most abundant in the road-center compared to roadsides (Eaton et al., 2021). While road removals can increase the diversity of fungal pathogens in redwood forests at first, our study showed that the fungal richness of pathogens was similar between old-growth, the 2005 roadside, and the 2013 road-center. Due to the relationship between fungal pathogens and stressed trees, the 2013 roadside may be an indication of stress. While fungal pathogens attributed to sudden oak death and canopy die back were not detected or where in low abundances, the results of our study provide an important baseline for the detection of fungal pathogens in and adjacent to road scars.

Our study demonstrated the diversity and relative abundance of saprotrophic fungi in the 2013 road-center was significantly lower compared to the 2013 roadside. In Costa Rica, roadsides associated with second-growth forests had a higher abundance of lignin degrading saprotrophic

fungi (*Hypocrea*, *Calonectria*, and *Cylindrodladium*) compared to the road-center (Eaton et al., 2021). The 2013 road-center in our study had a relatively high abundance of genera (*Geastrum* and *Geminibasidium*) involved in lignin degradation (Hewelke et al., 2020; Kuhar et al., 2016). In Costa Rica, when topsoil is replaced during road construction there is a lack of forested materials to support decomposition by saprotrophic fungi (McGee et al., 2019). A potential factor contributing to the decline of saprotrophic fungi in the 2013 road-center is the lack of leaf litter and forests debris (Seney & Madej, 2015). While the 2013 road-center contained lignin degrading fungi, the lack of forested materials may inhibit the prevalence of saprotrophic fungi indicative of old-growth and adjacent roadsides.

The development of inoculums to restore abandoned and reclaimed roads has been recommended (Allen et al., 2018; Coutinho et al., 2019; Cox & Allen, 2008; Lapointe, 2019; Lowe, 2005; Paschke et al., 2000). Ideally, inoculums derived from soil of the reference ecosystem, compared to commercial inoculums, are more effective at restoring mycorrhizal fungi (Maltz & Treseder, 2015; Rowe et al., 2007). Impeding the use of a reference inocula are the extensive studies required to screen mycorrhizal fungi for field applications (Coutinho et al., 2019; Wu et al., 2002). Aside from the low diversity of ERM, based on the results of our study, the full recountour led the gradual recovery of the mycorrhizal community without a reference inoculum. However, to curb the low diversity of saprotrophic fungi in the 2013 road-center, mulch and other forest debris can be applied to road-center to encourage saprotrophic fungi. While a reference inoculum may not be necessary for road scars to reintroduce mycorrhizal fungi, a reference inoculum from roadsides or old-growth will introduce saprotrophic fungi to the road-center.

The results of this study elucidate the varied effects of road removal on fungal guilds in redwood forests. While we expected fungal guilds to be affected by road removals, we saw mixed results.

Mycorrhizal fungi were not affected by the road scars, where EMF was most abundant in the 2013 road-center. Pathogenic fungi were highest in the 2013 roadside compared to other road scars and old-growth. Additionally, saprotrophic fungi were similar across road scars and old-growth, except for the 2013 road-center having a lower presence compared to the 2013 roadside. Aside from diversity of pathogenic fungi in the 2013 roadside being higher compared to old-growth, there was not a strong influence of time since removal for all fungal guilds. Lastly, the location on the roadside or the road-center did play a role in determining the presence of saprotrophic and mycorrhizal fungi. With only a fraction of roads having been removed in redwood forests (RR, 2019), considering the response of fungal guilds provides additional insight to gauge the success of restoration efforts.

Figures

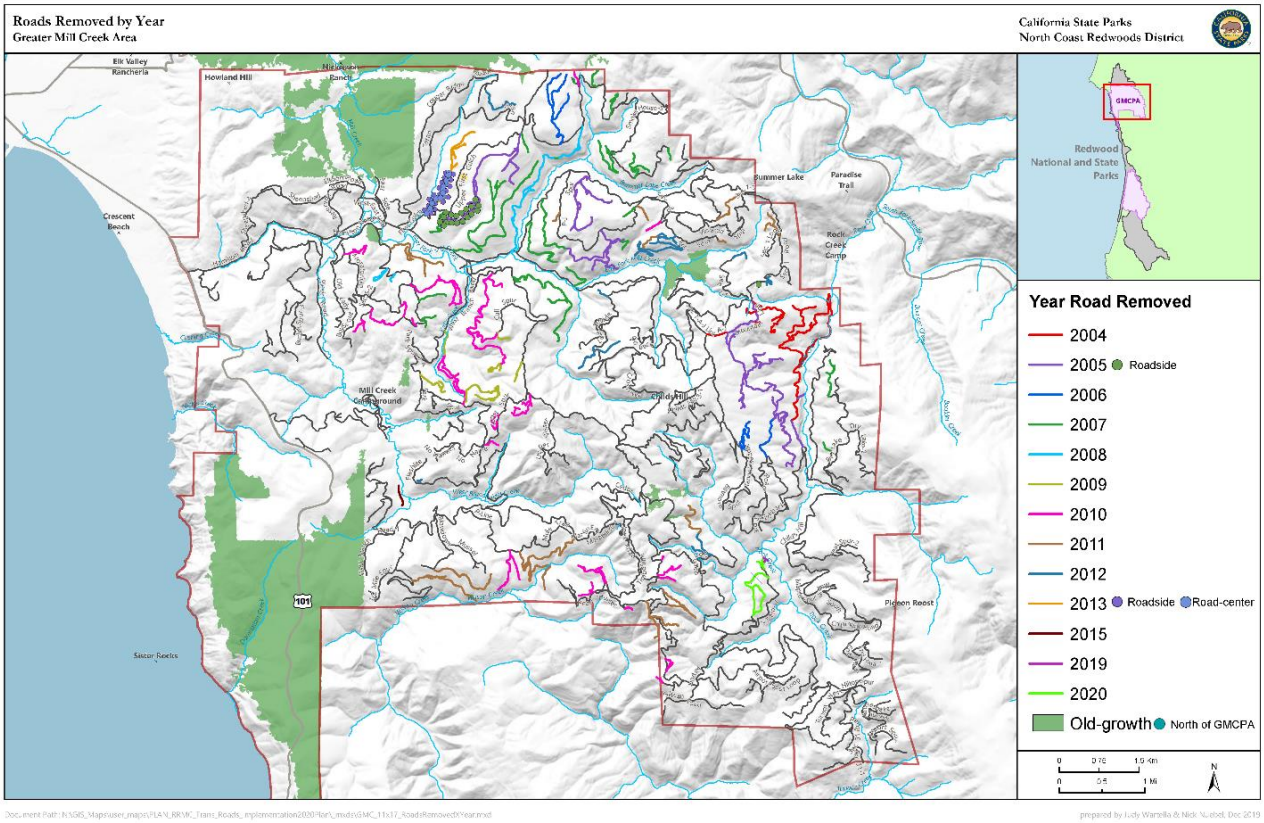

Figure 1. The various colors represent annual removal efforts in the Great Mill Creek Project Area of the Del Norte Coast Redwoods State Park. A total of 18 soil samples were collected from both the 2005 roadside and the 2013 roadside. Additionally, 18 soil samples were recovered from the 2013 road-center. The old-growth forest is depicted in green, where 18 soil samples were recovered from the Jedidiah Smith Redwoods State Park north of Howland Hill Road.

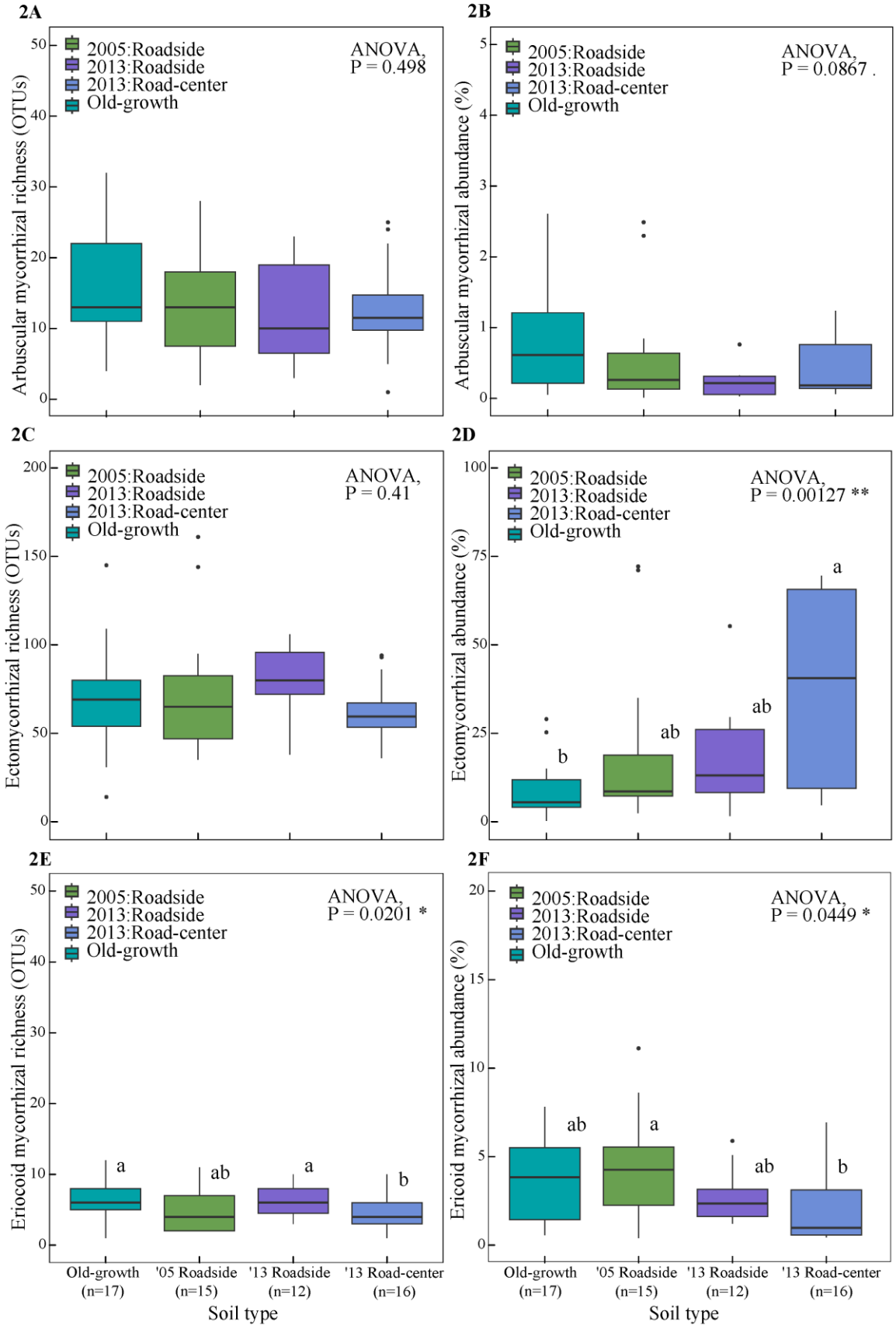
2A
2005:Roadside
2013:Roadside
2013:Road-center
Old-growth
ANOVA,
P = 0.498
Arbuscular mycorrhizal richness (OTUs)
2B
ANOVA,
P = 0.0867 .
Arbuscular mycorrhizal abundance (%)
2C
ANOVA,
P = 0.41
Ectomycorrhizal richness (OTUs)
2D
ANOVA,
P = 0.00127 **
Ectomycorrhizal abundance (%)
a
ab
ab
b
2E
ANOVA,
P = 0.0201 *
Eriocoid mycorrhizal richness (OTUs)
a
ab
a
b
2F
ANOVA,
P = 0.0449 *
Ericoid mycorrhizal abundance (%)
ab
a
ab
b
Old-growth (n=17)
'05 Roadside (n=15)
'13 Roadside (n=12)
'13 Road-center (n=16)
Soil type

Figure 2. Richness and relative abundance of mycorrhizal taxa, including arbuscular mycorrhizal richness (A), arbuscular mycorrhizal relative abundance (B), ectomycorrhizal richness (C), ectomycorrhizal abundance (D), ericoid mycorrhizal richness (E), and ericoid mycorrhizal abundance. Box plots denote the interquartile range between the 25 and 75 percentiles (boxes), medians (black lines), and potential outliers (dots).

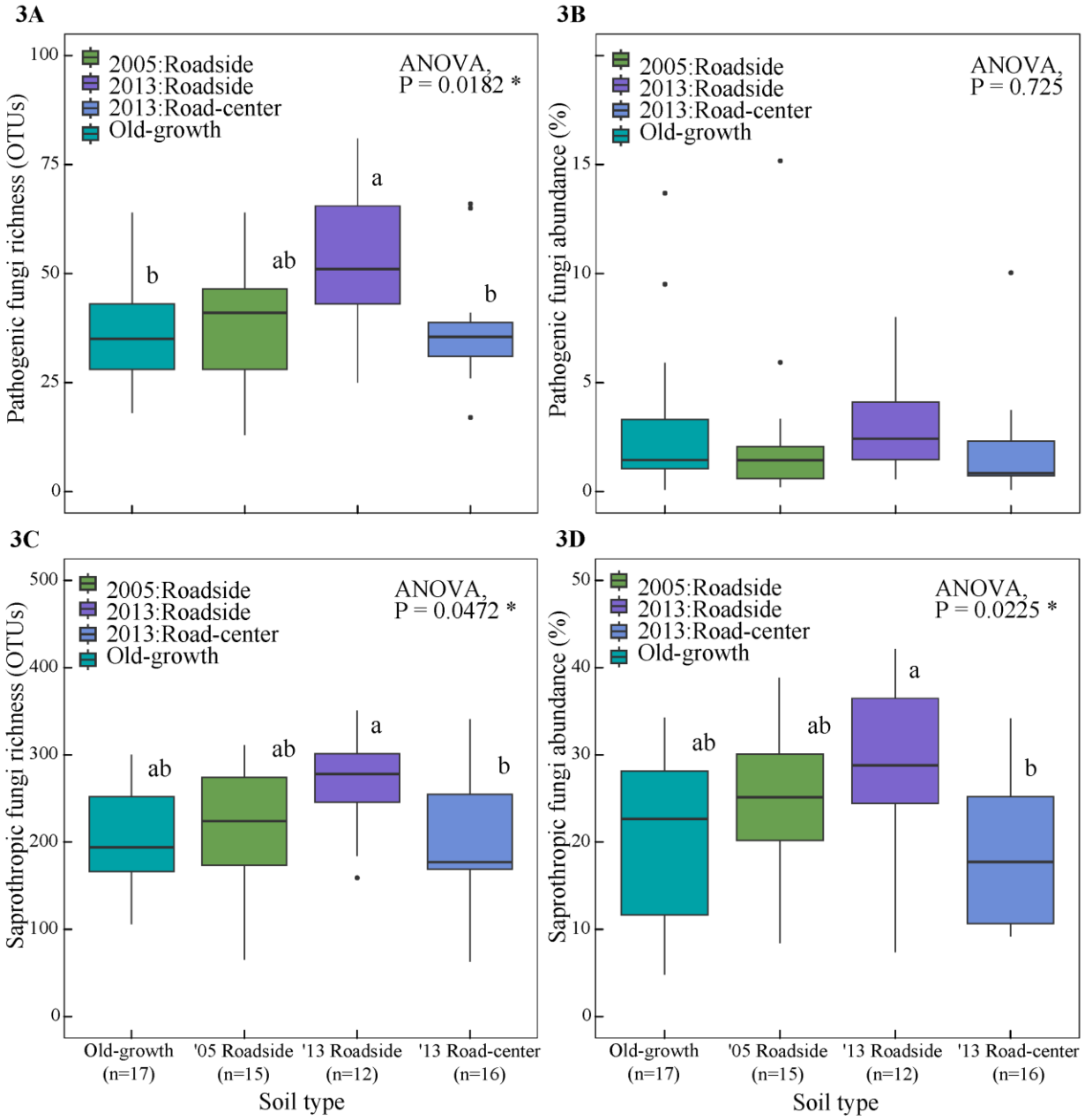

Figure 3. Richness and relative abundance of pathogenic and saprotrophic taxa, with (A), pathogenic relative abundance (B), saprotrophic richness (C), saprotrophic abundance (D). Box plots denote the interquartile range between the 25 and 75 percentiles (boxes), medians (black lines), and potential outliers (dots).

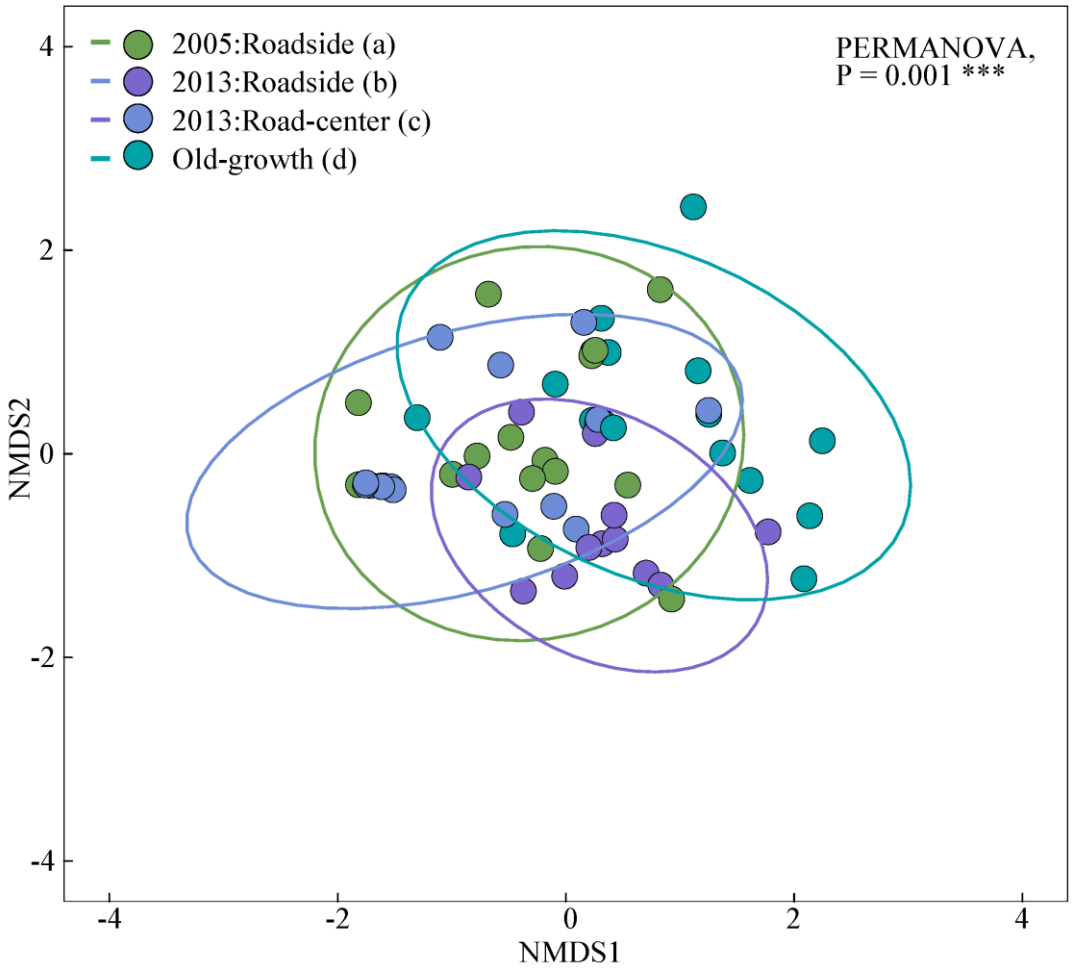

Figure 4. Non-metric multidimensional scaling (NMDS) plot of the community composition of the broader fungal community in soils. Symbols = samples. Asterisks (*) = significant differences resulting from a PERMANOVA test. Solid lines = 97% confidence intervals. Different letters denote significant differences between groups resulting from a pairwise comparison.

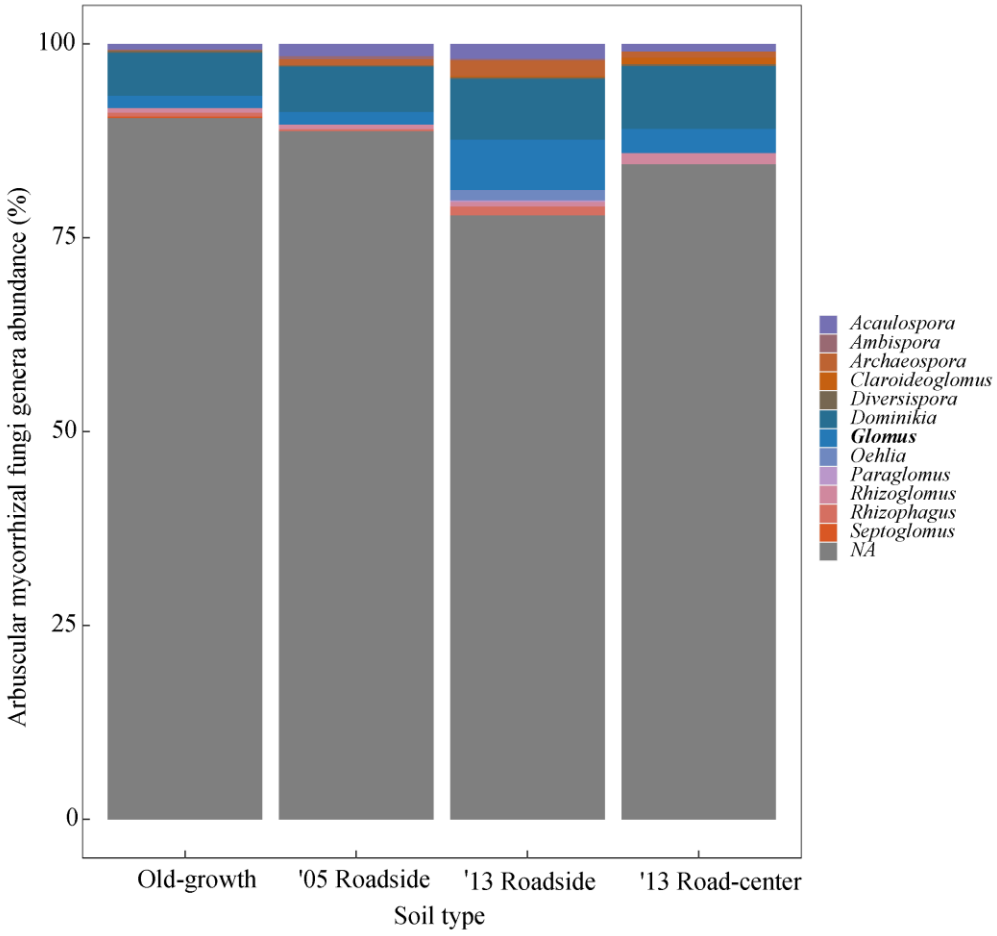

Figure 5. The relative abundance of 11 arbuscular mycorrhizal genera. Significant differences in the relative abundance of genera are indicated in bold ($P < 0.05$). NA = genera not assigned. *Glomus* spp. was significantly higher in the 2013 roadside compared to old-growth and the 2005 roadside.

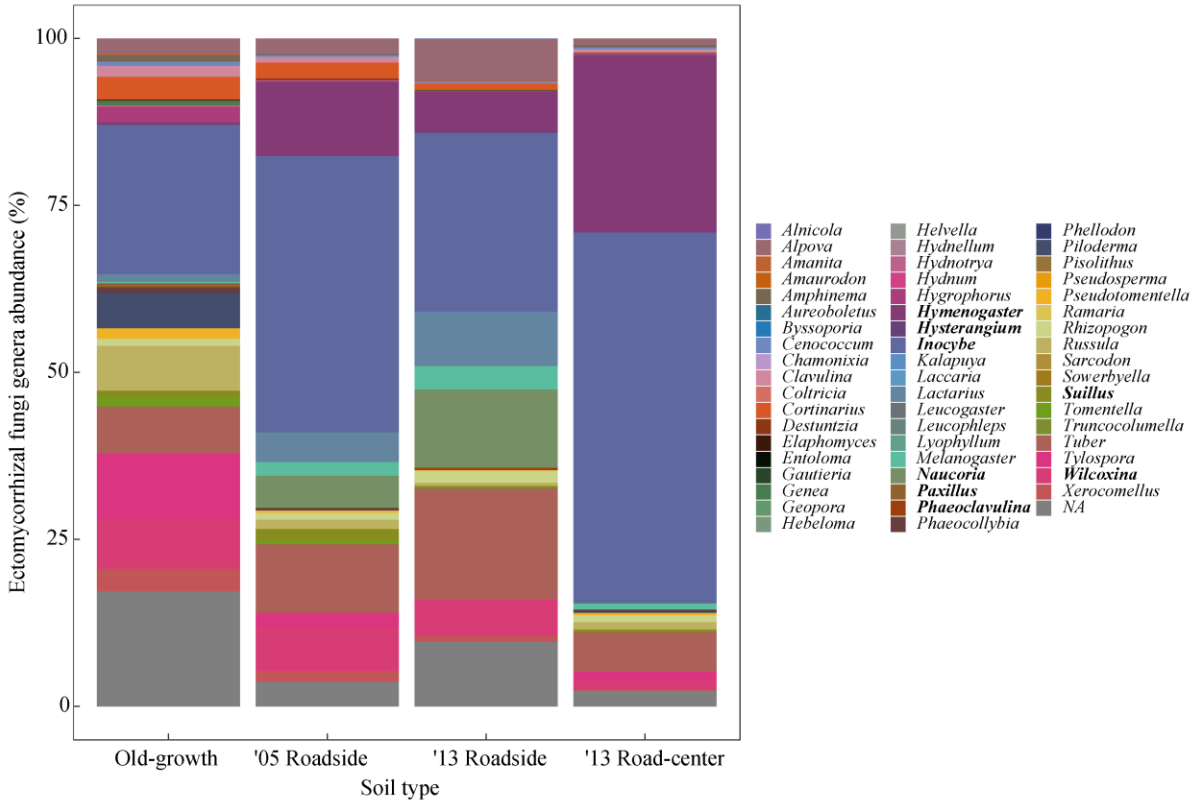

Figure 6. The relative abundance of the 11 ectomycorrhizal genera. Significant differences in the relative abundance of genera are indicated in bold ($P < 0.05$). NA = genera not assigned. *Hymenogaster* spp. and *Inocybe* spp. were significantly higher in the 2013 roadside compared to old-growth and the 2005 roadside. Whereas *Paxillus* spp. was highest in the old-growth compared to all other removal sites.

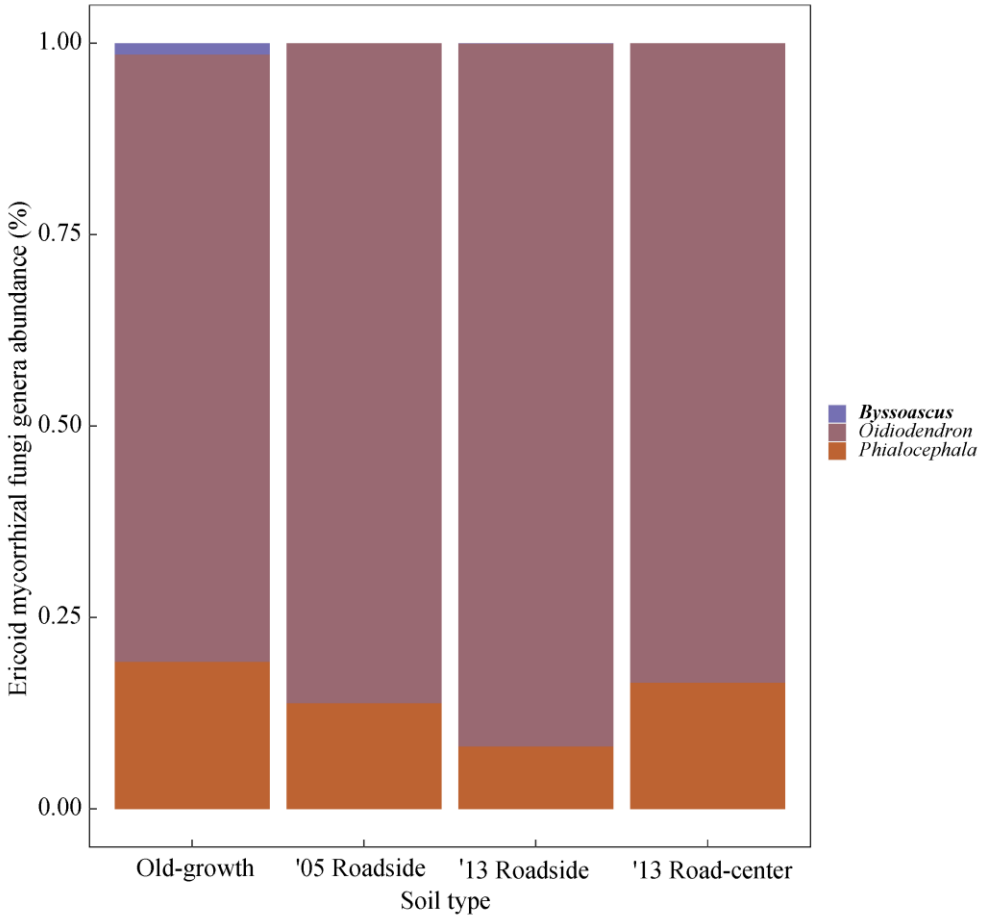

Figure 7. The relative abundance of three ericoid mycorrhizal genera. Significant differences in the relative abundance of genera are indicated in bold ($P < 0.05$). NA = genera not assigned. Byssoascus was significantly higher in old-growth forests compared to road scars.

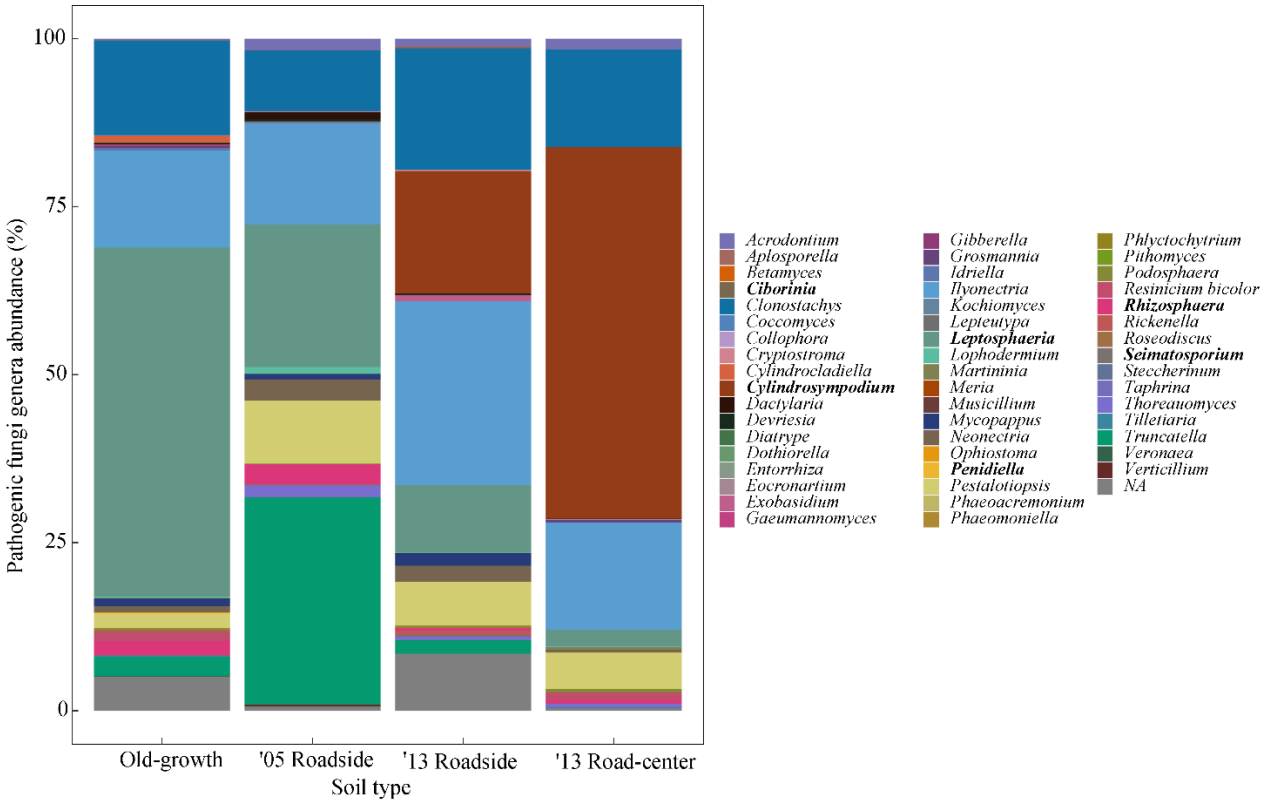

Figure 8. The relative abundance of the 33 pathogenic genera. Significant differences in the relative abundance of genera are indicated in bold ($P < 0.05$). NA = genera not assigned. *Cylindrosympodium* spp. was significantly higher in the 2013 roadside and road-center compared to the 2005 roadside and old-growth forests.

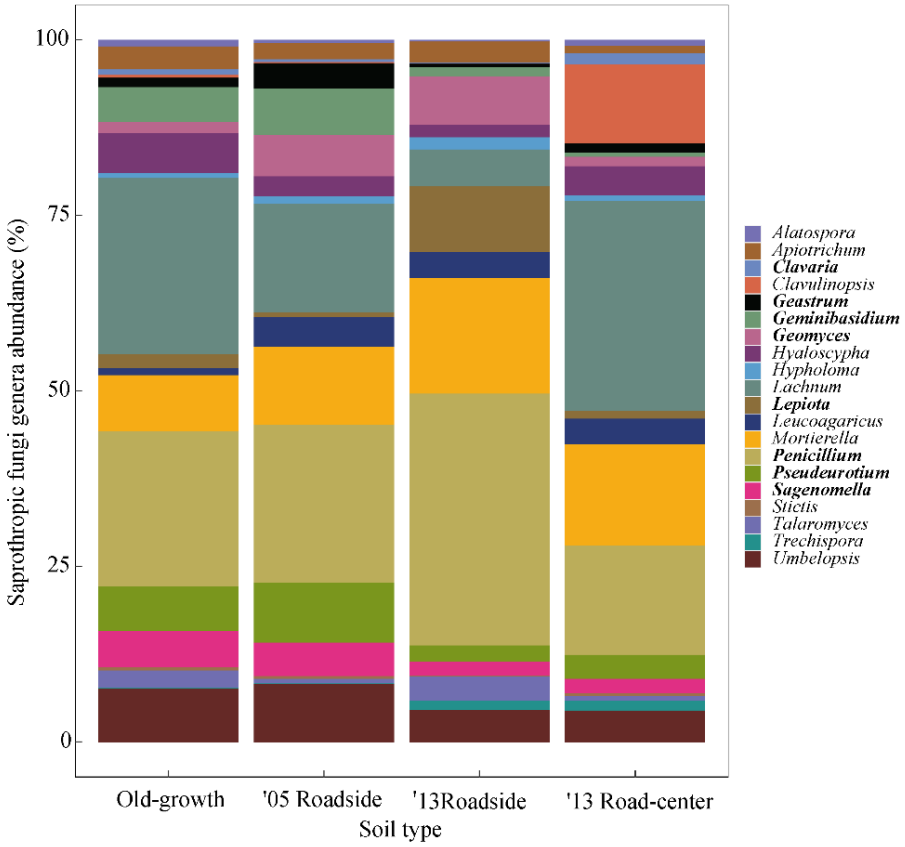

Figure 9. The relative abundance of the top 10 most abundant genera associated with saprotrophic fungi. Significant differences in the relative abundance of genera are indicated in bold ($P < 0.05$). NA = genera not assigned. *Geastrum* spp. and *Geminibasidium* spp. were significantly higher in the 2013 road-center. *Penicillium* spp. was significantly higher in the 2013 roadside compared to old-growth and the 2005 roadside. *Clavaria* spp. was highest in old-growth compared to all road removal sites.

Table 1. Significant genera associated with different fungal guilds are reported (mycorrhizal, pathogenic, and saprotrophic). Asterisks (*) = significant differences resulting from the indicator analysis.

Genus	Guild	P-Value
Glomus	Arbuscular mycorrhizal fungi	0.03516 *
Hymenogaster	Ectomycorrhizal fungi	0.002218 **
Hysterangium	Ectomycorrhizal fungi	0.034 *
Inocybe	Ectomycorrhizal fungi	0.003759 **
Naucoria	Ectomycorrhizal fungi	0.02759 *
Paxillus	Ectomycorrhizal fungi	0.01294 *
Phaeoclavulina	Ectomycorrhizal fungi	0.02302 *
Suillus	Ectomycorrhizal fungi	0.01972 *
Wilcoxina	Ectomycorrhizal fungi	0.01444 *
Byssoascus	Ericoid mycorrhizal fungi	0.00207 **
Ciborinia	Pathogenic fungi	0.01196 *
Cylindrosympodium	Pathogenic fungi	0.006381 **
Leptosphaeria	Pathogenic fungi	0.001902 **
Penidiella	Pathogenic fungi	0.0491 *

Rhizosphaera	Pathogenic fungi	0.01774 *
Seimatosporium	Pathogenic fungi	0.0005318 ***
Clavaria	Saprotrophic fungi	0.01782 *
Geastrum	Saprotrophic fungi	0.0003351 ***
Geminibasidium	Saprotrophic fungi	0.0004527 ***
Geomyces	Saprotrophic fungi	0.04365 *
Lepiota	Saprotrophic fungi	0.04969 *
Penicillium	Saprotrophic fungi	0.007562 **
Pseudeurotium	Saprotrophic fungi	0.03818 *
Sagenomella	Saprotrophic fungi	0.04642 *

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