INVESTIGATING THE INFLUENCE OF MANAGEMENT PRACTICES ON THE ASSEMBLY AND FUNCTION OF MICROBIAL COMMUNITIES

by

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DISSERTATION ABSTRACT

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Title: Investigating the Influence of Management Practices on the Assembly and Function of Microbial Communities

Microbial communities are integral to many ecosystem functions, including functions of interest to humans such as nutrient cycling and pathogenic infection. However, the influence of management intensity on microbial community assembly and functioning is poorly understood. High intensity management often reduces the overall diversity and biomass of fungi and bacteria; yet ecosystem function does not follow such a clear trend. Additionally, the timeframe in which management impacts microbial community and function is generally unknown. To address these gaps, I characterized microbial communities within logged and unlogged watersheds in the Western Cascade Mountain range and vineyards using different management intensities across a climate gradient in western Oregon, USA. Furthermore, I used wine fermentation as a proxy for microbial community functioning to measure functional differences between management intensities. I found that human management continued to have legacy effects upon microbial communities even five decades after cessation. I also found that management intensity had a clear influence on the organoleptic compounds found within Pinot noir wine samples. This work can deepen our understanding of the response of microbial communities and their functioning to human management.

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CHAPTER I

INTRODUCTION

Community assembly is a framework that explains how local communities of organisms are formed and shaped through the understanding of dispersal, abiotic conditions, biotic interactions, and dynamic changes over time (Figure 1) (Young et al., 2001; Nemergut et al., 2013; Leibold et al., 2016). Within the framework of community assembly there is a nebulous "regional species pool" that contains all species that could possibly disperse into the focus communities. In the real world the species pool might include species found in the surrounding environment as well as any species capable of airborne, waterborne, or other forms of long-distance dispersal (e.g., spores, seeds). Any species that manages to disperse into the focus communities must then survive abiotic and biotic "filters" to persist. Abiotic filters include all environmental parameters that may impede or aid the establishment of a species (nutrient pools and fluxes, climatic conditions, pH, light availability, etc.). Biotic filters include all direct or indirect interactions involving other individuals and/or species ranging from facilitation to competition. The community assembly process is ongoing; it is not a singular event. The species pool and filters can change over time as new species are introduced and the physical parameters of the environment change from species "functions" and discrete abiotic events (Leibold et al., 2016). Species' functions encompass all organismal processes and behaviors that alter the physical or chemical conditions of the environment. As those conditions change, so do the filters. However, linking community composition to function is difficult, as function is a vague concept shaped by the context of the study system and the interest of the researcher (Vellend et al., 2014; Bier et al., 2015; Leibold

et al., 2016). Simplified systems, such as agroecosystems, are excellent for studying the link between community composition and function. Managed systems are lower in diversity, producing less noise within data, are widely replicated, have well-defined complexity gradients, allow for large-scale manipulations, and have measurable functions relevant to the system managers (Perfecto and Vandermeer, 2008; Wolfe and Dutton, 2015).

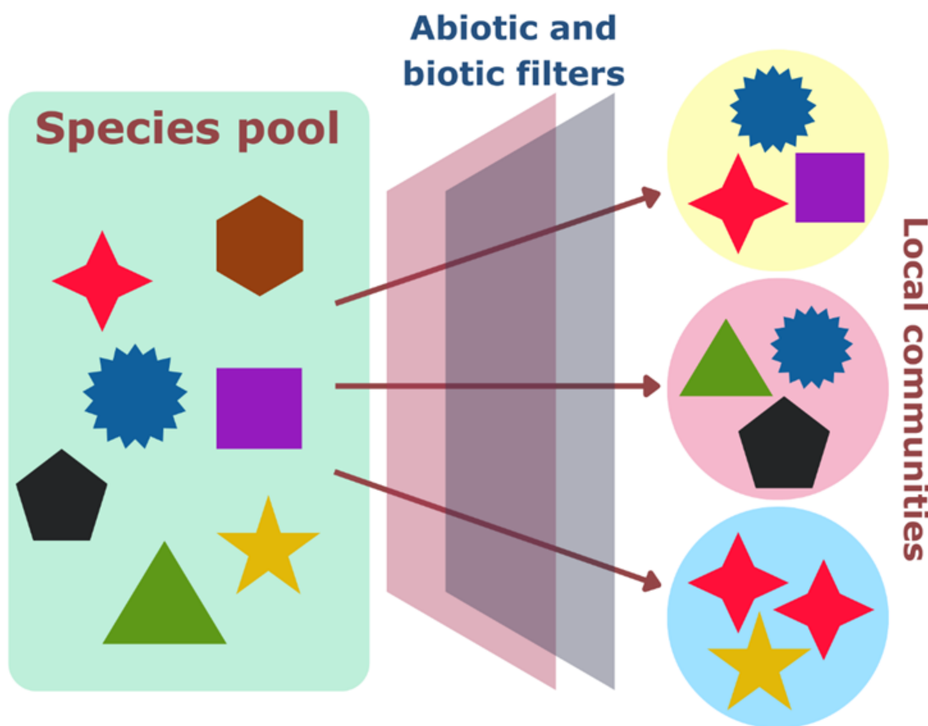

Figure 1. Graphic depiction of community assembly. Species disperse from a regional species pool into local communities where they encounter abiotic and biotic filters. A species must be able to survive the filter conditions to establish and persist within a local community.

Through the lenses of community assembly and ecological disturbance, human management can be viewed as an attempt to manipulate biotic and abiotic filters to

prevent the establishment of undesirable species (weeds, pathogens, herbivores, predators) and encourage the facilitation and growth of desirable species (crops, livestock, ornamentals, lumber trees, etc.). Management varies dramatically in both intensity and frequency, depending on the needs of the target species and the practices chosen by the manager. Management practices involve altering the physical and chemical properties of the environment, fitting within the definition of disturbance (Borics et al., 2013). Examples of physical alterations include the removal of biomass, compaction or disruption of soils, and irrigation; while chemical alterations include the addition of fertilizers, use of biocides, and changes to organic inputs. Ultimately, disturbances, including those caused by management, directly impact the process of community assembly and community functions by altering abiotic and biotic filters.

In this dissertation I discuss the influence of variation in management intensities on microbial communities in two managed plant communities common in Oregon, USA: logging in forest stands of the Western Cascades mountain range, and vineyards comprised of *Vitis vinifera* grape vines for wine making. This work is separated into three chapters. Chapter I focuses on the long-term legacy effects of logging on fungi in mineral soil and litter layers several decades after logging has ended at the Long-Term Ecological Research (LTER) forest known as H.J. Andrews. The second chapter explores the influence of management intensities (high and low) on bacteria, fungi, and their function in vineyards across several growing regions in Oregon. The third chapter is a case study of one vineyard from Applegate Valley that utilizes a timeseries of soil microbial data during and after converting the vineyard from high intensity management practices to low intensity management practices.

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CHAPTER II

THE LEGACY EFFECTS OF LOGGING ON SOIL AND LITTER MICROBIAL COMMUNITIES IN A WESTERN CASCADES FOREST STAND

This manuscript writing and core ideas are my own and resulted from discussions with co-authors. I led the fieldwork team including C.J. Paulino, G. Ridder, J. Cothern, and K. Evens. Tiffany Thornton and I performed all molecular laboratory work. Tiffany also wrote the methods section. Bitty Roy compiled the sporocarp data, wrote the sporocarp results, and critically revised the manuscript. Lucas Silva provided site selection assistance, put us in contact with the HJA rangers, and critically revised the manuscript. Krista McGuire provided funding, aided in developing the experimental design, and critically revised the manuscript.

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1. Introduction

Logging is one of the most pervasive disturbances to forests. Around 3.5 billion m3 of wood is harvested around the globe annually, affecting 29 million hectares of forest (Pepke, 2010; Crowther et al., 2015). While improvements to harvesting and processing technologies have made the logging industry more efficient, demands for timber products have increased in recent decades and that trend is predicted to continue (Zhou et al., 2005; Nepal et al., 2021). Logging not only affects tree cover, but also disrupts belowground soil communities that are essential for forest ecosystems. Fungi are

especially vulnerable to logging-associated disturbances due their formation of multicellular bodies known as mycelia that ramify through their substrate, and their reliance upon plant hosts—alive or dead, depending on the species—as their source of carbon. Fungal communities play critical roles in the functioning of forest ecosystems, including structuring plant communities (Gams, 2007), driving nutrient fluxes (DelgadoBaquerizo et al., 2016), influencing forest productivity, and sequestering carbon (Fr ˛ac et al., 2018).

Three main functional groups of fungi important to forest functioning are mycorrhizal fungi, pathogens, and decomposers (Ehrenfeld et al., 2005; Baldrian, 2016). Mycorrhizal fungi take up nutrients from the soil for their host plants in exchange for photosynthetically derived carbon (Bagyaraj and Ashwin, 2017). These fungi are also able to protect their hosts from pathogenic infections and increase stress tolerance (Jayne and Quigley, 2014; Kanekar et al., 2018; Gonthier et al., 2019). There are two broad categories of mycorrhizal fungi, arbuscular mycorrhizal fungi (AMF), and ectomycorrhizal fungi (ECMF). AMF are widely distributed and associate with >80% of plant species, while ECMF associate with ∼2% of plant species, primarily large woody species that dominate temperate and some tropical forests (Aerts, 2003). Pathogenic fungi attack their host plant species, which adds nutrients and carbon to resource pools (Maron et al., 2011). Decomposers—or saprotrophs—break down organic matter by releasing extracellular enzymes, increasing access to nutrients for other organisms (Bagyaraj and Ashwin, 2017). Decomposers can also suppress pathogenic fungi, reducing mortality of vulnerable host plants (Adnan et al., 2019). Many soil fungi also produce fruiting bodies (e.g., mushrooms) to generate and release sexual spores. Prior to next generation

sequencing, studying fungal communities primarily consisted of collecting these reproductive structures for identification. It is useful to compare sequences found in belowground environmental DNA (eDNA) studies with macrofungi collections to discover what proportion of species are shared between different methods. Not all fungi produce fruiting bodies, therefore sequencing of DNA in soil can reveal species absent from aboveground collections. On the other hand, aboveground collections can fill in gaps resulting from biases in DNA extraction, PCR amplification, and sequencing. Studies comparing fruiting body surveys to soil sequencing surveys typically find between 10 and 20% overlap, indicating the importance of utilizing both styles of survey (Gardes and Bruns, 1996; Dahlberg et al., 1997; Baptista et al., 2015).

The Pacific Northwest of the US is dominated by ectomycorrhizal trees and continues to be one of the most active logging regions in North America. Recent estimates of logging in the area show an annual average of seven million tons of lumber are harvested in Oregon (ORFI, 2020), and nine million tons are harvested in Washington per year (Arnold, 2004). Increases in efficiency of lumber use and conservation efforts have protected some old-growth areas in these areas, but demand continues to drive increases in harvesting (Pepke, 2010). Two major logging techniques are selective logging and clearcutting. The impacts of each technique on soils are similar, but clearcutting effects tend to be far more extreme. The use of large vehicles and machines in both techniques results in compaction of soil and heavy metal pollution (Hartmann et al., 2014). Openings in the canopy allow more light to reach the soil, which decreases moisture and increases temperature. The removal of trees also eliminates hosts for symbionts and alters the chemistry of soil as litter inputs are reduced. Erosion is also far

more likely, which can further alter soil chemistry and nutrient pools. Knowledge about the impacts of logging on belowground communities will not only increase understanding the role of disturbance in fungal community assembly but could also aid in active forest management and replanting efforts.

To evaluate the consequences of logging legacies on fungal composition, we sequenced fungi from soils in clearcut, selectively logged, and old-growth forest in the Pacific Northwest. We separately sampled soil (Oi—Oe) and litter because the litter layer is often ignored but contains a substantial amount of fungal biomass (Lim et al., 2010; Frey et al., 2011; Hu et al., 2019). Furthermore, the layers may differ in the composition of fungal functional guilds (McGuire et al., 2013). We also compared belowground communities to aboveground fruiting body composition. We addressed the following questions: (1) How do soil and litter fungal communities vary between forest stands with different logging histories? (2) What proportion of fungi are found both and belowground within the forest? (3) How do fungal communities in the litter layer differ from those in the soil?

2. Materials and methods

2.1 Site description

Our study sites were located within The H. J. Andrews Experimental Forest (hereafter referred to as HJA; 44.2◦N, 122.2◦W), a mid-elevation Pacific Northwest conifer forest dominated by *Pseudotsuga menziesii* (Douglas-fir) and *Tsuga heterophylla* (Western hemlock) with stands of varying age up to 700 years. The annual mean temperature is 8.6◦C, the mean annual precipitation is 2,289 mm/yr. HJA was established in 1948 by the United States Forest Service. The original focus of the research being

conducted within HJA was to manipulate forest operations such as logging and forest regeneration to optimize the efficiency of the timber industry. Due to this history, the watersheds within HJA were subjected to logging of varying intensity at different times. We sampled three watersheds with different logging legacies within HJA—watershed 6 (WS6), watershed 7 (WS7), and watershed 8 (WS8) (Figure 1). WS6 and WS7 were both previously logged: WS6 was clearcut in 1974 with 9% road coverage and was replanted with *P. menziesii* seedlings in 1976, while WS7 had 60% of its overstory harvested in 1974 with 0% road coverage, the rest of the canopy harvested in 1984, and a final thinning in 2001 to ensure ~4 m between each tree. WS8 was never logged, it was left as an old-growth forest control for comparison with other watersheds from the inception of HJA (Table 1). However, it did experience a fire that burned up to 70% of the standing trees in the 1850s, though this is still considered an old-growth forest within this system. The watersheds lie within the transition between western hemlock (*Tsuga heterophylla*) and silver fir (*Abies amabilis*) zones. WS6 was planted with 100% *P. menziesii* at a density of 383 stems/ha with a basal area of 70 ± 10 m2/ha. WS7 was planted with 100% *P. menziesii* at a density of 333 stems/ha with a basal area of 75 ± 4.8 m2/ha, which was reduced to 220 stems/ha in 2001. The understory of all three watersheds is comprised of vine maple (*Acer circinatum*), two Oregon grape species (*Mahonia aquifolium* and *M. nervosa*), whipplea (*Whipplea modesta*), salal (*Gaultheria shallon*), and rhododendron (*Rhododendron macrophyllum*). The overstory in WS8 is a mix of *P. menziesii*, *T. heterophylla*, *A. amabilis*, and western red cedar (*Thuja plicata*) with nearly one-third of individuals being greater than 450 years of age and the remaining around 125 years or younger (Andrews, 2022).

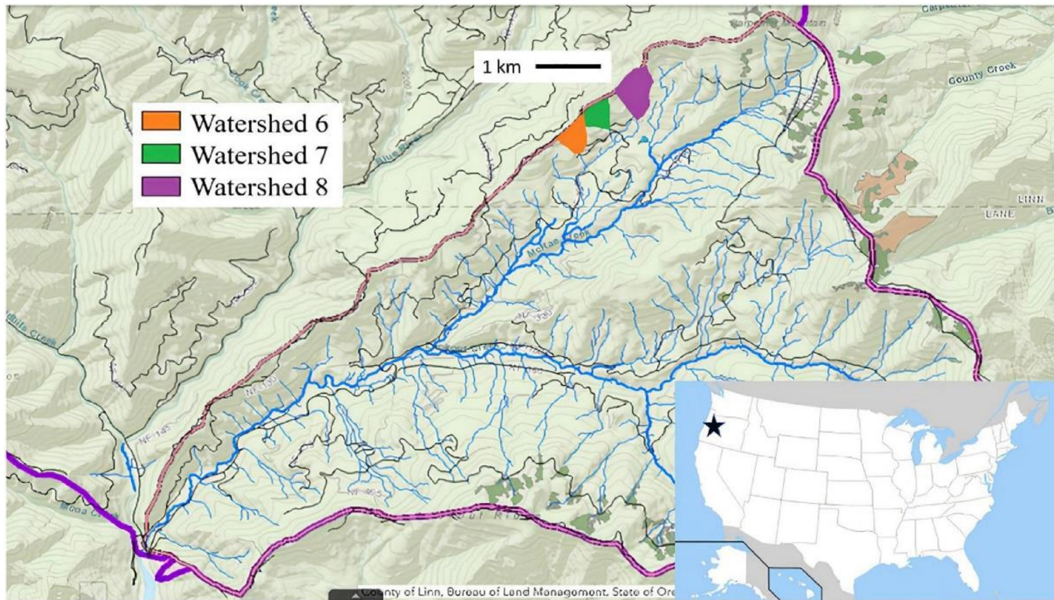

Figure 1. Map of H. J. Andrews Experimental Forest—a Long Term Ecological Research Site (LTER) in Lane and Linn County, Oregon, USA. Includes coloration of three watersheds with different management histories that were sampled for soil fungi. Watershed 6, clearcut in 1974; watershed 7, canopy harvest in 1974, 1984, and thinning in 2004; watershed 8, unlogged.

Watershed	WS6	WS7	WS8
Logging history	Clearcut in 1974 Residue burned in 1975	A total of 60% of basal area removed in 1974 Remaining canopy removed in 1984 Thinned in 2001	None
Removal strategy	High-lead cable system (90%) and tractor (10%)	High-lead cable system (40%) and tractor (60%)	N/A
Replanting history	*P. menziesii* in 1976 Canopy closure 2004	*P. menziesii* in 1976	N/A

Table 1. Management history for each watershed sampled within the H. J. Andrews experimental forest.

2.2 Sample collection

All samples were collected on 15 November 2018. Within each watershed we sampled along three 100 m transects running east to west that were separated by 50 m north to south. Along each transect we collected five litter and five soil samples at randomized points (soil n = 45, litter n = 45). Litter samples consisted of collecting organic litter around the target point by hand while wearing EtOH-sterilized gloves to fill a sterile 500 mL bag. Soil samples consisted of 2.5 cm × 20 cm cores taken using an EtOH sterilized corer after the litter layer was removed. Samples were stored on ice for up to 12 h before being transferred to a −20◦C freezer until homogenization and DNA extraction.

2.3 Soil and litter sample processing

Soil samples were homogenized using EtOH and UV-sterilized 2 mm aperture sieves, and litter samples were homogenized using EtOH and UV-sterilized ceramic mortar and pestles. To facilitate homogenization, liquid nitrogen was poured onto the litter samples immediately before pulverizing. For each sample, 0.25 g and 3.0 g were weighed in sterile 2 ml Eppendorf tubes for DNA extraction and moisture analysis, respectively. An additional sterile 2 ml Eppendorf tube was filled and kept as an archival sample within a −80◦C freezer. All tubes were stored at −20◦C before use.

The rest of each homogenized sample was air-dried at room temperature in a labeled paper bag. Air-dried soil samples were prepared for pH analysis by mixing a 1:2 ratio of sample to deionized water. For soil samples, 10 g of soil was weighed in a glass beaker and mixed with 20 ml of deionized water. For litter samples a ratio of 1:8 was

used to compensate for absorption, 2.5 g of litter was weighed in a glass beaker and mixed with 20 ml of deionized water. Three readings per sample were recorded using a benchtop pH meter with a glass electrode, and the average value was calculated. Moisture was calculated for each sample by recording the weight of 3.0 g of sample in an aluminum weighing dish, drying the sample in a drying oven at 130◦C for 48 h, re-weighing the sample, and subtracting the dried sample weight from the initial sample weight (Black, 1965).

2.4 Library preparation

High throughput sequencing was used to analyze fungal community composition for soil and litter samples. DNA was extracted from 0.25 g of each soil and litter sample using Qiagen DNeasy PowerSoil single-tube extraction kits, following the manufacturer's protocol. The ITS1 region of the fungal rRNA gene was amplified using the ITS1-F (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC) primer pair, the Nextra NX2 Illumina (San Diego, California) adapter, and Earth Microbiome barcoded primers. A master mix containing 12.5 µl GoTaq, 9.45 µl nuclease-free water, 0.5 µl forward (ITS1F) primer, 0.5 µl reverse (ITS2) primer, 0.5 µl Nextera (NX2) primer, and 0.05 µl BSA was added to the wells of a 96-well PCR plate. In each well, 1.0 µl of genomic DNA and 0.5 µl of a unique barcoded primer was added to the master mix for a total volume of 25 µl per well. PCR reactions were held in a thermal cycler at 94◦C for 1 min, with amplification proceeding for 35 cycles at 94◦C for 30 s, 52◦C for 30 s, and 68◦C for 30 s, and a final elongation of 7 min at 68◦C.

Amplicon concentrations were quantified according to a Quant-iT PicoGreen protocol (Thermo Fisher Scientific, Waltham, MA). DNA concentrations were quantified

on a Molecular Devices SpectraMax M5E multi-mode plate reader (San Jose, CA). Amplicons were pooled together according to quantification data from the PicoGreen assay to obtain an equimolar concentration of 1 ng of DNA per sample. The pooled library was purified using a Qiagen QIAquick PCR purification kit, according to the manufacturer's protocol. The amplicon pool was sequenced using an Illumina MiSeq (San Diego, CA) instrument at the University of Oregon's Genomics and Cell Characterization Core Facility (GC3F).

2.5 Sequence processing

Raw sequences were demultiplexed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010). We then assembled an amplicon single variant (ASV) table using the DADA2 pipeline (Callahan et al., 2016). Taxonomy was assigned using the naïve Bayesian classifier from the Ribosomal Database Project (RDP) against the UNITE database (Koljalg et al., 2005; Cole et al., 2014). We used variationstabilizing transformation to normalize ASV counts (Anders and Huber, 2010). To assign functional guild classifications to each ASV we used the annotation tool FUNGuild set to its default parameters (Nguyen et al., 2016). Many ASVs tend to have multiple guilds assigned to them (e.g., saprotroph and pathogen). When performing our downstream analyses, we allowed for ASVs to be represented in multiple categorical subsets (i.e., the previous example would be counted once as a saprotroph and once as a pathogen).

2.6 Statistical analyses

Statistical analyses were performed in R (v3.6.3) using the vegan package (Oksanen et al., 2019). We used the Bray-Curtis dissimilarity statistic to quantify the

compositional dissimilarity between our sample communities. This statistic allowed us to visualize the difference between communities within a two-dimensional plot using non-metric multidimensional scaling (NMDS). Additionally, to test for significance in our Bray-Curtis dissimilarity results we performed permutation analyses of variance (PERMANOVAs) comparing watershed, sample source (soil or litter), as well as physicochemical properties (pH, moisture). Additionally, to visualize compositional differences, we compared the relative abundances of functional guilds and the most abundant 15 genera found in each treatment. Figures were generated using ggplot2 (Wickham, 2016).

2.7 Compilation of fruiting (sporocarp) studies

To gain an understanding of the total fungal community in the forest and because eDNA and sporocarp surveys capture different parts of the fungal community (Frøslev et al., 2019) we wanted to compare the fungal species represented by sporocarps (sexual) vs. those in the soil eDNA samples (largely asexual). We limited the lists to macrofungi [mushrooms in the general sense (Thiers and Halling, 2018), including boletes and corals as well as larger ascomycetes such as Helvella] that were identified to species. This list was then compared to a list of macrofungi collected from the LTER using existing data from the H. J. Andrews from the following publications and reports, and their associated datasets in the H. J. Andrews datafiles [Rhoades, 1972; Luoma et al., 1991 (TP109); Smith et al., 1996 (SA01401)] and iNaturalist (2022) observations through February 2021. Sporocarps in the Rhoades (1972) study were collected opportunistically between October 1970 and April 1972, those in the Smith et al. (1996) study were collected twice per year for 4 years from three forest stands, one old growth, one rotation age and one

young-growth. The Luoma et al. (1991) study took place in 10 stands, with sampling occurring for about 6 weeks twice per year for 4 years. All the names in our final list (Supplementary Table 1) were verified in Index Fungorum; this process uncovered numerous typos and names changes which are enumerated in Supplementary Table 1. Many collections were identified only to genus; these were not included in our comparison with the soil eDNA because we had no way of determining which species they might match in the eDNA.

3. Results

3.1 Differences between management types

While keeping litter and mineral soil separate, the fungal communities form separate clusters by management histories (clearcut, canopy + thinned, unlogged) based on Bray-Curtis dissimilarity (Figure 2). The difference between management types was significant for both mineral soil and litter using PERMANOVA (soil: $df = 2$, $F = 1.84$, $R^2 = 0.0825$, $p < 0.001$; litter: $df = 2$, $F = 2.32$, $R^2 = 0.104$, $p < 0.001$). The composition of the genera that comprised each guild were distinct between management types (Figures 3–5A). Saprotrophs, pathogens, and ectomycorrhizal fungi were all significant when considered separately (Figures 3– 5C and Supplementary Table 2). Similarly, if the watersheds are grouped into a binary of logged and unlogged (Figure 6) all of these categories remain significantly different (all fungi: $df = 1$, $F = 2.55$, $R^2 = 0.0291$, $p < 0.001$; ECMF: $df = 1$, $F = 2.35$, $R^2 = 0.0275$, $p < 0.001$; saprotroph: $df = 1$, $F = 7.34$, $R^2 = 0.0795$, $p < 0.001$; pathogen: $df = 1$, $F = 3.08$, $R^2 = 0.125$, $p < 0.001$).

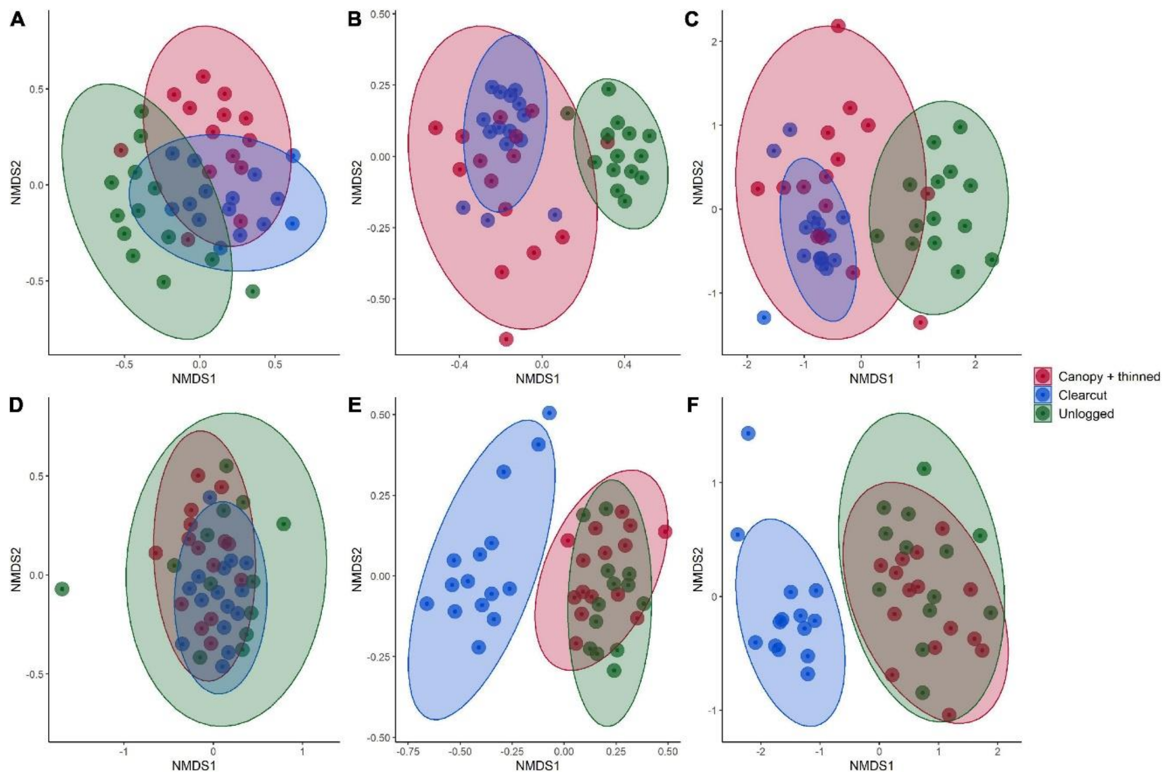

Figure 2. Non-metric multidimensional scaling ordinations based on Bray-Curtis dissimilarity between three forest stands with different logging histories sampled in H. J. Andrews Forest, Oregon. Included are ectomycorrhizal fungi, saprotrophs, and plant pathogens in soils (**A–C**), as well as ectomycorrhizal fungi, saprotrophs, and plant pathogens in litter (**D–F**). All PERMANOVA *p*-values are <0.001. Clearcut, watershed 6, canopy + thinned, watershed 7, unlogged, watershed 8.

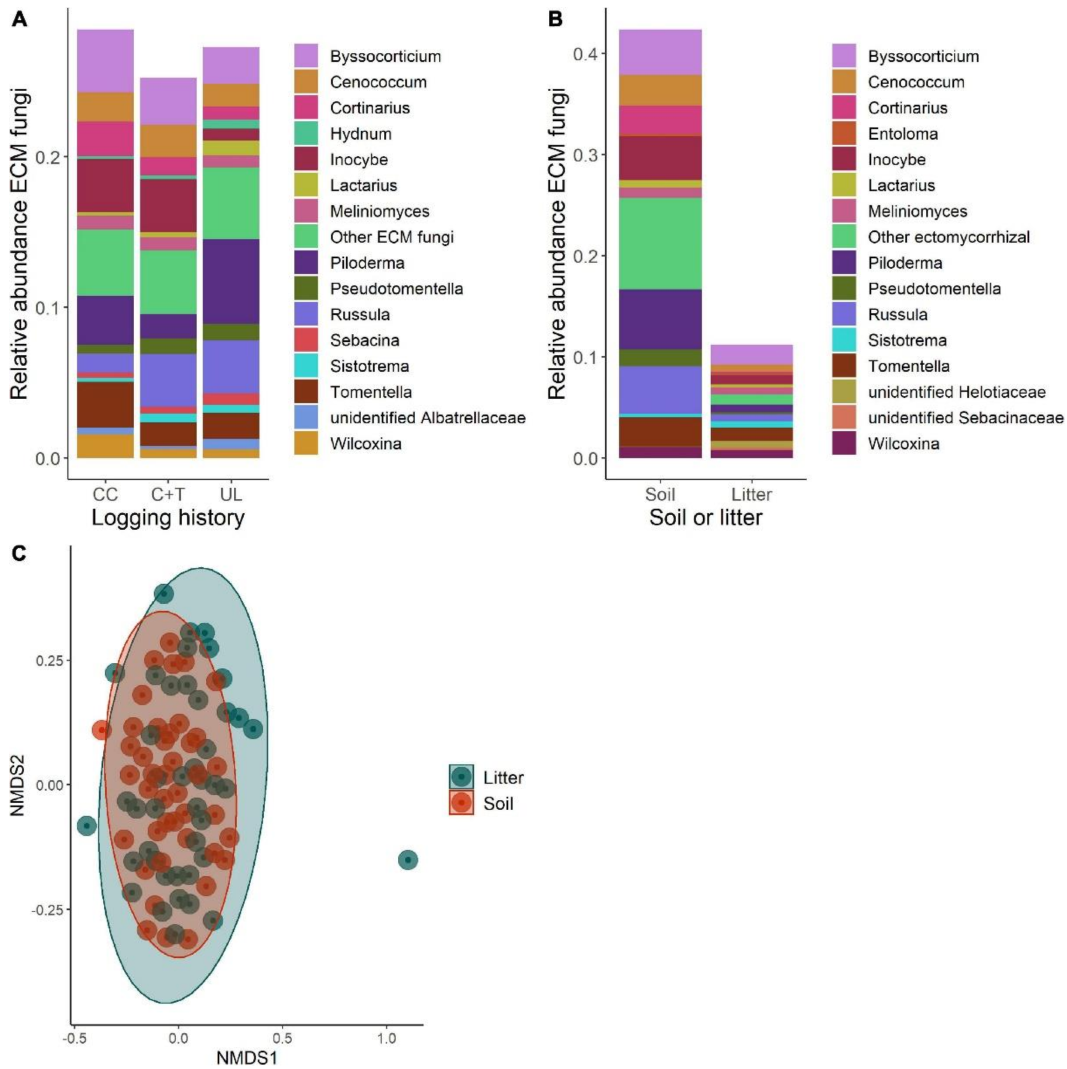

Figure 3. Comparisons of ectomycorrhizal fungal (ECM fungi) communities in soil and litter of logged and unlogged forest stands within the H. J. Andrews Forest, Oregon, USA. Clearcut (CC), watershed 6, canopy + thinned (C + T), watershed 7, unlogged (UL), watershed 8. **(A)** Relative abundances of ECMF genera between watersheds. **(B)** Relative abundances of ECMF genera between soil and litter. **(C)** NMDS ordination of ECMF communities based on Bray-Curtis dissimilarities between soil and litter (PERMANOVA, p = 0.578).

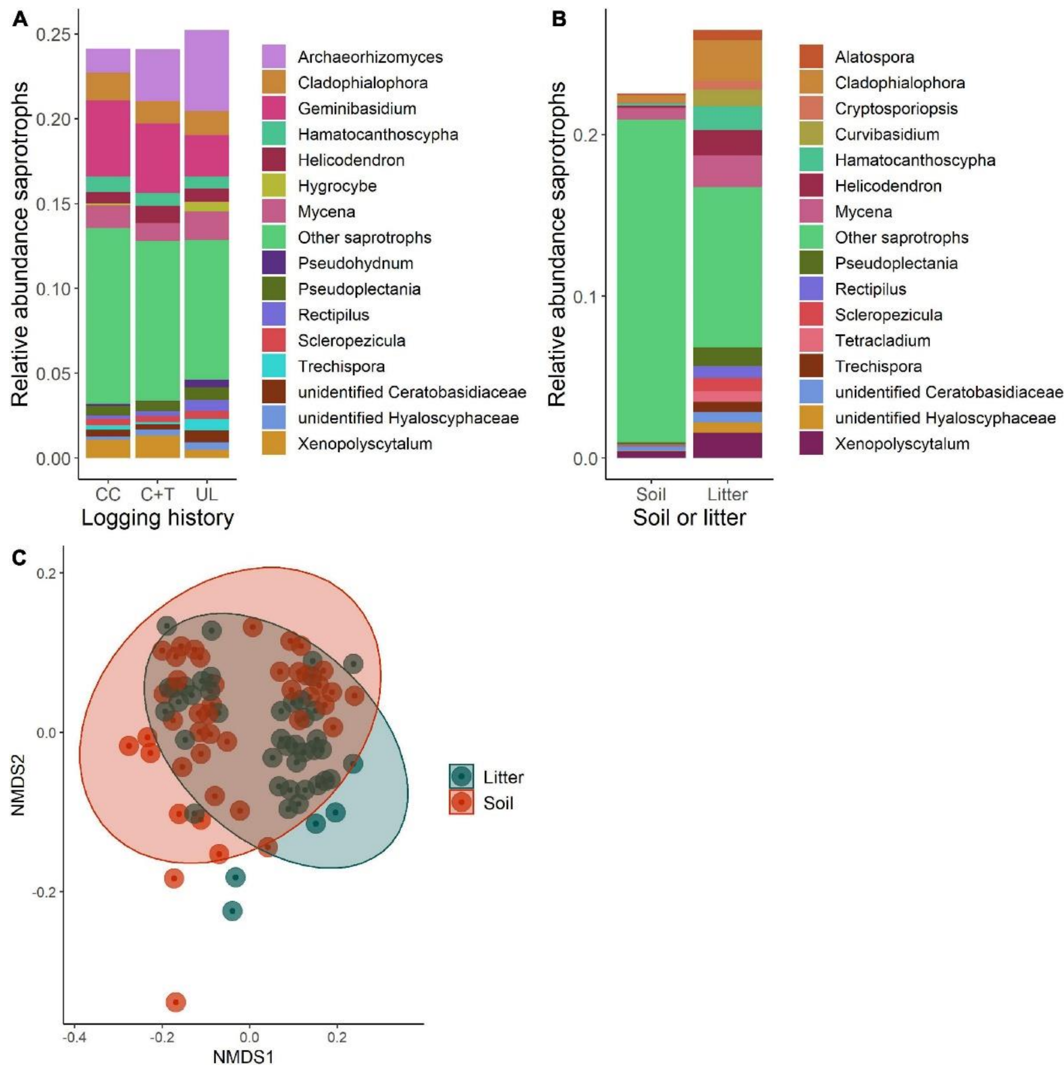

Figure 4. Comparisons of saprotroph communities in soil and litter of logged and unlogged forest stands within the H. J. Andrews Forest, Oregon, USA. Clearcut (CC), watershed 6, canopy + thinned (C + T), watershed 7, unlogged (UL), watershed 8. **(A)** Relative abundances of saprotroph genera between watersheds. **(B)** Relative abundances of saprotroph genera between soil and litter. **(C)** NMDS ordination of saprotroph communities based on Bray-Curtis dissimilarities between soil and litter (PERMANOVA, $p = 0.028$).

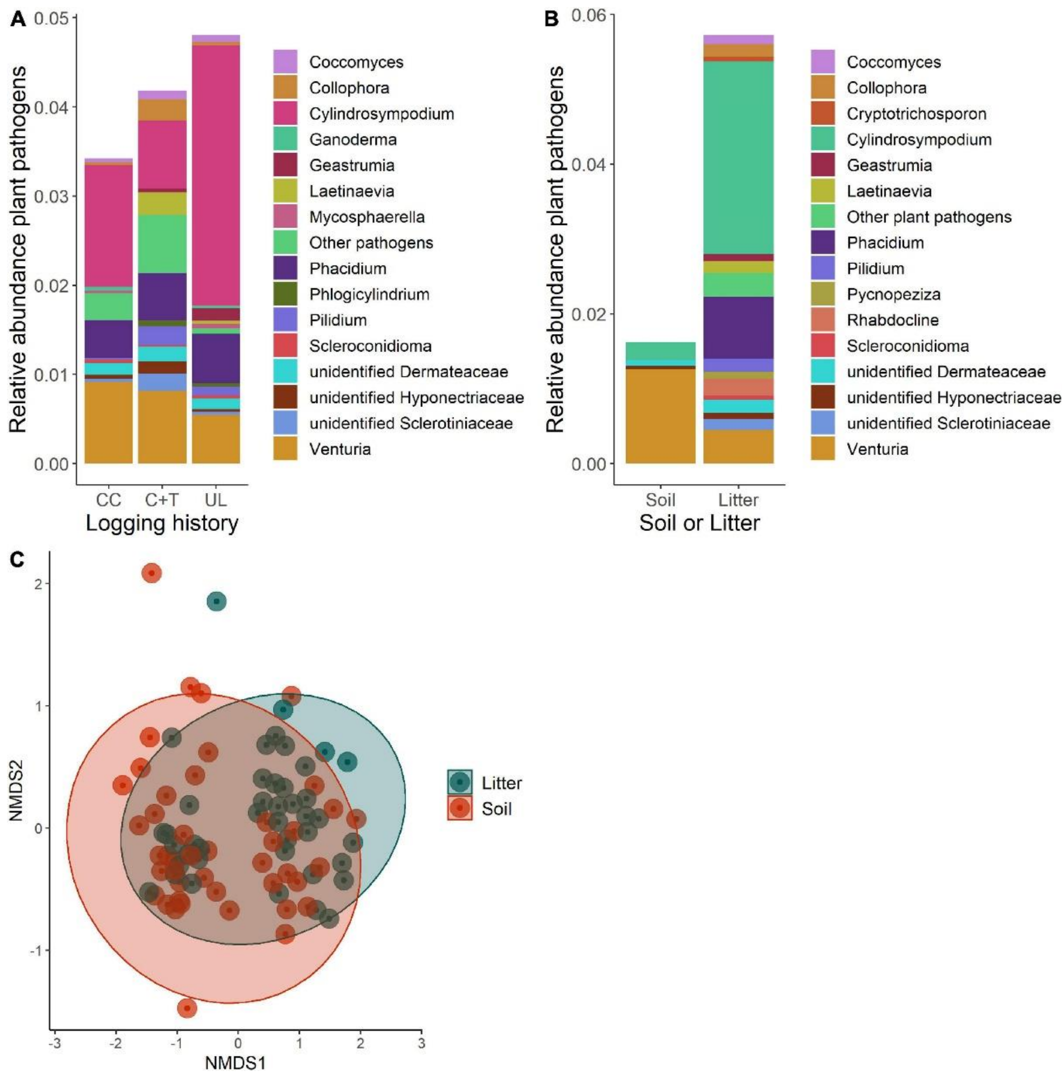

Figure 5. Comparisons of pathogen communities in soil and litter of logged and unlogged forest stands within the H. J. Andrews Forest, Oregon, USA. Clearcut (CC), watershed 6, canopy + thinned (C + T), watershed 7, unlogged (UL), watershed 8. **(A)** Relative abundances of pathogen genera between watersheds. **(B)** Relative abundances of pathogen genera between soil and litter. **(C)** NMDS ordination of pathogen communities based on Bray-Curtis dissimilarities between soil and litter (PERMANOVA, $p = 0.005$).

3.2 Mineral soil vs. litter

Based on Bray-Curtis dissimilarity, the overall fungal communities separated into distinct clusters for mineral soil and litter (Oi—Oe) samples (Figure 6C). We found that the difference between these groups is highly significant using PERMANOVA (df = 1, F = 13.92, R^2 = 0.141, p < 0.001). Some of these differences are apparent when comparing relative abundances of functional guilds and the genera that comprise them (Figures 3–5B). Litter communities tend to contain more pathogens, while mineral soil communities contain more ectomycorrhizal fungi (ECMF; Figure 6A). When comparing the saprotrophs and pathogens between soil and litter samples there are significant differences (df = 1, F = 2.42, R^2 = 0.028, p = 0.008 and df = 1, F = 3.08, R^2 = 0.035, p = 0.007, respectively). There are not significant differences between soil and litter for ectomycorrhizal fungi (df = 1, F = 0.944, R^2 = 0.011, p = 0.574)—however, it was significant when accounting for an interaction with management history (df = 1, F = 1.63, R^2 = 0.019, p = 0.007).

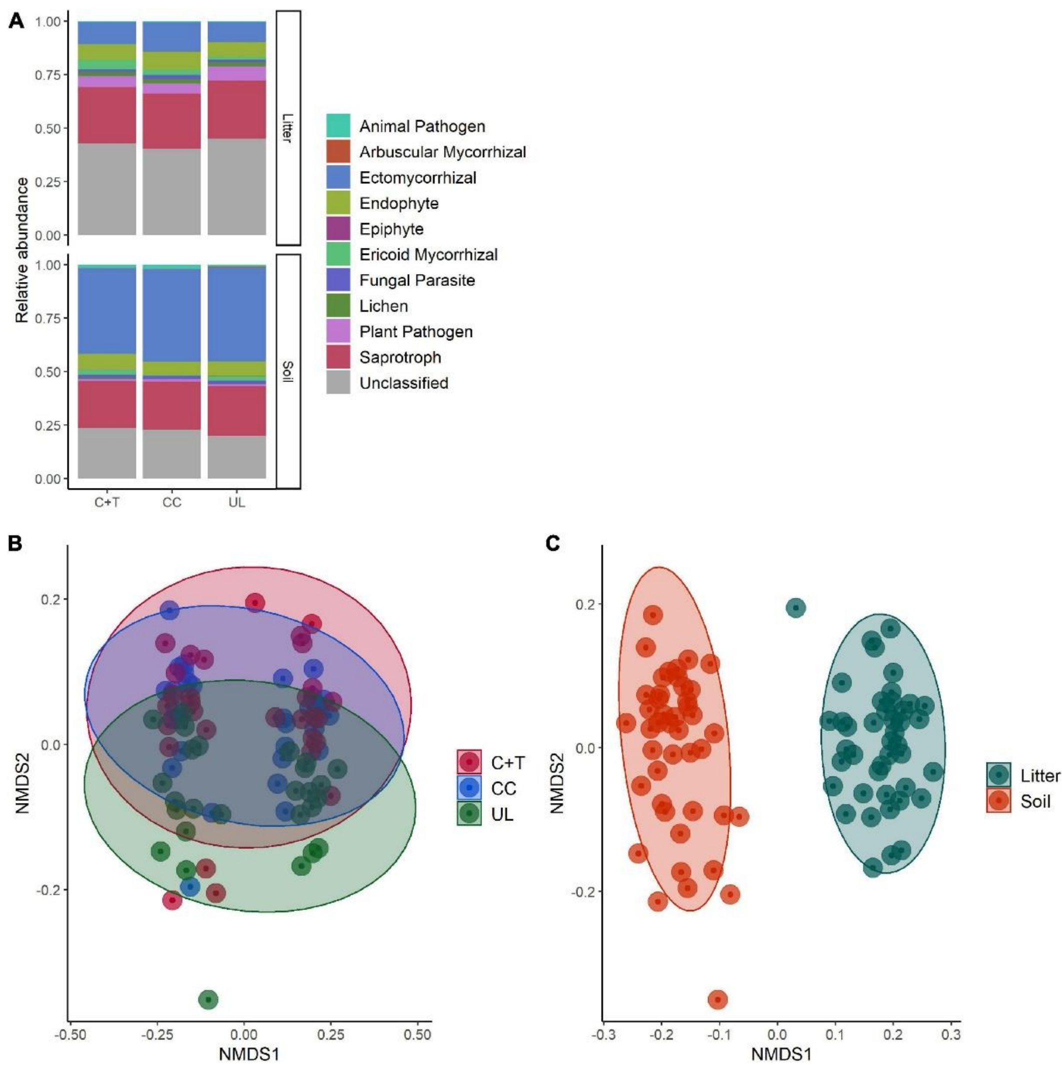

Figure 6. Comparisons of fungal communities in soil and litter of three watersheds with varying logging histories within the H. J. Andrews Forest, Oregon, USA. Clearcut (CC), watershed 6, canopy + thinned (C + T), watershed 7, unlogged (UL), watershed 8. **(A)** Relative abundances of functional guilds between watersheds for soil and litter. Functional guilds were assigned using FUNGuild analysis. **(B)** NMDS ordination of fungal communities based on Bray-Curtis dissimilarities comparing watersheds (PERMANOVA, $p < 0.001$). **(C)** NMDS ordination of fungal communities based on Bray-Curtis dissimilarities comparing soil and litter (PERMANOVA, $p < 0.001$).

3.3 Fruiting-body comparison

Our belowground sequences from the three sites yielded 144 macrofungi including those that form mushrooms, cups, crusts, and truffles that were identified to species. Our compilation of sporocarp data across the entire H. J. Andrews forest yielded 380 species; however, only 34 (6.49%) were shared between the sporocarp and eDNA datasets for a total of 524 species found. The entire list, indicating the kind of data (eDNA or sporocarp) and the source of the information can be found in Supplementary Table 1.

3.4 Physiochemical properties

We characterized the pH and moisture content of our samples. Based on PERMANOVAs of the Bray-Curtis dissimilarities of communities, we found that pH and moisture had a significant effect on soil fungi (pH: $df = 1$, $F = 2.63$, $R^2 = 0.0590$, $p < 0.001$; moisture: $df = 1$, $F = 3.07$, $R^2 = 0.0682$, $p < 0.001$) while it did not have a significant effect on litter fungi (pH: $df = 1$, $F = 1.19$, $R^2 = 0.0282$, $p = 0.121$; moisture: $df = 1$, $F = 1.16$, $R^2 = 0.0275$, $p = 0.128$).

4. Discussion

4.1 Legacies of logging

Pseudo replication is a potential concern with our dataset, as our logging treatments are confounded with watershed identity. This may limit the generalizability of our data to other logging systems. However, due to the close geographic proximity, shared climate, similar topography, identical understory composition, and limited physical barriers we are confident that this effect is minimal. Logging practices vary in their effects on aboveground forest structure, and we found that differences in historical

logging also had significant legacy effects on belowground fungal composition. We saw large differences in some taxa between logged and unlogged forests, and these differences depended on the functional groups of the fungi. We also saw differences in fungal composition between logged forests depending on whether the forest was clearcut or selectively logged. Logging causes several consistent ecological changes in forest structure, composition, and function that the fungi are likely responding to. For example, the early stages of succession are lost due to replanting and since the tree ages are the same in replated forests, there are no snags, few downed logs and the canopy is open allowing more sunlight to hit the forest floor for about 30 years post cut (Franklin et al., 2002). To simplify our discussion, we only consider the genera that had very large (2× or more) differences between sampling units in our soils and litter data.

In soils, we found that ECM fungi showed strong compositional responses to logging legacies, which may have implications for forest regeneration trajectories since most of the trees in these sites depend on ECM fungi for growth and survival. We found that some fungal genera were more abundant in early to mid-successional forests whereas others were more restricted to unlogged, old growth forests. Since the dominant overstory tree hosts (*P. menziesii*) are the same in these sites, these results suggest that environmental filtering structures ECM fungal communities across sites due to the abiotic changes from historical logging practices. We found greater abundance (≥2×) of *Piloderma* and *Russula* in unlogged forests, which is consistent with other studies (Smith et al., 2016; Kranabetter et al., 2018) and we found more of the early successional *Wilcoxina* in logged forests (Dickie et al., 2013). Studies of sporocarps (fruiting bodies) have generally found that *Inocybe* species are largely early successional (Cripps, 1997

and refs. therein), which is consistent with our data. However, a study by Norvell and Exeter (2004) on sporocarps in the Oregon Coast range found no relationship between stand age after logging and *Inocybe* fruiting. The differences between the Inocybe studies could be the result of differences in methods; studies of sporocarps can only detect fruiting, not abundance of the taxon in the soil, whereas we could only detect abundance in the soil and our fruiting body survey was not concomitant—it was a compilation of more than 40 years of data. Similarly, the genus *Cortinarius* is considered to be later successional (Nara et al., 2003; Sun et al., 2015), but we found them to be more than 2× more abundant and more diverse in the soils of logged watersheds, with only 3/37 taxa restricted to the soils of the unlogged forest. Another study compared ECMF in logged and unlogged forests in Malaysia, finding similar trends in relative abundances of genera—nearly twice as many *Russula* in unlogged forests, and more *Inocybe* in logged forests. Together, these results suggest that some fungal clades may show similar trends due to logging regardless of location (McGuire et al., 2015).

Plant pathogens were distinctly in their abundance and composition between the logged and unlogged sites, and our data suggest that these differences are potentially linked to differences in the abundance of litter. For example, *Cylindrosympodium* was greater than twice as abundant in the unlogged watershed, and this genus was also greater than twice as abundant in the litter. Post clearcutting, the logged watersheds were burned, which would have significantly reduced the litter relative to the unlogged watershed which also would have had larger and older trees. We note here that FUNGuild (Nguyen et al., 2016) classified *Cylindrosympodium* as a pathogen, but the particular species in our data were not determinable with the exception of *C. lauri*, which was not common. It was

originally isolated from *Laurus* leaves and was assumed to be plant pathogenic (Crous et al., 2007), but we found no evidence that it has been tested for pathogenicity in any study. While some species of *Cylindrosympodium* are thought to be plant pathogens (Crous et al., 2007), others appear to be involved in decomposition (Purahong et al., 2016; Hernandez-Restrepo et al., 2017). Deeper study of this abundant genus is warranted as its abundance in litter and in both our study and others (e.g., Jumpponen et al., 2010; Silva and Lambers, 2021) suggests it is important at the ecosystem scale. The higher abundance of pathogens could make seed recruitment difficult within the unlogged watershed but may also help maintain tree diversity as has been found in tropical systems (Song et al., 2021).

We also saw differences between the individual logging treatments. The soil fungal communities of watershed 7 (selectively logged) were similar to watershed 6 (clearcut), while the litter fungal communities were similar to watershed 8 (old growth). This may indicate that below-ground soil fungi are more vulnerable to impacts from logging than fungi found in the litter later. Another explanation may be that the process of selectively harvesting in watershed 7, rather than clearcutting, allowed for persistence of the original above-ground fungal community. This is in contrast with the above-ground fungal community of watershed 6, which was likely wiped out when clearcut and burned with a novel assembly replacing it, perhaps representative of where the seedlings were sourced. These differences indicate that there are alternative states depending on the type of logging and regeneration strategy, and that above-ground and below-ground communities may exhibit different legacy responses (Rodriguez-Ramos et al., 2020). Even following planting, and a cessation of logging in watershed 6, 43 years before our

sampling there remains a legacy effect. This is indicative of potential changes in the function of the communities between logged and unlogged watersheds.

Changes to the soil fungal community can impede or facilitate forest regeneration through their interactions with plants as they establish and mature. The seedlings of ectomycorrhizal plant species can rely upon common mycorrhizal networks (CMN) to assist in their establishment (Liang et al., 2021). Access to CMNs give seedlings a steady flow of nutrients, and—depending on the fungi involved—a source of fixed carbon from mature conspecifics in the network (Walder et al., 2012). Changes to the saprotroph community can further influence access to nutrients by altering decomposition. Thus, changes in the composition of these two guilds due to logging can influence the accessibility of nutrients and carbon for trees during early regeneration. This can ultimately slow down the process of regeneration and the storage of carbon within a forest (Allison and Treseder, 2008).

4.2 Mineral soil vs. litter

Soil and litter fungi had different compositional trends across the logging gradient due to the strong segregation of fungal functional groups across these horizons. Higher relative abundances of pathogens in the litter layer are likely due to the presence of pathogens within senesced host material that falls and constitutes the Oi layer (Wahdan et al., 2020). We saw the opposite trend with ECMF. ECMF can break down organic materials, but they are not as efficient as independent saprotrophs (Talbot et al., 2008). This explains why ECMF were detected in greater relative abundances in the mineral soil, as they forage for nutrient pools, rather than in the litter layer where the early stages of decomposition occur (Lindahl et al., 2007). This tendency may also explain why there

does not seem to be a distinct difference between the ECMF found in mineral soil and litter layers—it appears that the genera in the litter samples are comprised of a subset of the genera found in the mineral soil samples. The ECMF sequences found in the litter layer may be the exploratory hyphae of the belowground mycelium searching for nutrient sources (Rosinger et al., 2018) or may be spores from fruiting bodies.

Saprotrophic fungal composition was also distinct between soil and litter, likely due to the different forms of organic material in the layers (Bödeker et al., 2016). The relative abundance of saprotrophs was also higher in litter samples. Early colonizers of senesced organic material are likely found in the litter layer, while those that are responsible for later stages of decay may be found in or near the mineral soil. ECMF and saprotrophs compete for the same organic resources, which further explains their spatial segregation (Talbot et al., 2008; Bödeker et al., 2016).

4.3 Soil eDNA vs. sporocarp sampling

Fungal sporocarps, e.g., mushrooms, are produced when sexual reproduction has occurred (Pelkmans et al., 2016), whereas fungal DNA in soils is primarily composed of asexual hyphae and asexual spores (Camenzind et al., 2022). The two datasets combined suggest that the 16,000 acres of the HJ Andrews Forest contain at least 524 species of macrofungi. Of these only a small subset of the soil eDNA dataset shared species with the compilation of sporocarp data from HJ Andrews (6.49%), which is somewhat less than the 10% overlap typically found between sporocarp and sequencing surveys (Dahlberg et al., 1997; Richard et al., 2005; Porter et al., 2008; Baptista et al., 2015). Interestingly, there were more macrofungi in collections/observations than in the eDNA, which is unusual. However, given that we surveyed a much larger area (the whole LTER) for

sporocarps than we collected soil eDNA from (several points in each of three watersheds), and the sporocarp surveys covered several years, the larger number of species in the sporocarp group is less surprising. It could be argued that we can't compare the two data sets, even qualitatively, because they were not taken in exactly the same places and at the same time. However, studies of fruiting body formation indicate that a combination of environmental signals trigger fruiting and that these signals are species-specific (Pelkmans et al., 2016). For this reason, it can take dozens of years or more to completely sample a plot in a forest. For example, a study in Switzerland by Straatsma et al. (2001) sampled mushrooms over a 21-year period in the same plots. Only 8/408 were found in all years and many only fruited once during the 21 years. Future studies would benefit from collecting and sequencing fruiting bodies as well as soils because that is the only way to directly and accurately compare the datasets, as identification errors can occur with morphological studies, fungal diversity is poorly documented, and species identification with high-throughput sequence data can be error-prone (Thines et al., 2018; Nilsson et al., 2019).

5. Conclusion

Our data show that logging can have a legacy effect upon fungal communities lasting decades, including guilds that are important for forest ecosystem processes. Planting following logging, as was done in our sites, may minimize some of these legacy changes, as there is less of a lag between the loss of hosts for the soil fungi and their reintroduction. However, planting may introduce novel fungi into the system, which can change the trajectory of regeneration for the fungal community and add its own legacy effect. Changes to the fungal community due to legacy effects can have consequences for

recovery following disturbance. Forest regeneration is an ongoing process with no real end—yet forests can reach stable states where the turnover of species is minimal. The plant communities of the forests in the Western Cascades can take decades or even more than a century following a disturbance to reach such a state depending on the severity (Franklin et al., 2002). Long term studies that track the regeneration of fungal communities in parallel to the regeneration of plants are severely lacking, limiting our understanding of how the communities influence one another. Further research is required to determine the impacts from the loss of particular fungal species on tree establishment and recruitment.

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BRIDGE I

In chapter II, I demonstrated that management has long term effects on soil microbial community assembly, but I did not link those changes to ecological functions. We can make assumptions about functional capacity using the taxa present; however, that is not equivalent to measuring a target function. In chapter II we investigate how variation in vineyard management intensity across several grape growing regions throughout Oregon influences microbial communities in soils, plant tissues, and wine. We then link community differences to variation in a function of interest: the production of organoleptic compounds during fermentation.

CHAPTER III

THE INFLUENCE OF MANAGEMENT ON THE MICROBIAL TERROIR AND METABOLOME OF WINE FROM OREGON VINEYARDS

Contains unpublished co-authored material (with K. L. Shek, K.L. McGuire, K.M. Meyer, J.B. Weisz, and G.V. Jones). Conception of project by Krista McGuire, Katherine Shek, Jeremy Weisz, Kyle Meyer, and Greg Jones. Field collection was performed by me, Kaye Shek, or Jeremy Weisz. Lab work was performed by me and Kaye Shek. Data analysis was performed by me or Kaye Shek. The manuscript writing is mine with additions by Kaye Shek. Critical revision was provided by Krista McGuire. Editing assistance provided by Kyle Meyer, Jeremy Weisz, Greg Jones.

1. Introduction

An important aspect of wine, both commercially and scientifically, is the concept of *terroir*. *Terroir* refers to the environmental factors that contribute to the phenotype of a crop, including climate, topography, soil characteristics, and processing methods (Van Leeuwen and Seguin, 2007; González-Barreiro et al., 2015; Lucini et al., 2020). Not until recently have researchers considered the influence of microbial communities—the metabolic engines driving fermentation and shaping organoleptic properties of wine—as part of wine *terroir* (Bokulich et al., 2014; Gilbert et al., 2014; Bokulich et al., 2016; Belda et al., 2017; Morrison-Whittle and Goddard, 2018). It is now widely accepted that microbes affect the winemaking process from start to finish beyond merely creating ethanol (Morrison-Whittle and Goddard, 2018). Within the vineyard diverse communities of fungi and bacteria associated with the *Vitis* plant can alter nutrient availability, increase tolerance to stressors such as drought, lower rates of pathogenic infections, and

regulate the synthesis of plant metabolites (Bagyaraj and Ashwin, 2017; Kundu et al., 2022).

After grapes are harvested from the vineyards the entire process of fermentation is driven primarily by communities of microbes (Morrison-Whittle and Goddard, 2018). While most modern wineries rely on commercial yeasts, typically *Saccharomyces cerevisiae*, many are beginning to adopt spontaneous fermentation methods (sometimes referred to as “natural” wines) or some combination of the two (Jolly et al., 2014). Spontaneous fermentation relies on inoculation of post-harvest grape must with microbes from the environment. The source of these “wild” microbes is thought to be a combination of grapes, other plant tissues, airborne dispersal in the vineyard and winery, harvesting and winery equipment, and staff (Morrison-Whittle and Goddard, 2018; Pinto et al., 2015). Despite this assumption, few studies have sought to quantify the relative contribution of potential inoculation sources to the fermentation of wine throughout the fermentation process, with most to date primarily focused on the grape microbiome (Bokulich et al., 2016; Gilbert et al., 2014; Vitulo et al., 2019).

Bacteria and fungi, particularly yeasts, present during fermentation can have a massive influence on the organoleptic properties of wine. Through their diverse metabolic capacities microbes can convert flavorless precursors within grapes into volatile compounds, such as terpenoids and thiols, which contribute to aroma and flavor (González-Barreiro et al., 2015; Parker et al., 2017). They can also synthesize metabolites *de novo*, including alcohols, esters, phenols, and organic acids. These organoleptic compounds associated with microbial metabolisms are core to what we understand as the basic palate of wine and can include compounds that are characteristic of different

varietals of wine (Bokulich et al., 2014; Anesi et al., 2015; González-Barreiro et al., 2015; Parker et al., 2017). Furthermore, different consortia of microbes present in wine can result in distinct flavor profiles, which indicates that understanding microbial *terroir* is part of understanding wine *terroir* (Jolly et al., 2014).

Understanding how different aspects of terroir influence microbial communities is relatively unexplored—especially the effects of management (Agarbati et al., 2019; Burns et al., 2016; Hendgen et al., 2018; Vitulo et al., 2019). The common agricultural practices of tilling, biocide application, irrigation, addition of chemical fertilizer, and others disrupt the physical and chemical aspects of an environment (Peterson et al., 2018). Understanding how management practices influence microbial communities in agroecosystems is essential for understanding the organoleptic properties of wine, and, importantly, for improving the resiliency of modern crop production to further disturbance. As it stands, modern agriculture is not sustainable into the future due to the reliance upon fossil fuels, limited quantities of mineral fertilizers, use of synthetic biocides, loss of topsoil to erosion, water scarcity, and the emergence of novel pathogens (Cordell and White, 2011; Foley et al., 2005; McDonald and Stukenbrock 2016). Sicrobes can help alleviate some of these issues by reducing the reliance upon non-organic inputs for fertilization and biocide use, tilling, and irrigation if given the chance (Garcia-Orenes et al., 2016; Peterson et al., 2018; Nosheen et al., 2021). The use of microbes to increase crop resiliency is of special interest to perennial crops such as *V. vinifera*, which has seen a recent surge in managers switching to more sustainable practices within the United States (Castellini et al., 2017; Baiano, 2021).

In the state of Oregon, USA there are approximately 1000 wineries across 21 distinct growing regions known as American Viticultural Areas (AVAs) and sub-AVAs. The AVA system assigns appellation of origin to regions of wine grape growing that share geographic, climatic, and geological aspects that contribute to a shared identity of *terroir*. Oregon AVAs encompass a wide range of climates in a gradient from warm and dry in the south to cold and wet in the north. There also exists a wide variety of management styles that range from high to low intensity and input. Many low intensity vineyards follow strict standards to gain certifications such as Demeter Biodynamic or LIVE Sustainable, whereas high intensity vineyards frequently apply chemical fertilizers, biocides, and practice tillage. Biodynamic vineyards are certified through the Demeter International standard while other vineyards are certified through LIVE, which is a Pacific Northwest organization that bases its program on international Integrated Production standards (Castellini et al., 2017). This makes Oregon a perfect natural laboratory to test for the simultaneous influences of climate and management on *terroir* and microbes within vineyards and wineries. We set out to answer three main questions: 1) How does management intensity alter microbial communities in vineyards independent of climate? 2) What is the relative contribution of different microbial sources (soil, bark, leaf, and grape) to wine fermentation communities? 3) Are detectable differences in fermentation communities correlated with differences in microbial metabolite diversity?

2. Materials and methods

2.1 Site descriptions

Our sites consisted of seven pairs of vineyards in four different American Viticultural Areas (AVAs) throughout western Oregon, USA. Therefore, we ensured that

every AVA we sampled included a minimum of one high intensity management site and one low intensity management site to account for variation in abiotic conditions. The AVAs we selected were Rogue Valley (two pairs of vineyards; n = 4), Willamette Valley (three pairs; n = 6), Columbia Gorge (one pair; n = 2), and Umpqua Valley (one pair; n = 2). These AVAs represent a temperature and precipitation gradient ranging from warm and dry in the southernmost region to cool and wet in the north. Average temperatures decrease and average precipitation increases from our southernmost vineyard in Rogue Valley (~42.3°N, 64cm rainfall, mean of 13.3°C) to the northernmost vineyard in Columbia gorge (~45.6°N, 110cm rainfall, mean of 8.9°C).

2.2 Sample collection and processing

At each vineyard we sampled within a singular block to minimize any influence from variation in rootstock, grape varietal, or soil characteristics between blocks. We chose blocks of Pinot noir where available, and syrah if not due to its ubiquitous nature in southern Oregon, to keep the varietal consistent between vineyards. Within the selected block we sampled five random vines and took one leaf, one cluster of grapes, one strip of bark approximately 15cm in length, and a 10cm deep by 2.5cm wide soil core near the base. Samples were collected using either EtOH sterilized gloves or EtOH sterilized soil cores and placed in a sterile whirlpak bag. Once collected, samples were placed on dry ice before being stored in a -20°C freezer until processing and extraction. A subset of the sampled vineyards (n = 10) also harvested, crushed, and fermented grapes from the blocks we targeted separate from the rest of their crop. They provided five samples from must (freshly crushed grapes) as well as five samples from the end of fermentation (~0°Bx). This allowed us to sequence and track microbial community contributions of

both bacteria and fungi from tissue to wine; additionally, it allowed us to perform metabolomic assays on the must and wine to link microbial community to changes in metabolic products.

Soils were hand homogenized using EtOH and UV sterilized sieves prior to extraction to ensure even sampling from within each entire core. We used approximately 250mg of each soil sample for extraction with Qiagen (Venlo, Netherlands) Dneasy PowerSoil Pro Kits. Leaf and bark samples were crushed in sterile mortars using liquid nitrogen. We stripped the skins from three randomly selected grapes from each cluster for extraction. We extracted DNA from all plant tissues with Qiagen Dneasy Plant Pro Kits using approximately 100mg of homogenized material per sample.

Must and wine samples were prepared using a modified protocol (Bokulich et al., 2014). We centrifuged 10 mL of each must and wine sample at 4,000xg before decanting. We then mixed and rinsed the resulting pellet with 1000uL of cold PBS, each rinse followed by centrifuging at 8,000xg and decanting. Samples were then immediately run through the Qiagen Dneasy Plant Pro Kits protocol. Additionally, three 1.5 mL technical replicates of must, fermenting wine, and bottled wine from ten of the vineyards (n = 30) were sent to the West Coast Metabolomics Center at the University of California, Davis for primary metabolite analysis using gas chromatography with time-of-flight mass spectrometry (GC TOF-MS).

2.3 Library preparation and sequencing

All samples were prepared for amplicon sequencing using a two-stage PCR protocol referred to as iTag: the first stage amplifies the genomic region of interest using forward and reverse primers attached to heterogeneity spacers to increase base-call

diversity within a sequencing run; the second stage ligates combinatorial dual indexes for multiplexing, as well as i5 and i7 adapters for binding to the Illumina NovaSeq flow cell. We used the *ITS1f* and *ITS2* primer pair to amplify the internal transcribed spacer (ITS) amplicon to characterize fungi, the *515F* and *806R* primer pair to amplify the 16S ribosomal RNA (16S) amplicon to characterize bacteria, and the *1333* and *1647* primer pair to amplify the 18S ribosomal RNA (18S) amplicon to characterize yeasts.

We carried out PCR1 in 20µl reactions composed of 2µl of genomic DNA, 0.5µl of 10µM forward primer, 0.5µl of 10µM, 10µl GoTaq Green MasterMix (Promega), 0.1µl of 100µg/µl bovine serum albumin (BSA), and 6.9µl nuclease free water. ITS and 18S PCR1 conditions: Denaturation of DNA at 94°C for 3 mins; 30x amplification cycles of 45 sec at 94°C, 1 min at 54°C and 90 sec at 72°C; finished with a 10 min extension at 72°C. 16S PCR1 conditions: Denaturation at 94°C for 3 mins; 30x amplification cycles of 45 sec at 94°C, 1 min at 50°C and 90 sec at 72°C; finished with a 10 min extension at 72°C.

We conducted PCR2 in 25µl reactions composed of 3µl PCR1 product, 2µl of barcoded primers, 10µl GoTaq Green MasterMix (Promega), 0.1µl of 100µg/µl BSA, and 9.9µl nuclease free water. Conditions: denaturation at 94°C for 3 mins; 12x amplification cycles of 45 sec at 94°C, 1 min at 52°C and 90 sec at 72°C; finished with a 10 min extension at 72°C. PCR2 products were sequenced on an Illumina NovaSeq 6000 at the Genomics and Cell Characterization Center (GC3F) at the University of Oregon. To obtain the longest reads available on the NovaSeq 6000 we used paired end 2x 150 base pair chemistry.

2.4 Sequence processing and analyses

We demultiplexed and removed primers from raw sequences using the *HTStream* pipeline (v.1.3.3; Settles, 2022). We then processed trimmed reads through the *dada2* pipeline (v.1.24.0; Callahan et al., 2016) in the R environment; this pipeline filters, denoises, and merges the reads, assigns taxonomy to each unique amplicon sequence variant (ASV), and constructs an ASV counts by sample matrix for each dataset—ITS, 16S, 18S. For taxonomic assignment we used >97% alignment with the UNITE database for our ITS sequence data (v9.0; Nilsson et al., 2018), the SILVA database for our 16S sequences (v138.1; Quast et al., 2013), and the PR2 database for our 18S sequences (v4.14.1; Gillou et al., 2013). To normalize our microbial and metabolomic data, we used the negative binomial variance stabilizing function of the DESeq2 R package (v1.38.3; Love et al., 2014). We did not rarify prior to normalization, as rare taxa are known to influence fermentations in other studies (Zhao et al., 2022).

All analyses were performed in the R environment (v4.2.2; R Core Team, 2021). We used the *vegan* R package (v2.6.4; Oksanen et al., 2022) to calculate Bray-Curtis dissimilarities between our samples. We then used *vegan* to run permutation analyses of variance (PERMANOVAs) based on those previously generated Bray-Curtis dissimilarity matrices. We created Euler diagrams using the *ps_euler* function in the *MicEco* R package (v0.13.1; Liu et al., 2021). We also used the fast expectation-maximization microbial source tracking (FEAST) R package (v0.1.0; Shenhav et al., 2019) to calculate relative contributions from source samples (soils and tissues) to sink samples (must). FEAST uses an expectation-maximization algorithm to determine the most likely ratio of source contributions on a specified sink. To test the significance in community variation between treatments we used analysis of compositions of microbiomes with bias

correction (ANCOM-BC, v1.6.4; Lin and Peddada, 2020). ANCOM-BC is a form of differential abundance analysis created to account for sampling biases inherent to microbiome amplicon datasets by estimating the sampling fraction which is the ratio of true abundance versus sample abundance based on the observed counts using a linear regression approach. We removed any taxa that had <10 reads in our total data set before the ANCOM-BC analysis as rare taxa can easily produce false positives. We then used the differential abundance analysis function in DESeq2 to identify metabolites within Pinot noir must and wine samples that were significantly different between management types. In our analyses we used the metabolites identified by DESeq2 that were significant ($p < 0.05$ and $FDR < 0.05$), named, and had a $|\log_2|$fold change of ≥ 1. The *ggplot2* package (v3.4.0; Wickham, 2016) was used to generate figures.

3. Results

3.1 Sources of microbial taxa in grape must

Using the source tracking abilities of the FEAST package we found that within our data the majority of ITS and 16S ASVs from fresh must samples are likely derived from grape samples or “unknown” sources (Figures 1 and 2 respectively). Grapes had a mean contribution of 31% for fungi and 68% for bacteria while unknown had a mean contribution of 48% for fungi and 20% for bacteria. Additionally, based on the FEAST results and Euler diagrams using presence / absence, wine samples share fungal and bacterial taxa with all the tissues that we sampled as well as soil (Figure 3). 18S showed a similar pattern using the Euler diagram approach, though we were unable to run source tracking on that dataset due to the low richness of ASVs in the data.

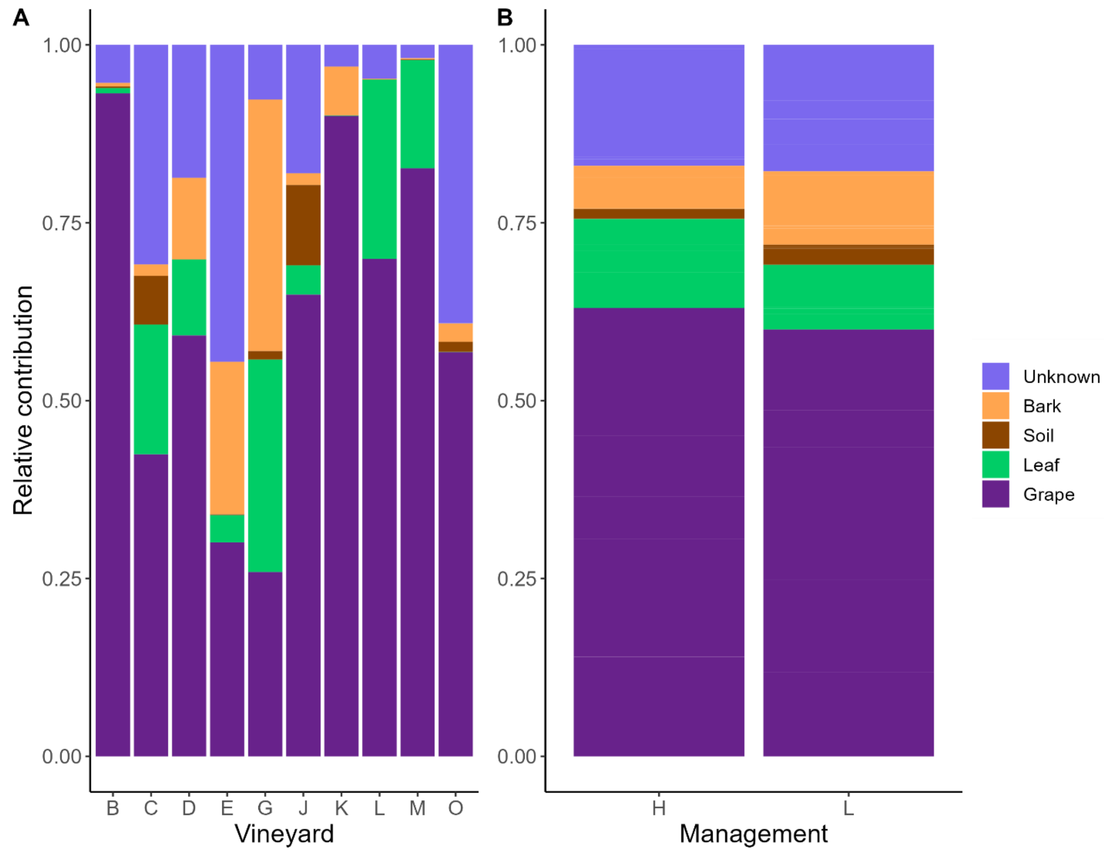

Figure 1. Relative contributions of source samples (bark, soil, leaf, grape, unknown) to sink samples (must) for bacteria using the 16S amplicon in Oregon, USA vineyards. Relative contributions were calculated using the fast expectation-maximization microbial source tracking (FEAST) package for R. **(A)** = individual vineyards; **(B)** = average by management (H for high intensity, L for low intensity).

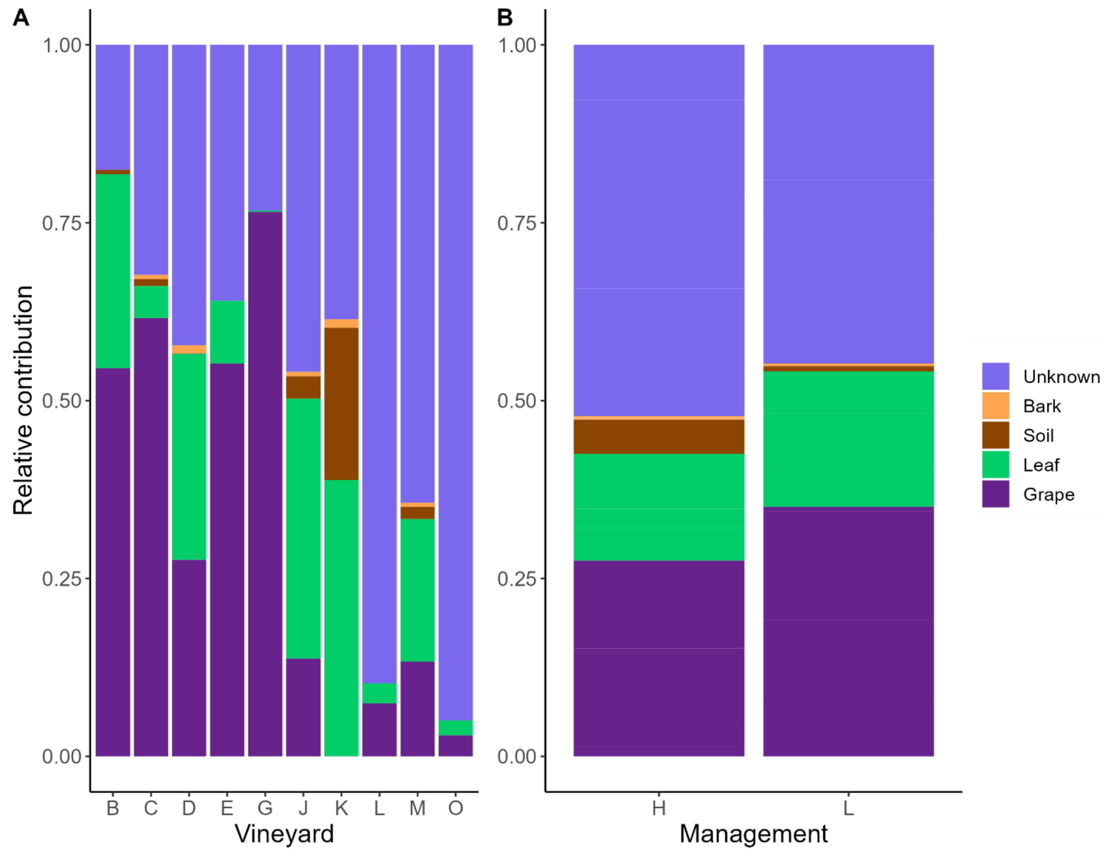

Figure 2. Relative contributions of source samples (bark, soil, leaf, grape, unknown) to sink samples (must) for fungi using the ITS amplicon in Oregon, USA vineyards. Relative contributions were calculated using the fast expectation-maximization microbial source tracking (FEAST) package for R. **(A)** = individual vineyards; **(B)** = average by management (H for high intensity, L for low intensity).

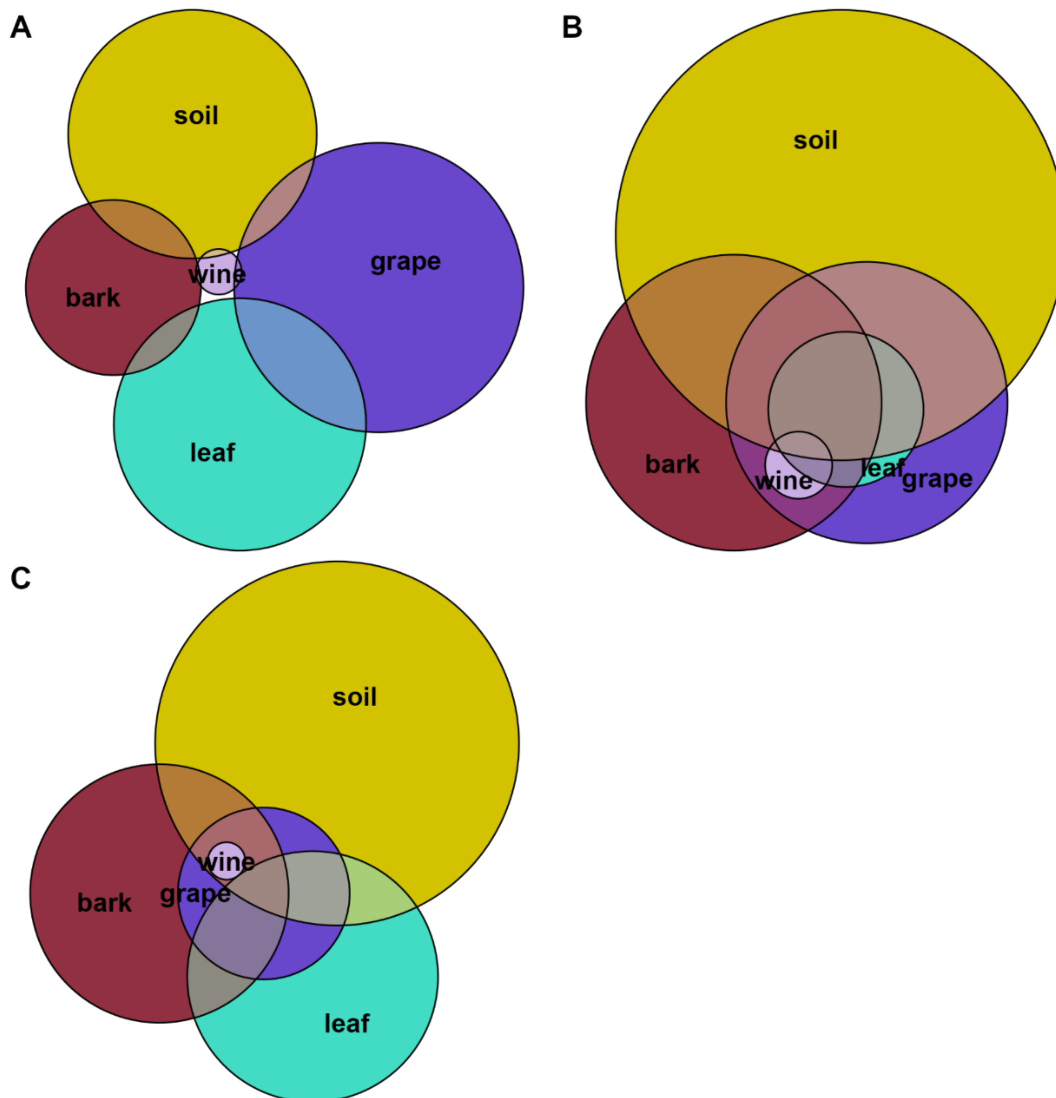

Figure 3. Euler diagrams showing overlap of microbial amplicon sequence variants (ASVs) between sample types taken from vineyards within Oregon, USA. The "wine" category is a mix of must samples and fermented samples. **(A)** = fungi (ITS), **(B)** = bacteria (16S), **(C)** = yeasts (18S).

3.2 Influence of management and American Viticultural Area on microbial taxa

Management and AVA had a significant effect, including a strong interaction between the two, on the composition of fungi, bacteria, and yeasts when considering each sample type separately (supplemental table 1). Euler diagrams based on the presence / absence of ASVs within our data showed several patterns of note. When considering AVAs, ITS and 18S data show a distinct geographic pattern with geographically adjacent AVAs and sub-AVAs overlapping, while there is little to no overlap between distant AVAs (Supplemental Figure 1). This is in contrast with the 16S data where there was overlap between all AVAs, regardless of geographic distance. All three amplicons showed large differences in ASVs between high and low management regardless of the varietal (Figure 4). Based on ANCOM-BC, four genera and eight species of fungi identified via ITS were differentially abundant within soil (Figure 5) and only one genus within wine. Two genera of bacteria were differentially abundant in soils and none in wine. No genera or species were differentially abundant for our 18S data.

and fermented samples. **(A)** = fungi (ITS), **(B)** = bacteria (16S), **(C)** = yeasts (18S).

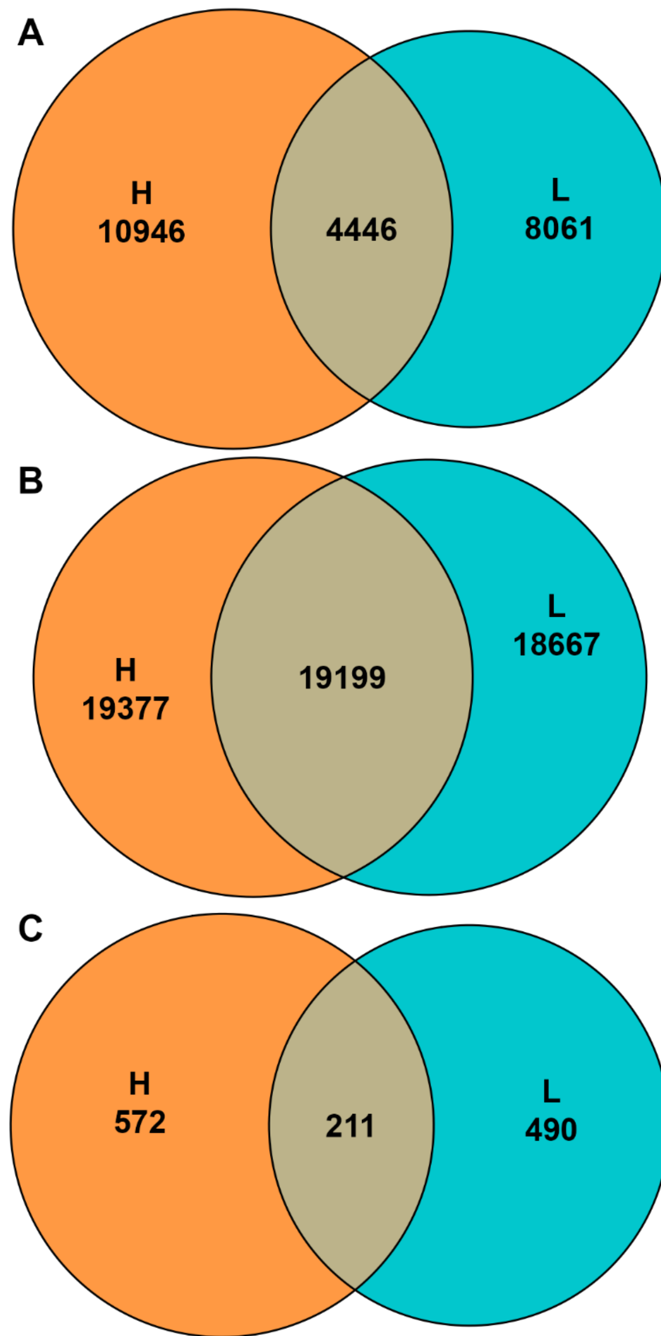

Figure 4. Euler diagrams showing overlap of microbial amplicon sequence variants (ASVs) between vineyards within Oregon, USA with high (H; n = 7) or low (L; n = 8) intensity management practices. **(A)** = fungi (ITS), **(B)** = bacteria (16S), **(C)** = yeasts (18S).

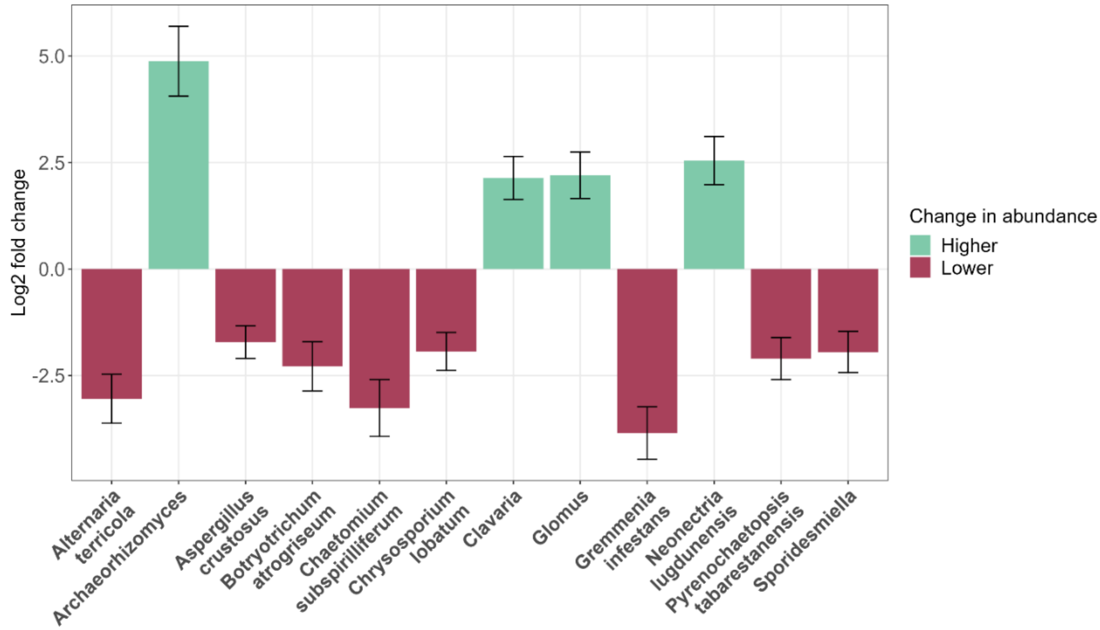

Figure 5. Differential abundance of fungal species and genera comparing the change in low management intensity vineyards relative to high intensity vineyards in Oregon, USA. Log fold change was calculated using analysis of compositions of microbiomes with bias correction (ANCOM-BC, v1.6.4; Lin and Peddada, 2020).

3.3 Changes in metabolites between treatments

Our differential abundance analysis on the metabolomic data of Pinot noir samples revealed named metabolites that are significantly different (adjusted p-value or FDR < 0.05) based on management intensity in fermented (Figure 6) and bottled (Figure 7) wine. In fermented wine we found ten metabolites that were more abundant in high intensity samples, and twelve metabolites more abundant in low intensity samples. In bottled wine we found eight metabolites more abundant in high intensity samples, and eighteen metabolites more abundant in low intensity samples.

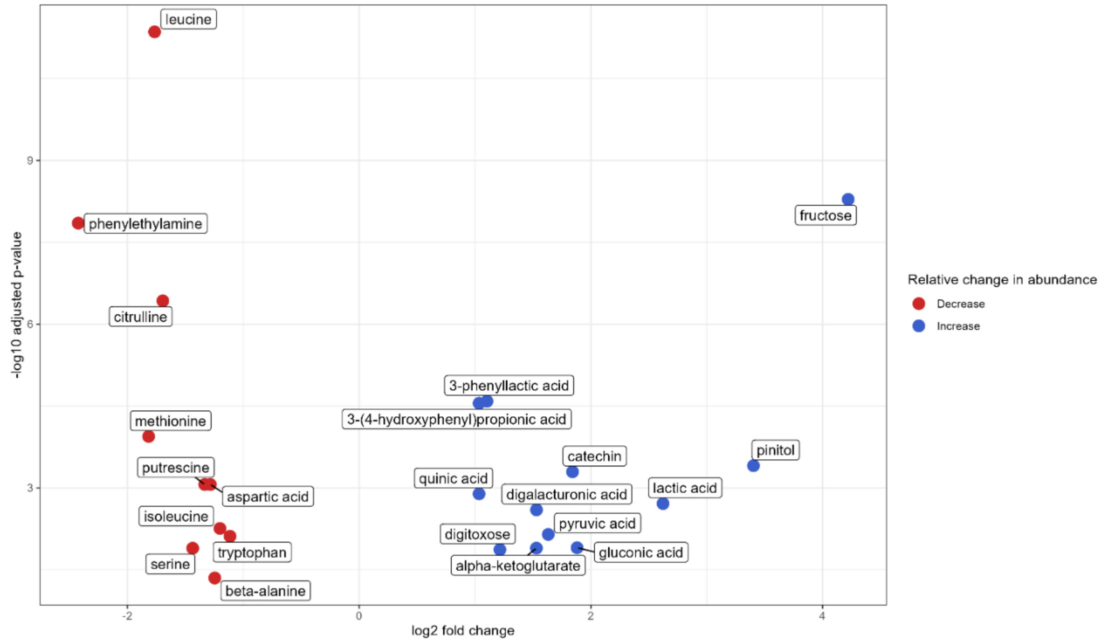

Figure 6. Volcano plot of differentially abundant metabolites in wine at the end of fermentation from Pinot noir grapes in Oregon, USA. Log_2 fold change is comparing the change in abundance of metabolites from low management intensity vineyards relative to the abundance in high management intensity vineyards. Adjusted p-value is also known as the false discovery rate (FDR). Blue indicates a greater abundance in low management intensity samples, red indicates a greater abundance in high management intensity samples. The cutoff for inclusion of metabolites is adjusted p-value < 0.05 and $|\log_2$ fold change$| \geq 1$.

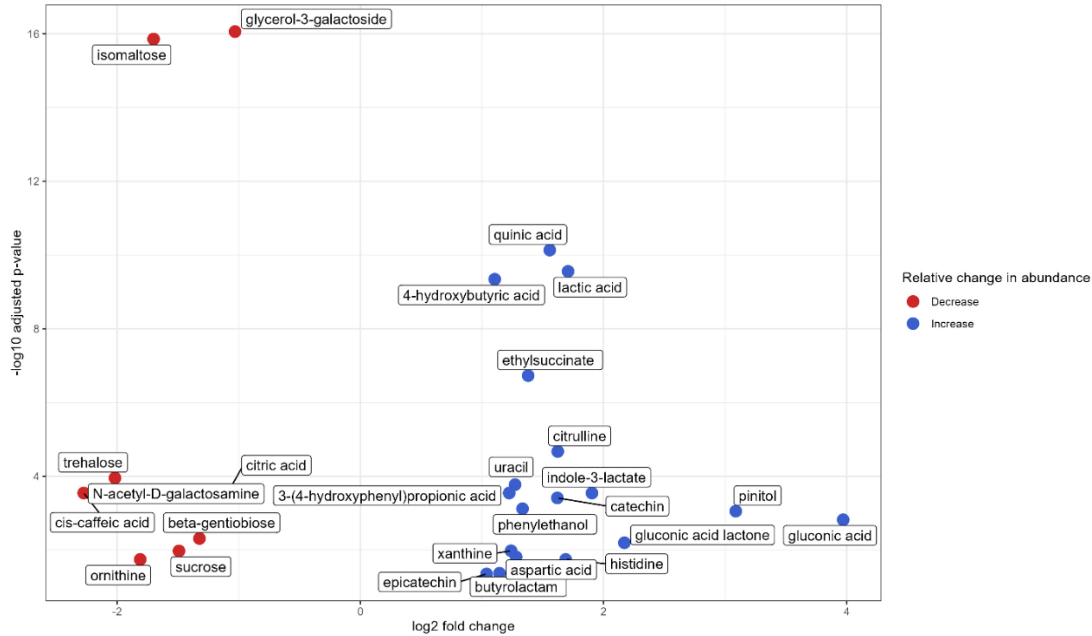

Figure 7. Volcano plot of differentially abundant metabolites in bottled wine from Pinot noir grapes in Oregon, USA. Log_2 fold change is comparing the change in abundance of metabolites from low management intensity vineyards relative to the abundance in high management intensity vineyards. Adjusted p-value is also known as the false discovery rate (FDR). Blue indicates a greater abundance in low management intensity samples, red indicates a greater abundance in high management intensity samples. The cutoff for inclusion of metabolites is adjusted $p\text{-value} < 0.05$ and $|\log_2 \text{fold change}| \geq 1$.

4. Discussion

4.1 Taxa present in wine must and their sources

We found that the primary source of microbial taxa in the fresh must is from grapes and unknown sources, implying that the grape microbiome is the most important to manipulate for fermentation purposes. However, our results also show that leaves and bark are sources of bacterial or fungal inoculum. These findings indicate that surveying the grape microbiome does not capture the full set of microbial taxa that can disperse into and influence the fermentation. Additionally, due to the high contribution of unknown

sources to must communities in our study, further sampling of alternative sources is necessary. These "unknown" sources are likely airborne dispersal, contact with equipment, and contact with personnel (Whittle and Goddard, 2017; De Roos et al., 2019). It is surprising that soil was estimated to contribute very little to must, as the overlap in ASVs between soil, plant tissues, and wine samples was large. This result indicates that while soil may not directly inoculate the must it may act as a refugia for microbial taxa during the yearly dormancy *V. vinifera* experiences during winter. Fungi and bacteria can lie in dormancy within soils for varying lengths of time, even if their typical substrate or host is not present (Warren et al., 2019; Joergensen and Wichern, 2018).

4.2 Microbial communities in high vs. low intensity vineyards

Our microbial data revealed that there were significant compositional differences between management intensities for soil, wine, and all plant tissues demonstrating that management influences microbial *terroir*, which provides a basis for the variation that we see in organoleptic compounds. While compositions were significantly different, the signal seems to be weak at a genus and species level based on differential abundance analysis. This indicates that any differences in composition are primarily due to species that are rare within our dataset. The one exception to this is soil fungi where the mycorrhizal genus *Glomus*, and several genera of pathogens and saprotrophs varied based on management. Multicellular fungi tend to have slow growth cycles relative to bacteria and single cell fungi, this makes them particularly susceptible to physical disturbances like tilling (Shi et al., 2019). *Glomus* and other arbuscular mycorrhizal fungi (AMF) are also composed of long, multi-nucleate hyphae. This physical structure likely explains

why *Glomus* was higher in abundance in low intensity vineyard soils (van der Heyde et al., 2017). AMF aid *V. vinifera* vines with nutrient uptake, water stress, and protection from plant pathogens (Jayne and Quigly, 2014; Bagyari and Ashwin, 2017). Higher abundance of *Glomus* signals that low intensity vineyards may be more resilient in the face of outside disturbance. Of the plant pathogens highlighted in our differential abundance analysis, the species *Neonectria lugdunensis* was the only one of major concern to vineyards. *Neonectria* was higher in abundance in lower intensity vineyards likely due to limited use of fungicides. *Neonectria* is part of suite of fungi that cause "black foot disease" which blocks vascular tissues and can kill vines under water stress (Agustí-Brisach and Armengol, 2013).

We found that the most abundant bacterial genera and fungal taxa tied to fermentation and organoleptic compound production did not vary between management intensity, implying that variation in metabolites are due to less common taxa or intraspecific variation. The relevant yeasts we found include several *Candida* spp., *Pichia* spp., *Metschnikowia* spp., *Saccharomyces* spp., *Schizosaccharomyces japonicus,* and *Wickerhamomyces* spp. The relevant bacteria in our data include *Acetobacter* spp., *Gluconobacter* spp., *Lactobacillus* spp., and *Oenococcus oeni*. None of these genera or species were significantly different between management intensities. However, some were excluded from the differential abundance analysis as they were rare within our dataset. This further supports that differences within our metabolomic data are due to rare taxa or variation in the chemical composition of the must prior to fermentation. Alternatively, differences in the organoleptic metabolites may be due to intraspecific variation. Most bacterial and fungal taxa have highly modular genomes and metabolisms,

which could explain differences in concentrations of metabolites associated with microbial metabolisms (Legras et al., 2018; McCarthy et al., 2019). Additionally, higher disturbance intensity can lower genetic diversity and cause functional differences between sites (Banks et al., 2013) Untargeted genomic sequencing is required to further investigate this possibility in the future.

4.3 Connecting management, microbial communities, and metabolites

We found a higher abundance of amino acids in high intensity wine ferment samples, perhaps due to higher levels of nitrogen in vineyards receiving chemical fertilizers (Ough and Bell, 1980; Smit et al., 2014; Döring et al., 2022). Amino acids can influence the flavor of wine by being catabolized into alternative compounds such as higher alcohols and carboxylic acids via transamination (Styger et al., 2011). The three primary amino acids in wine involved with these transformations are leucine, isoleucine, and valine—two of which are more abundant in our high intensity ferment samples, leucine and isoleucine (Styger et al., 2011). Within our data, higher concentrations of bacteria or yeast-derived carboxylic acids and higher alcohols were almost exclusively found in low intensity samples, except for citric acid in bottled high intensity wine. This seems counterintuitive, as amino acids were higher in the high intensity ferment. However, it may indicate that the "maturity" of the fermentation was further along in the low intensity wine samples and any free amino acids were already consumed. This explanation is supported by the higher concentration of α-ketoglutarate in low intensity ferment samples which is made in the first step of the transamination reaction. Another explanation may be that the yeasts present in high intensity fermented samples were not as effective at creating these compounds, as the amino acids can be used in other

pathways and protein synthesis (Ough et al., 1980; Styger et al., 2011). The compounds can also be made as a byproduct of glycolysis, avoiding the requirement of amino acids (Styger et al., 2011).

We also saw significant variation in organic acids between management intensities in both fermented and bottled wine samples. Organic acids play a key role in the organoleptic properties of wine. Being acids, many of them increase the perception of "sourness" in wine (Chidi et al., 2018; Valerio et al., 2004; Vicente et al., 2022). Sourness can take on different forms (i.e., vinegar versus lemon juice) which can add complexity and depth to a wine's palate depending on the identity and relative concentration of the acids. Two of the acids in our data come exclusively from bacterial and yeast metabolic processes (lactic acid and phenyllactic acid) two are exclusively from plant processes and three from either plant or microbial sources (citric acid, propionic acid, and pyruvic acid) which indicates strong microbial influence on the acid profile (Bauer et al., 2018). Most of these acids saw an increase in low intensity wine samples; all except caffeic acid and citric acid in the bottled wine samples. These variations in acid profile suggest that the perception of acidity and acid-associated flavors is different between high and low intensity wine in our study.

Several other miscellaneous bacteria and yeast derived metabolites highlighted by our analyses imply that low intensity bottled wine may possess stronger notes of fruit and flowers, while the high intensity bottled wine may possess a more complex bitterness—all of which are favorable in Pinot noir (Singleton and Ouch, 1962; MacNeil, 2022). These miscellaneous organoleptic relevant metabolites include the disaccharides trehalose and gentiobose which are produced via microbially derived enzymes acting on

carbohydrates present in must (Ruiz-Matute et al., 2009; López et al., 2018). These disaccharides are typically bitter and may add to the complexity of flavor. Disaccharides are higher in the high intensity bottled wine which indicates the presence of yeasts that produce more β-glycosidases and β-xylosidases or a greater abundance of complex carbohydrates (López et al., 2018). The only significantly different higher alcohol is phenylethanol, which is higher in our low intensity bottled samples. Phenylethanol confers a floral or fruity character to wine and is produced via yeast metabolism (Jolly et al., 2014). Higher concentration of phenylethanol is associated with the presence of non-*Saccharomyces* yeasts, such as *Hanseniaspora, Pichia*, *or Wickerhamomyces*, all of which were present in our wine samples (López et al., 2018). Ethyl succinate is the only ester we found varied between our samples with greater abundance in low intensity bottled samples. Esters within wine are perceived as fruity and are created through the metabolisms of lactic acid bacteria or yeast (Lasik-Kurdyś et al., 2018; Garde-Cerdán et al., 2021). Except for the disaccharides these compounds are higher in abundance in low intensity bottled samples.

5. Conclusions

Our data indicates that management intensity in vineyards has an influence on microbial community composition; furthermore, the differences in microbial composition are correlated to changes in metabolites related to the organoleptic properties of wine. As the wine industry is one that sells a product focused on flavor and aroma, understanding the nuances of variation in organoleptic compounds is important. While we cannot make judgements upon “quality”, as that is a deeply subjective concept, our results can inform winemakers of potential changes to expect when shifting management styles. However,

these results might not be the same for other varietals, as our metabolite analysis was entirely focused on Pinot noir samples. Future data on other varietals is essential to understanding whether the patterns in our data are consistent. Additionally, variation in the function of organoleptic compound production during fermentation seems to be linked to differences in rare microbial taxa rather than those more abundant. This reflects the72ominationn of fermentation by a select few species with rather cosmopolitan distributions. Grape must and wine are extremely harsh environments that favor a narrow range of microbial taxa. Any rare taxa or genotype that survives the beginning of fermentation can leave a unique signature on the final metabolome and organoleptic characteristics. Further study of rare or ”inconsequential” taxa in fermentations may lead to greater insight into the microbial contribution upon *terroir*.

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BRIDGE II

I demonstrated in chapter III that management intensity can significantly alter the compositions of bacterial and fungal communities present within vineyards and fermentations; furthermore, variation in the microbial communities was linked to changes in the organoleptic properties of fermented wine. In chapter IV we will explore how changes to management intensity can rapidly influence microbial communities within vineyard soils and the implications of those effects.

CHAPTER IV

THE INFLUENCE OF MANAGEMENT ON THE MICROBIAL TERROIR AND METABOLOME OF WINE FROM OREGON VINEYARDS

Contains unpublished co-authored material (With Blaine Pappa and Krista McGuire). Conception of project by myself and Krista McGuire. Fieldwork performed by me and Blaine Pappa. Molecular lab work performed by myself or Blaine Pappa under my supervision. Analysis by me or Blaine Pappa under my supervision. The writing is mine with critical revision by Krista McGuire.

1. Introduction

Wine is a multibillion-dollar international industry and commodity that plays a key role in cultural identity for many regions. Cultivating grapes (*Vitis vinifera*) for that industry, like all agricultural industries, is facing a growing concern of security in the face of threats such as climate change, topsoil erosion, dependence on chemical fertilizers, dependence on biocides, and water scarcity (Foley et al., 2005; McDonald et al., 2016; Peterson et al., 2018). Grape vines are particularly susceptible to these threats due to their perennial nature and long production lifespans. It takes up to three years for a *V. vinifera* vine to produce its first harvest and a vine can continue to produce fruit for up to fifty years (Strik, 2019). Because of this reality, grape producers must consider both short and long-term strategies for increasing crop security. Many have begun to adopt methods focusing on sustainability in a bid to increase the resistance and resiliency of their vines by treating their vineyards as ecosystems (Castellini et al., 2017; Nosheen et al., 2021). A key focus of modern sustainable agricultural movements falls under the umbrella of “soil health”. This nebulous concept shares common target parameters across

movements, including increasing biodiversity of microbes and invertebrates, increasing soil organic matter, increasing nutrient availability, as well as maximizing the benefits of other physical and chemical parameters (Tahat et al., 2022).

Most of the various aspects of soil health are invariably linked to and driven by the ecological functions of plants and microbial communities. Plants are the primary source of organic inputs into soil through the shedding of litter, release of exudates, and death. Those inputs can alter the chemistry of the soil and increase concentrations of carbon and nutrients (van der Putten et al., 2013). Soil microbial communities feed upon these inputs, breaking them down and freeing up recalcitrant nutrients (van der Putten et al., 2013). A group of mutualistic fungi known as mycorrhizal fungi can further increase plant access to nutrients by shuttling nitrogen and phosphorus to their hosts in exchange for carbon (Bagyari and Ashwin, 2017; Rillig et al., 2023). Furthermore, mycorrhizal fungi can reduce water stress by increasing the surface area of roots and preventing water loss to the soil (Kamayestani et al., 2019). Mycorrhizal fungi and saprotrophic, or decomposer, fungi also compete with pathogens for resources and host access, which can reduce rates of infections (Kardol et al., 2015). Some bacterial taxa can also fix nitrogen from the atmosphere creating bioavailable forms that can be accessed by other organisms including crop plants. Microbial biomass also tends to be recalcitrant, increasing the organic matter in the soil and acting as a carbon sink for carbon sequestration. Additionally, from a winemaking perspective, soil can act as a refugia for fungi and bacteria that are associated with fermentation (Setati et al., 2012). Many bacteria and fungi can enter states of dormancy for long periods of time (Joergensen and Wichern, 2018; Warren et al., 2019). Dormancy strategies include creating spores as well as

creating compounds that help resist environmental variation or attacks from other microbial taxa (Kearns and Shade, 2018). This allows bacteria and fungi to survive through the winter and persist year to year to act as a source of inoculum.

Despite their importance to all aspects of soil health and fermentation, little is known about the influence of management practices upon the composition and functioning of microbial communities within vineyards. Studies have found that the use of high intensity management practices such as tilling, biocide application, and chemical fertilizer application reduces the diversity and abundance of bacteria and fungi within agroecosystems (Kihara et al., 2012; Agarbati et al., 2019; Barreiro et al., 2022); furthermore, low intensity practices encourage microbial diversity and abundance, leading to soil that is more resistant to disturbances (Thiele-Bruhn, et al., 2012; Garcia-Orenes et al., 2016; Chen et al., 2020). We worked with a vineyard that shifted from high intensity to low intensity management practices in 2018 to see if there were detectable differences in soil fungi and bacteria by the 2021 harvest season. We wanted to address three main questions using the dataset: 1) are there detectable changes in communities of soil bacteria and soil fungi following a dramatic change in management strategy? 2) if there are changes, how quickly do they occur and are they temporally stable? and 3) how much intra-vineyard variation exists in communities of soil bacteria and soil fungi between blocks of the same grape varietals?

2. Methods

2.1 Site description

Troon Vineyard (42°18’18”N, 123°13’15”W) is located within the Applegate Valley, a sub-American Viticultural Area (AVA), nestled within the Rogue Valley AVA

of southern Oregon, USA. AVAs are a system of appellation in the USA that group vineyards with shared climate, geology, and other physical characteristics that sum up to a shared identity. The Applegate Valley AVA is situated around 500m above sea level on south facing slopes between the Cascade Mountains and the Siskiyou Mountains. This results in a warm-summer Mediterranean climate with a mean annual temperature of 12.5°C and a mean annual precipitation of 50-65cm. Troon has a unique management history. The first vines were planted in 1972, and until 2018 Troon used a high intensity "conventional" approach to management—including biocide, tilling, and chemical fertilizer use. After 2018, the vineyard began using a low intensity "biodynamic" approach. As of 2023, Troon is both Demeter Biodynamic certified and Regenerative Organic Certified, indicating practices that includes permaculture crop cover, presence of livestock, organic only inputs of fertilizer, no tilling, and extremely limited biocide use (Castellini et al., 2017; Regenerative Organic Certified, 2019).

2.2 Sample collection and processing

All sampling was performed during the harvest season on October 15th, 2021. To test for variation between blocks of vines we selected and sampled the two most common varietals grown by Troon, syrah (n = 4) and vermentino (n = 3). Within each block we took a 10 cm deep by 2.5 cm radius soil core using an EtOH sterilized soil auger within 0.5 m of the base of five random vines from five random rows (n = 5 per block). Row and vine were picked via a random number generator. Samples were placed on dry ice for transit and stored at -20°C at the University of Oregon until processing. We hand homogenized the soils using EtOH and UV sterilized gloves and scoops to increase

sampling evenness in each core. We then used approximately 250mg of each sample for extraction with Qiagen DNeasy PowerSoil Pro Kits.

2.3 Molecular methods

All 2021 samples were prepared using a two-stage PCR protocol known as iTag. The first stage uses paired forward and reverse primers attached to heterogeneity spacers to amplify regions of interest within the genome; the second stage ligates combinatorial dual indexes for multiplexing, as well as i5 and i7 adapters for binding to the Illumina NovaSeq flow cell. We characterized fungi using the *ITS1f* and *ITS2* primer pair to amplify the internal transcribed spacer (ITS) region, and bacteria using the *515F* and *806R* primer pair to amplify the 16S ribosomal RNA (16S) region.

We carried out PCR1 in 20µl reactions composed of 2µl of genomic DNA, 0.5µl of 10µM forward primer, 0.5µl of 10µM, 10µl GoTaq Green MasterMix (Promega), 0.1µl of 100µg/µl bovine serum albumin (BSA), and 6.9µl nuclease free water. ITS PCR1 conditions: denaturation of DNA at 94°C for 3 mins; 30x amplification cycles of 45 sec at 94°C, 1 min at 54°C and 90 sec at 72°C; finished with a 10 min extension at 72°C. 16S PCR1 conditions**:** Denaturation at 94°C for 3 mins; 30x amplification cycles of 45 sec at 94°C, 1 min at 50°C and 90 sec at 72°C; finished with a 10 min extension at 72°C.

We then carried out PCR2 in 25µl reactions composed of 3µl PCR1 product, 2µl of barcoded primers, 10µl GoTaq Green MasterMix (Promega), 0.1µl of 100µg/µl BSA, and 9.9µl nuclease free water. Conditions: denaturation at 94°C for 3 mins; 12x amplification cycles of 45 sec at 94°C, 1 min at 52°C and 90 sec at 72°C; finished with a 10 min extension at 72°C.

2.4 Timeseries data

Previous soil microbial data from the 2018, 2019, and 2020 harvest seasons were provided by Biome Makers and Troon. The data from these samples were originally used to look at the microbial profile of the soil for management purposes. 2018 was the last year that Troon used high intensity management practices before switching to Demeter Biodynamic and Regenerative Organic practices. For each block included in the timeseries data, three "spoonfuls" of soil were collected by Troon in a sterile tube, repeated three times per block. Samples were sent to WineSeq (now part of Biome Makers) for DNA extraction, amplification, and sequencing on an Illumina MiSeq. Collection and processing methods are described in further detail in Belda *et al.,* 2017. We ran the raw FASTQ files through the same pipelines as our 2021 data to reduce variation due to data processing technique. We set them to an equal truncation length as our data (200 bp) and merged the sequences before removing chimeras and assigning taxonomy. We then subset the samples to contain only syrah and vermentino blocks to match our 2021 data. This data includes 17 syrah samples (two from 2018, nine from 2019, six from 2020) and 14 vermentino samples (four from 2018, six from 2019, and four from 2020).

2.5 Sequence processing and analyses

We demultiplexed and removed primers from raw sequences using the *HTStream* pipeline (v.1.3.3; Settles, 2022). We then processed trimmed reads through the *dada2* pipeline (v.1.24.0; Callahan et al., 2016) in the R environment; this pipeline filters, denoises, and merges the reads, assigns taxonomy to each unique amplicon sequence variant (ASV), and constructs an ASV counts by sample matrix for each dataset—ITS,

16S, 18S. We then assigned taxonomy with a cutoff of 98% similarity. For taxonomic assignment we used the UNITE database for our ITS sequence data (v9.0; Nilsson et al., 2018), the SILVA database for our 16S sequences (v138.1; Quast et al., 2013), and the PR2 database for our 18S sequences (v4.14.1; Gillou et al., 2013).

All analyses were performed in the R environment (v4.2.2; R Core Team, 2021). We created Euler diagrams to reveal the overlap of shared microbial taxa between factors using the *MicEco* package (v0.13.1; Liu et al., 2021). To normalize our microbial data for comparisons between varietals and blocks in our 2021 samples we used the negative binomial variance stabilizing function of the DESeq2 R package (v1.38.3; Love et al., 2014). We used the *vegan* package to calculate Bray-Curtis dissimilarity matrices and ran permutation analyses of variance (PERMANOVAs) on the resulting matrices.

To normalize our microbial data for timeseries analysis we performed a centered log ratio transformation and then detrended the result, also known as regression normalization (Coenen et al., 2020). We excluded any taxa that were not present in a minimum of 80% of samples or had an average relative abundance of less than 0.5% of the community for the rhythmicity analysis. We then used a combination of the R packages *RAIN* (Rhythmicity Analysis Incorporating Non-parametric methods, v1.30.0; Thaben and Westermark, 2014) and *BiomeHorizon* (v1.0.0; Fink, 2023) to test for statistically relevant rhythmic patterns in microbial community composition and to create longitudinal horizon plots for individual taxa across the four years of sampling. Originally developed for analyzing circadian rhythm transcriptomic data, *RAIN* can be used for any longitudinal or independent repeated measurement dataset. Traditional longitudinal statistics, such as Fourier, are often difficult to apply to large biological

datasets (Thaben and Westermark, 2014; Coenen et al., 2020). These datasets typically do not match traditional distribution families, even once log transformed, contain many structural zeroes, and can contain large amounts of noise (Coenen et al., 2020). Unlike other methods, *RAIN* is non-parametric and allows for independently rising and falling datasets that do not fit frequency patterns (e.g., sine, cosine). *RAIN* uses a rank test based on Jonckheere-Terpstra that detects monotonic slopes between rising and falling sections of data regardless of shape or symmetry. The primary assumption of *RAIN* is that the data is stationary (i.e., has the same mean sequences across all samples). This can be an issue if there is seasonality in sampling, or if sequencing runs have dramatically different performance. Our analysis attempts to avoid this issue in two ways: first, samples from all years were taken during the harvest season in the vineyard to avoid seasonal effects; second, the normalization method we used stabilizes the means across samples (Coenen et al., 2020).

3. Results

3.1 Inter-varietal and inter-block variation in soil microbial community composition

Neither varietal nor block had a significant effect on the community composition for our 2021 data ($p > 0.5$ for both) and there was no significant interaction between the two. Furthermore, soil microbial communities showed a large amount of overlap between all vermentino and syrah samples from all four years (Figure 1) out of 1,417 fungal taxa 767 were shared, 292 were unique to vermentino, and 358 were unique to syrah. For bacteria, out of a total of 953 taxa 651 were shared, 163 were unique to vermentino, and 139 were unique to syrah.

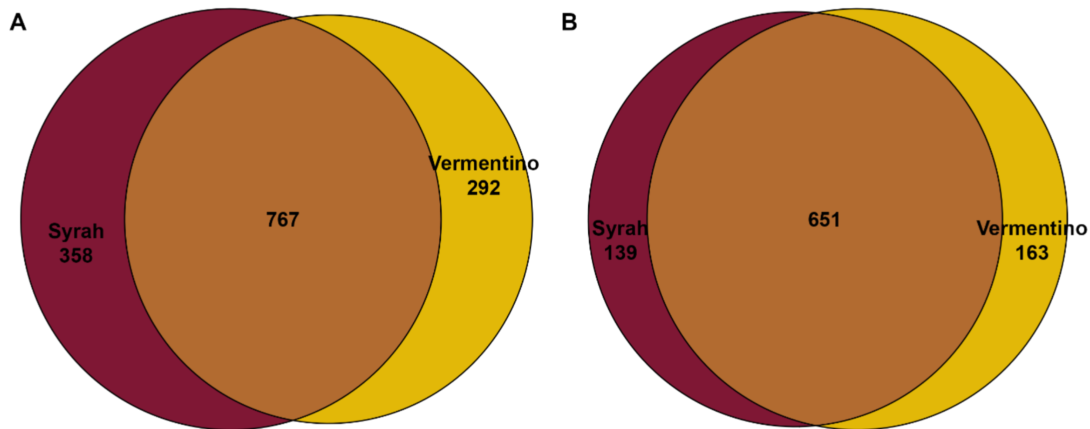

Figure 1. Euler diagrams of taxonomic overlap in soil microbial communities between blocks of vermentino (yellow) and syrah (red) wine grape varietals. The values are of ASVs identified to species and agglomerated at the species level. **(A)** overlap of fungal taxa using ITS sequencing. **(B)** overlap of bacterial taxa using 16S sequencing.

3.2 Time series of soil microbial communities in vermentino and syrah

There were many unique fungal and bacterial taxa in each year of syrah and vermentino soil samples (Figure 2). Our 2021 samples appear to have the largest number of unique taxa for both bacteria and fungi with 155 unique bacteria and 550 unique fungi—85% and 96% of 2021 taxa respectively. 2018-2020 showed far more overlap in taxa, but each year still had between 22% and 45% unique taxa for fungi and bacteria.

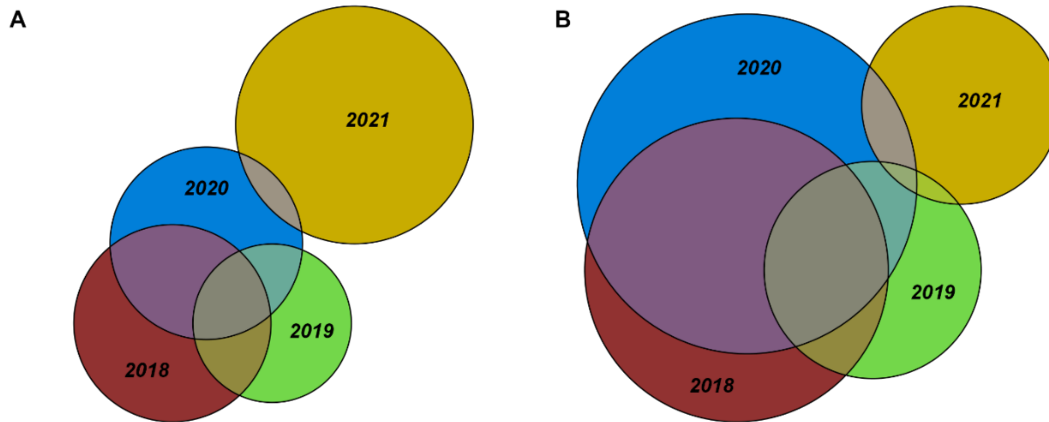

Figure 2. Euler diagrams of taxonomic overlap in soil microbial communities between years of sampling syrah and vermentino varietal blocks. The values are of ASVs identified to species and agglomerated at the species level. **(A)** overlap of fungal taxa using ITS sequencing. **(B)** overlap of bacterial taxa using 16S sequencing.

Because of this large separation of 2021 from the other years, we performed our periodicity analysis twice; once with the data for all four years (Figures 3 and 4; Supplemental Figures 1-2), and again excluding the data from 2021 (Supplemental Figures 3-6). Based on our analysis vermentino had 14 fungal taxa and 31 bacterial taxa that significantly varied between all four years (p-value < 0.01 from *RAIN* analysis), while syrah had 19 fungal taxa and 36 bacterial taxa that significantly varied between all four years. Significant taxa decreased to three fungi and seven bacteria for vermentino and three fungi and eight bacteria for syrah after restricting the results to taxa present in $>75\%$ of samples. Restricting the analysis to 2018-2020 removed two fungal taxa and ten bacterial taxa from vermentino, while it removed nine fungal taxa and removed fifteen bacterial taxon from syrah.

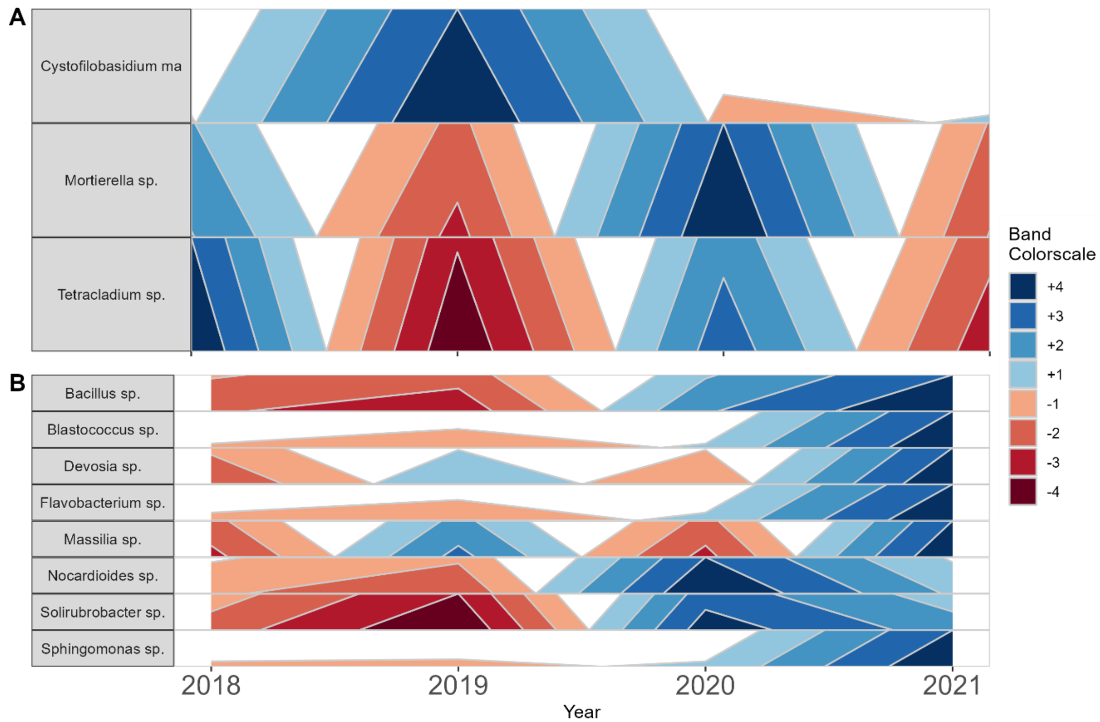

Figure 3. Fungal **(A)** and bacterial taxa **(B)** present in >75% of samples that significantly vary ($p < 0.05$, FDR < 0.05) between sampling years according to rhythmicity analysis incorporating non-parametric methods (RAIN) in blocks of *Vitis vinifera* var. syrah grape vines. Visualized using *BiomeHorizon* package. Band color and thickness indicates percent changes of relative abundances. Negative values are mirrored above the x-axis.

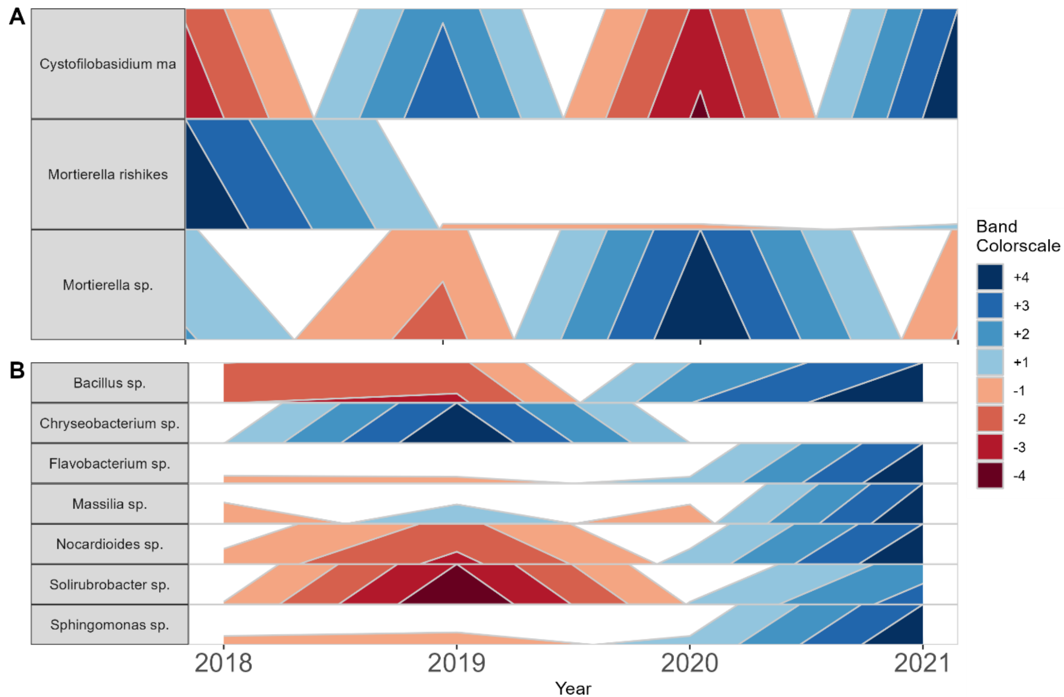

Figure 4. Fungal **(A)** and bacterial taxa **(B)** present in >75% of samples that significantly vary ($p < 0.05$, FDR < 0.05) between sampling years according to rhythmicity analysis incorporating non-parametric methods (RAIN) in blocks of *Vitis vinifera* var. vermentino grape vines. Band color and thickness indicates percent change of relative abundances. Negative values are mirrored above the x-axis.

4. Discussion

We found that neither the varietal nor the block number itself had a significant effect upon the community composition, indicating that intra-vineyard variation of soil microbial communities can be negligible. Other studies have found intra-vineyard variation is primarily due to sampling depth rather than block location—the exception being blocks with soils from different origins (Liang et al., 2019). This seems to indicate that when sampling soil for inter-vineyard studies it is not necessary to sample every block—unless there are considerable differences in edaphic characteristics between blocks.

We found that the *RAIN* package and analysis were easy to implement with our dataset. *RAIN* has quickly become a widely used method for analyzing longitudinal and repeated measurement data within the microbiological community to analyze community composition, genetic expression, and protein production (Coenen et al., 2020). *RAIN* has been implemented in human gut microbiome studies to test for periodic changes in community composition (Reitmeier et al., 2020), in marine and freshwater studies to examine diel effects on phototrophic and heterotrophic microbes and their gene expression (Linz et al., 2019; Coesel et al., 2021), and in studies focusing on the interactions of microbes with insects (Trinh et al., 2021). These studies all use similar sequencing-based datasets across longitudinal or repeated sampling schemes with varying windows—some 24 hours, some days, some weeks. Based on our use, we think that *RAIN* is a usable alternative to more traditional parametric methods; however, it is far more computationally expensive. Users may find it difficult to run without access to high-end hardware or access to a high-performance computing cluster. The major concern for our dataset, and other microbial datasets, is that *RAIN* was designed to be used for datasets with moderate amounts of time points (10-100) whereas our dataset contained fewer time points (Thaben and Westermark, 2014). Further experiments and comparative analyses are necessary to understand if *RAIN* is suitable for such low numbers of time points.

While there is clear variation in microbial communities between years, it is difficult to determine if those differences are due to management or climatic factors. The 2019 and 2021 samples display similar shifts in abundance for many taxa relative to 2018 and 2020. While some of these changes were likely caused by adopting new management

practices, the more dramatic shifts can also be explained by the fact that Troon experienced severe to extreme droughts during the 2019 and 2021 growing seasons (Supplemental Figure 7). However, the 2020 growing season also experienced drought and does not show these patterns. Some taxa show a steady increase or decrease in abundance over the four years without massive shifts during drought and are better candidates for further exploration. These taxa include the fungi *Seimatosporium vitis, Cystofilobasidium macerans*, and the bacterial genera *Bacillus, Blastococcus, Mesorhizobium, Methylobacterium, Nocardiodes, Paenibacillus, Solirubrobacter,* and *Sphingomonas.* The fungus *S. vitis* increased in later years and is related to several grapevine trunk diseases (Camele and Mang, 2019) which may be of concern as fungal pathogens can increase in concentration following the cessation of fungicide use. The other fungal species that decreased in 2020 and 2021, *C. macerans*, may play a role in releasing pectinases during pressing the must and early fermentation, which assists fermentation and clarification (Bezus et al., 2022).

We found several bacterial and fungal taxa only present in the 2020 and 2021 samples that are related to the functions of nutrient cycling, pathogenic infection, and fermentation; all of which influence soil "health" and the production of wine. The nitrogen fixing bacterial genus of *Rhizobium* (van Rhyn and Vanderleyden, 1995) and nitrifying genus of *Nitrospira* (Daims et al., 2015) were only found in 2020 and 2021 samples. Further, two species of the mycorrhizal genus *Glomus* (Kamayestani et al., 2019) were only found in 2021 samples. These groups together indicate the potential for higher N fixation and uptake in the later soils. Several species of yeast related to fermentation and organoleptic compound production were only present in 2020 and 2021

samples, including five *Candida* spp., two *Metschnikowia* spp., two *Mrakia* spp., and *Schizosaccharomyces pombe*. These four genera are known to release flavor precursor compounds from *V. vinifera* during fermentation and synthesize *de novo* flavor compounds (Jolly et al., 2014; Parker et al., 2018). The presence of these species indicates that the flavor and aroma profile of wine produced in 2021 from Troon may contain a higher diversity of organoleptic compounds. Several pathogenic fungi from the genera *Alternaria*, *Botrytis*, *Colletotrichum*, and the taxon *Armillaria mellea* were also only found within the latter two years of sampling—potentially increasing the risk of crop loss. *Alternaria, Botrytis* and *Colletotrichum* can cause rot of leaves and grape bunches (Steel et al., 2011; Peng et al., 2012; Stranska et al., 2022), while *A. mellea* attacks the roots of *V. vinifera* vines (Devkota and Hammerschmidt 2020). The invasion of novel pathogens into the vineyard is most likely due to the decreased use of fungicides and is of concern for future grape production. However, the dynamics of pathogenic infection are difficult to predict solely on the presence of pathogenic taxa due to the presence of taxa that are known to lower rates of infection. Fungal saprotrophs, mycorrhizal fungi, and rhizosphere associated bacteria have all been shown to reduce infection rates; all of which are common within our dataset (Reynolds et al., 2003; Richards et al., 2020). Further study is required before making strong predictions about pathogen dynamics following a shift in management intensity within vineyards.

5. Conclusions

While severe drought conditions in 2019 and 2021 made it difficult to find other trends, our data does reveal that several taxa associated with nutrient cycling and organoleptic compound production increased from 2018 to 2021. These results indicate

that the ability of the microbial community in soil to cycle nitrogen is greater after shifting to low intensity practices. Further, the soil was more hospitable to a broader range of microbes beneficial to fermentation and the production of organoleptic compounds based on the presence of several unique taxa in later samples. However, the greater abundance of pathogens in the soil may be a concern for grape production over time. In the future, incorporating additional vineyards that shift management practices is essential for increasing the generalizability of our results.

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CHAPTER V

CONCLUSION

In this dissertation I have demonstrated that the intensity of human management practices influences the composition and function of microbial communities within logged forests and *Vitis vinifera* vineyards. Further, we saw that those impacts can persist for decades after cessation but can be reversed using practices that promote the recruitment and growth of microbial taxa. Understanding the correlation between management intensity and microbial community functioning is essential for increasing the resilience of contemporary human managed ecosystems. Additionally, changes to microbial functions can be of direct interest to producers and consumers of agricultural products, as those functions can change the organoleptic characteristics. Results from other microbial studies reveal that management intensity has similar effects in other systems, such as grazed grasslands (Piton et al., 2019; Zhang et al., 2022) and wheat agroecosystems (Hartman et al., 2018). These studies imply that the relationship between management intensity and microbial community composition may be universal.

In future studies a finer gradient of management intensity could reveal the trend of the relationship between intensity and changes to microbial community composition and/or function within these systems. Additionally, functions other than organoleptic compound production may not have the same correlation with management intensity, including nutrient cycling, water stress mitigation, and pathogen protection.

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