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MS Environmental Biology Capstone Project

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MS ENVIRONMENTAL BIOLOGY
CAPSTONE PROJECT

by

Klaudia Sowizral

A Project Presented in Partial Fulfillment
of the Requirements for the Degree
Masters of Science
in Environmental Biology

REGIS UNIVERSITY
May, 2024

MS ENVIRONMENTAL BIOLOGY
CAPSTONE PROJECT

by

Klaudia Sowizral

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May, 2024

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Table Of Contents

CHAPTER 2, LIST OF TABLES

CHAPTER 3, LIST OF FIGURES

CHAPTER 3, LIST OF TABLES

CHAPTER 1. LITERATURE REVIEW

Changes in arbuscular mycorrhizal fungi associations with soybean plants under atmospheric climate change projections

Introduction

Anthropogenic sources of carbon emissions that lead to climate change, such as the burning of fossil fuels and deforestation, have contributed to elevated atmospheric carbon concentrations, which are expected to double in the next 50 years (Long, 2004). These elevated levels of carbon have not only enhanced plant growth and photosynthesis, particularly for C3 plants, but also carbon fluxes from above ground plants to soil. Changes in atmospheric carbon directly impact carbon fluxes which impact soil microbial communities and their associations between plants and arbuscular mycorrhizal fungi (AMF) symbiosis. Approximately 90% of plant species are colonized by AMF and rely on these symbionts for nitrogen uptake, drought resistance, pathogen resistance, and heavy metal tolerance, while AMF receives carbon from plants (Cotton, 2015; Finlay 2008; Brundrett, 2009). AMF symbiosis plays a large role in plant and ecosystem health. However , the mechanisms by which this symbiosis is impacted by increased carbon are somewhat elusive.

There is a need to understand how elevated atmospheric carbon influences changes in carbon fluxes below ground, and how that impacts associations between plants and AMF is especially crucial in agroecosystems that provide people and livestock with food. In the United States, soybeans (*Glycine max* (L.) Merrill) are the second most planted crop, covering approximately 87 million acres, and are the top agricultural US export generating $25.7 billion in 2020 (USDA, 2023). Soybeans are a C3 plant with a high phosphorus requirement, and like many legumes, interact symbiotically with AMF. For this reason, the AMF association with soybeans may be more sensitive to changes in atmospheric CO_2 concentrations (Adeyemi, 2020). Elevated carbon concentrations have increased plant growth and yields for soybeans (Sakariyawo, 2017);however, the role that microbial associations play in modulating these effects on plants remains poorly understood. Because increasing global temperatures are expected to contribute to plant stress due to heat and drought (Thomas, 2003; Gavito 2005), understanding how AMF-plant associations will change under elevated carbon and increased temperature may help the agricultural industry better understand how to plan for an uncertain climate future (Sakariyawo, 2017; Ziska, 2000, Soretire, 2020). Current literature suggests that under projected future climate scenarios, robust AMF communities can provide plants with heat and drought resistance as well as increase plant growth and yield. However, if temperatures exceed the upper temperature tolerance of AMF, long-term sustainability in agroecosystems is threatened. In these cases, agroecosystems may require AMF inoculation to overcome the negative consequences of annual harvesting and temperature extremes that surpass 35 °C.

AMF composition changes under elevated CO_2

The potential implications of community compositional shifts include changes in functional roles of AMF. For example, species from the family Glomeraceae arepreferred under elevated CO_2 (eCO_2);based on current studies suggesting that this may increase pathogen resistance; nevertheless,   plants phosphorous (P) uptake may be less efficient. The exact significance of these changes has yet to be studied with soybean associations, and would be beneficial to understand whether changes in agricultural practices such as Gigsporaceae inculcation, P enrichment, or less intensive pathogen resistance strategies are necessary.

AMF fungal communities comprise several fungal taxa in diverse families. Analogous to plant ecosystems, increasing the limiting resource that shapes microbial communities can decrease species evenness when rare species are disproportionately lost from the community (Stevens 2004). A global rise in atmospheric CO_2 increases below-ground carbon availability through plant photosynthesis, thus indirectly increasing C which is the limiting resource, for fungal communities. Therefore, increased carbon is predicted to alter fungal community composition and decrease evenness (Cotton, 2015; Waldrop, 2006).

Understanding the long-lasting effects of increasing atmospheric carbon on AMF community changes is important because it will allow agricultural managers to comprehensively understand the impacts on crop growth and soil health as atmospheric carbon increases. Differences in fungal families translate to functional changes in plant processes which will impact human agriculture. Due to the functional differentiation in these taxa, the composition of these fungi can impact plant nutrient cycling and productivity (Cotton, 2015). AMF communities are primarily composed of three species within the *Glomus* genus: *G. mosseae*, *G. claroideum*, *G. intrarardices*, as well as species belonging to the Gigasporaceae family, all of which differ functionally from each other. *G. claroideum* and *G. intraradices* acquire more phosphorus when in mixtures compared to monocultures, and fungal composition was primarily determined by plant identity (Jansa, 2008; Munkvold, 2004). The literature primarily distinguishes between the two functional families: Glomeraceae and Gigasporacaeae. Gigasporacaeae increased plant P uptake and significantly increased plant biomass, while Glomeraceae decreased pathogen abundance (Sikes, 2010). These results however largely depended on the identity of the host plants, and the relative importance and impact of pathogen protection positively correlated with root surface area (Sikes, 2010). Increased root surface area makes plants more likely to benefit from pathogen resistance. In addition, Gigasporacaea fungi tend to grow less dense mycelia which may decrease soil stability and structure (Leifheight, 2014). Whether these functional differences translate to soybeans remains an open question and requires further investigation to understand accurate implications.

Under elevated levels of carbon dioxide (eCO_2) in field experiments, AMF communities colonizing became dominated by Glomeraceae over Gigasporaceae, to nearly 99% monoculture (Cotton, 2015). These field results are consistent with sterilized pot experiments where plants were subjected to eCO2 for extended durations. These controlled experiments employed real-time PCR and C pulse-chase techniques alongside fatty acid biomarker analysis to assess and characterize microbial communities (Klironomos et al., 2005; Drigo et al. 2010). The decline in the impacts of Gigasporacea remains under varied nutrient compositions (Johnson, 2005). There are a few competing hypotheses regarding the mechanisms by which Glomeraceae dominates under eCO_2. One study predicts that AMF increase nutrient concentrations to plant roots, and in turn the plant allocates more carbon to the fungus. This suggests that increased nutrient cycling to the host plant will benefit relative abundance of AM fungal species (Fitter, 2006). If this hypothesis is accurate, it alludes to the ability of Glomeraceae to more effectively use additional carbon outputs from plants towards nutrient cycling and optimizing symbiosis. Another reason why Glomeraceae may dominate communities under eCO_2 is that these fungi are particularly efficient at obtaining carbon from host plants and colonizing plant roots under reduced soil P concentrations (Cotton, 2015). These changes in fungal community composition as a result of enhanced CO_2 allude to differences in life strategies between fungal families and specific species. Gigasporaceae are known to grow more slowly and establish hyphae before exchanging phosphorus with plants, exhibiting signs of K-selected strategies compared to Glomeraceae which display r-selected strategies including rapid growth that provides host plants with phosphorus more readily (Boddington and Dodd, 1999), particularly under eCO_2.

AMF growth responses to elevated CO2

Anthropogenic activities have increased atmospheric carbon concentrations, which have significantly increased photosynthesis and plant biomass, particularly of C3 plants (Poorter, 2003). The pathways involved in carbon cycling with plants are well understood; however, the implications of CO_2 enrichment on microbial community growth are less clear.

Elevated atmospheric carbon has been shown to shift carbon flow and impact the microbial community in the rhizosphere (Drigo, 2013). The pathways of carbon within the rhizosphere have been documented through the use of fatty acid biomarkers analysis, and confirmed that carbon deposited into soil was first processed by AMF and then bacterial communities. As elevated CO_2 becomes more common, the relationships between atmospheric carbon, soil microbial communities, and plants should be further investigated. Current literature suggests that CO_2 enrichment increases the growth and colonization of AMF for many species, and literature revolving around legumes has consistent results. The specific plant interactions with soybeans requires further investigation.

While literature specific to the effects of eCO2 on soybeans is limited, existing studies on AMF associations with legume plants provide insights. AMF exhibits augmented growth and improved nutrient exchange in response to eCO2 (Gamper, 2004). A meta-analysis investigating AMF responses to eCO2 revealed a noteworthy positive increase (21%) in AM fungal growth (Alberton, 2005). Notably, variations in AMF response were observed primarily between herbaceous and woody species, demonstrating more pronounced impacts on AMF communities in woody species under eCO2 conditions. Specific investigations focusing on legume plants corroborate the findings of eCO2 impacts on AMF growth. When testing pea plants under ambient and elevated levels of carbon there were significant positive impacts on AMF colonization and colonization intensity, as well as spore density (Gamper, 2004). The duration of eCO2 exposure emerged as a significant factor ($p<0.01$) across all AMF studies, indicating a greater response over extended periods compared to shorter-term studies of under a year. Furthermore, no significant disparity was noted between experimental settings (field, pot, laboratory), suggesting the relevance of ex situ experiments in reflecting field conditions (Alberton, 2005). Changes in AMF colonization due to eCO2 might influence carbon storage in soil, trigger larger ecological feedback loops, and be subject to variations in nutrient availability (Johnson, 2005).

AMF growth responses to elevated temperatures

In addition to its fertilization effects on plants, enhanced CO_2 also leads to increased mean global temperatures, which increases abiotic stress on plants. Temperature increases can cause heat stress which leads to yield loss, as well as injury to the photosynthetic mechanism of plants (Thomas, 2003). Many plants respond to increased heat by closing their stomata to prevent water loss and desiccation. There is a trade-off between this water efficiency mechanism and the efficacy of gas exchange since closing the stomata reduces gas exchange negatively affecting the net primary productivity. This problem has been already documented in India, where soy production reported a significantly decreased yield due to heat stress (Jumanari et al. 2017).

Elevated temperature can also indirectly affect plant production by impacting AMF symbiosis. Under elevated temperature, AMF growth is significantly impacted. Optimal growth of AMF occurs in the 18-30 °C temperature range, with temperatures outside this range (< 18°C or > 30°C) negatively impacting fungal biomass and growth (Gavito, 2005). Through temperature manipulation in growth chambers, Gavito (2005) documented linear increases in fungal growth from 24°C to 30°C, which may indicate favorable conditions until a temperature threshold is reached beyond which AMF growth decreases. This effect of increased temperature on AMF growth was echoed in field experiments that showed decreased fungal growth above 30 °C (Gavito, 2005). Such loss of AMF under increased temperatures is concerning because AMF may be crucial to ensure plants remain resistant to abiotic stress. Soybean plants inoculated with AMF under various soil moisture conditions were found to have reduced oxidative damage, and considerable drought resistance (Grümberg, 2015). Furthermore, the presence of AMF can up regulate genes that increase drought and heat tolerance of plants (Roger, 2008). When soybeans inoculated with AMF were exposed to extreme temperatures (35°C), the plants exhibited increased growth and greater photosynthetic rates compared to control soybean plants. The mechanisms behind these improvements indicate that AMF enhances gas exchange, improves water efficiency, and decreases stomatal resistances, which is consistent with other studies that isolated AMF nutrient exchange in greenhouse settings through the use of bio markers and Stomatal analysis (Jumrani et al. 2022; Zhu, 2012).

Understanding how rising temperatures additionally impact AMF symbiosis is critical. Decreased growth of fungal networks in high temperature environments is aggravated by intensive agricultural practices. Annual tilling, planting, and harvesting of crops create disturbances that negatively impact fungal growth (Cotton, 2015). The disruptions to fungal networks due to agricultural practices and elevated temperature stress indicate the need to preserve AMF communities in order to continue to sustainably support food production. The use of AMF inoculation supports AMF communities and the resilience and productivity of soybean crops.

Inoculation efficacy of AMF in agroecosystems and future management

As atmospheric CO2 and global temperatures increase and droughts become more common, finding solutions to ensure food security under these conditions is a top priority. The significant ability of AMF to increase soybean growth, yield, extreme temperature resistance, and pathogen resistance has expanded the interest in the use of AMF biofertilizers.. Literature regarding the success of AMF inoculation for soybeans in agroecosystems shows promising results for soybean cultivation under future climate scenarios.

Studies have converged on the increased growth of AMF, after inoculation of soybeans in agricultural settings (Adeyemi, 2020; Soretire, 2020; Faggioli, 2022). The use of biofertilizers typically involves the co-inoculation of AMF and rhizobia to enhance microbial communities originally present in the soil. In P poor soils, AMF inoculation significantly increased plant productivity (seed weight, yield, and number of pods) (Adeyemi, 2021). Additionally, the impact of AMF inoculation led to higher productivity compared to P-rich soils that were not inoculated by AMF (Adeyemi, 2021). AMF Inoculation also promoted soil fertility post-harvest (Soretire, 2020), which highlights its role in prolonging the longevity of agricultural fields. Adeyemi (year) investigated specific soybean cultivar responses to AMF in collation and found that TGx 1448-2E had the highest yield and performance with AMF inoculation.

The use of biofertilziers with AMF have also been shown to reduce drought stress and decrease the inhibitory impacts of drought stress on cell division and productivity. When drought stressed soybean plants were inoculated with AMF, there were improved levels of plant productivity (biomass, leaf chlorophyll, and seed yield) (Sheteiwy, 2021). The mechanisms of this relationship seem to be within the gene expression of soybeans. AMF improved the relative expression of catalase and peroxidase under drought and downregulated genes involved in proline metabolism and protein degradation (P5CS, P5CR, PDH, and P5CDH) (Sheteiwy, 2021).

Inoculation of soybean plants not only led to plants that were more resilient to climactic conditions, but also resistant to pathogens. The addition of AMF in a soybean field significantly decreased the severity and occurrence of red soybean rot, and gene analysis of inoculated plants showed increased pathogen defense-related genes (Gao, 2012). Inoculation combined with an appropriate fertilization regime could significantly control pathogen occurrence. Even though N fertilization reduces overall fungal growth, inoculation of AMF was able to reduce the occurrence of charcoal root rot in soybeans with and without N fertilization (Spagnoletti, 2020). These results are promising for the application of AMF biofertilizers in agricultural settings where N fertilization and harvesting will impact fungal communities. There has been increasing studies on the regulation of genes by AMF, however the exact mechanisms that make this possible has yet to be identified.

Discussion and conclusion of literature

AMF has been shown to have significant positive impacts on soybean yield and growth. Fungal communities are responsive to a variety of environmental and regional differences as well as host plants. Under elevated atmospheric carbon, the majority of studies point to a shift in fungal communities toward Glomeraceae dominant communities. This family of fungi displays greater pathogen resistance, less P uptake, and more effective carbon allocation from the plant and fungal symbiosis. The homogenous nature of fungal communities under elevated carbon may have other consequences or vulnerabilities that requires further studies. Under elevated temperatures, AMF growth may suffer for temperaturesabove 35°C. We expect to see fungal growth until 35°C, which may improve soil communities in the short term; however, as temperatures begin to surpass this threshold, this may cause declines in fungal growth and colonization. Future studies are needed to identify the cumulative impacts of temperature and carbon on AMF growth. This would allow for better analysis of future fungal community projections and study the potential of inoculation of biofertilizers that may replenish the reduced growth of AMF under high temperatures and elevated CO2.

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CHAPTER 2. GRANT PROPOSAL

Understanding the impacts of AMF inoculation on soybeans under eCO2 and temperature in sterilized pot experiments in Boulder County

A proposal submitted to Boulder County Parks and Open Space 2023 Small Grants Program

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Section 1. Abstract

Anthropogenic stressors such as the burning of fossil fuels and deforestation contribute to higher atmospheric carbon concentrations. C3 plants in particular are sensitive to elevated carbon, as the rate of photosynthesis and plant growth increase due to carbon fertilization. Increased nutrient requirements due to carbon fertilization has made agriculture particularly vulnerable to heat and water stress posed by elevated temperatures and drought conditions, respectively. Understanding how elevated carbon and increased temperature impacts agroecosystem has received attention due to dependence on large scale practices; however, the interactive effects of many climate change variables are still unknown.

Soybeans (*Glycine max*) are one of the most important agricultural crops in the United States, accounting for $25.7 billion in export (USDA, 2023). Soybeans are a C3 plant, heavily relying on arbuscular mycorrhizal fungi (AMF) associations to meet the high phosphorus demand. There is a need to understand how elevated CO2 and elevated temperatures (both expected to increase under a changing climate will impact AMF associations in soybeans, . Elevated carbon has been shown to cause a change in AMF diversity, leading to fungal communities dominated by family Gigasporacea. On the other hand, increased temperature has resulted in decreased AMF community colonization and growth. Understanding how projected climate change variables interact and impact fungal communities in agricultural settings will allow for a more comprehensive understanding of both heat and carbon increases. I propose to conduct a full factorial experiment to assess the impacts of heat and elevated carbon on soybean plants' fungal communities.

Section 2. Objectives, Hypotheses, Anticipated Value, Literature Review

Objectives

The goal of this project is to understand how AMF communities respond to climate change. I propose to conduct a full factorial pot experiment to understand the joint effects of temperature and elevated atmospheric carbon on fungal communities that colonize soybean plants. Data from this experiment will allow for the assessment of how AMF communities are impacted by multiple interacting climate change factors.

Questions and Hypothesis

Q1. How do increased temperature and elevated CO_2 jointly affect AMF fungal community composition? How strong is the interaction between temperature and eCO2?

H1. Higher carbon levels are likely to favor Glomeraceae fungi in AMF communities. Temperature changes, amplified by increased CO2, are expected to significantly impact fungal communities, potentially reducing the overall fungal community.

Q2. How do increased temp and eCO_2 jointly impact AMF growth and total biomass?

H2.Due to the known impacts of carbon and temperature on AMF communities, I expect to see less fungal biomass overall under elevated temperatures at every carbon level

Anticipated Value

Because soybeans are an important economic crop as well as a source of calories and nutrients for humans and farm aninmals , this study will be important to inform agricultural management. The need for a baseline understanding of how fungal communities will respond to climate change projections will suggest best management practices for soybean crops under a changing climate.

Literature Review

Anthropogenic sources of carbon emissions that lead to climate change, such as the burning of fossil fuels have contributed to elevated atmospheric carbon concentrations, which are expected to double within 50 years (Long, 2004), and increase ambient temperatures due to the greenhouse effect. These elevated levels of carbon have not only enhanced plant growth and photosynthesis, particularly for C3 plants, but also carbon fluxes from above ground plants to soil. Changes in atmospheric carbon directly impact carbon fluxes, which impact microbial communities and their associations with plants, most notably AMF symbiosis.

Soybeans are a C3 plant with a high phosphorus requirement and interact symbiotically with AMF. the AMF association with soybeans may be sensitive to changes in atmospheric CO_2 concentrations (Adeyemi, 2020). Elevated carbon concentrations have increased plant growth and yields (Sakariyawo et al., 2017); however, the role that microbial associations play in modulating these effects on plants remains poorly understood. Because increasing global temperatures are expected to contribute to plant stress due to heat and drought (Thomas, 2003; Gavito 2005), understanding how AMF-plant associations will change under elevated carbon and increased temperature is crucial (Sakariyawo et al., 2017; Ziska et al., 2000, Soretire et al., 2020). Current literature suggests that under projected future climate scenarios, robust AMF communities can provide plants with heat and drought resistance as well as increase plant growth and yield. However, if temperatures exceed the upper-temperature tolerance of AMF, long-term sustainability in agroecosystems is threatened.

Increases in atmospheric CO2 have significant impacts on the community of AMF. The literature primarily distinguishes between the two functional fungal families in AMF: Glomeraceae and Gigasporacaeae. Gigasporacaeae increases plant P uptake and significantly increases plant biomass, while Glomeraceae decreases pathogen abundance because increased root surface area makes plants more likely to benefit from pathogen resistance. (Sikes, 2010). Under elevated levels of carbon dioxide (eCO_2) in field experiments, AMF communities colonizing became dominated by Glomeraceae over Gigasporaceae, to nearly 99% monoculture (Cotton et al., 2015). These field results are consistent with sterilized pot experiments where plants were subjected to eCO2 for extended durations. These experiments employed real-time PCR and C pulse-chase techniques alongside biomarker analysis to assess microbial communities (Klironomos et al., 2005; Drigo et al. 2010). This work suggests that Glomeraceae may more effectively use additional carbon outputs from plants towards nutrient cycling, optimizing symbiosis (Cotton et al., 2015).

Enhanced CO_2 also leads to increased mean global temperatures, which increases abiotic stress on plants. Temperature increases can cause heat stress, which causes yield loss, and injury to the photosynthetic mechanism of plants (Thomas, 2003). Many plants respond to increased heat by closing their stomata to prevent water loss and desiccation, but this increase in water efficiency decreases the efficacy of gas exchange and, consequently net primary productivity. The impacts of heat stress on soybeans are already documented in parts of India that cultivate soy through decreased yields (Jumanari et al. 2017).

Under elevated temperatures, AMF growth is significantly impacted. Optimal growth of AMF occurs in the 18-30 °C temperature range, with temperatures outside this range (< 18°C or > 30°C) negatively impacting on fungal biomass and growth (Gavito, 2005). Through temperature manipulation in growth chambers Gavito (2005) documented linear increases in fungal growth from 24 to 30°C, after which AMF growth declines. This effect of increased temperature on AMF growth was echoed in field experiments that showed decreased fungal growth above 30 °C (Gavito, 2005). Such loss of AMF under increased temperatures is concerning as AMF may be crucial to ensure plants remain resistant to abiotic stress. The presence of AMF can downregulate genes that increase drought and heat tolerance of plants (Roger, 2008) and reduce oxidative damage of heat stress (Grümberg, 2015).

While previous studies have evaluated the impacts of temperature and elevated carbon on AMF communities separately, there is no robust understanding of how these variables may interact with each other on fungal growth and composition. Baseline data is necessary to assess how multiple interactions of temperature and carbon will impact fungal communities.

Section 3. Methods

Detailed Sampling and Analysis Plan

I will use a full factorial approach to consider multiple interactions between a range of temperatures (15 ℃, 22 ℃, 32 ℃), and atmospheric carbon range (385ppm, 460ppm, 550ppm). I will use growth chambers containing sterilized pots of soybeans grown from seed. Plants will be grown in Petri dishes in an agar solution until large enough for transplant (7 days). Seedlings will then be moved into sterilized soil in pots, which will be placed in growth chambers and maintained at 100% field capacity for 62 days. There will be a total of 9 trials, each containing 30 replicates (pots). Once plants have been grown in respective temperatures and carbon combinations (table 1), roots samples will be taken and sent out to a laboratory to be prepared and perform QPCR and standard MOTHUR analysis. QPCR will ensure that relative abundances are maintained, and MOTHUR practices will provide community composition. Since there are two available growth chambers, seedlings will be grown concurrently with plants in their 62-day growth period, and factorial combinations will be replaced every 62 days after sterilization of chambers.

Specific Aim 1. Full factorial sterilized pot experiment, analysis of fungi communities

I will compare richness between treatments to evaluate if there are significant differences. I will create a generalized linear model to analyze the strength of carbon, temperature and their interactive effect on fungal communities. In order to further analyze community data, I will perform an NMDS. The environmental projections of carbon and temperature will be overlayed on the analysis to determine the impact of each variable. I will analyze the significance of temperature and carbon on NMDS1 and NMDS2 scores. Additionally, to look at functional families (Gigasporacea and Glomeracea), I will use the relative abundance of families and generalized linear models to determine the significance of each predictor on the relative abundance of each family. Boxplots and a MANOVA will be used to assess significant differences between treatments.

Specific Aim 2. Analysis of fungal biomass

Mean fungal biomass will be quantified for each treatment based on QPCR data. Generalized linear models will be created to assess the significance of temperature and carbon. A model selection process will be performed to assess the best-fitting model. This model will be used to identify the strength and impact of each variable. Additionally, a MANOVA will be performed to quantify significant differences between all treatments.

Timeline

Dates	Activities	Deliverables
January 2023- February 2023	• Complete the first two factorial experiments • Grow soybean seeds on sterile agar solutions • Transplant the first two soybean seedlings into sterilized pots in growth chambers	• Soil samples ready
March 2023	• Harvest soil and root samples • Send samples to the lab for qPCR and TRFLA testing	• Raw data from the first set of samples
March 2023- December 2023	• Repeated process for replicated 3-10	• Raw data from all samples
December 2023	• Analyze data	• Statistical Analysis
December 8th, 2023	• Report writing	• Final Report

Section 4. Budget

Item	Justification	Cost (Source)	Quantity	Total Cost
Soybean seeds	Experimental plants	$75 (per 50lb bag)	1	$75
Soil	Growth Medium	$38 (price per yard)	2	$76
Plastic nursery pots	Growing Medium, easily sterilized	$20 (100 pack)	3	$60
Sequencing Services	Fungal community Analysis in soybean roots	$53/ batch (University of Connecticut)	270	$14,310
Faculty stipend	For conducting experiments, report writing, statistical analysis and modeling	$25/ hour	150	$3,750
Laboratory Consumables Budget	For growing plants from seed, sterilization material	$200	1	$200
Total Proposal Request				**$18,471**

Section 5. Qualification of Researcher (see Attached CV)

Appendices

Table 1. Combinations of temperature and atmospheric carbon conditions

	385 ppm	**460 ppm**	**550ppm**
15 °C	15 °C + 385 ppm	15 °C+460 ppm	15 °C+550ppm
22 ℃	22 ℃ + 385 ppm	22 ℃+460 ppm	22 ℃+550ppm
32 ℃	32 ℃ + 385 ppm	32 ℃+460 ppm	32 ℃+550ppm

Klaudia Sowizral
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EDUCATION

University of Connecticut, Storrs CT
Bachelor of Science in Environmental Science and Sustainable Plant and Soil Systems May 2022
Concentrations: *Sustainable Systems and Environmental Horticulture* GPA: 3.7

Regis University, Denver CO May 2024
Candidate for a Masters of Science in Environmental Biology GPA: 3.8
Biology Fellow

RESEARCH EXPERIENCE

Dept. of Natural Resources and the Environment, University of Connecticut Storrs, CT
Laboratory Technician for Dr. Beth Lawrence April 2021-May 2022

- Worked with PhD student to pilot a design for a sterile hydroponic experiment with wetland grasses
- Researched and employed experimental design on hydroponic systems, nutrient solution, seed germination, and sterilization procedures
- Constructed shade tents, hydroponic jar systems, and monitored greenhouse data

Laboratory/Field Technician for Dr. Beth Lawrence May 2021-August 2021

- Collaborated with a team of 5 researchers in saltwater marshes to identify plant community composition, survey elevation, and prepare treatment sites for thin layer placements studies
- Conducted experiments on soil, water and plant samples for studies relating to saltmarsh biogeochemistry
- Analyzed data using specific procedures to extract information regarding carbon fluxes, pH, biomass

Sustainable Turf and Landscape Program, University of Connecticut Storrs, CT
Research Assistant for Victoria Wallace May 2020 – May 2022
Sustainable turf and landscaping IPM treatments with Victoria Wallace

- Analyze growing degree day and pest management research with Victoria Wallace
- Collect data from local municipalities regarding IPM management and treatment practices
- Collected data from every city in the state in order to accurately distribute information and send surveys

Department of Ecology and Biology, Regis University Denver, CO
Graduate Research Assistant July 2022-Present

- Research, design an experiment, and collect data on the effects of Indaziflam on plant community composition in three Denver Mountain Parks with different levels of grazing management
- Interpret data and run statistical models through R Studio software

PROFESSIONAL EXPERIENCE

Connecticut River Conservancy Middletown, CT
Seasonal Manager May-August, 2019-2021

- Managed a team of 3 interns and coordinated volunteers and partner organizations to host 3-5 water chestnut removal events per week in different locations on the Connecticut Rive
- Lead and educated volunteer groups on identifying invasive plants and effective removal methods

Seasonal Intern (2019)

- Identified invasive plant species in Connecticut and Massachusetts waterways and kayaked through rivers and watersheds to remove specific plant species with volunteers
-

UConn Extension Sustainable Turf and Landscaping Intern May 2020- May 2021

- Collaborated with researchers on invasive species control for land and turf managers in Connecticut
- Collected data from every city in the state in order to accurately distribute information and send surveys
- Determined content and authored informational IPM documents about for UConn online resources
- Organized and researched data about insect lifecycles and growing degree day information

Department of Ecology and Biology, Regis University
Adjunct Faculty/Biology Fellow August 2023-May 2024

- Facilitate laboratory exercises for a class of 20 undergraduate students
- Lecture on organismic biology topics that are relevant to laboratory practices and lectures
- Coordinate with lead professors on laboratory procedures and student standards and grading

United States Department of Agriculture Plant Physiology Dec 2023-Present

- Conducted a comprehensive meta-analysis of scientific papers in the field of agriculture, extracting relevant data and synthesizing findings to provide a comprehensive impact analysis
- Utilized strong analytical skills to extract meaningful data from scientific literature, aiding in the development of evidence-based impact assessments for USDA contributions to plant physiology.
- Engaged in plant tissue culture activities, demonstrating proficiency in handling and maintaining plant cultures for experimental purposes.

Mycology Research Intern, Denver Botanic Gardens November 2023-Present

- Design and execute a statistical analysis of Thelephorales distribution in North America based on LIDAR sequence data, under the supervision of 16 collaborators
- Work with researchers on CLIMUSH data in order to access and process mycological distributions
- Use R studio and ArcGIS in order to visualize data and research results, to create posters and travel for mycological and ecological symposiums funded by Denver Botanic Gardens

LEADERSHIP EXPERIENCE

UConn Women's Varsity Volleyball Team (Division 1) July 2018- May 2022

- Completed demanding 30+ hour/week in-season practice and travel schedule, off season community responsibilities and practices, and full-time academic responsibilities
- Collaborated with and motivated a team of 15 other players in a competitive environment

Team Impact Coordinator October 2018- May 2022

- Served as the liaison between Team Impact, an NGO that pairs children with chronic illnesses with college athletic teams and the UConn volleyball team
- Organized events for the team impact player, created a supportive environment for the child and family

Regis Women’s Volleyball (Division 2) Team Captain July 2022-Present

- Completed demanding practice and travel schedule, and acted as a liaison between coaching staff, strength and conditioning staff, and a team of 16 other players.

SKILLS

- Computer Competency: Microsoft Excel, PowerPoint, Publisher, R Studio, GIS modeling, ArcPro GIS
- Data visualization and management in R software
- Lab Experience: DNA Isolation & Amplification; Microscopy; Gel Electrophoresis; Spectrophotometry; Titrations; Staining techniques and slide preparation; plant identification; soil analysis
- Wetland Delineation experience
- Professional and scientific writing; Scientific manuscripts; literature reviews; grant proposals; management report writing
- Language: Bilingual in English and Polish, rudimentary Spanish

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CHAPTER 3. JOURNAL MANUSCRIPT

Ecology, diversity, and distribution of Thelephorales across North America

Abstract

Thelephorales fungi are considered important ectomycorrhizal fungi (EMF) in forest ecosystems, yet the patterns of diversity and distribution and the processes that shape them remain poorly understood. This study aims to address this research gap by conducting an exploratory analysis of Thelephorales fungi in soil and litter across various habitat types and environmental characteristics. To do so, we used data collected and synthesized by the Climush project, a multi-year, systematic sampling of macrofungal diversity across North America, that aims to assess sexual (sporocarp and spore trap data) and asexual (soil and endophyte metabarcode data) diversity and distribution in relation to environmental factors including fire disturbance. Using metabarcoding data from soil samples, Thelephorales diversity was quantified at the genus level by patterns of molecular operational taxonomic units (MOTUs). Notably, richness of these fungi was negatively correlated with nitrogen and carbon. Additionally, we observed significant species-level diversity within the genus *Tomentella*, supporting the concept of genus-level hyper-diversity within Thelephorales. Genus- and species-level linear regression models across habitats and ecoregions provided valuable insights into Thelephorales diversity and distribution. These models identify significant cryptic diversity in the Thelephorales associated with late-stage forest ecosystems that is not apparent in fire-disturbed habitats. These findings have significant implications for our understanding of Thelephorales life cycles and their role as EMF in terrestrial ecosystems.

Introduction

Soil fungi, which are integral to the health and functionality of terrestrial ecosystems on a global scale, play a fundamental role that, until recently, has been insufficiently understood. Emerging evidence from recent studies has shed light on the critical importance of soil fungi in maintaining ecosystem stability (Liu et al. 2022). The richness of soil fungi communities directly correlates with enhanced nutrient cycling, efficient soil decomposition, and a decrease in plant pathogenicity (Delgado-Baquerizo et al., 2020; Liu et al., 2022). These impacts on soil processes in turn affect above-ground plant communities creating a feedback loop to the soil ecosystem (Kadowaki, 2018). The distribution and diversity of soil fungi are partially controlled by abiotic characteristics such as pH, climate, and organic carbon (Tedersoo, 2013). However, the complex nature of understanding fungal-plant associations remains a challenge, given the reciprocal interactions between soil fungi and plant communities. Soil fungi influence plant community composition, while plants, in turn, exert both direct and indirect impacts on soil microbes (Fierer, 2017).

Mycorrhizal fungi associate with approximately 75% of all terrestrial plant species (Van der Heijden et al., 2015). Basidomycota and Ascomycota are the only fungal divisions that contain ectomycorrhizal fungi (EMF), which play a vital role in maintaining the health of forest ecosystems. This specialized group predominantly associates with angiosperms (flowering plants) and gymnosperms (coniferous trees). While community composition varies across the landscape, EMF are more common in nutrient-poor soils and coniferous habitats (Kranabetter et al., 2009). In exchange for carbon exudates from plants, EMF facilitate nutrient cycling by making nitrogen and phosphorus readily available to plant roots (Liu et al., 2020). Notably, a comprehensive meta-analysis highlighted that the host plant family exerts the strongest influence on EMF distribution, emphasizing that EMF reach their greatest richness in boreal and temperate forests (Tedersoo et al., 2012). Given their integral role at the interface of carbon and nutrient cycles, understanding the mechanisms controlling the geographic distribution of EMF has become a critical area of research in the context of global climate change. EMF, unlike many other soil fungi primarily controlled by soil chemistry, exhibit sensitivity to temperature, and therefore global climate change may contribute to a decline in EMF diversity (Liu et al., 2020). Together, their functional roles in nutrient cycling and sensitivity to temperature underscore the crucial role that EMF play in maintaining the ecological balance of forest ecosystems in the face of global change.

The fungal order Thelephorales (Basidiomycota) is primarily characterized by its ectomycorrhizal nature, and in most cases a distinctive thelephoroid fruiting body form. First documented by mycologist Franz Oberwinkler in 1976, the Thelephorales includes 250 recognized species within the order (Kirk et al., 2008); however, the order is thought to contain many more cryptic species that are challenging to accurately identify (Svantesson et al., 2020). For this reason, studies are needed to elucidate the environmental factors that shape the distribution and diversity of these fungi. A comprehensive understanding of this order holds the potential to provide insights into optimizing forest health and resilience, as well as informing management strategies in the face of global change. Given their presumed ectomycorrhizal nature, we expect their diversity to be higher in conifer and oak forests, as well as in nitrogen-poor soils (Tedersoo et al, 2003; Bauman, 2016) even though some EMF can be adapted to high rates of nitrogen mineralization which enhance soil N (Kranabetter et al., 2009).

Many EMF communities are impacted by site-specific variation in environmental conditions, and therefore the factors influencing their diversity and distribution requires continued research. Similar to other EMF, the distribution of Thelephorales fungi correlates with the presence of coniferous trees, whose roots form strong mutualistic relationships with EMF (Koljalg et al., 2000). Although Rinaldi et al. (2008) classify many Thelephorales species as EMF, Tedersoo et al. (2009) question that assertion and categorize many species as needing further review for classification. Understanding the environmental factors that influence diversity and distribution in the Thelephorales will allow us to better understand the life cycle of these species and how they will respond to global climate change and nutrient deposition.

This study will take a first step at filling the existing knowledge gap regarding the distribution of Thelephorales across the United States through the use of metabarcoding data from soil samples taken by Climush project investigators who conducted a comprehensive sampling across eight US states in diverse habitat types including oak forest, coniferous forest, and grassland, both burned and unburned. In the evolving field of mycology and soil fungi, there is a growing emphasis on utilizing genomic data to unravel the intricacies of microbial taxa and their distribution (Fierer, 2017). By leveraging this genomic data and integrating environmental data, our aim is to understand environmental factors influencing the presence and distribution patterns of Thelephorales. If these fungi follow the patterns of EMF seen in the literature, we expect to see higher presence, richness and relative abundances of Thelephorales reads in nutrient poor soils as well as oaks and conifer habitats, as well as unburned sites. By synthesizing genomic information with detailed environmental assessments, we anticipate gaining a nuanced understanding of the ecological drivers shaping the dynamics of Thelephorales in the studied regions and the role they play in these ecosystems.

Methods

Field Sites and Sample Collection

Data for this study was obtained from the Climush project (VanGalder, 2023). From 2022 to 2023, this project systematically sampled macrofungi (plant symbiotic and sexual sporocarps) from multiple media including air, organic minerals and soils in eight North American ecoregions in the US: Desert Southwest (DSW), Great Lakes (GRTL), Northeast (NE), Pacific Northwest (PNW), Prairie Peninsula (PRAI), Southeast (SE), Southern Rockies (SROC), and Taiga (TAIG).

Each ecoregion is represented by a single site (with the exception of two for PNW) consisting of multiple (~10-30) plots to represent each combination of habitat (grassland, oak forest, conifer forest) and disturbance regimes (unburned and burned). Plots consist of three 25-meter long parallel transects spaced 25 meters apart. Each transect consists of three subplots at 0 m, 12.5 meters, and 25 meters, for a total of nine subplots per plot. In every plot, we sampled soil cores from all nine subplots in Spring 2022. In Fall 2022 and Spring and Fall 2023, we reduced sampling in each plot to five subplots specified for each plot. We collected soil and litter samples from subplots twice per year in both the organic and mineral soil horizons. Carbon, nitrogen, and soil pH were sampled for each of the subplots, with carbon and nitrogen being quantified as percent by weight using flash combustion of soil samples.

Sample Sequencing and Processing

At each plot, 5 samples of litter and 5 samples of soil were collected from subplots as described above. Fungal DNA from these soil and litter samples was extracted using Qiagen

DNeasy PowerSoil Pro Kits. PCR amplification of fungal DNA targeted the nuclear ribosomal internal transcribed spacer 1 sequence (nrITS1), a standard DNA barcode for fungi (Schoch et al., 2012). The nrITS1 region was targeted using primers ITS1F (Gardes and Bruns, 1993) and ITS2 (White et al., 1990). These were linked to Illumina MiSeq adapters and indexes for sequencing with variable spacers to create primer pools. After PCR amplification, multiplexed libraries were built for sequencing on an Illumina MiSeq platform.

In order to analyze Illumina MiSeq data from soil and litter, samples were processed separately using standard DADA2 pipelines (Callahan, 2016). Once data had gone through demultiplexing and removal of chimeras, samples that were not classified to the genus or species level were assigned as “unknown x” placeholders for further analysis. Individual MOTUs were classified by 100% match for DNA sequences, and taxonomy was assigned through a 97.5% match to reference taxa taken from UNITE (Nilsson et al., 2019). We processed data to include species reads per site as well as environmental data for fire history, pH, carbon and nitrogen. Relationships between environmental variables were analyzed separately for soil and litter because fungal communities differ between the two media.

Statistical Analysis:

Binomial generalized linear models were fit to assess presence of Thelephorales in soil and litter. Subplots with any occurrence of Thelephorales (cumulative reads of Thelephorales > 0 per subplot) were denoted as sites with Thelephorales present. Models were fit using presence as a response variable individually for each different predictor including ecoregion, habitat, elevation, pH, carbon, and nitrogen. Barplots were created for ecoregion and habitat to identify total number of subplots where Thelephorales fungi were present at each category.

For both litter and soil samples, Thelephorales species richness was calculated per subplot. Generalized linear models with a Poisson distribution were fit using richness as the response variable. Ecoregion, habitat, elevation, pH, carbon, and nitrogen as well as fire history were used individually as predictor variables. Differences in mean richness between ecoregion and habitat types were analyzed using a Tukey's post hoc analysis using the R package emmeans (Lenth, 2024). Richness of Thelephorales was compared between soil and litter samples using a paired t-test. Due to the fact that Thelephorales was not observed at any subplot in two ecoregions (SROC and DSW), a separate model of ecoregion omitting those two sites was fit in order to complete a post hoc analysis between sites.

For both soil and litter communities, binomial linear models were fit to assess the impact of chemical and physical site characteristics on Thelephorales relative abundance of reads. The number of Thelephorales species reads per site were summed and divided by the total number of reads of all species present at the site as a proxy for relative abundances (Bellemain et al., 2010). Models were fit with relative abundance as a response variable, weighted by the total number of reads at each subplot, and the chemical (nitrogen, carbon, and pH) as well as physical (fire, ecoregion and habitat) site characteristics individually as predictor variables.

To visualize community composition of Thelephorales by ecoregion and habitat type, stacked barplots at the genus level were used for both soil and litter plots. Using the phyloseq package (McMurdie, 2013) in R, we subset MOTUs which were classified as the order Thelephorales. A stacked barplot was used to visualize genus occurrences within the order, with black bars used to visualize frequency of species within each genus. These plots were constructed for both habitat types (conifer, grassland, and oak) as well as ecoregion. All data analyses were conducted in R studio version 4.3.2 (R Core Team, 2023).

Results

Thelephorales Presence:

Contrary to our hypotheses, soil and litter chemistry did not significantly influence Thelephorales distribution. Although the odds of Thelephorales presence in soils were not significantly related to soil pH (p=0.387), soil carbon (p=0.596), and soil nitrogen (p=0.430), Thelephorales presence in soil was negatively related to carbon and nitrogen concentrations. Similar to soil samples, Thelephorales presence in litter was not significantly affected by pH (p=0.607) although it was marginally less likely to occur at higher carbon (p=0.142) and nitrogen (p=0.066) concentrations. The odds of Thelephorales presence decreased by 2.3% (95% CI: 5.2% decrease to 0.8% increase) for a one percent increase in litter carbon, while the odds of observing Thelephorales decreased by 69.5% (95% CI: 92.2% decrease - 1.2% increase) for a one percent increase in litter nitrogen.

Although Thelephorales was more commonly observed in some ecoregions than others, habitat type and burn status did not significantly influence the order's distribution even though we predicted that they would (Table 1). Ecoregion had a significant ($p<0.001$) impact on the incidence of Thelephorales in soils, with SE and PNW sites having the highest incidence and SROC having the lowest. Incidence of Thelephorales in DSW (0.200, SE 0.089) was significantly lower than both SE (0.750, SE 0.097, p = 0.020) and PNW (0.655, SE 0.088, p =0.059), with SE having marginally higher incidence than PNW (p = 0.095). No other significant differences ($p < 0.05$) were observed in Thelephorales incidence among ecoregions. Contrary to our predictions, we observed no significant differences in incidence between plots in different habitat types (p = 0.893, Table 2). Furthermore, unburned sites had similar (p = 0.966) Thelephorales incidence (0.471, SE = 0.112) to burned sites (0.475 , SE= 0.095).

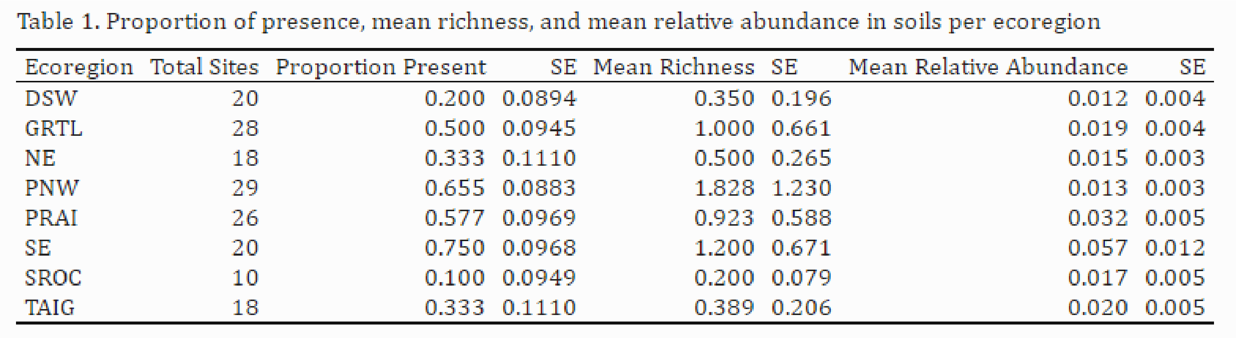


Table 1. Proportion of presence, mean richness, and mean relative abundance in soils per ecoregion

Ecoregion	Total Sites	Proportion Present	SE	Mean Richness	SE	Mean Relative Abundance	SE
DSW	20	0.200	0.0894	0.350	0.196	0.012	0.004
GRTL	28	0.500	0.0945	1.000	0.661	0.019	0.004
NE	18	0.333	0.1110	0.500	0.265	0.015	0.003
PNW	29	0.655	0.0883	1.828	1.230	0.013	0.003
PRAI	26	0.577	0.0969	0.923	0.588	0.032	0.005
SE	20	0.750	0.0968	1.200	0.671	0.057	0.012
SROC	10	0.100	0.0949	0.200	0.079	0.017	0.005
TAIG	18	0.333	0.1110	0.389	0.206	0.020	0.005

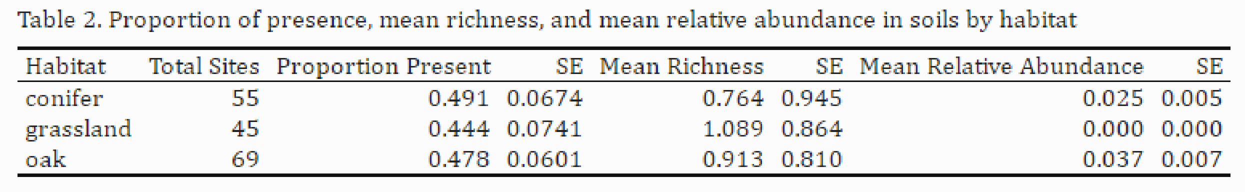


Table 2. Proportion of presence, mean richness, and mean relative abundance in soils by habitat

Habitat	Total Sites	Proportion Present	SE	Mean Richness	SE	Mean Relative Abundance	SE
conifer	55	0.491	0.0674	0.764	0.945	0.025	0.005
grassland	45	0.444	0.0741	1.089	0.864	0.000	0.000
oak	69	0.478	0.0601	0.913	0.810	0.037	0.007

Similar to soil samples, we observed significant differences in Thelephorales incidence in litter by ecoregion (p<0.001) but not habitat type or burn status. These differences were driven by the fact that DSW and SROC ecoregions had no litter occurrences of Thelephorales in subplots (Table 3). No other ecoregions differed significantly in Thelephorales incidence (p > 0.05) in litter. Also like soil samples, neither habitat type (p = 0.432) nor fire (p = 0.480) significantly influenced Thelephorales incidence in litter (Table 4). Thelephorales taxa in litter were observed in 28.4% (SE 7.6%) of burned subplots and 23.5% (SE 5.4%) of unburned subplots.

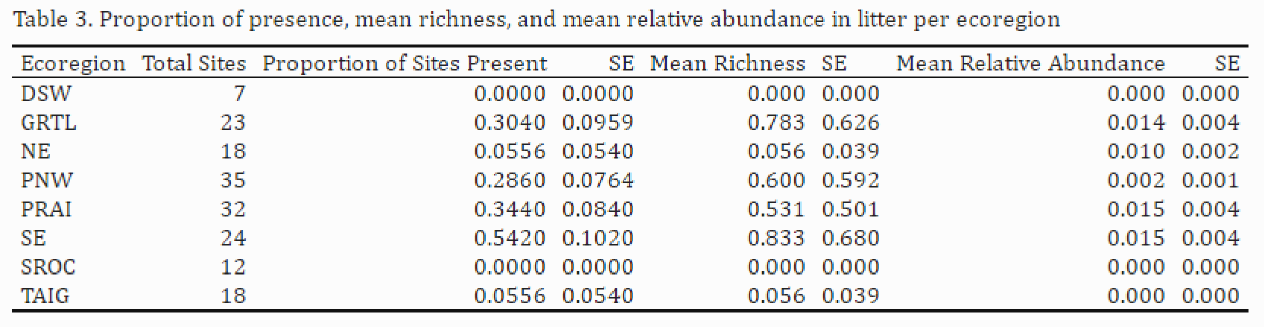


Table 3. Proportion of presence, mean richness, and mean relative abundance in litter per ecoregion

Ecoregion	Total Sites	Proportion of Sites Present	SE	Mean Richness	SE	Mean Relative Abundance	SE
DSW	7	0.0000	0.0000	0.000	0.000	0.000	0.000
GRTL	23	0.3040	0.0959	0.783	0.626	0.014	0.004
NE	18	0.0556	0.0540	0.056	0.039	0.010	0.002
PNW	35	0.2860	0.0764	0.600	0.592	0.002	0.001
PRAI	32	0.3440	0.0840	0.531	0.501	0.015	0.004
SE	24	0.5420	0.1020	0.833	0.680	0.015	0.004
SROC	12	0.0000	0.0000	0.000	0.000	0.000	0.000
TAIG	18	0.0556	0.0540	0.056	0.039	0.000	0.000

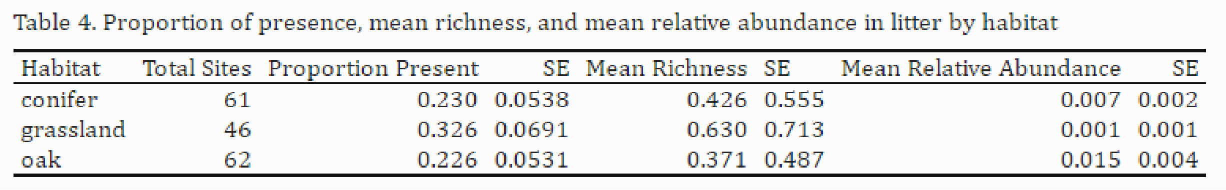


Table 4. Proportion of presence, mean richness, and mean relative abundance in litter by habitat

Habitat	Total Sites	Proportion Present	SE	Mean Richness	SE	Mean Relative Abundance	SE
conifer	61	0.230	0.0538	0.426	0.555	0.007	0.002
grassland	46	0.326	0.0691	0.630	0.713	0.001	0.001
oak	62	0.226	0.0531	0.371	0.487	0.015	0.004

Thelephorales richness:

Species richness of Thelephorales in subplots was higher in soil than in litter (paired t-test, $p < 0.001$) and tended to be driven by similar chemical and ecoregional gradients. Although soil pH did not significantly ($p = 0.335$) influence local (i.e. at the subplot-level) Thelephorales species richness (Figure 1), soils higher in nitrogen and carbon tended to have fewer taxa from the order as we predicted they would (Figure 2) . Plots with one percent higher nitrogen concentration had 28.0% fewer taxa (95% CI: 2.2%-52.7%, p=0.084) while plots with a one percent higher soil carbon concentration had 1.4% fewer taxa (95% CI: 0.1%-2.8%, p=0.057).

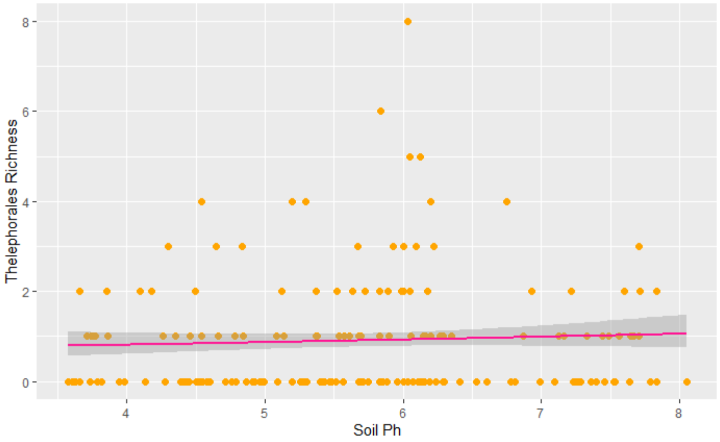
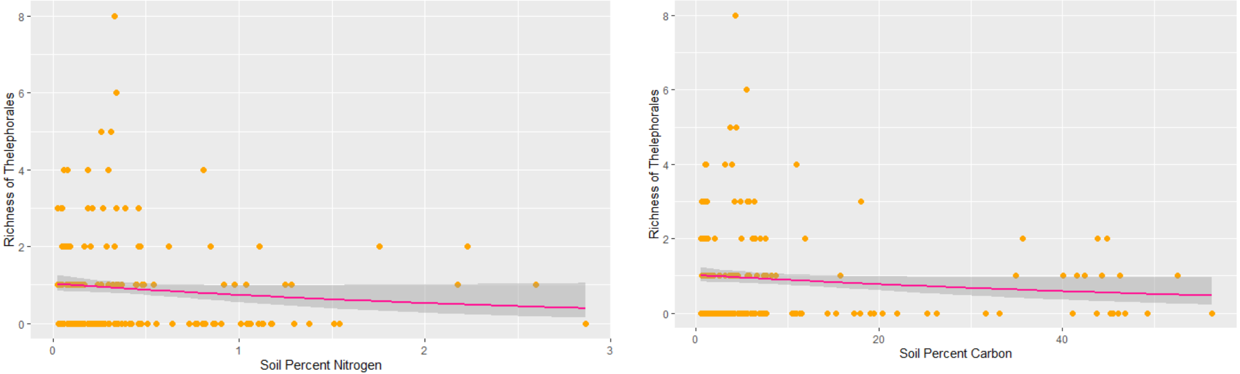


Figure 1. A generalized linear model depicting the negative relationship between Thelephorales richness in soil as a function of pH. The pink line indicates the model regression with a 95% CI in grey. Orange



dots represent individual soil plotFigure 2. A generalized linear model depicting a marginally significant negative relationship between Thelephorales richness in soil as a function of percent soil nitrogen (left) (p=0.084) and *percent soil carbon (right)* (p=0.057). *The pink line indicates the model regression with a 95% CI in grey. Orange dots represent individual soil samples.*

As we predicted, litter carbon and nitrogen had significant negative impacts on Thelephorales species richness at the local (subplot) level (Figure 3). Nitrogen had a strong negative impact on richness (p=0.001), where a one percent increase in litter nitrogen resulted in a 72.9% (95% CI: 40.9%-88.3%) decrease in mean richness. Carbon also had a strongly negative relationship with Thelephorales richness (p=0.005), where a one percent increase in litter carbon resulted in a 2.6% decrease (95% CI: 0.1%-4.3% decrease) in richness. Litter pH showed an insignificant impact on richness per subplot (p=0.316).

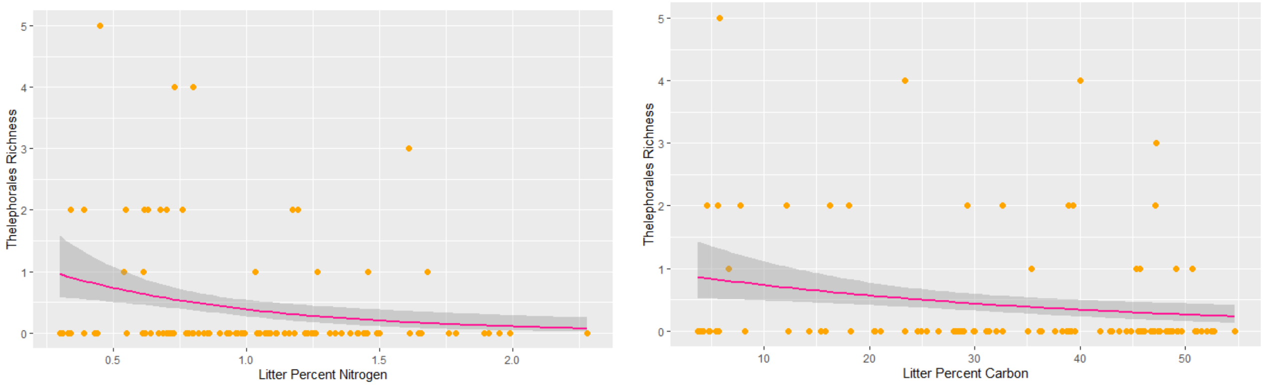
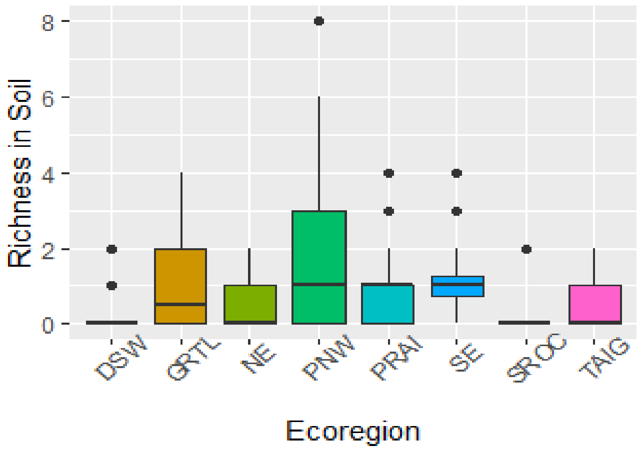


Figure 3. A generalized linear model depicting a significant negative relationship between Thelephorales richness in litter as a function of percent litter nitrogen (left) (p=0.0016) and *percent litter carbon (right)* (p=0.0048). *The pink line indicates the model regression with a 95% CI in grey. Orange dots represent individual litter samples*

While species richness of Thelephorales differed significantly by ecoregion in both soil and litter communities, we did not observe similar significant effects of habitat type or fire (Figure 4). Thelephorales species richness in soils differed by ecoregion (p<0.001) with PNW having higher subplot-level richness than DSW (p = 0.001), NE (p = 0.0078), SROC (p=0.0443) and TAIG (p = 0.003). All other combinations of ecoregions showed no significant differences (p > 0.05). Litter communities also differed by ecoregion with differences driven by DSW and SROC where Thelephorales was not observed. A Tukey's post hoc analysis did not indicate any other ecoregions with significant differences in richness. Average subplot richness in both soil and litter communities did not significantly differ by habitat type (soil: p=0.234, litter: p=0.142) or with the presence of fire (soil: p=0.434, litter: p=0.162).



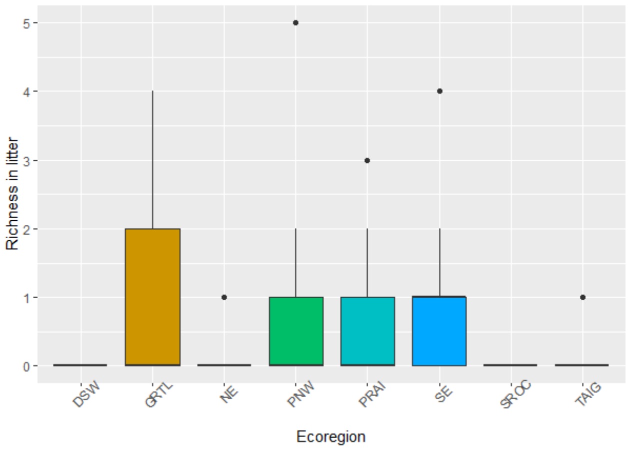


Figure 4. A boxplot depicting mean richness of soil subplots per ecoregion in soil (left) and litter (right) communities. Boxes represent the inter-quantile range, black bars represent the medians, and the whiskers represent a 99.3% confidence interval. Black dots represent outliers

Thelephorales Relative Abundance:

Relative abundances of Thelephorales reads in soil were significantly impacted by all physical and chemical factors. Soil nitrogen, carbon, and pH had positive and significant relationship with relative abundance ($p<0.001$ for all). A one percent increase in soil nitrogen resulted in a 8.7% increase in the odds of a Thelephorales read (95% CI: 6.5% to 10.9%), and a one percent increase in soil carbon resulted in a 0.02% increase in the odds of a Thelephorales read (95% CI: 0.0041% to 0.0055%), and a one unit increase in soil pH resulted in a 2.2% increase in the odds of a Thelephorales read (95% CI: 1.3% to 3.1%). There were also significant differences between ecoregion and habitat types in Thelephorales relative abundance. Ecoregion pairwise estimates are depicted in Table 5, with significant differences between nearly every site. Conifer habitats had the highest percentage of Thelephorales reads (3.1%) compared to oak habitats (2.8%) and grassland habitats (0.04%). All habitat types were significantly different in paired comparisons ($p<0.001$ for all). Unburned sites had a significantly higher ($p<0.001$) percentage of Thelephorales reads (2.42%) compared to burned sites (1.9%).

Mirroring soil samples, relative abundance of Thelephorales reads in litter differed significantly based on physical and chemical site characteristics. Nitrogen had a significant ($p<0.001$) increase in the odds of a Thelephorales read. A one unit increase in litter nitrogen resulted in a 254.6% (95% CI: 242.6% - 265.2%) increase in the odds of a Thelephorales read. Carbon had a small but significant ($p<0.001$) increase in the odds of a Thelephorales read, with a one unit increase in litter carbon resulting in a 0.0061% increase (95% CI: 0.0051% to 0.0072%)

in the odds of a Thelephorales read. A one unit increase in pH resulted in a 6.1% increase (95% CI: 4.6%-7.6%) in the odds of a Thelpohorales read (p<0.001).

Similar to soil communities unburned sites had a significantly higher (p<0.001) percentage of Thelephorales reads (0.01%) compared to burned sites (0.006%). Ecoregion pairwise estimates are depicted in Table 6. Habitat types were all significantly different in a pairwise comparisons (p<0.001n for all). Unlike soil habitats grasslands had the highest number of Thelephorales reads (0.008%) compared to oak (0.0006%) and conifer habitats (0.003%).

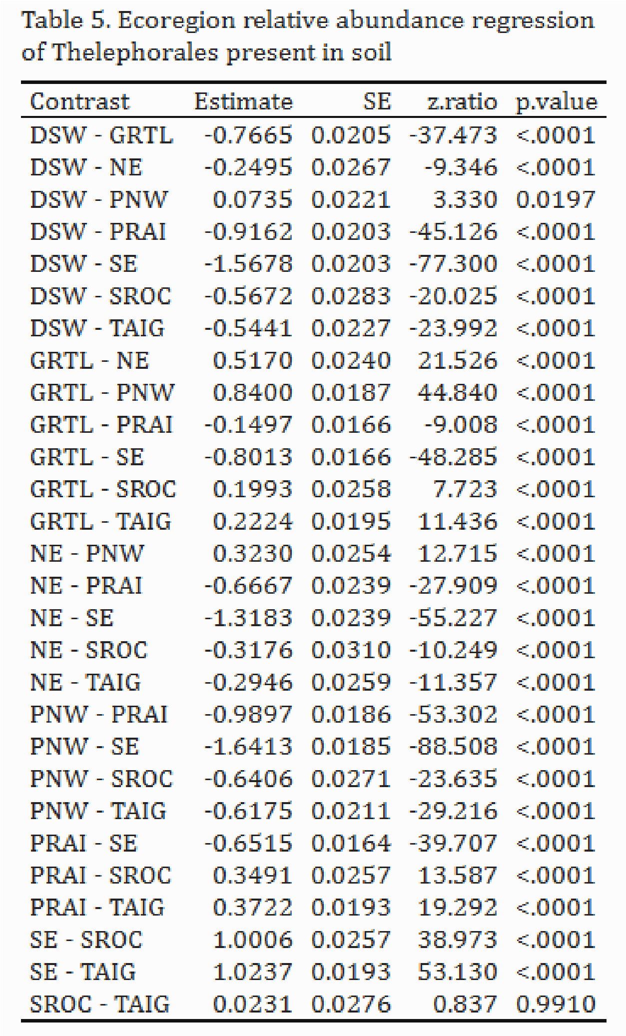


Table 5. Ecoregion relative abundance regression of Thelephorales present in soil

Contrast	Estimate	SE	z.ratio	p.value
DSW - GRTL	-0.7665	0.0205	-37.473	<.0001
DSW - NE	-0.2495	0.0267	-9.346	<.0001
DSW - PNW	0.0735	0.0221	3.330	0.0197
DSW - PRAI	-0.9162	0.0203	-45.126	<.0001
DSW - SE	-1.5678	0.0203	-77.300	<.0001
DSW - SROC	-0.5672	0.0283	-20.025	<.0001
DSW - TAIG	-0.5441	0.0227	-23.992	<.0001
GRTL - NE	0.5170	0.0240	21.526	<.0001
GRTL - PNW	0.8400	0.0187	44.840	<.0001
GRTL - PRAI	-0.1497	0.0166	-9.008	<.0001
GRTL - SE	-0.8013	0.0166	-48.285	<.0001
GRTL - SROC	0.1993	0.0258	7.723	<.0001
GRTL - TAIG	0.2224	0.0195	11.436	<.0001
NE - PNW	0.3230	0.0254	12.715	<.0001
NE - PRAI	-0.6667	0.0239	-27.909	<.0001
NE - SE	-1.3183	0.0239	-55.227	<.0001
NE - SROC	-0.3176	0.0310	-10.249	<.0001
NE - TAIG	-0.2946	0.0259	-11.357	<.0001
PNW - PRAI	-0.9897	0.0186	-53.302	<.0001
PNW - SE	-1.6413	0.0185	-88.508	<.0001
PNW - SROC	-0.6406	0.0271	-23.635	<.0001
PNW - TAIG	-0.6175	0.0211	-29.216	<.0001
PRAI - SE	-0.6515	0.0164	-39.707	<.0001
PRAI - SROC	0.3491	0.0257	13.587	<.0001
PRAI - TAIG	0.3722	0.0193	19.292	<.0001
SE - SROC	1.0006	0.0257	38.973	<.0001
SE - TAIG	1.0237	0.0193	53.130	<.0001
SROC - TAIG	0.0231	0.0276	0.837	0.9910

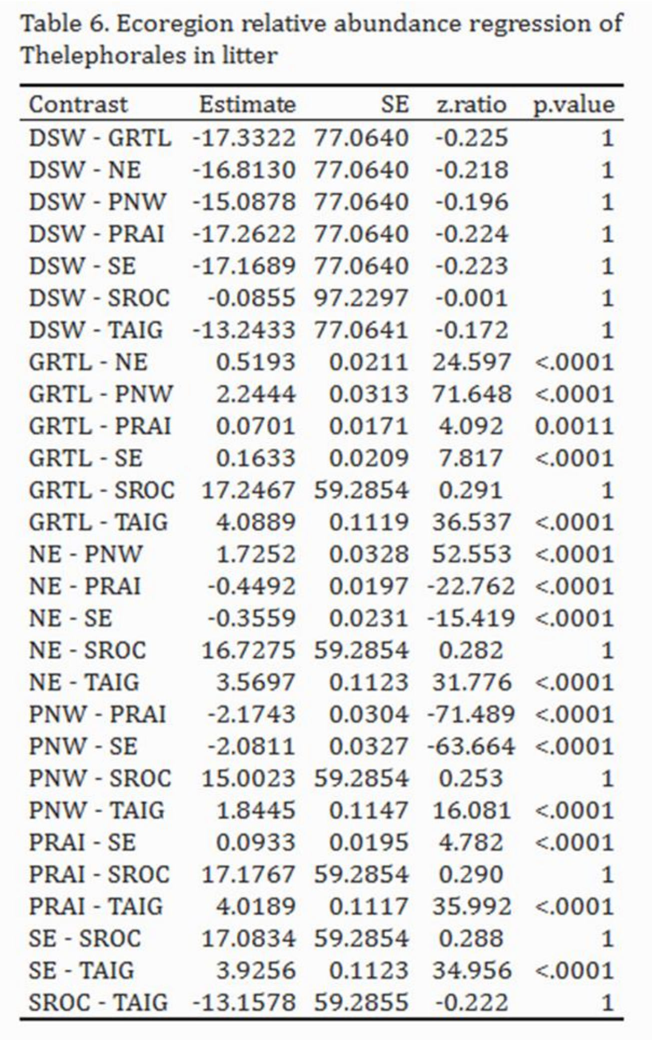


Table 6. Ecoregion relative abundance regression of Thelephorales in litter

Contrast	Estimate	SE	z.ratio	p.value
DSW - GRTL	-17.3322	77.0640	-0.225	1
DSW - NE	-16.8130	77.0640	-0.218	1
DSW - PNW	-15.0878	77.0640	-0.196	1
DSW - PRAI	-17.2622	77.0640	-0.224	1
DSW - SE	-17.1689	77.0640	-0.223	1
DSW - SROC	-0.0855	97.2297	-0.001	1
DSW - TAIG	-13.2433	77.0641	-0.172	1
GRTL - NE	0.5193	0.0211	24.597	<.0001
GRTL - PNW	2.2444	0.0313	71.648	<.0001
GRTL - PRAI	0.0701	0.0171	4.092	0.0011
GRTL - SE	0.1633	0.0209	7.817	<.0001
GRTL - SROC	17.2467	59.2854	0.291	1
GRTL - TAIG	4.0889	0.1119	36.537	<.0001
NE - PNW	1.7252	0.0328	52.553	<.0001
NE - PRAI	-0.4492	0.0197	-22.762	<.0001
NE - SE	-0.3559	0.0231	-15.419	<.0001
NE - SROC	16.7275	59.2854	0.282	1
NE - TAIG	3.5697	0.1123	31.776	<.0001
PNW - PRAI	-2.1743	0.0304	-71.489	<.0001
PNW - SE	-2.0811	0.0327	-63.664	<.0001
PNW - SROC	15.0023	59.2854	0.253	1
PNW - TAIG	1.8445	0.1147	16.081	<.0001
PRAI - SE	0.0933	0.0195	4.782	<.0001
PRAI - SROC	17.1767	59.2854	0.290	1
PRAI - TAIG	4.0189	0.1117	35.992	<.0001
SE - SROC	17.0834	59.2854	0.288	1
SE - TAIG	3.9256	0.1123	34.956	<.0001
SROC - TAIG	-13.1578	59.2855	-0.222	1

Taxonomic Diversity in Soil and Litter:

We observed strong differences in both soil and litter Thelephorales diversity across the entire region (Figures 5, 6). A total of 434 MOTUs in the order Thelephorales were identified from soil samples. These taxa were distributed among three families in the order and one taxon ranked to an unidentified family in the UNITE reference bank (Abarenkov et al., 2024). Additionally, 12 genera were identified including one taxon ranked to genus, but similarly unidentified in the UNITE reference bank (Abarenkov et al., 2024). *Tomentella*, represented by 342 distinct MOTUs was the most common genus we observed in Thelephorales. In litter, 93 species-level MOTUs were identified and they were distributed across 5 genera, while soil had 222 species-level MOTU's across 12 genera. Mean Thelephorales richness per subplot in soil was significantly higher (paired t-test, p < 0.001) than in litter by 0. 5 (95% CI: 0.2-0.7) species on average per subplot. Similarly relative abundance of Thelephorales reads was significantly higher (paired t-test, p<0.001) in soil than in litter by 0.015% (95% CI: 0.006%-0.023%)

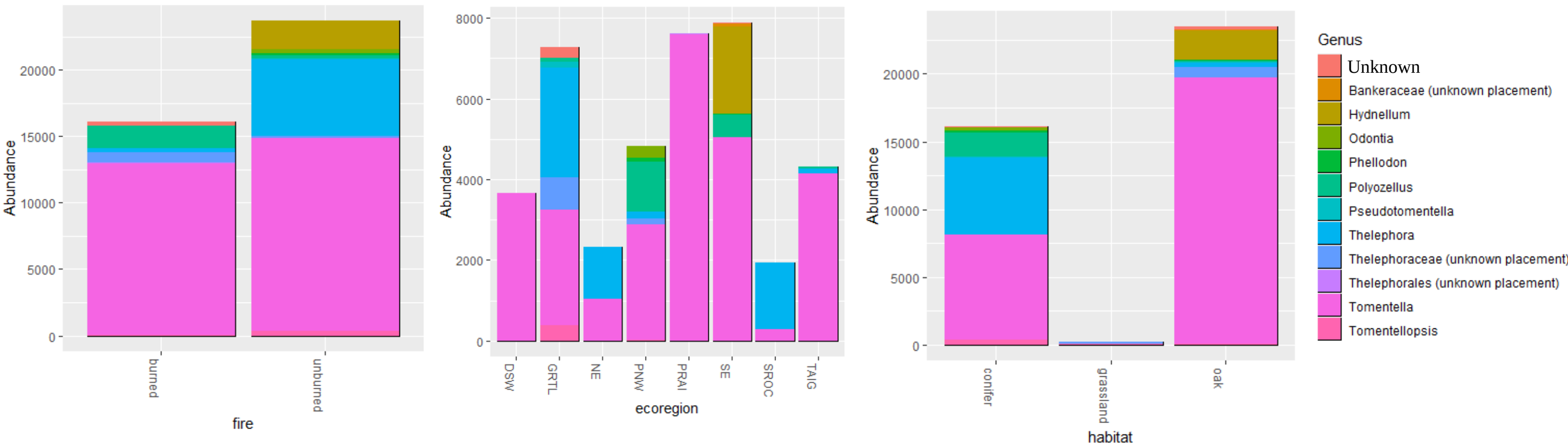


Figure 5: Stacked bar plot of Genus richness in soil samples in the order Thelephorales by fire, ecoregion, and habitat type

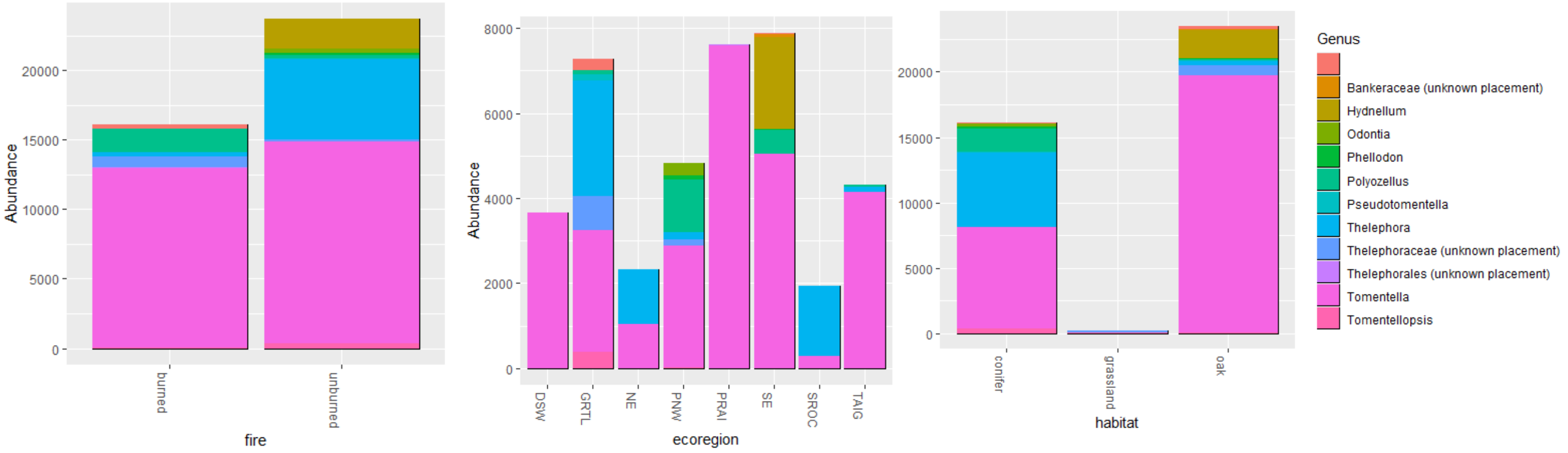


Figure 6: Stacked bar plot of genus richness in litter samples in the order Thelephorales, by fire, ecoregion and habitat

Although local diversity of Thelephorales at the subplot-level tended to only differ by ecoregion in both soil and litter communities, community structure and region-wide diversity also strongly differed by habitat type and burn status. Additionally, strong differences in genus-level community structure occurred across all site types. While the genus *Tomentella* was ubiquitous across all site types, *Thelephora* was most abundant in conifer habitats and unburned sites for both soil and litter. *Thelephora* was also not equally prevalent in all ecoregions. reaching higher MOTU diversity in GRTL (soil: 6 *Thelephora* MOTUs; litter: 4 *Thelephora* MOTUs) and NE (soil: 9 MOTUs; litter: 8 *Thelephora* MOTUs) . Burned sites in soil have greater diversity of *Polyozellous* (8 MOTUs; 1,652 reads) while *Thelephora* (19 MOTUs; 5,718 reads) and *Hydnellum* (4 MOTUs; 2,160 reads) were more prominent in unburned sites. *Hydnellum* was also notable only in soil communities where it prevailed more so in oak habitats (5 MOTUs; 2,166 reads) in SE ecoregion. The unknown genus was abundant in burned soil sites (3 MOTUs; 289 reads), and unburned litter sites (2 MOTUs; 1,571 reads).

Discussion

Our study aimed to understand the drivers of Thelephorales incidence, richness, and community structure at local and regional scales. Thelephorales richness in both soil and litter communities declined significantly as carbon and nitrogen concentrations increased and was higher in some ecoregions than in others. On the other hand, relative abundance of reads increased with both carbon and nitrogen concentrations, differed strongly across ecoregions. When considering all sites together, we observed stark differences in genus-level community composition based on dominant vegetative cover and fire history. While some Thelephorales genera like *Tomentella* were more cosmopolitan, others like *Thelephora* and *Hydnellum* were localized to certain habitat types. In both cases, Thelephorales genera exhibited high levels of MOTU diversity at the species level, indicating significant cryptic diversity in the order across different site characteristics. The fact that we did not observe similar changes in local richness but dramatic differences in regional diversity highlights the high degree of species turnover across the United States.

As predicted based on the presumption of an ectomycorrhizal trophic strategy, we observed negative effects of nitrogen and carbon on both local richness and incidence in soil and litter, and we found higher richness and abundance of *Thelephorales* in soil compared to litter Studies find that EMF incidence is negatively correlated with nitrogen levels (Lilleskov et al., 2002) and pH gradients (Walker, 2014) because EMF symbiosis is selected for by plants in nutrient-poor soils when they allocate a larger proportion of their carbon resources to belowground structures, fostering greater EMF associations (Hobbie & Högberg, 2012). Because Thelephorales is presumed to be ectomycorrhizal, we would expect incidence and richness to be greater in soils with lower N, and our results support that pattern. However, although the relative abundance of *Thelephorales* within the community was significantly impacted by nitrogen and carbon in both the litter and the soil, the effect occurred in the opposite direction to that of richness and incidence, indicating that a common Thelephorales species, perhaps not ectomycorrhizal in nature dominates in nutrient-rich soils . Changes in local dominance of Thelephorales coupled with the increase in richness and incidence at low N values highlights that a more diverse, likely ectomycorrhizal community is at play in nutrient-poor soils, while a dominant taxon with a different trophic lifestyle is present in nutrient-rich soils. These results highlight the need to examine which species are present in nutrient-rich plots to re-examine their ectomycorrhizal ecology.

Contrary to our hypothesis, local Thelephorales richness and distribution were not negatively impacted by burning. Burned plots did not differ from unburned plots in local richness or incidence of Thelephorales. This may indicate that the order is resilient to burning. Fire tends to decrease fungal richness but the precise strength of the effect depends on fire frequency and intensity. Fires can alter soil pH and nutrient concentrations due to volatilization, changes in bulk density, and increased hydrophobicity of soil particles which decreases soil biota (Agbeshie et al., 2022; Panico et al., 2020). Spore banks of EMF communities may stay intact and their presence may allow these taxa to restore community structure quickly after fire occurs (Glassman, 2016). Recent studies have shown that *Tomentella* increases after fire (Cheng et al., 2023) suggesting that some Thelephorales taxa may possess adaptations that allow them to persist in a range of soil properties after burning. Potential correlation between fire and nutrient availability at our sites requires further investigation as nitrogen has been documented to decrease with fire and raise C/N ratios (Panico, 2020). Our study did not assess whether the fire regime differed due to intensity of fire or frequency of fires events per plot, but additional information regarding fire history may provide for more robust understanding of these relationships.

Although we did not observe effects of burning on local richness and incidence in Thelephorales, overall we did find that unburned plots had higher overall diversity than burned plots and greater relative abundance. Similarly, contrary to our predictions, we did not find higher Thelephorales incidence and richness in oak and conifer habitats compared to grasslands at the local scale, but we did observe higher numbers of Thelephorales MOTUs and abundance of reads in conifer and oak habitats relative to grasslands overall. Large differences in regional diversity and community structure across different site categories but minimal differences in local diversity at the subplot level indicates that the rate of species turnover across local sites is high. Thus, individual subplots in different habitats may not vary strongly in the local number of species present, but the taxonomic composition of those species differs considerably. Even the relatively ubiquitous genus *Tomentella* differed in the number of MOTUs across site categories, corroborating literature documenting their hyper diversity (Svantesson, 2019). *Thelephora* was also more abundant and diverse in conifer habitats and unburned plots overall but comparatively lacking in grassland and oak habitats and sites that experienced recent fire. *Thelephora's* success in evergreen ecosystems is expected with EMF nutritional strategies (Phillips et al. 2013), but highlights the genus's sensitivity to fire.

This continental analysis of community composition corroborates other studies that have demonstrated hyper diversity of the genus *Tomentella* (Tedersoo et al., 2009; Tedersoo et al, 2014). *Tomentella* and *Thelephora* are two of the most abundant and diverse genera of EMF, and isotopic studies demonstrated that these two genera live characteristic EMF lifestyles, hallmarked by their association with nutrient poor soils and forested ecosystems (Tedersoo et al., 2014; Svantesson et al., 2019). Tedersoo et al. (2003) showed preferential associations of *Tomentella* and *Thelephora* with decayed wood compared to mineral soils which is consistent with our finding of a lack of nutrients (N and C) predicting higher incidence and richness of Thelephorales. We also found that grassland habitats had lower *Tomentella* abundance and richness overall than woody habitats (Fig. 5 & Fig. 6), even though the Thelephorales species richness was not significantly lower in grassland subplots in soil or litter. Therefore, the characteristics which support high *Tomentella* turnover across local sites leading to high diversity regionally requires further investigation.

Because we saw such shifts in large-scale community composition across habitats, burn status, and ecoregions with diversity controlled at the local-scale by nutrients, some Thelephorales species may occur in a wider diversity of conditions than expected. In EMF-plant symbioses, fungi provide nitrogen and phosphorous to plant species, typically gymnosperms, which in turn provide carbon to fungi. Many of the 20,000 species of EMF exhibit a wide range of morphological and metabolic diversity (Tedersoo et al., 2012), and many species can, in part, be saprotrophic. Ectomycorrhizal relationships are believed to have convergently evolved multiple times from saprotrophic lineages of fungi that decompose wood and litter (Van der Heijden et al., 2015, Tedersoo & Smith, 2013). The two dominant Thelephorales genera that we observed, *Thelephora* and *Tomentella*, are currently believed to be ectomycorrhizal but are closely related to the non-mycorrhizal wood decay fungus *Odontia* (Tedersoo et al., 2014; Floudas et al., 2012). Our results highlighting the abundance and diversity of *Thelephora* in conifer soil habitats (Fig. 5) mirror those of *Odontia* which relies on wood. The large-scale turnover implied by our findings in conjunction with the local-scale decline in richness and higher relative abundance of a dominant taxon as nutrient concentrations increase, suggest that while the EMF classification of Thelephorales is accurate, future work should determine whether wholesale classification is accurate for all Thelephorales species, or whether some Thelephorales species may rely more so on saprotrophy than previously thought.

Our findings that drivers of EMF community composition, dominance, and richness differ by scale mirror those of other studies. Gavitto et al. (2019) assert that at a local scale EMF are impacted by microclimates and conditions that cause community composition to change continuously and increase turnover. The most significant drivers of local level composition are edaphic characteristics such as soil nitrogen, carbon, phosphorous and soil porosity (Gua et al., 2020, Kranabetter et al., 2009). Our results showing significant relationships between local (i.e. subplot-level) Thelephorales richness and soil nitrogen and carbon are consistent with this literature. At the regional scale, Guo et al. (2020) found geographic distance and large-scale nitrogen cycling to be a driver of community structure and stated that moisture and temperature play a significant role in large-scale spatial differences, which is consistent with our analysis of genus level changes in diversity by ecoregion and relative abundance relationships. Other factors can influence rapid community structure at a broader scale such as endemism and dispersal (Talbots et al., 2014), which adds to our understanding of significantly different relative abundances and richness by ecoregion.

Understanding local and regional differences is crucial to our understanding of ecological drivers of Thelephorales, however current reference banks and sequencing techniques create limitations in our study. Our MOTUs were referenced using the UNITE database with 97.5% sequence match, which serves as a valuable and consistent resource for fungal identification (Abarenkov et al., 2023). The UNITE database is limited to sequences submitted by researchers which may disproportionately represent certain taxonomic groups or ecosystems. This bias presents a limitation on the database's capacity to truly capture fungal diversity in the environment. Future steps in our research include cross-referencing our sequence data with additional databases, including Genbank nucleotide database and refseq database, both of which are more comprehensive in sequence representation and independently verified through vouchered specimens. This would allow us to compare the composition of the community structure under multiple databases and validate the trends we see in our results.

When using the DADA2 pipeline for amplicon sequence analysis, biases may arise due to primer choice, PCR amplification, and sequencing errors. These biases can influence the observed fungal community composition, particularly when relying on relative abundance of reads (Bellemain et al.; 2010, Lindahl et al.; 2013). Consequently, caution must be exercised when interpreting our results that found significant relationships between relative abundance of Thelephorales and nearly all environmental predictors. In future research, measuring relative abundances by weight using stable isotope probing would provide a clearer understanding of dominance. Addressing these limitations and integrating additional databases can enhance the robustness of future research on the specific environmental drivers of Thelephorales dominance in fungal communities.

Despite the limitations of using metabarcoding data to conduct diversity analyses, our work demonstrates that local diversity and incidence of Thelephorales is controlled by nutrient concentrations with lower diversity at higher nutrient concentrations in accord with the expectation that the order exhibits an ectomycorrhizal trophic strategy. However, high turnover across local sites results in high regional diversity especially in forested ecosystems and unburned sites that is not apparent at local scale. Consequently, this work demonstrates that Thelephorales exhibits a larger percentage of cryptic taxa than previously known, especially in cosmopolitan genera like *Tomentella*. In other genera like *Thelephora* and *Hydnellum*, diversity is also high and we observe fidelity to unburned and forested sites. Despite the general corroboration of Thelephorales as ectomycorrhizal, the presence of many taxa across a wide array of gradients highlights the need for future studies to understand alternate trophic strategies like saprotrophy. Additionally, the local-scale resilience of Thelephorales diversity to fire highlights the potential ability of some taxa to rebound quickly. Together, these findings provide ample room for subsequent exploration into the natural history of this group of fungi.

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CHAPTER 4: STAKEHOLDER ANALYSIS

Integrating Multiple Management Perspectives in Bristol Bay Alaska

Introduction and background

Bristol Bay, Alaska, stands out as a rural region renowned for its robust salmon fisheries and unspoiled natural environment. These fisheries serve as the cornerstone of the regional economy, comprising nearly the entire private sector (Duffield, 2007). Moreover, the abundance of wild-caught foods holds significant cultural and nutritional value, serving as a primary protein source for local residents and playing a central role in the traditions of the indigenous community. However, public health concerns have emerged due to increasing levels of pollutants in the water shed. Recent toxicology studies have revealed relatively low levels of heavy metal contamination, yet consistently elevated arsenic levels due to the movement of industrialization related pollutants have raised alarm (Hartwell, 2018). The potential consequences of heightened habitat degradation, increased biomagnification, loss of critical ecosystem functions, and a decline in local biodiversity set the context for management. Adding to the complexities of the situation is the proposed Pebble Mine project, which aims to extract copper and gold from the surrounding landscape and has been at the forefront of legal conflict since its proposal in 2001. This project would comprise the largest active mine in North America and would result in the disposal of dredge material into local waterways. While proponents argue that the mine would provide much-needed jobs and revenue for the community, the US Environmental Protection Agency (EPA) has intervened, halting construction due to concerns over potential adverse effects on the ecosystem (EPA, 2023). EPA’s decision has sparked legal battles and litigation, particularly regarding the EPA's jurisdiction over the watershed, especially in light of shifting definitions of protected waters following the 2023 Sackett vs. EPA. Based on the economic and nutritional needs of the local community, as well as the likely environmental impacts of mining on public health and tourism, I recommend that the ilimina lake should be designated as critical habitat under the endangered species act for harbor seals, and management changes are reinforced with stricter policy enforcement and focus on monitoring. The resilience of the pebble mine project which has come back many times requires long term solutions to ensure the environmental and social welfare of Bristol Bay.

Stakeholders

The creation of the Pebble Mine will likely create jobs for members of the community. Proponents also suggest that the mine will allow for more financial resources to be given back to the community based on the sale of mineral material and job opportunities (Northern Dynasty, n.d). The creation of the mine is supported by Republican Governor of Alaska, Mike Dunleavy, who has acknowledged multiple times that his support of the mine would be controversial given the disagreement among his constituents and the volatility of the debate. However, his administration's views are based on the potential of the mine to economically benefit a community that has been beset by economic inequity and high poverty rates of the community. Consequently, Governor Dunleavy argues that EPA's decision to halt the mine is unjust, and his administration has pursued legal action to sue the EPA over its decision to deny a permit to Pebble Mine. This pushback is due to several factors including the premature nature of the EPA's decision before project plans were finalized (Office of Mike Dunleavy, 2022). Additionally, EPA's action was based on denial of 404 permits to dredge and fill wetlands; however following the 2023 Sackett vs. EPA decision that removed federally protection from many wetlands, Governor Dunleavy argues that the EPA does not have jurisdiction. Recently the Supreme Court has rejected the lawsuit issued by Governor Dunlevey, which means that the case must go through a regular federal appeals process.

The Pebble Mine project aims to be completed by the Pebble Limited Partnership which is now wholly owned by Northern Dynasty, a Canadian mining company ("Mine Proposal," Save Bristol Bay, 2020). Northern Dynasty states that it is progressing through the permitting process and has plans to potentially increase mine development beyond the initial mine to create a secondary recovery gold plant (Northern Dynasty Minerals, n.d.). The project plans currently involve developing an open pit mining operation that will span over a mile in length and 1,750 feet in depth primarily to extract and process gold and copper (Bristol Bay Native, 2023). The project would also require the construction of tailings storage, treatment ponds, and a private two-lane road which spans 83 miles (SaveBristol Bay, 2018). If built, the Pebble Mine would be the largest active mine in North America, and mining operations would take place for 20 years with plans for future expansion (Northern Dynasty Minerals, nd). An independent financial assessment funded by Northern Dynasty, predicted that $7.7 billion in cash flow would result from the project as well as $1.5 billion in mining taxes and government royalties and $1.9 billion in corporate income tax. The company claims that copper production would allow for the creation of greener energy infrastructure by reducing US reliance on imports, and that the residents of Bristol Bay would be compensated directly through royalties through the Pebble Performance Dividend while the mine was active (Northern Dynasty Minerals, n.d.). The pebble mine project planned to employ Alaskan residents, creating 4,350 mining jobs (Pebble Partnership, n.d.). Following the EPA’s halting of the project by rejecting applications for 404© permits, Pebble Limited Partnership sued the EPA for delaying the project.

Majority of the surrounding community opposes the development of the mine. A poll from 2020 showed that 62% of Alaska voters oppose the mine, and 48% of respondents “strongly oppose” the mine (Heimer, 2020). The consensus of the community is that the benefits do not outweigh the potential threats to fishing and recreation. There is a lack of current baseline conditions and monitoring, which was an area of concern for residents during public comment periods for mining permits (Trout Unlimited, 2018). The lack of baseline data was made apparent when proposals for mine construction lack robust mitigation strategies over the proposed 20 year mining activities (Earthjustive, n.d.)

The EPA currently has the greatest authority over the progression of the project. Northern Dynasty applied for permitting, and in January 2023 the EPA issued a final determination under section 404(c) of the Clean Water Act that the recommended determination would have “unacceptable adverse effects on salmon fishery areas in the South Fork Koktuli River, North Fork Koktuli River, and Upper Talarik Creek watersheds of Bristol Bay.” The EPA deemed these watersheds in Bristol Bay to be Waters of the United States, and therefore prohibited the discharge of dredge and fill material as was described in the Pebble Mine plan. The technical report cited the loss of aquatic habitats critical to anadromous fish due to contamination associated with dredge material, which would extend beyond the local confines of the mine farther downstream. EPA’s conclusion were corroborated by the Army Corps of Engineers following the lawsuit Pebble filed against the EPA (United Tribes of Bristol Bay [UTBB], n.d.). In addition to the US EPA, a coalition of environmental associations which comprise the Bristol Bay Defense Fund also oppose the project on environmental grounds. Groups involved include: United Tribes of Bristol Bay, SalmonState, Natural Resource Defense Council, Native American Rights Fund among others.

The watershed area is an important habitat for many aquatic and potentially endangered species such as harbor seals (*Phoca vitulina*). In Bristol Bay, Iliamna Lake serves as a crucial habitat for seals, representing a unique and ecologically significant enclave within the broader marine ecosystem. Seals inhabiting this area, considered part of the Bristol Bay harbor seal stock, play essential roles in local food webs and ecosystem dynamics (Bovenf, 2018). The seals rely on Iliamna Lake for various aspects of their life history, including breeding, pupping, and foraging. However, despite their importance, these seals face uncertainties due to limited scientific knowledge about their population dynamics, habitat requirements, and interactions with human activities (Hauser, 2008). An evaluation was completed by NOAA in 2016 to evaluate the distinctiveness of Harbor seals in ilimina lake. There is preliminary evidence from this study that suggests that the species are a distinct population (Boveng, 2016). The study underscores the need for a comprehensive assessment of their population status, which can be used to determine ilimina lakes potential as a critical habitat delineation if the population was found to be distinct.

Opponents of the mine are concerned not only about the direct environmental impacts on water quality and salmon harvesting and quality, but also the implications for indigenous community practices (Lewis, 2014). Many community members rely on fishing, and the mine may degrade the quality of the salmon fishery through reduced harvest and contamination from mine activity. Opponents of the mine include the EPA and various conservation organizations like trout unlimited and the nature conservancy. Currently Bristol Bay Alaska is one of the most productive salmon fisheries, supplying 50% of wild sockeye salmon worldwide (Ruggerone, 2010). Virtually the entire community economically depends on the fisheries, and hunting and subsistence fishing provide much of the community with food as well as important cultural practices within the indigenous community. Even without the threat of large-scale mining, Bristol Bay salmon habitat is threatened by hydrological alteration due to climate change (Wobus, 2016). Baseline hydrological conditions are likely to change due to climate change, and these changes could alter burial depth of eggs and scour depths which could reduce reproduction (Wobus, 2016). Additionally, pollutants known as metal mining effluent (Al, Ba, B, Fe, Mn, Sr, Tl, Ti, and Zn) threaten sockeye salmon populations because they reduce salmon spawning and increase mortality through chemical discharge, hydrology changes, and temperature alterations due to construction (Dube, 2005). Salmonid species in this area are particularly vulnerable to pollutant exposure because they migrate between freshwater and marine environments which increases the variety of pollutants they are exposed to (Sergeant, 2022). Local mining pollutants include heavy metals (copper, aluminum, selenium) which can be leached from ore, tailings and exposed rocks which can persist in the environment if not managed properly. Additionally, these contaminants can bioaccumulate in fish which increases the risk of contaminant exposure via human consumption (Sergeant, 2022).

The potential threats of the Pebble Mine to the sustainability of salmon fisheries have caused opposition to the creation of the mine from those invested in the ecological longevity of the fishery including recreational anglers and those employed by the fishing industry. The fisheries in Bristol Bay have generated $1.5 billion in sales across the US (Alaska.edu), and in 2010 alone the Bristol Bay fishery accounted for 6% of total US seafood exports. Recreational angling is the next most important economic activity in the watershed (Neher, 2012) resulting in $48 million in revenue from non-residents who visit and fish in the Bristol Bay region (Neher, 2012). The building of the mine may disrupt the experience of recreationalists not only due to the ecological effects on prime fishing habitat, but also because of the degradation of visual and audio aesthetics. The creation of the mine would likely introduce increased auditory pollution with construction and mining equipment, as well as potentially impede aesthetic views both of which may reduce tourism in the area, as well as degrade the recreational value of the watershed for local residents and tourists alike (U.S. Environmental Protection Agency, 2023).

Salmon subsistence fishing plays an important role in the community both calorically and culturally. Currently residents of Alaska are eligible to engage in subsistence salmon fishing which requires permits that authorize fishing with specific gear (5 Alaska Admin. Code § 01.320). Alaskan residents rely on 329 pounds of annual subsistence harvest per capita including salmon (56%), other fish, land mammals, with subsistence harvest being more pronounced in smaller communities (Alaska Department of Fish and Game, 2013). This practice not only sustains food intake but also upholds cultural traditions, especially significant to the Yup'ik people.in the Kvichak River watershed, which includes Iliamna Lake and Lake Clark being the largest locations for subsistence fishing (Alaska Department of Fish and Game, 2013). Notably, both water bodies are linked to Upper Talarik Creek and the Koktuli River, areas targeted for potential mining discharge by the Pebble Mine project (Northern Dynasty Minerals, n.d).

The issue of access to subsistence fishing has historically sparked debate, particularly with the historical preference for indigenous communities. Changes in policy, allowing all residents access to fishing, have raised concerns from indigenous communities regarding the erosion of indigenous rights in the area (Endter-Wada, 1996). During the public hearing period of the Pebble Mine project, residents spoke on the lack of baseline data for the watershed, the importance of fishing, and the history of degrading indigenous land (Environmental Protection Agency, n.d.). Currently section 1203 of the Alaska National Interest Lands Conservation Act

(ANILCA) provides a framework for cooperative land use management, however implementation varies depending on the specific project, funding and stakeholder collaboration. In Bristol Bay this includes indigenous tribes, local residents and businesses, Alaska Department of Natural Resources, BLM and other state authorities. The history of threats to indigenous practices coupled with the necessity for improved integration of ANILCA highleights the need for solutions that incorporate diverse perspectives.

Recommendations and Conclusions

Based on the considerations of multiple stakeholders and their motivations, I recommended a two-part plan which includes that sections of the watershed be subject to research for critical habitat designation and designated if applicable, and management changes be implemented to protect the longevity of the fishery while maintaining community access to subsistence fishing. Due to the longevity of the mine proposal, and multiple resurfacing attempts to for construction, long term solutions are necessary for the future of the watershed.

Reclassification: The Endangered Species Act (ESA) of 1973 provides a framework for the conservation and protection of species facing the risk of extinction due to human activities. Under the ESA, species can be listed as "endangered" or "threatened," triggering various protections and conservation measures. Section 7 of the ESA mandates consultation by federal agencies to ensure that actions they authorize, fund, or carry out do not jeopardize the continued existence of listed species or result in habitat destruction (1973, § 7). Section 9 prohibits the "take" of endangered species (1973, § 9), but exemptions are provided under Section 10 for certain activities, such as those conducted by Alaskan Natives, which preserves the considerations and priorities of the indigenous community (1973, § 10). Iliamna Lake seals, currently considered part of the Bristol Bay harbor seal stock, require further research to determine their distinct population designation. ESA designation would provide longer term safeguards for harbor seal habitat. Tribal subsistence activities would be exempted under Section 10, ensuring continuity of cultural practices. Collaborative efforts between local tribes and the National Marine Fisheries Service (NMFS) would enhance understanding of the seals' ecology and inform management strategies. Establishing partnerships between tribes and NMFS not only validates traditional knowledge but also ensures the preservation and sustainable management of species for future generations in Alaska's diverse ecosystems. The classification as critical habitat will prevent harmful activities such as mining and dumping to occur on these designated areas (Arce, 2014).

Management changes: The State of Alaska currently has a policy for the management of sustainable salmon fisheries (Alaska Department of Fish and Game, 2013), which I recommend be adopted by the Alaska Department of Fish and Game and the Board of Fisheries for the watersheds in this region. The state areas shall better enforce these policies and land not under the jurisdiction of the state will be encouraged to comply with this policy. Areas under the jurisdiction of the Department of Natural Resources require increased communication with the Board of Fisheries and coordinated policy implementations would allow for more consistent management. This policy would specifically mandate the protection of salmon spawning, rearing and migratory habitats. This policy applies a precautionary approach which prompts corrective measures to conserve stock and habitats. While the ANILCA provides a framework for cooperative land use management, implementation is variable I recommend that this policy be reimplemented with changes that prioritize monitoring and preserving local ecology, as well as the creation of cooperative management units in the Nushag and Kvichak drainages. Including local research institutions such as the University of Alaska, Anchorage, in cooperative land use management and monitoring would allow for more data to be collected. Ecological standards can be implemented as a goal of ANILCA, and continuous monitoring can inform future management plans. I recommend increased monitoring with multiple approaches to address baseline data gaps: The use of LIDAR to assess steam courses to update information from the national hydrography dataset, streams should be monitored for fish presence including species, life stages and abundance, sediment coring to estimate historic magnitudes of salmon return to create a baseline, and aquatic macroinvertebrate studies. Accurate monitoring and results can allow for better informed management and decision making. The creation of cooperative management units should prioritize native coalitions and would establish decision making with multiple stakeholders. Public meetings can be introduced to discuss potential legislation and development plans and engage local residents.

Considering the multifaceted values of the stakeholders in Bristol Bay, Alaska, the region is at a critical point where economic interests intersect with environmental preservation and public health concerns as they relate to the Pebble Mine project. The reliance of the local community on the salmon fisheries for sustenance and livelihood underscores the importance of safeguarding the pristine natural environment. Additionally, the emerging public health risks associated with elevated arsenic levels and the potential exacerbation of habitat degradation due to mining activities further highlights the need for a proactive approach. Other sources of contaminants in the Nushagak and Kvichak Bay include, waste from marine activities, septic tanks, stormwater runoff and ldeposition of atmospheric pollutants (Hartwell, 2018). The intervention by the Environmental Protection Agency to halt the Pebble Mine project was a first step in preserving the long-term health of not only the ecosystem but also the social fabric of

Bristol Bay. It is recommended to implement management changes and land classification to mitigate potential adverse impacts on the environment, public health, and the cultural practices of the region. Preserving Bristol Bay's natural ecosystem not only aligns with the interests of the local community but also upholds broader ecological integrity and ensures the continued uses for future generations.

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