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MICROBIOME RECOVERY OF THE UPPER CLARK FORK RIVER RESTORATION SITE

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MICROBIOME RECOVERY OF THE UPPER CLARK FORK RIVER RESTORATION SITE

by

Danella Stapley

A thesis submitted in partial fulfillment of the requirements for the degree of

Ecological Restoration, M.S.

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Abstract

Soil microorganisms provide ecosystem functions and services critical for life, can have lasting effects on aboveground plant communities, and can serve as indicators of soil quality. However, biotic indicators are underrepresented in soil health assessments. Incorporating microbial soil evaluations in ecological restoration studies are important in order to obtain a more comprehensive understanding about their role in healthy soil development. In this study, we used three bioindicators to assess the post-restoration soil microbiome recovery of the Upper Clark Fork River: extracellular soil enzyme activity, microbial biomass, and microbiome community and diversity. We found that microbiome recovery rate varied at different phases of the restoration site, with one section still exhibiting low microbial biomass and enzyme activity after eight years of recovery. Prokaryotic community compositions of the different sites were compared. Prokaryotic communities from the study site separated into two groups based on Bray-Curtis measurements. These groups were unique compared to communities from the control site. We further tried to assess soil function through a soil inoculation experiment. Sandbar willow cuttings were grown in sterile soil inoculated with live or sterilized samples from different phases of the restoration site. Inoculation treatment (live or sterilized) and collection site did not influence willow growth, however, willow cutting mass had a significant effect. Extracellular soil enzyme activity was higher in soils inoculated with live inoculant than those inoculated with sterile inoculant. Inoculation with live soil from all collection sites increased enzyme activity over soil inoculated with unsterilized backfill soil. Inclusion of biotic indicators in soil health assessments may elucidate environmental factors and management actions that contribute to variance in microbiome recovery rates observed, leading to more effective restoration practices, and increasing the likelihood of self-sustaining, resilient ecosystems.

Keywords: soil microbiome, soil health, bioindicators, restoration, bioinformatics, extracellular soil enzymes

Dedication

I wish to dedicate this thesis to my family: to Mom and Dad who never let me believe that there were limits to my capabilities; to Vance, Caleb, Bennett, and Enoch who bring so much joy in my life and keep me humble; and especially to Ryan who supports me in all the ways, even though graduate students are the worst.

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1. Introduction

To assist in the recovery of mine-damaged sites, the ecology of the site needs to be considered as a whole. Soil health is a crucial component of that ecology. The physical and chemical attributes of soil such as particle size, pH, and nutrient availability are often assessed during restoration planning, implementation, and post-restoration monitoring. However, soil is not a sterile growing medium. There is a rich and complex ecosystem living within the soil that is frequently overlooked. Soil contains one of the most diverse ecosystems in the world, and it is teeming with life. One gram of soil can contain 10 million to 1 billion bacteria, over 7000 taxa, and 200 meters of fungal hyphae (Wagg et al., 2014). The soil microbiome is a subset of all soil biota which includes a vast variety of microorganisms, from 20 nm to 10 μm in diameter. Microbial populations consist of many taxa, including bacteria, fungi, archaea, protozoa, algae, and viruses (Islam et al., 2020; Swift et al., 1979).

Soil microorganisms provide ecosystem functions and services critical for life including nutrient cycling, decomposition, carbon sequestration, soil structure maintenance, water quality and supply, pollutant degradation, and pest and disease control (Decaëns et al., 2006; *State of Knowledge of Soil Biodiversity - Status, Challenges and Potentialities*, 2020). Mycorrhizal fungi and nitrogen-fixing bacteria can contribute up to 75% of phosphorus and 80% of nitrogen that is acquired by plants, and are important regulators of plant diversity and abundance (Van Der Heijden et al., 2008).

Aboveground and belowground biota are ecologically linked, influencing each other through positive and negative feedback mechanisms (Jain et al., 2020; Lyu et al., 2021; van der Putten et al., 2013; Wardle et al., 2004). Introduction of microbes through soil inoculations affect these feedbacks and can have long-lasting impacts on restored sites, affecting plant growth and

influencing plant compositions. Some microbes aid in plant establishment. For example, microbes have been shown to promote growth and increase heavy metal tolerance in plants (Grandlic et al., 2009; Hao et al., 2014). Microbes can affect successional trajectories of plant communities of restoration sites. Inoculation of soil from late secondary succession grasslands increased performance of late successional plants (Carbajo et al., 2011) and suppressed ruderal plant species giving late-successional plants a competitive advantage (Wubs, Van Heusden, et al., 2019). When a mixture of native and invasive grasses was grown in sterile soil inoculated with either soil from abandoned farmland (old-field) or a remnant grassland, the biomass of the invasive grass species increased equally for each treatment. However, soil inoculation from the old-field site resulted in higher mortality of native grasses, which may give invasive species a competitive advantage (Smith et al., 2018). The effects of soil inoculations can be long lasting. Inoculations from different soil sources changed long-term trajectories of plant communities in a degraded grassland (Han et al., 2022), and a one-time addition of soil biota to an arable field had sustained effects on the trajectories of aboveground and belowground biota, remaining distinct from uninoculated controls after 20 years (Wubs, Van der Putten, et al., 2019).

Various soil biotic factors have been evaluated as possible indicators of soil health such as extracellular soil enzyme activity, nitrogen mineralization, presence of pathogens, primary productivity, soil organic matter, and microbial biomass. (Chen et al., 2020; Doran & Zeiss, 2000; Lehmann et al., 2020; Sullivan et al., 2019). The characteristics of the site, ecosystem services being monitored, and practicability of the biotic indicator should be assessed when choosing which indicators to use in soil health assessments. Interpretation of these indicators should be context specific. Comparing changes at a particular site over time or due to management activities can help inform future management decisions (Fierer et al., 2021).

Extracellular soil enzymes catalyze many of the reactions involved in nutrient cycling, and microorganisms are considered the main source of those enzymes. The activity of these enzymes change promptly in response to shifts in environmental factors or management actions, allowing for early detection of altered microbiome function (Alkorta et al., 2003). Enzymes involved in nutrient cycling include β-glucosidases and phosphatases. β-glucosidases are involved in the carbon cycle as it catalyzes the final, and rate-limiting, step in cellulose degradation by hydrolyzing cellobiose into glucose (Yeoman et al., 2010). Phosphatases hydrolyzes organic phosphorus to inorganic phosphorus, making bioavailable phosphorus (Eivazi & Tabatabai, 1977).

Microbial biomass is the living component of soil and is often used as a bioindicator of soil health. It plays an important role in soil fertility, serving as both a source and a sink of nutrients (Brookes et al., 1984). It is also an indicator of heavy metals, decreasing as metal concentrations increase (Brookes & McGrath, 1984). Soil DNA can be used as an estimate of soil microbial biomass as it has a high correlation to other estimates of soil microbial biomass such as those obtained using chloroform fumigation based assays (Marstorp et al., 2000). Soil DNA extraction is quick and simple, and can be analyzed through microbiome sequencing (Bloem et al., 2005).

Microbiome analysis is becoming more accessible as advances in DNA sequencing have significantly reduced the cost. This increased ability to characterize microbiota and correlate them with environmental outcomes opens opportunities to use them as bioindicators of soil conditions (Hermans et al., 2017, 2020). Analysis of soil microbial biodiversity can serve as an indicator of soil ecosystem functions. Many studies have correlated biodiversity levels of microbial communities with these important ecosystem functions (Bach et al., 2020; Delgado-

Baquerizo et al., 2016; Philippot et al., 2013; Powell & Rillig, 2018; van Elsas et al., 2012). Incorporation of microbiome biodiversity data strengthened predictions in models of ecosystem process rates over environmental variables alone (Graham et al., 2016). It was shown by Wagg *et al.* (2014) that artificially reducing soil biodiversity and species abundance in grassland microcosms (closed growth chambers) resulted in lower plant diversity and the loss of multiple ecosystem functions, suggesting that soil biota are important in maintaining these functions. It was further shown that diversity both within and between fungal and bacterial communities enhanced ecosystem functioning (Wagg et al., 2019). Soil is a heterogeneous environment on both a spatial and temporal scale. Availability of resources, such as carbon, can vary greatly on a nanoscale level, limiting microbe activity. In order to cope with environmental variability, microorganisms can enter a state of dormancy where they are not metabolically active (Lennon & Jones, 2011). While there is a great abundance of microorganisms in the soil, most of the microbes are in a dormant state. It is estimated that only 0.1 – 2 % of soil microbial biomass are active, though 10 – 40% may become active due to substrate inputs (Blagodatskaya & Kuzyakov, 2013). Soil microorganisms tend to form biofilms and groups of colonies, only spreading to uncolonized surfaces under favorable conditions. This aggregation of organisms; ready to become active when resources become available results in hotspots of microbial activity (Ekschmitt et al., 2005). One active microbial hotspot is the rhizosphere, as roots release nutrients and energy sources into the soil, increasing carbon availability for microbial growth (Kuzyakov & Blagodatskaya, 2015). Since rhizospheres are centers for increased microbial activity, they are often targeted for bioindicator analysis.

Previous research had shown variability in the recovery time of soil microbiota after restoration. For example, revegetation of an old field with native plant species resulted in

increased microbiota recovery over a short time, with microbial communities becoming more similar to remnant stands within a 10 year period (Gellie et al., 2017), while other restoration sites may take 40-60 years to recover (Liddicoat et al., 2022). More research is needed to determine factors that influence recovery time of the soil microbiome in order to improve restoration recovery rates.

The biotic component of soil serves critical roles in soil ecosystem functions, can affect aboveground plant compositions, and can serve as indicators of soil quality. Including biotic indicators to soil health assessments can increase understanding of the interplay between restoration activities and the soil microbiome, leading to more effective restoration practices and long-term success. Inclusion of soil's biological component in soil evaluation is necessary for a more comprehensive picture of soil health, yet biotic components typically make up less than 20% of the indicators in soil assessment schemes (Doran & Zeiss, 2000; Lehmann et al., 2020; Swati et al., 2020). Given the crucial role of soil organisms in supporting many ecosystem functions and enhancing resiliency, this omission may significantly impede long-term restoration success. Therefore, it is essential to incorporate microbial soil evaluations in ecological restoration studies in order to obtain a more comprehensive understanding about their role in healthy soil development.

If the end goal for many of the remediated and restored sites impacted by our history of mining in western Montana is to establish self-sustaining, native plant communities, efforts should be made to assess recovery of soil microbiome communities and functions for the purpose of gauging the likelihood for the successful establishment of resilient native plant communities.

2. Purpose and Aim of Study

The Upper Clark Fork River has been impacted by over a hundred years of mining and smelting activities, including a historic flooding event in 1908 that washed over six million cubic yards of mine tailings from Butte, Montana. The event deposited heavy metal contamination throughout the flood plain. This area is currently targeted by a massive ecological remediation and restoration project being completed in phases. Interestingly, phases 1-5 are at different stages of the remediation and restoration process, with each of the phases being initiated in different years ranging from 2013 to the current year. This spread, in terms of years since remediation initiation and completion, presents an opportunity to investigate ecosystem events such as post-reclamation establishment of microbial communities and the recovery of soil functions at a mine-damaged site.

The specific objectives of this study are to compare both microbial community function and microbial population diversity within soil samples collected from willow rhizospheres along the Upper Clark Fork Restoration Site in phases 1, 2, 4, and 5 to each other and to a reference site in Thompson Park, a reference site within the same watershed that has not sustained extensive mine waste contamination. The potential for confounding effects due to differences in 1) the variable environmental biotic and abiotic conditions at the different phases of the Clark Fork restoration project and 2) the potential for unique microbial communities forming within the rhizosphere of different plant species, was minimized by narrowing this study to the populations of soil life associated with the rhizosphere of sandbar willows (*Salix exigua*). Sandbar willow is a native shrub that grows along the banks of the Upper Clark Fork River and is actively installed during restoration actions as it can establish quickly and stabilize riverbanks (Bentrup & Hoag, 1998; Polster, 2003).

The biotic soil indicators we used for assessment of microbiome recovery were soil enzyme analysis, microbial biomass as determined by DNA yield per gram of soil, microbiome composition analysis, and primary productivity as measured by total mass of willow growth.

The two experimental aims of this research were:

1) Assess post-restoration microbiome recovery of the Upper Clark Fork River in the field at the site known as Reach A with the following biotic soil indicators:
    a. Extracellular soil enzyme activity of phosphatases and β-glucosidases
    b. Relative microbial biomass as estimated by soil DNA yield per gram soil
    c. Microbiome analysis of the composition and diversity of the sandbar willow rhizosphere. This was accomplished through 16S, 18S, and ITS microbiome sequencing.

Analyses were performed on soil samples taken from phases 1, 2, 4, and 5 of the Upper Clark Fork River restoration site and from a reference site located at Thompson Park.

2) As an additional measure of microbial community function, we compared the primary productivity of willow cuttings planted in sterilized soil inoculated with soil samples from willow rhizospheres collected at the different phases. Extracellular enzyme activity of phosphatases and β-glucosidases were also measured after 2 months of willow growth.

We sought to address the following questions:

1) Are soil microbiome communities and functions recovering with time post-restoration, as measured by microbial biomass, microbiome diversity and composition, and extracellular soil enzyme activity?

2) Can we measure soil microbiome function by comparing primary productivity and extracellular enzyme activity of sandbar willow cuttings grown in inoculated soil from restoration and remnant sites against each other and with cuttings grown in sterile soil?

As remediation actions include the removal of contaminated soil and backfilling with clean sediment, mainly subsoils borrowed from non-riparian sites, we expect that both the soil biomass and the microbiome biodiversity levels would be low. Because of this, we hypothesized that restored sites would have lower biomass, biodiversity, and enzyme activity levels than the control site at Thompson Park. Microbial inputs from revegetation and upstream locations like the reference site would contribute to soil repopulation, so we hypothesized that microbial biomass, diversity, and soil enzyme activity would be positively correlated with time post-restoration and that the microbiome composition would become more similar to the reference site. Because soil samples collected from unremediated sites had heavy metal concentrations low enough to allow plant growth, we hypothesized that soil biomass and biodiversity would be similar to the control site yet would have unique microbiome composition in response to the presence of heavy metals.

We hypothesized that since soil microbes produce enzymes that make nutrients bioavailable to plants, willows grown in inoculated soil would exhibit greater growth and soil enzyme activity than willows grown in sterile soil. We also hypothesized that willow growth and enzyme activity would be higher for plants grown in soil inoculants with longer post-restoration times as the microbiomes would have had greater time to recover.

3. Materials and Methods

3.1. Study Site

The Upper Clark Fork River Basin is located in southwest Montana, USA and is comprised of the uppermost 43 miles of the Clark Fork River, from the headwaters near Butte, MT to the confluence of the Little Blackfoot River near Garrison, MT (Clark Fork Coalition, 2016). The Upper Clark Fork has been negatively impacted by mining and smelting activities in Butte and Anaconda, including a historic flooding event in 1908 that deposited metal-contaminated tailings throughout the floodplain. It is estimated that 100 million tons of mine tailings entered the Upper Clark Fork from 1880 to 1982 (Pascoe & Dalsoglio, 1994). The Clark Fork River was added to the Superfund National Priorities List in 1983 and the Upper Clark Fork was designated as Reach A of the Clark Fork River Operable Unit (CFROU). Remediation of Reach A is being completed in phases over the course of several years, beginning with Phase 1 in 2013 (US EPA, 2016). Remediation of the phases is occurring non-sequentially to minimize potential adverse effects of the remediation process and protect newly remediated areas from potential flood events (Clark Fork Coalition, 2016). Remediation and restoration activities include removal of contaminated sediment and backfilling with clean sediment, reconstruction of the floodplain and streambanks, and revegetation. Backfill soils must meet certain chemical and physical specifications: soil texture must be sandy loam or finer, but not clay; have pH between 6.5 and 8.5; metal concentrations of arsenic < 30 ppm, cadmium < 4 ppm, copper < 100 ppm, lead < 100 ppm, zinc < 250 ppm; contain > 1.5 percent organic matter; and a specific conductance < 4.0 deciSiemens per meter (US EPA, 2004).

This study focuses on riparian shrubland within phases 1, 2, 4, and 5 of Reach A, from Warm Springs to Racetrack (46.222738 N, 112.759402 W, 1450 m ASL elevation). This study

uses the same phase divisions as the CFROU but has relabeled them to correspond with the year that woody revegetation was completed at the soil collection sites. Revegetation in Phase 1 was completed in 2014; Phase 5 in 2015, and Phase 2 in 2016. Remediation and restoration actions had not been implemented for Phase 4 and is labeled as 'unremediated.' Phase 3 was undergoing remediation and was excluded from the study.

The area can be characterized by a semi-arid climate with 11-12 inches annual precipitation (*WRCC*, 2023). Between 1981 and 2010, average temperatures for Anaconda (the nearest weather station) were -3.6°C in January and 18°C in July.

For a nearby reference site, we picked the Blacktail Creek in Thompson Park, Montana (45.904211 N, 112.466460 W, 1700 m ASL elevation) which is eight miles south of Butte, Montana, USA. It is part of the Clark Fork Watershed and is a tributary of the Upper Clark Fork River. At this site, riparian shrubland is present and it has not sustained extensive mining damage nor been remediated (Figure 1).

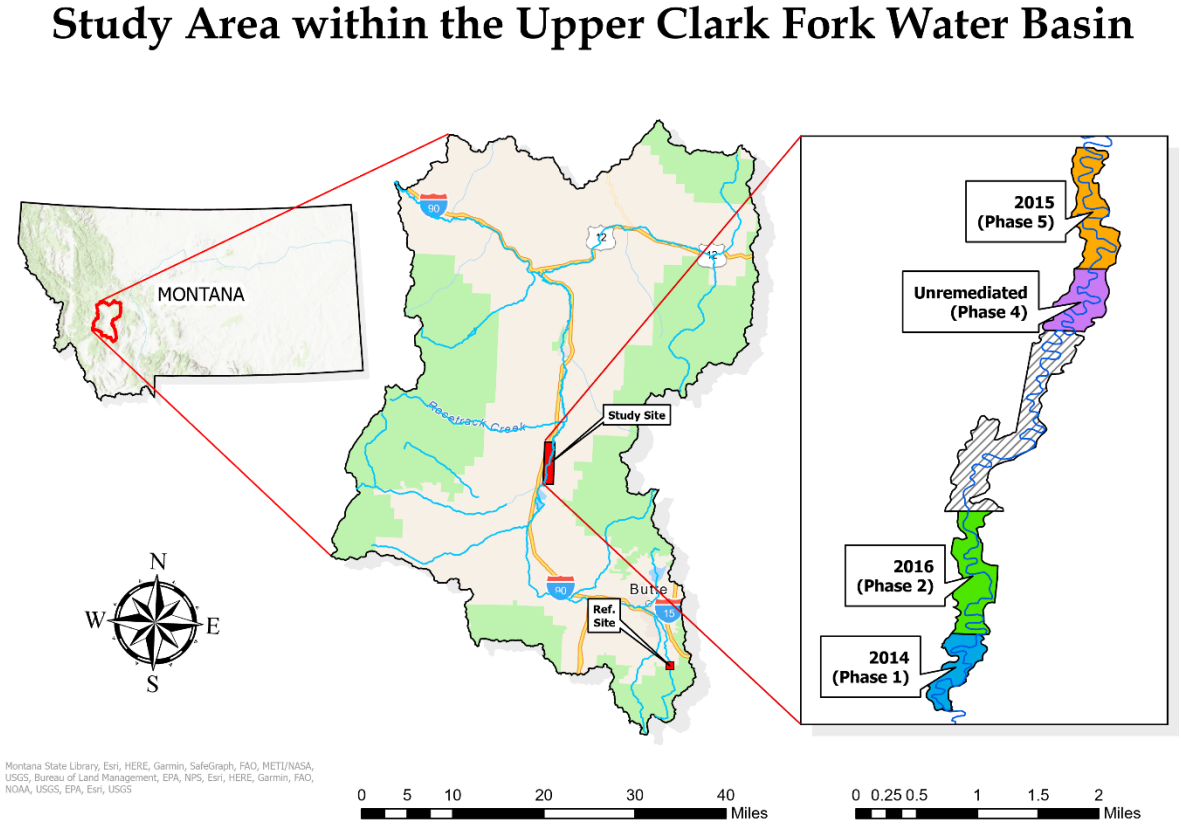

Figure 1: Map of the study site

3.2. Physical and Chemical Characterization of Soil

All samples and measurements were taken within a one-meter quadrat that was placed around a sandbar willow shrub with stem bases less than 2 cm in diameter. Sample sites were restricted to a maximum distance of 10 m from the river within riparian shrub habitat having at least 40% shrub cover.

Soil moisture was measured with a FieldScout TDR 150 Moisture Meter. The water volume content was determined by taking the average of five readings from each site. Soil compaction was measured with a FieldScout SC 900 Soil Compaction Meter. Soil samples from one site within each phase was sent to the Agricultural and Environmental Services Laboratories at the University of Georgia Extension for analysis. To determine heavy metal concentrations,

soil was acid digested following EPA method 3051 (US EPA, 2007), then analyzed by ICP-OES (Spectro Arcos) following EPA method 200.2 (US EPA, 1996a). Nitrate concentrations were measured using a continuous flow analyzer (I/O Analytical FS3100) following EPA method 353.2 (US EPA, 1996b). Ammonium concentrations were measured using a discrete analyzer (Astoria) following EPA method 350.1 (US EPA, 2019). Nutritional, salt, and pH levels of soil were determined by the soil media extract method (Kissel & Sonon, Leticia, 2011).

3.3. Sandbar Willow Rhizosphere Collection

Three sites were randomly selected within each phase (1, 2, 4, and 5) and the reference site (Thompson Park). A sandbar willow plant was exhumed to a max depth of 30 cm. Soil was shaken from the willow roots onto a clean sheet of paper. Two 1.5 mL samples for DNA and soil extracellular enzyme analysis were collected and stored on ice until transferred to -20°C. For use in microbiome inoculation experiments, 2 L of soil were collected and stored on ice until transferred to 4°C. All equipment was cleaned with water and sterilized with 70% EtOH between sample collections.

Soil for sequencing, enzyme analysis, and microbiome inoculation experiment was collected from the study and reference sites in May of 2022. Additional samples for enzyme analysis were collected in October of 2022.

3.4. Microbiome Sequencing

DNA was extracted from 250 mg of soil using a Qiagen DNeasy PowerSoil Pro Kit. DNA was checked for quality and quantified using ThermoScientific NanoDrop 2000c spectrophotometer. Bacterial 16S rRNA genes were amplified using primers 515f (Parada et al., 2016) and 926r (Quince et al., 2011). Eukaryotic 18S rRNA genes were amplified using primers 1391f and 1510r (Amaral-Zettler et al., 2009). Fungal internal transcribed spacer (ITS) regions

were amplified using primers ITS1f and ITS2 (White et al., 1990). include the TruSeq adaptor sequence (Table I).

Table I: Primer sequences for microbiome sequencing.

Target-Organisms		Gene	Region	Location	Length (bp)
Prokaryotes		**16S**	**V4-V5**	**515-926**	**~410**
	515f	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGYCAGCMGCCGCGGTAA			
	926r	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCGYCAATTYMTTTRAGTTT			
Microbial Eukaryotes		**18S**	**V9**	**1391F-1510**	**~210 - 310**
	1391f	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTACACACCGCCCGTC			
	1510r	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTGATCCTTCTGCAGGTTCACCTAC			
Fungal and Micro Eukaryotess		**ITS**	**ITS1-ITS2**	**ITS1F-ITS2**	**~250 - 600**
	ITS1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTTGGTCATTTAGAGGAAGTAA			
	ITS2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCTGCGTTCTTCATCGATGC			
TruSeq sequences adaptors are indicated in blue, and gene specific sequences are orange.					

PCR reaction mixtures are listed in Table II. 10 ng of template DNA was used in the 16S and ITS reactions, while 20 ng of template DNA was used in the 18S PCR reactions as it improved product yields. 10 µL of template DNA was used for samples with DNA concentrations below the limit of detection.

Table II: PCR reaction mixtures

PCR Reaction Mixture	
Reagent	**Volume**
PCR-grade water	17.4 µL
Taq DNA Polymerase (1U/µL)	0.6 µL
Taq Buffer with KCl (10x)	2.5 µL
$MgCl_2$ (25 mM)	2.0 µL
dNTP Mix (10 mM each)	0.5 µL
Forward primer (10 µM)	0.5 µL
Reverse primer (10 µM)	0.5 µL
Template DNA	1.0 µL
Total reaction volume	25.0 µL

All thermocycler conditions were performed according to the Earth Microbiome Project standard protocols (Thompson et al., 2017) (Table III). Three 25 μL PCR reactions for each sample were pooled and cleaned with the ThermoScientific GeneJET PCR Purification Kit (Section 7.2), and the resulting amplicons were barcoded and sequenced at the University of New Hampshire Hubbard Center for Genome Studies (HCGS) using the NovaSeq 6000 platform. Sequences were demultiplexed and primer sequences were trimmed by HCGS.

Table III: Thermocycler conditions

16S		
Temperature	**Time**	**Repeat**
94 °C	3 min	
94 °C	45 s	x35
50 °C	60 s	x35
72 °C	90 s	x35
72 °C	10 min	
4 °C	hold	

18s		
Temperature	**Time**	**Repeat**
94 °C	3 min	
94 °C	45 s	x35
57 °C	60 s	x35
72 °C	90 s	x35
72 °C	10 min	
4 °C	hold	

ITS		
Temperature	**Time**	**Repeat**
94 °C	3 min	
94 °C	45 s	x35
57 °C	60 s	x35
72 °C	90 s	x35
72 °C	10 min	
4 °C	hold	

3.5. Microbiome Bioinformatics

The sequencing data were uploaded to the Galaxy web platform, and we used the public server at usegalaxy.org to analyze the data (The Galaxy Community, 2022). Sequences were trimmed, quality filtered, and de-replicated prior to generating amplicon sequence variant (ASV) tables using DADA2 (Callahan et al., 2016). The 16S, 18S, and ITS rRNA gene amplicon reads were trimmed to 240 bases for both forward and reverse. Read lengths less than 60 bp were removed. The dada tool was used to remove sequencing errors, the mergePairs tool was used to merge sequences, and the makeSequenceTable tool was used to de-replicate the data. Chimeric sequences were removed using the removeBimeraDenovo tool with its default settings, and

actual sequence variant (ASV) tables were constructed with both the assignTaxonomy and addSpecies tools with their default settings. Training databases were Silva 138.l dada formatted (McLaren & Callahan, 2021), Silva v128 and v132 dada2 formatted (Morien & Parfrey, 2018), and UNITE general FASTA release for eukaryotes (Abarenkov et al., 2022) for 16S rRNA, 18S rRNA and ITS genes, respectively. Beta diversity was calculated using the scikit-bio Beta Diversity tool with the Bray-Curtis diversity index (Galaxy Version 0.4.2.0). Principle components analysis (PCA) was performed using the Multivariate tool on the Bray-Curtis diversity matrix (Galaxy Version 2.3.10).

3.6. Extracellular Soil Enzyme Analysis

To assess soil microbial functioning, the potential enzyme activity of two soil extracellular enzymes, β-glucosidase and phosphatase, were measured photometrically according to Jackson et al (2013) with modifications.

Enzyme assays were conducted at pH 8.0, the average pH of the soil at willow harvest as suggested by German et al (2011). Approximately 150 µL of soil was weighed then added to a 96-well deep-well plate. The soil was suspended in 400 µL of Tris buffer (50mM, pH 8.0) and mixed with 200 µL of para-Nitrophenol (pNP)-based substrate solutions: 5mM pNP-β-glucopyranoside for β-glucosidase and 5mM pNP-phosphate disodium salt hexahydrate for phosphatase. The reactions were incubated at 20°C for 18 – 24 h (greenhouse experiment samples) or 2 – 3 h (field samples) under continuous shaking. The reactions were centrifuged at 1.2 x *g* for 8 minutes and 100 µL of supernatant was transferred into a transparent 96-well plate and mixed with 200 µL of Tris buffer. Absorbance was measured by spectrophotometer at 410 nm and 500 nm using a ThermoScientific Varioskan LUX plate reader.

The 500 nm reading was subtracted from the 410 nm reading to correct for soil contamination (Section 7.1), then the mean substrate control absorbance was also subtracted to obtain the final absorbance value.

Approximately 9 g of soil from each sample was dried at 60° C for 48 hours. The ratio of the masses of the dry and wet soil samples was determined. The dry mass of the soil in each reaction was calculated by multiplying the wet mass of the soil in the assay by the dry/wet mass ratio.

The pNP concentration was calculated from an absorbance calibration curve included on each 96-well plate. pNP concentrations ranged from 0 to 0.4 mM. Concentrations in the standard curve were multiplied by 0.6 to get μmoles pNP per 600 μL reaction volume. Absorbance was plotted against μmole pNP to obtain a best fit line. The slope of the line was the conversion factor (C) to convert absorbance values to pNP concentration for each enzyme reaction.

Enzyme activity (EA) in μmoles hr^{-1} g dry $mass^{-1}$ was calculated from the equation:

$$EA = \frac{FA}{(C * incubation\ time * sample\ dry\ mass)} \quad (1)$$

where EA is enzyme activity, FA is final absorbance, and C is the pNP conversion factor.

The enzyme activity for a sample was equal to the mean of three technical replicates.

3.7. Willow Cutting Collection and Storage

Sandbar willow (*Salix exigua* L.) cuttings were collected from Thompson Park, Montana in April of 2022. The diameter of the stakes ranged from 7.66 mm to 14.02 mm with a mean of 10.48 mm. Stakes were kept dormant, wrapped in damp burlap, at 13°C until planting. Stakes were then cut into 30 cm segments in preparation for planting.

3.8. Sterile Growth Medium

Soil was collected from the backfill excavation site for CFROU Reach A Phase 3 in May 2022. Soil high in sand content was chosen to better approximate sandbar willow habitat. Soil was sifted through a 0.5-inch metal screen to remove large rocks. Perlite was incorporated into the soil (30% by volume) to lighten the soil and aid in drainage. The soil was then sterilized by autoclaving at 121°C and 20 psi for 20 minutes on regular setting. The resulting soil and perlite mix was used as sterile growth medium.

3.9. Soil Inoculant Preparation

Sandbar willow rhizosphere soil (Section 3.3) was sifted through a 5 mm sieve and homogenized. One liter of the soil was sterilized by autoclaving at 121°C and 20 psi for 20 minutes on regular setting. Another liter of soil was stored at 4°C until needed. All equipment was cleaned with water and sterilized with 70% EtOH between samples.

3.10. Greenhouse Microbiome Inoculation Experiment

To test whether soil microbiome inoculation had an effect on willow growth and extracellular soil enzymes, willow stakes were planted in sterile growth medium with a band of either live soil inoculant or sterilized soil inoculant. In a 655 cm^3 pot, 400 cm^3 of sterilized growth medium was added. Then 59 cm^3 of sterilized growth medium was of soil inoculant was layered on top of the growth medium. Finally, the inoculant soil band was covered with sterilized growth medium until soil was 1.5 cm below the lip of the pot (Figure 2). Willow stakes were planted in the soil so that two-thirds of the stake was within the soil. Equipment was cleaned with water and sterilized with 70% EtOH between samples. There were 15 replicates for each of the two treatments (live inoculant and sterilized inoculant) for each of the three sites from phases

1, 2, 4, 5, the reference site, and backfill for a total of 480 pots. Pots were randomized to avoid positional effects.

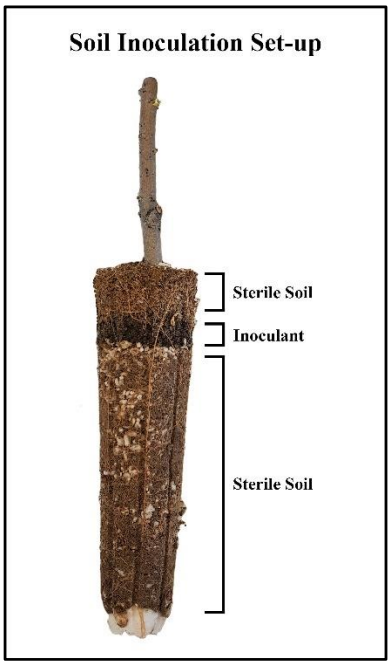

Figure 2: Soil set-up for the soil inoculation experiment. A band of 59 cm^3 of inoculant was placed between two layers of sterile soil.

The experiment was conducted in a naturally lit greenhouse at Montana Technological University. Standard temperatures settings were 24° C during the day and 18° C at night. The pots were watered three times daily for 5 minutes. The watering regime was increased to 10 minutes as temperatures increased. After 8 weeks, the plants were harvested (Figure 3). Roots, shoots, and cuttings were dried at 60°C for 48 hours, and their biomass was determined. The soil was collected and stored at -20° C to reduce enzyme degradation before soil extracellular enzyme analysis (Lee et al., 2007).

Figure 3: Willow growth at 8 weeks.

3.11. Statistical Analysis

All statistical analyses were performed in Minitab® (2023); an α level of 0.05 was accepted as significant. Data was checked for normality using the Anderson-Darling Normality Test. Prior to analysis, non-normal data were normalized using natural logarithm transformations. Non-parametric analyses were performed on data that did not normalize after transformation. Variance was checked via Levene's method.

To determine whether there was a difference in phosphatase activity between live and sterilized inoculated soils, the two-sample t-test was used with equal variances not assumed.

To determine whether there was a difference in β-glucosidase activity between live and sterilized inoculated soils, the Mann-Whitney test was used.

Differences in phosphatase activity for live inoculated samples were analyzed with one-way ANOVA with equal variances not assumed. Differences in β-glucosidase activity for live inoculated samples were analyzed with the Kruskal-Wallis Test. Grouping information was determined by the Games-Howell Method using 95% confidence.

Regression analysis was performed in Minitab with two-sided confidence intervals.

4. Results

4.1. Study Site Assessments

Soil moisture content of soil collection sites ranged from 23.2 – 35.8 % water volume content (% WVC), with lower moisture levels found at the 2015 and 2016 year of revegetation (YOR) sites (Figure 4).

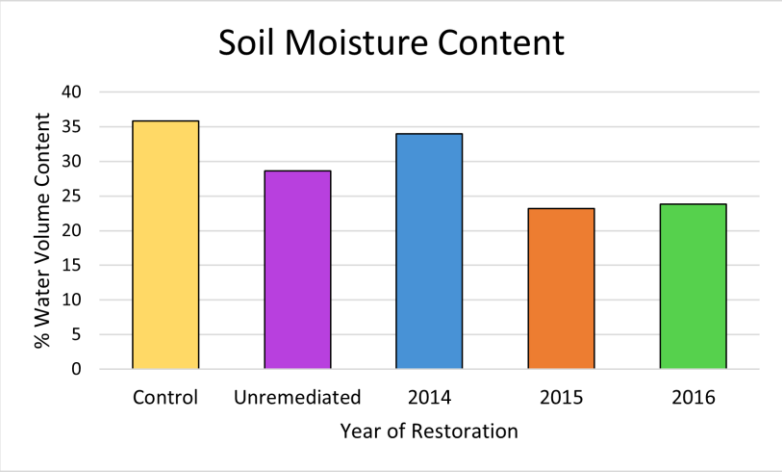

Figure 4: Soil moisture levels (% WVC) at soil collection sites.

Penetration resistance of 200 PSI is a root-limiting level of compaction. Average penetration depth to 200 PSI compaction ranged from 16.0 – 45.7 cm. The maximum measurement depth for the FieldScout soil compaction meter is 45.7 cm. The mean penetration depths of the restored sites were 49.5% of the control and non-remediated sites (Figure 5).

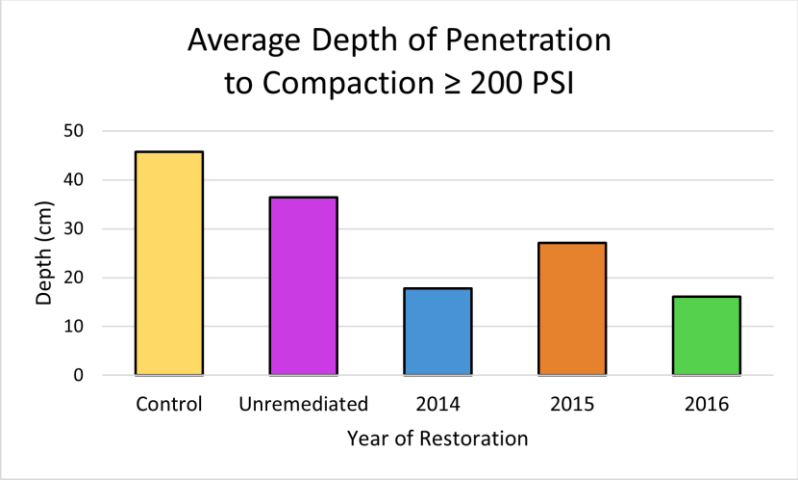

Figure 5: The average depth of 200 PSI compaction. Larger measurements indicate less compaction.

Heavy metal concentrations of the five contaminants of concern were measured, after acid digestion, on an ICP-OES. One soil collection site from each phase was analyzed. Higher levels of heavy metals were present at the unremediated, slicken, and 2016 YOR sites (Figure 6).

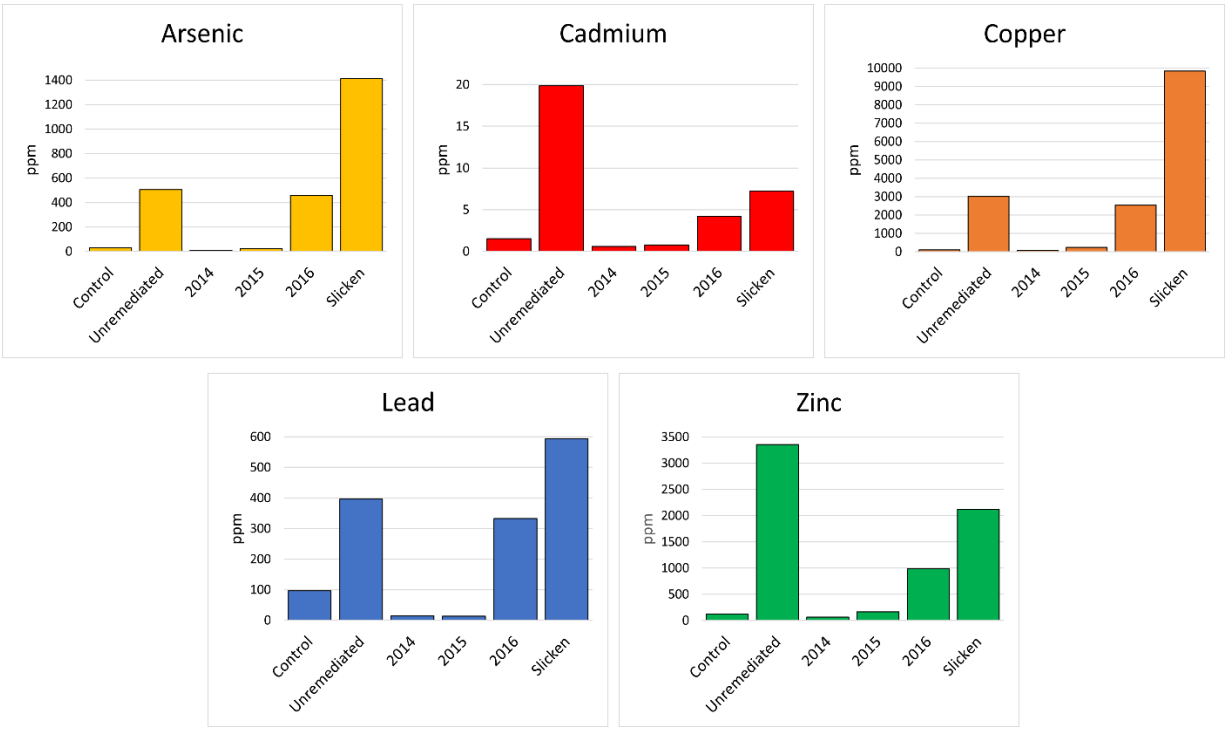

Figure 6: Heavy metal concentrations in parts per million (ppm) of field soil samples. Metal concentrations from slickens were included for comparison. Minerals were analyzed by ICP-OES after acid digestion.

Nutritional levels of soil were determined by the soil media extract method followed by analysis on an ICPS. The concentrations of nitrate, phosphorus, and potassium were below optimal ranges for all sites except for slickens, concentrated deposits of mine tailings. Soluble salt concentrations were above optimal ranges for the unremediated and slicken sites. The pH for all sites was neutral except for the 2014 YOR and slicken sites which were basic and acidic respectively (Table IV).

Table IV**: Nutritional analysis of field soil samples**

	Soluble Salts	NO3-N	NH4-N	P	K	Ca	Zn	pH
Control	1.35	1.15	0.49	0.21	10.02	162.51	0.44	7.22
Unremediated	4.21	2.72	<0.02	0.11	52.08	566.97	14.79	7.04
2014	1.22	0.28	<0.02	0.12	11.69	117.31	0.27	8.01
2015	2.07	3.62	<0.02	0.15	11.07	373.55	1.29	7.29
2016	1.38	<0.05	<0.02	0.07	7.46	108.4	0.55	7.85
Slicken	19.34	42.34	34.35	9.13	56.29	440.23	844.78	4.55
Optimal Ranges	0.75 - 3.49	40 - 199		3 - 10	60 - 249	80+	5 - 30	

Soil DNA yields are used to estimate relative abundance of microbial biomass. The control and unremediated sites had the highest DNA yields. The 2015 YOR site had the lowest DNA yield of the soil collection sites, with one of the sites within 2015 YOR and the backfill soil did not have detectable levels of DNA (Figure 7).

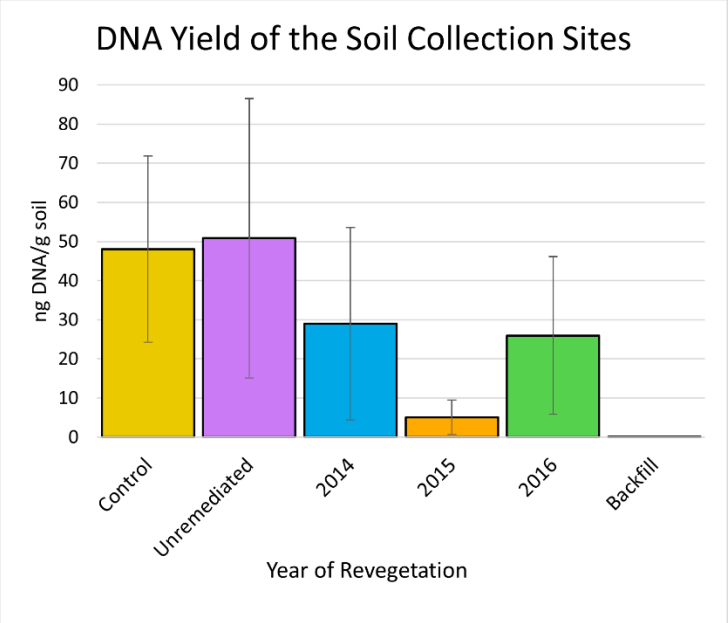

Figure 7: Effect of soil location on DNA yield. Error bars represent standard deviation.

Extracellular soil enzyme analysis of field collected samples showed seasonal variation, with higher enzyme rates in fall as compared to spring. Differences in enzyme activity between sites were more prominent in the fall samples. The control and unremediated sites had the highest enzyme activity rates for both enzymes. The 2014 YOR site had intermediate levels of enzyme activity, while 2015 YOR and 2016 YOR sites had low activity levels. Backfill soil samples were inert (Figure 8). Soil samples collected in May were utilized in the soil inoculation experiments (Section 4.2).

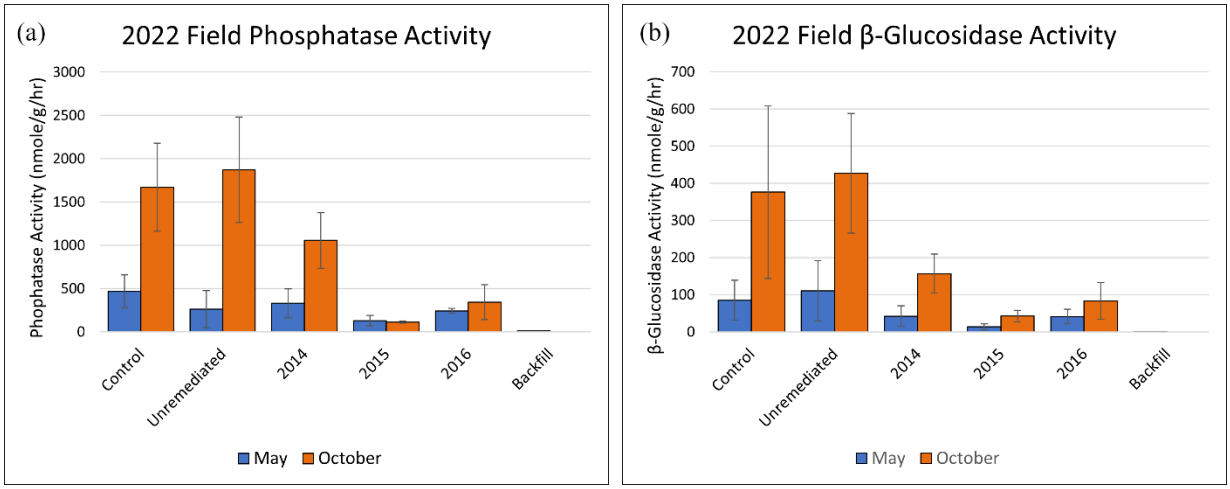

Figure 8: Seasonal transitions and the effect of restoration status on soil extracellular enzyme activities. Potential enzymatic activity of (a) phosphatase and (b) ß-glucosidase. Error bars represent standard deviations.

Sequencing of the 16S, 18S, and ITS rRNA gene amplicons resulted in 5,531,499 total reads. The number of reads were not evenly distributed between the amplicon types. 16S rRNA gene amplicons had the fewest number of initial raw reads with 941,755 counts and exhibited the greatest reduction of read counts during data processing (Table V). Optimization of the data processing steps may increase the amount of usable data, however a large number of read lengths too short to come from the 16S rRNA gene region were observed.

Table V: 16S Amplicon sequencing read counts.

16S Sample	Initial	Post-processing	Percentage Kept
P1A	11122	1357	12.20
P1B	44688	10151	22.72
P1C	28856	6788	23.52
P2A	58120	14652	25.21
P2B	79550	17145	21.55
P2C	60536	12189	20.14
P4A	55592	20713	37.26
P4B	66913	18926	28.28
P4C	37010	13803	37.30
P5A	66446	16279	24.50
P5B	57480	13231	23.02
P5C	55897	10240	18.32
TPA	81006	18938	23.38
TPB	69269	16320	23.56
TPC	81932	16264	19.85
VB	87338	12049	13.80
Total	**941755**	**219045**	

Sequencing data from the eukaryotic amplicons 18S and ITS had higher read counts: 2,994,758 and 1,445,001 respectively. Higher percentages of data were also maintained during data processing (Table VI).

Table VI: 18S and ITS amplicon sequencing read counts

18S Sample	Initial	Post-processing	Percentage Kept
P1A	53919	45007	83.47
P1B	110231	96057	87.14
P1C	132336	117327	88.66
P2A	104485	95122	91.04
P2B	156595	139151	88.86
P2C	239271	215529	90.08
P4A	153868	133838	86.98
P4B	184330	166673	90.42
P4C	213158	197953	92.87
P5A	163813	129798	79.24
P5B	237734	220679	92.83
P5C	313996	257487	82.00
TPA	202313	182656	90.28
TPB	198709	180298	90.73
TPC	255700	229627	89.80
VB	274300	198866	72.50
Total	**2994758**	**2606068**	

ITS Sample	Initial	Post-processing	Percentage Kept
P1A	108074	97451	90.17
P1B	89856	80391	89.47
P1C	137974	124712	90.39
P2A	77329	72079	93.21
P2B	132327	118537	89.58
P2C	136231	127055	93.26
P4A	140115	126344	90.17
P4B	73373	67659	92.21
P4C	107100	85978	80.28
P5A	123996	114643	92.46
P5B	65193	60190	92.33
P5C	79731	71524	89.71
TPA	72825	67560	92.77
TPB	100576	91143	90.62
TPC	40685	37261	91.58
VB	109601	102474	93.50
Total	**1594986**	**1445001**	

Rarefaction curves for all samples, except 2014-A, plateaued indicating sufficient sampling depth to reach saturation (Appendix B: Rarefaction Curves). Sequencing data from sample 2014-A was removed from further analysis as sufficient sampling depth was not achieved.

Sequence count tables were generated, and amplicon sequence variants (ASV) were assigned for all samples. Random rarefied community matrices were generated from the sequence count tables for downstream diversity analysis.

A Bray-Curtis dissimilarity matrix was produced from random rarefied community matrices of the 16S amplicon data in order to generate a PCA ordination of prokaryotic communities (Figure 9). Preliminary analysis of the prokaryotic community compositions via

PCA ordination using Bray-Curtis distance shows distinct patterns. Communities from the control site form a grouping that is separate from the study site communities. The study site communities form two groupings. 2015 YOR sites are grouping together with the unremediated sites and backfill communities. The 2016 YOR sites form a separate group. The 2014 YOR sites are split between the two groups. Interestingly, 2014-C was collected from an area of vegetation that was preserved during remediation activities and it is grouping with the unremediated sites. 2014-B was collected from a site where soil was removed and backfilled and is grouping with the 2016 YOR sites with the same treatment. The 2015 YOR sites were also restored, however their prokaryotic communities are more similar to the unremediated community assemblages.

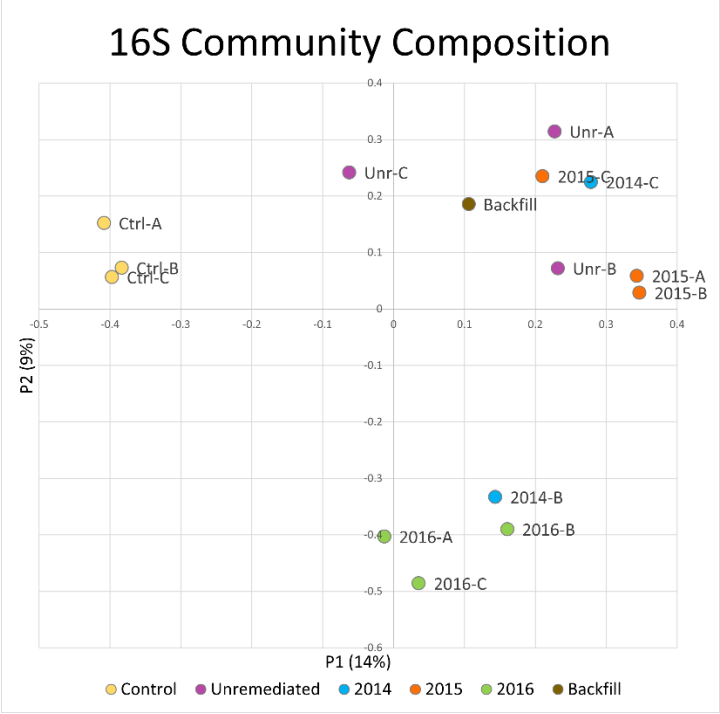

Figure 9: Effects of site location on prokaryotic communities. PCA ordinations of Bray-Curtis distance are presented for the 16S amplicon data.

PCA ordination analysis was not performed on the 18S and ITS libraries. The 18S library identified many eukaryotes that are not considered part of the microbiome such as centipedes, mites, and nematodes (Table VII). Further filtering of the data needs to occur before a meaningful analysis of the eukaryotic microbiome can be completed.

Table VII: Top 18 ASV Assignments for 18S library

Eukaryote	Counts
Green Algae	75136
Centipede	57215
NA	30112
NA	26078
Boletaceae (fungal family)	21316
Green Algae	17112
Green Algae	15675
Centipede	15493
Fungi	15058
Boletaceae (fungal family)	14778
Mite	13569
Green Algae	12975
Protist	12408
Green Algae	11915
NA	10768
Green Algae	9699
Mortierella (fungal genus)	9563
Nemotode	9423

4.2. Soil Inoculation Experiment

Neither the collection site nor treatment (live or sterilized) of inoculant had a significant effect on primary productivity of the sandbar willows, as measured by total dry mass of roots and shoots. Age of willow cuttings, as determined by number of rings, also had no effect on primary productivity, however, their dry mass had a significant effect (Figure 10).

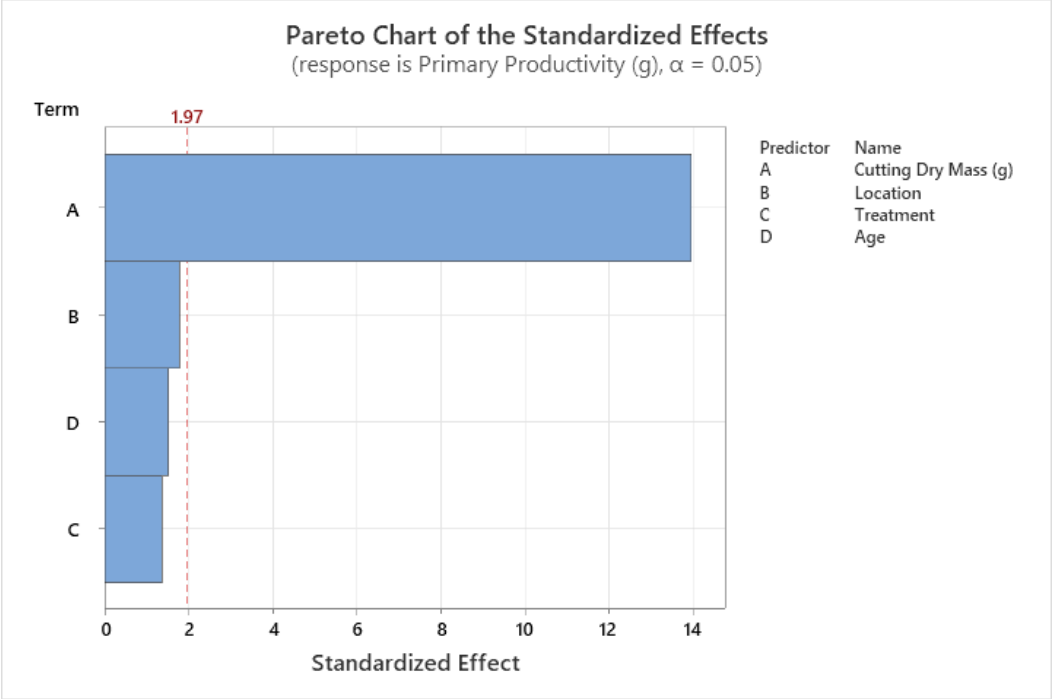

Figure 10: Cutting mass had a significant effect on primary productivity. Regression analysis of the effect of willow cutting dry mass, location, treatment, and willow age on primary productivity. Only bars that cross the reference line are statistically significant.

Primary productivity, in terms of willow root and shoot growth during the soil inoculation greenhouse study, had a positive correlation with the dry mass of the willow cutting, explaining 36% of the observed variation (Table VIII, **Figure 11**).

Table VIII: Results for regression analysis of willow cutting mass and primary productivity.

Coefficients					
Term	**Coef**	**SE Coef**	***t*-Value**	**P-Value**	**VIF**
Constant	0.4445	0.0997	4.46	<0.0001	
Cutting Dry Mass (g)	0.1299	0.0079	16.51	<0.0001	1
Model Summary					
S	**R-sq**	**R-sq(adj)**	**R-sq(pred)**		
0.603006	36.46%	36.32%	35.90%		

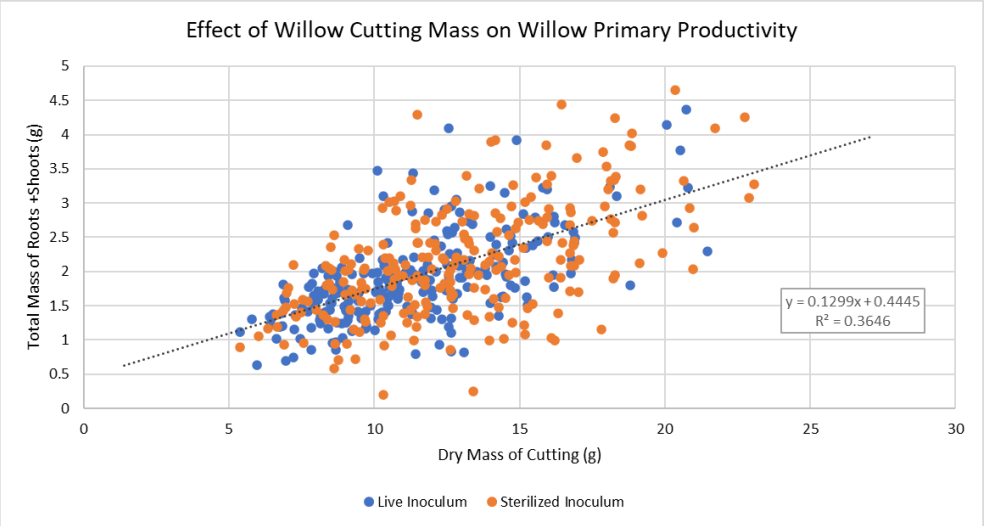

Figure 11: Effect of willow cutting mass on primary productivity, which was equal to the total dry mass of the roots and shoots.

Enzyme activity levels of soils from plants inoculated with live inoculant were significantly higher than those inoculated with sterilized soils for both phosphatase and β-glucosidase (Table IX). Statistic outcomes comparing the enzyme activity of soils from live and sterilized inoculants.

Table IX: Statistics table for pairwise comparisons of enzyme activity.

Phosphatase Assay			
Test	***t*-Value**	**DF**	**p-value**
2-Sample t-test	11.3	247	<0.0001
β-Glucosidase Assay			
Test	**W-Value**		**p-value**
Mann-Whitney	20023.0		<0.0001

Samples from the live treatment had more variance than those from the sterilized treatment (Figure 12).

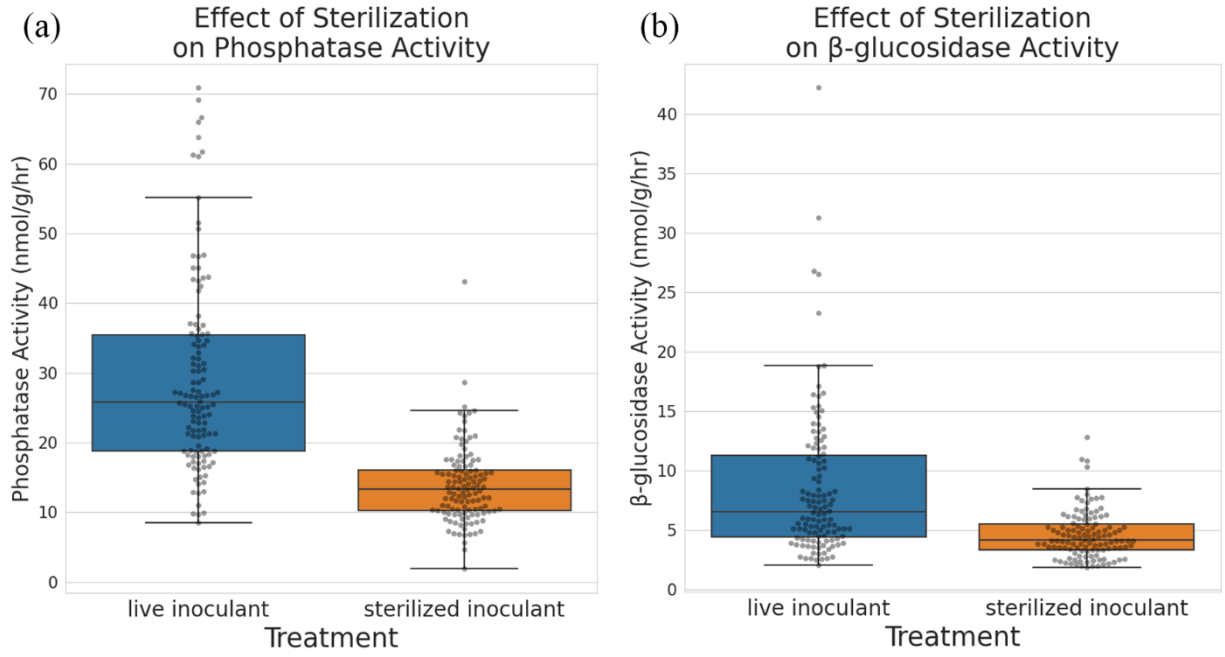

Figure 12: Effect of inoculant treatment on soil enzyme activity after 8 weeks of willow growth. Differences in enzyme activity were significantly different for both a) phosphatase and b) β-glucosidase ($p < 0.0001$).

Significant differences were observed in the level of soil enzyme activity between the different collection sites (Table X).

Table X: Results for One-way ANOVA of soil enzyme activity by soil collection site.

Welch's Test					
Enzyme Assay	**Source**	**DF Num**	**DF Den**	**F-Value**	**P-Value**
Phosphatase	Soil Collection Site	5	42.3071	17.56	<0.0001
β-Glucosidase	Soil Collection Site	5	47.6392	20.91	<0.0001

For both phosphatase and β-glucosidase activity, the reference and 2014 YOR sites displayed the highest levels of activity, while the 2015 YOR site had low enzymatic activity. Addition of live soil inoculant from any collection site significantly increased soil enzyme activity over backfill (Figure 13).

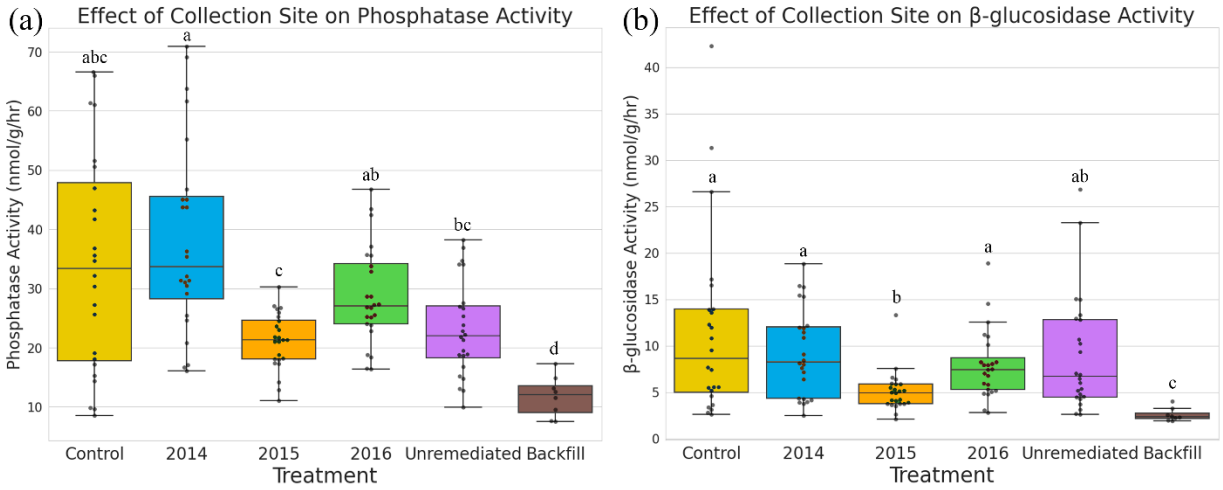

Figure 13: Effect of soil collection site on soil enzyme activity for a) phosphatase and b) β-glucosidase after 8 weeks of willow growth. Grouping information using the Games-Howell Method and 95% confidence. Means that do not share a letter are significantly different.

5. Discussion

As restoration activities at the Upper Clark Fork River involves replacement of large amounts of contaminated soil with subsoil backfill, we expected that soil microbiota and functions would initially be at low levels following remediation. Our data supports this idea as the soil enzyme activity levels for backfill were near zero and no detectable amounts of DNA were obtained from the DNA extraction process.

Our data also show that soil microbiome biomass and functions are exhibiting dissimilar rates of recovery at the different restoration sites. The 2015 YOR site consistently had the lowest bioindicator measurements of all restored sites, even though it did not have the shortest recovery period. There are several factors that may contribute to microbiome recovery rate. Microbial biomass and diversity is influenced by both environmental soil factors, such as pH, particle size, nutrient and water availability, and oxygenation (Hansel et al., 2008; Sessitsch et al., 2001; Tripathi et al., 2018), and biological factors, such as aboveground plant communities (Bulgarelli et al., 2013; Marschner et al., 2004). Different restoration approaches and histories, such as flooding events, may also affect microbiome recovery. Further research needs to be done to determine what factors may be hindering microbiome recovery at the 2015 YOR site.

Preliminary analysis of the prokaryotic community compositions via PCA ordination of principal components 1 and 2 using Bray-Curtis distance shows distinct patterns (Figure 9). Communities from the Thompson Park control site form a grouping that is separate from the study site communities. The Warm Springs settling ponds are located downstream from Thompson Park and immediately upstream to the Upper Clark Fork restoration site. If soil microbiota spread throughout the flood plain via sediment transport, then the settling ponds could be blocking upstream microbial inputs. The Thompson Park control site may also be too

different from the study sites. Thompson Park is found at a higher elevation and has lower soil pH than the study sites. Thompson Park exhibits soil texture and vegetation characteristics more similar to the emergent wetlands than the riparian shrublands found at the study site. These factors may contribute to the microbial community differences observed.

The study site communities form two groupings. 2015 YOR sites are grouping together with the unremediated sites and backfill communities. The 2016 YOR sites form a separate group. The 2014 YOR sites are split between the two groups. Interestingly, 2014-C was collected from an area of vegetation that was preserved during remediation activities and it is grouping with the unremediated sites. 2014-B was collected from a site where soil was removed and backfilled and is grouping with the 2016 YOR sites with the same treatment. The 2015 YOR sites were also restored, however their prokaryotic communities are more similar to the unremediated community assemblages. A possible explanation for this pattern may be positional. 2015 YOR sites are positioned immediately downstream of the unremediated sites, and the microbiome may be influenced by the upstream communities. There also may be different microbial inputs as the river moves downstream. Further analysis of microbiome communities could help us better understand what factors contribute to differences in community assemblages. Microbiome analysis of the 16S, 18S, and ITS communities and diversity are ongoing.

Willow growth did not respond to differences in treatment or collection site of soil inoculant but was found to have a significant positive correlation with cutting mass. There are several possible explanations for why soil inoculations had no noticeable effect on willow growth. One, sandbar willow is a pioneer plant species, adapted to grow in undeveloped soils (Iowa State University Extension, 2023). Some willow species have been shown to harbor

nitrogen fixing endophytes that allow them to grown in nitrogen poor soils, and it is suggested that this may be extended to other pioneer riparian willow species (Doty et al., 2009). These adaptations may allow willows to establish rapidly without reliance on soil microbiome communities and function. Two, soil microorganisms have been shown to protect plants from stress (Lau & Lennon, 2012; Poudel et al., 2021). The willows were grown in a controlled environment with regular access to water. This stress-free environment may have masked any benefit that the soil microbiome provided. Three, the effects of soil inoculation often become apparent over a period of years (Han et al., 2022). The eight-week growth period of the willow plants may have been too short for differences in productivity to be observed.

Extracellular soil enzyme activity was higher in soils that were inoculated with live inoculant than those inoculated with sterilized inoculant, and inoculation with live soil from any collection site increased enzyme activity over those inoculated with back fill soil. This suggests that soil inoculation at restoration sites may boost soil function in the short term. However, soil inoculation sources should be carefully considered as they can have lasting effects on plant communities and may introduce pathogenic species (Han et al., 2022; van de Voorde et al., 2012; van der Putten et al., 2013; Wubs, Van der Putten, et al., 2019). A more conservative approach would be to incorporate areas of preserved vegetation and soil into the restoration plan. This may provide a source for rewilding the remediated soils with local microbiota.

This study showed varying recovery rates and community compositions for the different restoration phases of the Upper Clark Fork River. Given the key roles that microorganisms have in ecosystem functions and resiliency, it is important to understand what factors affect soil microbiome recovery after restoration. Inclusion of biotic indicators in soil health assessments may elucidate environmental factors and management actions that contribute to the variance in

the microbiome recovery rates observed, leading to more effective restoration practices and increasing the likelihood of self-sustaining, resilient ecosystems.

6. References Cited

Abarenkov, K., Zirk, A., Piirmann, T., Pöhönen, R., Ivanov, F., Nilsson, R. H., & Kõljalg, U. (2022). *UNITE general FASTA release for eukaryotes* [Application/gzip]. UNITE Community. https://doi.org/10.15156/BIO/2483913

Alkorta, I., Aizpurua, A., Riga, P., Albizu, I., Amezaga, I., & Garbisu, C. (2003). Soil Enzyme Activities as Biological Indicators of Soil Health. *Reviews on Environmental Health*, *18*, 65–73. https://doi.org/10.1515/REVEH.2003.18.1.65

Amaral-Zettler, L. A., McCliment, E. A., Ducklow, H. W., & Huse, S. M. (2009). A Method for Studying Protistan Diversity Using Massively Parallel Sequencing of V9 Hypervariable Regions of Small-Subunit Ribosomal RNA Genes. *PLOS ONE*, *4*(7), e6372. https://doi.org/10.1371/journal.pone.0006372

Bach, E. M., Ramirez, K. S., Fraser, T. D., & Wall, D. H. (2020). Soil Biodiversity Integrates Solutions for a Sustainable Future. *Sustainability*, *12*(7), Article 7. https://doi.org/10.3390/su12072662

Bentrup, G., & Hoag, J. C. (1998). *The Practical Streambank Bioengineering Guide*. Interagency Riparian/Wetland Project. https://efotg.sc.egov.usda.gov/references/public/NM/BIO-48_The_Practical_Streambank_Bioengineering_Guide.pdf

Blagodatskaya, E., & Kuzyakov, Y. (2013). Active microorganisms in soil: Critical review of estimation criteria and approaches. *Soil Biology and Biochemistry*, *67*, 192–211. https://doi.org/10.1016/j.soilbio.2013.08.024

Bloem, J., Hopkins, D. W., Benedetti, A., & Benedetti, A. (2005). *Microbiological Methods for Assessing Soil Quality*. CABI. http://ebookcentral.proquest.com/lib/mtech/detail.action?docID=289436

Brookes, P. C., & McGrath, S. P. (1984). Effect of metal toxicity on the size of the soil microbial biomass. *Journal of Soil Science*, *35*(2), 341–346. https://doi.org/10.1111/j.1365-2389.1984.tb00288.x

Brookes, P. C., Powlson, D. S., & Jenkinson, D. S. (1984). Phosphorus in the soil microbial biomass. *Soil Biology and Biochemistry*, *16*(2), 169–175. https://doi.org/10.1016/0038-0717(84)90108-1

Bulgarelli, D., Schlaeppi, K., Spaepen, S., van Themaat, E. V. L., & Schulze-Lefert, P. (2013). Structure and Functions of the Bacterial Microbiota of Plants. *Annual Review of Plant Biology*, *64*(1), 807–838. https://doi.org/10.1146/annurev-arplant-050312-120106

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), Article 7. https://doi.org/10.1038/nmeth.3869

Chen, Q.-L., Ding, J., Zhu, Y.-G., He, J.-Z., & Hu, H.-W. (2020). Soil bacterial taxonomic diversity is critical to maintaining the plant productivity. *Environment International*, *140*, 105766. https://doi.org/10.1016/j.envint.2020.105766

Clark Fork Coalition. (2016). *Upper Clark Fork Aquatic Restoration Strategy II*. Clark Fork Coalition. https://www.usbr.gov/watersmart/cwmp/docs/plans/ClarkForkWRP.pdf

Decaëns, T., Jiménez, J. J., Gioia, C., Measey, G. J., & Lavelle, P. (2006). The values of soil animals for conservation biology. *European Journal of Soil Biology*, *42*, S23–S38. https://doi.org/10.1016/j.ejsobi.2006.07.001

Delgado-Baquerizo, M., Maestre, F. T., Reich, P. B., Jeffries, T. C., Gaitan, J. J., Encinar, D., Berdugo, M., Campbell, C. D., & Singh, B. K. (2016). Microbial diversity drives

multifunctionality in terrestrial ecosystems. *Nature Communications*, *7*(1), Article 1. https://doi.org/10.1038/ncomms10541

Doran, J. W., & Zeiss, M. R. (2000). Soil health and sustainability: Managing the biotic component of soil quality. *Applied Soil Ecology*, *15*(1), 3–11. https://doi.org/10.1016/S0929-1393(00)00067-6

Doty, S. L., Oakley, B., Gang, X., Kang, J. W., Singleton, G., Khan, Z., Azra, V., & Staley, J. T. (2009). Diazotrophic endophytes of native black cottonwood and willow. *Symbiosis*, *47*(1), 23–33. https://doi.org/10.1007/BF03179967

Eivazi, F., & Tabatabai, M. A. (1977). Phosphatases in soils. *Soil Biology and Biochemistry*, *9*(3), 167–172. https://doi.org/10.1016/0038-0717(77)90070-0

Ekschmitt, K., Liu, M., Vetter, S., Fox, O., & Wolters, V. (2005). Strategies used by soil biota to overcome soil organic matter stability—Why is dead organic matter left over in the soil? *Geoderma*, *128*(1), 167–176. https://doi.org/10.1016/j.geoderma.2004.12.024

Fierer, N., Wood, S. A., & Bueno de Mesquita, C. P. (2021). How microbes can, and cannot, be used to assess soil health. *Soil Biology and Biochemistry*, *153*, 108111. https://doi.org/10.1016/j.soilbio.2020.108111

Gellie, N. J. C., Mills, J. G., Breed, M. F., & Lowe, A. J. (2017). Revegetation rewilds the soil bacterial microbiome of an old field. *Molecular Ecology*, *26*(11), 2895–2904. https://doi.org/10.1111/mec.14081

German, D. P., Weintraub, M. N., Grandy, A. S., Lauber, C. L., Rinkes, Z. L., & Allison, S. D. (2011). Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. *Soil Biology and Biochemistry*, *43*(7), 1387–1397. https://doi.org/10.1016/j.soilbio.2011.03.017

Graham, E. B., Knelman, J. E., Schindlbacher, A., Siciliano, S., Breulmann, M., Yannarell, A., Beman, J. M., Abell, G., Philippot, L., Prosser, J., Foulquier, A., Yuste, J. C., Glanville, H. C., Jones, D. L., Angel, R., Salminen, J., Newton, R. J., Bürgmann, H., Ingram, L. J., … Nemergut, D. R. (2016). Microbes as Engines of Ecosystem Function: When Does Community Structure Enhance Predictions of Ecosystem Processes? *Frontiers in Microbiology*, *7*. https://www.frontiersin.org/articles/10.3389/fmicb.2016.00214

Grandlic, C. J., Palmer, M. W., & Maier, R. M. (2009). Optimization of plant growth-promoting bacteria-assisted phytostabilization of mine tailings. *Soil Biology and Biochemistry*, *41*(8), 1734–1740. https://doi.org/10.1016/j.soilbio.2009.05.017

Han, X., Li, Y., Li, Y., Du, X., Li, B., Li, Q., & Bezemer, T. M. (2022). Soil inoculum identity and rate jointly steer microbiomes and plant communities in the field. *ISME Communications*, *2*(1), Article 1. https://doi.org/10.1038/s43705-022-00144-1

Hansel, C. M., Fendorf, S., Jardine, P. M., & Francis, C. A. (2008). Changes in bacterial and archaeal community structure and functional diversity along a geochemically variable soil profile. *Applied and Environmental Microbiology*, *74*(5), 1620–1633. https://doi.org/10.1128/AEM.01787-07

Hao, X., Taghavi, S., Xie, P., Orbach, M. J., Alwathnani, H. A., Rensing, C., & Wei, G. (2014). Phytoremediation of Heavy and Transition Metals Aided by Legume-Rhizobia Symbiosis. *International Journal of Phytoremediation*, *16*(2), 179–202. https://doi.org/10.1080/15226514.2013.773273

Hermans, S. M., Buckley, H. L., Case, B. S., Curran-Cournane, F., Taylor, M., & Lear, G. (2017). Bacteria as Emerging Indicators of Soil Condition. *Applied and Environmental Microbiology*, *83*(1), e02826-16. https://doi.org/10.1128/AEM.02826-16

Hermans, S. M., Buckley, H. L., Case, B. S., Curran-Cournane, F., Taylor, M., & Lear, G. (2020). Using soil bacterial communities to predict physico-chemical variables and soil quality. *Microbiome*, *8*, 1–13. https://doi.org/10.1186/s40168-020-00858-1

Iowa State University Extension. (2023, April 17). *Sandbar Willow*. Natural Resource Stewardship. https://naturalresources.extension.iastate.edu/forestry/iowa_trees/trees/sandbar_willow.html

Islam, W., Noman, A., Naveed, H., Huang, Z., & Chen, H. Y. H. (2020). Role of environmental factors in shaping the soil microbiome. *Environmental Science and Pollution Research*, *27*(33), 41225–41247. https://doi.org/10.1007/s11356-020-10471-2

Jackson, C. R., Tyler, H. L., & Millar, J. J. (2013). Determination of Microbial Extracellular Enzyme Activity in Waters, Soils, and Sediments using High Throughput Microplate Assays. *Journal of Visualized Experiments : JoVE*, *80*, 50399. https://doi.org/10.3791/50399

Jain, A., Chakraborty, J., & Das, S. (2020). Underlying mechanism of plant–microbe crosstalk in shaping microbial ecology of the rhizosphere. *Acta Physiologiae Plantarum*, *42*(1), 8. https://doi.org/10.1007/s11738-019-3000-0

Kissel, D., & Sonon, Leticia. (2011). *Soil Test Handbook for Georgia*.

Kuzyakov, Y., & Blagodatskaya, E. (2015). Microbial hotspots and hot moments in soil: Concept & review. *Soil Biology and Biochemistry*, *83*, 184–199. https://doi.org/10.1016/j.soilbio.2015.01.025

Lau, J. A., & Lennon, J. T. (2012). Rapid responses of soil microorganisms improve plant fitness in novel environments. *Proceedings of the National Academy of Sciences*, *109*(35), 14058–14062. https://doi.org/10.1073/pnas.1202319109

Lee, Y. B., Lorenz, N., Dick, L. K., & Dick, R. P. (2007). Cold Storage and Pretreatment Incubation Effects on Soil Microbial Properties. *Soil Science Society of America Journal*, *71*(4), 1299–1305.

Lehmann, J., Bossio, D. A., Kögel-Knabner, I., & Rillig, M. C. (2020). The concept and future prospects of soil health. *Nature Reviews Earth & Environment*, *1*(10), Article 10. https://doi.org/10.1038/s43017-020-0080-8

Lennon, J. T., & Jones, S. E. (2011). Microbial seed banks: The ecological and evolutionary implications of dormancy. *Nature Reviews Microbiology*, *9*(2), 119–131. https://doi.org/10.1038/nrmicro2504

Liddicoat, C., Krauss, S. L., Bissett, A., Borrett, R. J., Ducki, L. C., Peddle, S. D., Bullock, P., Dobrowolski, M. P., Grigg, A., Tibbett, M., & Breed, M. F. (2022). Next generation restoration metrics: Using soil eDNA bacterial community data to measure trajectories towards rehabilitation targets. *Journal of Environmental Management*, *310*, 114748. https://doi.org/10.1016/j.jenvman.2022.114748

Lyu, D., Msimbira, L. A., Nazari, M., Antar, M., Pagé, A., Shah, A., Monjezi, N., Zajonc, J., Tanney, C. A. S., Backer, R., & Smith, D. L. (2021). The Coevolution of Plants and Microbes Underpins Sustainable Agriculture. *Microorganisms*, *9*(5), 1036. https://doi.org/10.3390/microorganisms9051036

Marschner, P., Crowley, D., & Yang, C. H. (2004). Development of specific rhizosphere bacterial communities in relation to plant species, nutrition and soil type. *Plant and Soil*, *261*(1/2), 199–208.

Marstorp, H., Guan, X., & Gong, P. (2000). Relationship between dsDNA, chloroform labile C and ergosterol in soils of different organic matter contents and pH. *Soil Biology and Biochemistry*, *32*(6), 879–882. https://doi.org/10.1016/S0038-0717(99)00210-2

McLaren, M. R., & Callahan, B. J. (2021). *Silva 138.1 prokaryotic SSU taxonomic training data formatted for DADA2* [Data set]. Zenodo. https://doi.org/10.5281/zenodo.4587955

Minitab, LLC. (21.4). (2023). https://www.minitab.com

Morien, E., & Parfrey, L. W. (2018). *SILVA v128 and v132 dada2 formatted 18s "train sets"* [Data set]. Zenodo. https://doi.org/10.5281/zenodo.1447330

Parada, A. E., Needham, D. M., & Fuhrman, J. A. (2016). Every base matters: Assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environmental Microbiology*, *18*(5), 1403–1414. https://doi.org/10.1111/1462-2920.13023

Pascoe, G. A., & Dalsoglio, J. A. (1994). Planning and implementation of a comprehensive ecological risk assessment at the milltown reservoir-clark fork river superfund site, montana. *Environmental Toxicology and Chemistry*, *13*(12), 1943–1956. https://doi.org/10.1002/etc.5620131209

Philippot, L., Spor, A., Hénault, C., Bru, D., Bizouard, F., Jones, C. M., Sarr, A., & Maron, P.-A. (2013). Loss in microbial diversity affects nitrogen cycling in soil. *The ISME Journal*, *7*(8), Article 8. https://doi.org/10.1038/ismej.2013.34

Polster, D. F. (2003). *Soil Bioengineering for Slope Stabilization and Site Restoration*.

Poudel, M., Mendes, R., Costa, L. A. S., Bueno, C. G., Meng, Y., Folimonova, S. Y., Garrett, K. A., & Martins, S. J. (2021). The Role of Plant-Associated Bacteria, Fungi, and Viruses in Drought Stress Mitigation. *Frontiers in Microbiology*, *12*. https://www.frontiersin.org/articles/10.3389/fmicb.2021.743512

Powell, J. R., & Rillig, M. C. (2018). Biodiversity of arbuscular mycorrhizal fungi and ecosystem function. *New Phytologist*, *220*(4), 1059–1075. https://doi.org/10.1111/nph.15119

Quince, C., Lanzen, A., Davenport, R. J., & Turnbaugh, P. J. (2011). Removing Noise From Pyrosequenced Amplicons. *BMC Bioinformatics*, *12*(1), 38. https://doi.org/10.1186/1471-2105-12-38

Sessitsch, A., Weilharter, A., Gerzabek, M. H., Kirchmann, H., & Kandeler, E. (2001). Microbial Population Structures in Soil Particle Size Fractions of a Long-Term Fertilizer Field Experiment. *Applied and Environmental Microbiology*, *67*(9), 4215–4224. https://doi.org/10.1128/AEM.67.9.4215-4224.2001

Smith, M. E., Delean, S., Cavagnaro, T. R., & Facelli, J. M. (2018). Evidence for species-specific plant responses to soil microbial communities from remnant and degraded land provides promise for restoration. *Austral Ecology*, *43*(3), 301–308. https://doi.org/10.1111/aec.12567

State of knowledge of soil biodiversity—Status, challenges and potentialities. (2020). FAO. https://doi.org/10.4060/cb1928en

Sullivan, D. M., Moore, A. D., & Brewer, L. J. (2019). *Soil organic matter as a soil health indicator: Sampling, testing, and interpretation*.

Swati, M., Abraham, J. S., Sripoorna, S., Ravi, T., Gupta, R., & Seema, M. (2020). Indicators for assessment of soil quality: A mini-review. *Environmental Monitoring and Assessment*, *192*(9). https://doi.org/10.1007/s10661-020-08556-z

Swift, M. J., Heal, O. W., Anderson, J. M., & Anderson, J. M. (1979). *Decomposition in Terrestrial Ecosystems*. University of California Press.

The Galaxy Community. (2022). The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2022 update. *Nucleic Acids Research*, *50*(W1), W345–W351. https://doi.org/10.1093/nar/gkac247

Thompson, L. R., Sanders, J. G., McDonald, D., Amir, A., Ladau, J., Locey, K. J., Prill, R. J., Tripathi, A., Gibbons, S. M., Ackermann, G., Navas-Molina, J. A., Janssen, S., Kopylova, E., Vázquez-Baeza, Y., González, A., Morton, J. T., Mirarab, S., Zech Xu, Z., Jiang, L., … Knight, R. (2017). A communal catalogue reveals Earth's multiscale microbial diversity. *Nature*, *551*(7681), Article 7681. https://doi.org/10.1038/nature24621

Tripathi, B. M., Stegen, J. C., Kim, M., Dong, K., Adams, J. M., & Lee, Y. K. (2018). Soil pH mediates the balance between stochastic and deterministic assembly of bacteria. *The ISME Journal*, *12*(4), Article 4. https://doi.org/10.1038/s41396-018-0082-4

US EPA. (1996a). Sample Preparation Procedure for Spectrochemical Determination of Total Recoverable Elements. In *Methods for the Determination of Metals in Environmental Samples* (pp. 12–23). Elsevier. https://doi.org/10.1016/B978-0-8155-1398-8.50008-2

US EPA. (2004). *Clark Fork River Operable Unit. Record of Decision. Part 2: Decision Summary*. https://dojmt.gov/wp-content/uploads/CFROU-ROD-2004-P2-Decision-Summary.pdf

US EPA. (2007). *Method 3051A: Microwave Assisted Acid Digestion of Sediments, Sludgs, Soils, and Oils*. https://www.epa.gov/sites/default/files/2015-12/documents/3051a.pdf

US EPA. (2016). *Second Five-Year Review Report for the Milltown Reservoir/Clark Fork River*. https://dojmt.gov/wp-content/uploads/2nd-Five-Year-Review-Rpt-Milltown-CFR-Superfund-Site-2016-reduced-1-E.pdf

US EPA. (2019, June 10). *EPA Method 350.1: Determination of Ammonia Nitrogen by Semi-Automated Colorimetry* [Data and Tools]. https://www.epa.gov/esam/epa-method-3501-determination-ammonia-nitrogen-semi-automated-colorimetry

US EPA, J. W. (1996b). Determination of Nitrate-Nitrite Nitrogen by Automated Colorimetry. In *Methods for the Determination of Metals in Environmental Samples* (pp. 464–478). Elsevier. https://doi.org/10.1016/B978-0-8155-1398-8.50026-4

van de Voorde, T. F. J., van der Putten, W. H., & Bezemer, T. M. (2012). Soil inoculation method determines the strength of plant–soil interactions. *Soil Biology and Biochemistry*, *55*, 1–6. https://doi.org/10.1016/j.soilbio.2012.05.020

Van Der Heijden, M. G. A., Bardgett, R. D., & Van Straalen, N. M. (2008). The unseen majority: Soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters*, *11*(3), 296–310. https://doi.org/10.1111/j.1461-0248.2007.01139.x

van der Putten, W. H., Bardgett, R. D., Bever, J. D., Bezemer, T. M., Casper, B. B., Fukami, T., Kardol, P., Klironomos, J. N., Kulmatiski, A., Schweitzer, J. A., Suding, K. N., Van de Voorde, T. F. J., & Wardle, D. A. (2013). Plant–soil feedbacks: The past, the present and future challenges. *Journal of Ecology*, *101*(2), 265–276. https://doi.org/10.1111/1365-2745.12054

van Elsas, J. D., Chiurazzi, M., Mallon, C. A., Elhottovā, D., Krištůfek, V., & Salles, J. F. (2012). Microbial diversity determines the invasion of soil by a bacterial pathogen. *Proceedings of the National Academy of Sciences*, *109*(4), 1159–1164. https://doi.org/10.1073/pnas.1109326109

Wagg, C., Bender, S. F., Widmer, F., & van der Heijden, M. G. A. (2014). Soil biodiversity and soil community composition determine ecosystem multifunctionality. *Proceedings of the National Academy of Sciences*, *111*(14), 5266–5270. https://doi.org/10.1073/pnas.1320054111

Wagg, C., Schlaeppi, K., Banerjee, S., Kuramae, E. E., & Heijden, M. G. A. van der. (2019). Fungal-bacterial diversity and microbiome complexity predict ecosystem functioning. *Nature Communications*, *10*, 1–10. https://doi.org/10.1038/s41467-019-12798-y

Wardle, D. A., Bardgett, R. D., Klironomos, J. N., Setala, H., & al, et. (2004). Ecological Linkages Between Aboveground and Belowground Biota. *Science*, *304*(5677), 1629–1633.

Western Regional Climate Center. (2023). https://wrcc.dri.edu

White, T., Bruns, T., Lee, S., Taylor, F. J. R. M., White, T. J., Lee, S.-H., Taylor, L., & Shawe-Taylor, J. (1990). *Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: A guide to methods and applications*. https://www.scinapse.io/papers/2591172920

Wubs, E. R. J., Van der Putten, W. H., Mortimer, S. R., Korthals, G. W., Duyts, H., Wagenaar, R., & Bezemer, T. M. (2019). Single introductions of soil biota and plants generate long-term legacies in soil and plant community assembly. *Ecology Letters*, *22*(7), 1145–1151. https://doi.org/10.1111/ele.13271

Wubs, E. R. J., Van Heusden, T., Melchers, P. D., & Bezemer, T. M. (2019). Soil Inoculation Steers Plant-Soil Feedback, Suppressing Ruderal Plant Species. *Frontiers in Ecology and Evolution*, *7*. https://www.frontiersin.org/articles/10.3389/fevo.2019.00451

Yeoman, C. J., Han, Y., Dodd, D., Schroeder, C. M., Mackie, R. I., & Cann, I. K. O. (2010). Chapter 1—Thermostable Enzymes as Biocatalysts in the Biofuel Industry. In *Advances in Applied Microbiology* (Vol. 70, pp. 1–55). Academic Press. https://doi.org/10.1016/S0065-2164(10)70001-0

7. Appendix A: Supplementary Information

7.1. Normalization of Absorbance Readings

Occasional contamination by soil particles in the supernatant was a problem as it increased absorbance readings. It was found that the difference in absorbance between soil contaminated and uncontaminated buffer was similar at both the 410 nm and 500 nm wavelengths, while absorbance at the 500 nm wavelength was not affected by pNP concentrations allowing it to serve as a control for absorbance by soil particles (Figure 14).

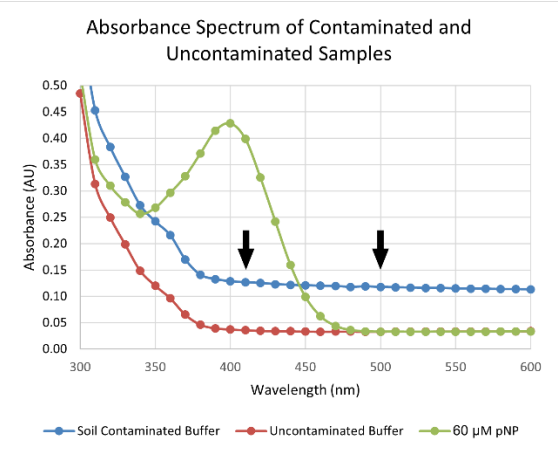

Figure 14: Absorbance spectrum comparing soil contaminated and uncontaminated buffer. The difference in absorbance between soil contaminated and uncontaminated buffer was similar at both the 410 nm and 500 nm wavelengths indicated with black arrows. Absorbance at 500 nm is not affected by pNP concentrations (green line). Inflated absorbance at 410 nm due to soil contamination can be corrected by subtracting the absorbance at 500 nm.

Two pNP Standard curves were set up, one with soil contamination added and one without. Subtracting the 500 nm absorbance from the 410 nm absorbance corrected inflated absorbance values from soil contamination as shown in Figure 15.

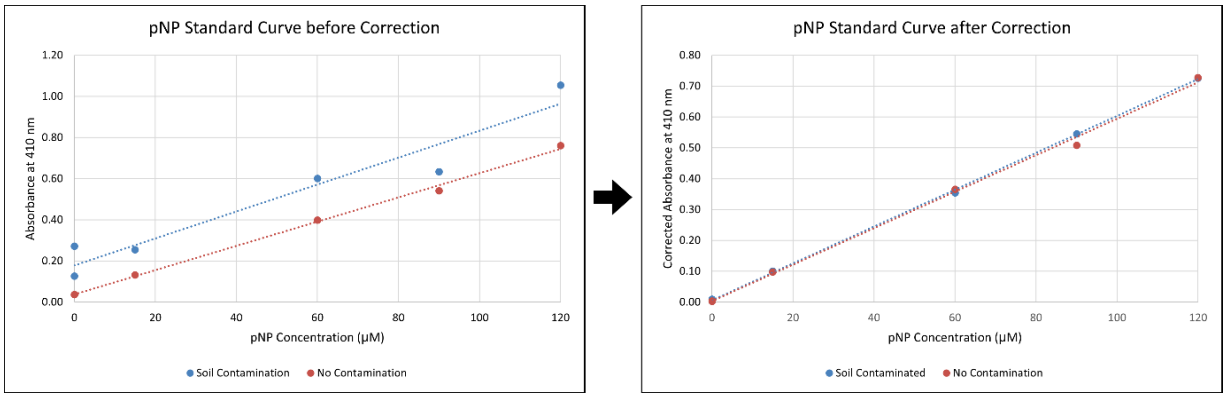

Figure 15: Correction of soil contaminated pNP standard curve. Subtracting the 500 nm absorbance from the 410 nm absorbance corrected the inflated absorption readings obtained from soil contaminated samples.

7.2. Representative ITS, 16S, and 18S PCR products

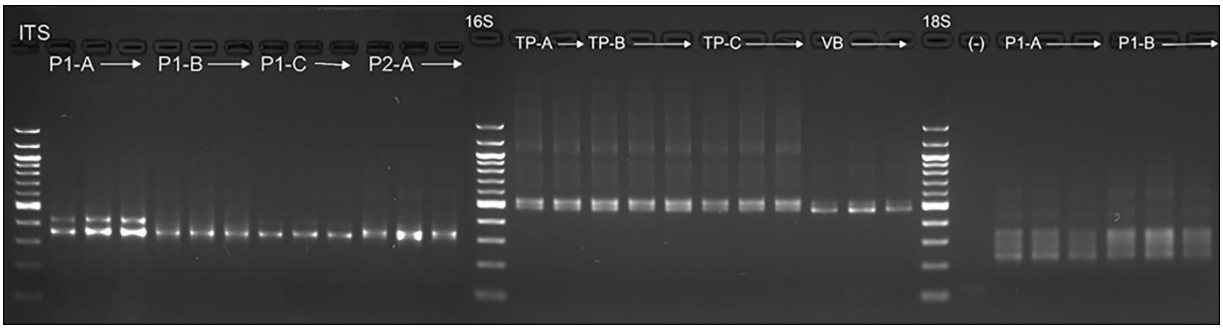

Figure 16: DNA gel of representative ITS, 16S, and 18S PCR products.

8. Appendix B: Rarefaction Curves

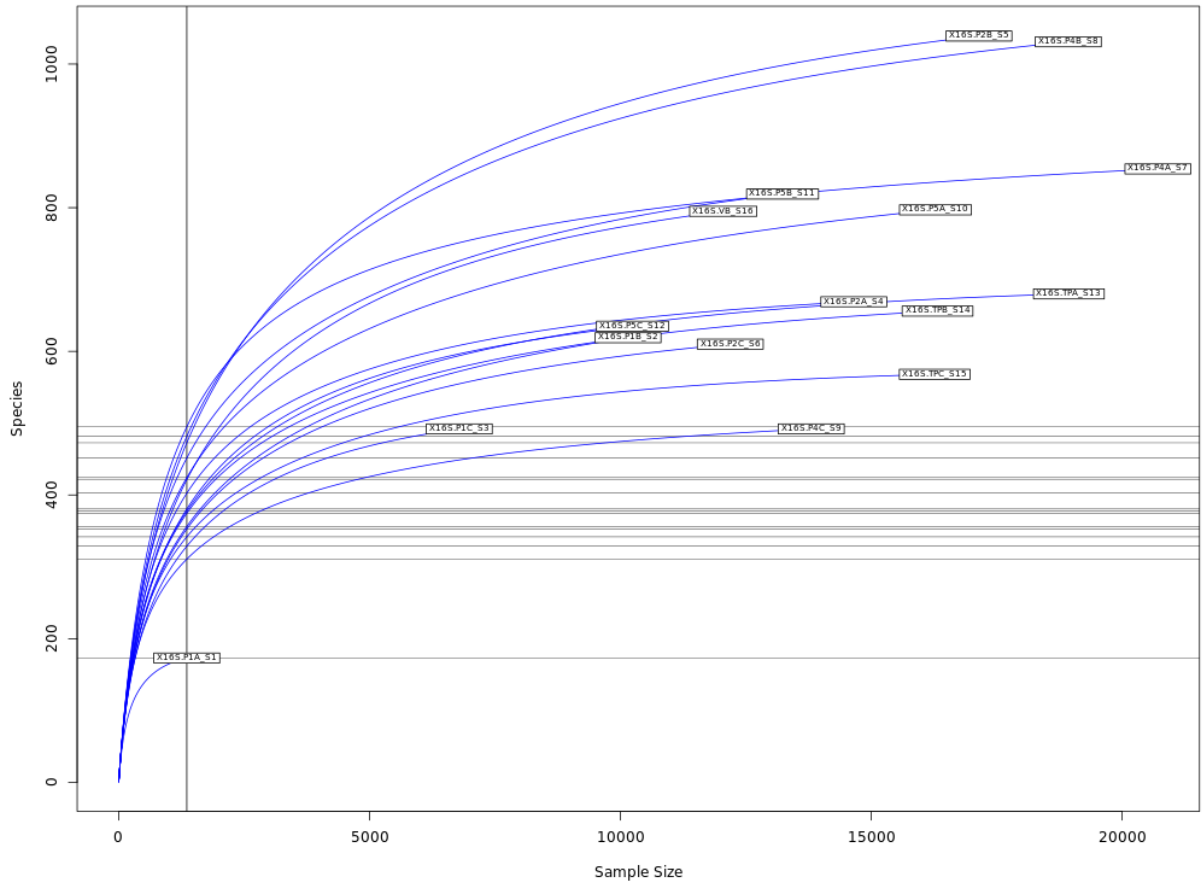

Figure 17: Rarefaction curves for 16S amplicons. Sample 2014-A (P1A) was removed from further processing.

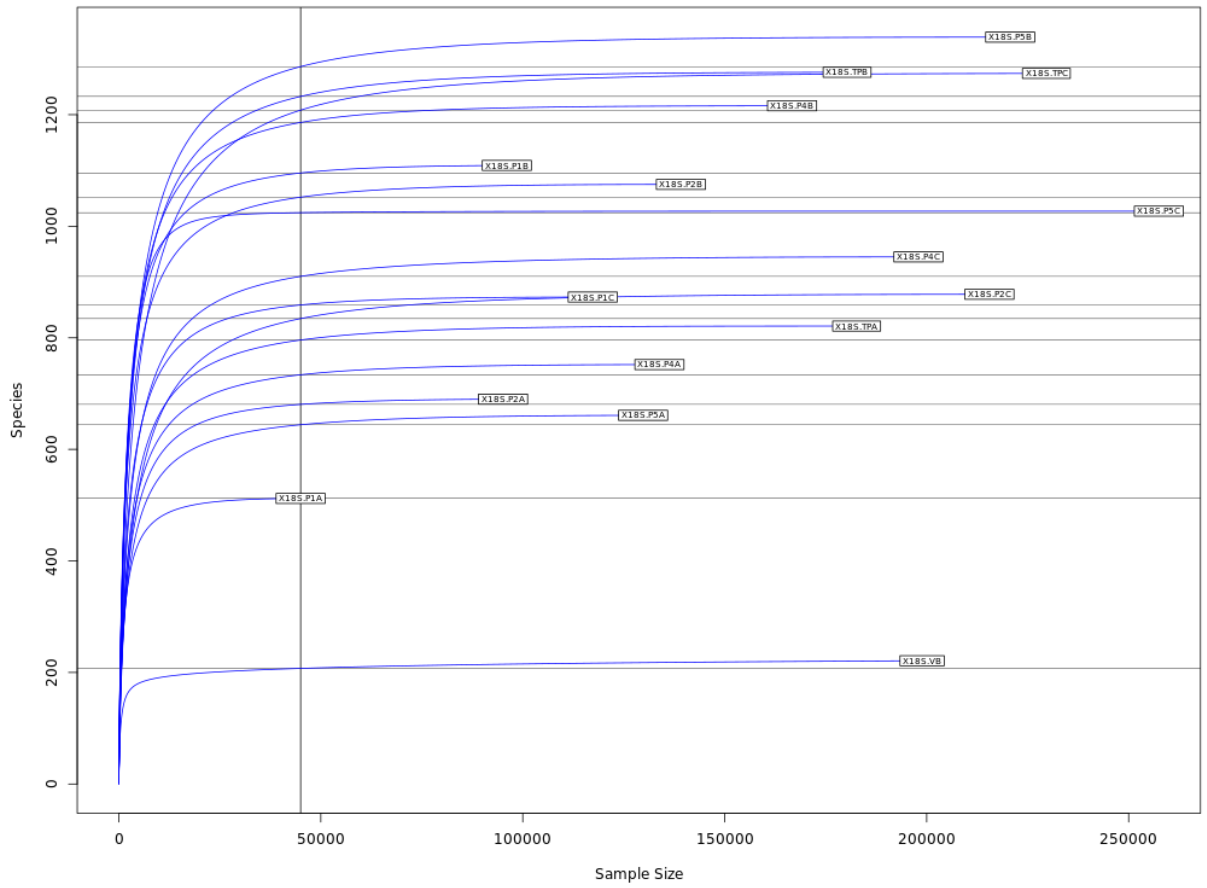

Figure 18: Rarefaction curves for 18S amplicons.

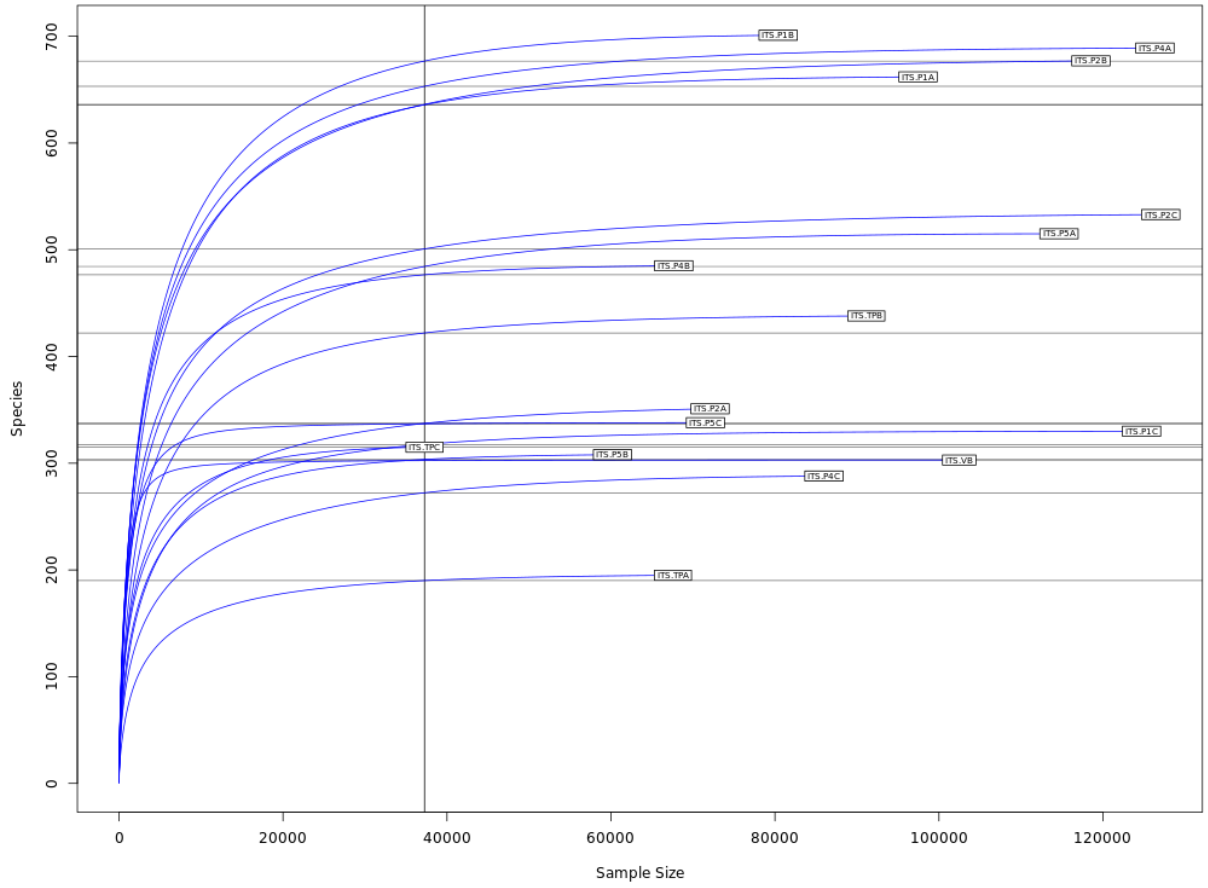

Figure 19: Rarefaction curves for ITS amplicons. Sample 2014-A (P1A) was removed from further processing.