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Linking Decomposition Reactions In Arctic Soils To Microbial Enzyme Production

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Dedication

It is with deep gratitude that I dedicate this dissertation to my parents, Victor and Virginia Martinez, and my husband, Jonathan Kwong, for their support and encouragement over the years. Also, I want to dedicate this to my kids, Jolene, Jian, and Jun. I hope that one day they get to read this dissertation

LINKING DECOMPOSITION REACTIONS IN ARCTIC SOILS TO MICROBIAL ENZYME PRODUCTION

by

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DISSERTATION

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Abstract

Microbial processes such as soil enzyme production are a major driver of decomposition and a current topic of interest in arctic soils due to the effects of climate warming. Despite the advances in understanding soil enzymes, there are still knowledge gaps regarding the role of enzymes in decomposition. In this dissertation, I addressed three of those gaps in the following chapters: (Ch.2) to explore the location of enzymes within the soil matrix, (Ch.3) to identify peptides matched to soil enzymes produced by microorganisms for organic matter decomposition, and (Ch.4) understand the longevity of enzymes in the soil after microbial production. For this project, I examined the soils from Utqiaġvik (Barrow), located in the North Slope of Alaska, north of the Arctic circle. Chapters 2, 3, and 4 measured the activities of potential hydrolytic and oxidative enzymes. Chapter 2 focused on various separation techniques to determine the location of enzymes in the soil matrix. Chapter 3 enhances protein extraction techniques used in proteomics to remove humic substances and achieve improved data without humic interference. With the optimized method, I examine the proteins from the soil samples to enable the identification of soil enzymes. In addition, I examined the association between the peptides seen in the proteome and the activity of enzymes. In chapter 4, enzyme activity was monitored weekly to assess the decline in activity after the eradication of microbial organisms by chloroform ($CHCl_3$) fumigation. Additional microscopy techniques were implemented in this chapter to confirm cellular death after chloroform fumigation. My findings demonstrate that, in chapter 2, soil enzymes tend to be predominantly linked with larger organic matter particles. Enzyme activity was not detected in the soil pore water, suggesting that enzymes have a limited persistence in the soil pore water over an extended period. Chapter 3 determined that using FASP (filter-aided sample preparation) for soil enzyme extractions during proteomics yields higher peptide counts. Following this, I identified

our typically assayed soil enzyme in the proteome and two additional enzymes with a high peptide count (β-galactosidase and arylsulfatase), and was able to measure substantial enzyme activity for each of these. Finally, in chapter 4, I observed that soil enzymes have a prolonged lifespan following their production. While the exact timeframe was not determined, my results indicate their longevity extends beyond 12 weeks. Overall, this dissertation provides evidence that a variety of microbes produce soil enzymes that have a relatively long lifespan and are most likely associated with large organic matter particles they decompose.

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Chapter 1: Introduction

1.1 Decomposition

Decomposition is the natural process of breaking down organic material into smaller particles and compounds. The process of decomposition is important because as organic material is decomposed, nutrients are released, making them accessible for other organisms. Without this process, nutrients are locked in the organic material resulting in poor soil health and plant growth. Biochemically, decomposition is driven by microbial activity, specifically by the production of hydrolytic and oxidative enzymes in the soil (Burns, 1982; Skujiņscaron; and Burns, 1976). Microbes produce soil enzymes to access nutrients required for metabolic processes and growth (Allison and Vitousek, 2005; Dick et al., 2011). The primary function of soil enzymes is to catalyze the breakdown of polymers into smaller, more digestible molecules. Despite the importance of enzymatic processes to decomposition and the biology of soil microbes, there are still several important missing pieces of information about how enzymes function in the soil. First, though their potential activity levels can be assayed (Sinsabaugh et al., 2005), it is not completely understood where the soil enzymes operate within the soil matrix. Second, matching specific genes and enzymes to the microbes that produce them is largely unexplored (Burns et al., 2013), and these connections will eventually be made possible by new omics techniques. Lastly, the longevity of enzymes in the soil has not been completely determined (J.Schimel et al., 2017), which is important in modeling work (Allison, 2005; Allison and Vitousek, 2005). By searching for these missing pieces, we can better understand microbial ecology and function in soils.

1.2 Soil Enzymes

The foundation for biochemical enzyme assays came from an experiment looking at yeast fermentation (Brown, 1902) and then was further developed by focusing on enzyme kinetics by

Michaelis and Menten (Michaelis et al., 1913). The first report of extracellular enzymes was recording oxidative enzymes, such as peroxidases, that came from decaying roots and plants (Dick et al., 2011; Woods, 1899). Due to their importance in decomposition, soil enzymes have continued to remain a topic of interest. Multiple methods have been developed and optimized to detect potential enzyme activity in soils. An early method of soil enzyme assays using colorimetric measurements relied on conjugates of *p*-nitrophenol after hydrolysis (Tabatabai and Bremner, 1969). Later, fluorometric techniques were introduced and are now the most widely used method for measuring potential enzyme activity under natural conditions that mimic the soil environment (Burns et al., 2013; Darrah and Harris, 1986). In the Darrah & Harris protocol (1986), 4-methylumbelliferone (MUB) conjugates were used as artificial substrates for soil hydrolases. Since then, several modifications have been made to improve the assay. Some methods start by sonicating the sample (Marx et al., 2001), while others use a blending technique (Saiya-Cork et al., 2002; Sinsabaugh et al., 2003). Later, calculations of potential enzyme activity were improved to ensure the enzyme activities were adequately performed. Due to methodological differences between researchers and labs, modern techniques now allow for standardized enzyme assays (German et al., 2011).

1.2.2 Hydrolytic Enzymes

Hydrolytic enzymes are a class of enzymes that catalyze the breakdown of complex molecules into smaller ones with the addition of water by a process called hydrolysis (Speir and Ross, 2002). This dissertation focuses primarily on seven hydrolytic enzymes contributing to carbon (C), nitrogen (N), and phosphorus (P) cycles, namely: β-glucosidase, α-1,4-glucosidase, cellobiohydrolase, xylosidase, leucine aminopeptidase, and β-1,4-N-Acetyl-glucosaminidase, and acid phosphatase (Table 1.1).

These seven enzymes are among the most common and consistently measured in previous studies (Hoppe, 1983; Marx et al., 2001; Saiya-Cork et al., 2002; Sinsabaugh et al., 2009). Each enzyme breaks specific chemical bonds of the decomposing material, making them substrate-specific (Caldwell, 2005). Soil organic matter (SOM) is formed from plant litter, microbial biomass sources, and stabilized organic matter (humus). Compounds of humus are the remaining part of organic matter that has been processed and transformed by several microorganisms; these include humic acids, fulvic acids, and humins (Pettit, 2014; Tan and Binger, 1986). The major constituents of non-humic substances include carbohydrates (cellulose, hemicellulose, and polysaccharides), proteins (amino acids), and lipids (fats, waxes, and resins) Stevenson, 1994). Soil enzymes specifically degrade non-humic substances, making these nutrient sources available for uptake by plants and microbes. Lately, there has been a discussion regarding the occurrence of humic substances in soils. Some researchers argue that humic substances are a diverse mixture of organic compounds with no distinct chemical structure, while others claim that they are chemically distinct and can be characterized by their molecular weight and functional groups (Kelleher and Simpson, 2006; Orlov, 2020). Humic substances are thought to constitute merely a minor portion of the total organic matter (Schmidt et al., 2011).

Enzymes that can hydrolyze one or more biomolecules in soils (*hydrolytic enzymes*) play a significant role in decomposition. In soil and organic material, biomolecules include carbohydrates, proteins, nucleic acids, and lipids. One of the most abundant of the four biomolecules that soil enzymes target is carbohydrates for which the primary functions are energy storage and maintenance of structure in living organisms (Deng and Popova, 2015). Essential carbohydrates in soil include, for example, cellulose, hemicellulose, starch, chitin, sucrose, glucose, glucosamine, galactose, fructose, and xylose. Cellulose and hemicellulose are broken

down by cellulases, enzyme systems consisting of glycosyl hydrolases, each with distinct functions that degrade cellulose, also referred to as a type of carbon-acquiring enzymes. For example, β-glucosidase is an enzyme that breaks down cellulose and releases glucose by acting on sugar polymers and oligosaccharides (Moss, 2010). Similarly, cellobiohydrolase breaks down cellulose but releases disaccharides. Another category of C-acquiring enzymes is xylanases, which include β-1,4-Xylosidase, a type of soil enzyme that cleaves a side chain of the xylan backbone (Sunna and Antranikian, 1997). Xylan is a component of plant cell walls and the most abundant hemicellulose (Ebringerová et al., 2005; Sun et al., 2004). The end product of this enzymatic degradation is often referred to as wood sugar, a reducing sugar that contains an aldehyde functional group (Deng and Popova, 2015).

Nitrogen acquisition via enzymes is also important because it is a critical limiting nutrient for plant growth. Enzymes associated with microbial N-acquisition include β-1,4-N-Acetyl-glucosaminidase (NAG) and Leucine-aminopeptidase (LAP) (Moorhead et al., 2012). β-1,4-N-Acetyl-glucosaminidase monomers make up chitin, which is an amino sugar-containing nitrogen. Chitin is similar in structure to cellulose, but D-glucose is substituted in the C-2 amino group (Tronsmo and Harman, 1993). Chitin is a significant biopolymer as it does not originate from plant biomass (Baldrian et al., 2011). Chitinases are significant in the breakdown of the structural component of cell walls of fungi and insects (e.g., arthropod's exoskeletons). Leucine-aminopeptidase is a metalloproteinase, a catalytic mechanism that involves a metal atom that breaks N-terminal amino acid residues (Matsui et al., 2006; NACHLAS et al., 1957). Although there are other classes of aminopeptidases, LAP is primarily used in environmental samples as this enzyme hydrolyzes peptide bonds associated with the two most abundant protein amino acids, leucine, and alanine, with leucine having the highest occurrence at 9.1 %, followed by alanine at

7.8 % (Sinsabaugh and Foreman, 2001); the other 83.1 % is dispersed in the remaining 18 amino acids lson & Cox, 2008).

When plants are deficient in phosphorus, they can control their acquisition via several mechanisms, including secretion of organic acids, formation of symbiotic association with mycorrhizal fungi, and, most importantly to this research, the production of phosphatase enzymes (Crain et al., 2018; Turner, 2008). Phosphatases are soil enzymes involved in releasing inorganic phosphorus from ester–phosphate bonds (Eivazi and Tabatabai, 1977). Phosphorus is significant for all organisms as it transports energy and stimulates plant growth. Phosphatases are abundant in plant roots, having higher activities in the rhizosphere than in bulk soil, indicating this nutrient's importance to plants (Nannipieri et al., 2011). Numerous enzymes secreted by microbes target organic P, including: phosphomonoesterase, phosphodiesterase, and phytase (Turner, 2008) .Soil phosphatase activities depend on the P availability and pH can alter enzyme activity (DeForest & .Moorhead, 2020). This study will focus on acid phosphatases, which are included in the phosphomonoesterase group; this enzyme thrives in acidic soils.

1.2.3. Oxidative Enzymes

In addition to hydrolytic enzymes, oxidative enzymes were assessed in my studies. Peroxidases are enzymes with iron (Fe) containing prosthetic groups, typically using H_2O_2 as an electron acceptor to degrade aromatic compounds (Hofrichter, 2002; Yu et al., 2020). Phenol oxidases consume oxygen (Sinsabaugh, 2010a), and they have copper (Cu) containing prosthetic groups that extract electrons from the phenols (Mayer and Staples, 2002). One primary function of phenol oxidases is to degrade lignin, a significant SOM component (Ander and Eriksson, 1976; Eriksson, 1984). Although peroxidase and phenol oxidase activities are less stable than hydrolytic enzymes because they are more dynamic (Sinsabaugh, 2010a), they correlate with decomposition

reaction rates. When performing potential colorimetric enzyme assays, L-DOPA (L-3,4-dihydroxyphenylalanine) is used as a substrate (Bach et al., 2013). However, it is important to note here that the oxidation of this substrate can be abiotic due to several factors, such as the presence of oxygen, transition metals, or other oxidizing agents in the sample. (Hall and Silver, 2013; Jones et al., 2020). Abiotic oxidation can occur more rapidly than enzymatic oxidation and can produce a similar colorimetric response, leading to an overestimation of enzyme activity.

Table 1.1: Summary of hydrolytic enzyme functions and substrates

Hydrolytic Enzymes	Abbreviation	Function	Substrate	Fluorescent Standard	Acquisition
β-1,4-Glucosidase	BGLUC	Releases glucose from cellulose, optimal activity at 85°C	4-MUB-β-D-Glucosidase	MUB	Carbon
α-1,4-glucosidase	AGLUC	Releases glucose from soluble saccharides	4-MUB- α -D-Glucosidase	MUB	Carbon
β-D-1,4-Cellobiohydrolase	CELL	Releases disaccharides from cellulose	4-MUB-β-D-Cellobioside	MUB	Carbon
β-1,4-Xylosidase	XYLO	Degrades hemicellulose	4-MUB-β-D-Xyloside	MUB	Carbon
β-1,4-N-acetyl-glucosaminidase	NAG	Degrades chitin	4-MUB-N-acetyl-β-D-glucosaminide	MUB	Nitrogen
Leucine-amino-peptidase	LAP	Degrades protein into amino acids	7-amino-4-methylcoumarin	MC	Nitrogen
Acid Phosphatase	PHOS	Releases phosphate ions from the phosphate group	4-MUB-phosphate	MUB	Phosphorus

1.3 METAPROTEOMICS

The recently established *"omics"* approaches within biology involve the comprehensive analysis of biological molecules. The most used methods include genomics, transcriptomics, proteomics, and metabolomics (Horgan and Kenny, 2011). In detail, genomics is the study of an organism's entire genome. Transcriptomics involves the examination of the organism's complete set of RNA (ribonucleic acid) molecules produced by the genome (Dong and Chen, 2013). Proteomics looks at the complete set of proteins (proteome) that are expressed by the genome (Shah and Misra, 2011). Metabolomics is the study that involves all small molecules involved in

metabolism (Klassen et al., 2017). Other "omics" sciences are emerging and have gone further in depth with other compound classes, such as lipidomics (lipids) (J. Wang et al., 2019; Züllig et al., 2020) and microbiomics (microbiomes) (Bokulich et al., 2020; Kumar, 2021).

Metaproteomics, which I focus on in this dissertation, is an approach within proteomics that looks at the collective protein profiles of entire microbial communities in a given environment (Herbst et al., 2016). While it has tremendous potential, studying soil metaproteomics is challenging due to spectral interference (likely from humic substances), spatial complexity, high diversity, and seasonal variability (Wilmes et al., 2015). Despite this, soil was among the first environmental samples to be studied using metaproteomics. For example, in a pioneering study, it was demonstrated that proteomic analysis was possible, and although there were a few limitations (fewer databases), this was the starting point for a deeper understanding of environmental proteomics (Schulze et al., 2005). Obtaining high-quality metagenomic data has proven challenging, even in recent years, and many researchers have been working to achieve the best extraction method for soil proteomes (Chacha et al., 2023; Quinn et al., 2022; Salvato et al., 2022). Although metaproteomics has been successful in the large-scale study of proteins, it is challenging to use proteomics in environmental samples due to the samples' high complexity and lack of metagenomic sequences to create an adequate reference protein database (Schneider et al., 2012).

1.4 ARCTIC ECOSYSTEMS AS A MODEL FOR EVALUATING SOIL ENZYME FUNCTION

In this dissertation, I focus on soil enzymes in the soils of the arctic tundra. Arctic ecosystems are critical for several important climate system feedbacks in which warming will likely drive more warming (Bradford et al., 2016; Hinzman et al., 2013; Pearson et al., 2013). In 2018, NOAA reported that the Arctic was warming at more than twice the rate of global mean

temperatures causing the phenomenon known as arctic amplification (Osborne et al., 2018). By 2021, data suggested that the trend had accelerated three times faster than the global average (AMAP, 2021; Rantanen et al., 2022). Arctic amplification refers to the surface air temperatures (Serreze and Barry, 2011). Factors known to contribute to this warming are positive feedback loops, permafrost melt, and the decrease of jet stream high winds trapped in the polar regions (Miller et al., 2010; Previdi et al., 2021).

Moreover, several carbon release models have been explored (Koven et al., 2011; Schaefer et al., 2011; Schuur et al., 2013), estimating the cost of permafrost degradation, predicting a 29–59% decrease in permafrost extent by 2200 (Schaefer et al., 2011). Furthermore, carbon dioxide release from 2000-2300 is expected to keep increasing in permafrost soils (Schneider and Riedel, 2010). Therefore, monitoring and studying decomposition in arctic ecosystems is of global relevance.

The work of this dissertation is focused on Utqiaġvik, which is in the northernmost point of Alaska (71°19′N, 156°36′W) (Figure 1.1). Most of Alaska's north slope is composed of predominantly gelisols, which are permafrost soils with an active layer that thaws during the summer. Soils north of the Alaska Range are underdeveloped even though they have been under continuous soil formation over millennia due in part to the extreme cold weather (Chapin et al., 2006). Below the active layer, the permafrost acts as a barrier where there is little root or water movement. This part remains frozen throughout the year. The active layer has increased in depth in recent years, allowing for more root formation and carbon release. These trends are projected to continue and affect physical soil characteristics such as soil moisture, a key challenge for accurately estimating soil dynamics (Seneviratne et al., 2010).

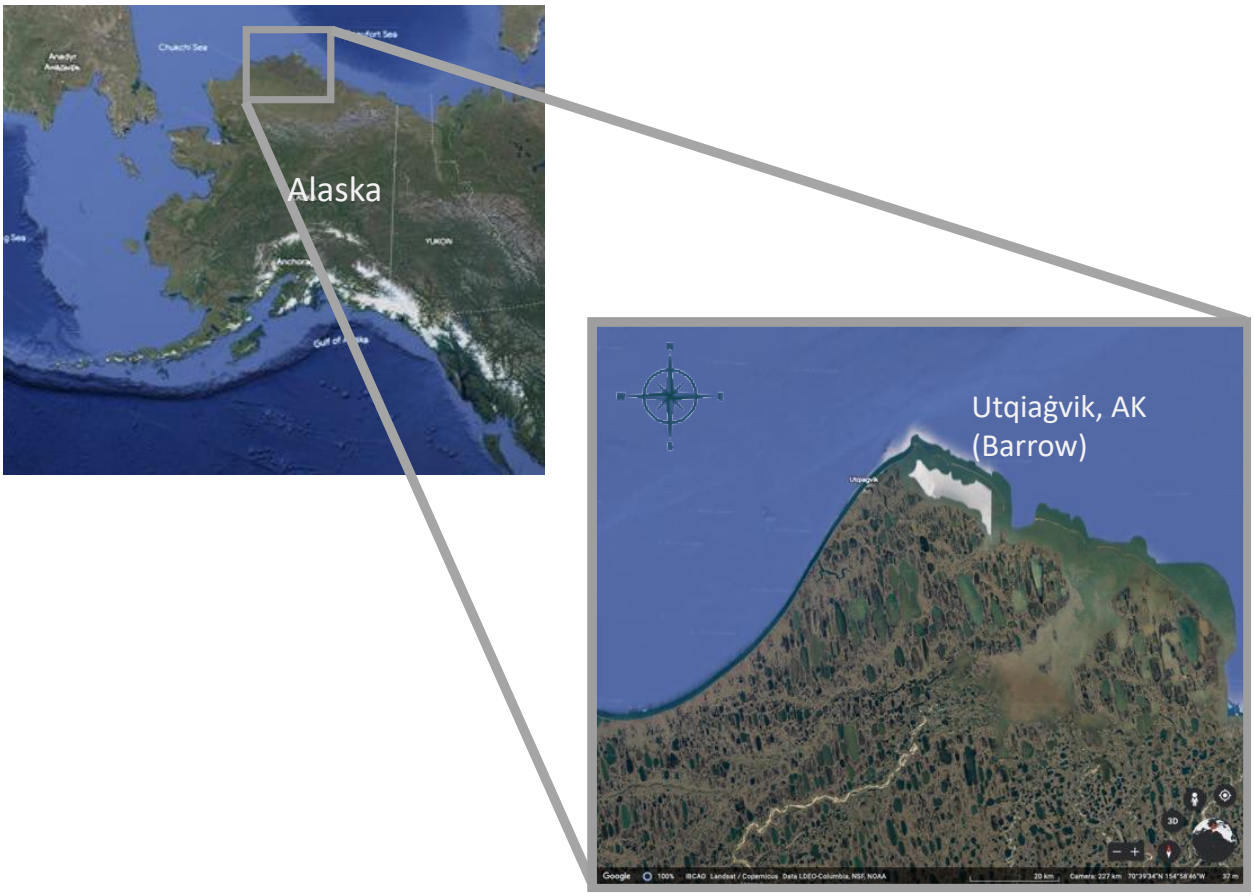


Figure 1.1: Map of Alaska focused on the research site used for this dissertation, Utqiaġvik (formally known as Barrow).

Furthermore, continuous thawing and freezing creates a talik zone where unfrozen conditions remain below the active layer. This zone accelerates permafrost melt and increases decomposition reactions below the active layer surface (Parazoo et al., 2018; Romanovsky and Osterkamp, 2000), thus making decomposition a critical factor in carbon loss and affecting microbial communities and their soil enzyme productions and compositions. Linking these larger scale processes with the microscale biochemical processes is essential for addressing and modeling these emerging and accelerating problems in the Arctic.

1.5 Dissertation Summary

This dissertation targets several longstanding major knowledge gaps in enzyme function in the arctic tundra of Alaska. While enzymes are found in all soils, studying enzymes in arctic ecosystems is important for understanding how these unique and vulnerable ecosystem function and respond to environmental change. In arctic ecosystems, there is concern that with climate change, the large soil carbon reserves built up in the permafrost are vulnerable to decomposition and release from the biosphere to the atmosphere (Lawrence et al., 2015). Arctic soils contain approximately 50% of the world's soil carbon (Tarnocai, 2009). Soil enzymes directly control decomposition; hence their function is essential. At the biochemical level, decomposition will increase, and therefore enzymes will catalyze more organic matter that is then respired to CO_2. It is thus helpful to consider the ecological response to these trends by focusing on the chemical reactions belowground that could potentially change the function of the ecosystem.

This dissertation research aims to identify and uncover missing information about soil enzyme activities by understanding their location, identities, and longevity in arctic ecosystems. These knowledge gaps will be addressed in the following chapters:

Chapter 2. Soil enzymes are preferentially associated with larger particles in highly organic arctic tundra soils.

Chapter 3. Characterizing enzyme profiles of arctic tundra soils through metaproteomic analysis

Chapter 4. Assessing the possible persistence of soil enzymes in arctic soil subsequent to the use of chloroform fumigation for microbial activity removal.

In Chapter 2, we investigate the location of soil enzymes in the soil matrix. Previous studies have touched on this topic to understand the dynamics of decomposition and their potential to decompose organic matter (Burns, 1982; Burns et al., 2013; Perez Mateos and Gonzalez Carcedo,

1985; Wallenstein and Burns, 2015). The specific enzyme location may not be uniform in the soil matrix (Ladd et al., 1996). It is not completely understood if there are free enzymes attached to living/dead cells or attached to a complex. By being able to localize enzymes, we can determine their distribution, which is relevant for soil function and the process of decomposition. Understanding this could then identify areas of soil that are most important for nutrient cycling and carbon sequestration and predict how soil ecosystems will respond to environmental change.

In Chapter 3, we explored the proteome and identified peptides associated with decomposition. Connecting enzymes with their producing microbes has been challenging; 90% of soil organisms are not culturable (Amann, 1995), and millions of organisms can perform these processes. Proteomics may shed some light on this question, but a critical limitation in proteomics is the inability to separate the peaks in the data that can identify soil proteins from humic substances (Chourey et al., 2010). For non-environmental samples, it is easy to grow cells in culture for proteomic analyses. However, soil samples are masked by humic substances that inhibit the peptide signals. By creating an optimized protocol to remove humics accurately, we can start answering questions regarding the origin of soil enzymes, which microbes produces them, and if certain species are focused on enzymatic production. Thus, my goal in this dissertation is to advance methods in this area and work toward these identifications.

Chapter 4 focuses on the lifespan of soil enzymes in the soil. Production of the peptides that form soil enzymes is energetically demanding for the microbes (J. P.Schimel & Weintraub, 2003). Therefore, determining their longevity is critical to understanding how microbes perform decomposition. The benefits to the microbe of producing these proteins should be greater than the energetic losses, and the persistence of the proteins in the soil should not be short-lived. Additionally, knowing a specific rate of decay would strengthen enzyme modeling systems, like

the EnzModel, that explore resource acquisition through enzyme production (Allison, 2005; Allison and Vitousek, 2005).

Chapter 2: Soil Enzymes Are Preferentially Associated With Larger Particles In Highly Organic Arctic Tundra Soils

ABSTRACT

Microbial processes, including extracellular enzyme (exoenzyme) production, are a major driver of decomposition and a current topic of interest in arctic soils due to the effects of climate warming. While enzyme activity levels are often assessed, we lack information on the specific location of these exoenzymes within the soil matrix. Identifying the locations of different soil enzymes is needed to improve our understanding of microbial and overall ecosystem function. Using soil obtained from Utqiaġvik, Alaska, our objectives in the study are: (1) to measure the activity of enzymes in soil pore water; (2) to examine the distribution of activity among soil particle size fractions using filtration; and (3) to cross these particle size fraction analyses with disruption techniques (blending to shred and sonication to further separate clumped/aggregated soil materials) to assess how tightly bound the enzymes are to the particles. The results of the soil pore water assays showed little to no enzyme activity (< 0.05 nmol g $soil^{-1}$ hr^{-1}), suggesting that enzymes are not abundant in soil pore water. In the soil cores, I detected activity for most of the hydrolytic enzymes, and there were clear differences among the particle size and disruption treatments. Higher activities in unfiltered and 50 μm filters relative to much finer 2 μm filters suggested that the enzymes were preferentially associated with larger particles in the soil, the organic material that makes up the bulk of these arctic soils. Furthermore, in the sonication + blending treatment with no filter, five of six hydrolytic enzymes showed higher activity compared to blending only (and much higher than sonication only), further indicating that enzyme-substrate complexes throughout the organic matter component of the soil matrix are the sites of hydrolytic enzyme activity. These results suggest that the enzymes are likely bound to either the producing microbes,

which are bound to the substrates, or directly to the larger organic substrates they are decomposing. This close-proximity binding may potentially minimize transport of decomposition products away from the microbes that produce them.

INTRODUCTION

Decomposition of organic matter in soils is driven by microbial biochemical activity, specifically by producing hydrolytic and oxidative enzymes (Burns, 1982; Skujiņscaron; and Burns, 1976). Microbes produce soil enzymes to access nutrients required for metabolic processes and growth (Allison and Vitousek, 2005; Dick et al., 2011). Despite the importance and ubiquity of this process, the location of soil enzyme activity within the soil matrix is incompletely known. There are many distinct locations within the soil matrix where these enzymes might operate, such as: free-floating in soil pore water, attached to the microbial cells, linked with enzyme-substrate complexes, associated with soil organic matter (SOM) (Burns, 1982), or associated with clay-enzyme or tannin-enzyme complexes (Burns, 1986; Marx et al., 2005). These different locations vary in their mobility within the soil and their proximity to the enzyme-producing microbes. Identifying the location of soil enzymes is needed to improve our basic understanding of soil microbial functioning, helping to illuminate how microbes interact with their substrates (Burns et al., 2013) and how they guarantee a return on investment for the microbes that create them (Treseder and Vitousek, 2001). In addition, less work has been done on assessing enzyme activity in soil pore water than on potential association with soil particles, yet it is an important exchange depot for soil materials, and thus worth quantifying.

Arctic soils are an especially relevant system in which to study the location of enzyme activities because of the possibility for accelerated decomposition due to climate change (Schuur et al., 2015). These soils remain frozen for most of the year, and the low temperatures preserve

decomposed material in the surface layers (Walz et al., 2017; Weintraub and Schimel, 2003). The accumulation of large stores of decomposed material has created a thick layer of organic soils, with tundra soils containing approximately 50% of the world's soil carbon (Shaver et al., 2006; Tarnocai, 2009). Due to temperature increases, large soil carbon reserves in the permafrost are vulnerable to decomposition, the increase of nutrient availability, and the release of CO_2 to the atmosphere (Nadelhoffer et al., 1991; Schuur et al., 2013). With these climate change effects, gaining better knowledge of enzyme activity locations within the soil matrix will help to enable a better mechanistic understanding of how enzymes will respond to increasing temperatures in cases such as deeper active layers, longer growing seasons, and changing moisture conditions in the soil.

In arctic soils, enzymes could be associated with various soil matrix components such as soil pore water, soil organic matter, higher molecular weight compounds such as tannins, or microbial cells, (Wallenstein and Burns, 2015). Investigations of enzyme locations often point to particle size as a determinant of enzyme location. For example, substantial differences between particle sizes were observed in a grassland ecosystem depending on enzyme identity (Marx et al., 2005). Also, a fertilization experiment on crop soils determined that particle sizes 63-200 μm had the highest enzyme activities (Zhang et al., 2015). However, these more mineral soils differ substantially from arctic soils, which are highly organic and frequently water saturated. Besides particle size, aggregation of soil materials can also affect enzyme location, given that it can physically prevent enzymes from being exposed, thus limiting our ability to measure activity. For example, disaggregation by disruption of soils by grinding has for example shown an increase in the measurable activity of both protease and β-glucosaminidase, suggesting that the enzymes were inside the aggregates (Fukumasu and Shaw, 2017). Moreover, the disruption of soils can release enzymes and microorganisms entrapped in clustered soil particulates by sonication (Kaiser and

Asefaw Berhe, 2014). Another factor to consider is that separating the soil particles by performing physical disturbances, such as microwaves, waterbath, blending, ultrasonic and laser treatment to soils, may lead to cell lysis (McMillan et al., 2013). This cellular damage can expose intracellular or cell-bound enzymes (Wallenstein and Burns, 2015). Because of the different nature of the soil matrix in organic horizons of arctic soil, further investigations of enzyme locations are warranted.

This study aims to explore the possible location of arctic soil enzymes within the soil matrix using various separation techniques. Our objectives in the study are: (1) to measure the activity of enzymes free-floating in the soil pore water; (2) examine the effect of the distribution of activity among soil particle size fractions using filtration; and (3) to cross these particle size fraction analyses with disruption techniques within the soil matrix (blending to shred and sonication to separate further clumped/aggregated soil materials) to assess how tightly bound the enzymes are to the particles. These investigations of particle size crossed with varying levels of disruption will help to narrow down where in the soil matrix the greatest soil potential enzyme activity is occurring. We hypothesize that the soil enzymes are associated with the large organic matter substrates, and thus, fractions containing these substrates will have the highest potential activities. To examine this hypothesis, we assayed free-floating enzyme activities in soil pore water samples and assessed hydrolytic and oxidative enzyme activities from soil cores using blending, filtration, and sonication techniques separately and in combination.

MATERIALS AND METHODS

Research site

Soil cores were collected in Summer 2016 from field locations near Utqiaġvik, Alaska, located on the northern coast and Atqasuk (70.47778 °N, 157.41806 °W), a village located 60 miles southwest of Utqiaġvik. The mean annual air temperature is -12 °C (Meisel et al., 2021).

During the summer months, June-August, the temperature can rise to 4 °C (Gädeke et al., 2021). The climate from the last 30 years (1991- 2020) is reported by the National Oceanic and Atmospheric Administration (NOAA NCEI, 2020): mean annual temperature (MAT) is -10.7 °C and mean annual precipitation (MAP) is 15.7 cm. Additionally, recent years have shown that the soil temperatures have been increasing over time (Hollister et al., 2006; May et al., 2020). Historical data show that the changes in mean annual air temperature are controlled by the winter months. From 1921 – 1995, the long-term means were 3.3 °C during the winter and 1.6 °C in the summer months (Zhang and Stamnes, 1998). The general mean soil temperature can vary in the summer months from 3.6 – 5 °C (Hinkel et al., 2001). This ecosystem is dominated by ice-wedge polygonal tundra spanning drained thaw-lake basins and interstitial tundra (Sulikowska et al., 2019). Vegetation at this site consists of moist acidic tundra plant communities, including lichens, bryophytes, graminoids, and shrubs, which fluctuate in vegetation cover with herbivory outbreaks (Haugen and Brown, 1980; Lara et al., 2015). Additionally, it has been shown that there was continuous permafrost at Utqiaġvik from 1950 through the late 1970s, classifying them as Gelisols (Nachtergaele, 2001). More recently, soils have generally warmed (Villarreal et al., 2012), thus making it relevant to study decomposition processes in this area. In Utqiaġvik, the young (<10 cm deep) and medium-aged (10-15 cm) basins contain an organic layer, and soils are classified as Aquorthels and Aquiturbels (Hinkel et al., 2003; USDA, 1999). In this region, the active layer, which is the maximum thaw depth by the end of the summer (Schaefer et al., 2011), is between 40-60 cm deep (Clayton et al., 2021). It has been shown that in soil from the Utqiaġvik Peninsula, roughly 10% can be classified as being clay material (<2 μm size) (Mueller et al., 2017). For the purpose of this study, we sampled across a wide geographic area to represent the spatial variation in soils across the North Slope shown in Table 2.1. We designed this study with the expectation

that the loci of enzyme activities in the soil are relatively general across space within these arctic systems. Thus, while this widely dispersed geographic design risks the variation being high, if we can demonstrate effects, this would be evidence that the documented trends are more general across different soil locations and types. The soil core samples were 5 cm in diameter and 15 cm in depth, made from cylindrical metal and hammered with a rubber mallet into the soil. To have a wide range of site representation of disturbed and undisturbed areas, we collected from inland moist acidic tundra regions and close to the coast (~2-5 meters away), snow fences, and within vehicle track disturbances (Table 2.1).

Table 2.1: Description of soil samples. Areas used to identify the soil collected: The Barrow Environmental Observatory (BEO), Barrow Arctic Research Facility (BARC), Mobile Instrumented Sensor Platforms (MISP), International Tundra Experiment (ITEX).

Soil Sample	**Collection Site**	**Location on Collection Site**	**Date**	**Tundra Class**	**Vegetation**	**Latitude/Longitude**
S1	Utqiaġvik, AK	ITEX/ BEO	07/21/16	Dry-moist dwarf shrub-graminoid tundra	*Sphagnum* spp.	71.31371 -156.58887
S2	Utqiaġvik, AK	Gaswell Rd	08/13/16	Moist graminoid tundra	Eriophorum scheuchzeri	71.25814 -156.33311
S3	Utqiaġvik, AK	BEO	07/16/16	Moist graminoid tundra	Eriophorum scheuchzeri *Eriophorum angustifolium*	71.31403 -156.59401
S4	Utqiaġvik, AK	Gaswell Rd	08/08/16	Moist graminoid tundra	*Eriophorum scheuchzeri* *Eriophorum angustifolium*	71.28325 -156.43034
S5	Utqiaġvik, AK	Snow Fence/ BARC	07/14/16	Moist graminoid tundra	*Eriophorum scheuchzeri*	71.31303 -156.592662
S6	Utqiaġvik, AK	Hospital Vehicle Tracks	07/30/16	Wet graminoid tundra	Eriophorum scheuchzeri	71.29814 -156.7361
S7	Utqiaġvik, AK	Hospital Vehicle Tracks	07/30/16	Dry dwarf shrub-graminoid tundra	*Eriophorum angustifolium*	71.29793 -156.73387
S8	Utqiaġvik, AK	Cake Eater Rd	07/20/16	Successional-dry dwarf shrub-graminoid tundra	*Salix rotundifolia*	71.25736 -156.53288

S9	Utqiaġvik, AK	Vehicle Tracks BARC	07/11/16	Successional-dry dwarf shrub-graminoid tundra	*Potentilla hyparctica* Eriophorum scheuchzeri	71.32113 - 156.67395	
S10	Atqasuk, AK	MISP GRID	08/06/16	Successional-dry dwarf shrub-graminoid tundra	*Papaver hultenii* *Eriophorum angustifolium* *Vaccinium Vitis-idaea*	70.501957 157.434615	-

Figure 2.1: Map of Research Site. Map of Alaska showing the collection sites in the vicinity of Utqiaġvik (Barrow), Alaska. Symbols represent sites where soil samples were collected

Soil Pore Water Assays

To examine free-floating enzymes in the soil pore water, we used rhizon samplers (Eijkelkamp North America Inc, Morrisville, North Carolina, part no. 192101) for soil pore water collection. These allow access to the continuous water within the soil matrix but not necessarily water-bound at low matric potential (Darrouzet-Nardi and Weintraub, 2014; Dick et al., 2011; K. R. Saiya-Cork et al., 2002). The rhizon samplers consist of four main parts: a hydrophilic porous polymer, stainless steel wire, PVC (polyvinylchloride) tube, and the lock connector. We attached a needle to the lock connector, and soil pore water was acquired using a 6 ml vacuette (Greiner

Vacuette No. 456089 (Darrouzet-Nardi and Weintraub, 2014). Rhizon sampler samples were collected near the plots from the International Tundra Experiment, ITEX, site located in Barrow, Alaska (samples shown in Supplemental Material Table S1). The tubes were attached to the needles and collected within 24 hours. Once the sample was recovered, it was placed with the soil samples in the freezer at -20 °C. From these samples, fluorometric measurements were conducted as described above to test the potential enzyme activity (Henry and Molau, 1997). In addition, to compare with the potential enzyme activities measured in the soil core extractions, we performed a dimensional analysis based on the amount of soil mass represented by the measured soil water volume in both cases (Supplemental material S1).

Separation Treatments

We applied eight different treatments (Table 2.3) to separate subsamples for each of the ten samples collected using our standard protocol (blending with no filter). To test if enzymes were associated with SOM or with other particles, after blending, we then applied two separation techniques factorially: sonication (with and without sonication) and filtering (no filter, 50 µm, and 2 µm). The filters separate the soil by particle size fractions. To assess the total soil matrix including all organic matter particles and their constituents, no filter was used. The 50 µm filter removed larger (primarily organic) particles. Consequently, to separate most particles sized 2-50 µm from particles sized < 2 µm (German et al., 2011), a 2 µm filter was used. Similar soils from this arctic region have been analyzed by physical soil fractionation, where it was determined that there is a high amount of particulate organic matter not associated with soil minerals (Mueller et al., 2017).

Alongside particle size fractionation, sonication was used as a physical disruption technique to clumped and aggregated soil from itself and possibly separate enzymes or cells from

soil materials. Sonication is generally less harsh and does not shred soil particles as blending does. Sonication generates pressure waves and can disperse soil aggregates at low energy applications without damaging the microbes or causing cell lysis (Suslick, 1990; Amelung and Zech, 1999; Kaiser et al., 2012).

Treatment 1 (blend + 50 µm filter) and treatment 2 (blend + 2 µm filter) were run through the treatment's respective filter after the soil was blended. The filtration was to distinguish the particle size at which enzyme activities could be detected within the soil matrix and possibly determine location by removing larger particles (reference Figure 2 for visual details). Treatment 3, 4, and 5 (No blend + sonicate, no blend + sonicate + 50 µm filter, no blend + sonicate + 2 µm filter) did not get shredded by the blender. These treatments were sonicated with a VWR Ultrasonic bath (VWR Ultrasonic Cleaner, Radnor, PA) at a low amplitude of 20-30 kHz for 5 minutes per sample. Sonication was at a low setting to avoid cell lysis but allow for dispersion of soil aggregates (Hunter and Busacca, 1989; Kaiser et al., 2012). After the sonication process took place, treatment 3 was transferred to the 96-well plate for fluorometric analysis.

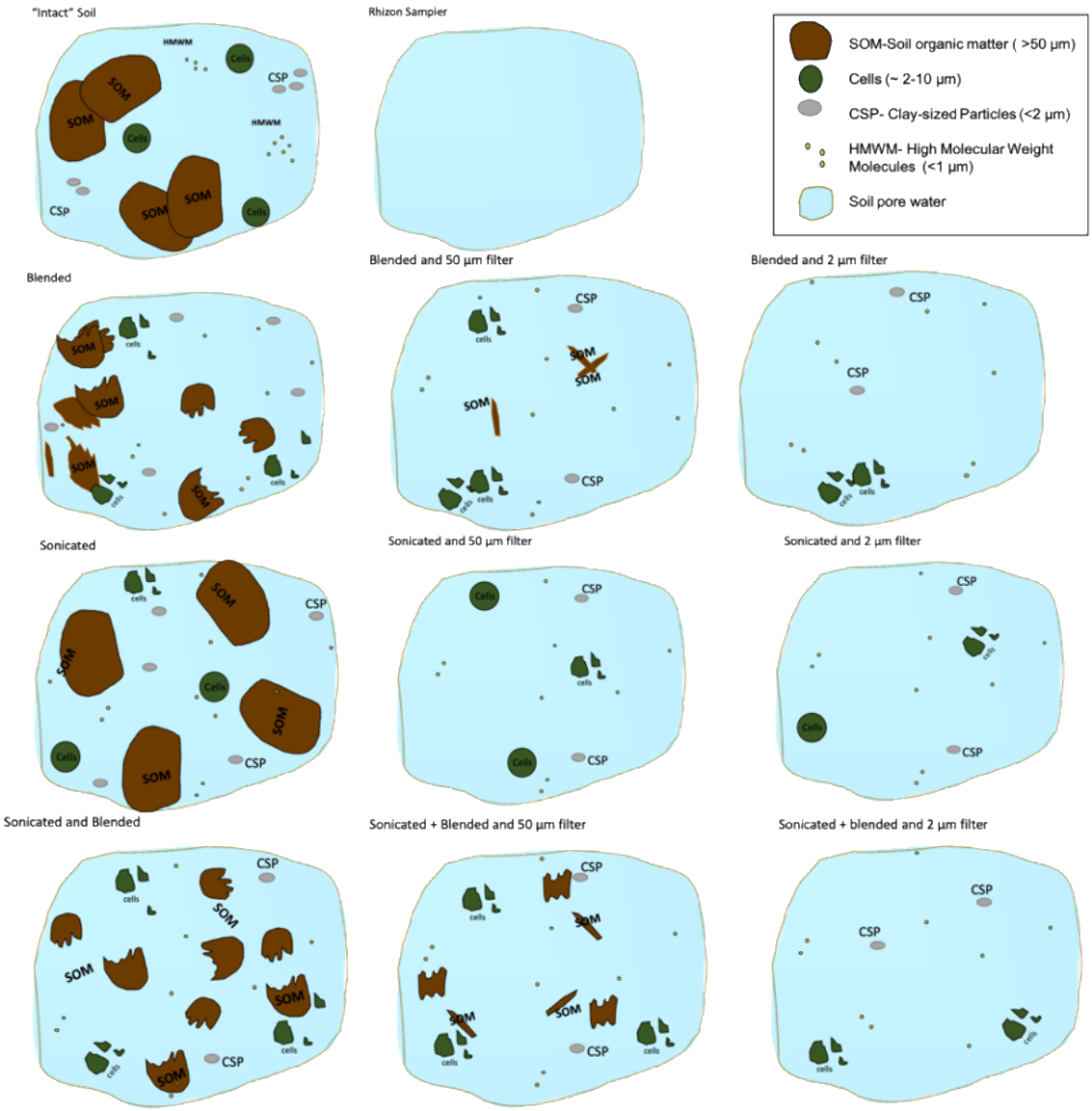


Figure 2.2 Conceptual diagram of the soil matrix. The soil disruption and separation techniques are shown in the diagram above. The soil pore water (in light blue) is always present in the arctic soils; therefore, it will always be in the background of each of the treatments. The dark brown represents the SOM being affected by the treatment. We also included in gray the clay-sized particles (<2 µm), in green we show the cell particles, and in yellow are the high molecular weight particles. In the top left, an "intact" soil is included to compare against each treatment. Theoretically, in the diagram, the disruptions of blending are demonstrated by the breaking of the SOM and cells. In sonication, SOM is not broken, it is dispersed. Filtration shows the separations by particle size.

Soil potential enzyme activity assays

We used a fluorometric soil activity assay protocol to quantify soil decomposition enzyme activity within the soil matrix (Romanovsky and Osterkamp, 2000). Enzyme assays were performed with a high-throughput microplate reader technique. We analyzed the samples from the activity of nine enzymes: two oxidative and seven hydrolytic (Table 2.2). It is important to note that the enzyme activity we measure is potential enzyme activity in the laboratory and not *in situ*. *In situ* activity refers to enzymes under natural conditions, as opposed to potential enzyme activity, which measures the maximum reaction rate of an enzyme-catalyzed reaction at a specific temperature with no substrate limitation (Wallenstein and Weintraub, 2008). Soil slurry samples were prepared by mixing 1 g soil and 125 ml of 50 µM sodium acetate buffer (pH 5). The samples were kept stirring with a magnetic plate until transferred to the black (hydrolytic) and translucent (oxidative) 96 microwell plates. There were two types of fluorescent standards for the hydrolytic enzymes: 7-amino-4-methyl-coumarin (MC) and 4-methylumbelliferyl (MUB). The MC standards were used for Leucine-amino-peptidase (LAP) and MUB for the other enzymes, (see Table 2.2). Control quench samples were made (soil slurry and MUB/MC) to correct MUB/MC fluorescence intensity due to soil turbidity (Marx et al., 2001). Additionally, measurements were taken of the background fluorescence of soils, substrates, and MUB/MC standard curves (Marx et al., 2001; McLaren et al., 2017; K.R Saiya-Cork et al., 2002; Sinsabaugh et al., 2003). The microplate reader read the black plates to check the fluorescent activity, and the oxidative plates were incubated for 24 hours, then run in a BioTek Synergy H.T. microplate reader (BioTek Instruments Inc., Winooski, VT, USA) (German et al., 2011).

Table 2.2 Summary of enzyme functions and substrates.

HYDROLYTIC ENZYMES	ABBREVIATION	FUNCTION	SUBSTRATE	FLUORESCENT STANDARD *
***B*-1,4-GLUCOSIDASE**	BGLUC	Releases glucose from cellulose, optimal activity at 85°C	4-MUB-β-D-Glucosidase	MUB
A-1,4-GLUCOSIDASE	AGLUC	Releases glucose from soluble saccharides	4-MUB-α-D-Glucosidase	MUB
***B*-D-1,4-CELLOBIOHYDROLASE**	CELLO	Releases disaccharides from cellulose	4-MUB-β-D-Cellobioside	MUB
***B*-1,4-XYLOSIDASE**	XYLO	Degrades hemicellulose	4-MUB-β-D-Xyloside	MUB
***B*-1,4-N-ACETYL-GLUCOSAMINIDASE**	NAG	Degrades chitin	4-MUB-N-acetyl-β-D-glucosaminide	MUB
ACID PHOSPHATASE	PHOS	Releases phosphate ions from phosphate group	4-MUB-phosphate	MUB
LEUCINE-AMINO-PEPTIDASE	LAP	Degrades protein into amino acids	7-amino-4-methylcoumarin	MC

OXIDATIVE ENZYMES	ABBREVIATION	FUNCTION	SUBSTRATE
PHENOL OXIDASE	PHENOL	Oxidizes phenols and consume oxygen Degrades lignin and humus to gain carbon	L-Dopa (L-3,4-dihydroxyphenylalanine)
PEROXIDASE	PEROX	Use H_2O_2 as an electron acceptor Lignin degradation	L-Dopa (L-3,4-dihydroxyphenylalanine)

*Fluorescent Standards: 7-amino-4-methylcoumarin (MC) and 4-methylumbelliferyl (MUB)

Table 2.1 Separation treatments and soil pore water

IDENTIFIER	TREATMENT	PURPOSE OF TREATMENT	BLEND	SONICATE	FILTER
CONTROL	Blend	Shred soil organic particles and compare disruption to sonication	Yes	No	No Filter
TREATMENT 1	Blend + Filter 50 µm	Remove larger particles, mostly organic particles	Yes	No	50 µm
TREATMENT 2	Blend + Filter 2 µm	To separate silt-sized particles (2-50 µm) from clay-sized particles (<2 µm)	Yes	No	2 µm
TREATMENT 3	Sonicated	Separate organic particles Compare the disruption with blending.	No	Yes	No Filter
TREATMENT 4	Son. + Filter 50 µm	Compare with treatment 1 and 7	No	Yes	50 µm
TREATMENT 5	Son. + Filter 2 µm	Compare with treatment 2 and 8	No	Yes	2 µm
TREATMENT 6	Son. +Blend	Potentially extract the enzymes and bacteria from bulk soil or soil fractions	Yes	Yes	No Filter
TREATMENT 7	Son. +Blend + Filter 50 µm	Same as Treatment 6 + removed larger particles mostly organic particles (larger than 50 µm)	Yes	Yes	50 µm
TREATMENT 8	Son. +Blend + Filter 2µm	Same as Treatment 6 + to separate silt-sized particles (2-50 µm) from clay-sized particles (< 2 µm)	Yes	Yes	2 µm
SOIL PORE WATER	Lysimetry	Determine if enzymes are free-floating in the soil matrix	No	No	No Filter

Data Analysis

Using R 3.6.2 (R Core Team 2019, 2019), we used a linear mixed-effects model (Pinheiro and Bates, 2000), with treatment analyzed as a fixed and soil core as a random effect because each of the 10 cores were subsampled for use in each of the 8 treatments. Following this, Tukey post-hoc pairwise comparisons were made across treatments for each enzyme using the 'Multicomp' package ($p<0.05$) (Hothorn et al., 2008).

RESULTS

Soil Pore Water Enzyme Assay

Activities of four of the six enzymes were detectable from the assays conducted on soil water from the rhizon sampler technique used to collect soil pore water (Figure 2.3). N-acetyl-glucosaminidase and xylosidase had extremely small means, close to non-detectable. We observed measurable activity in α-1,4-glucosidase, β-glucosidase, cellobiohydrolase, and acid phosphatase. However, after adjusting for the mass of soil represented, activities were very low compared to the soil core samples, with averages less than 0.05 nmol g^{-1} soil hr^{-1} l (Figure 3; Supplemental material S1). Thus, there was no significant difference between the enzymes.

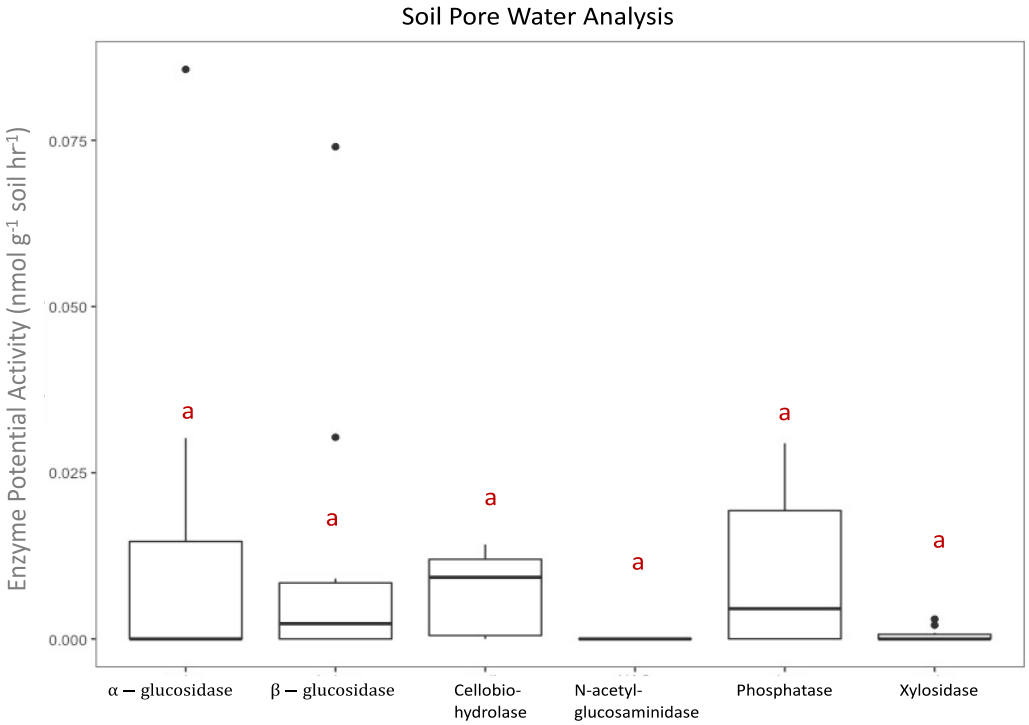


Figure 2.3 Soil pore water potential enzyme activities (nmol g-1 soil h-1) for arctic soils assayed. The bottom and top boxes represent 25th and 75th percentiles. The boxplot whiskers represent the values that are 1.5 times interquartile range (IQR) above and below quartile (Q1) and quartile 3 (Q3), respectively (Q1 – 1.5 × IQR; Q3 + 1.5 × IQR). The dots represent outlier values. Letter a represents that there is no difference between enzymes.

Soil Cores – Hydrolytic Enzymes

In the soil core samples, measurable activity was present for most hydrolytic enzymes assayed with blending and the various separation techniques. In most cases, the activity dropped when a filter was added to a treatment. It stayed consistent from highest to lowest according to the corresponding filter size. Although activity was low at 2 µm across all enzymes, activity was still measurable. In α-1,4-glucosidase, there is a significant difference between the sonicated and the sonicate + blend treatment, the filters had less of an effect. The means of five out of the six hydrolytic enzymes (all except cellobiohydrolase) at blend + sonicate showed higher enzyme activity. Cellobiohydrolase has its highest activity with just blending; additional treatments did not show higher activity. The activity of most enzymes did not increase with the sonication treatment alone (Figure 2.4)

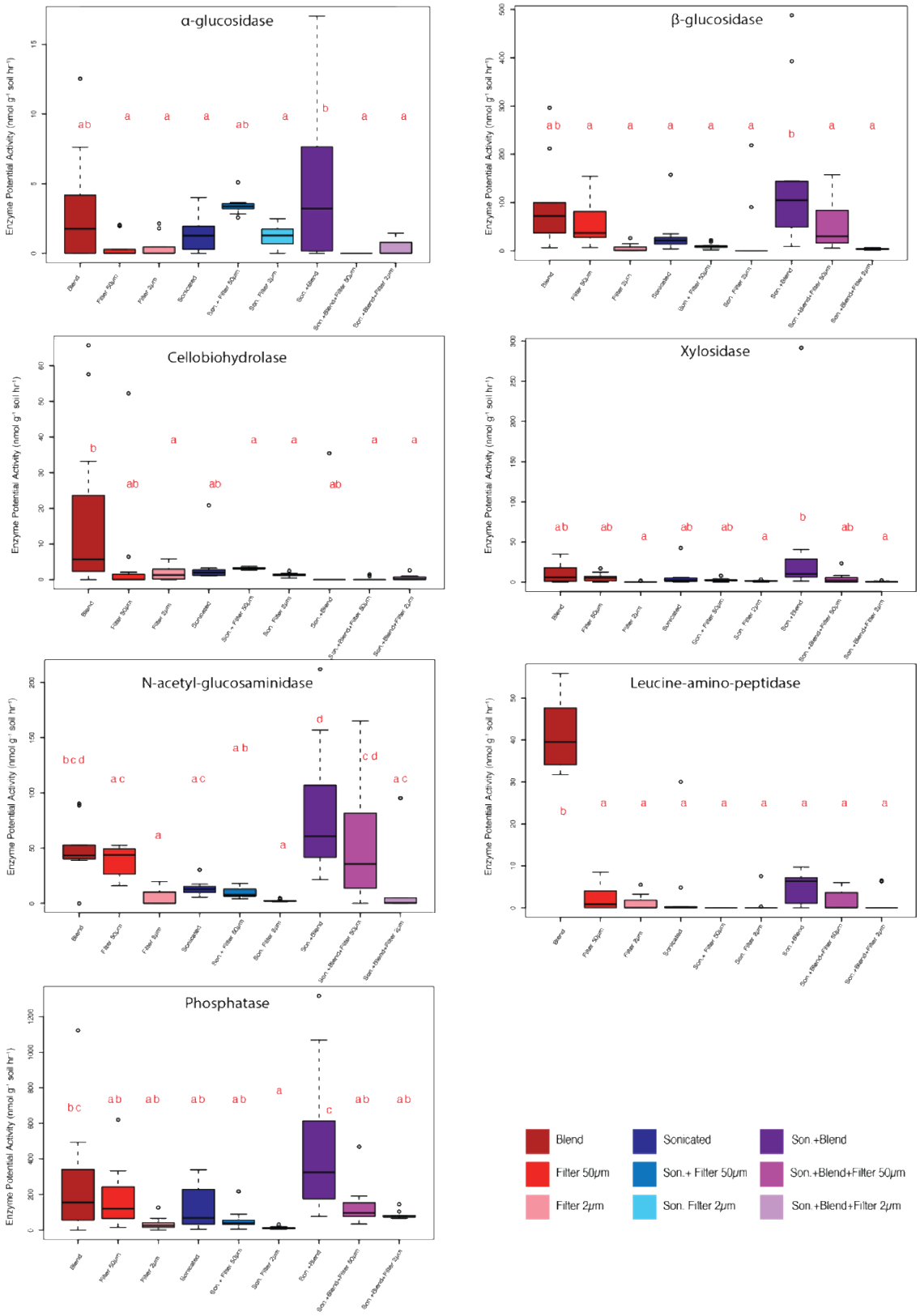

α-glucosidase
β-glucosidase
Cellobiohydrolase
Xylosidase
N-acetyl-glucosaminidase
Leucine-amino-peptidase
Phosphatase
Enzyme Potential Activity (nmol g⁻¹ soil hr⁻¹)
Blend
Filter 50µm
Filter 2µm
Sonicated
Son.+ Filter 50µm
Son. Filter 2µm
Son.+Blend
Son.+Blend+Filter 50µm
Son.+Blend+Filter 2µm

Figure 2.4. Hydrolytic Enzyme Potential Activity. Enzyme activities were calculated in units of nmol g^{-1} soil hr^{-1} and are color-coordinated by treatment. Letters denote significant differences among treatments based on estimated marginal means with Tukey adjusted p-values.

Oxidative Enzyme Assays

For phenol oxidase, sonication did not affect the enzyme activity ($p > 0.05$). Additionally, when blending was performed on phenol oxidase, it showed no activity (mean= 0.0). When sonication is applied, there was a slight average activity of 2.4 nmol g^{-1} soil hr^{-1}. In peroxidase, sonication had a significant effect compared to the blended treatment ($p < 0.05$). In both phenol oxidase and peroxidase, when looking at blend + 50 µm filter, the means are higher for both than in blending with filtration of 2 µm ($p<0.05$). However, there is no significant difference between most of the sonicated treatments (Figure 2.5).

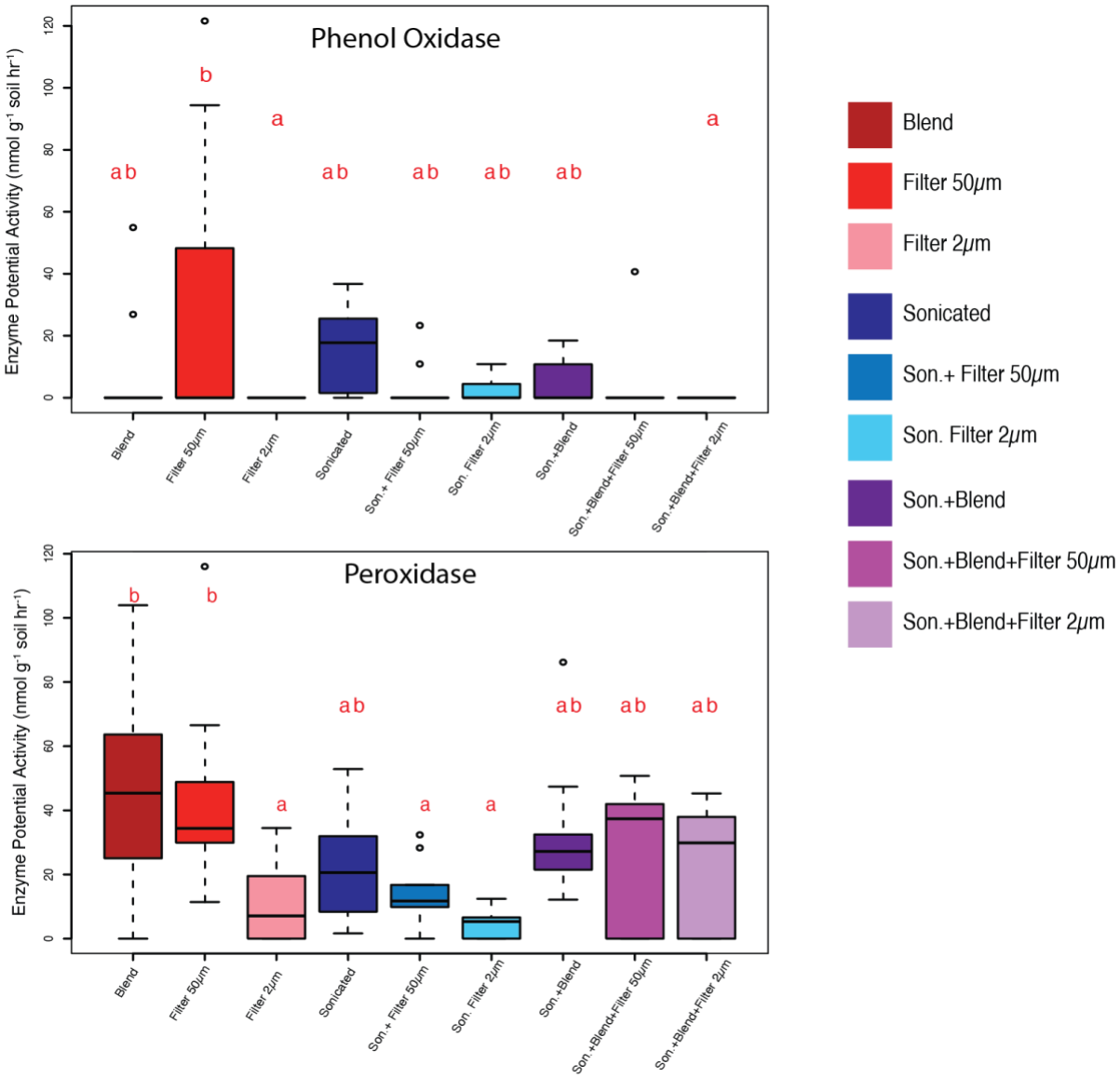


Figure 2.5 Oxidative Enzyme Potential Activity. Enzyme activities were calculated in units of nmol g^{-1} soil hr^{-1} and are color-coordinated by treatment. Letters denote significant differences among treatments based on estimated marginal means with Tukey adjusted p-values.

DISCUSSION

We sought to better understand enzyme location in arctic tundra soils. To do this, we quantified the enzyme activity of two oxidative and seven hydrolytic enzymes by the distribution of activity among size fractions and disruption of soil aggregates. Our results suggest that most of the enzymes in this region are associated with large particles of SOM and are not highly abundant

in soil pore water. Additionally, there were clear differences among the particle size and disruption treatments. In the following sections, we will discuss why enzymes are not free-floating in the soil pore water, why the enzymes are not located in the small size fraction (<2 μm), and how enzymes are likely tightly bound to the organic matter in tundra soils. Overall, we will make the case that the enzymes are likely bound either directly to the decomposition substrate (the large partially decomposed organic materials in the soil matrix) or to microbial cells that are bound to the substrate.

Soil pore water

The results of soil pore water assays showed very low potential enzyme activities once corrected for the soil volume represented (<0.05 nmol g soil^{-1} hr^{-1}), suggesting that enzymes are not abundant in soil pore water. Microorganisms might secrete the enzymes into the soil water, but enzyme allocation has been shown to shift with changes in dissolved organic carbon and nutrient availability (Allison and Vitousek, 2005; Seifert-Monson et al., 2014). Changes in the soil may potentially explain why they do not remain in the pore water for a long time. Our pore water results show some low but detectable enzyme activity on some enzymes like α-1,4-glucosidase and acid phosphatase, where we may observe the activity of highly transitory enzymes moving toward the substrate. Since α-1,4-glucosidase and acid phosphatase were still low in potential activity, the slight increase does not suggest that these enzymes are unusual with respect to the others tested. Furthermore, a recent study using microdialysis worked to analyze free soil enzymes *in situ,* it was demonstrated that 91% of the total hydrolytic activity were not free-floating enzymes (Buckley et al., 2019), thus, supporting our results and agreeing that the free enzyme pools are rapidly changing. Moreover, in our study, when using soil core samples, typically there are higher enzymatic activities than when using the soil pore water samples, which is why our data suggest

that enzymes are physically bound to soil particles (Bell et al., 2013; Steinweg et al., 2012) or cells (Burns, 1982), and are not free-floating in the pore spaces.

Enzyme activity among particle sizes in the soil

Our filtration treatment results showed higher activities in unfiltered and 50 µm filters relative to much finer 2 µm filters, suggesting that all the enzymes we measured related to C, N, and P- acquisition were preferentially associated with larger particles in the soil, likely the organic material that makes up the bulk of these arctic soils. The oxidative enzymes related to lignin degradation had a similar association (Sinsabaugh, 2010a). This suggests that the microbes are likely attached or located near the organic matter, which could be important for the producing microbes to ensure return on investment for the enzyme production. Our data does not allow us to discern whether the enzymes are attached to substrates or the microbes. However, the data does show that arctic soils have higher enzyme activity in the large coarse fraction, supporting our hypothesis of a physical connection between enzyme and substrate.

As we expected based on the known textural differences between these arctic soils and more mineral soils that have been investigated in other studies, we did not see a significant amount of enzyme activity in the < 2 µm fraction. All the hydrolases had higher activities in the coarser fractions (>50 µm). In contrast with Alaskan soils, there is a higher amount of weathering products, such as clays in warm and humid areas (Hobbie et al., 2002; Jonasson et al., 2001). Soils containing higher clay content, such as a tallgrass prairie, demonstrate abundant enzyme activity in all type of particle size fractions (Borden et al., 2010). Therefore, our data suggests a substantial structural difference in where enzyme activity can expect to be found in these highly organic soils as compared to more typical mineral soils.

High molecular weight particles, such as tannins and humic substances, are also included in the <2 µm fraction and are sometimes thought to be associated with enzymes. For example, one of the main challenges in protein extraction for soil proteomics has been humic substances, inhibiting protein signaling (Chourey et al., 2010; Chourey and Hettich, 2018). Humic substances are substances formed by the secondary synthesis of a reaction, typically the end product of decaying organic matter (Huang and Hardie, 2009). When decomposition occurs, the complexity of organic material increases, and enzymes have fewer sites available for them to attach to (Ladd et al., 1996; Marx et al., 2005). This possibly explains why enzymes are not in the smaller fractions. It has been shown that large fractions hold higher microbial abundance and conditions for microorganisms (Zhang et al., 2015), and enzymes tend to stay close to the microbes that produce them. Nevertheless, our study does show that enzyme proteins are not attached to the freely available humic molecules such as dissolved or suspended organic materials, which would be found in the <2 µm fraction. While we cannot dismiss that humic substances are attached to large particles, we can suggest that enzymes are not primarily located in suspended high molecular complexes (<2 µm).

The role of soil structure in isolating enzyme activity

We used two disruption effects, sonication, and blending, to potentially expose enzymes entrapped in the soil matrix (Fukumasu and Shaw, 2017). Sonication and blending together increased the measured enzyme activities of these arctic soils the most, indicating that enzymes are likely bound inside undecomposed material. Our results suggest that enzymes in arctic soils require severe disruption, like blending, to break apart the clumped undecomposed soil material to see more activity. Sonication alone did not have the force to separate the particles due to the relatively high structural integrity of the organic material, which is a notable difference with more

mineral soils that can be better dispersed by sonication. For example, xylanase increases with low-energy sonication in a clay loam forest soils (De Cesare et al., 2000a), and acid phosphatase increases by 156% (Rojo et al., 1990) in colluvial Rendzina from a grassland ecosystem. In contrast, in our samples, xylosidase activity was nearly the same if using blending or sonication but using them together resulted in higher activity. Because sonication was used alone at a low energy level, substrate-bound enzymes may not have been widely dispersed, and this technique may only be releasing a fraction of the enzymes attached to clumped soil materials. The disruption effects we observed are consistent with the hypothesis that enzymes are tightly bound to the relatively undecomposed organic matter or microbial cells that are themselves bound to the organic matter.

Finally, a noteworthy topic to touch on is the interaction between cell walls and enzymes. Some soil enzymes have been suggested to be attached to cell walls of soil microbes (Stemmer et al., 1998). For example, β-glucosidase, when isolated from culture, has been found to be bound to fungal cell walls (Lynd et al., 2002). While it would require further research, it is possible that blending lyses cells and releases these cell-bound enzymes into the slurry, resulting in higher contact and higher measured enzyme activities. Our results showed that using sonication without blending, we still see either equal or slightly less enzyme activity than with blending. Because sonication is a less invasive method than blending, sonication may not lyse cells and release the inside material, including enzymes. When comparing oxidative and hydrolytic enzymes, there is a slight difference when looking at the oxidative enzymes, particularly in peroxidase, in which sonication and blending together do not show the same synergistic increase we observed in the hydrolytic enzymes. This could be because the oxidative enzymes are less stable in the environment (Sinsabaugh, 2010a), and they are negatively correlated to hydrolytic enzymes

(Sinsabaugh et al., 2005). Our results suggest that further investigation of whether cellular-bound enzymes contribute to activity would be worth pursuing.

CONCLUSION

Our results revealed a clear trend in which enzymes exhibited minimal (but not zero) activity in the soil pore water. Additionally, we can rule out high enzyme activities in small size fractions (<2 µm) that include clays, high molecular weight molecules such as tannins and humic substances, and cellular debris. Using sonication amplified the enzyme activities in conjunction with blending, suggesting that this technique is causing enzyme release otherwise not seen by blending, suggesting that enzymes are heavily interlaced within the organic substrate and hence more exposure and de-clumping leads to higher measured activities. Overall, our results suggest that soil enzymes are mostly associated with larger particle sizes, likely organic matter. Further studies might focus on differentiating and confirming the type of materials in these larger particle sizes (>2 µm).

Chapter 3: Characterizing Enzyme Profiles of Arctic Tundra Soils through Metaproteomic Analysis

ABSTRACT

Metaproteomics is a growing area of interest for describing complete protein profiles of microbial organisms in soils. One of the biggest challenges in investigating soil metaproteomes is low protein counts due to spectral interference by high molecular weight interferents such as humic substances. In this study, we work to overcome this challenge and examine two soil metaproteomes in arctic tundra soils. Microbial function in arctic soils, especially microbial control of decomposition, is of particular interest due to warming conditions, permafrost thaw, and the possibility of C loss from these soils. In this study, we: 1) explored protein extraction methods to improve the number of peptide matches; 2) explored the metaproteome of two soil samples (a sample from a long-term fertilization experiment in moist graminoid tundra near Utqiaġvik AK and a nearby unfertilized sample) with a focus on hydrolases and oxidases that include the soil enzymes relevant for decomposition; and 3) compared the relative abundance of peptides with laboratory assays of potential enzyme activities in these soils. We identified FASP (Filter Aided Sample Preparation) as a crucial methodological step for removing interferents, providing an average of 17,613 peptide matches per sample vs 1721 without. We categorized the dominant groups of enzymes in these samples using the Enzyme Commission (EC) system. Enzymes belonging to all seven major classes were observed, with a high quantity of transferases that are likely used for intracellular purposes and hydrolases that may consist of both intra- and extracellular constituents. We further compared our metaproteomic results with enzyme activity assays for nine soil enzymes we had previously measured in these samples. We found evidence that all nine enzyme types were present in the proteome, with bacteria being the main producers.

We also identified two additional soil enzymes, β-galactosidase and arylsulfatase, that exhibited a large count of peptides in the proteome. To assess whether these would have high activity, we acquired fluorescent substrates for these enzymes and assayed their activity. Both showed high activity, with β-galactosidase particularly showing high rates. However, across all 11 enzymes, there was not a clean one-to-one correlation between peptide counts and enzyme activity levels. Our research revealed that FASP clearly improved peptide yields, thereby enhancing our comprehension of the enzymes present in the proteome and their compatibility with metabolic processes. We were able to assess some taxonomic information about the microbial enzyme producers, including evidence that enzymes of a single type can be produced by multiple taxonomically distant organisms in a given soil sample. However, further research is required as more than 75% of the organisms matched to the peptides we observed are currently unclassified.

INTRODUCTION

Arctic soil microbial communities are composed of bacteria, fungi, and archaea, many of which can produce enzymes that degrade organic matter, thus playing crucial roles in nutrient cycling and decomposition (Burns, 1982; Øvreås, 2000). Up to 65% of prokaryotic genomes are capable of soil enzyme production (Berlemont and Martiny, 2015; Zimmerman et al., 2013). However, not all organisms produce enzymes at the same time. Among soil proteins, there are two major categories: detrital proteins, composed of proteins released by deceased organisms, and functional proteins, which include proteins in live organisms, soil enzymes, and microbial surface-active proteins (Rillig et al., 2007). While potential enzyme activity levels in soils are regularly assayed (Burns et al., 2013), metaproteomic profiles for soil function have only recently started to be thoroughly explored (Quinn et al., 2022; Salvato et al., 2022). Moreover, the relative abundance of enzymes produced by these different soil organisms has been difficult to quantify (Bastida et

al., 2009). Thus, investigations in this area have fallen short of a complete characterization of microbial function.

While these approaches and questions are relevant to any soil, we here chose to investigate arctic soils for several reasons. First, they are highly organic and contain significant amounts of humic substances. Second, due to climate change factors such as increased warming, the arctic soils will continue to experience reduced snow cover and increased permafrost thaw (AMAP - Arctic Monitoring and Assessment Programme, 2019). These changes impact ecosystem processes and belowground microbial communities responsible for the stabilization/destabilization of carbon (Hartley and Singh, 2018) and nitrogen (Schimel et al., 2004; Weintraub and Schimel, 2003). Recent changes in plant species composition and abundance have been shown to affect both above and belowground ecosystem processes (Leffler et al., 2016; McLaren et al., 2017; Mekonnen et al., 2018). Belowground, enzymes from these arctic microbial communities are temperature sensitive and driven by seasonal patterns (Darrouzet-Nardi et al., 2019; Wallenstein et al., 2009). Furthermore, the location of the soil enzymes within the soil matrix has been associated with the large particulate organic substrates found in these soils, likely in proximity to the microbes that produce them (Martinez et al., 2021). The activity of these enzymes can provide valuable insight into the microbial activity in these soils and the nutrient availability of substrates for microbial use (Schimel and Weintraub, 2003). Thus, working to reveal the proteome in arctic soils will further understanding of the main functions of the proteins, both intra- and extracellular.

To explore the metaproteome in arctic soils and begin to connect it with soil enzyme function, we aim in this study to compare and optimize techniques for extracting, quantifying, and identifying arctic soil enzymes. Our main objectives are to 1) explore protein extraction methods that may improve the number of peptide matches; 2) characterize and classify soil proteins and

their functions, especially those related to decomposition; and 3) compare the relative abundance of peptides with potential enzyme activities to determine whether high-activity enzymes are represented by significant components in the proteome that are not currently measured. To work toward these objectives, we examined two arctic tundra soil samples (historically fertilized and unfertilized soils) under a range of extraction techniques.

MATERIALS AND METHODS

Site Description

We collected soil from one of Alaska's northernmost villages, Utqiaġvik (formerly Barrow; 71.2906°N, 156.7886°W), in summer 2016. Utqiaġvik has a mean annual temperature of −12 °C and a mean annual precipitation of 106 mm (Kurek et al., 2022). Soils in this area remain frozen and under ice for about nine months of the year and have an active layer of <1 m (Bockheim et al., 1999). We sampled during months when the upper 15-25 cm of soil was fully thawed. This allowed us to capture the period when soil microbes were highly active. Utqiaġvik has a short growing season during the cold summer months, during which vegetation includes bryophytes, lichens, graminoids, forbs, and a few shrubs (Villarreal et al., 2012).

Sample collection

The soil samples used in this study measured 5 cm in diameter and 15 cm in depth, and due to the methodological objectives as well as the complex metaproteomic measurements and analyses, we focus on just two soils samples: one was from a legacy fertilized site, and the other was from a non-fertilized site. Given the limited number of studies of fertilization experimental sites in tundra ecosystems that been abandoned for decades then restudied recently (Werner et al., 2021), we believed this comparison to be particularly interesting. The sampling site is a drained thaw lake basin, the tundra vegetation is dominated by aquatic graminoids (Villarreal et al., 2012),

with *Carex aquatilis* as a dominant species in this area (Figure 3.1). We chose these samples to create a basis for comparison, even though we could not perform a statistical analysis because of the low sample number. The legacy fertilized sample (Sample 1) was obtained from an abandoned nutrient addition experiment (71.2890822°N, 156.5874145°W) conducted between 1960-1970. The non-fertilized soil (Sample 2) was obtained on the Barrow Environmental Observatory (BEO) near the fertilization site but had no nutrient addition (71.32339°N, 156.66821°W). Immediately after collection, the soil samples were taken to the lab and frozen at -20 °C. All samples remained frozen until they were ready to be sampled.

Figure 3.1. Soil collection sites in Utqiaġvik, AK. Showing the unfertilized (BEO) and fertilization site.

Protein extraction

Soils were removed from the freezer and left to thaw for approximately 10 hours at room temperature. Once the sample was completely thawed, we removed the visible plant residues (root

segments > 1 cm) with tweezers. Four tubes were prepared with 25 g of soil, each dissolved in 30 ml of 1% SDS-resuspension buffer (sodiumdodecylsulfate, pH 8.5). 100 µl of bovine serum albumin (BSA) and 100 µl apomyoglobin was added to the soil mixes as a recovery standard (following Chourey et al. 2010).

To begin the protein extraction procedure (Figure 3.2), we incubated samples under four different treatments to test the efficacy of humic acid removal: (1) 100 °C for 10 mins (boiling), (2) 37 °C for 10 mins (non-boiling), (3) 100 °C for 12 hrs (overnight, in the dark), (4) 37 °C for 12 hrs (overnight, in the dark). The tubes were placed in boiling water to boil the samples, and the non-boiling samples were kept in a 37 °C water bath. Theoretically, the boiling treatment was used to denature proteins, including intracellular and cell-bound proteins. In contrast, the non-boiling treatment left the cell undisturbed and solely analyzed the proteins outside the cell membrane (Table 1). One set of samples (A-D) was incubated for 10 mins in Lab Armor bead bath (Lab Armor, Cornelius, Oregon, USA), and the other set of four samples (E-H) was incubated overnight in the dark to inactivate proteases that are released due to cell lysis. All samples were then vortexed vigorously and centrifuged to remove large particles that would not dissolve and could clog filters. The supernatant was transferred to a glass centrifuge tube for further processing.

Table 3.1 Description of Samples and Treatments

Sample Name	Treatment/Incubation time	Soil type
A	Boiling/ 10 minutes	S1 Fertilized
B	Boiling/ 10 minutes	S2-Non-fertilized
C	37 °C/ 10 minutes	S1 Fertilized
D	37 °C / 10 minutes	S2-Non-fertilized
E(A*)	Boiling/overnight in the dark	S1 Fertilized
F(B*)	Boiling/overnight in the dark	S2-Non-fertilized
G(C*)	37 °C /overnight in the dark	S1 Fertilized
H(D*)	37 °C /overnight in the dark	S2-Non-fertilized

Protein Precipitation

Samples were adjusted to 20% trichloroacetic acid (TCA) and placed in an ice bath for 10 hours to precipitate proteins, after which they were centrifuged for 10 minutes. The supernatant was discarded, and we only kept the concentrated protein pellet. The pellet was washed with 10 ml of acetone (20 °C) and re-suspended gently, followed by centrifugation for 25 minutes, with three repetitions, to remove the excess TCA. The pellets were dried with nitrogen gas and frozen for 24 hours.

Trypsin Digestion

Protein pellets from boiled samples were resolubilized in 10 ml of 6 M Guanidine in Tris buffer. For the 37 °C samples, 5 mls of 6 M Guanidine was added in Tris buffer. The samples were vortexed and incubated for 30 minutes at 37 °C. After freezing overnight, we heated all the samples for 10 mins and vortexed. On the 37 °C samples, we took 2 ml for direct extraction and 1 ml from the remaining samples. We then added 10 mM Dithiothreitol (DTT) in Tris buffer to all and centrifuged for 5 minutes. From the 37 °C samples, we took 200 µl, and from the boiled samples, we took 100 µl for FASP (Filter Aided Sample Preparation). The direct extraction samples were vortexed. The concentrate samples were raised to 5 ml in Tris buffer, and the remaining samples were raised to 10 ml. 5 µg of trypsin was added to all samples and then incubated overnight in the bead bath (37 °C). DTT was added to stabilize the proteins, then followed by centrifugation. The supernatant was frozen and desalted by C18 solid phase extraction.

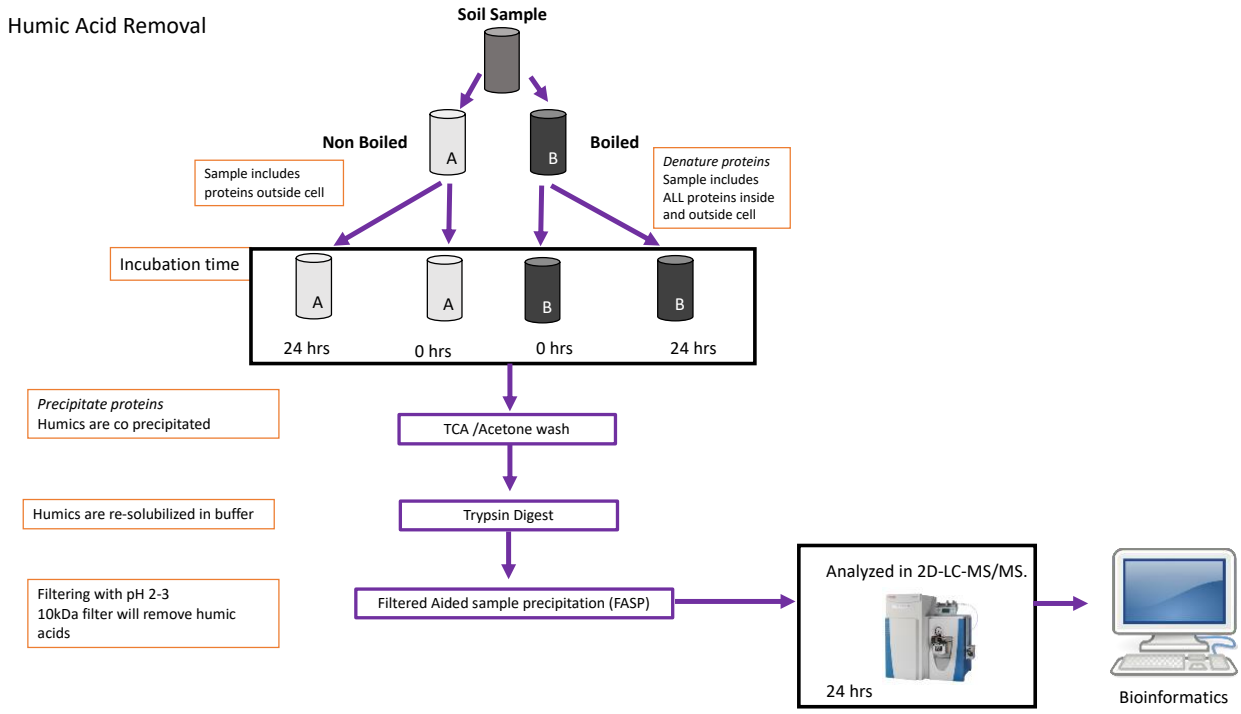


Figure 3.2 Proteomic approach to extract proteins using different treatments for one soil sample.

FASP (Filter Aided Sample Preparation)

The samples set aside for the Filter Aided Sample Preparation (FASP) were unfrozen and continued using the FASP protocol (Chourey et al., 2010; Wiśniewski et al., 2009). This method allows for detergents to be entirely removed and enables unbiased clean sample preparation without adding protease inhibitors. Samples that had high liquid-based digestion (C, D, G, H) did not have iodoacetamide (IAA) treatment, and they are accounted for in the proteome searches. The IAA treatment allows for trypsin to access cleavage sites within the protein. On the trypsin digestion, one tube of Trypsin stock solution was diluted with 2 ml of ammonium bicarbonate to create the working solution. Of this, 200 µl was added to each sample and incubated overnight. The samples were eluted with 100 µl 1% Formic Acid and centrifuged. The filters were removed, and the peptide samples were frozen at -20 °C.

Measurement by 2d-LC-MS/MS

Following extraction, 250 µl of each sample were loaded for HPLC-MS (High-Performance Liquid Chromatography-Mass Spectrometry) analysis onto the Strong Cation Exchange (SCX) back column (Luna SCX, 5 µm particle size, 100 Å pore size, Phenomenex, CA, USA) and washed offline twice for fifteen minutes with 100% aqueous solvent (95% H_2O / 5% acetonitrile / 0.1% formic acid) followed by 100% organic solvent (30% H_2O / 70% acetonitrile / 0.1% formic acid). This procedure reduces residual SDS or other substances that could clog the column. The front HPLC (High-Performance Liquid Chromatography) column was connected to the Q Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) and ran via online 2D-LC-MS/MS for 24 hours.

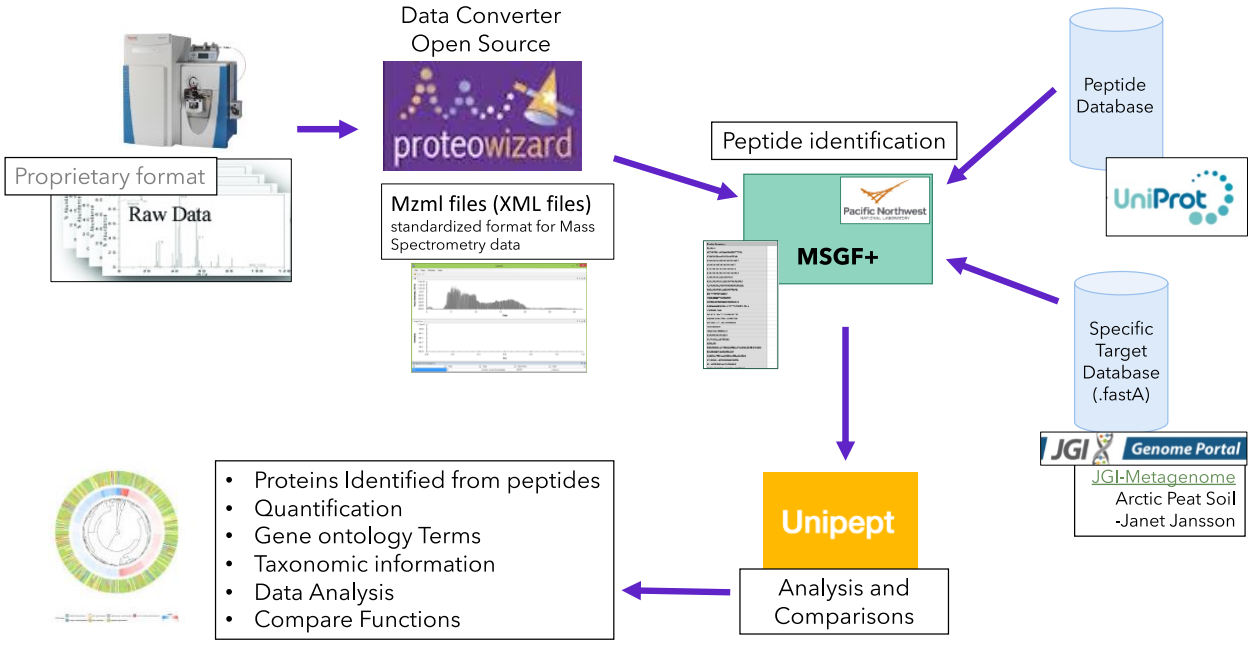


Figure 3.3. Bioinformatics Pipeline. The mass spectrometer gives the data as a raw spectrogram, which is then converted to an open source format using Proteowizard. The data is then loaded in MFGF+ software along with the peptide database (UniProt) and specific target database (JGI (Joint Genome Institute)). The files created in MSGF can then be transferred to Unipept to get the analysis and comparison results, such as peptide identification or the gene ontology (G.O.). This G.O. characterization system shows all the information available about a particular sequence it identified. It separates the information into biological processes, cellular components, and molecular functions. Using the molecular function to look at potential linkage to any enzyme, we performed searches to identify peptides that link back to the soil enzymes.

Proteome Bioinformatics

The bioinformatics pipeline is shown in Figure 3.3. Once the raw data (MS/MS spectra) was obtained from the mass spectrometer, we converted the files to .mzml format using Proteowizard (Adusumilli and Mallick, 2017). By using this formatting software, the files can then be used by various open source programs to identify peptides. One database was downloaded from JGI's Integrated Microbial Genomes server (http://img.jgi.doe.gov). Our database included metagenomic data from arctic soils from the Next Generation Ecosystem Experiment (NGEE) (Jansson, 2012). In addition to that, we also used UniProt's proteome database (Bateman et al., 2021; The Uniprot Consortium, 2019) to be able to identify the peptides and proteins.

Additionally, contaminants such as trypsin and keratin were included in the databases to be removed from the results. We then used MSGF+ software (Kim and Pevzner, 2014), which scores MS/MS spectra against peptides derived from protein sequence databases to identify peptides. Decoy analysis was performed in MSGF+ to estimate the peptide matches better and remove false positives. The false discovery rate (FDR) was also calculated by comparing the number of false positive identifications with the number of true positive identifications. To interpret the data, we used Unipept version 4.0 (https://unipept.ugent.be/datasets) with default parameters to perform protein functional analysis (Mesuere et al., 2015; Verschaffelt et al., 2021).

In Unipept, we searched for the E.C. numbers (Enzyme Commission numbers), the numerical classification for each enzyme assigned by the chemicals they catalyze (Tipton and Boyce, 2000). This nomenclature allowed us to find target enzymes in our proteomic data.

Enzyme Assays

Before the metaproteomic work, we had measured nine potential enzyme activities on these same samples (Table 3.2). The potential enzyme activity assays were performed using high-throughput microplate methods described in Martinez et al. (2021). We measured fluorescence in hydrolytic enzymes after 2 hour incubation (German et al., 2011; McLaren et al., 2017; K.R Saiya-Cork et al., 2002; Sinsabaugh et al., 2003) and performed colorimetric assays for oxidative enzymes (Sinsabaugh, 2010b). After obtaining the proteomics data, we identified several relatively frequent peptides that were potentially organic matter-degrading enzymes in the samples. Fluorescent substrate availability (typically 4-methylumbelliferone) limited us to finding two enzymes from the proteome data: β-galactosidase and arylsulfatase. We performed additional enzyme assays with these to test the hypothesis that high peptide counts could indicate active hydrolases in the soil.

Table 3.2. Enzyme description includes the names, functions, and the assigned E.C. number. The enzymes highlighted in gray are the enzymes added after proteomics.

Enzymes	Type	Function	Acquisition	E.C. Number
β-Glucosidase	Hydrolytic	Releases glucose from cellulose, optimal activity at 85°C	Carbon	3.2.1.21
α-1,4-glucosidase	Hydrolytic	Releases glucose from soluble saccharides	Carbon	3.2.1.20
Cellobiohydrolase	Hydrolytic	Releases disaccharides from cellulose	Carbon	3.2.1.91
Xylosidase	Hydrolytic	Degrades hemicellulose	Carbon	3.2.1.37
N-acetyl-glucosaminidase	Hydrolytic	Degrades chitin	Nitrogen	3.2.1.52
Leucine-amino-peptidase	Hydrolytic	Degrades protein into amino acids	Nitrogen	3.4.11.1

Acid Phosphatase	Hydrolytic	Releases phosphate ions from the phosphate group	Phosphorus	3.1.3.2
Phenol-oxidase	Oxidative	catalyze polyphenol oxidation in the presence of oxygen (O2) by removing phenolic hydrogen	Recalcitrant Carbon & Nitrogen	1.10.3.2
Peroxidase	Oxidative	use H2O2 as an electron acceptor	Recalcitrant Carbon & Nitrogen	1.11.1.7
β-galactosidase	Hydrolytic	cleaves the disaccharide lactose to produce galactose and glucose	Carbon	3.2.1.23
Arylsulfatase	Hydrolytic	Hydrolyses C-O-S ester bonds of organic S forms and releases inorganic SO4	Sulfur	3.1.6.1

STATISTICAL ANALYSIS

We used R (R Core Team, 2022) for all analyses. To assess differences between (a) FASP vs. non-FASP treatments (i.e., temperature and incubation) and (b) fertilized and unfertilized samples, two-sided *t*-tests were performed to determine the statistical significance of the observed difference in protein expression between samples. Though we had only two samples, we analyzed the total peptide counts of the four FASP samples as if they were replicates, as FASP methodology had a significant impact compared to other treatments (boiling and incubation). While this is not a replicated statistical approach with respect to the field-collected samples, the subsamples give at least some indication of variability among metaproteomic runs. The *p*-value was calculated using Student's *t*-distribution with a significance level of $\alpha = 0.05$. Additionally, the correlation between enzyme activity and total peptide counts was analyzed using standard linear regression and the coefficient of determination (r^2).

RESULTS

Comparison of extraction techniques and fertilization treatment

Of the three treatments performed in this study, temperature (boiling and non-boiling) ($p = 0.75$) and incubating (10 minutes and overnight incubation) treatments ($p = 0.84$) did not

significantly differ in the acquisition of proteins. FASP identified 24,132 peptide matches compared to 2,402 without FASP (Table 3.4). The false discovery rate (FDR) was 0.05 of those peptides, 73% were matched with a known peptide, and 27% remained unknown. When we focused on only the enzymes (as opposed to all proteins), we found that 1,152 peptides matched without FASP, while with FASP, there were 11,857 enzymes matched (Table 3.4). Additionally, there was no significant difference (p = 0.79) in total peptides between the fertilized (Sample 1) and the unfertilized sample (Sample 2). The calculated fold change was 0.97 (p = 0.79), indicating strong similarity in peptide abundance in the fertilized samples compared to the unfertilized samples.

Exploring the Proteome: Classifications and abundances of observed enzymes

From the FASP samples, we found that most enzymes are categorized in the transferases (EC 2), hydrolases (EC 3), and oxidoreductase (EC 1) categories (Figure 3.5; Figure 3.6). The oxidoreductases category (EC 1) had a large number (~600) of peptides matched (Figure 3.6), but our oxidative enzymes assayed, peroxidase (EC 1.10.3.2) and phenol-oxidase (EC 1.11.1.7), had a low percentage (< 3.77%) in both fertilized and unfertilized samples. From this category, most of the oxidoreductases were involved in cellular function, such as metabolic and signaling pathways (Supplemental Table 3.2); we compared our results to known microbial biochemical redox reactions (Jelen et al., 2016). We identified many proteins that perform cellular respiration, such as glycolysis, enzymes involved in the citric cycle (TCA), and oxidative phosphorylation. We observed low abundance in oxygenic photosynthesis and methane oxidation EC numbers. We also detected many peptides involved in sulfur assimilation, but fewer for sulfur reduction and oxidation (Supplemental Table 3.2.). Proteins involved in many parts of the nitrogen cycle were

also identified, including those for fixation, assimilation, ammonification, denitrification, and nitrification.

The hydrolases category (EC 3) had a total peptide count of 570 (Figure 3.5), making it the third highest of the seven categories. We identified peptides involved in intra and extracellular function. The highest subclass was acid anhydrides (EC.3.6), with the highest number of identified enzymes being involved in DNA and RNA repair, such as DNA helicase and RNA helicase (Supplemental Table 3.3). The next most abundant subclass was EC 3.1, which includes enzymes that act on ester bonds; the peptide count was 2,447 for the fertilized sample and 3,067 for the unfertilized sample. The least common enzymes in these soil samples were EC 3.9- 3.13, with less than three total peptide counts in both samples, these enzymes are involved in phosphorus-nitrogen bonds (EC 3.9), sulfur-nitrogen bonds (EC 3.10), carbon-phosphorus bonds (EC 3.11), sulfur-sulfur bonds (EC 3.11), and carbon-sulfur bonds (EC 3.13).

Table 3.3 Means on all samples showing the difference between FASP and no-FASP.

	Total peptides count	Matched Peptides	Matched Enzymes
FASP	*24132*	*17613*	*11857*
No FASP	*2402*	*1721*	*1152*
Total% Matched peptides	73%		

Exploring the Proteome: Total peptide counts of enzymes involved in decomposition.

Out of the peptides detected in our samples, we identified commonly studied enzymes: β-glucosidase, α-1,4-glucosidase, cellobiohydrolase, xylosidase, leucine aminopeptidase, N-acetyl-glucosaminidase, and acid phosphatase (Table 3.5). The most abundant soil enzyme detected was β-glucosidase in both fertilized (mean number of peptides = 43.5 ± 2.60) and unfertilized samples (39.75 ± 3.99). Out of the assayed enzymes, cellobiohydrolase had the lowest peptide counts in the fertilized (mean = 1.5 ± 0.29) and unfertilized sample (mean = 1.75 ± 0.75) (Table 3.5).

Table 3.4. Total peptide counts are shown for each sample, and FASP samples are shown in gray (FDR=0.05).

Sample	Treatments	Matched Peptides (Total Peptide Counts)
Fertilized	Boiling/ 10 minutes	1897
Unfertilized	Boiling/ 10 minutes	2607
Fertilized	37 C/ 10 minutes	1910
Unfertilized	37 C/10 minutes	1957
Fertilized	Boiling/overnight in the dark	1796
Unfertilized	Boiling/overnight in the dark	1465
Fertilized	37C/overnight in the dark	540
Unfertilized	37C/overnight in the dark	1592
Fertilized	Boiling/ 10 minutes /FASP	18156
Unfertilized	Boiling/ 10 minutes /FASP	18892
Fertilized	37C/ 10 minutes /FASP	17916
Unfertilized	37C/ 10 minutes /FASP	14496
Fertilized	Boiling/overnight in the dark/FASP	20202
Unfertilized	Boiling/overnight in the dark/FASP	17782
Fertilized	37C/overnight in the dark/FASP	20729
Unfertilized	37C/overnight in the dark/FASP	12728

We identified no taxonomically significant difference between fertilized and unfertilized samples ($p = 0.99$) for the enzymes involved in decomposition (Figure 3.4). Not all of the matched enzymes were associated with an organism. In the β-glucosidase sample, the database was able to detect four bacterial organisms in the unfertilized sample, while in the fertilized sample, we identified four distinct bacterial organisms and one archaean. Furthermore, N-acetyl-glucosaminidase had one unspecified eukaryote organism and three bacteria in the fertilized sample, while we only identified one bacterial domain in the unfertilized sample (Supplemental Table 3.1).

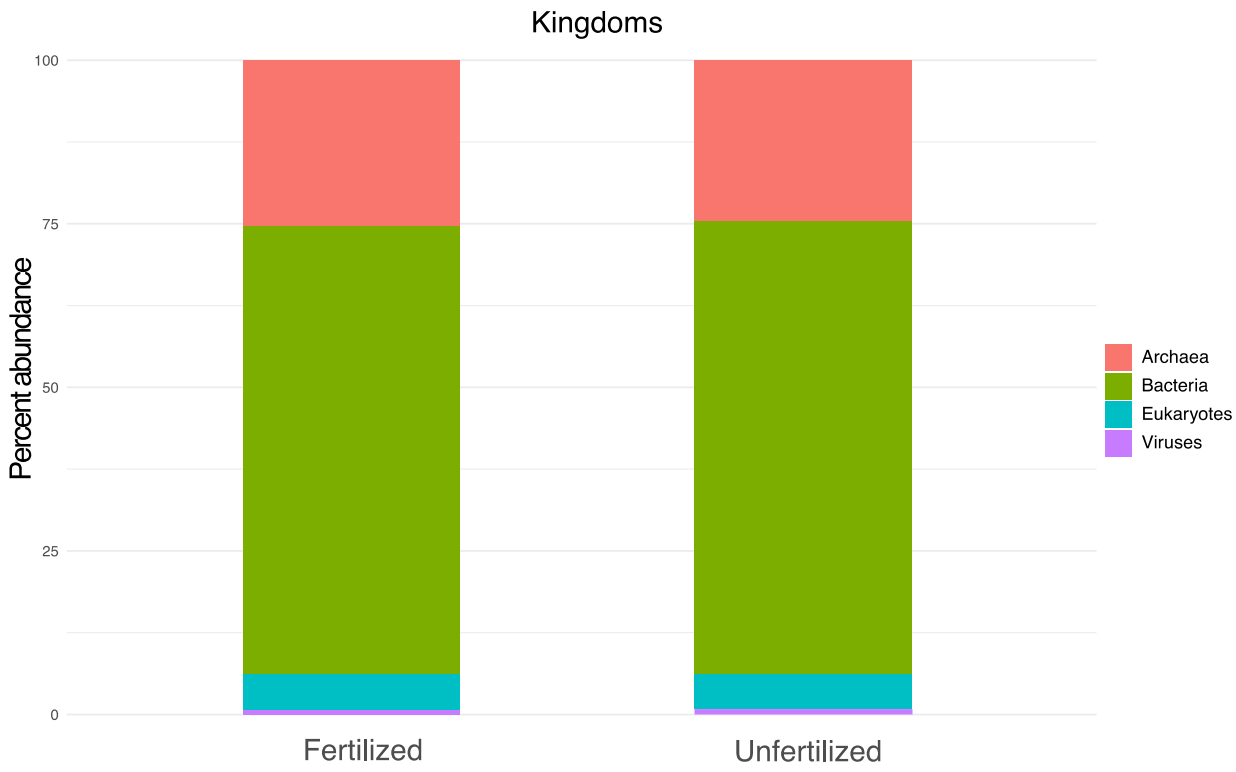


Figure 3.4. Taxonomic annotation at the kingdom level.

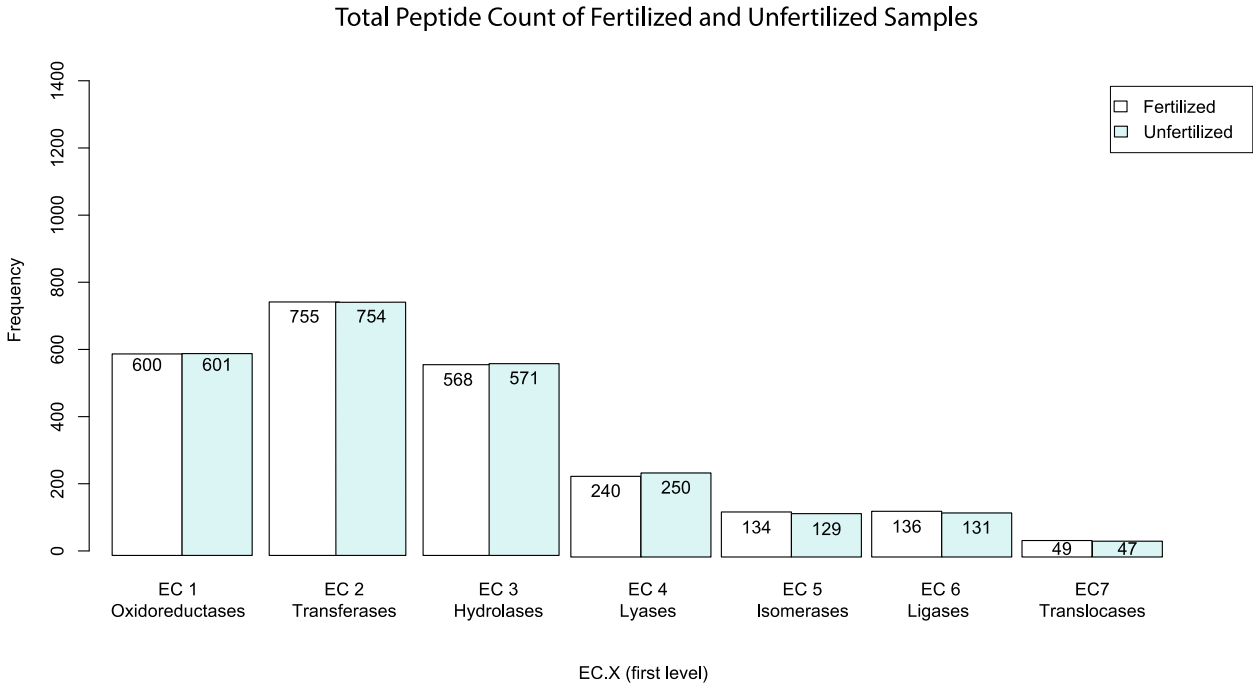
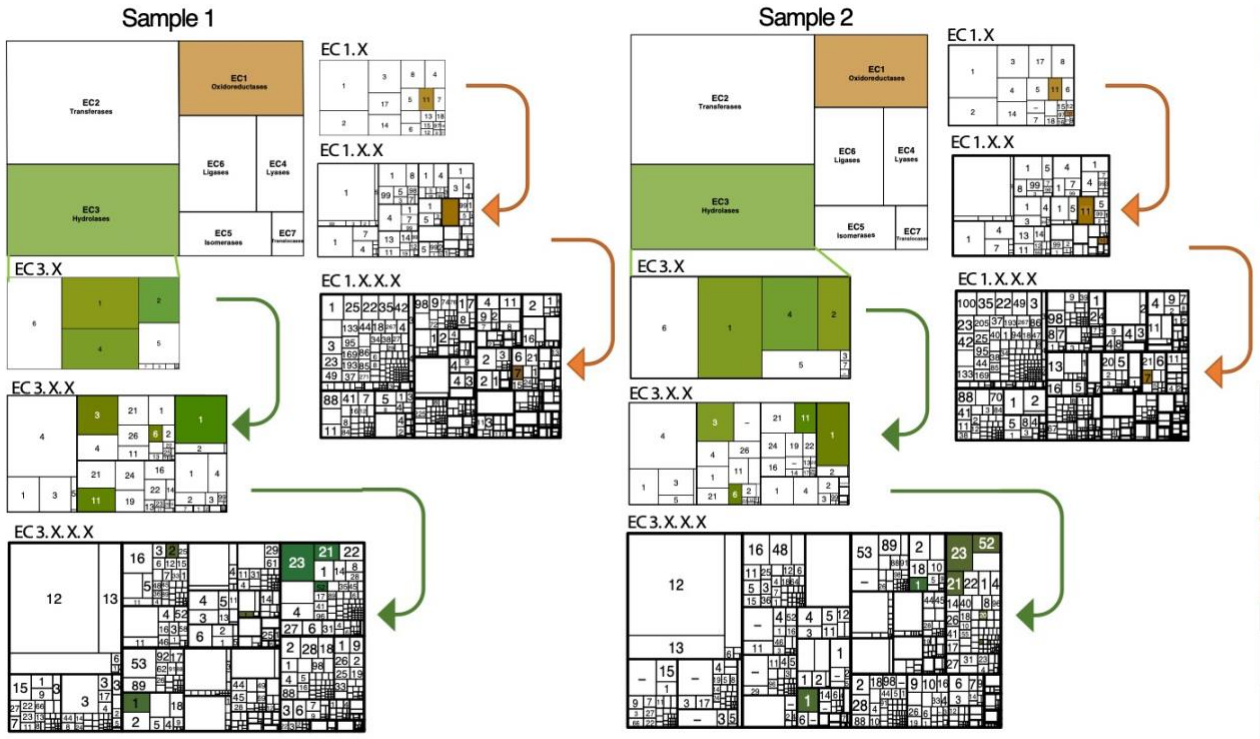


Figure 3.5. Frequency of peptides per sample for each EC category.



Hydrolytic Enzymes for Assays	EC Number
β-Glucosidase	3.2.1.21
α-Glucosidase	3.2.1.20
Cellobiohydrolase	3.2.1.91
Xylosidase	3.2.1.37
N-acetyl-glucosaminidase	3.2.1.52
Leucine-amino-peptidase	3.4.11.1
Phosphatase	3.1.3.2
Galactosidase	3.2.1.23
Arylsulfatase	3.1.6.1
Other Hydrolytic Enzymes	**EC Number**
DNA helicase	3.6.4.12
RNA helicase	3.6.4.13
Deoxyribonuclease	3.1.21.3
Oxidoreductase Enzymes	**EC Number**
Phenol-oxidase	1.10.3.2
Peroxidase	1.11.1.7
Other Oxidoreductase Enzymes	**EC Number**
Ribonucleoside-diphosphate reductase	1.17.4.1
Oxoglutarate dehydrogenase (succinyl-transferring)	1.2.4.2
Pyruvate dehydrogenase (acetyl-transferring)	1.2.4.1

Figure 3.6. Summary of total peptide counts by EC number. Sample 1 (fertilized) and 2 (unfertilized) are categorized by the Enzyme Commission (EC) numbers. Hydrolases are classified as EC 3 (green), and oxidoreductases are EC 1 (orange). All subclasses (EC1.X, EC3.X), sub-sub classes (EC1.X.X, EC3.X.X), and enzymes (EC1.X.X.X, EC3.X.X.X) are shown separately

Table 3.5. Total peptide counts for enzymes in fertilized (S1) and unfertilized (S2) samples with FASP.

Fertilized	*Treatment description*	*β-glucosidase (3.2.1.21)*	*α-1,4-glucosidase (3.2.1.20)*	Xylosidase *(3.2.1.37)*	Cellobiohydrolase *(3.2.1.91)*	β-1,4-N-Acetyl-glucosaminidase *(3.2.1.52)*	Acid Phosphatase *(3.1.3.2)*	Leucine-aminopeptidase *(3.4.11.1)*	*Phenol oxidase (1.10.3.2)*	*Peroxidase (1.11.1.7)*
A	S1/Boil/10minutes Incubation	42	10	6	1	37	15	43	10	17
E (A*)	S1/Boil/Overnight Incubation	39	16	6	2	43	26	50	5	20
C	S1/37C/10minutes Incubation	51	10	13	1	39	25	41	7	22
G (C*)	S1/37C/Overnight Incubation	42	10	8	2	26	21	34	3	15
	Means	43.5	11.5	8.25	1.5	36.25	21.75	42	6.25	18.5
	Standard Error	2.598076211	1.5	1.652018967	0.288675135	3.63719214	2.495829855	3.291402943	1.493039406	1.55456318
Unfertilized	*Treatment description*	*β-glucosidase (3.2.1.21)*	*α-1,4-glucosidase (3.2.1.20)*	Xylosidase *(3.2.1.37)*	Cellobiohydrolase *(3.2.1.91)*	β-1,4-N-Acetyl-glucosaminidase *(3.2.1.52)*	Acid Phosphatase *(3.1.3.2)*	Leucine-aminopeptidase *(3.4.11.1)*	*Phenol oxidase (1.10.3.2)*	*Peroxidase (1.11.1.7)*
B	S2/Boil/10minutes Incubation	47	9	8	3	42	18	37	5	13
F (B*)	S2/Boil/Overnight Incubation	35	5	12	0	41	18	34	2	17
D	S2/37C/10minutes Incubation	46	11	5	3	43	18	47	8	19
H (D*)	S2/37C/Overnight Incubation	31	6	10	1	27	21	26	1	17
	Means	39.75	7.75	8.75	1.75	38.25	18.75	36	4	16.5
	Standard Error	3.986957905	1.376892637	1.493039406	0.75	3.772156766	0.75	4.339738855	1.58113883	1.25830574

Comparison between peptide counts in proteome with potential enzyme activity assays

Upon obtaining a peptide list through the initial proteomic analysis of our samples, we selected two additional enzymes, β-galactosidase, and arylsulfatase, with high peptide counts to examine whether they likewise would show high enzyme activity assays. β-galactosidase exhibited elevated levels of both enzyme activity and peptide counts, whereas arylsulfatase showed high enzyme activity levels when compared to most commonly-assayed enzymes, except for acid phosphatase. The correlation analysis between enzyme activity and total peptide counts showed a weak positive correlation between the two variables ($r = 0.43$, $p = 0.25$), indicating that only 18.4% of the variation in total peptide counts could be explained by the variation in enzyme activity. The scatter plot (Figure 3.7) illustrates the weak relationship between the variables. In particular, the figure shows the two highest potential enzyme activities, β-galactosidase, and acid phosphatase, outliers in the regression, with high peptide counts for β-galactosidase and low peptide counts for acid phosphatase.

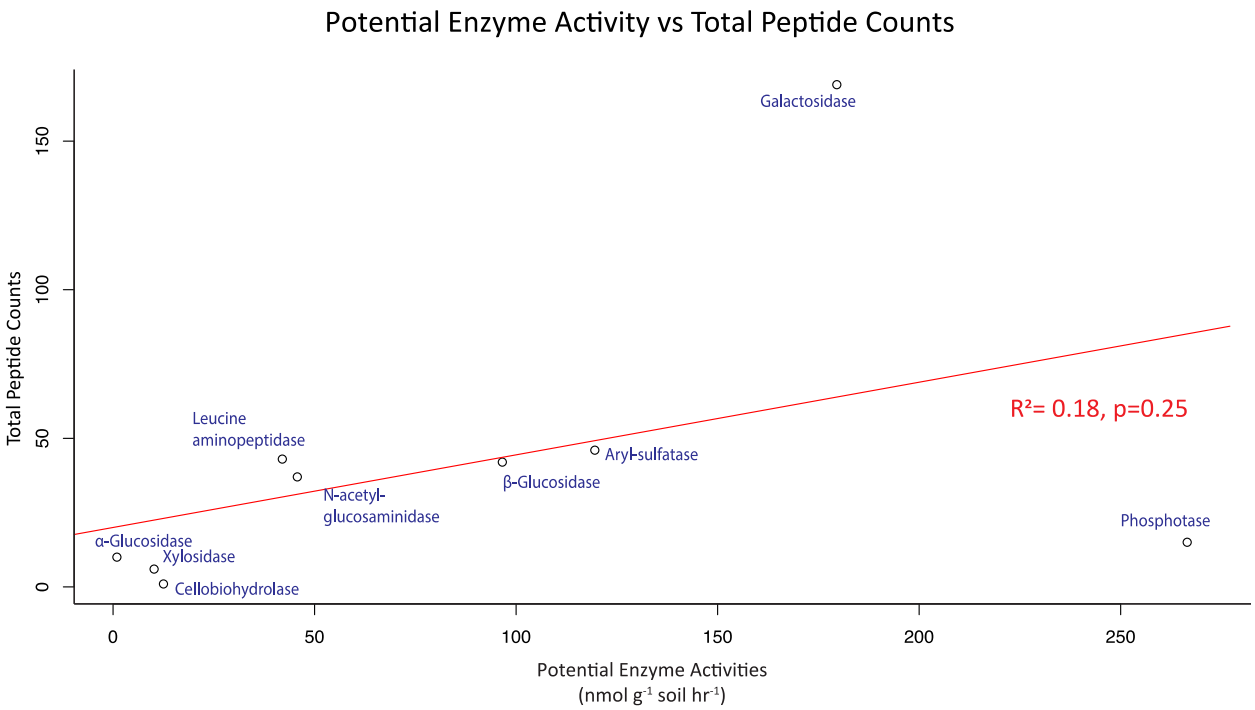


Figure 3.7. Relationship between hydrolase enzyme activity and total peptide counts. There is no observable correlation between the two variables (r^2 = 0.18, p = 0.25), with only 18.4% of the variation.

DISCUSSION

We aimed to optimize the protein extraction method for metaproteomic analysis on fertilized and unfertilized arctic soil samples with a variety of methodological variations to investigate the protein profiles. Using FASP clearly yielded higher peptide counts and was thus the most important component to the sample preparation. Using these samples that were prepared with FASP, we explored the proteome by classifying the significant groups of enzymes and further investigating soil enzyme decomposition. Our results suggested that there was not a significant difference between our fertilized and unfertilized samples. Lastly, we found no evidence of a correlation when we compared the enzyme activities to the total peptide counts, however, there seems to be an observable effect. We will discuss why there was no difference in fertilized and unfertilized samples, why FASP is worth using as an improved methodology, what the major group

classifications of enzymes are, and why they are important. Overall, we will focus on identifying enzymes and providing a further understanding of the proteome in these soils.

Protein Extraction

We implemented several procedural changes to improve the removal of high molecular weight compounds, such as humic acids, that interfere with proteomic analysis in the soil. Our results show that by using FASP, we can get a much higher number of peptide matches, suggesting that with this processing step, protein detection is possibly less hindered by the interferents, such as humic acids in the soil. Incidentally, other treatments were attempted initially but were unsuccessful in preparing the protein extraction, such as bead beating, temperature changes, and various incubation times. Our findings demonstrated that we could get a high peptide count (24,132 total peptide counts). Similarly, a study used topsoil to enhance protein extraction by attempting to remove humic interferences, identifying approximately 16,000 peptides in microbial spiked samples. This study closely resembles ours by using a 10 kDa filter similar to FASP (Qian and Hettich, 2017). On the other hand, a study carried out in a dense Amazon forest that did not use a 10 kDa filter to eliminate soil interferences reported their maximum matched proteins to be 336 (Trindade et al., 2021), while our study was able to detect 17,613. Thus, using FASP or other filtration techniques can efficiently extract protein. Additionally, a study of prairie soil metaproteome aiming to improve the collection of the proteins present in the soil microbial community was able to identify less than 8,000 peptides identified by using offline 2D LC-MS/MS and approximately 3000 peptides with online 2D LC-MS/MS (Callister et al., 2018). In our study we used online 2D LC-MS/MS because it allows for continuous and automated separation an analysis of the samples, if we took the time to do an offline 2D LC-MS/MS we would most likely yield a higher peptide count. Nevertheless, offline 2D LC-MS/MS requires manual steps which could also lead to potential errors.

A study that attempted the removal of humics from moa cortical bones recovered from a New Zealand cave used FASP to prepare their sample for proteomic analysis and found that by using this filtration, they could physically see the sample go from brown to light brown (almost colorless) hue. Interestingly, they were able to remove most of the humics, but because the sample was not completely colorless, the investigators concluded that humics were not completely removed from the sample (Schroeter et al., 2019). Similarly, our study observed that the pre-FASP samples appeared in a dark brown color, whereas the post-FASP samples were colorless. This observation supports our above suggestion that interference from humic substances was reduced with FASP, thus improving the yield of identifiable peptides. Future research should implement this methodology to get a highly identifiable peptide count and further our understanding of the soil proteome.

Fertilized vs. unfertilized comparisons

Our analysis did not reveal a significant contrast between the fertilized and unfertilized samples (see. Fig 5). Although we only had two samples, we expected an enzyme difference between the fertilized and unfertilized soil. Numerous studies show the effects of nutrient availability on carbon-degrading enzyme activities and found that nutrient addition affects enzyme activities, resulting in increased enzyme activities related to carbon degradation (Allison and Vitousek, 2005; Dong et al., 2015; Koyama et al., 2013; Xiao et al., 2018). The implication is that alterations in the availability of nutrients may have significant effects on the function of ecosystems and the cycling of carbon in arctic tundra soils. However, one possible explanation for the lack of difference observed in the samples could be attributed to the duration since the plot was fertilized, and the fact that the fertilization site was abandoned. In a study that investigated subarctic heath, they investigated the long-term effects of a 28-year-old fertilization site, where

they did not see a significant difference in the activity of microbial activity (Hicks et al., 2020). This supports our claim that it is unlikely to be a discernible difference in the soils after more than 50 years without consistent fertilization.

Enzyme abundance in soils

Our results on taxonomic classification of the organisms from the metagenome to which the proteins matched suggest that bacteria produced most of the enzymes found in our proteome, followed by archaea, eukaryotes, and lastly, viruses. A metagenomic–metatranscriptomics study from arctic soil systems suggested that bacteria were 70 - 80% abundant in the active layer (Tveit et al., 2013). Conversely, in an Austrian forest, it was determined that fungi were the leading producers of extracellular hydrolytic enzymes, with no bacterial hydrolases detected by their metaproteomics approach (Schneider et al., 2012). This could be because, unlike bacteria, fungi can form extensive networks of hyphae that allow them to efficiently scavenge for nutrients and interact with other soil organisms, which could be challenging for fungi in permafrost soils. Besides bacteria and eukaryotes, our results showed a high abundance of archaea associated with our peptides. Our findings align with a high arctic tundra study investigating the diversity and prevalence of bacterial, archaeal, and fungal communities. The study revealed that fungi are less abundant than bacteria and archaea (Blaud et al., 2015). Also, we have to consider that sampling was done in two soil samples, which may misrepresent the fungal abundance.

Moreover, our proteome analysis showed that most of the oxidoreductases were involved in cellular function, such as metabolic and signaling pathways. According to a study where metagenomic datasets were collected from diverse microbial communities, they concluded that one of the major ways to classify the functional diversity of soil microbial communities is by analyzing their oxidoreductase profiles (Ramírez-Flandes et al., 2019). Our results are consistent

with this study because oxidoreductases from our soil proteome had high total peptides and pathways it was linked to aerobic cellular respiration. We also saw some evidence of enzymes associated with denitrification and methanogenesis, which require less oxic conditions. We saw no manganese (EC 1.10.3.9) that are part of oxygenic photosynthesis (Supplemental Table 3.2). In our hydrolase examination of the proteome, we found the subclasses EC 3.6 and EC 3.1 to have more peptides associated with them. Similar to the oxidoreductase enzymes, the enzymes in these categories are mostly related to intracellular function, except the hydrolases, which are involved in the breakdown of lipid or nucleic acids (Sacher et al., 2009). Most decomposition enzymes that were part of our suite of assayed enzymes (Table 3.5) are in the subclass EC 3.2. Thus, we can suggest that most of our soil proteome is associated with cellular function.

Identification of decomposition enzymes

We focused on soil decomposition enzymes to further understand the microbial communities involved in the breakdown of organic matter in soil in the Arctic ecosystem. A study that used sequenced prokaryotic genomes to identify taxa with the genetic potential to produce soil enzymes determined that nearly 50% of microorganisms were capable of producing soil enzymes, such as alkaline phosphatases, chitinases, and β-N-acetyl-glucosaminidase (Zimmerman et al., 2013). This suggests that soil microbial communities are likely to be rich in extracellular enzymes and that the breakdown of organic matter in soil is likely to be mediated by a diverse array of enzymes produced by many different microbial taxa.

In our research, we used the EC numbers of the decomposition enzymes commonly assayed in microbial ecology to guide our proteome investigation. Our findings indicated that the proportion of these typical decomposition enzymes was less than 1.54% of total proteins (Table 3.5). This is not unexpected, considering the substantial portion of the genome allocated to

enzymes needed for basic cellular function. However, few studies have attempted to perform this quantification of decomposer enzymes, offering a glimpse into the level of investment that microbes allocate to produce these enzymes (Keiblinger et al., 2012; Schneider et al., 2012). A forest soil rich in humics and clay was spiked with enzymes to determine the protein recovery, where they found lower extracellular recovery compared to the intra-cellular enzymes (Keiblinger et al., 2012). In fact, numerous extracellular enzymes contribute to the decomposition process, and many of these enzymes have not been fully characterized.

Our results showed β-glucosidase to have high peptide counts compared to the initial seven hydrolytic enzymes we commonly assess, whereas cellobiohydrolase had the lowest peptides in the proteome. One possible explanation is that β-glucosidase is more broadly involved in degrading a wider range of carbohydrates than cellobiohydrolase, including the breakdown of cellobiose, a product of cellobiohydrolase activity (Deflandre et al., 2022; Saritha Mohanram, 2015). As such, β-glucosidase may play a more important role in the overall degradation of plant biomass in arctic soils, even in the absence of high levels of cellobiohydrolase.

Post-proteomic soil enzyme assays

The enzyme with the highest peptide count and potential enzyme activity was β-galactosidase, which we identified for assay after the proteomic analysis. The high relative abundance (at least qualitatively via peptide counts) and activity may be due to arctic soils possibly having a high number of microbes that participate in the breakdown of complex carbohydrates such as melibiose and lactose (Eivazi and Tabatabai, 1988). Moreover, microbes in arctic soils have adapted to cold temperatures by producing enzymes that function optimally at low temperatures. β-galactosidase is one such enzyme known to be active at low temperatures (Park et al., 2006), which may contribute to its abundance in arctic soils. Generally, the abundance of β-

galactosidase in arctic soils is likely a result of a combination of factors, including its role in cycling organic matter and adaptation to cold temperatures. In an agricultural research site in Oregon, β-glucosidase was found to have higher enzyme activities than β-galactosidase (Bandick and Dick, 1999). In agricultural sites, there is a greater abundance of plant biomass, and β-glucosidase may be more abundant to facilitate the decomposition of cellulose.

Our second enzyme in the post-proteomic analysis was aryl sulfatase. This enzyme is important in sulfur acquisition (S. Wang et al., 2019) and is less often measured in typical suites of soil enzymes compared to carbon and nitrogen-acquiring enzymes (Chen et al., 2019). According to our findings, this enzyme had a count of 47 peptides, ranking second to β-galactosidase. The prevalence of sulfate esters in these environments may contribute to high aryl sulfatase activity in arctic soils. Sulfate esters are commonly found in plant and animal tissues, and their breakdown is a key component of the sulfur cycle in soils (Fitzgerald, 1976). A study suggested that in arctic ecosystems, sulfate esters are prevalent in soils that have not experienced any vehicle track disruptions (Neal and Herbein, 1983); they also found a higher amount of sulfatase activity in areas with high organic matter, which coincides with our study. The sulfate esters can provide a rich substrate for aryl sulfatase to act upon, which can further stimulate its activity.

Our findings indicated a weak correlation between peptide counts and enzyme activity when we compared our enzyme assay to the total peptides. It is possible that the outliers, namely phosphatase and β-galactosidase, may have contributed to this outcome. A potential reason why this could be happening is the substrate incubation times of these two enzymes could be different than the other enzyme measured in this set of samples and further investigation of this could strengthen this comparison (Deng et al., 2011; Nannipieri et al., 2011). A metaproteomics study

of forest sites linked enzyme activities and proteomic protein profiles of xylanases and cellulases (hydrolytic enzymes) found faster litter decomposition in sites with high abundances of soil enzymes (Schneider et al., 2012). This implies that the amount of enzyme activity limits the decomposition process, and there could possibly be a link between the activity and the abundance of enzymes in the soil. A higher number of assayed enzymes would potentially make the correlation of this study stronger. It would be worthwhile to investigate these associations in additional ecosystems and with a wide range of enzyme substrates for future studies (Sinsabaugh et al., 2008).

CONCLUSIONS

Efficient protein extraction for arctic soil metaproteomic study was observed in the FASP (Filter-Aided Sample Preparation) samples, and thus we recommend this for future proteomic analyses. The comparison between fertilized and unfertilized soils did not yield any major differences, which was likely due to the long time period since fertilization. The identified proteomes were consistent with expected metabolic processes such as aerobic respiration, denitrification, and methanogenesis. Additionally, an interesting correlation between enzyme abundance and activity was observed, highlighting the need for further investigation. Finally, we suggest future work should perform metagenomics and metaproteomics simultaneously to yield a higher number of peptide matches and more robust data analysis to identify more enzyme producers and include soils from other ecosystems to improve our understanding of soil enzymes in highly complex soils.

Chapter 4: Assessing the possible lifespan of soil enzymes in arctic soil subsequent to the use of chloroform fumigation and sonication for microbial activity removal.

ABSTRACT

Soil microbes act as decomposers of soil organic matter (SOM) due to their production of enzymes. In arctic tundra ecosystems, SOM decomposition tends to be slow because the cold temperatures limit microbial activity and enzymatic reactions. Over the past few decades, global temperatures have risen rapidly, contributing to the acceleration of decomposition, with likely impacts on soil enzyme activities. One of the aspects of enzyme function that is less understood is their longevity in the soil, which matters for microbial return on investment for enzyme production. Evidence suggests that enzymes survive after cell death, but the time enzymes are active is poorly understood. The first objective of this study was to inhibit microbial activity by preventing new soil enzyme production using chloroform fumigation. Second, we wanted to determine the lifespan of soil enzymes in the Arctic by measuring the enzyme activities throughout a 12-week period. For our third objective, we investigated the impact of sonication on microbial activities using microscopy to detect cell death. Our results indicate that chloroform lyses approximately 75% of the microorganisms but does not entirely eradicate them. During the entire 12-week observation period, we observed maintenance of potential soil enzyme activity, with no significant decrease in enzyme activities, except for leucine aminopeptidase, which exhibited a decline after week 4. Furthermore, the findings from the microscopy analysis indicate that sonication does not cause additional cellular death. We conclude that enzyme production could still be happening in the chloroform-resisting microorganisms, which could be why there is no significant decline in enzyme activities. Additionally, we suspect the resilience of enzymes and microbes in coastal tundra soil enables enzymes to endure extended periods even without microbial cells. These results

highlight the importance of continued research on soil enzyme longevity, particularly to improve our understanding of the function and stability of enzymes in these ecosystems.

INTRODUCTION

Microbes, such as bacteria and fungi, are the main decomposers of soil organic matter (SOM) due to their production of hydrolytic and oxidative enzymes involved in catabolism (Burns, 1982). Due to northern Alaska's extreme, often below-freezing climate, microbial activity and enzyme reactions are limited, leading to slow soil decomposition (Chapin et al., 1995; Mack et al., 2004; Wallenstein et al., 2009). During the last few decades, the temperature has increased, which is expected to accelerate decomposition, likely in part via effects on soil enzyme activities (Nowinski et al., 2008; Sistla and Schimel, 2013). In addition, the relationship between decomposition and enzyme production is complex and can depend on various environmental factors, such as soil temperature, moisture, pH, and nutrient availability, as well as the types of microorganisms involved (Allison and Vitousek, 2005).

Enzyme longevity, that is, the length of time an enzyme remains active in the soil, is important in enzyme-driven models (Allison, 2005); therefore, understanding the functional lifespan of enzymes is crucial for understanding decomposition. The size of an enzyme pool is critical because it affects the efficacy of enzymatic reactions, the rate of carbon, and nutrient cycling (Arnosti et al., 2014). However, the abundance of functional enzymes within the enzymatic pool at any given time is poorly understood, largely because our understanding of enzyme longevity and production rate is lacking (Schimel et al., 2017). There is evidence that enzymes survive after cell death (Skujiņscaron; and Burns, 1976), but the total amount of time enzymes are active is still not completely understood. The lifespan of enzymes could be influenced by substrate availability, temperature, and soil moisture. By estimating the lifespan of the enzymes, we can

better understand the impacts of environmental change on ecosystem function and develop strategies to mitigate or adapt to these changes.

In arctic ecosystems, it is especially important to understand the longevity of soil enzymes given the severe temperature fluctuations from freezing winters to cold summers (Kane et al., 1991). Soils from higher latitudes associated with permafrost might have different resistance rates compared to enzymes that go through different fluctuations, such as desert soils. Soil enzyme production is costly (Schimel and Weintraub, 2003; Sinsabaugh and Moorhead, 1994), and identifying which soil enzymes persist after cellular death can give us an estimate of the longevity of soil enzymes.

Moreover, the investigation of microbial viability following physical disruption is an area of research that has not been fully explored in the laboratory setting. Very few studies go in depth to look at bacteria viability after physical disruption methods like blending, sonication, or chemical treatment (Kaiser and Asefaw Berhe, 2014; Lindahl and Bakken, 1995). Sonication is a physical disruption technique that extracts proteins, DNA, or RNA from cells (De Cesare et al., 2000b; Fykse et al., 2003; Shrestha et al., 2012). It uses high-frequency sound waves to disrupt cellular membranes (Kaiser and Asefaw Berhe, 2014; Suslick, 1990). Two commonly used methods of sonication are probe sonication and bath sonication. Probe sonication involves inserting a small probe into the cell suspension and vibrating it at a high frequency, while bath sonication involves placing the cell suspension in a sonication fluid-filled bath and vibrating the fluid to produce sound waves (Capelo et al., 2004). Bath sonication is ideal for larger samples, while probe sonication is more target specific with higher energy (Mason, 1999). The extent to which sonication disrupts viable cells remains uncertain, where studies suggest that sonication could lyse cell membranes causing the DNA (Deoxyribonucleic acid) to be released (Fykse et al., 2003; Zhang et al., 2007).

Furthermore, chemical treatments, such as chloroform ($CHCl_3$) fumigation, are also used to disrupt and lyse bacteria (Inubushi et al., 1991; Witt et al., 2000). A study in Mediterranean-type soils looked at the separation of cellular metabolism from soil enzyme activity (Blankinship et al., 2014), using several methods to lyse microorganisms and keep enzymes from breaking down. From the methods tested, chloroform fumigation had low biochemical disruption and high cellular disruption.

This study aims to determine the amount of time a soil enzyme persists in the soil without further microbial production. Our previous work (Martinez et al., 2021) showed that applying sonication and blending would increase potential enzyme activity, potentially maximizing the amount of enzyme activity we see in each sample. In this study, our objectives were 1) to induce microbial cell death while preserving the integrity of the enzymes when fumigating the samples, 2) to examine the decay time for arctic soil enzymes, and 3) to determine if sonication and/or chloroform causes microbial cellular damage by using fluorescent microscopy to visualize microorganisms. We hypothesize that most soil enzymes will decay at a similar time scale and that sonication will not have an effect on cellular death.

MATERIALS AND METHODS

Research Site and sampling

Soil samples for this research were collected from Utqiaġvik, Alaska (71°16′48.26″N; 156°36′33.12″W). The collection site is in a coastal ice wedge tundra ecosystem on the North Slope of Alaska. Utqiaġvik is susceptible to the effects of climate change, and its upper soil horizon is vulnerable to thawing (Irrgang et al., 2022; Painter et al., 2023). It has had a recent community composition shift from dominant deciduous shrubs to aquatic mosses and forbs (Jorgenson et al.,

2022; Moon et al., 2021). The mean annual temperature is -12 °C with a mean annual precipitation of 114.3 mm (Dengel et al., 2021).

Soil cores were collected in and around the f International Tundra Experiment (ITEX) and the Barrow Environmental Observatory (BEO). For this research, we used 12 soil core samples (Table 4.1), which were approximately 5 cm in diameter and 15 cm in depth. We sampled disturbed and undisturbed areas to represent a wide range of coastal tundra. The samples were stored in -20 °C after collection.

Table 4.1. Description of soil sample collection. Highlighted in gray are the chloroformed treated samples.

Soil Sample	Description	GPS Coordinates	
S1	Moist graminoid tundra	71.31447	-156.58342
S2	Dry-moist dwarf shrub graminoid tundra	71.31493	-156.58475
S3	Dry-moist dwarf shrub graminoid tundra	71.31498	-156.58453
S4	Moist graminoid tundra	71.31439	-156.58444
S5	Dry-moist dwarf shrub graminoid tundra	71.31461	-156.58487
S6	Dry-moist graminoid tundra	71.3146	-156.58426
S7	Dry-moist dwarf shrub graminoid tundra	71.31509	-156.5851
S8	Dry-moist graminoid tundra	71.3148	-156.58408
S9	Dry-moist graminoid tundra	71.31435	-156.58455
S10	Wet graminoid tundra	71.31403	-156.58926
S11	Moist graminoid tundra	71.3145	-156.58406
S12	Dry-moist dwarf shrub graminoid tundra	71.31501	-156.58504

Microbial death

Using the standard chloroform fumigation technique to determine microbial biomass, we used chloroform ($CHCl_3$) to kill microbial organisms (Brookes, 1985). This technique disrupts cellular activities while leaving biochemical processes intact (Blankinship et al., 2014). Half of the 12 soil core samples (S7- S12) were used with $CHCl_3$, while the remaining six (S1- S6) did not undergo

this process (Table 4.1). To begin the fumigation treatment, soil samples S7- S12 (~5 g each) were exposed to $CHCl_3$ continuously for two weeks.

To assess the $CHCl_3$ fumigation's effects, we measured the enzyme activities of all samples pre-$CHCl_3$ fumigation and after the samples were fumigated for 24 hours. All soil samples were incubated at room temperature in the fume hood in Erlenmeyer flasks sealed after ensuring the rubber stopper would not pop off within 24 hours. After the first two weeks, we checked cell viability and ran an enzyme assay on all samples. Every week, the lid was opened to check the beakers with chloroform. Subsamples were then taken for soil enzyme assays weekly and frozen to prevent any enzyme decay if not run immediately after collection.

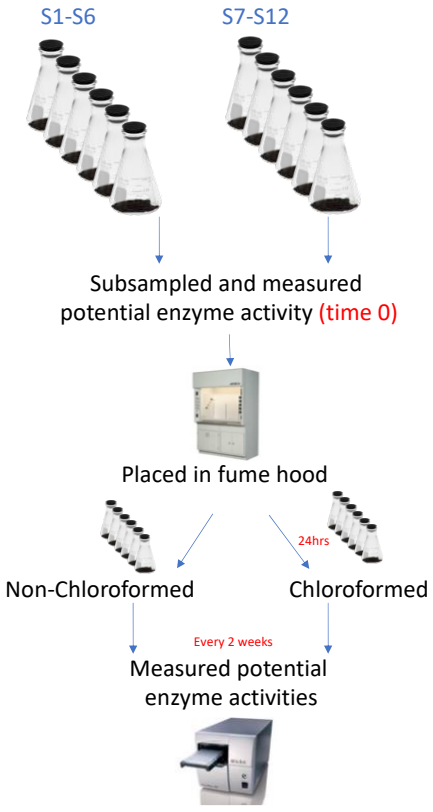


Figure 4.1 Measuring potential microbial cell death by chloroform fumigation.

Confocal Microscopy

A cell viability assay was performed to monitor microbial sterilization efficacy, confirming that living cells had been eliminated from the soil. This assay allows observation and quantification of live vs. dead cells under a fluorescent microscope (LIVE/DEAD BacLight bacterial viability kit; Life Technologies, Inc., Grand Island, NY). Live cells are stained with SYTO™ 9, which fluoresces green, while dead or dying cells are stained with propidium iodide, which fluoresces red. Inverted confocal-laser-scanning microscopy (model LSM 700; Zeiss, Thornwood, NY) was used to capture high-resolution digital fluorescent images with 63× immersion-oil DIC objective and assisted with ZEN software (Zeiss, Thornwood, NY).

Sonication

Following the procedure from Martinez et al., 2021, sonication was used with the enzyme assay with a VWR Ultrasonic bath (VWR Ultrasonic Cleaner, Radnor, PA) at 20-30 kHz low amplitude (6 W ml^{-1}). The samples were placed in the bath for 5 minutes and transferred to the 96-well plate for fluorometric analysis. Immediately after sonication, we used the LIVE/DEAD cell kit to observe the cells and check for additional cellular death. The microscopy component of this technique allowed us to examine and determine the disruption of living cells via sonication waves.

Soil enzyme activity assays: Hydrolytic and Oxidative

The potential activities of nine enzymes were measured before and after sterilization every two weeks, seven hydrolytic and two oxidative (Chapter 2, Table 2.2). Hydrolytic enzymes were measured using the standard soil activity assay (Marx et al., 2001; K. R. Saiya-Cork et al., 2002). Using 1 g of soil per sample, we prepared a soil slurry to blend the samples with 125 ml of 50 µM sodium acetate buffer adjusted to a pH of 5, blending for 1 minute. Samples were transferred into a container where they remained, constantly mixing with a small magnetic plate. The sample was

then transferred to the 96 microwell plate. 7-amino-4-methylcoumarin (MC) and 4-methylumbelliferyl (MUB) were used as the fluorescent standards, which attach to the enzyme substrates to identify the enzyme being tagged. MC is used for leucine aminopeptidase (LAP) and MUB for the remaining hydrolytic enzymes (β-glucosidase, α-1,4-glucosidase, cellobiohydrolase, xylosidase, and B-1,4-N-Acetyl glucosaminidase, and acid phosphatase). The enzyme substrates used for each enzyme are located in (Chapter 2, Table 2.2). These substrates attach to the active enzymes in the soil. 100 µl of substrates were added at 200 µm accordingly. To correct the reduction in fluorescence by soil particles, quench samples were made (soil slurry and MUB/MC) to measure the background fluorescence of soils, substrates, and MUB/MC standard curves. The microplate reader (BioTek Instruments Inc., Winooski, VT, USA) read the plates to check the fluorescent activity at 360 nm excitation and 460 nm emission after a 2 hour incubation period.

Subsequently, oxidative activities were measured using a standard colorimetric assay (Sinsabaugh and Linkins, 1988; Sistla and Schimel, 2013). The same soil slurry was used for the fluorometric and oxidative enzyme assays, and L-Dopa (L-3,4-dihydroxyphenylalanine) was used as a substrate. 600 µl of soil slurry and 400 µl of L-Dopa were added to the deep well plates. For the peroxidase, 30 µl of hydrogen peroxidase was added. They are incubated for 24 hours, 200 µl transferred to clear bottom plates without disturbing the soil set at the bottom of the well. The plates were visualized using a microplate reader (BioTek Instruments Inc., Winooski, VT, USA) to determine the absorbance (460 nm) after 24 hours of incubation.

DATA ANALYSIS

Statistical analysis was carried out using R version 4.2.2 (R Core Team, 2022). A mixed effects model was used for the soil enzyme activity assays, with treatment analyzed as a fixed and soil core as a random effect because each of the 12 cores were subsampled for each of the four

treatments. Additionally, we performed a repeated measures ANOVA to determine the effect of treatment on enzyme activity over 12 weeks. Samples were measured for enzyme activity across the four treatment groups. The 'aov' function was used to fit the repeated measures ANOVA model, with the 'Error' function used to account for the within-subjects variability across time.

Microbial death and sonication

We used the mean percent (%) from the fluorometric microscopy technique to calculate microbial sterilization. For the live/dead analysis of microscope counts, a one-way ANOVA was conducted to analyze the differences in "percentage alive" among the treatment groups. Tukey post-hoc pairwise comparisons were made across treatments for each enzyme using the 'multcomp' package ($p < 0.05$) (Hothorn et al., 2008).

RESULTS

Potential enzyme assay

Soil enzymes remained active during the measured 12-week timeframe in both the chloroform and non-chloroformed (alive) samples. The enzyme trends were the same for both alive and chloroformed samples During the first three weeks, there was an increase in activity for all enzymes except leucine aminopeptidase. After week 3, the enzymes that declined in potential enzyme activity were β-glucosidase (45% decrease), cellobiohydrolase (27% decrease), B-1,4-N-Acetyl glucosaminidase (61.5% decrease), and xylosidase (71.6% decrease). On week 4, α-1,4-glucosidase (45% decrease). By the sixth week, there is an increase again; by the seventh week, it decreases for most of the measured enzymes. Leucine aminopeptidase ($F (1, 276) = 51.122$, $p < 0.001$), after day 35, significantly decreased (Supplementary Table 4.1). We did not observe a significant steady decline in enzyme activity throughout the 12 weeks by six hydrolytic enzymes assessed (Figure 4.2, Supplemental Table 4.1). At the same time, for the oxidative enzymes,

peroxidase ($F(1, 276) = 7.65, p < 0.001$) does show significance throughout the days, while phenol-oxidase ($F(1, 276) = 2.326, p = 0.128$) does not (Supplementary Table 4.1).

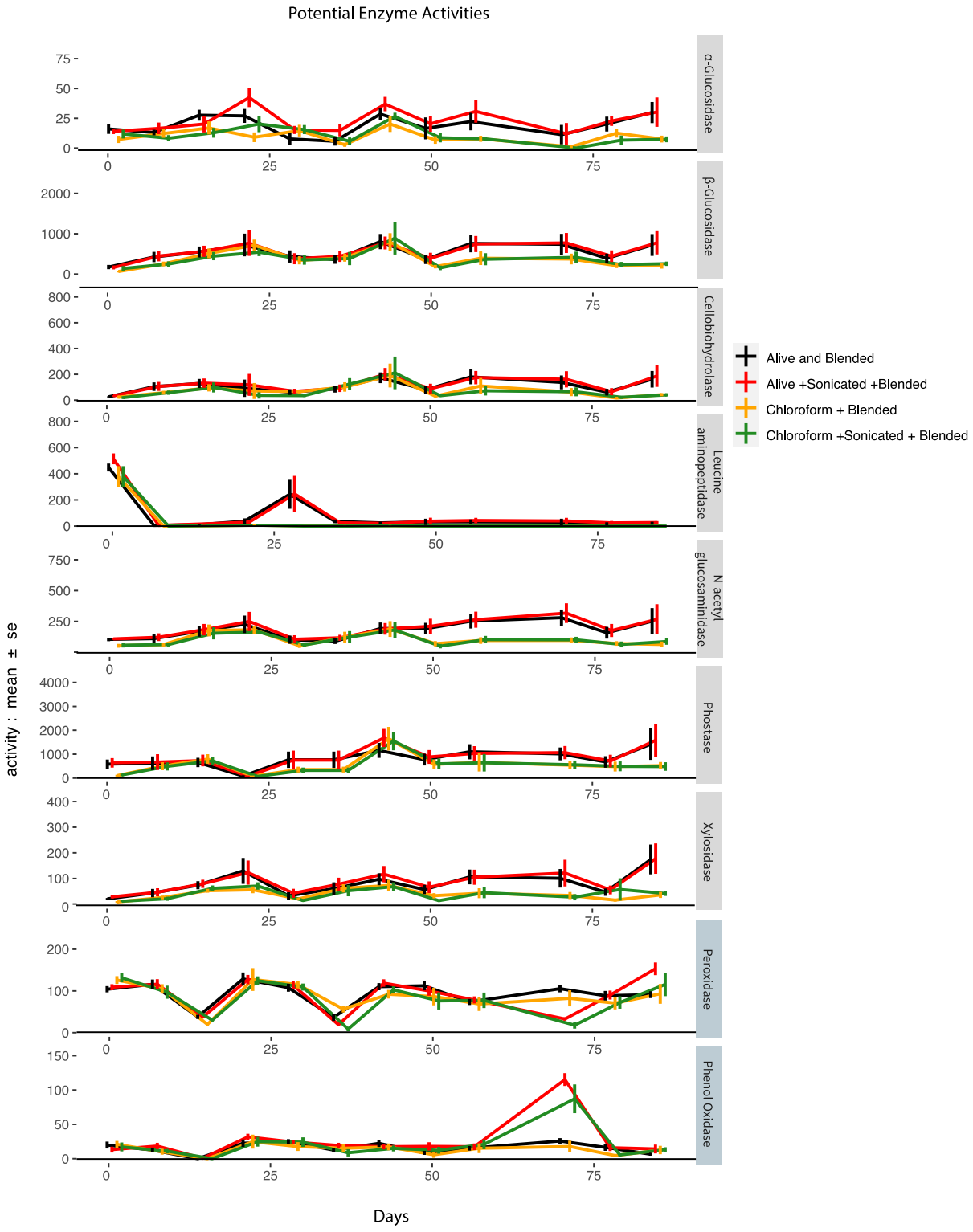


Figure 4.2. Potential enzyme activities by time (in days), including the four treatments. The treatments are color-coded: in black alive and blended, shown in red, alive +sonicated + blended, yellow, chloroform+ blended, and in green, chloroform +sonicated +blended).

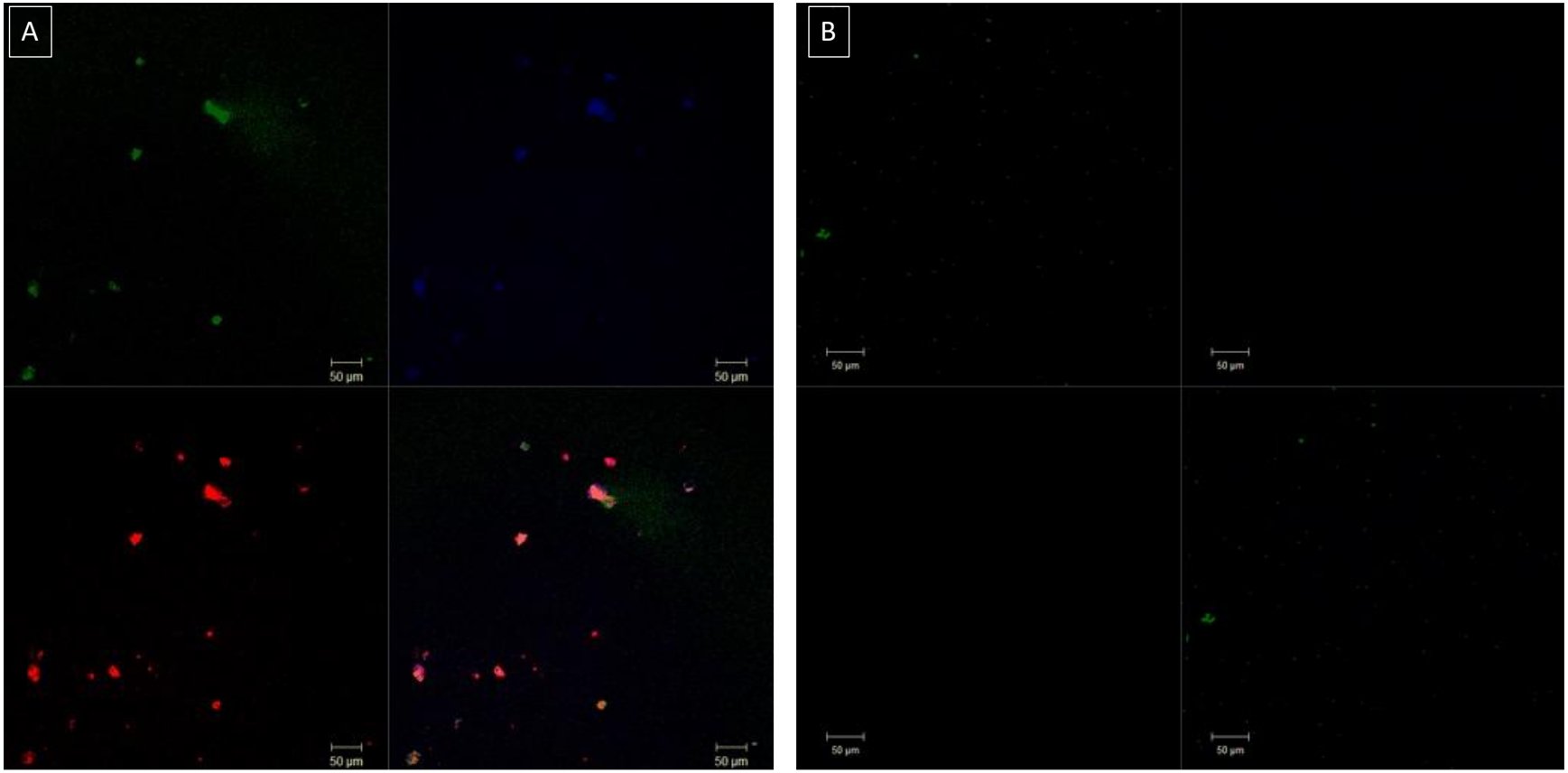


Figure 4.3. Confocal microscope images, at week 5 (day 35), were obtained to show dead cells in sample 6 following chloroform treatment (A) and live cells in sample 1 without chloroform treatment (B). Green fluorescence marks alive cells, while red fluorescence shows dead cells. If both colors overlap, the cell is dead/dying. Scale bars are 50 µm.

Live/Dead Analysis

Microscopy revealed a greater abundance of cells in the samples classified as alive than those that underwent chloroform treatment (Figure 4.3). Additionally, the weekly microscope cell counts show a difference between chloroform and non-chloroform. The results of the One-Way ANOVA indicated a significant effect of the treatment groups on "Percent alive" ($F(3, 284) = 344.9$, $p < .0001$). The post-hoc Tukey test showed that chloroform and chloroform + sonication had significantly lower "Percent alive" rates compared to no chloroform and sonication ($p < .05$). No other significant differences were found between the groups. However, throughout the weeks, it did vary. Chloroformed samples ranged below 25% alive, while the ones that did not receive the treatment were over 70% all 84 days. Furthermore, based on the data presented in Figure 4, it can be suggested that there is no total cell death. Sonication does not appear to have any impact on live/dead counts. There was no significance in treatments between chloroform (and chloroform +sonication ($p = 0.989$), as well as between no chloroform and sonication ($p = 0.986$).

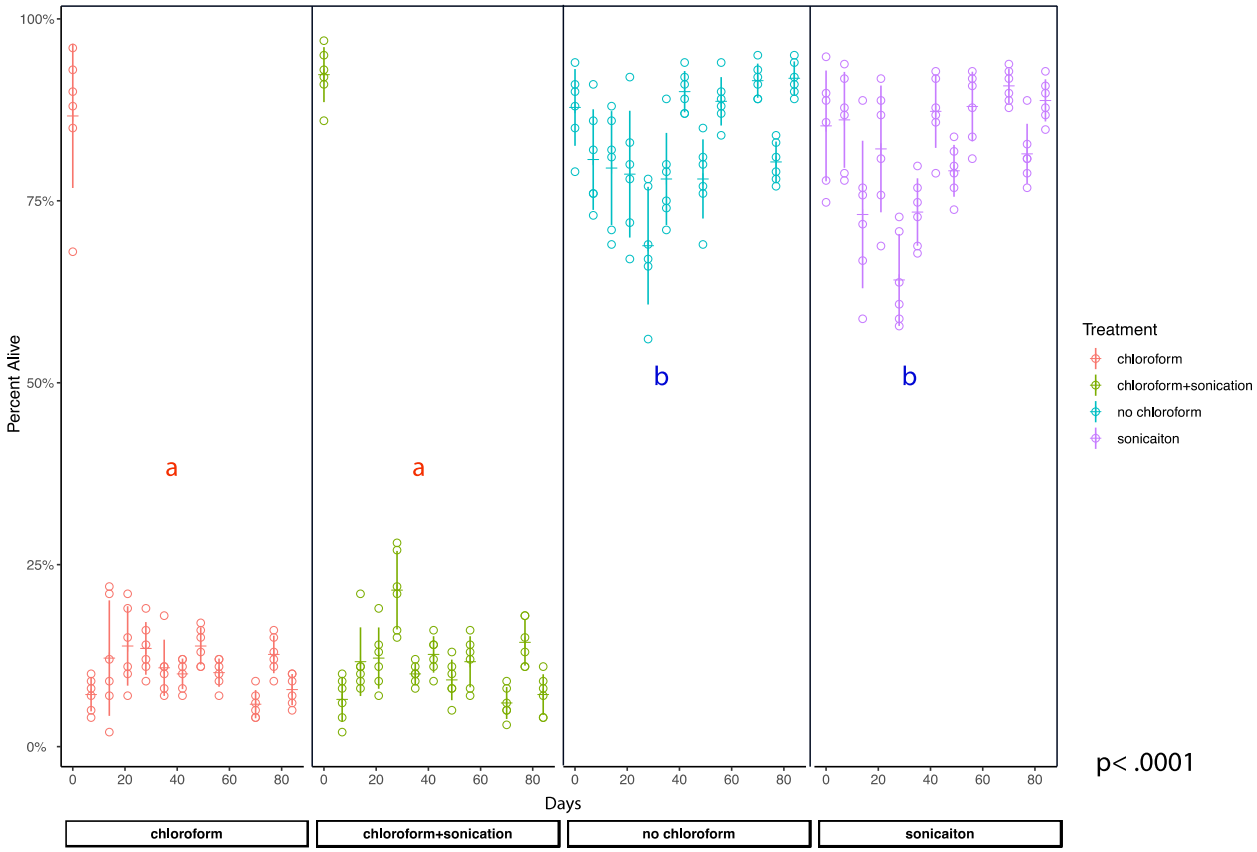


Figure 4.4. Microscope analysis by treatments. Red samples are chloroformed only, green samples are chloroformed/sonicated, blue samples are not chloroformed/not sonicated, and purple was not chloroformed, but it was sonicated. Letters denote significant differences among treatments Tukey adjusted p-values. Error bars represent the standard error of the mean.

DISCUSSION

Our study aimed to determine the time a soil enzyme persists in the soil without further microbial production by using chloroform fumigation on soils to suppress microbial activity while preserving enzyme integrity for a period of 12 weeks. When observing cells under a confocal microscope, we saw a clear significant difference between the chloroform treatment (dead cells) and the no-chloroform treatment (live cells). Despite the apparent death of cells, we continued to observe enzyme activity. The results of our study showed enzyme activity was largely maintained during the 12-week observation period. Additionally, our results suggest no difference between

sonicated and unsonicated potential enzyme activities samples. From our microscopy observations, sonication samples did not show increased cellular death compared to the unsonicated samples. These observations support our hypothesis that sonication does not lyse cells and can be used as an additional extraction method when measuring soil enzyme activity, as suggested in Chapter 2, without releasing additional intracellular enzymes into the sample (Martinez et al., 2021).

Microbial Death

Chloroform fumigation is typically successful at killing microorganisms (Allison, 2006); however, in some studies, enzyme activities do not decrease immediately (Renella et al., 2002). In a research study, enzyme activities were measured for various soil types over a period of ten days. The findings revealed that the enzyme activity of β-glucosidase did not significantly decrease; protease exhibited a slight decrease, while arylsulfatase increased after fumigation (Renella et al., 2002). Our results align with this observation, as we also found that enzyme activity does not decrease during the initial days of chloroform fumigation. This could be due to chloroform-resistant bacteria. The ability of certain bacteria to form spores has been proposed as a reason for their resistance to chloroform fumigation (Toyota and Kimura, 1996; Zelles et al., 1997). This can potentially suggest that our samples might have contained some spore-forming microorganisms. The Arctic is known to have these types of bacteria that lay dormant in permafrost soils (Boyd and Boyd, 1964; Soina et al., 2004).

Enzyme Longevity

A similar study that measured the decay of enzyme activities in tundra soil obtained from the Toolik field station (North Slope of the Brooks Range in Alaska) reported a significant decrease within the initial few weeks of their experiment (Schimel et al., 2017). However, our study did not

report a steady decline in decay times, which could potentially be explained by the slight variation between the tundra ecosystems, as the soil we used was from Utqiaġvik, AK, a coastal tundra ecosystem. Coastal tundra soil remains frozen longer and has different vegetation types than inland tundra (III and Shaver, 1981; Michaelson et al., 1996). We speculate soil enzymes in Utqiaġvik have adapted to more extreme environments and survive extended periods of time, due to most enzymes behaving similar in all tests. Our study used five grams of soil, while this contrasting study used two grams. This could have had an impact on our results and a reason why there were higher enzyme activities in the contrasting study. Nevertheless, we anticipate that continued monitoring of enzyme activity in our samples over a 12-week period may reveal a reduction in soil enzyme activities.

Ultimately, understanding the longevity of these arctic soil enzymes is important, especially given the extreme temperature variations; enzyme decay is also important in enzyme-driven models. Enzyme decay can affect microbial turnover. If enzymes degrade rapidly, microbial populations may not have access to enough nutrients to sustain growth and reproduction (Hagerty et al., 2014), leading to decreased microbial turnover and reduced nutrient cycling.

Enzymes in arctic soils may also have unique properties due to extreme environmental conditions, such as low temperatures and limited nutrient availability (Schnecker et al., 2014). It has been discussed that enzymes produced by fungi, such as acid phosphatase, acid phosphomonoesterase, and proteases, have adapted to cold environments and, therefore, elevated concentrations of these enzymes when recorded in isolates (Ferrer et al., 2007). The enzymes' prevalence observed in our study could be attributed to their adaptation to the cold environment, which could potentially slow down their decay rate and lead to their long persistence.

Sonication

Our findings indicated that sonication does not cause significant cell lysis compared to the unsonicated samples. We agree with the studies with comparable sonication techniques used for dispersion (Janajreh et al., 2022; Kaiser and Asefaw Berhe, 2014) and disagree with using this technique to completely lyse cells (Fykse et al., 2003; Zhang et al., 2007), though the strength of the sonication device could be a factor. Microscopic imaging was critical in visualizing the effects of sonication on cells, as we had previously established in Chapter 2 that adding an extra sonication step to enzyme assays resulted in increased enzyme activity without lysing microorganisms at 6 W ml^{-1}. Low-energy sonication (<5 W ml^{-1}) has been suggested not to disturb cellular organisms and soil aggregates (Stemmer et al., 1998). At the same time, according to some, high-intensity sonication (> 20 W ml^{-1}) can be used as an alternative to even chloroform fumigation (Qin et al. 1998).

Enzyme degradation

Our results (Figure 4.4) showed that not all microbes were dead, even in the presence of chloroform, when comparing the different treatments. This suggests that some enzymes, such as proteases, may still be created and capable of degrading other enzymes over time. Proteases break down proteins into smaller peptides and amino acids (Cryns and Yuan, 1998). These proteases can impact the cycling of nutrients by degrading soil enzymes. Other factors affecting the inactivation and degradation of enzymes include moisture change and oxidation reactions (Selmer, 2005). Oxidation reactions can degrade enzymes by causing changes in the chemical structure of enzymes (Farooq et al., 2009). In arctic soils, cold temperatures and limited microbial activity can accumulate organic matter, including dead plant and animal material, which can undergo oxidation reactions. Additionally, reactive oxygen species (ROS), produced in response to environmental

stressors, can interact with and damage the enzyme's protein structure, leading to denaturation and loss of enzymatic activity (Hossain and Dietz, 2016; Salehi-Lisar and Bakhshayeshan-Agdam, 2016).

CONCLUSIONS

It can be concluded that chloroform fumigation does not completely lyse cells, implying that there are chloroform-resisting microorganisms in the soil that survive after the treatment. The resilient microorganisms could produce additional enzymes, so we do not see a decrease in enzyme activity. Additionally, we speculate coastal tundra soil enzymes could have a resilience that allows them to survive prolonged periods even without microbial cells, mainly because they are cold-adapted enzymes (Siddiqui and Cavicchioli, 2006). Furthermore, our study demonstrated that sonication does not induce cellular mortality at a low intensity, making it a valuable technique for potential enzyme assays. Taken together, our results suggest enzyme activity is robust in these high organic matter soils and may provide relatively long-lived benefits for the microbes that produce them.

Chapter 5: Conclusions

SUMMARY OF FINDINGS

This dissertation emphasized the importance of extracellular enzymes in soil, specifically in arctic ecosystems. Microorganisms produce and release soil enzymes to decompose organic material and utilize the nutrients they release (Burns, 1982). I emphasized the significance of soil enzymes that play a vital role in decomposition and contributed to filling some gaps in our knowledge regarding their enzymatic functions. Additionally, I worked on developing methods to address key questions to provide insights into the functioning of the arctic ecosystem. This dissertation presents several conclusions regarding our ability to locate enzymes in the soil matrix (Chapter 2), uncover the soil proteome (Chapter 3), and demonstrate the durability and resilience of enzymes in arctic soils (Chapter 4).

THE ROLE OF ENZYMES IN ARCTIC ECOSYSTEMS

Enzymes play a key role in the decomposition process and are very resilient in arctic soils, as this dissertation (Chapter 4) and previous research shown (Burns et al., 2013; Dick et al., 2011; Siddiqui and Cavicchioli, 2006). Focusing on these activities can help us further understand their extremely specific functions. As temperatures rise, these ecosystems are likely to experience significant shifts in biogeochemical processes, including changes in the rate of nutrient cycling and organic matter decomposition (Sistla and Schimel, 2012; Sterner and Elser, 2017). Understanding the role of enzymes in arctic ecosystems can aid in comprehending the intricacies of biogeochemical cycles and enable us to make more accurate predictions regarding how these cycles might be influenced by environmental change.

The investigation of enzyme location within the soil matrix has been an area of interest that has yet to be extensively investigated (Burns, 1982; Burns et al., 2013; Wallenstein and Burns,

2015). My dissertation explored the location of enzymes in the soil matrix in harsh arctic environments (Chapter 2). The findings presented in this chapter suggest that enzymes in arctic ecosystems tend to be mostly associated with large particles, such as organic matter, potentially influencing the cycling of carbon and nitrogen. Additionally, I found that additional physical disruption, such as sonication, can serve to break apart the relatively high structural integrity of the organic material in these highly organic soils. I learned that sonication is a valuable tool for enhancing enzyme activity (Chapter 2) and demonstrated through microscopy there is no increase in cell lysis with this application (Chapter 4). The findings indicate that soil enzymes could be either secreted or attached to microbial cells.

Metaproteomics allows us to analyze the complete proteome, allowing for a more detailed picture of the microbial community's metabolic processes and functional capabilities. In one of my chapters, we assessed the total peptide counts of soil enzymes (Chapter 3). Upon comparison with other proteins identified in the proteome, it became apparent that the numbers of decomposing enzymes are relatively low, while those involved in cell function are high. While not unexpected, it is noteworthy that our protocol was able to attain peptide matches and exhibit their prevalence in the samples. This rich data uncovered the role of enzymes in metabolic pathways for arctic soils. Metaproteomics has the potential to revolutionize our understanding of soil microbial communities and their functions. The application of metaproteomics in soils will likely continue to grow in the coming years, influencing and expanding our knowledge of the role of enzymes in the soil.

CHALLENGES AND CONSIDERATIONS

During the metaproteomics analysis, I encountered obstacles in developing and navigating a practical bioinformatics pipeline. It is known that there is little standardization of bioinformatics for detecting peptides and annotating proteins (Schiebenhoefer et al., 2019; Timmins-Schiffman

et al., 2017). Because various steps can be taken to get results, it is challenging to identify the best pipeline for the data. Moreover, we discovered that conducting FASP on soil samples makes detecting spectral data simpler than previous techniques (as highlighted in Chapter 3). To further validate the efficacy of our methodology, it would be interesting to increase the number of samples tested. Future studies examining metaproteomics in soils should also consider performing metagenomic analysis on their samples. This would have benefited our samples by providing a more robust metagenome for identifications. By doing this, we could potentially be able to answer questions regarding the origins of enzymes and possibly explore the major microbial producers of the set of commonly assayed enzymes that I studied.

For my final experiment, I sought to determine the longevity of the enzymes in the soil. Despite the absence of a decrease in enzyme activity as an indicator of lifespan, our findings indicate that enzymes can last longer than 12 weeks in these arctic soils. Surprisingly, this chapter was the most challenging to interpret because of the minimal to no reduction in soil enzyme activities, indicating that the degradation of soil enzymes was not occurring as expected. Nevertheless, I believe the enzyme activities will eventually decrease with more time, indicating that an extended observation period is necessary.

FUTURE DIRECTIONS

My dissertation contributes to the understanding of enzymes in arctic ecosystems. This investigation on soil enzymes serves as a foundation for further research. Future research directions could include exploring the impact of environmental factors on soil enzyme activity and investigating the potential role of microbial communities in enzyme production. The findings of this research can be used in decomposition ecology and to pursue further metaproteomic studies.

Enhancing our comprehension of cellular-bound enzymes would aid in better understanding enzyme decomposition. As discussed in Chapter 2, some enzymes are probably attached to the producing microbes, which are bound to the substrates or directly to the larger organic substrates being decomposed. The close proximity binding between enzymes and substrates may reduce the transportation of decomposition products away from the microbes that generated them. Identifying these enzymes and conducting further research could be important in determining the specific location of certain enzymes.

Despite Chapter 3 (metaproteomics) being a high-risk project, our findings were intriguing and revealed an interesting potential link between the abundance of peptides associated with particular enzymes and the activity of those enzymes in fluorescent assays; the encouraging findings suggest that further research is warranted in this area. Understanding the relationship between the abundance of enzymes and their activity in the soil, for example, may provide valuable insights into the functioning of the soil ecosystem. Furthermore, incorporating multi-omics studies by adding metagenomic or metametabolomic approaches would provide significant benefits in revealing the microbial community functions and diversity. Metaproteomics and metagenomics can identify which enzymes are present in the soil and who produced them, while metabolomics can provide information on which metabolic pathways are active. This information can help us understand how enzymes contribute to the breakdown and transformation of organic matter in the soil and their role in nutrient cycling.

Additionally, exploring the resilience of enzymes to different biotic and abiotic factors could help expand our research findings. Further research could investigate how these environmental factors affect enzyme persistence and how they interact with microbial communities and other soil components. As previously stated, I believe these enzymes are subject

to degradation over time. Therefore, undertaking extended monitoring of the enzymes would significantly advance this study. Such an approach would strengthen enzyme models, increasing their reliability and accuracy in predicting resource acquisition (Allison, 2005).

Finally, because an arctic ecosystem was used in this dissertation as a model to begin answering biogeochemical questions, future studies could benefit from a broader approach that examines enzymes in various ecosystems to enhance our knowledge of the ecological relevance and compare it with our findings. It would be interesting to examine the location of enzymes in soil systems such as deserts, where soil organic matter is not as abundant as in arctic soils (Clapp et al., 2018), and determine and analyze the soil proteome of other ecosystems.

CONCLUSION

Arctic tundra soils contain a large portion of Earth's soil carbon, and as the tundra continues to warm in upcoming years, more carbon will be released due to decomposition (Michaelson et al., 1996). My dissertation contributes to the growing interest in the implications and roles of soil enzymes in this decomposition. The conclusions drawn from the findings and data in this dissertation can serve as a foundation for further investigations, including but not limited to comprehending the precise location of enzymes within the soil matrix, identifying the various microbial producers of soil enzymes, and estimating the rate of production and death of enzymes to update decomposition models. Taken together, my studies provide evidence that multiple microorganisms generate relatively long-lived soil enzymes, which are likely in close association with the large particles of organic matter that they are working to decompose.

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Supplemental Tables

Table S2.1. Description of Rhizon Samplers

Soil Sample	*Collection Site*	*Location on Collection Site*	*Date*	*GPS Coordinates*	
L1	Barrow, AK	BEO- Itex	07/16/16	71.31358	-156.59419
L2	Barrow, AK	BEO- Itex	07/16/16	71.31364	-156.59366
L3	Barrow, AK	BEO- Itex	07/16/16	71.31366	-156.59343
L4	Barrow, AK	BEO- Itex	07/16/16	71.31369	-156.59315
L5	Barrow, AK	BEO- Itex	07/16/16	71.31374	-156.59288
L6	Barrow, AK	BEO- Itex	07/16/16	71.31381	-156.59253
L7	Barrow, AK	Snowfence	07/14/16	71.32385	-156.66846
L8	Barrow, AK	Snowfence	07/14/16	71.32375	-156.66806
L9	Barrow, AK	Snowfence	07/14/16	71.32338	-156.66772
L10	Barrow, AK	Snowfence	07/14/16	71.32385	-156.66872

Table 3.1 Taxonomic Classification of Enzymes

FERTILIZED

Enzyme	Peptide sequence	Lowest Common Ancestor	Super kingdom	Phylum	Class	Order	Family	Genus	Species
β-glucosidase (3.2.1.21)	MEGDDVVQLYVSK	Sulfolobus islandicus	Archaea	Crenarchaeota	Thermoprotei	Sulfolobales	Sulfolobaceae	Sulfolobus	*Sulfolobus islandicus*
	QVGLALGEECR	Chloroflexi	Bacteria	Chloroflexi					
	VGVLLGPGANLK	Bacteria	Bacteria						
	AALGLFEDPYR	Proteobacteria	Bacteria	Proteobacteria					
	GELTDEMLNER	Fervidobacterium	Bacteria	Thermotogae	Thermotogae	Thermotogales	Fervidobacteriaceae	Fervidobacterium	
	RPELEELK	Fervidobacterium	Bacteria	Thermotogae	Thermotogae	Thermotogales	Fervidobacteriaceae	Fervidobacterium	
	DPEYEDYYLWR	Haloarcula	Archaea	Euryarchaeota	Halobacteria	Halobacteriales	Haloarculaceae	Haloarcula	
α-1,4-glucosidase (3.2.1.20)	WQDGLAADGWNTLYWENHDQPR	Haloarcula	Archaea	Euryarchaeota	Halobacteria	Halobacteriales	Haloarculaceae	Haloarcula	

Xylosidase (3.2.1.37)	TPQNEDWFLHFQDVGVMGR	Bacteroidetes	Bacteria	Bacteroidetes
B-1,4-N-Acetylglucosaminidase (3.2.1.52)	LFTDDTLK	Bacteria	Bacteria	
	FDEELWNSLLEEMPR	Armatimonadetes	Bacteria	Armatimonadetes
	CSLSLSAR	Eukaryota	Eukaryota	
	QLLNNLMR	Bacteria	Bacteria	
Leucine-aminopeptidase (3.4.11.1)	QDFNLDK	Bacteria	Bacteria	
	FGEVFVTK	Candidatus Woesearchaeota	Archaea	Candidatus Woesearchaeota

UNFERTILIZED

Enzyme	Peptide sequence	Lowest Common Ancestor	Super kingdom	Phylum	Class	Order	Family	Genus	Species
β-glucosidase (3.2.1.21)	LYAEEWR	Bacteria	Bacteria						
	HFAANNQER	Bacteria	Bacteria						
	MGVNAHVSER	Flavobacterium	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	
	DNPQVVVYDEDLYVGYR	Fervidobacterium pennivorans	Bacteria	Thermotogae	Thermotogae	Thermotogales	Fervidobacteriaceae	Fervidobacterium	*Fervidobacterium pennivorans*
α-1,4-glucosidase (3.2.1.20)	DWYWWR	Bacteria	Bacteria						
	DWYWWRPPR	Actinobacteria	Bacteria	Actinobacteria					
Xylosidase (3.2.1.37)	TPQNEDWFLHFQDVGVMGR	Bacteroidetes	Bacteria	Bacteroidetes					
B-1,4-N-Acetyl	LYYTLDGR	Bacteria	Bacteria						

glucosaminidase (3.2.1.52)									
Leucine-aminopeptidase (3.4.11.1)	GPSGYDAR	Bacteria	Bacteria						
	ASEGAVGYR	Luteitalea pratensis	Bacteria	Acidobacteria	Vicinamibacteria		Vicinamibacteraceae	Luteitalea	*Luteitalea pratensis*
Phenol oxidase (1.10.3.2)	GEDVYVTWK	Halalkalicoccus paucihalophilus	Archaea	Euryarchaeota	Halobacteria	Halobacteriales	Halobacteriaceae	Halalkalicoccus	*Halalkalicoccus paucihalophilus*
Peroxidase (1.11.1.7)	LSFLLDEQGK	Gammaproteobacteria	Bacteria	Proteobacteria	Gammaproteobacteria				

Supplemental Table 3.2 Redox Pathways in Arctic Soil Proteomes (Oxidoreductases)

Process	EC number	Fertilized (total peptide count)	Unfertilized (total peptide count)
Ammonification	1.7.1.15	0	2
Ammonification	1.7.2.2	10	9
Ammonification	1.7.99.4	25	25
Nitrogen Assimilation	1.7.1.4	4	6
Nitrogen Assimilation	1.7.7.1	2	2
Nitrogen Assimilation	1.7.7.2	1	1
Denitrification	1.7.2.1	8	5
Denitrification	1.7.1.15	0	2
Denitrification	1.7.2.1	8	5
Denitrification	1.7.2.2	10	9
Denitrification	1.7.2.4	5	3
Denitrification	1.7.2.5	0	0
Denitrification	1.7.5.2	0	0
Denitrification	1.7.99.4	25	25
Nitrification	1.14.99.39	1	0
Nitrification	1.7.2.6	1	2
Nitrification	1.7.3.6	0	0
N-fixation	1.18.6.1	22	22
N-fixation	1.19.6.1	0	0
Sulfur Metabolism	1.8.2.1	0	2

Sulfur Metabolism	1.8.3.1	2	1
Sulfur Metabolism	1.12.98.4	1	0
Sulfate reduction	1.8.4.10	3	3
Sulfate reduction	1.8.4.9	1	0
Sulfate reduction	1.8.99.2	7	10
Sulfite reduction	1.8.99.1	1	0
Sulfur assimilation	1.8.1.2	16	22
Sulfur assimilation	1.8.4.8	28	19
Sulfur assimilation	1.8.7.1	11	14
Sulfydrogenase	1.12.1.5	0	0
Thiosulfate Oxidation	1.8.2.2	0	0
Thiosulfate Oxidation	1.8.5.2	0	0
Thiosulfate Oxidation	1.8.5.4	1	0
3-Hydroxypropionate bi-cycle	1.1.1.-	55	46
3-Hydroxypropionate bi-cycle	1.1.1.298	1	1
3-Hydroxypropionate bi-cycle	1.1.1.35	55	73
3-Hydroxypropionate bi-cycle	1.1.1.37	27	27
3-Hydroxypropionate bi-cycle	1.1.1.42	54	48
3-Hydroxypropionate bi-cycle	1.2.1.12	13	12
3-Hydroxypropionate bi-cycle	1.2.1.13	0	2
3-Hydroxypropionate bi-cycle	1.2.1.43	0	0
3-Hydroxypropionate bi-cycle	1.2.1.59	3	4
3-Hydroxypropionate bi-cycle	1.2.1.75	0	0
3-Hydroxypropionate bi-cycle	1.2.1.76	1	2
3-Hydroxypropionate bi-cycle	1.2.7.-	7	6
3-Hydroxypropionate bi-cycle	1.2.7.1	18	27
3-Hydroxypropionate bi-cycle	1.3.5.1	50	45
3-Hydroxypropionate bi-cycle	1.3.5.4	15	16
acetyl-CoA pathway (Wood-Ljungdahl pathway)	1.2.7.4	11	18
acetyl-CoA pathway (Wood-Ljungdahl pathway)	1.2.99.2	4	3
Carbon fixation pathways in prokaryotes	1.3.1.6	1	2
Carbon fixation pathways in prokaryotes	1.3.4.1	0	1
Hydroxy propionate hydroxy butylate cycle	1.3.1.84	1	0
Reductive acetyl-CoA pathway (Wood- Ljungdahl pathway)	1.5.1.20	47	40
Reductive acetyl-CoA pathway (Wood- Ljungdahl pathway)	1.5.1.5	30	40
Reductive citrate cycle (Arnon-Buchanan cycle)	1.2.7.3	13	17
Fatty acid degradation	1.3.8.8	24	25
Fatty acid degradation	1.3.8.9	0	1
Glycerophospholipid metabolism	1.1.1.156	0	0
Glycerophospholipid metabolism	1.1.1.21	1	2

Glycerophospholipid metabolism	1.1.1.6	6	4
Glycerophospholipid metabolism	1.1.1.8	12	7
Glycerophospholipid metabolism	1.1.3.15	10	8
Glycolysis /Gluconeogenesis	1.1.1.2	3	4
Glycolysis /Gluconeogenesis	1.1.1.27	14	10
Glycolysis /Gluconeogenesis	1.12.1.2	9	6
Glycolysis /Gluconeogenesis	1.2.1.12	13	12
Glycolysis/Gluconeogenesis/Fatty acid degradation	1.1.1.1	18	21
Glycolysis/Gluconeogenesis/Fatty acid degradation	1.2.1.3	16	16
Glycolysis/Gluconeogenesis/Fatty acid degradation	1.2.1.59	3	4
Glycolysis/Gluconeogenesis/Methane metabolism	1.1.2.7	1	2
Glycolysis/Gluconeogenesis/Pentose Phosphate	1.2.1.9	4	5
Glycolysis/Gluconeogenesis/TCA Cycle	1.2.4.1	76	85
Glycolysis/Gluconeogenesis/TCA Cycle	1.2.7.1	18	27
Glycolysis/Gluconeogenesis/TCA Cycle	1.8.1.4	51	66
Glycolysis/Gluconeogenesis/TCA Cycle	1.8.1.9	31	37
Pentose Phosphate	1.1.1.49	42	58
TCA Cycle	1.1.1.286	0	0
TCA Cycle	1.1.5.4	21	15
TCA Cycle	1.2.4.2	90	84
TCA Cycle/Oxidative Phosphorylation/ Reverse TCA Cycle	1.3.5.1	50	45
TCA Cycle/Reverse TCA Cycle	1.2.7.3	13	17
TCA Cycle/Reverse TCA Cycle	1.3.5.4	15	16
catalase	1.11.1.6	39	35
catalase-peroxidase	1.11.1.21	34	38
eosinophil peroxidase	1.11.1.18	0	0
Na Translocating	1.6.5.8	0	0
Oxidative Phosphorylation	1.10.3.12	0	0
Oxidative Phosphorylation	1.10.3.13	0	0
Oxidative Phosphorylation	1.10.3.14	0	0
peroxidase	1.11.1.7	24	23
Oxidative Phosphorylation	1.10.2.2	0	1
Oxidative Phosphorylation	1.6.5.3	18	21
Oxidative Phosphorylation	1.6.99.3	0	0
Oxidative Phosphorylation	1.6.99.5	11	10
Oxidative Phosphorylation	1.9.3.1	13	11
Methanogenesis	1.12.98.1	1	0
Methanogenesis	1.2.7.4	11	18
Methanogenesis	1.2.99.2	3	4

Methanogenesis	1.2.99.5	5	3
Methanogenesis	1.5.98.2	0	2
Methanogenesis	1.8.98.1	11	8
Methane Oxidation	1.14.13.25	0	0
Methane Oxidation	1.14.18.3	0	0
Methane Oxidation	1.14.99.39	1	0
Methane Oxidation	1.1.3.13	0	0
Anoxygenic Photosynthesis	1.97.1.12	3	2
Oxygenic Photosynthesis	1.97.1.12	3	2
Oxygenic Photosynthesis : Manganese Co-factor	1.10.3.9	0	0
Oxygenic Photosynthesis	1.10.9.2	0	0
Oxygenic Photosynthesis	1.18.1.2	0	0
Hydrogen Oxidation	1.12.1.2	9	6
Hydrogen Oxidation	1.12.1.3	7	13
Arsenate reduction	1.20.4.1	5	6
Arsenate reduction	1.20.4.1	5	6
Mercuric reductase	1.16.1.1	5	4
Selenate reduction	1.97.1.9	2	2

Supplemental Table 3.3 Hydrolases subclasses. Total peptide count of fertilized and unfertilized samples.

EC3.X subclass	**Fertilized**	**Unfertilized**
EC 3.1: ester bonds (esterases: nucleases, phosphodiesterases, lipase, phosphatase)	2447	3067
EC 3.2: sugars (DNA glycosylases, glycoside hydrolase)	1199	1135
EC 3.3: ether bonds	38	47
EC 3.4: peptide bonds (Proteases/peptidases)	1905	1946
EC 3.5: carbon-nitrogen bonds, other than peptide bonds	965	1032
EC 3.6 acid anhydrides (acid anhydride hydrolases, including helicases and GTPase)	3043	3238
EC 3.7 carbon-carbon bonds	43	41
EC 3.8 halide bonds	10	10
EC 3.9: phosphorus-nitrogen bonds	1	1
EC 3.10: sulfur-nitrogen bonds	1	0
EC 3.11: carbon-phosphorus bonds	2	3

EC 3.12: sulfur-sulfur bonds	0	0
EC 3.13: carbon-sulfur bonds	1	2

Vita

Jane K. Martinez earned her Bachelor of Science in Biological Sciences from The University of Texas at El Paso in 2012. In 2016, she was accepted into the doctoral program in Ecology and Evolutionary Department of Biological Sciences at The University of Texas at El Paso to study the effects of soil enzymes in arctic ecosystems with Dr. Anthony Darrouzet-Nardi. Ms. Martinez was a recipient of The Hispanic Alliance for Graduate Education and the Professoriate (H-AGEP) Fellowship. She received a travel award from this fellowship to present a poster at the City College of New York. As part of H-AGEP, Ms. Martinez was part-time lecturer from 2020- 2022, where she worked alongside Dr. Jeff Sivils at El Paso Community College.

While pursuing her degree, Ms. Martinez also worked as a Graduate Teaching Assistant for Human Anatomy and Physiology I, Topics of Biology, and Organismal Biology. While in the program, Ms. Martinez had the opportunity to do research in Utqiaġvik, Alaska, under the supervision of Dr. Craig Tweedie. Ms. Martinez is working on her second publishing of a first-author article. Her first published article, "Soil enzymes are preferentially associated with larger particles in highly organic arctic tundra soils," was published in 2021 with the help of Dr. Jennie McLaren, Dr. Craig Tweedie, and Dr. Anthony Darrouzet-Nardi.

Ms. Martinez's dissertation, "Linking Decomposition Reactions in Arctic Soils to Microbial Enzyme Production," was supervised by Dr. Anthony Darrouzet-Nardi. Ms. Martinez plans to pursue a teaching position at El Paso Community College or a position in a government agency.

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This dissertation was typed by Jane K. Martinez