5-2016

The Effects of Heavy Metals and Microbes on Extracellular Enzyme Activity at Liberty State Park

Eleanor Urenna Ojinnaka

Thesis Abstract

The overall goals of this research were to 1) understand the influence of microbes and heavy metals on extracellular enzyme activity in soil environments and 2) explore the possibility of using living organisms, such as microbes, to improve the enzyme activity of contaminated, poor-functioning soil—bioremediation. Microbes exude enzymes into the soil, which are vital in the cycling of nutrients in soil communities. Thus, measuring extracellular enzyme activity can be used to quantify the health of soil. In this experiment, phosphatase enzyme was measured as a proxy for enzyme activity. The study site is a closed-off section of Liberty State Park (LSP), located in Jersey City, NJ. LSP was previously a rail yard and industrial dumping ground, yet it sustains a robust forest. This thesis contains four individual chapters, each with a purpose and objective(s) that contribute to the overall goals:

1. Chapter 1 (*Thesis Introduction*) details the importance of this research and provides necessary background for the thesis.
2. Chapter 2 (*Enzyme Activity and Metal Concentrations at LSP and HMF*) provides preliminary research that quantified the metal concentration and enzyme activity at LSP, in comparison to a reference site, Hutcheson Memorial Forest (Franklin Township, NJ), with no history of heavy metal contamination. LSP has a higher concentration of heavy metals than HMF, as expected; but it also exhibits higher enzyme activity than HMF.
3. Chapter 3 (*Extracellular Enzyme Activity at LSP during Bioremediation*) contains two parts that contribute to the second goal of this thesis, using the microbes at LSP to improve the enzyme activity of contaminated poor-

functioning soil. Both parts suggest that LSP's microbes could be used to increase enzyme activity of poor functioning soil, and that the success of this was dependent on both the living and non-living contributors of soil environments. Finally,

4. Chapter 4 (*The Effect of Storage Conditions on Enzyme Activity*) is a physical characterization study that determined that the optimum storage condition to minimize changes in enzyme activity over time was the fridge (20 °C) or the freezer (4 °C).

This research will give insight into the extracellular enzyme activity of microbes that are able to survive in heavy metal contaminated sites, as well evaluate LSP's potential as a source for these unique microbes used to increase the enzyme activity of contaminated soils in the field of bioremediation.

MONTCLAIR STATE UNIVERSITY

The Effects of Heavy Metals and Microbes on Extracellular Enzyme Activity at Liberty State Park

by

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A Master's Thesis Submitted to the Faculty of

Montclair State University

In Partial Fulfillment of the Requirements

For the Degree of

Master of Science

May 2016

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2016

Acknowledgements

This thesis is dedicated to my entire support system. My sincerest gratitude to my committee: Dr. Nina Goodey, Dr. Jennifer Krumins and Dr. Jim Dyer. I cannot possibly communicate my appreciation for the valuable time you have dedicated towards the completion of this thesis. In addition to my committee, I would like to thank Diane Hagmann, who helped complete a lot of the preliminary research for this thesis, and Jay Singh, who was solely responsible for the incubation of samples in Chapter 3 and was very helpful in the moisture analysis and phosphatase enzyme assays. I would also like to thank other students that supported me in our lab group, including Yacoba Minnow, Romy Perez, Thamanna Misbah and Danielle Le Roux.

I am indebted to the organizations that have supported me financially throughout my collegiate career. The American Chemical Society (ACS) Project Seed Scholarship and the ACS/BASF Scholar Program has continuously blessed me with financial assistance and career development programs since my first year as an undergraduate. The Reed-Lehmkuhl Scholarship, the Margaret E. Herman Sokol Graduate Summer Research Fellowship, both from Montclair State University (MSU), and the Research Award from the American Society of Biochemistry and Molecular Biology allowed me to conduct research towards my thesis. Without the 2015 scholarship from the Executive Women of New Jersey (EWNJ), I would have had to defer my program for a semester; and I am eternally grateful for their support.

I am also thankful to the faculty/staff at MSU for advising me beyond the classroom walls. Without Dr. Hans Schelvis or Dr. Marc Kasner, I would have been unable to transition into or complete this BS/MS program. To Dr. Krumins, who

introduced me to the world of soil biology and microbes, I am grateful for your time and influence towards this project. I am especially grateful to Dr. Goodey, who took a considerable amount of time to advise me on every aspect of my experience at MSU. Thank you for the long hours you've dedicated to my personal and professional growth. You are truly a Godsend and this entire thesis or my graduation would have been impossible without your countless contributions.

Finally, I would like to express my gratitude to my family, whose endless support, motivation and encouragement never fail. I am eternally grateful to my parents, Clement and Lawritha Ojinnaka, whose diligence and perseverance are continuous inspiration to my siblings and I. I appreciate my older brothers, Arinzechukwu and Elochukwu Ojinnaka for reminding me of the importance of family and hard work. There are not enough words to thank my younger brothers, Clemente and Henry Ojinnaka, my cousins, Gerald, Joshua and Faith Ogbennaya, my best friends, Akosua Baah, Samantha Hanciles and Shuanub Alhassan, and especially my younger sister, Sarah Ojinnaka, for being THE best hype-men. They were my number one cheerleaders and relentless motivators. Sarah, your admiration and love are constant and I am blessed that you are my twin sister. I am particularly grateful to my best friend, Ose Pius, for his relentless love and continuous words of encouragement. He never failed to remind me that I am beautiful, intelligent and loved, especially in my hardest moments. Most importantly, I am grateful to God for giving me the strength to complete this project and blessing me with such an amazing support system. The completion of this project is a result of his endless blessings to me, and I am thankful. I have learned and grown a great deal throughout this project and I am grateful to everyone for his or her contributions.

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1. Chapter 1: Thesis Introduction

1.1. Microbes have Important Interactions Within the Soil

Soil microbes are paramount in most of the processes that allow ecosystems to exist. They are believed to be the most influential factors that increase the availability of nutrients and the efficiency of plants in soil environments. Van der Heijden et al.[1], in a 2007 review of the importance of microorganisms, called microbes "the unseen majority" that are the "drivers" of plant diversity and productivity[1]. There is a large variety of individual microbial species in soil, including bacteria, fungal and protozoal species; and they perform a wide range of functions in the soil (Table 1). These include transportation and translocation of water, metabolism of compounds, and even photosynthesis—the conversion of sunlight to energy.[1,2]

Table 1: Microbial Roles in Key Biogeochemical Cycles (modified from Gadd[2])

Elements (s) involved	Role of Microbes
C, H, O	Degradation and metabolism, photosynthesis, respiration, hydrocarbon degraders, biomethylation, demethylation, water uptake, water transport/translocation, hummus formation, etc.
N	Ammonia and nitrite oxidation, nitrogen fixation, decomposition of nitrogenous compounds.
P	Decomposition of P-containing organic compounds, transformation of inorganic P, P transfer to plants
Co, Zn, Ni, Mg, Ca	Bioweathering of minerals in rocks and soil; biosorption;
Cu	Mobilization from Cu-containing minerals in rocks and soils

Table 1: Microbial Roles in Key Biogeochemical Cycles (modified from Gadd[2]). Lists a few known functions of soil microbes, and the elements/metals that are involved.

Researchers estimate that up to 10 billion microorganisms, containing more than 5,000 different bacterial species, can be harbored in just one gram of soil[1,3]. For

example, an image from the work of Torsvik and Orvreas[3] (Figure 1, *copied from Torsvik and Orvreas*[3]) shows the micrography of a soil sample with a total bacterial count of 4.2 $\times$ 10^{10} cells/gram $soil_{dry\ weight}$ (represented by the white dots). This characteristic magnitude and complexity of microbes make it easy for them to establish beneficial interactions within their soil environment.[1,3,4,5,6]

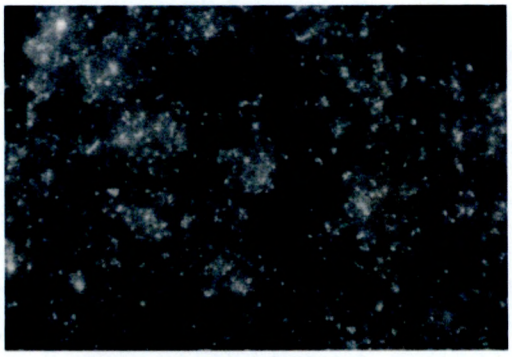


Figure 1. Epifluorescence Micrography of Soil Microorganisms (*copied from Torsvik and Orvreas*[3]).

A 4′,6-diamidino-2-phenylindole (DAPI) stain, which binds strongly to the Adenine-Thymine base pair of DNA, was used to highlight bacterial species. Photo saturation was reduced to 0 % to avoid color; so, bacterial species are indicated by the white dots.

1.2. The Vital Role of Microbes and Enzymes in Nutrient Cycling

Microbes are largely responsible for processes that influence the productivity of plants because of the beneficial interactions they form within their soil environment *(see examples of microbial roles in Table 1, Section 1.1)*. Perhaps, one of the most significant functions of microbes is their role in biogeochemical processes—the physical, chemical and biological interactions required for the availability and cycling of nutrients in the soil. Elemental nutrients such as nitrogen, carbon and phosphorous, are crucial for the growth of plants. The mineralization of these nutrients from organic matter as they transfer through a soil's ecosystem is referred to as nutrient cycling[4,7].

During nutrient cycling, soil microorganisms, such as bacteria and fungi, break down organic matter into elemental nutrients for plant uptake—releasing enzymes as hydrolytic catalysts in the process. Therefore, the presence of specific enzymes in soil is a representation of active nutrient cycling. For example, the enzyme $_{L}$-leucine-amino peptidase is representative of the Nitrogen cycling, cellobiohydrolase is representative of the Carbon cycling, and alkaline phosphatase enzymes are representative of Phosphorous cycling. As a result, extracellular enzyme activity assays are reliable indicators of soil health and microbial function[5,8,9,10]. Microbes would not be able to participate in any of these processes without the relationships they form with minerals in the soil. [2,4,5,6,7,8,11,12]

1.3. Interactions between Metals and Microbes

Another interaction present in soil is between microbes and metals. According to a study released by Gadd in 2010[2], metals can be directly or indirectly involved in the growth, metabolism and differentiation of microorganisms[2]. Additionally, most microbial processes necessitate the presence of certain metals—including Co, Cu, Ni, or Zn—in very low concentrations *(see examples of microbial roles in Table 1, Section 1.1)*. However, there are other metals—heavy metals such as Hg and Pb—that have no known favorable function in soil. Regardless of the nature of their interactions with microbes, metals can quickly become toxic to microbes and their soil environments.[2,4,13]

1.3.1. Heavy Metal Contamination in Soil

Once metals exceed their acceptable threshold, the soil is rendered unhealthy mainly because the necessary functions of its microbes are hindered[13,14]. With a half-life reaching 1,000 years and the inability to self-degrade, metals can remain in the soil for extremely long periods of time[4]. They induce obstructive stress upon microorganisms by

disrupting metabolic activities, reducing diversity, and preventing microbial functions[5,15]. Additionally, metals from contaminated sites can transport easily. They can disperse from their origin to contaminate surrounding soils, streams and groundwater, consequently spreading their toxicity potential. Heavy metals are not just a danger to soil; rather, the sites that they contaminate present detrimental consequences for surrounding ecosystems and human populations. A majority of heavy metal contaminated sites are a result of human activities, including the run-off from mining, the dumping of industrialized waste, and the use of chemical fertilizers. As a result, there is a worldwide effort to stabilize and/or eliminate the growing number of contaminated sites.

1.4. Bioremediation: A Soil Restoration Strategy

The multiple disadvantages of current treatment methods of contaminated sites necessitate the search for other more efficient methods. A majority of existing methods are either physical or chemical, such as the excavation or transfer of contaminated soils to landfills, the use of chemical reagents, or incineration of entire sites. Unfortunately, these methods have environmental concerns. This includes leachate from landfills to ground water wells, formation of hazardous chemical byproducts, or generation of air pollution from incineration/excavation. In addition to the inefficiency, conventional soil restoration methods are also very expensive and labor intensive. Global remediation efforts cost between $25 and $50 billion annually, and restoration of all currently contaminated sites in the United States has an approximate cost of $1.7 trillion[16]. The search for an alternative, more sustainable soil restoration strategy that will save money, energy and the health of the environment has led to the field of bioremediation.[16,17]

1.4.1. The importance of Microbes in Bioremediation Strategies

Bioremediation is the application of living organisms to degrade, detoxify or stabilize hazardous materials in the environment. It is more cost-effective and energy efficient than current conventional soil restoration methods. Unlike conventional methods, bioremediation strategies do not require any transportation of polluted soil—a major disadvantage that increases the pollution consequences of current methods.[16,17,18] The innate abilities of microbes, discussed earlier, are at the core of bioremediation strategies. Recall that microbial communities form interactions with their soil environments—minerals and pollutants such as metals, alike. When exposed to contamination, the composition of microbial communities may change. The novel community will be better adapted to the contaminated environment with selective biochemical traits[16,18,19,20]. Specifically, metal-resisting or pollutant-consuming bacteria may be able to out-compete their undeveloped counterpart and proliferate to stabilize their environment[13,16,21,].

Exploitation of these adapted microbial species forms the three main bioremediation strategies—natural attenuation, biostimulation and bioaugmentation. Also known as intrinsic remediation, natural attenuation allows the contaminated site to develop its own competitive microbes for soil restoration. Over time, certain sites may be able to stabilize. However, given that this approach can span a length of decades, it is useless in contaminated areas that are close to human populations or are extremely toxic.[16] Similar to natural attenuation, biostimulation allows the indigenous microbes to restore its soil environment. In biostimulation, the site is 'stimulated' via the addition of the appropriate nutrients to the soil and by maintaining optimized physical conditions (pH, moisture,

etc.). Finally, bioaugmentation is the introduction of adapted microbial species to the contaminated site.[18] Among all three bioremediation strategies, the main limiting factor is the identification of competitive microbial species that have adapted to heavy metal contamination.

1.5. The Importance and History of Liberty State Park *(Thesis Study-Site)*

This research attempts to understand the relationship between the microbes and metals at Liberty State Park (LSP) because despite a history of heavy metal contamination, LSP's soil biota was able to undergo the necessary geochemical processes that regenerated its currently flourishing forest. For many years leading up to the industrial revolution, LSP was known to its natives as Communipaw Cove[22,23]. In 1860, it was purchased by the Central Railroad of New Jersey (CRRNJ) and filled with waste from New York City, dredge spoil and ship's ballast.[22] During the next 68 years (1860-1928), as the need for expansion grew, so did the amount of fill and waste that was dumped on the land that is now LSP. For another 40 years (1928 – 1964), LSP became a major transportation hub and received consequential waste from the hundreds of daily activity of trains, ferries, barges, tugboats and travelers.[22,24]

It was not until 1975 that the massive cleanup campaign for LSP began, approximately 7 years after all train traffic was rerouted to another Station and all operations were ceased[22]. The ensuing systematic clearing of railroad tracks, dumps and abandoned industrial buildings stripped LSP of most of its vegetation. Yet, LSP currently boasts a variety of vegetation including phragmites, birch trees, cherry trees, oak trees, and Japanese knotweed. Although a portion of the land is now an open park, the most metal-contaminated section was fenced off and is still closed to the public.[20,22,23,33,

The focus of this study is the fenced-off area of LSP that was never remediated, but still managed to regenerate a robust forest. Located within a 251-acre plot in Jersey City, NJ (40° 42" 16 N, 74° 03' 06 W), it contains a variety of heavy metals (As, Cr, V, Cu, Zn, Pb) that are above ambient concentrations for New Jersey Soils[24,25]. The variation of metal concentrations within this area was recorded by Gallagher et. al.[33], and he generated an arbitrary numerical index to describe the distribution of metals at the site (Figure 2, *copied from Gallagher et. al.*[33]).

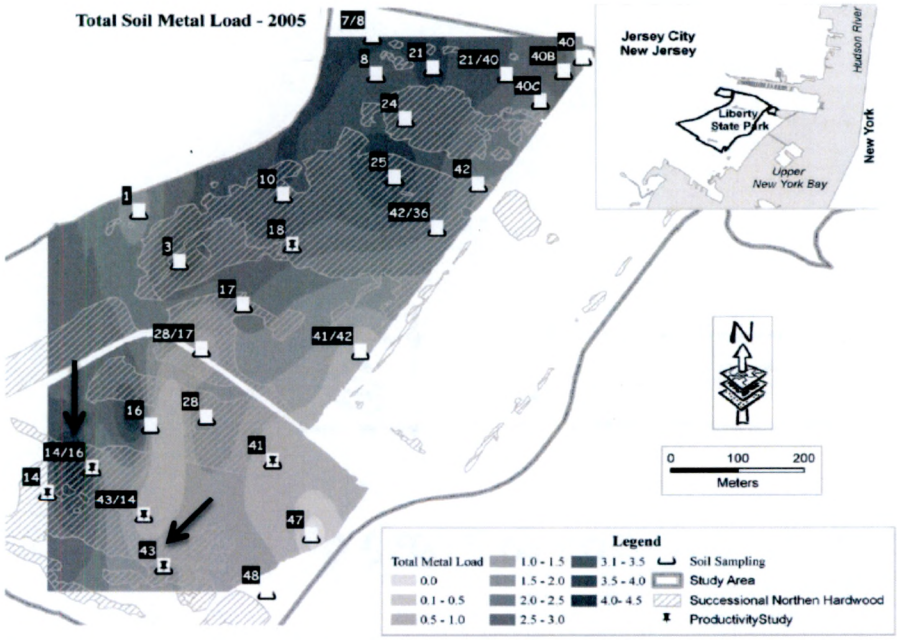


Figure 2. Map of Liberty State Park (*copied from Gallagher et. al.*[33]).
A map of Liberty State Park taken from Gallagher et. al.[33] that numerically classifies the metal gradient. The total metal load increases with the shade of gray. Two sites (indicated with the black arrows) were studied: a low metal load (LSP 43, light shade) and a high metal load (LSP 146, dark shade).

Indicated with a black arrow in Figure 2 (*copied from Gallagher et. al.*[33]), only two sites from LSP were observed in this study. In increasing metal loads, they were sites 43 (LSP 43) and 14/16 (LSP 146), which represented a low and high metal gradient with

similar physical characteristics of moisture and pH. The vegetation within these sites were identified by the United States Army Corps of Engineers as successional northern hardwood,[26] including species of *Betulla populifolia* (grey birch), *Populous deltoids* (cottonwood) and *P. tremuloides* (quaking aspen)[24].

1.6. Overall Purpose and Chapter Synopsis

The presence of a forest at LSP, despite its history of heavy metal contamination makes the soil biota an interesting potential source for the competitive microbial species used in bioremediation techniques. In order to understand the unique phenomenon at LSP, we have to understand the enzymatic capabilities of its microbes, the "drivers" of its plant diversity and productivity[1]. Accordingly, this thesis is a collection of three individual chapters that uses extracellular enzyme activity to characterize the influence of the microbes at LSP. It begins in Chapter 2—*Enzyme Activity and Metal Concentrations at LSP and HMF*—with the quantification of the metals and enzyme activity of the two study sites, compared to a reference site that has no history of heavy metal contamination. The subsequent chapter, Chapter 3: *The Effect of Cross-Inoculation on LSP's Microbial Community*, observes the behavior of LSP's microbes when introduced to a different environment via cross-inoculation and biostimulation. And finally, Chapter 4 (*The Effect of Storage Conditions on Enzyme Activity*) is a physical characterization study to determine the optimum storage conditions that minimizes changes in enzyme activity over time. Collectively, this research provides insight into the interactions between the metals and microbes at LSP and interesting findings that can be applied to the sustainable bioremediation of heavy metal contaminated sites.

2. Chapter 2: Enzyme Activity and Metal Concentrations at LSP and HMF

2.1. Introduction

The productivity of soil is dependent on a variety of natural processes, most of which are mediated by microorganisms in the soil. As a result, any repressive changes to soil microbes, caused by pollutants or toxic agents, can damage their innate ability to produce enzymes and stimulate plant growth.[4,5,19] Heavy metals are classified as the most toxic inorganic pollutants to soil microbes[4]. According to Leita et. al.[14], some adverse effects of heavy metals on microorganisms include the disruption of function, reduction of metabolic rate and significant decrease in diversity[14]. The purpose of this section of the research was to identify the relationship between the microorganisms and metals at LSP by quantifying the extracellular enzyme activity and heavy metal concentration.

As discussed in Chapter 1 (*see Table 1, Chapter 1, Section 1.1*), some of the processes facilitated by microbes include the transfer of organic matter throughout the soil ecosystem and the mineralization of elemental nutrients—N, C, P and S—necessary for plant growth[4,5,8]. During these processes, microorganisms exude requisite enzymes—that can be representatives of specific nutrients—as reaction catalysts[27,28,29]. Due to this important function of enzymes in the soil, extracellular enzyme assays have been used to understand soil productivity and microbial function[5,9,10,30]. Assays have also been used to analyze the effect of heavy metal pollutants on soil microbes in numerous research projects, including the works of Kandeler et. al.[5], Nannipieri, P.[29], Baath, E.[31], and Tyler, G.[32]. In accordance with a majority of other studies, these authors concluded that while there are other determinative factors—such as the soil environment and the specific metal or enzyme measured—the presence of heavy metals in the soil severely damages the

function and diversity of soil microbes. Therefore, an increase in heavy metal concentration dictates a decrease in extracellular enzyme activity.[5,29,31,32].

Previously researched enzymes in soil include L-leucine-amino peptidase, cellobiohydrolase and alkaline phosphatase, respectively representative of the N, C and P nutrient cycling in soil[5,8]. Multiple studies have shown that of the three most studied enzymes, the most dramatic change in enzyme activity was observed in alkaline phosphatase activity[5,20]. During catalysis, phosphate groups are released from phospho-monoesters. In this experiment, alkaline phosphatase assay is used to understand the function of enzymes in the P-cycling of LSP's metal laden soil.

The heavy metals found at LSP—namely V, Cr, Cu, Zn, As, and Pb—have different concentrations across the site *(see Figure 2, Chapter 1, Section 1.5)*[20,33]. The concentration of metals that are present in the soil strongly influences the environmental impact that the metals can have on soil enzymatic function. As explained in Chapter 1, Section 1.3.1, extremely high concentrations of heavy metals are detrimental to soil environments.[13,14] When quantifying heavy metal contamination, most studies extract and measure the total concentration of metals in the soil. However, according to Shivakumar et. al.[35], most researchers now believe that determining the total metal concentration is not an accurate estimation of the environmental impact of the contamination[35]. This is because simply using the total concentration analysis incorrectly implies that the different forms a metal can adopt in soil impacts and interacts with soil microorganisms equally.[35,36,37]

There are five common geochemical forms that contribute to the total metal concentration; and metals can persist in soil in any one or more of these forms, which are

classified by the metal's solubility and mobility. Both solubility and mobility affect how easily a metal is released into the soil and its interactions with microorganisms within the soil. Therefore, the specific form in which metals dwell in soil influences their level of toxicity to soil microorganisms and enzymes. The more mobile or soluble the metal form, the more available and accessible the metal is to soil microorganisms; thus, the more harmful its effects.[37,38,39,40]

The classification of metal forms is known as metal speciation, which is defined as "the identification and quantification of the different, defined species, forms or phases in which a metal occurs"[41]. The environmental variables that affect metal speciation or forms in soil include temperature, adsorption capability of the metal, time of metal contamination, and the pH of the soil. The latter—pH—is the most influential factor because changes in pH can have significant impact on metal solubility.[41,42] Therefore, most research projects that analyze the different forms of metal use sequential selective extraction (SSE), a method first developed by Tessier et. al.[36] in 1979—modified by multiple authors since—that uses extracting reagents of varying pH to sequentially isolate the metal forms[35,36,37,38,40,43]. In increasing order of mobility, or availability to enzymes and microbes, metal forms are classified as residual, organic matter bound, Fe—Mn oxide bound, carbonate bound, and exchangeable[37,38,39,40].

The least mobile fraction is the residual fraction. It is also known as the crystalline fraction because it consists of metals that are contained within the crystal structure of chemically inert minerals. For this reason, the residual fraction is not easily available to soil microorganisms and enzymes.[35,39,42] In fact, when a majority of the metals in the soil are in the residual form, the immediate hazard they present to their environment is

negligible[42,44]. Excluding the residual metal form, the sum of the other four fractions—organic matter bound, Fe—Mn oxide bound, carbonate bound, and exchangeable—is known as the non-residual form (Figure 3, *modified from John and Leventhal*[42]). [39,40,42,45,46]

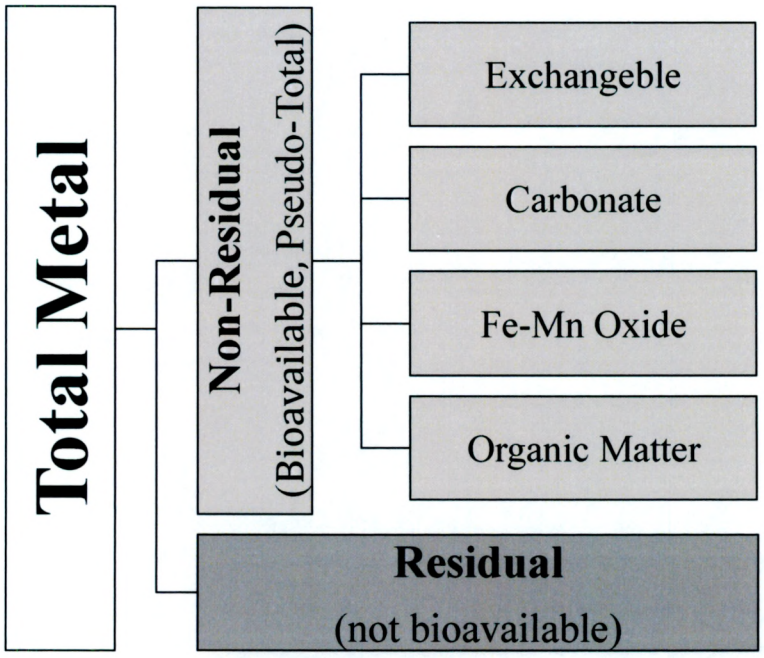


Figure 3. The Chemical Forms of Metals *(modified from John and Leventhal*[42]*).*

The two different chemical forms that constitute the total metal concentration (white rectangle), are the non-residual (light gray) and residual (dark gray) forms. Non-Residual is easily available to the environment and can further be separated into organic matter, Fe-Mn oxide, carbonate and exchangeable, in increasing order of availability. Residual is not easily available to the environment.

The metal fractions that constitute the non-residual metal forms are more available and mobile than the metals of the residual metal form. Additionally, any slight modifications in environmental factors—including pH and temperature as mentioned previously—can make non-residual metal forms readily available for microbes and

enzymes. Since they're easily available to their environment, non-residual metals are commonly referred to as environmentally available or bioavailable metals. [35,42] In this experiment, a single step extraction technique—EPA method 3050*b*[34]—was used to quantify the total metal concentration for six metals (*detailed method forthcoming in Section 2.2.5.2*). According to the EPA, this method is not a "total digestion technique". It was designed to dissolve most "environmentally available" metals. The EPA also cautioned that another method should be employed for absolute total digestion.[34] Thus, our analysis of total metals is referred to as 'pseudo-total metal' because it only consists of the bioavailable, non-residual metal forms; rather than both the residual and non-residual metal forms (*see Figure 3, Section2.1*). Note that any mention of total metals hereafter is referring to pseudo-total metals, unless specified otherwise.

Since the pseudo-total metal concentration includes just the four bioavailable metal forms (*See Figure 3, Section 2.1*), it represents the maximum amount of metals that could be mobilized in its environment[35,39,42,45]. Table 2 briefly identifies unique properties for each bioavailable metal form and their relative availability to soil biota. Within the pseudo-total metal fractions, possible interactions between the metal and soil particles are highest for the exchangeable fractions and decreases respectively[39,40,45,46] (Table 2). Numerous studies—including work conducted by Ma and Rao[40], and Sanghoon[47]—coherently report that the exchangeable metal fraction is the most mobile and bioavailable form of the pseudo total metals. In this experiment, the first step of the sequential selective extraction—modified by Hass and Fine[39]—was used to isolate the exchangeable metal fraction; and on a different unaltered soil sample, a single step

extraction[34] was used to isolate the pseudo-total metal (detailed method forthcoming in Section 2.2.5.1 and 2.2.5.2, respectively).

Table 2: Relative Mobility and Availability for Pseudo-Total Metals (Bioavailable Forms) (*modified from John and Leventhal[42]*)

Relative Mobility *(Availability of metals to enzymes)*	**Metal Form**	**Form Property**
Most Available	Exchangeable	Bound to particulate matter by electrostatic exchange mechanisms
	Carbonate	Bound to carbonate minerals and sedimentary rocks
	Fe-Mn oxides	Adsorbed to iron-manganese oxide particles
Least Available	Organic Matter	Bound to various forms of organic matter

Table 2: Relative Mobility and Availability for Bioavailable Metal (Pseudo-Total) Forms (*modified from John and Leventhal[42]*). Shows each metal form, their unique properties, and their relative availability to soil microbes and enzymes.[36,42] The pseudo-total metal form represents only the bioavailable or non-residual forms of metals. The residual metal form is not listed here since metals in this form are not bioavailable; rather, they are stuck within crystal lattices.

Overall, this chapter details the phosphatase assay and metal concentrations measured at LSP, in comparison to a reference site with no history of heavy metal contamination, Hutcheson Memorial Farm (HMF). The purpose of this chapter was to understand the relationship between extracellular enzymes produced by microbes and metals at LSP. The objectives were to:

A. Measure the exchangeable metal concentrations of metals (V, Cr, Cu, Zn, As, Pb) at LSP 43, LSP 146 and HMF,

B. Measure the total metal concentrations of metals (V, Cr, Cu, Zn, As, Pb) at LSP 43, LSP 146 and HMF and

C. Use extracellular enzyme activity assay to quantify the phosphatase activity of LSP 43, LSP 146 and HMF.

The exchangeable metal fraction, which are the metals loosely bound to the soil, were extracted by changing the ionic composition of the soil[43] *(see detailed method in forthcoming Section 2.2.5.1)*. The pseudo-total metal concentration, which includes the exchangeable metal fraction and three other metal forms—organic matter bound, Fe—Mn oxide bound, carbonate bound, and exchangeable—was determined using EPA method 3050*b*[34] *(see detailed method in forthcoming Section 2.2.5.2)*. Extracted metal samples were analyzed using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). After quantification of the heavy metals, enzymatic assay analysis was used to measure the phosphatase activity of the soil. These parameters were measured for soil samples from LSP site 43, LSP site 146, and reference site HMF.

2.2. Protocol

2.2.1. Study Sites

Three sites were studied in this chapter: two sites at Liberty State Park (LSP)—Site 43 (LSP 43) and Site 146 (LSP 146) and the reference site Hutcheson Memorial Farm (HMF).

2.2.1.1. Liberty State Park (LSP)

As detailed in the thesis introduction (Chapter 1, Section 1.5), the area observed within LSP (40° 42' 16" N, 74° 03' 06" W) was fenced off and abandoned in 1975 due to severe heavy metal contamination. It was never cleaned up; yet, there is currently an active and diverse forest. *Please refer to Chapter 1, Section 1.5 for a description of LSP.*

2.2.1.2. Hutcheson Memorial Farm (HMF)

The area used as a reference site to compare results from LSP is located (40° 30' N, 74° 34' W) within Rutgers University Hutcheson Memorial Forest (HMF). HMF (Franklin Township, NJ) is approximately 40 mi from LSP. They share equivalent exposure to climate and geographic influences. Both have also undergone natural succession for a similar length of time. The main difference is HMF has no history of exposure to heavy metals. Administered and protected by Rutgers University, HMF serves as a comparable indicator of average enzyme activity and heavy metal concentration in an uncontaminated site.[20]

2.2.2. Soil Collection

Soil collection from HMF was completed on October 3, 2014 and 10 days later, on October 13, 2014, soil was collected from both sites at LSP. Each site is systematically divided into 3 transects, labeled A, B and C, with 5 pins constituting each transect (Figure 4). In this experiment, soil was cored from each pin in a site, resulting in a total of 15 distinct soil samples from each site. Only the top 1 – 5 cm of soil inside the core was used. At the lab, each soil sample was sieved separately through a 2 mm sieve. Then equivalent amounts from each of the five pins were amassed into a representative sample for its corresponding transect. Thus, each site was characterized by three unique

samples—A, B and C. Samples were stored in a refrigerator (4 °C) to be used for enzyme activity and metal concentration measurements.

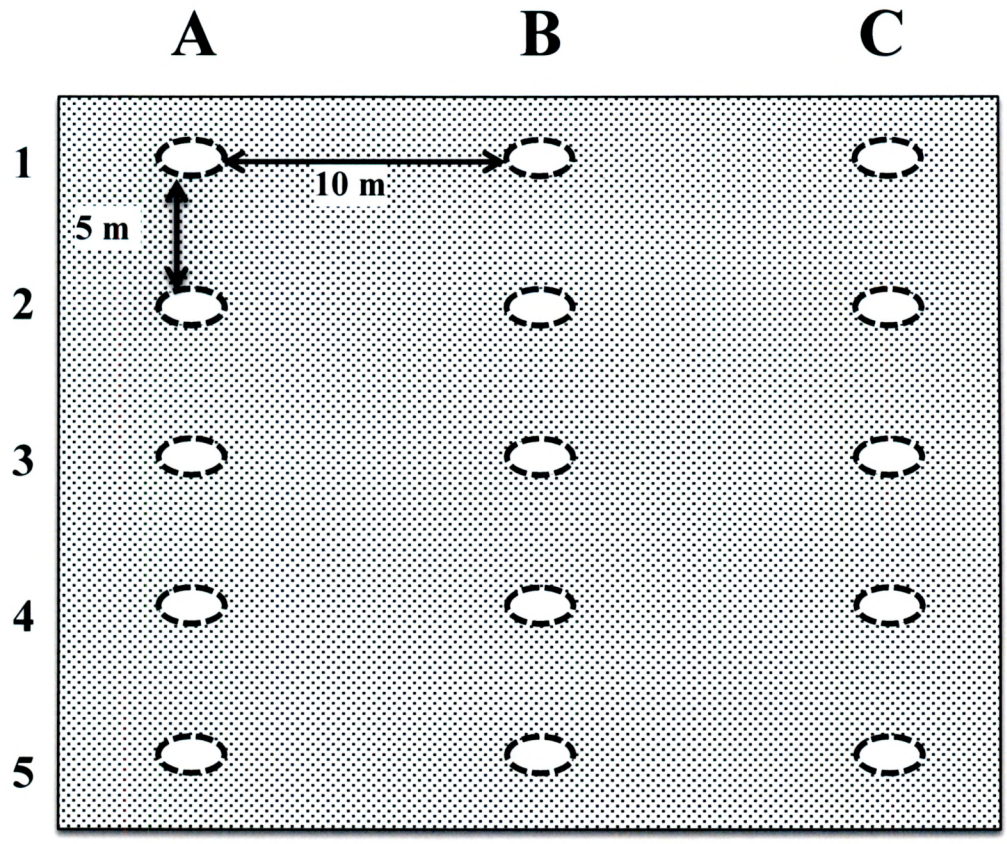


Figure 4. Layout of the Soil Sites.

Each soil site at both HMF and LSP is organized into three fixed transects, labeled A, B and C, that are approximately 10 m apart. Subsequently, each transect is divided into five distinct pins that are spaced out by about 5 m (represented by dotted ovals in the figure). During collection, soil samples are cored from the pins. This allows for sampling that is thoroughly representative of the site, and a relative consistency in soil samples from different collection dates.

2.2.3. Phosphatase Activity Assay

Phosphatase activity was measured for each of the three samples—A, B, and C—at each of the three sites—HMF, LSP 43, and LSP 146. A slightly modified fluorometric

assay protocol from Marx et al.[9] and Morrissey[48] was used. All Reagents were acquired from Sigma-Aldrich Co. LLC. 2-(N-morpholino)-ethanesulfonic acid, commonly known as MES buffer (0.1 M, pH = 6.0, 100 mL), was added to a 125 mL flask containing soil (0.1g). While enzyme activity was measured with unaltered soil, the result was reported in grams of dry soil, calculated via gravimetric analysis (See 'Percent Moisture' Chapter 2, Section 2.2.4). The soil slurry, mixture of soil and buffer, was homogenized via continuous sonication for 3 minutes at an output setting of 25 W. Afterwards, as the sonicated soil slurry stirred on a stir plate, 160 μL was pipetted into 8 wells of a 96-well black plate for a total of 1 column for each sample.

After all the sonicated soil samples were added to the plate, dilutions of 4-methylumbelliferone (MUB) were prepared for the standard curve required to analyze the fluorescent products. An MUB stock solution (200 μM) was made by mixing MUB (20 mg/mL, 8.81 μL) and Deionized (DI) water (4,991.2 μL) in a 50 mL falcon tube. From the 200 μM MUB stock, 4 dilutions (5 mL) were prepared for the standard curve, targeting approximate product concentrations of 0 pmol, 500 pmols, 1000 pmols, 1500 pmols, and 2500 pmols. A distinct standard curve was measured for each soil sample, using 5 of the 8 wells designated for each sample on the plate.

Each sample's enzyme activity was measured in replicates of three, using the remaining 3 of the sample's 8 wells. The substrate analog, 4-MUB-phosphate (Sigma-Aldrich #M8883, 350 μM in well) was prepared by adding 189.91 μL of a stock solution (10 mg/mL in DI water) to DI water (1,810.09 μL).

The substrate (4-MUB-phosphate), each dilution of the product (MUB), and DI water, were poured into labeled plastic troughs. The computer and instrument (Molecular

Devices M3) were turned on and the instrument temperature was set to 30 °C. The plate reader was set to kinetic fluorescence (excitation: 320 nm, emission: 450 nm) with a low PMT setting; a total run time of 6 hours with readings every 15 minutes (for a total of 24 reading time points); and shaking before and after each reading. Then, 40 μL of the prepared solutions were added to the plate in this order: DI water was added to the fourth row; the MUB dilutions were added in increasing concentrations to the 5^{th} through 8^{th} rows; and finally, MUB-phosphate was added to the first three rows. During addition to the plate, solutions were mixed thoroughly with the soil slurry by pipetting up and down a few times. The plate was placed in the instrument immediately after the MUB-phosphate was added and reading of the samples began.

For each soil sample (single column on the well plate), and for each of the 24 time points, a standard curve of fluorescence emissions versus concentration was generated. These standard curves were used to calculate the amount of product produced by each of the 3 sample wells at each corresponding time point. The resulting value was the phosphatase activity of the soil: the amount of product generated by a sample of dried soil over time. The final unit for the activity was $pmols/g_{dry}/hr$.

2.2.4. Percent Moisture

Percent moisture measurements gave insight into the moisture levels of the soil. The procedure also provided the soil's dry-weight, which was needed to complete enzyme activity calculations. The weight of an empty crucible was recorded. A sample of soil, approximately 2 – 3 g, was placed into the crucible to give the "crucible + soil" value. The "crucible + soil" was placed into an oven (~ 70 °C) for at least 24 hours, then weighed again to give the "crucible + dry soil" value. The moisture was the difference

between the dry and original soil for each sample, divided by the amount of original soil. Percent moisture is the product of this value and 100, giving a unit of percentage.

2.2.5. Heavy Metal Determination

The unusual abundance of metals at LSP is at the forefront of this research. Therefore, two different analysis of metal concentration was used: the exchangeable metal analysis measured the bioavailable metal fraction, while the total metal analysis quantized the maximum amounts of soluble metals in the soil. The concentrations of all six metals (V, Cr, Co, Zn, As, Pb) were determined for each analysis.

2.2.5.1. Exchangeable Metal Analysis

The concentration of exchangeable metals was determined based on a protocol by Hass et. al.[43]. Soil samples were dried ($\geq$ 24 hrs, 70 °C) and the dry weight was recorded. The dried soil sample (1 g) and a solution of Magnesium Nitrate [$Mg(NO_3)_2$] (0.1 M, pH = 6, 10 mL) was mixed in a 50 mL falcon tube. The resulting slurry was placed horizontally on a shaker for approximately 2 hours at medium speed, and then centrifuged (30 mins, 2500 rpm). The supernatant was decanted through a Whatman 42 filter paper into a 15 mL falcon tube. This filtered solution was used for exchangeable metal analysis. The exchangeable concentration of metals in the samples was measured using ICP-MS.

2.2.5.2. Pseudo-Total Metal Analysis

The total metal concentration was determined using EPA method 3050*b*[34]. HNO_3 (50%, 10 mL) was added to a sample of dried soil (1g) and the mixture was heated to 90 °C. Immediately after, it was allowed to cool for approximately 15 min. Then, 5 mL of

concentrated HNO_3 was added. The solution was heated again to 90 °C. If brown fumes formed during heating, an additional 5 mL of concentrated HNO_3 was added. The solution was then reduced to 5 mL, without boiling; afterwards, DI water (2 mL) and H_2O_2 (3 mL) were added. After addition, the solution was heated again. Small amounts of H_2O_2 (not exceeding 10 mL) were added periodically to the heating solution until bubbling stopped. Finally, the solution was reduced again to 5 mL, allowed to cool, and then diluted to 50 mL. For HMF soil samples, 15 mL of this solution was used for metal analysis; for LSP soil samples, the 50 mL solution was again diluted (0.75 mL:15 mL) with 1 % Nitric acid to reduce its salt content because highly concentrated solutions could clog the instrument. The total concentration of metals in the samples was measured using ICP-MS.

2.3. Results

The purpose of this chapter was to understand the relationship between extracellular enzymes produced by microbes and metals at LSP. The objectives were to use extracellular enzyme activity assay to quantify the phosphatase activity of LSP 43, LSP 146 and HMF, measure the exchangeable metal concentrations of metals (V, Cr, Cu, Zn, As, Pb) at LSP 43, LSP 146 and HMF and measure the total metal concentrations of metals (V, Cr, Cu, Zn, As, Pb) at LSP 43, LSP 146 and HMF.

2.3.1. Metal Concentrations

The concentrations of six heavy metals (V, Cr, Cu, Zn, As, and Pb) were determined at all three sites (LSP 43, LSP 146 and HMF). Two types of metal forms were measured, the exchangeable and the total metal.

2.3.1.1. *Exchangeable Metals*

Exchangeable metals—metals that are loosely bound to the soil particles—were measured for six metals, V, Cr, Cu, Zn, As, and Pb, by changing the ionic composition of the soil[43, 39]. The exchangeable metal concentrations were highest at LSP 146 for all the metals, except Pb (Figure 5). Additionally, there were negligible concentrations of exchangeable metals at HMF, as expected, since HMF has no history of heavy metal contamination.

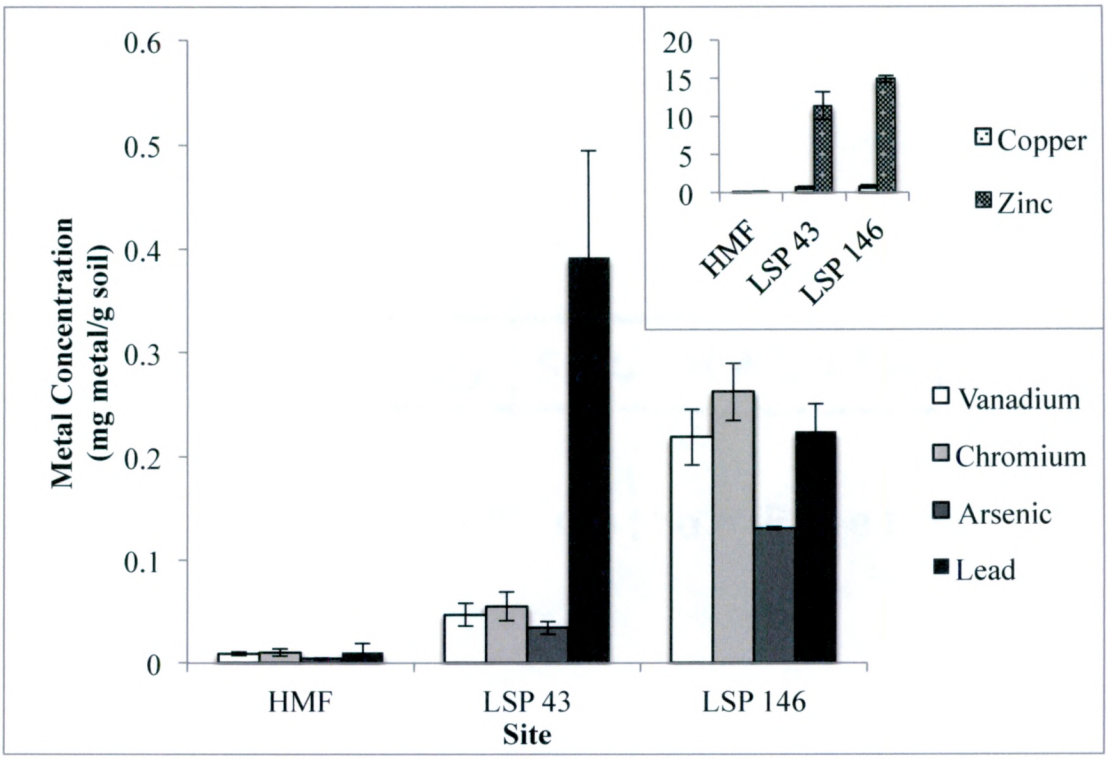


Figure 5. Concentration of Exchangeable Metals for all the sites.

The average exchangeable metal concentration and standard error (n = 3). Exchangeable metals are the most bioavailable form of metal, and thus the most toxic. The concentration of exchangeable metals generally increases from HMF to LSP 43, with LSP 146 exhibiting the highest concentrations. For most of the metals, the exchangeable metal concentrations are below 1 mg/g; however, Zn (inset) has over 10 mg/g at both LSP 43 and LSP 146.

2.3.1.2. Total Metals

The total metal concentration (V, Cr, Cu, Zn, As, and Pb) was analyzed for soil samples from each of the three sites (LSP 43, LSP 146 and HMF). The metals were extracted via acid digestion and analyzed using ICP-MS. Similar to exchangeable metals, the total metal concentrations are greater at LSP than at HMF (Figure 6). For most metals, including Vanadium, Chromium, Arsenic and Lead, LSP 146 has the highest metal concentrations; however, for Copper and Zinc (Figure 6 Inset), LSP 43 and 146 have comparable metal concentrations.

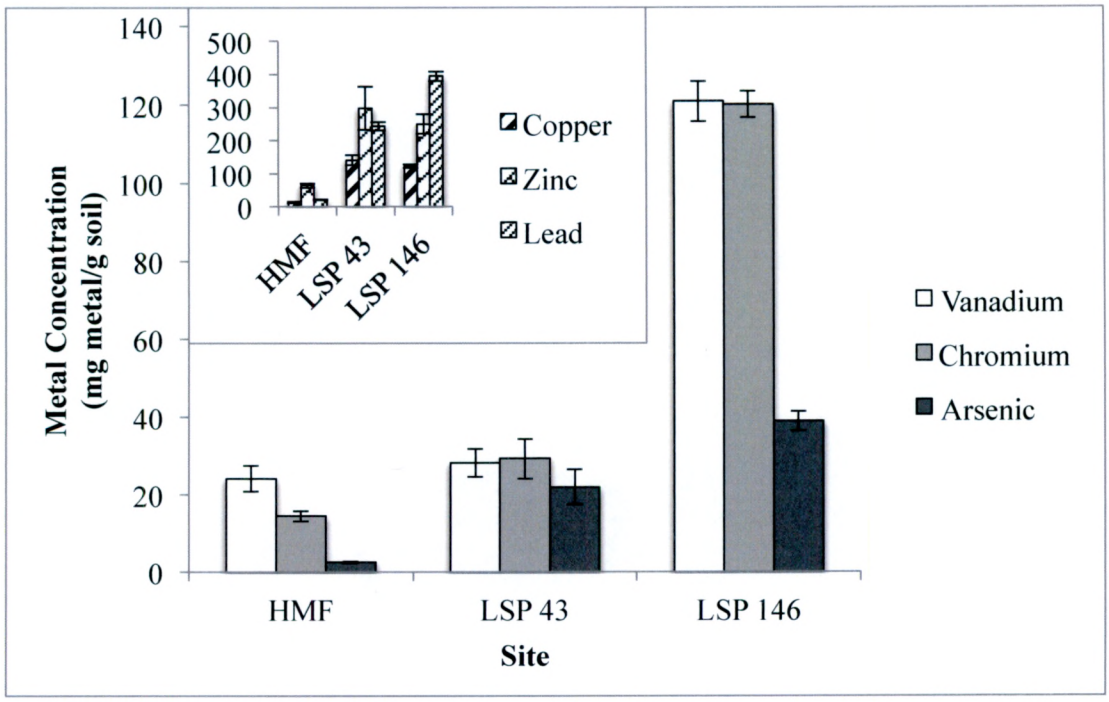


Figure 6. Total Metal Concentration for all the sites.

The average total metal concentration and standard error (n = 2) for all three sites. LSP has more metals than HMF. For Vanadium, Chromium, Arsenic and Lead, LSP 146 has the highest amount of total metal concentration. The total metal concentrations are highest for the metals Zinc and Lead, with values over 200 mg/g. *Thanks to Diane Hagmann for metal isolation and partial analysis of data in this figure*

Furthermore, recall from the chapter introduction (*see Section 2.1*) that the pseudo-total metal concentration are the metals that are easily available to soil organisms. There are four geochemical metal forms classified as pseudo-total metal. In increasing order of availability, they are carbonate bound, Fe—Mn oxide bound, organic matter bound and exchangeable. Since the concentration of both the pseudo total metal and one of its constituents-exchangeable metal fraction—was measured, we can also quantify the percentage of metals that can be classified as '*non*-exchangeable, yet mobile'. The '*Non*-Exchangeable Yet Mobile' metal form (referred to as NEYM for our purposes) is the difference between the pseudo-total metal and the exchangeable metal form. For example:

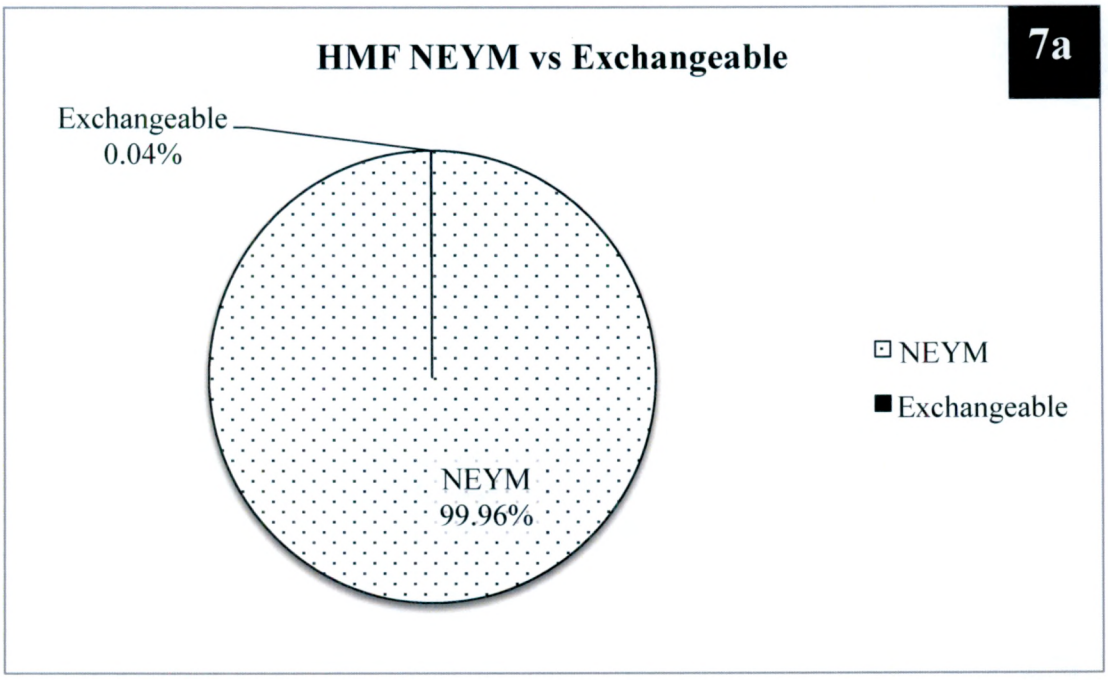
$$NEYM_{(Carbonate,Fe-Mn,OrganicMatter)} = Total\ Metal - Exchangeable\ Form$$

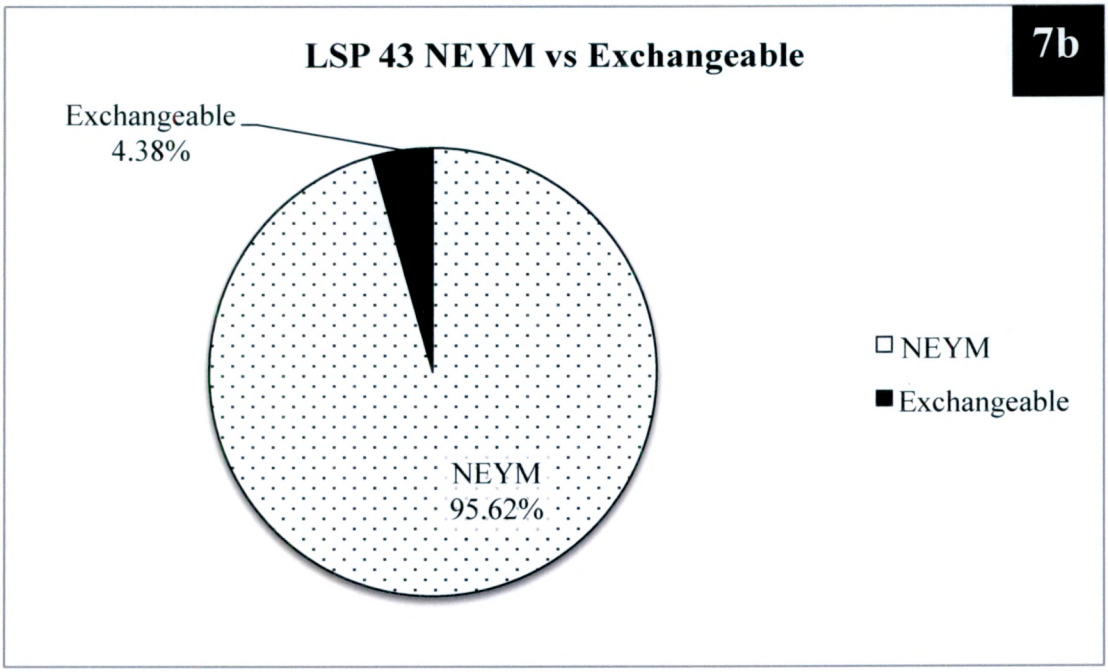
Equation 2.1: Calculation for the 'Non-Exchangeable Yet Mobile' Heavy Metal Forms. It is the difference between the pseudo total metal (total metal) and the exchangeable metal fraction. NEYM is less available and thus less toxic than the exchangeable metal form.

NEYM is an approximate representation of the three "other" bioavailable forms that metals can exist in soil—carbonate bound, Fe—Mn oxide bound, and organic matter bound. The metal forms included in NEYM are less bioavailable, and thus less toxic, to soil organisms than the exchangeable metal forms.

Figure 7 shows three graphs for HMF, LSP 43 and LSP 146, respectively, that analyzes the distribution of pseudo-total heavy metals between the NEYM form (polka-dot) and the exchangeable form (black). The entire circle represents the pseudo-total

metal concentration for all of the six metals measured. The white-filled, polka-dotted portion of each graph displays the NEYM forms, all of which are collectively less toxic than the exchangeable metal form (black-filled portion). This analysis assumes that the extraction of exchangeable metals was performed accurately.





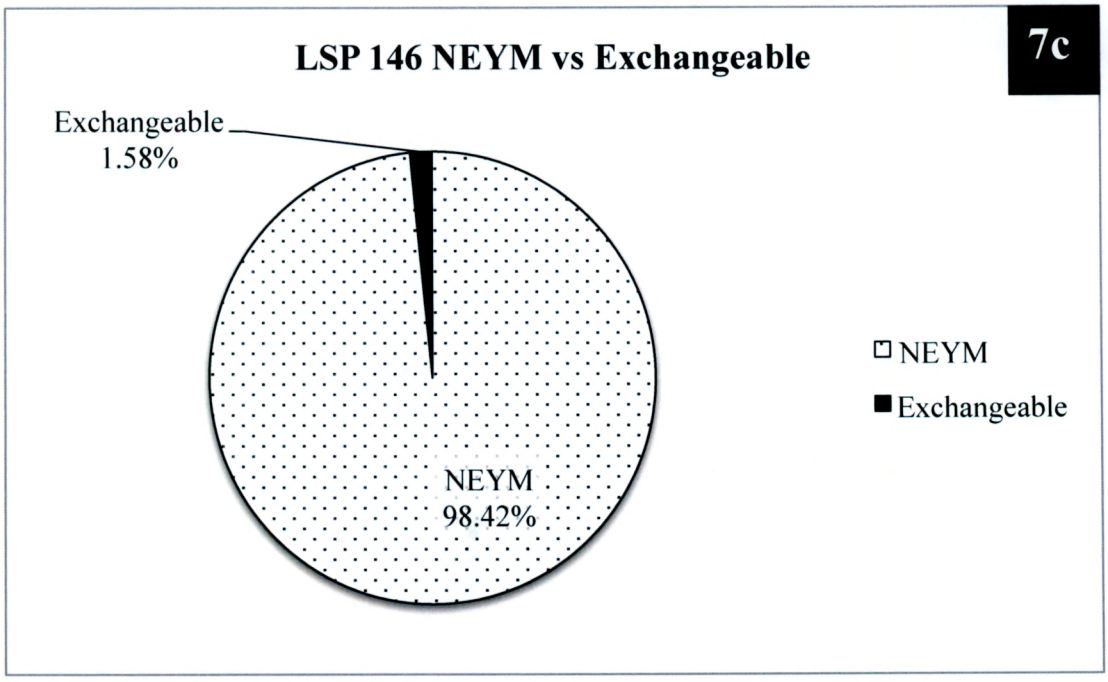


Figure 7. The distribution of Pseudo Total Heavy Metals at A) HMF B) LSP 43 and C) LSP 146.

This graph shows the relative amount of exchangeable metal (EX) and *non*-exchangeable yet mobile metal (NEYM) fractions that constitute the pseudo-total metals, for all six metals observed (unit is percent). The full circle represents the pseudo-total metal concentrations reported in Figure 6 (*see Section 2.3.1.2)*, the lowest percentages from the circle are magnified in the accompanying rectangle and "Other" in the dashed box is the total percent contribution of exchangeable metals. Among the pseudo total metal, the contribution of exchangeable metals is less than 0.03% at HMF, **B)** 4.49 % at LSP 43, and **C)** 1.53 % at LSP 146.

At HMF (See Figure 7a) a majority of the metals were in the NEYM form. The exchangeable metal fraction (black line in Figure 7a) only contributed about 0.04 % to the pseudo-total concentration; thus, 99.96 % of the metals at HMF were the less toxic NEYM metal forms (white polka-dot in Figure 7a).

LSP's ratio of exchangeable metal to total metal was slightly higher than at HMF. At LSP 43, the percentage of exchangeable metals was 4.49 % (black piece in Figure 7b);

and at LSP 146, the exchangeable metal concentration (black piece in Figure 7c) was a little lower at 1.52 %. Similar to HMF, the major form of metal at both LSP sites were the less bioavailable NEYM, which was approximately 95 % and 99 % for LSP 43 and LSP 146, respectively.

2.3.2. Phosphatase Activity

A phosphatase assay was conducted on soil samples from all three sites. This measure of enzyme activity is representative of the soil's ability to cycle nutrients, namely phosphorous, which is indicative of the overall health of the soil[8,9]. According to Figure 8, the soil at LSP 146 shows the highest amount of phosphatase activity, almost 4 times more than HMF, which has the lowest phosphatase activity. Additionally, LSP 43 exhibits phosphatase activity that is twice the activity at HMF.

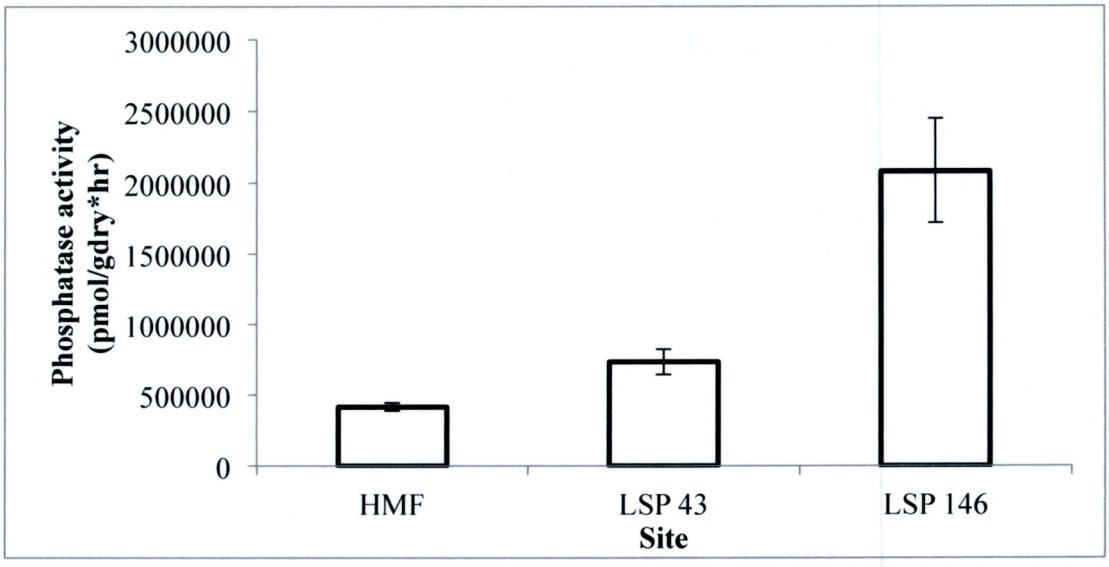


Figure 8. The Average Phosphatase Activity for all Soil Sites.

The average phosphatase activity and standard error (n = 3) of the three transects from each site. Phosphatase activity is significantly higher at LSP than at HMF. LSP 146 exhibits the highest amount of phosphatase activity, approximately 4 times more than the activity at HMF. *Thanks to Diane Hagmann for partial measurement and analysis of enzyme data in this figure.*

2.4. Discussion

There is an interesting phenomenon occurring at LSP because despite its history of heavy metal contamination, it still sustains an active and diverse forest (*see Chapter 1, Section 1.5 for a description of LSP).* In this chapter the concentration of heavy metals and the phosphatase enzyme activity was analyzed for two sites at LSP—Site LSP 43 and LSP 146. Results from both LSP sites were also compared to a reference site with similar natural succession as LSP, called HMF (*see Section 2.2.1.2 for a description of HMF).*

The first objective was to measure the pseudo-total metal concentration for six metals—V, Cr, Cu, Zn, As, Pb—at LSP 43, LSP 146 and HMF. Recall from the chapter introduction (*Section 2.1)* that the pseudo-total metal measured in our experiment is not the same as the total metal concentration. There are five fractions within the total metal concentration, and they can be separated into two forms: residual (1 of 5 forms) and non-residual (remaining 4 forms) (*See Figure 3*[42]*, Section 2.1).* Metals in the residual metal form are not easily available and are relatively harmless to soil organisms because they are contained within the crystal structure of chemically-inert minerals[35,39,42]. Since the residual metal form cannot easily interfere with microbial and enzymatic functions[42,44], it was not observed in this study.

On the other hand, the non-residual metal form interacts very easily with the soil environment, and is appropriately known as the bioavailable metal forms. The non-residual form is made up of fractions that differ by solubility and mobility. In order of increasing mobility they are organic matter bound, Fe—Mn oxide bound, carbonate bound, and exchangeable metal fractions[39,40,42,45,46]. The method used in our analysis of the total metal concentration (EPA method 3050*b*[34]) is not a "total digestion technique",

so it only extracted the non-residual metal form[34], rather than both the residual and non-residual forms collectively referred to as total metal. Therefore, we refer to our total metal digestion as 'pseudo-total metal' because it only consists of the bioavailable or non-residual metal forms *(see detailed method in Section 2.2.5.2)*.

The data showed that LSP had a higher concentration of pseudo-total heavy metal than the reference site, HMF *(see Figure 6, Section 2.3.1.2)*. The pseudo-total metal concentration was generally higher for all metals at both LSP 43 and LSP 146 than at HMF, with LSP 146 exhibiting the highest concentrations (4x the reference). HMF, which has no history of metal contamination, had the lowest concentration of heavy metals, as expected.

The second objective was to isolate and quantify the exchangeable metal concentration of metals (V, Cr, Cu, Zn, As, Pb) at LSP 43, LSP 146 and HMF. Recall that the exchangeable metal is the most mobile and bioavailable form among the non-residual metal forms[22,47]; and thus, it is the most toxic to soil microbes and enzymes[36,42]. In our experiment, the exchangeable metal concentration was isolated using the first step of a sequential selective extraction that was modified by Hass and Fine[39] *(see detailed method in Section 2.2.5.1)*. Similar to the results from the pseudo-total metal concentration, LSP 146 had the highest concentration of exchangeable metal, followed by LSP 43 and finally HMF, with negligible amounts of exchangeable metal fractions.

The third objective was to use extracellular enzyme activity assay to quantify the phosphatase activity of LSP 43, LSP 146 and HMF. A slightly modified fluorometric assay protocol from Marx et al.[9] and Morrissey[48] was used. The highest enzyme activity

was exhibited by soil samples from LSP 146, followed by LSP 43 and finally, HMF showed the lowest levels of enzyme activity (*see Figure 8, Section 2.3.2)*.

Overall, the purpose of this chapter was to understand the relationship between extracellular enzymes produced by microbes and metals at LSP. The extracellular enzyme measured was the phosphatase activity; and the two types of metal concentrations quantified were the pseudo-total form and exchangeable fraction. Pseudo-total form includes all four bioavailable forms a metal can persist in soil, and the exchangeable fraction is just the most bioavailable of the four forms contained in the pseudo-total metal.

The following figure (Figure 9) shows correlation graphs between the phosphatase activity and the exchangeable metal fraction for each of the six metals analyzed: **A)** Vanadium **B)** Chromium **C)** Copper **D)** Zinc **E)** Arsenic and **F)** Lead. For all metals, except for lead, the site with the highest exchangeable metal concentration (LSP 146, dark gray triangle) also has the highest phosphatase activity.

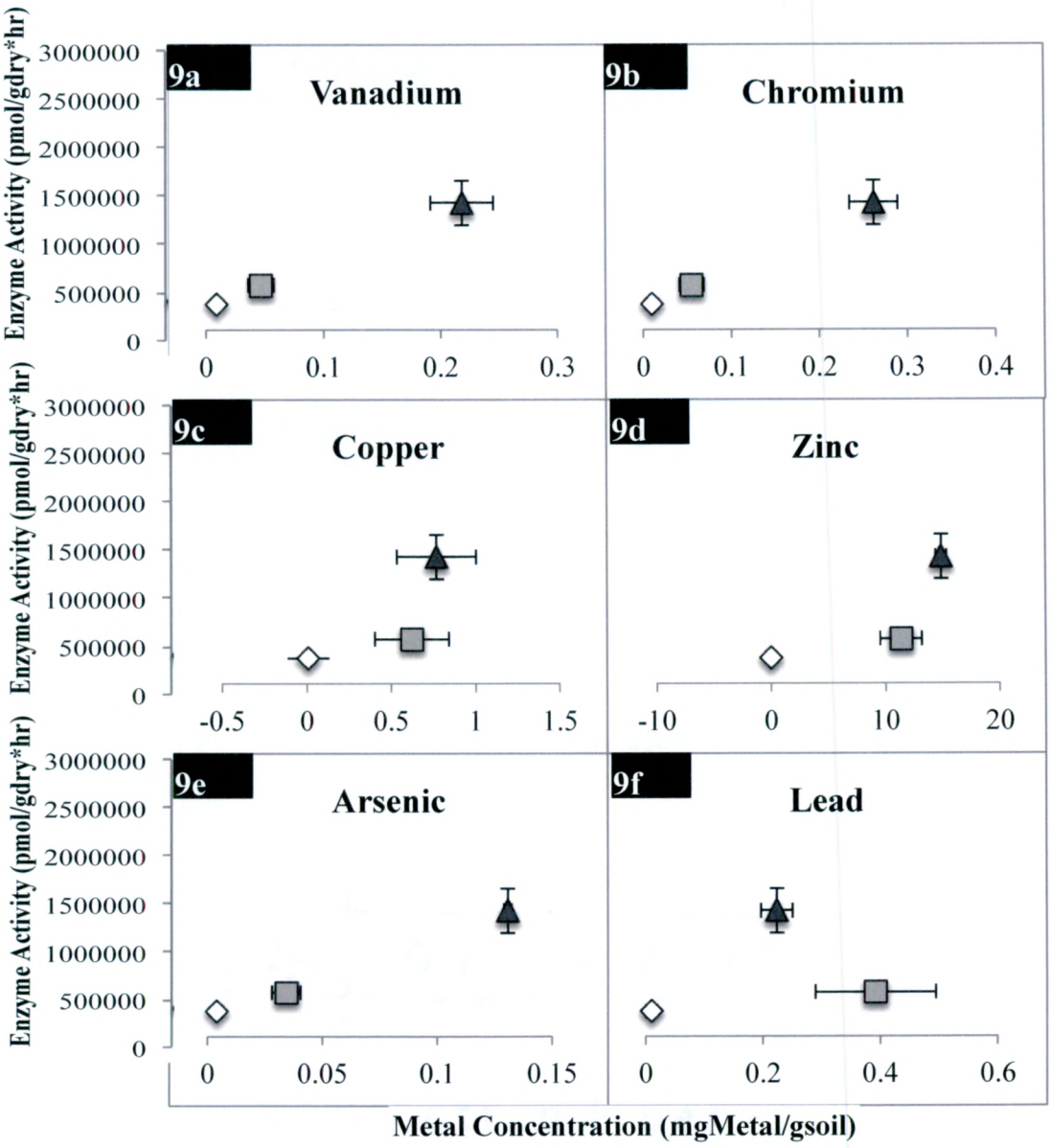


Figure 9. Correlation between Phosphatase Activity and Exchangeable Metal.
This graph shows the phosphatase activity (y-axis) and exchangeable metal concentration (x-axis) for all six metals: **A)** Vanadium **B)** Chromium **C)** Copper **D)** Zinc **E)** Arsenic and **F)** Lead in HMF (white diamond), LSP 43 (light gray square) and LSP 146 (dark gray triangle).

Similarly, Figure 10 shows correlation graphs between the phosphatase activity and the pseudo-total metal for each of the six metals analyzed: **A)** Vanadium **B)** Chromium

C) Copper **D)** Zinc **E)** Arsenic and **F)** Lead. For all metals, except for copper and zinc, the site with the highest pseudo-total metal concentration (LSP 146, dark gray triangle) also has the highest phosphatase activity.

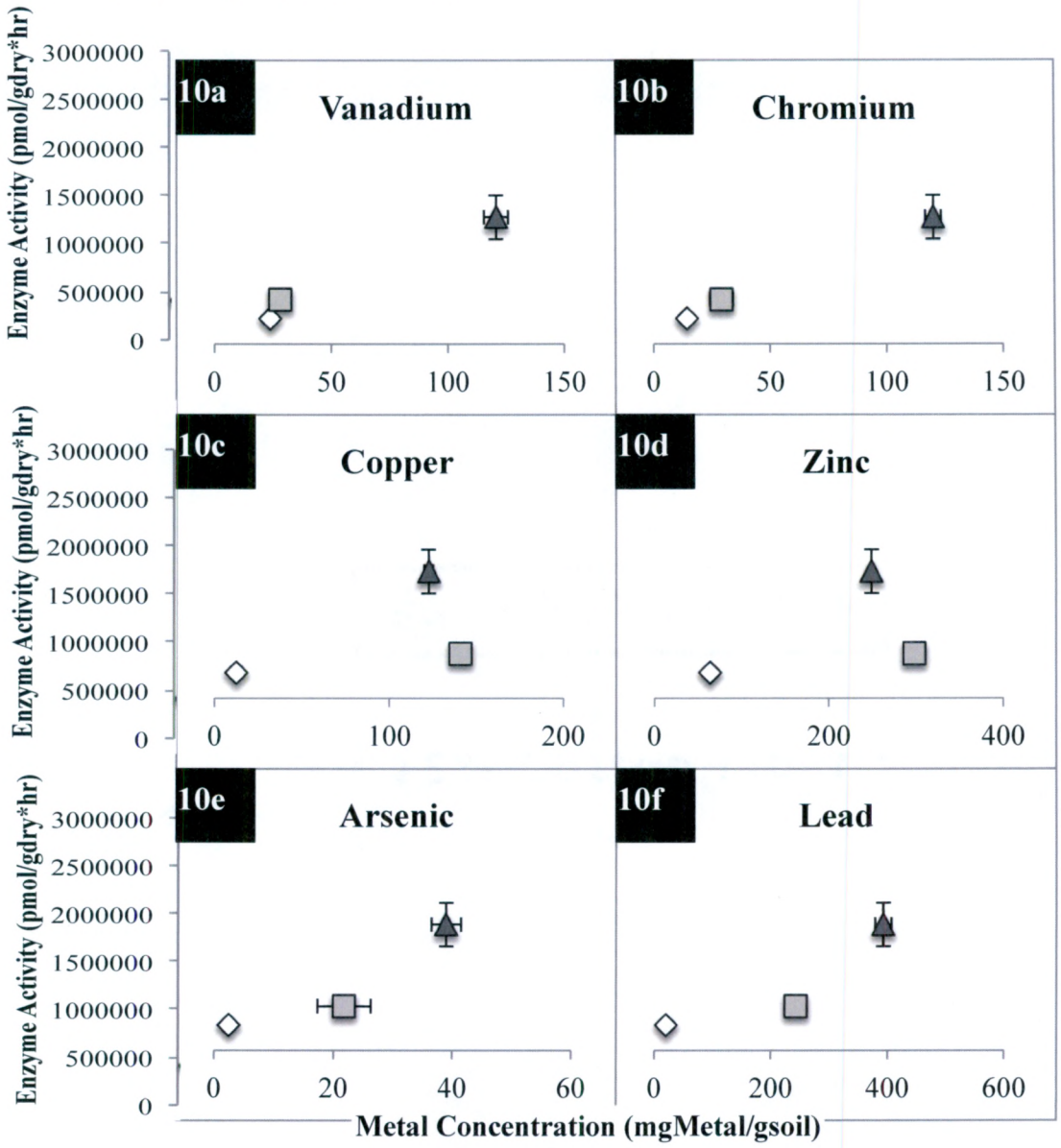


Figure 10. Correlation between Phosphatase Activity and Pseudo-Total Metal.
This graph shows the phosphatase activity (y-axis) and pseudo-total metal concentration (x-axis) for all six metals: **A)** Vanadium **B)** Chromium **C)** Copper **D)** Zinc **E)** Arsenic **F)** Lead in HMF (white diamond), LSP 43 (light gray square) and LSP 146 (dark gray triangle).

For both exchangeable metals and pseudo-total metals (see *Figure 9 and 10, respectively*), both sites at LSP have a higher metal concentration and phosphatase activity than HMF, the reference site with no history of metal contamination. This is very interesting because several studies conclude that an excessive amount of heavy metals has adverse effects on the health, population, microbial community and enzymatic activity of contaminated soil[8,19,39,49]. Since the soil at LSP is highly contaminated, HMF is expected to have much higher enzyme activity than any soil at LSP. Yet, the opposite is observed *(see Figure 9 and 10)*.

Between LSP 43 and LSP 146, there was one hypothesis that was proven true. Recall that the potential level of toxicity associated with a metal site increases with the bioavailability of its metal pollutants[42,44]. Also recall that the exchangeable metal fraction, which was measured in this study, is the most bioavailable and thus most toxic fraction of the pseudo-total metals. Even though LSP 146 has a higher concentration of pseudo-total metals than LSP 43 (*see Figure 6, Section 2.3.1.2*), the portion of its pseudo-total metals that are exchangeable (1.54 %) is lower than LSP 43 (4.49 %) (*see Figure 7b and 7c, Section 2.3.1.2*). Since LSP 43 has more exchangeable metals—the most available metal fraction to soil microbes and enzymes—relative to its pseudo total metals—the total concentration of available metals—than LSP 146, this strongly supports the likelihood that the activity at LSP 43 will be lower than LSP 146, as is reported in Figure 8 (*see section 2.3.2)*.

This interesting phenomenon at LSP necessitates further research so that the soil can be understood and characterized. There are more complex interactions occurring among

the soil biota at LSP. Thus, in the following chapters, a variety of experiments are employed to further understand the relationship between the metals and microbes at LSP.

2.5. Conclusion

The heavy metals and phosphatase activity at LSP was quantified to explain LSP's flourishing forest, despite a history of severe heavy metal contamination. Accordingly, the most metal contaminated site at LSP, LSP 146, exhibits the highest levels of phosphatase activity. This is a unique phenomenon because many research studies show that the presence of heavy metals is known to be toxic to the health and life of soil microbes and enzymes[4,5,19]. It is possible that over time, the microbes at LSP have adapted into metal-resisting or pollutant-consuming forms[13,16,22], as discussed in Chapter 1 (*see section 1.4.1)*, that can still exude enzymes for nutrient cycling. Recall that competitive microbes that developed selective biochemical traits to adapt to their polluted environments are at the forefront of bioremediation[16,18,19,20], a more efficient and sustainable soil restoration strategy. The following chapter (Chapter 3) further explores the effect of LSP's microbes on enzymatic activity by observing their behavior when applied to bioremediation strategies.

3. Chapter 3: Extracellular Enzyme Activity at LSP during Bioremediation

3.1. Introduction

Soil habitats, such as LSP, can be characterized by both the biotic and abiotic factors. The abiotic factors are non-living, physical aspects of soil habitats, including sunlight, moisture, temperature, pH, pollutants, etc. The biotic factors are the total collection of living organisms such as plants, animals and microbes, which are small microorganisms in the soil[50]. Since both factors contribute to the development of soil environments, it is important to understand their influence in the bioremediation of contaminated sites. Bioremediation is the application of living organisms, such as microbes, to degrade, detoxify or stabilize hazardous materials, such as heavy metals, in the environment.[17,18,16] This chapter observes the application of bioremediation only in improving the enzyme activity of heavy metal contaminated soil. Heavy metal contamination hinders nutrient cycling—the enzyme-catalyzed mineralization of complex polymers into elemental nutrients for plant uptake. One consequence is a dramatic reduction in soil enzymatic activity because the ability of microbes to exude catalytic enzymes into the soil is disrupted. Recall from Chapter 2 (*see Section 2.4)* that LSP shows very high enzyme activity, despite contamination with heavy metals. The purpose of this chapter was to evaluate LSP's potential as a source of microbes that can improve the enzyme activity of contaminated soil by characterizing the influence of both the biotic (living organisms such as microbes) and abiotic (non-living such as metal contamination) factors on its enzyme activity during bioremediation.

Recall from Chapter 1 (*see Section 1.2)* that microbes are an integral part of nutrient cycling because they exude requisite enzymes that catalyze food-producing reactions in

the soil *(see other examples of microbial roles in Table 1, Chapter 1, Section 1.1)*. The term microbe, or microorganism, is used to broadly classify the living, microscopically-small organisms in a habitat. This includes bacteria, fungi, protozoa, etc. Similarly to plants, microbes need nutrients to survive. They get their nutrients from organic matter, such as dead leaves, twigs or animal remains. Another nutrient source for microbes are complex organic molecules such as amino acids and sugars that are released from plants roots—known as root exudates, which are discussed later in this chapter. The organic matter and complex organic molecules—cellulose, protein, ATP, chitin, etc.—are made of elements that plants need to survive—including P, S, C and O. However, plants are unable to break down these compounds to sequester these elemental nutrients. Fortunately, during decomposition of organic matter, microbes exude enzymes into the soil that hydrolytically catalyze the breakdown of these complex polymers—ATP, cellulose—into monomeric subunits—glucose, inorganic phosphate—for plant uptake. The cycling of both inorganic and organic nutrients for the soil would be impossible without the presence of microbes and enzymes in soil habitats. For these reasons, enzyme activity assays are reliable indicators of soil quality and health. [2,5,6,12,20]

While some microbial processes require certain metals (*see examples of microbial roles in Table 1, Chapter 1, Section 1.1)* in very low concentrations, most other metals—especially heavy metals such as Pb and Hg—are toxic to soil biota even at very low concentrations. With a half-life reaching 1,000 years and the inability to self-degrade, metals can remain in the soil for extremely long periods of time[4]. They induce obstructive stress upon microorganisms by disrupting metabolic activities, reducing diversity, and preventing microbial release of enzymes[5,15]. As a result, a soil with extremely high

concentrations of metals can quickly become unhealthy because the necessary functions of its microbes, similar to the ones previously mentioned in Table 1 *(see Chapter 1, Section 1.1)*, are severely hindered.[2,4, 5,13,14,15]

Given that metals are often thought to decrease extracellular enzyme activity in soil, it is surprising that at LSP, the site with the highest concentrations of heavy metal also exhibits the highest levels of enzyme activity (see *Figure 9 and Figure 10, Chapter 2, Section 2.4).* This suggests that LSP's microbial community have undergone changes that allowed them to adapt to their harsh environment. Sometimes, the pressure of metal contamination can incite major genetic changes in microbes that result in heavy metal tolerance by the entire ecosystem[2,51]. Multiple studies—including the works of Rau et al.[52], Dimkpa et al.[53], and Gupta et al.[54]—previously reported that the formation of metal-resistant root bacteria caused a reduction in the uptake of heavy metals by plants[52,53,54]. Certain microbes can also decompose pollutants, release enzymes and stimulate plant growth in contaminated sites[12]. Known as heavy metal-resistant microbes, they produce supplements that can increase tolerance to stress caused by heavy metal contamination. They also reduce the toxicity of metals by modifying the solubility and bioavailability of heavy metals in the soil (Recall from Chapter 2 that heavy metals can persist in soil in different forms and that the most toxic form is the one that is most mobile and bioavailable). Most importantly, these microbes are able to exude enzymes into the soil to promote the production of elemental nutrient and the subsequent growth of plants.[13,17,22,55] These extraordinary microorganisms are at the core of a growing, more sustainable, environmental restoration strategy called bioremediation[17,18].

Bioremediation is the application of living organisms to degrade, detoxify or stabilize hazardous materials in the environment. While there are bioremediation strategies that can eliminate the source of contamination in contaminated sites, this chapter only observes the application of bioremediation strategies to improve the enzyme activity of the microbial community. As described in the thesis introduction (*see Chapter 1, section 1.4*), bioremediation is more cost-effective and energy efficient than current conventional remediation methods.[16,17,18] Of the three types of bioremediation strategies previously discussed, this chapter only explores two: biostimulation and bioaugmentation. Both strategies involve the addition of a 'substance' to the contaminated site in order to encourage the development of metal-resistant microbes and improve the enzyme activity. The following paragraphs explain the main difference between these two bioremediation strategies, which is based on the constituents of the 'substance' that is added to the contaminated soil.

In biostimulation, the microbes in the contaminated soil are excited or 'stimulated' by the addition of a mixture of complex organic compounds made up of oxygen, carbon, phosphorous and nitrogen[18]. This complex mixture mimics the role of root exudates—mentioned earlier in this chapter—which are compounds that are released by roots into their proximate soil environment as food for microbes.[18,56] In nature, the compounds collectively called root exudates include: "inorganic ions, amino acids, amides, sugars, aliphatic acids, aromatic acids, volatile aromatic compounds, gases such as ethylene, vitamins, peptides, proteins, enzymes, plant hormones, alcohols, ketones, olefins, urea, etc"[56]. The variety of compounds classified as root exudates make them an important nutrient source for surrounding microbes.[56,57,58]

Laboratory studies of interactions between root exudates and their soil environments use a synthesized mixture containing a few of the aforementioned compounds. Referred to as synthetic root exudates, these mixtures usually contain sugars, organic acids and amino acids in varying amounts *(refer to Appendix A-2 for the composition of root exudates used in this thesis)*. Although they do not nearly reproduce the complexity of *in situ* root exudates, synthetic root exudates are a good representation.[56,57,58] Note that for the remainder of this chapter, the use of root exudates refers to synthetic root exudates, unless otherwise specified.

During biostimulation, root exudates are added to the contaminated soil to increase the amount of nutrients that are available to microbes. The theory of biostimulation is that the addition of root exudates provides a nutrient source, which may have been deficient, for the native microbes of the contaminated soil. It is assumed that the microbes of the contaminated soil have already developed metal-resistive properties; or, that by providing the microbes with essential nutrients through root exudates, they are more equipped to facilitate genetic changes for resistive properties among themselves. Essentially, microbes, possibly with resistive properties, that are indigenous to the contaminated site are 'stimulated' with root exudates. The expected result is their proliferation into substantial amounts, enough to bring back or increase the enzyme activity of the formerly contaminated soil. Time is the main limiting factor of biostimulation.[18] In Part 1 (*Section 3.3*) of this chapter, we explore whether the microbes of LSP's contaminated soil can be 'stimulated' by observing the type of influence—positive or negative—that root exudates have on the enzyme activity of soil samples over time.

The second bioremediation strategy observed in this chapter is bioaugmentation. Unlike biostimulation, which only adds nutrients (root exudates) to the contaminated soil, bioaugmentation introduces a solution of new microbes, also known as an inoculum, to the contaminated soil. During bioaugmentation, an inoculum of microbes with metal-resistant capabilities, is introduced to the contaminated site. Usually, these microbes are from soil habitats, similar to LSP, which have a thriving diversity of living organisms and high enzymatic activity, despite a history of contamination. The two main concerns accompanying bioaugmentation is uncertainty in whether the donated microbes will be able to establish themselves or if they will dominate and possibly kill their host's indigenous microbes.[18]

During bioaugmentation, microbe-containing inoculant from the donating site is usually introduced to an unaltered contaminated site[18]. As a result, the outcome of the experiment is dependent on only the biotic factors—living organisms such as bacteria, fungi, and protozoa—from both the contaminated site and the inoculum-producing site. In Part 2 of this chapter, inoculum from one LSP site is introduced to the other LSP site to understand each site's response when exposed to living organisms from the other site. This transfer of microbe-containing inoculum from one site to another is referred as cross inoculation.

Biostimulation (*in Part 1, Section 3.3*) and bioaugmentation (*in Part 2*, Section 3.4) are two different ways we observe the influence of biotic factors on enzyme activities. Another form of cross inoculation, different from bioaugmentation, is used to observe the influence of abiotic factors on enzyme activity, the other part of our purpose. During the cross-inoculation performed during bioaugmentation, microbe-containing inoculum from

one site is added to a contaminated site with its native microbes. In order to study the effect of abiotic factors, microbe-containing inoculum from one site is added to a contaminated site that has been sterilized. Sterilization theoretically eliminates all living organisms (biotic factors), so the inoculation of sterilized soil means that we can observe the abiotic effects of the contaminated soil on the microbes of the donating soil. These experiments aimed to understand how microbial inoculum from one LSP site behaves in a foreign environment that has been striped of all its biotic factors. For purposes of simplification, cross-inoculation in this chapter only refers to the addition of inoculum to sterilized soil samples. Cross-inoculation experiments were conducted in both Part 1 (*Section 3.3*) and Part 2 (*Section 3.4*) to understand how abiotic factors influenced the enzyme activity.

The overall purpose of this chapter is to evaluate whether LSP's microbes can be used in bioremediation strategies to improve the enzyme activity of contaminated soil. Two different bioremediation strategies were observed. Biostimulation, the addition of root exudates, was studied in Part 1 (*Section 3.3*)—"The Cross-Inoculation and Biostimulation of Microbes at LSP with Root Exudates". Meanwhile, bioaugmentation, the addition of inoculum-containing microbes, was studied in Part 2 (*Section 3.4*)—"The Cross-Inoculation and Bioaugmentation of Microbes at LSP without Root Exudates". Both chapters had two main objectives: To characterize how

1) Biotic factors (living organisms such as microbes), and
2) Abiotic factors (non living organisms such as metal contamination)

Influence the enzyme activity of two LSP sites (LSP 43 and LSP 146) during bioremediation (biostimulation in Part 1 and bioaugmentation in Part 2). To study our

objectives, the parameters mentioned earlier—sterilized and non sterilized, inoculated and non-inoculated, and root-exudates and no root exudates—were used to create multiple combinations of soil samples between LSP 43 and LSP 146; for example, sterilized soil from LSP 43 inoculated with microbes from 146. These combinations were referred to as inoculation conditions and the conditions used are specified within each part. It is important to understand that even though soil samples from both LSP sites are contaminated, the soil donating its microbes is referred to as the "donor" soil, and the soil receiving either the root exudates or the inoculum, is the "contaminated" soil. The main difference between both parts of this chapter is the use (*Part 1, Section 3.3*), or absence (*Part 2, Section 3.4*), of root exudates. Other differences between both parts are detailed in Table 3 below.

Table 3: Differences between Part 1 and Part 2 Inoculation Studies

Characteristic	Part 1	Part 2
Experiment duration *Length of experiment from first sampling to final sampling*	2 months	8 months
Root Exudates *A mixture of amino acids, organic acids and sugars*	Yes	No
Inoculation Conditions *The different combinations of sterilized soil, the use of root exudates and the use of inoculum between LSP 43 and LSP 146*	12	10
Pot Sampling *The way soil samples for each condition are harvested for enzyme analysis over time. For the experiment duration, soil for each inoculation condition can be harvested from one pot—non-destructive—or from a different pot at each time point—destructive.*	Non-destructive	Destructive

Following soil collection and experiment set up, each combination was nurtured inside a pot; then over time, soil samples were harvested—collected from the pot—and phosphatase activity was measured to observe the effects of cross inoculation, biostimulation, or bioaugmentation on enzyme activity (Figure 11). This chapter was a collaborative study with associate professor, Dr. Jennifer Krumins and doctoral candidate, Jay Singh—who was mainly responsible for pot set-up and sample harvest. Without them, this chapter would have been impossible.

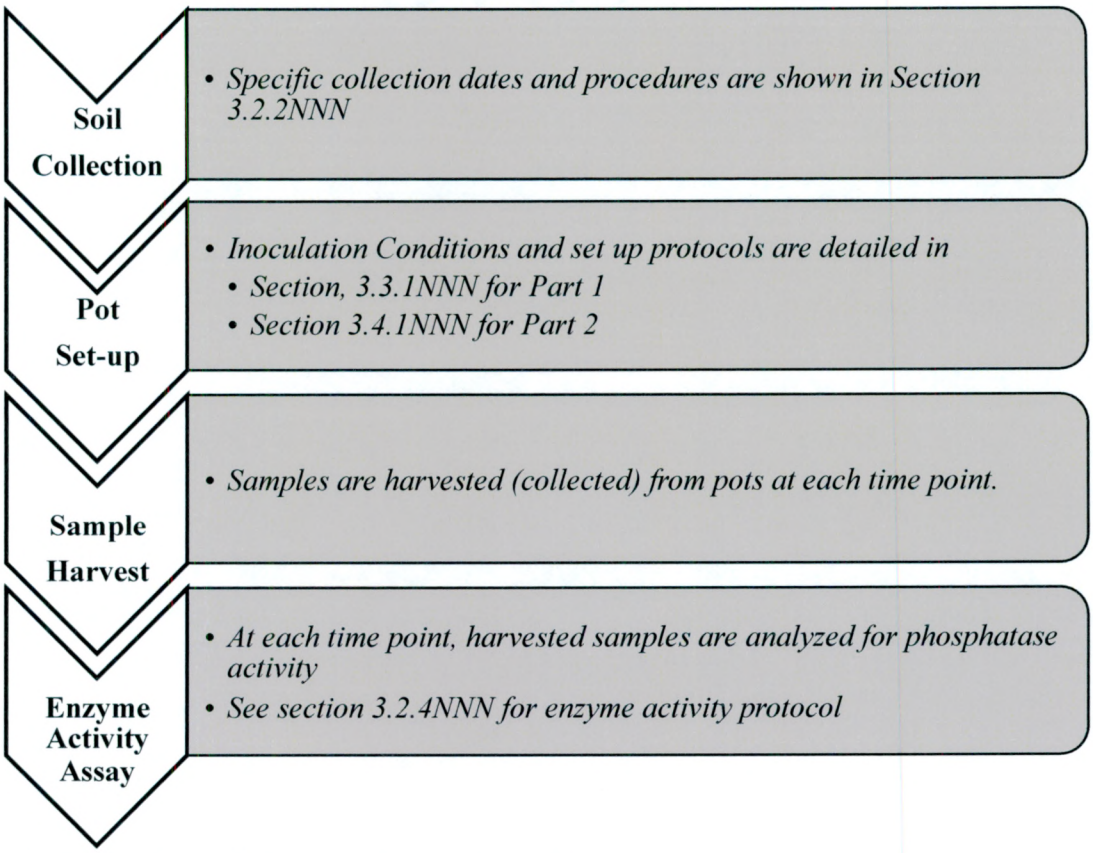


Figure 11: Overall Process of Cross Inoculation Study.

This shows the general process followed for both studies in this chapter. Any specific information for each part is found within their corresponding section.

3.2. Protocol

3.2.1. Study Sites

Only sites LSP 43 and LSP 146 were observed in this study. *Please refer to Chapter 1, Section 1.5 for information regarding LSP.*

3.2.2. Soil Collection & Preparation

Soil collection from LSP 43 and 146 were completed on June 4, 2015 for the study with root exudate (*Part 1, Section 3.3)*. For the study without root exudates (*Part 2, Section 3.4)*, soil from both sites was collected on October 4, 2015. For both studies, a small shovel was used to collect soil from each point of transect B only (*See layout of soil sites, Figure 4, Chapter 2, Section 2.2.2*), resulting in a total of 5 soil samples from each site. Each soil sample was sieved separately through a 2 mm sieve. Then equivalent amounts from each of the five pins were amassed into one representative sample for each site. Only the representative soil sample from each site was analyzed.

3.2.3. Phosphatase Activity Assay

Phosphatase assays were used as a proxy to measure extracellular enzyme activity in the soil samples. *Please refer to Chapter 2, Section 2.2.3 for the procedure.*

3.2.4. Percent Moisture

It was necessary to maintain a consistency in moisture over time and among all the pots. Percent moisture measurements were needed to monitor the moisture levels of the soil and to get the dry weight of the soil used in enzyme activity calculations. *Please refer to Chapter 2, Section 2.2.4 for the procedure.*

3.3. Part 1: The Cross-Inoculation and Biostimulation of Microbes at LSP with Root Exudates

The overall purpose of this chapter is to evaluate whether LSP's microbes can be used in bioremediation strategies—biostimulation and bioaugmentation—to improve the enzyme activity of contaminated soil. Biostimulation, the addition of root exudates as a source of nutrients, is the focus of this part. There were 4 objectives explored in this part of the chapter: to 1) determine whether root exudates have a positive or negative influence on the enzyme activity of LSP soil samples over time, to 2) understand the behavior of LSP's microbes when stimulated with root exudates, and to 3) characterize how biotic factors (living organisms such as microbes) and 4) abiotic factors (non living organisms such as metal contamination) influence the enzyme activity of LSP samples during biostimulation. To study these objectives, the enzyme activity was measured at different time points for sterilized and non-sterilized soil samples that were injected with root exudates and/or microbes. It is referred to as a cross-inoculation study because it involves the addition of microbes, in the form of an inoculum, from one site in LSP to another.

3.3.1. Inoculation Conditions

Inoculation conditions are the different combinations of sterilized (S) and non-sterilized (NS) soil, the use of root exudates (RE) or no root exudates (NRE) and the use of inoculum (I) or no inoculum (NI) from both LSP 43 and LSP 146 (Figure 12). There were a total of 12 conditions analyzed in this study. Eight of the soil samples were sterilized; the remaining four conditions used non-sterilized soil. The first pair of non-

sterilized conditions (dark gray) in Figure 12 is biostimulated samples—non-sterilized soil with the addition of only root exudates.

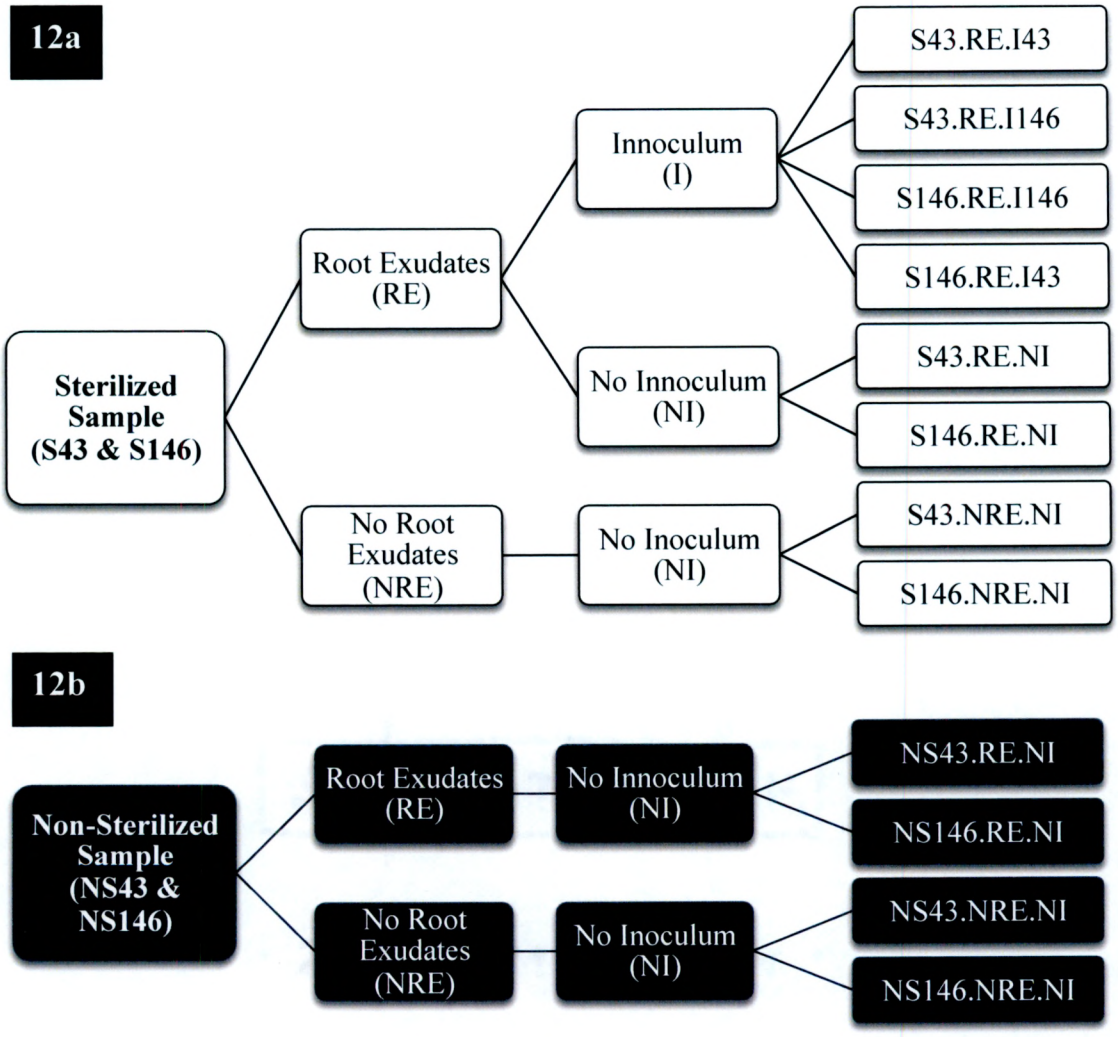


Figure 12: Experimental Conditions for Cross-Inoculation with Root Exudates.
A tree of the 12 conditions observed for this experiment: **(A)** sterilized (white) and **(B)** non-sterilized soil samples (dark gray). The right-most column is the label and letters represent specific characteristics: Sterilized (S), Non-Sterilized (NS), Root Exudates (RE), No Root Exudates (NRE), Inoculum (I) and No Inoculum (NI).

Each condition was prepared in a medium-sized pot (3" round × 2.5" deep) in replicates of three – Pot 1, 2 and 3 – producing a total of 36 pots (*See Appendix A-1 for Inoculation protocol*). Over a two-month time span, soil was non-destructively harvested from each pot three times. This means that at each of the three harvesting time points, soil was collected from the same pot. Approximately 3 g of soil was procured from the pots during each harvest.

3.3.2. Experimental Chronology

Soil was collected on June 4, 2015. The experimental chronology detailed here is listed in Table 4. Pots containing soil with each of the inoculation conditions were set up on June 16, 2015, 12 days after collection from the site (*See Appendix A-1 for Inoculation protocol)*. The first month after setting up all the pots was the period of microbial reactivation, which involved maintaining the moisture levels and adding the root exudates daily (*See Appendix A-2 for Composition of Root Exudates)*. For the first two weeks, root exudates were added at a concentration of 1 µg carbon / g of soil; then it increased to approximately 100 µg carbon / g of soil for the remainder of the study. While root exudates were no longer added after the first sampling date, DI water was continuously added throughout the course of the experiment to maintain a moisture balance. This study was conducted over a two-month period with three harvest days—time points when soil samples are collected from the pots and sampled for moisture and enzyme activity. The first sample was harvested a month after set up on July 13 2015, followed by July 20 and August 4. In addition to non-destructive sampling, this section is characterized by the use of root exudates in some of the inoculation conditions *(see inoculation conditions in Figure 12, Section 3.3.1)*.

Table 4: Experimental Chronology for The Cross-Inoculation and Biostimulation Study with Root Exudates

Date	Activity
6/04/2015	Soil collection
6/16/2015	Pot set up, Inoculation with 10 % soil slurry, Homogenization of soil in pots
6/17/2015 – 8/2/2015	Microbial reactivation including moisture level maintenance and addition of Root Exudates
6/22/2015 – 7/2/2015	Addition of Root exudates (1 µg Carbon / g of soil)
7/3/2015 – 8/2/2015	Addition of Root exudates (100 µg Carbon / g of soil)
7/13/2015	1st Harvest
7/20/2015	2nd Harvest
8/03/2015	Final Harvest

3.3.3. Results and Discussion

This section emulates biostimulation via the use of root exudates, compounds such as amino acids and sugars added to stimulate and increase microbial production of enzymes. The four objectives of this experiment were to 1) determine whether root exudates have a positive or negative influence on the enzyme activity of LSP soil samples over time, to 2) understand the behavior of LSP's microbes when stimulated with root exudates, and understand how LSP's 3) biotic (living organisms such as microbes) and 4) abiotic factors (non-living such as metal contamination) influence the enzyme activity. This was done via cross inoculation of soil samples from LSP 43 and LSP 146 and the addition of root exudates. Recall that the phosphatase activity at both LSP 43 and 146 is high, despite the presence of heavy metals (*see Figure 9 and 10, Chapter 2, Section 2.4).*

In fact, LSP 146, which has the higher concentration of heavy metals, also exhibits more phosphatase activity than LSP 43. A total of twelve conditions, in replicates of 3—Pot 1, 2 and 3—were analyzed over a period of 2 months. The pots were set up and inoculated on 6/16/2015. Soil samples were harvested and the phosphatase activity and moisture levels were measured 3 times on 7/13, 7/20 and 8/04.

3.3.3.1. Percent Moisture

The percent moisture was calculated to ensure that the moisture level of the soil was consistent throughout the duration of the project. Since phosphatase activity was reported in activity per gram of dry soil, the moisture data was also used in the phosphatase activity calculation. According to Figure 13 below, the moisture level on 7/13 was between 25 % and 45 %. A week later, on 7/20, the lower limit of the moisture level reduced to 15 %, while the upper limit remained the same. Similarly, on the final harvest date, the moisture limits were from 20 % to 45 %. Throughout the course of the study, variation in soil moisture among the conditions increased, from 15% on 7/13 to 25 % by 8/4. A majority of the soil samples had moisture ranging between 20 % and 30 %.

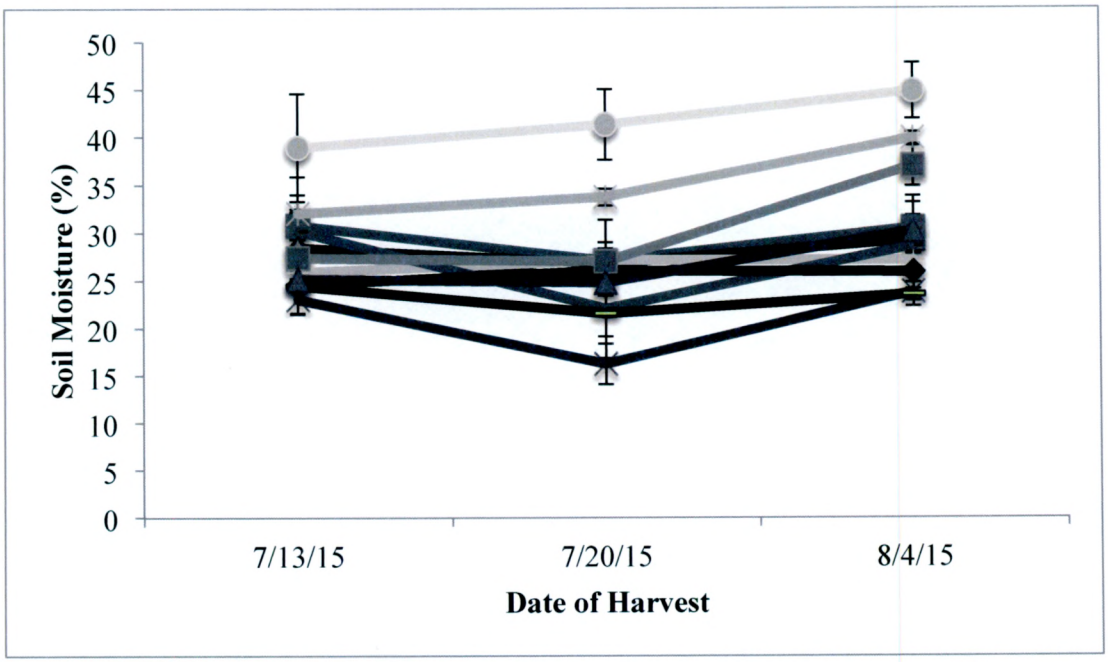


Figure 13. Average Percent Moisture of all Inoculation Conditions Over Time for Cross Inoculation Study with Root Exudates

This graph shows the average and standard error of the three pots for each inoculation condition harvested throughout the course of the experiment. On the Y-axis is the percent moisture with units of percentage and on the x-axis, the date of harvest. Points show standard error of the mean, n=3. Moisture levels were relatively consistent throughout the duration of the experiment ranging from a minimum of 15 % to approximately 45 %.

Figure 14 below shows only minor differences in average moisture between inoculation conditions over time. The sterilized soils are shown as clear bars with no patterns and the non-sterilized soils are striped. Generally, soils that are not inoculated (NI, pair of dark gray bars) for both sterilized (clear bars) and non-sterilized (striped bars) soils have slightly lower moisture than inoculated soils (white and light gray bars). There is no major difference in moisture between soils inoculated with 146 (I146, light-gray bar) and soils inoculated with 43 (I43, white bar); the former (I146, light-gray bar) is slightly moister than the latter (I43, white bar).

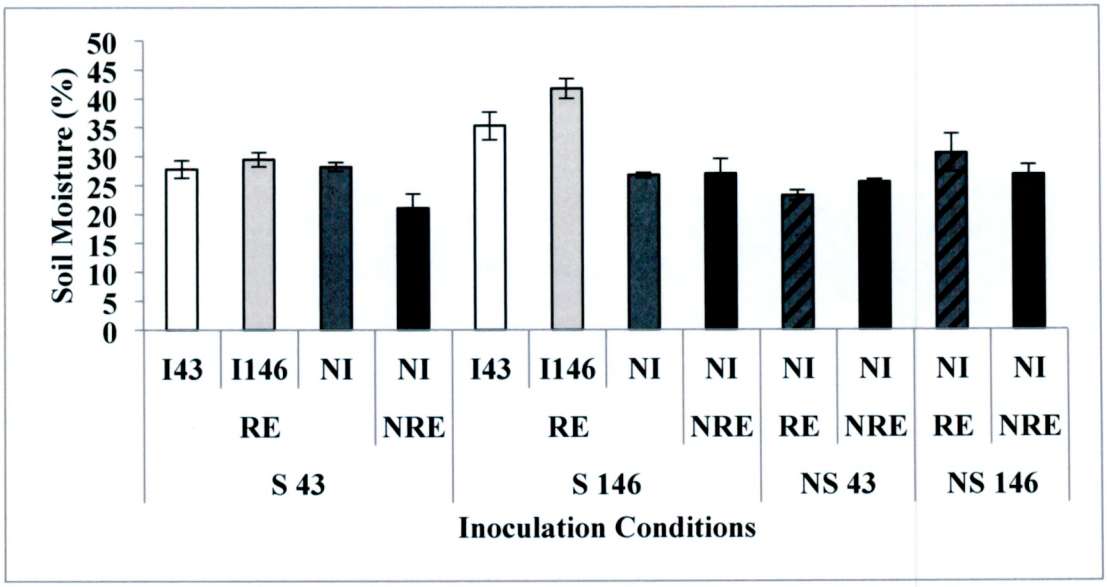


Figure 14. Average Over Time of Percent Moisture for All Inoculation Conditions in Cross-Inoculation study with Root Exudates.

This graph is percent moisture for each inoculation condition with each bar representing the average of the three time points. On the Y-axis is the percent moisture with units of percentage and on the x-axis, the inoculation conditions. The primary label is the sterilized (S) and non-sterilized (NS) contaminated soils. The secondary labels identify the use (RE) or absence (NRE) of root exudates and the specific soil inoculum (I or NI). Similar colored bars have the same secondary labels (RE, NRE, I, NI) and the striped bars are the non-sterilized soil samples.

3.3.3.2. Phosphatase Activity

The phosphatase activity was measured for all 3 replicates of the harvested soil to quantify the effect of the cross-inoculation. These three replicates were averaged and the standard error of all three values is reported in the following graphs, unless specified otherwise.

The first objective was to determine whether root exudates have a positive or negative influence on the enzyme activity of LSP soil samples over time. Root exudates were added to soil samples from LSP. Since root exudates are a source of nutrients for

the microbial community, and microbes exude the enzymes into the soil, the hypothesis is that samples with root exudates should show higher enzyme activity than soil samples from the same site without the added assistance of root exudates.

Figure 15 is a time course graph of both LSP samples with root exudates (light gray lines) and without root exudates (dark gray lines). For LSP 43, (circle marker) soil with root exudates (light gray, circle marker) had higher enzyme activity than soil samples without root exudates (dark gray, circle marker), as expected. However, for LSP 146 samples (triangle marker), soil with root exudates (light gray, triangle marker) did not have higher enzyme activity than soil without root exudates (dark gray, triangle marker), as was hypothesized. It is important to note that while LSP 146 with root exudates (light gray, triangle) is not higher than its counterpart without root exudates (dark gray, triangle) it still shows an increase in enzyme activity over time.

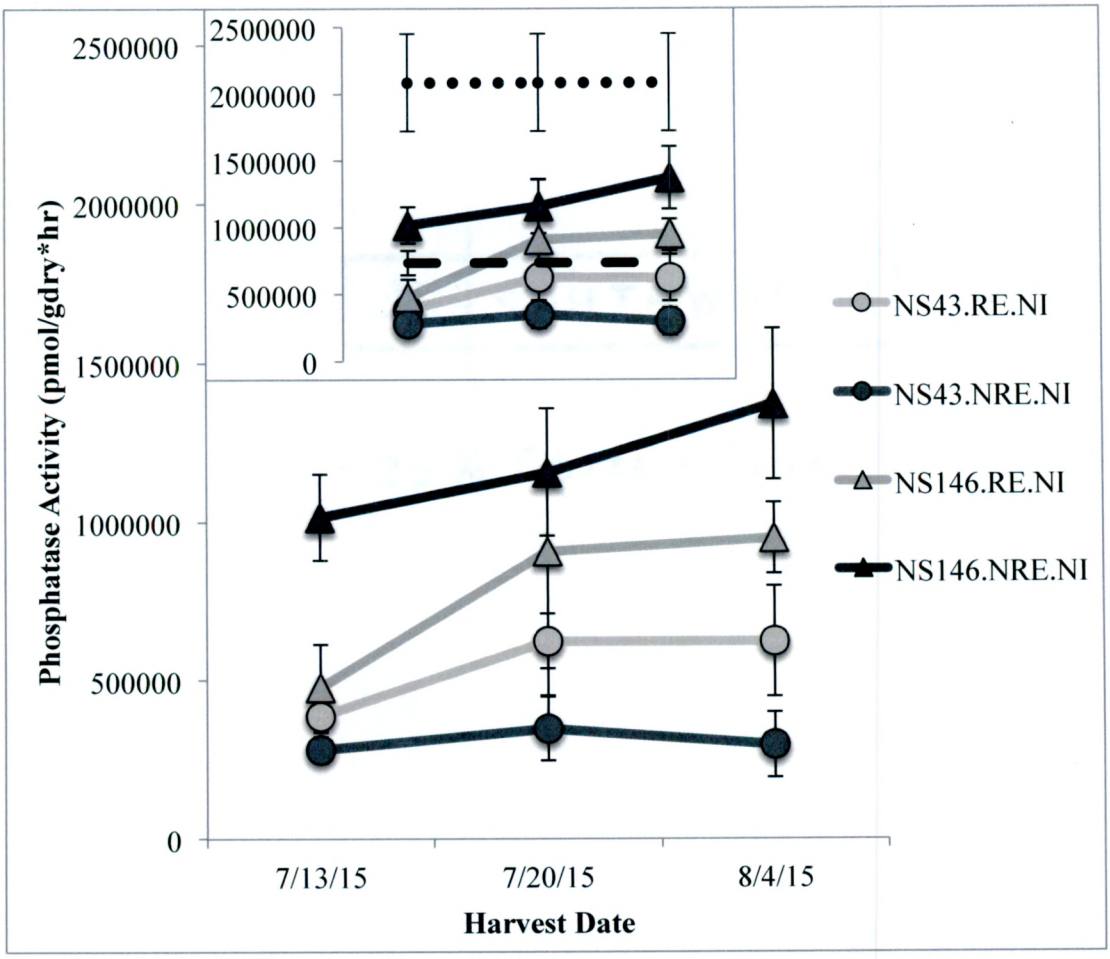


Figure 15. Average Phosphatase activity of All Non-Sterilized Soil with Root Exudates Over Time.

This shows the average phosphatase activity for only the non-sterilized conditions at each of the harvest date. The light gray lines represent the biostimulated conditions with root exudates and the dark gray lines the non-stimulated controls. LSP 43 has circle markers and LSP 146 has triangular markers. The inset shows the same graph and has the same x- and y-axis labels. But it also includes the average phosphatase activity (horizontal lines) for LSP 43 (dotted) and LSP 146 (striped), respectively from Chapter 2 (*See Figure 8, Section 2.3.2*), measured separately from this cross-inoculation study. Measured once, less than a week after collection, they are the most accurate representation of *in situ* enzyme activity; thus, they act as a standard for comparison.

This leads right into the second objective of this experiment: to understand the behavior of LSP's microbes when biostimulated. The biostimulation of a soil's indigenous microbes, through addition of nutrients, promises a surge in the number of metal-resistant bacteria that can reverse the effect of metal pollution[18]. There were two biostimulated conditions: the non-sterilized samples with root exudates (light gray pair); and two controls: the non-sterilized without root exudates (dark gray pair), for LSP 43 (circle marker) and LSP 146 (triangle marker).

Stimulated soil conditions (RE) were expected to show a higher phosphatase activity than unstimulated soil conditions (NRE) because they had the added benefits of root exudates, which provide nutrients for microbial growth. As mentioned earlier, Figure 15 shows that the biostimulated condition at LSP 43 (with root exudates, light gray, circle marker) showed higher activity than the non-biostimulated condition (no root exudates, dark gray, circle marker), as hypothesized. Additionally, by the final harvest date, biostimulated LSP (light gray, circle marker) had comparable activity to the standard, *in situ* activity of LSP 43 (dashed line).

However, for NS146 (triangular markers), our hypothesis did not hold: phosphatase activity was actually higher for the unstimulated soil samples without root exudates (dark gray, triangle marker) than for stimulated soil samples with root exudates (light gray, triangle marker), at any given harvest date. Throughout the course of the experiment, neither the activity of stimulated 146 with root exudates (light gray, triangle marker) nor the activity of its non-stimulated counterpart (dark gray, triangle marker) reached the standard *in situ* activity of LSP 146 (dotted line)—although by the final date, the non-stimulated soil sample (dark gray, triangle) was surprisingly close. Even though

stimulated NS 146 (light gray, triangular marker) did not meet the expectations, on each given harvest day, its activity was higher than stimulated NS 43 (circle marker), a pattern that was observed in the activity of the standard *in situ* soil (horizontal lines).

As noted earlier, while LSP 146 with root exudates (light gray, triangle) is not higher than its counterpart without root exudates (dark gray, triangle) it still shows an increase in enzyme activity over time. This can be further explored by the third objective of this study, which was to characterize how abiotic factors influence the enzyme activity of LSP samples during biostimulation. Recall that abiotic factors are non-living organisms, such as the level of metal contamination. Also recall that the concentration of heavy metal is lower at LSP 43 than at LSP 146 *(see Figure 5 and 6, Chapter 2, Section 2.3.1.1 and 2.3.1.2)*. One hypothesis is that the higher concentration of metals at LSP 146 means that LSP 146 soil is a harsher environment than LSP 43, meaning that the microbes at LSP 146 will initiate slower than microbes at LSP 43. Stimulated LSP 146 (light gray, triangle) did not behave as expected, since its unstimulated counterpart (dark gray, triangle) showed higher enzyme activity (Figure 15); yet, by the final harvest date, its activity (light gray, triangle marker) had doubled from its initial magnitude. This increase is much higher than the rate of increase of the un-stimulated LSP 146 soil (dark gray, triangular marker) and any of the LSP 43 soil (circle marker). This suggests that the microbes at LSP 146 might need more time to readjust, supporting the notion that its higher concentration of heavy metals (abiotic factor) make it a harder environment to colonize. However, additional time points will be required to draw such a conclusion.

The sterilized soil samples and their controls for each time point are shown in Figure 16. The controls were sterilized soil samples with root exudates, but without

inoculum (dark gray bars); and sterilized soil samples with neither root exudates nor inoculum (black bars). Both conditions were expected to exhibit no activity, since the soil was sterilized and there was no inoculum added, meaning that all the living organisms, including microbes were killed and no additional microbes were added. The lack of living organisms, especially microbes, which release the enzymes, means that there should be no phosphatase activity; unless the sample was contaminated. The sterilized soils with neither root exudates nor inoculum (black) showed minimal phosphatase activity. The soil conditions without inoculation, but with root exudates (dark gray) showed an unexpected level of activity that was just as high as samples that were inoculated with microbes (white and light gray bars). This suggests that there was possible contamination in the controls, which was further amplified by the application of root exudates.

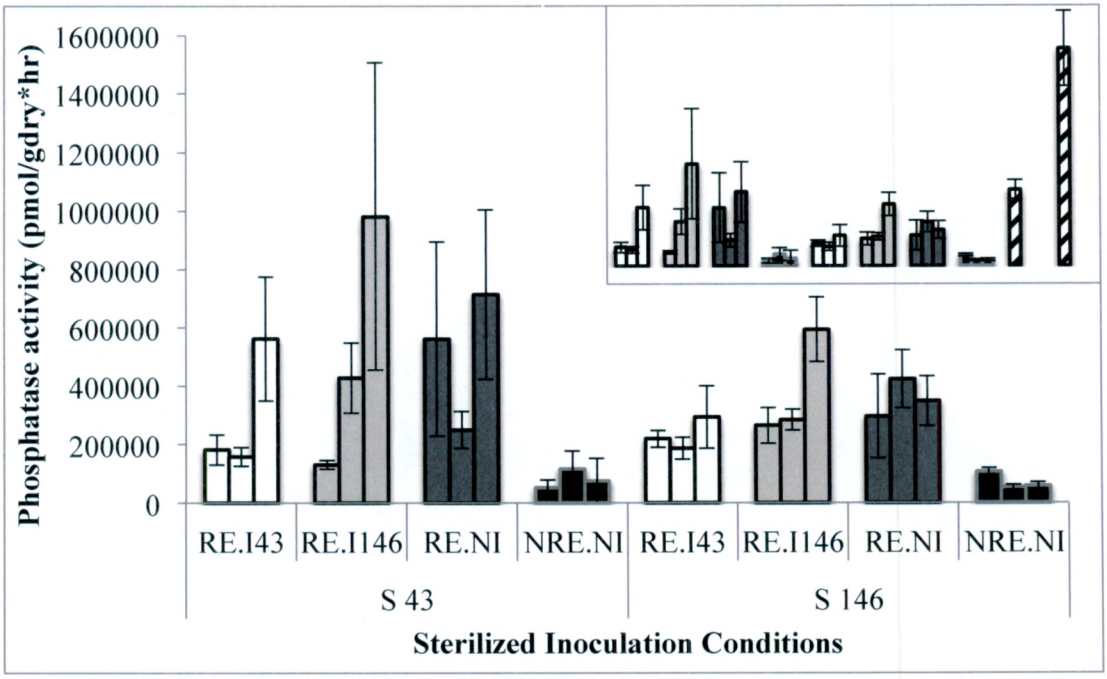


Figure 16. Average Phosphatase activity of All Sterilized Soil with Root Exudates. This shows the phosphatase activity—average and standard error of the 3 pots—for only the sterilized conditions at each of the harvest date. Each set of three bars from left to right is 7/13, 7/20 and 8/04. The primary label on the x-axis is the sterilized (S) parent soil. The secondary labels identify the use (RE) or absence (NRE) of root exudates and the specific soil inoculum (I) or non-inoculum (NI). Similar colored bars have the same secondary labels (white—RE.I43, light gray—RE.I146, dark gray—RE.NI and black—NRE.NI). The inset shows the same graph and has the same x- and y-axis labels. But it also includes the average phosphatase activity for LSP 43 and 146, respectively (striped bars) (*see Figure 8, Chapter 2, Section 2.3.2)*. Measured once, less than a week after collection, they are the most accurate representation of *in situ* enzyme activity; thus, they act as a standard for comparison.

The influence of abiotic factors on enzyme activity was further observed by inoculating sterilized soil samples with microbes. Sterilization theoretically kills all living organisms, so this means that the result in enzyme activity is affected by two factors. The first is the abiotic factors of the contaminated soil, including the level of contamination.

Since LSP 43 has a lower concentration of heavy metals than LSP 146, we hypothesized that regardless of what soil the donating microbes originated from, the enzyme activity at sterilized LSP 43 will be higher than at sterilized LSP 146.

In Figure 16 the enzyme activities of sterilized 43 (S 43, left set) appear to be larger on the whole, although not statistically significant, than the activities of sterilized 146 (S 146, right set). This supports the hypothesis, suggesting that the abiotic environment—collection of non-living factors including metal concentrations, pH, moisture, temperature, etc.—at LSP 43 is a bit easier for microbes to colonize than at LSP 146. This is not a surprising finding since LSP 146 has a higher heavy metal concentration than LSP 43 (*see Figure 5 and 6, Chapter 2, Section 2.3.1.1 and 2.3.1.2*).

This hypothesis is further supported by Figure 17, which shows the information from Figure 16 (without the controls) in a line graph. The dark colored pair represents inoculation with 146, the light colored pair represents inoculation with 43. The only difference between each same colored pair is the sterilized soil, allowing for analysis of the effects of abiotic factors. For the light gray pair (RE.I43), activity was usually higher when the sterilized soil is 43 (circle marker) than when it is 146 (square marker), although not significantly. Similarly for the dark gray pair (RE.I146) the enzyme activity was higher at 43 than at 146, although not significantly. Both figure 6 and 7 suggest that the abiotic factor of the contaminated soil does have some level of influence on the enzyme activity.

Recall that there are two factors that affect the addition of microbes to sterilized soil. The first is the abiotic factor of the contaminated soil, which was just discussed. The second is the donated microbes of the inoculum, and it is explored in the fourth objective

of this study—to characterize how biotic factors (living organisms such as microbes) influence the enzyme activity of LSP samples during biostimulation. Given that LSP 146 has higher enzyme activity than LSP 43, we hypothesized that inoculation with 146 microbes should exhibit higher enzyme activity than inoculation with 43 microbes.

Overall, except at the first time point, inoculation with 146 (dark gray pair) was usually higher than inoculation with LSP 43 (light gray pair), as hypothesized. At the first time point (Figure 17), sterilized 43, inoculated with 43 (S43.RE.I43, circle, light gray), had a higher activity when compared to sterilized 43, inoculated with 146 (S43.RE.I146, triangle, dark gray). Also, for sterilized 146, inoculation with 43 (square, light gray) and inoculation with 146 (diamond, dark gray) were within error of one another. This does not support our hypothesis that inoculation with 146 will always show higher activity than inoculation with 43; but it was only the first time point. By the 2nd and 3rd harvest time, inoculation with 146 (dark gray) exhibited more activity than inoculation with 43 (light gray), whether it was for the sterilized 43 (circle or triangle) or the sterilized 146 soil (square or diamond).

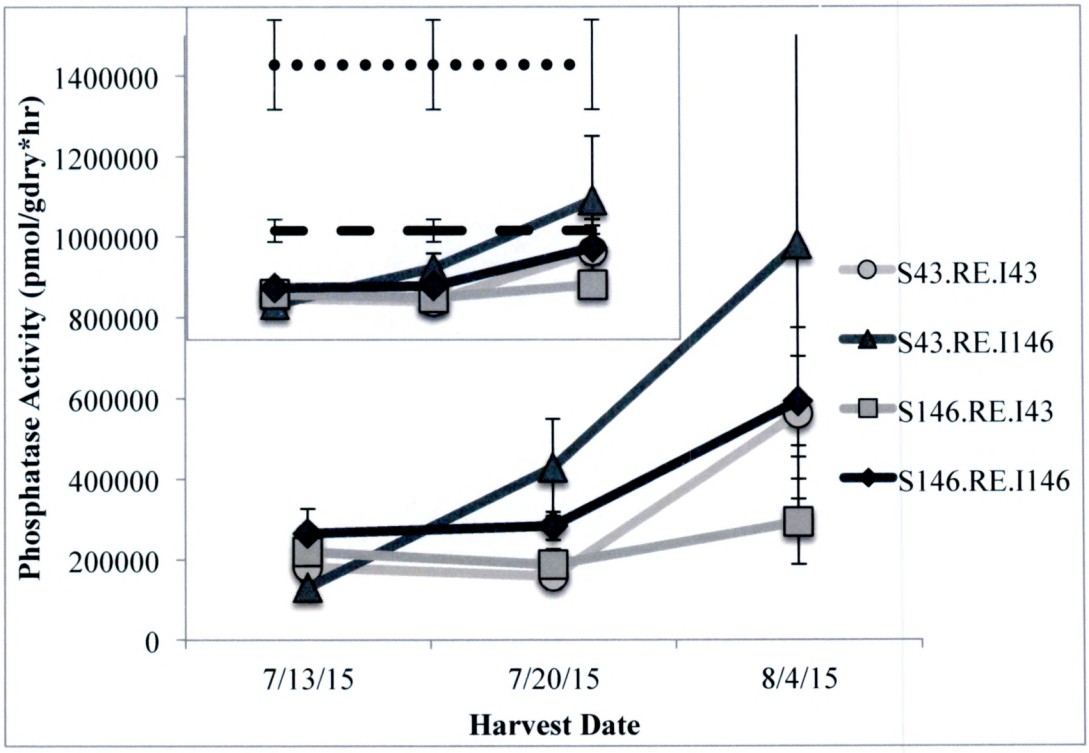


Figure 17. Average Phosphatase activity of Sterilized and Inoculated Soil with Root Exudates Over Time.

This shows the phosphatase activity—average and standard error of the 3 pots—for only the sterilized and inoculated conditions at each of the harvest date. The light gray bars are inoculated with 43 (I43) and the dark gray bars with 146 (I146). The inset shows the exact same graph with additional horizontal lines. The horizontal lines are the phosphatase activity for LSP 43 (dashed) and LSP 146 (dotted) from Chapter 2 (*See Figure 8, Chapter 2, Section 2.3.2*), measured separately from this cross-inoculation study. Activities of these samples (horizontal lines), which were measured once, less than a week after collection, are the most accurate representation of *in situ* enzyme activity; thus, they act as a standard for comparison.

In a comparable study applying bioaugmentation to the nitrification of soils, Nugroho et al.[59] concluded that in sterilized soils, the inoculum determined the behavior of the bacterial community and not the origin of the sterilized soils[59]. In our study, the origin of the inoculum did impact the enzyme activity: inoculation with 146 (dark gray)

was overall higher than inoculation with 43 (light gray). But so did the abiotic factors of the sterilized soil, as discussed in objective 3.

The inset shows that the relationship observed between inoculation with 146 (dark gray) and inoculation with 43 (light gray) followed the normal pattern observed at LSP (horizontal lines) where activity was higher at 146 (dotted) than at 43 (dashed). The inset of Figure 17 also showed that inoculation did not yield soil with activity as high as the *in situ* phosphatase activity for either LSP 43 (light gray vs. dashed line) or LSP 146 (dark gray vs. dotted line).

3.3.4. Conclusion

Recall that LSP 146 has an overall higher heavy metal concentration and phosphatase activity than LSP 43 *(Figure 9 and 10, Chapter 2, Section 2.4)*. In this section, phosphatase activity was determined after cross inoculating soil samples from these two sites. Sterilized samples from LSP 43 were inoculated with microbes from LSP 146; and sterilized samples from LSP 146 were inoculated with microbes from LSP 43. Additionally, non-sterilized samples of both LSP 43 and 146 were biostimulated with root exudates, which are a source of nutrients for soil microbial community. Combined with the controls, there were a total of 12 soil conditions prepared in replicates of three. Samples were harvested and analyzed at three time points (7/13, 7/20 and 8/04) over the course of two months.

The first two objectives were 1) to determine whether root exudates have a positive influence on the enzyme activity of soil samples over time, and 2) to understand the effect of LSP's microbes on enzyme activity when stimulated with root exudates. The enzyme activity was measured at different time points for sterilized and non-sterilized

soil samples that were injected with root exudates. Biostimulation is the addition of nutrients, in the form of root exudates, to contaminated soils. The increased nutrient source for microbes means that they can grow and function more readily than soils without the added benefits of root exudates. Thus we hypothesized that soils with root exudates, or stimulated soils should exhibit higher phosphatase activity than soils without root exudates. For LSP 43 soil conditions, stimulated soil showed higher activity than un-stimulated soil, as expected (*see Figure 14, Section 3.3.3.2*). In fact, the phosphatase activity to stimulated LSP 43 was comparable to the standard *in situ* activity of LSP 43 by the end of the study, confirming that addition of root exudates had a beneficial influence on its microbes.

Contrary to our hypothesis, stimulated LSP 146 had phosphatase activity that was lower than un-stimulated LSP 146, and 50% lower than its standard *in situ* phosphatase activity. Yet, it had the highest rate of increase than any other non-sterilized condition, suggesting the microbes at 146 are slower at reactivating or responding to the root exudates than the microbes at 43. This supports the notion that LSP 146 is a harsher environment than LSP 43, as was previously mentioned.

The last two objectives were to understand any relationships between biotic—living—and abiotic—non-living—factors of contaminated soil and inoculum of foreign microbes. To accomplish this, microbes from one LSP site (the inoculum) was used to inoculate sterilized soil samples from another LSP site. Sterilization kills a majority of the organisms in a soil, including the microbes. Thus, there were two factors that could influence the enzyme activity: either the microbes of the inoculating soil (biotic) or the level of contamination of the inoculated soil (abiotic)

Recall from Chapter 2 that the standard *in situ* phosphatase activity of LSP 146 is greater than that of LSP 43 (*see Figure 8, Chapter 2, section 2.3.2*). Therefore, in this cross inoculation study, we expected that inoculating any sterilized soil with LSP 146 microbes would show higher enzyme activity than inoculating with microbes from LSP 43. At the first time point, inoculation with LSP 43 exhibited higher activity than inoculation with LSP 146 (*see Figure 17, Section 3.3.3.2*), which contradicts our hypothesis. However, by the second and final harvest time, inoculation with LSP 146 showed greater activity. This supports our hypothesis that the microbes of the inoculating soil have an influence on enzyme activity. Furthermore, the level of heavy metal contamination is also greater at LSP 146 than at LSP 43. Thus, we hypothesized that, regardless of the origin, it would be more difficult for the new microbes to establish themselves at LSP 146 than at LSP 43. The data showed that activity was generally higher, although not significantly, for the sterilized 43 soils than for the sterilized 146 soils (*see Figure 16, Section 3.3.3.2*). This suggests that the abiotic factors of LSP 146 create a harsher environment than that of LSP 43, making it harder for microbes to establish themselves.

Overall, there was a consistency in the moisture levels—which ranged between 20 % and 30 %—and a generally consistent increase in phosphatase activity over time. However the brief duration of the study introduced a curiosity of how the behavior of can change over a longer period. Additionally, there was unexpectedly high enzyme activity within the sterilized, non-inoculated soils with root exudates—which were supposed to exhibit minimal activity since all the living organisms were killed during sterilization. This called into question the function of root exudates within the cross-inoculation study.

The next section is a slightly larger extension of this study that explores similar soil conditions over a longer period of time, while omitting the use of root exudates completely (*See Table 3, Section 3.1 for experimental comparison between this study—Part 1—and the next—Part 2*).

3.4. Part 2: Cross-Inoculation and Bioaugmentation at LSP without Root Exudates

The purpose of this entire chapter was to understand the relationship between LSP's microbe and its unusually high enzyme activity; and evaluate the potential of these microbes for use in increasing the enzyme activity during the bioremediation of other contaminated sites. Two cross-inoculation studies, in which inoculum from one LSP site is injected into another, and vice versa, were conducted. The first study (*Part 1, Section 3.3*) analyzed the stimulation of LSP soil samples via the addition of root exudates, or nutrients. In this study, we observe bioaugmentation, the other bioremediation strategy, by inoculating one LSP site with microbes from the other LSP site, without the use of root exudates for nutrients.

As discussed earlier in the chapter, the two main concerns accompanying bioaugmentation is uncertainty in whether the donated microbes will be able to establish themselves, or if they will dominate and possibly kill their host's indigenous microbes [18]. Thus, one of the objectives of this part was to observe the enzymatic response of each LSP site (43 or 146) when exposed to microbes (inoculum) from the other—bioaugmentation. It is important to note that in exploring this particular objective, soil samples will not be sterilized; this allows us to observe the effect on enzyme activity

caused by any the interactions between microbes from the donor site and the contaminated site.

The last two objectives were to characterize how 2) Biotic factors (living organisms such as microbes), and 3) Abiotic factors (non living organisms such as metal contamination) influence the enzyme activity of LSP samples. This was observed by measuring the enzyme activity of sterilized soil samples inoculated with microbes. These last two objectives were also observed in Part 1 (*See Section 3.3: Cross Inoculation and Biostimulation at LSP with Root Exudates)*; the difference is that in this study, root exudates were omitted from all soil conditions (*See Table 3, Section 3.1 for experimental comparison between this study—Part 2—and the previous—Part 1*).

3.4.1. Inoculation conditions

Inoculation conditions are the different combinations of sterilized (S) and non-sterilized (NS) soil, and the use of inoculum (I) or no inoculum (NI) from both LSP 43 and LSP 146 (Figure 18). There were a total of 10 conditions using soil samples from LSP 43 and LSP 146. Six of the soil samples were sterilized (white rectangles); the remaining four conditions used non-sterilized soil (dark gray rectangles). Each condition was prepared in a small pot (1.5" square × 2.25" deep) in replicates of three – Pot 1, 2 and 3—resulting in 10 sets of triplicates (*See Appendix B-1 for Inoculation protocol*). Pots were destructively harvested, meaning a different pot had to be prepared for each time point and each soil condition. Therefore, each set of triplicates was also prepared six times for each of the six harvesting time points. Consequently at time zero, there were a total of 180 pots: ten conditions in triplicates, reproduced six times *(Figure 18 depicts the ten conditions used in this study).*

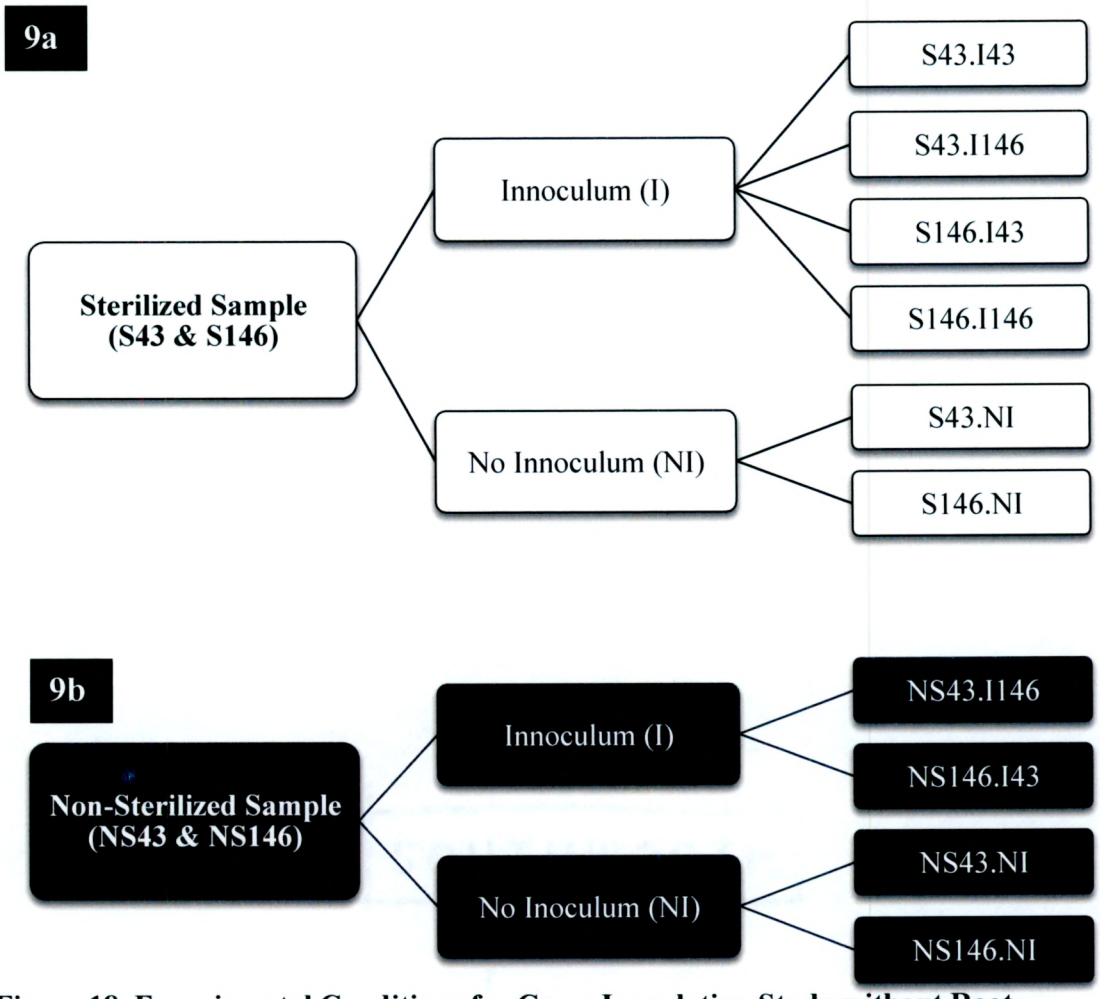


Figure 18. Experimental Conditions for Cross-Inoculation Study without Root Exudates.

The ten inoculation conditions for the experiment consisting of **(A)** six sterilized and **(B)** four non-sterilized soil samples. The final column on the right is the abbreviated name of each condition with the letters, S, NS, I and NI, representing Sterilized, Non Sterilized, Inoculated and Non Inoculated.

3.4.2. Experimental Chronology

Soil was collected on June 4, 2015. The experimental chronology detailed here is listed in Table 5. Pots containing soil with each of the inoculation conditions were set up on October 13th, 2015, 9 days after collection from the site (*See Appendix B-1 for*

Inoculation Protocol Without Root Exudates). The first 14 days after pot set up was the period of microbial reactivation, which involved maintaining the moisture levels daily.

This study was conducted over a five-month period, with one initial measurement and six harvest days—time points when soil samples are collected from the pots and sampled for moisture and enzyme activity. The first sample was harvested 15 days after set up on October 28th 2015. Pots were destructively sampled—unlike in the previous study—meaning that a different pot was set up for each harvest time point and each soil condition. In addition to destructive sampling, this part is characterized by the absence of root exudates in all the inoculation conditions.

Table 5: Experimental Chronology for The Cross-Inoculation and Bioaugmentation Study without Root Exudates

Date	Activity
10/4/15	Soil Collection
10/11/15	Sterilization of pots and measurement of initial enzyme activity
10/13/15	Pot set up, Inoculation with 10 % soil slurry, Homogenization of soil in pots
10/28/15	Harvest: 15 Days
11/13/15	Harvest: 1 Month
12/13/15	Harvest: 2 Months
1/13/16	Harvest: 3 Months
2/13/16	Harvest: 4 Months
3/13/16	Harvest: 5 Months

3.4.3. Results and Discussion

The purpose of this experiment was to study the effect of microbial cross inoculation and bioaugmentation on the phosphatase activity of metal contaminated soil. The effects of abiotic factors on LSP's microbial community, and the behavior of LSP's microbes when introduced to another contaminated site were analyzed—without the addition of nutrients (root exudates). Recall that the phosphatase activity at both LSP 43 and 146 is not weakened by the high concentrations of heavy metals, as is expected. Instead, LSP 146, which has the highest concentrations of heavy metals, also exhibits the highest levels of phosphatase activity (*see Figure 9 and 10, Chapter 2, Section 2.4*). A total of ten conditions, in replicates of 3—Pot 1, 2 and 3—were analyzed over a period of 5 months. The pots were set up and inoculated on 10/4/2015. Soil samples were harvested and the phosphatase activity and moisture levels were measured seven times over the five-month experimental period. (*See Table 5, Section 3.4.2 for the experimental chronology)*

3.4.3.1. Percent Moisture

The percent moisture was calculated to ensure that the moisture level of the soil was consistent throughout the duration of the project. Since phosphatase activity was reported in activity per gram of dry soil, the moisture data was also used in the phosphatase activity calculation. According to Figure 19 below, there was a gradual increase in moisture levels over time. At the beginning of the experiment, moisture was at approximately 20 %. By the 5th month, it was approximately 25% percent. The highest moisture level (35 %) occurred in the fourth month; and the lowest (15 %) occurred during the 3rd month. Additionally, the variation in moisture among soil samples at each

time point also increased with time, until the 5th month. At the first time point, variation was less than 5 %; by the 4th month, the difference between the lowest and highest moisture values increased to about 15 %. During the 5th month, variation in moisture among the samples had decreased to about 5 %.

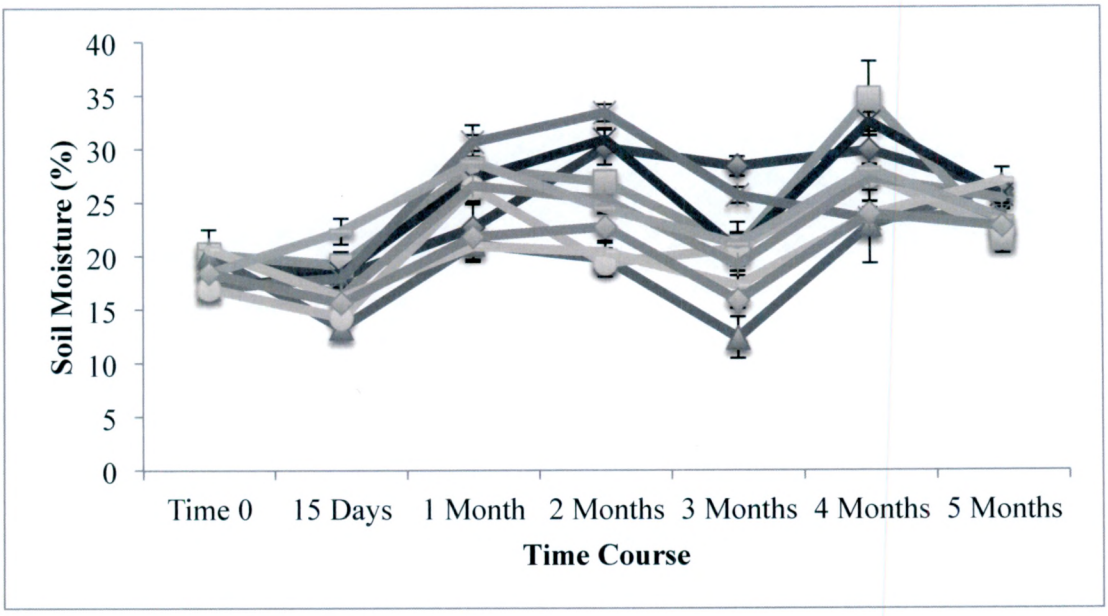


Figure 19. Average Percent Moisture of All Soil Conditions Over Time for the Cross Inoculation Study without Root Exudates.

This graph shows the average and standard error of the three pots for each inoculation condition harvested throughout the course of the experiment. There is no legend clarifying individual soil conditions because the purpose of this graph is to see the dependence of moisture on time, not on specific conditions. On the Y-axis is the percent moisture with units of percentage; and on the x-axis, the date of harvests. Moisture levels were relatively consistent throughout the duration of the experiment ranging from a minimum of 10 % to approximately 35 %.

While the previous graph focused on the effect of time on the moisture levels, the following graph (Figure 20) analyzes how moisture is affected by each soil condition. As shown in Figure 20, the differences in overall soil moisture among the different soil

conditions are not significant. By comparing the set of sterilized soil conditions (clear bars) to the non-sterilized ones (striped bars), it is apparent that sterilized soils (clear) are slightly moister than non-sterilized soil samples (striped). Among the sterilized samples (clear bars), the non-inoculated soils (dark gray, S43.NI and S146.NI) are lower than their inoculated counterparts (white and light gray, S43.I43, S43.I146 and S146.I43, S146.I146). Similarly, within the non-sterilized inoculation conditions (striped bars), soil samples without inoculum (dark gray, NS43.NI and NS146.NI) have lower moisture than their inoculated counterparts (white and light gray, NS43.I146 and NS146.I43). An overview of the graph also shows that in both sterilized and non-sterilized inoculation conditions, there is no significant difference in moisture levels between inoculation with 43 (white) and inoculation with 146 (light gray).

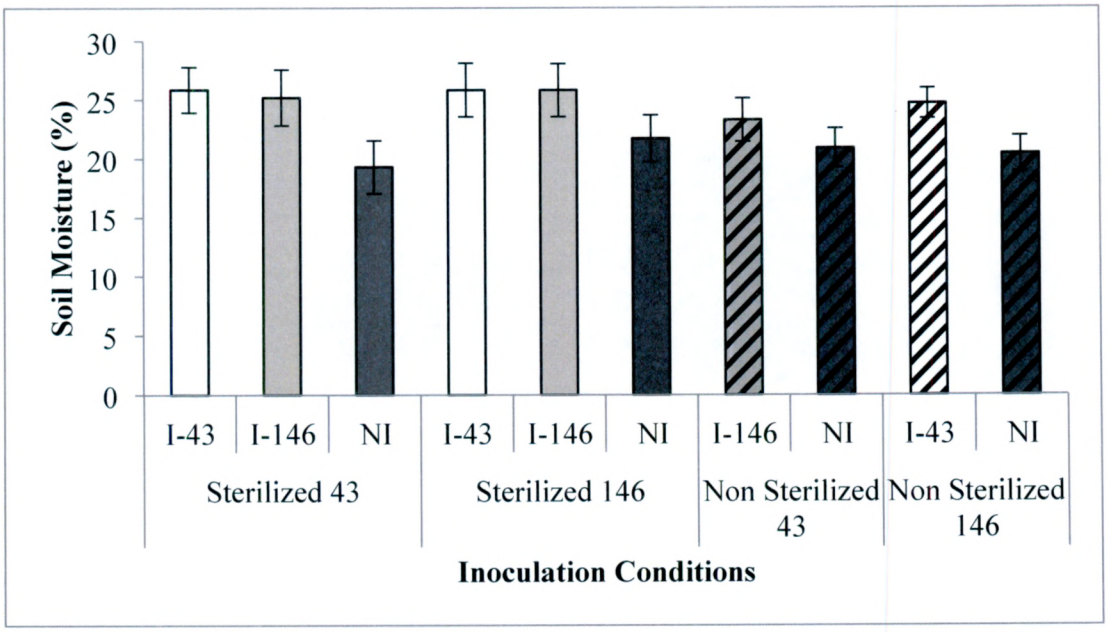


Figure 20. Average Over Time of Percent Moisture for All Inoculation Conditions in Cross-Inoculation study without Root Exudates.

This graph is the percent moisture for each inoculation condition with each bar representing the average of six time points (excluding time 0). On the Y-axis is the percent moisture with units of percentage and on the x-axis, the inoculation conditions. The primary label is the sterilized (S) and non-sterilized (NS) contaminated soils. The secondary labels identify the specific soil inoculum (I or NI). Similar colored bars have the same secondary labels (I43, I46, NI) and the non-sterilized soil samples (NS) are striped.

3.4.3.2. Phosphatase Activity

The phosphatase activity was measured for all replicates of the harvested soil to quantify the effect of the cross-inoculation. Recall from the chapter introduction (*See Section 3.1)* that there are two factors that can influence the phosphatase activity of sterilized, inoculated soil. Since all the living organisms, including microbes, are killed during sterilization, the abiotic factors—non-living such as pH, metal contamination, temperature, etc., will strongly influence the enzyme activity of the soil. Furthermore, the

death of all the microbes in the native contaminated soil also means that the only living organism will be from the inoculum—making the microbes of the inoculating soil the second factor that influences phosphatase activity.

The effects of the abiotic factors—more specifically the heavy metal contamination—on the phosphatase activity can be seen in Figure 21. Recall from Chapter 2 (*see Figure 5 and 6, Chapter 2, Section 2.3.1.1 and 2.3.1.2*) that the concentration of heavy metals was lower at LSP 43 than at LSP 146. Thus we expected that inoculating LSP 43 would yield higher enzyme activity than inoculating LSP 146, regardless of the source of the microbes. The data in Figure 21 below supports our hypothesis; it shows that inoculated, sterilized LSP 43 soil (light gray pair) generally exhibited higher activity than inoculated, sterilized LSP 146 soil (dark gray pair).

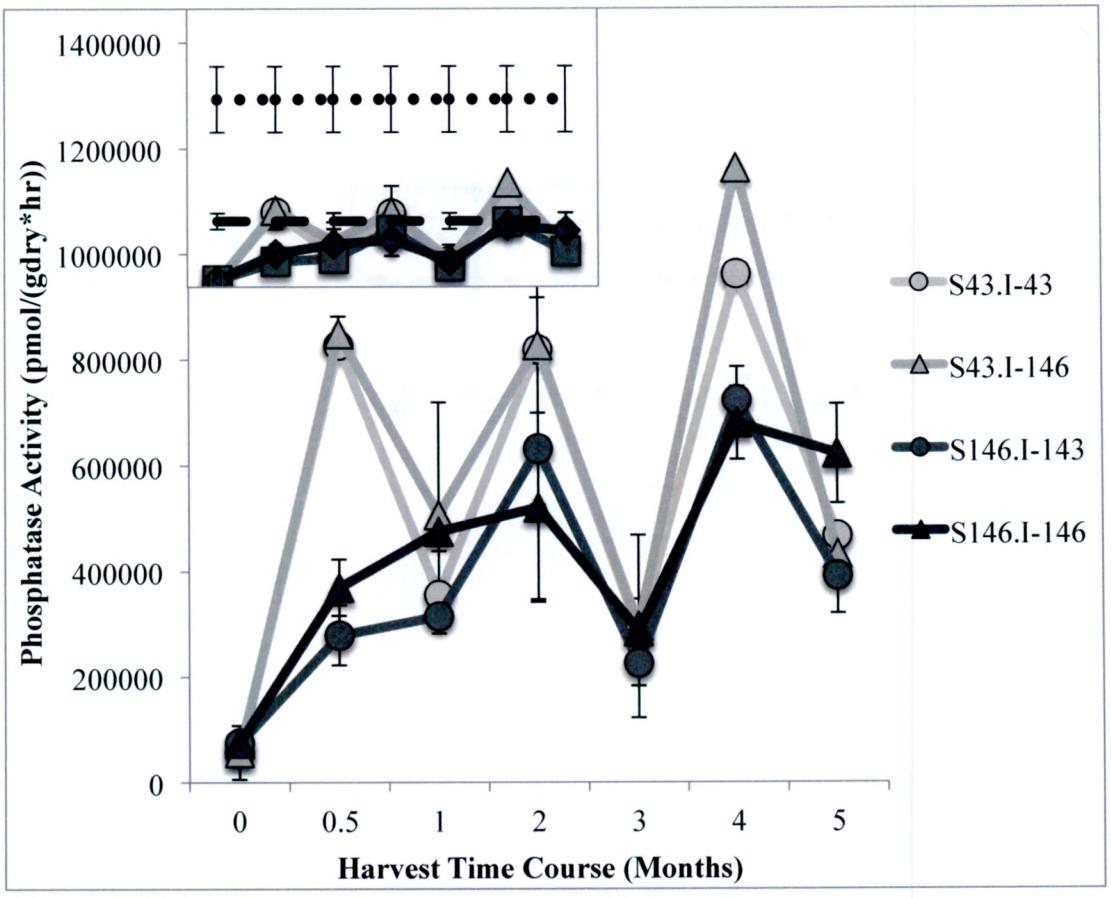


Figure 21. Average Phosphatase activity of Sterilized and Inoculated Soil without Root Exudates Over Time.

This shows the phosphatase activity—average and standard error of the 3 pots—for only the sterilized and inoculated conditions at each of the harvest date. The light gray bars are inoculated with 43 (I43) and the dark gray bars with 146 (I146). The inset shows the exact same graph with additional horizontal lines. The horizontal lines are the phosphatase activity for LSP 43 (dashed) and LSP 146 (dotted) from Chapter 2 (*See Figure 8, Chapter 2, Section 2.3.2*), measured separately from this cross-inoculation study. Activities of these samples (horizontal lines), which were measured once, less than a week after collection, are the most accurate representation of *in situ* enzyme activity; thus, they act as a standard for comparison.

Furthermore, when compared to their respective standards, which represents the *in situ* phosphatase activities (Figure 21 inset), only sterilized LSP 43 (light gray) exhibits

activity that is comparable to the standard. At 0.5 months, 2 months, and 4 months sterilized 43 showed activity that was equal to its standard *in situ* activity (dashed line; however, sterilized LSP 146 soil, regardless of the source of inoculum, showed activity well below its standard *in situ* activity (dotted line) and even below the standard activity of LSP 43 (dashed line). Neither sterilized LSP 43 (light gray) nor LSP 146 (dark gray) exhibited activity that was near the activity of standard *in situ* LSP 146, the site with the highest amount of phosphatase activity.

This following graph (Figure 22) is the average over time of the phosphatase activity of sterilized soil conditions, and it shows a better depiction of the relationship between sterilized 43 and sterilized 146. Each bar is the average of 6 time points, excluding time 0. As stated earlier, the overall activity is higher when the sterilized soil is LSP 43 (compare adjacent bars). For example, inoculation of sterilized 43 with 43 microbes (I-43, white) is greater than inoculation of sterilized 146 with 43 microbes (I-43, white, striped). A possible argument is that inoculating a soil with its own microbes ensures higher activity than inoculating a soil with foreign microbes because the native microbes are already acclimated to their own abiotic environment. However, inoculation of sterilized 43 with 146 microbes (I-146, light gray) is still greater than inoculation of sterilized 146 with its own microbes (I-146, light gray, striped).

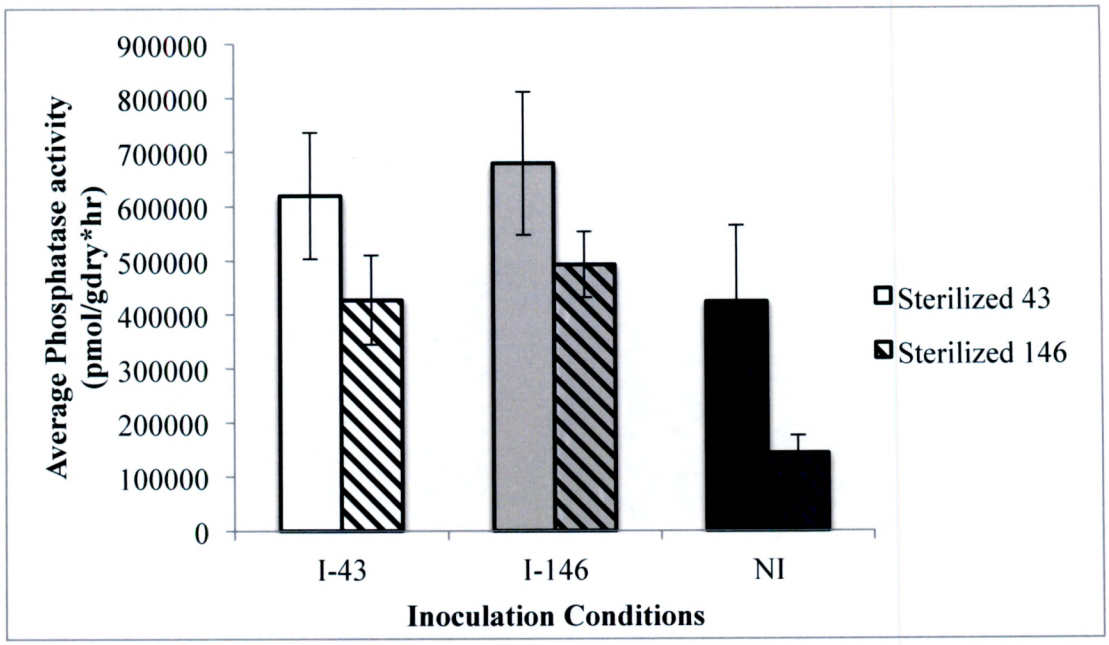


Figure 22. Average Over Time of Phosphatase activity for Sterilized Inoculation Conditions in Cross-Inoculation study without Root Exudates.

This graph shows the phosphatase activity averaged over time for only the sterilized inoculation condition, meaning that each bar represents the average and standard error of the six time points (excluding point zero). The sterilized LSP 43 soils are clear and the sterilized 146 are striped (represented by the legend). Similar colors match the x-axis labels, which shows the source of the inoculum: inoculation with 43 (white), inoculation with 146 (light gray) or no inoculum (dark gray).

Recall that the second factor that influences sterilized, inoculated soil is the microbial community of the inoculating soil. Inoculation of sterilized soil means that the inoculating microbes are the only living organisms in the soil, since the native microbes were killed during sterilization. Since LSP 146 exhibits phosphatase activity that is approximately twice the activity at LSP 43, the expectation for this experiment is that inoculating sterilized soil with LSP 146 inoculum would be significantly higher than inoculating with LSP 43 inoculum. However, Figure 23 shows that there is no significant difference in phosphatase activity between the different inoculum (compare adjacent

bars). In sterilized 43 (left) the activity of inoculating with 43 (I-43, white) is within error of inoculating with LSP 146 microbes (I-146, light gray). Similarly, in Sterilized 146 (right) the activity of inoculating with LSP 43 microbes (I-43, white) is similar to the phosphatase activity of samples inoculated with LSP 146 microbes (I-146, light gray).

Moreover, sterilized soils with no inoculum (dark gray) should show minimal activity because all living microorganisms were theoretically killed during sterilization and no additional microbes, native or foreign was added. For sterilized 146 soils (right, dark gray), there is relatively minimal activity, as expected. However, for sterilized 43 soil samples (left, dark gray), the activity is unexpectedly high; it is even more than half the activity of inoculated soil conditions, suggesting possible contamination in the sterilized LSP 43 soil.

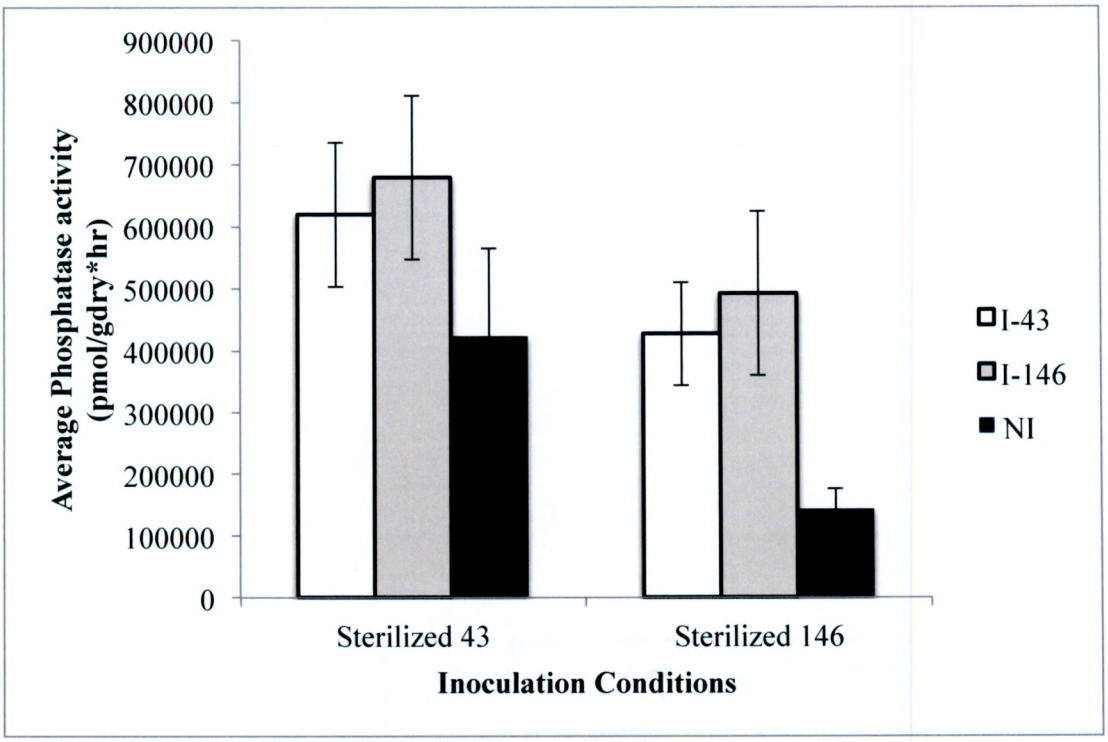


Figure 23. Average Over Time of Phosphatase activity for Sterilized Inoculation Conditions in study without Root Exudates.

This graph is phosphatase activity for only the sterilized inoculation condition with each bar representing the average and standard error of the six time points (excluding point zero). The x-axis is the specific sterilized soil. Each bar represents the inoculating soil, with similar colored bars representing the same: inoculation with 43 (white), inoculation with 146 (light gray) or no inoculum (dark gray).

Thus far, the direction of enzyme activity over time—increase or decrease—has not been addressed. The following figure (Figure 24) shows the phosphatase activity at each time point for all the sterilized conditions. Each set of seven bars in the graph show the activity for each condition in consecutive time points. Even though the activity at sterilized 146 (right 3 sets) is generally lower than activity at sterilized 43 (left 3 sets), there is a time-dependent pattern at sterile 146 that is not apparent at sterilized 43. All three sets of bars at sterile 146 (white, light gray and dark gray) show an overall gradual

increase in activity. Within each set of bars for sterilized 146, for example the light gray bars, there is an increasing trend from left to right. However, at sterile 43, there is no obvious increasing or decreasing pattern. Rather activity is inconsistent, going up-down-up-down, with each consecutive time point.

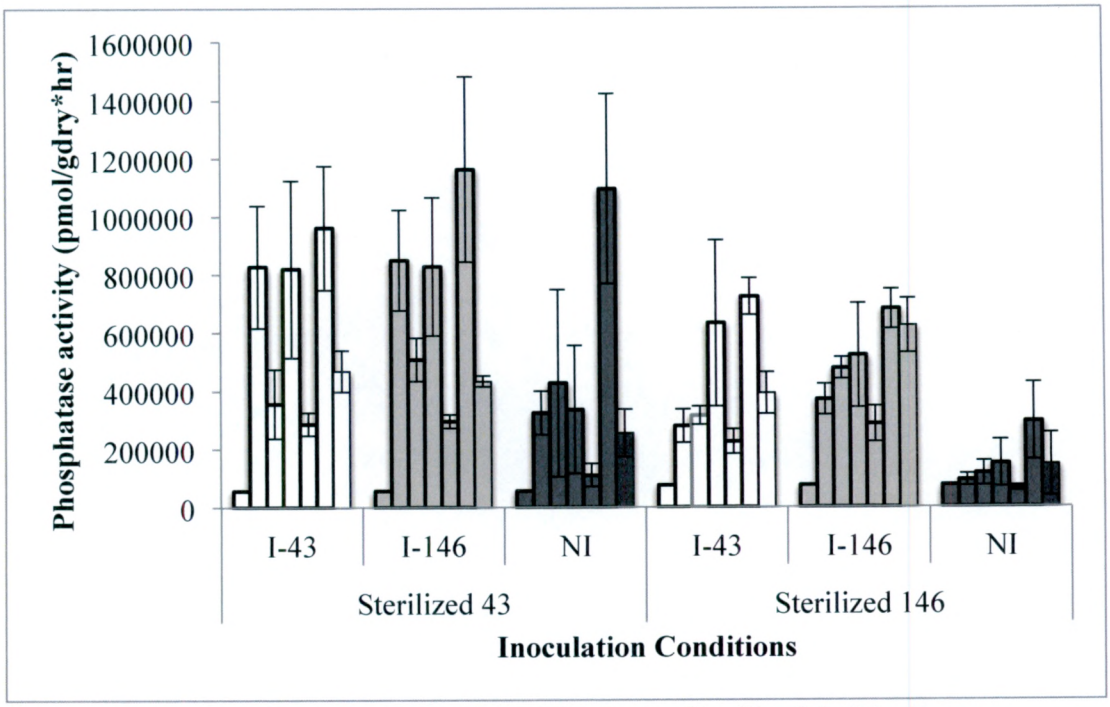


Figure 24. Average Phosphatase activity of only the Sterilized Soil in Cross-Inoculation Study without Root Exudates.

This shows the phosphatase activity—average and standard error of the 3 pots—for only the sterilized conditions at each of the harvest date. Each set of seven bars from left to right is: 0, 0.5, 1, 2, 3, 4 and 5 months. The primary label on the x-axis is the sterilized (S) parent soil. The secondary labels identify the inoculating soil (I43, I146) or non-inoculum (NI). Similar colored bars have the same secondary labels (white—I43, light gray—I146, dark gray—NI).

Contrary to the sterilized soil samples previously shown, in which sterilized 146 soil showed lower phosphatase activity than sterilized LSP 43, Figure 25 shows that

among the non-sterilized samples, non-sterilized 146 (dark gray) showed higher enzyme activity than LSP 43 throughout the experimental period. This follows standard *in situ* pattern observed between these two LSP sites, in which LSP 146 (dotted line) is higher than that of LSP 43 (dashed line).

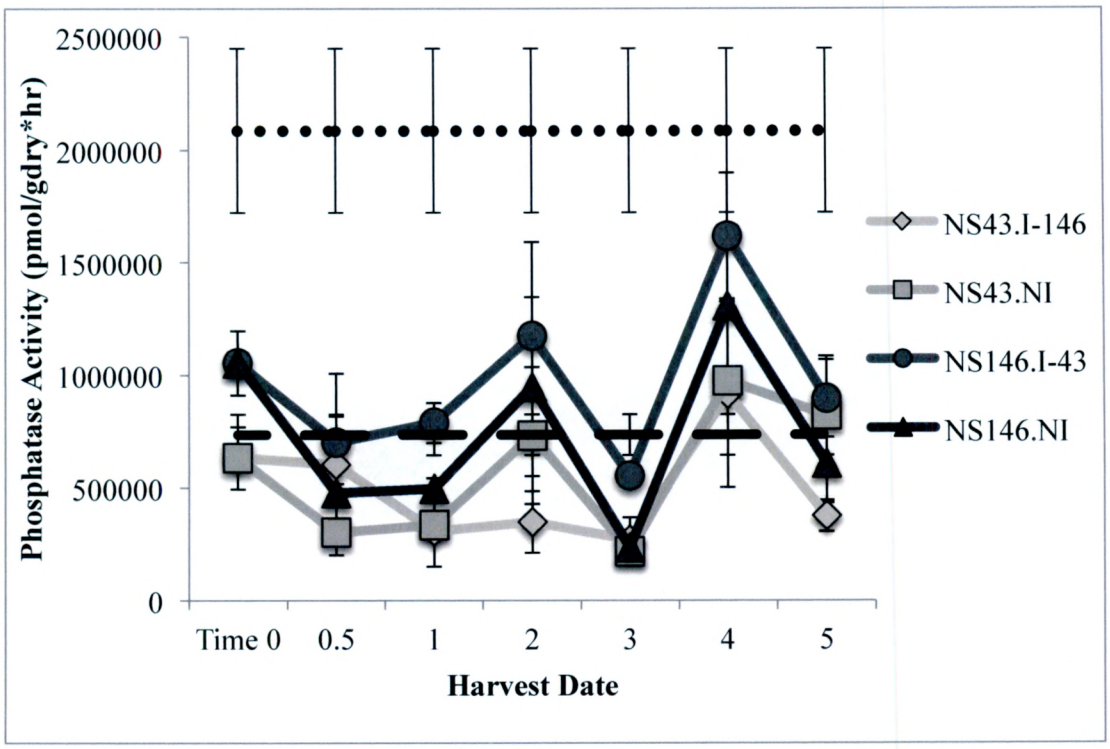


Figure 25. Average Phosphatase Activity of Non-Sterilized Soil Over Time in Cross-Inoculation Study without Root Exudates.

This shows the phosphatase activity—average and standard error of the 3 pots—for only the non-sterilized conditions at each of the harvest date. The light gray lines represent the non-sterilized LSP 43 soil and the dark gray lines, the non-sterilized LSP 146 soil. The horizontal lines are the phosphatase activity for LSP 43 (dashed) and LSP 146 (dotted) from Chapter 2 (*See Figure 8, Chapter 2, Section 2.3.2*), measured separately from this cross-inoculation study. Measured once, less than a week after collection, they (horizontal lines) are the most accurate representation of *in situ* enzyme activity; thus, they act as a standard for comparison. The horizontal lines are the average phosphatase activity measured for LSP 43 (dashed) and 146 (dotted) without any inoculation.

The non-sterilized samples were used to observe the enzyme activity when microbes from one LSP site were used to inoculate another LSP site. This is analogous to bioaugmentation, a bioremediation strategy in which an inoculum of microbes with metal-resistant capabilities is introduced to a contaminated site. Since LSP 43 and LSP 146 are both contaminated, this cross inoculation study was designed to observe how LSP 43 microbes would affect the enzyme activity of contaminated, non-sterilized LSP 146, and vice-versa. Note that non-sterilized soil samples with inoculum from another site are referred to as "augmented soil".

Figure 26 shows that the augmented soil (white and dark gray bar), non sterilized samples induced with foreign microbes from the other site, exhibits the same pattern as the standard *in situ* soils (striped) of LSP. This means that augmented LSP 43 (white) is lower in phosphatase activity than augmented LSP 146 (dark gray). LSP 146 usually shows higher activity than LSP 43; thus we suspected that augmented LSP 43—non-sterilized LSP 43 with the addition of microbes from LSP 146—would show higher enzyme activity than non-sterilized LSP 43 with no inoculum. However, Figure 26 shows that inoculating LSP 43 with LSP 146 microbes (augmented, white) exhibits comparable activity to LSP 43 without inoculum (NS43.NI, light gray). This suggests that the microbes of the inoculating soil (LSP 146) may not have been able to establish themselves in their new environment (non sterilized LSP 43). Recall that the two main concerns accompanying bioaugmentation is uncertainty in whether the donated microbes will be able to establish themselves or if they will become dominant and kill their host's indigenous microbes.

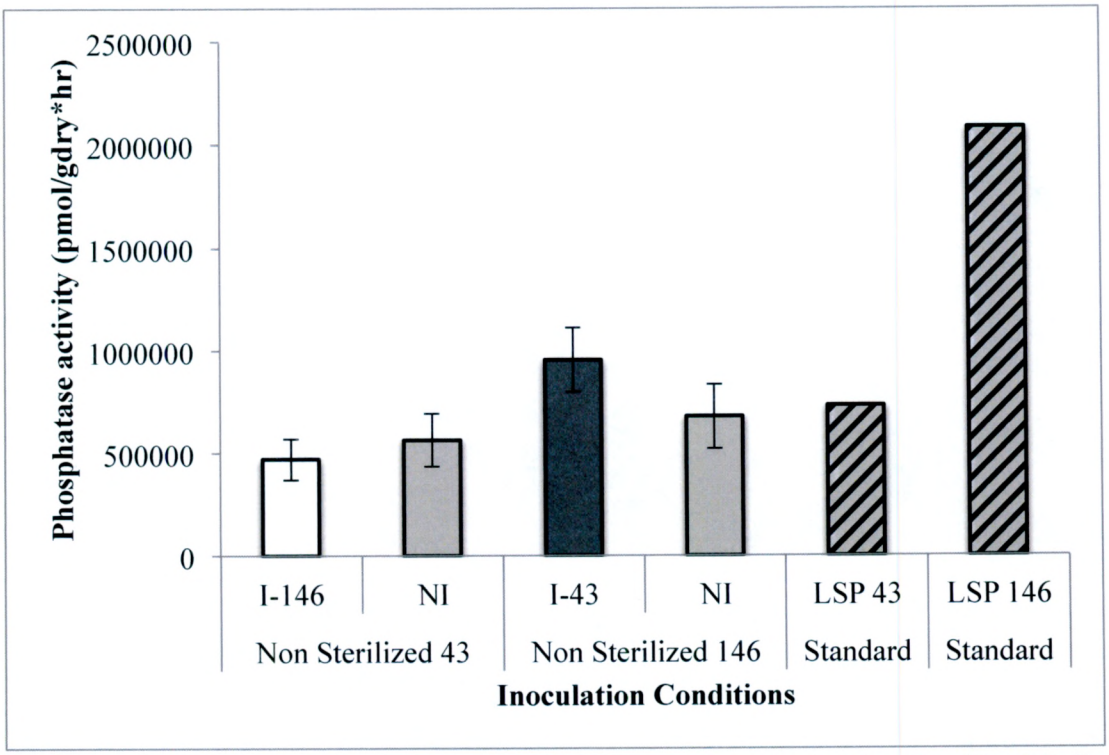


Figure 26. Average over time of the Phosphatase activity of only Non-Sterilized Soil in Cross Inoculation Study without Root Exudates.

This shows the phosphatase activity for only the non-sterilized conditions with each bar representing the average and standard error of the six time points (excluding point zero). The inoculation conditions are on the x-axis with similar inoculating soils sharing a color—I146 is white, I43 is dark gray and NI is light gray. The striped bars are the phosphatase activity for LSP 43 and LSP 146 from Chapter 2 (*See Figure 8, Chapter 2, Section 2.3.2*), measured separately from this cross-inoculation study. Measured once, less than a week after collection, they (striped bars) are the most accurate representation of *in situ* enzyme activity; thus, they act as a standard for comparison. The horizontal lines are the average phosphatase activity measured for LSP 43 (dashed) and 146 (dotted) without any inoculation.

Figure 26 also shows the relationship between augmented LSP 146 (dark gray)—non-sterilized LSP 146 soil with microbes from LSP 43—and its non-inoculated counterpart (NS146.NI). The standard *in* situ phosphatase activity of LSP 43 is lower

than that of LSP 146 (striped bars); therefore we hypothesized that augmenting LSP 146 by adding LSP 43 microbes to non-sterilized LSP 146 soil will not have any effect on the enzyme activity of LSP 146. However, Figure 26 showed that augmented LSP 146 (dark gray) actually has higher phosphatase activity than its non-inoculated counterpart, throughout the experimental period. While this implies that the addition of LSP 43 microbes may have improved the phosphatase activity of LSP 146, there's no straightforward method for attributing this behavior to either the native microbes of non-sterilized 146 or the microbes of the inoculum from LSP 43.

3.4.4. Conclusion

Recall from Chapter 2 that LSP 146 and LSP 43 have very high heavy metal concentration, and, despite convention[8,20,39,49], they also have high enzyme activity (*see Figure 9 and 10, Section 2.4*). In this section, phosphatase activity was used to observe the effects on phosphatase enzyme activity of cross inoculating soil samples from these two sites. Cross inoculation in this experiment meant adding microbes from one LSP site, in the form of an inoculum, to a soil sample from a different LSP site. Both sterilized and non-sterilized samples from LSP 43 were inoculated with microbes from LSP 146; similarly, sterilized and non-sterilized samples from LSP 146 were inoculated with microbes from LSP 43. Combined with the controls, there were a total of 10 soil conditions, prepared in replicates of three for each of the 6 harvest time points. Samples were harvested and analyzed at six time points (0.5, 1, 2, 3, 4, and 5 months) over the course of five months.

The first objective was to understand any relationships between the abiotic factors—non-living such as pH, metal contamination, temperature—of contaminated soil and the

enzyme activity of microbes from a different soil. To accomplish this, microbes from one LSP site (the inoculum) was used to inoculate sterilized soil samples from another LSP site. Sterilization kills a majority of the organisms in a soil, including the microbes; resulting in soil that is still contaminated with heavy metals, but now without enzymes of its own. Thus we expect that when microbes from another site is added to the sterilized soil, the phosphatase activity will be affected by the level of heavy metal contamination at the sterilized site—abiotic factor—as well as the level of enzyme activity of the microbes from the inoculating soil.

Recall from Chapter 2 that both the standard *in situ* phosphatase activity and the level of heavy metal contamination are greater at LSP 146 than at LSP 43 (*see Figure 9 and 10, Chapter 2, Section 2.4*). Therefore, in this cross inoculation study, we hypothesized that inoculating any sterilized soil with LSP 146 microbes would show higher enzyme activity than inoculating with microbes from LSP 43. However, Figure 21 (*Section 3.4.3.2)* showed that the origin of microbes of the inoculating soil does not significantly affect the phosphatase activity. When introduced to sterile soil, inoculation with 43 shows very similar phosphatase activity to inoculation with 146, contrary to our hypothesis. Furthermore, *sterilized LSP* 43 exhibits higher activity than sterilized LSP 146, regardless of the source of the inoculating soil. This suggests that sterilized LSP 43 is an easier environment for microbes to establish themselves than sterilized LSP 146, since the metal concentration—an influencing abiotic factor of sterilized soil—is lower at LSP 43 than at LSP 146 (*see Figure 21, Section 3.4.3.2*).

The other objective of this part of the chapter was to observe the response of each LSP site (43 or 146) when exposed to microbes (inoculum) from the other. This is a

replication of bioaugmentation, in which the metal-resistant microbial community from one contaminated site is injected as an inoculum into another contaminated site. Since the soil was not sterilized, the phosphatase activity is influenced by two microbial communities—the native microbes from the contaminated soil, and the foreign microbes from the inoculating soil. As discussed earlier in the chapter, the two main concerns accompanying bioaugmentation is uncertainty in whether the inoculating microbes will be able to establish themselves, or if they will dominate and possibly kill the native microbes of host soil [18].

To investigate this objective, the enzyme activity was measured at different time points for non-sterilized samples that were injected with inoculum containing microbes from a different soil sample—augmented soil samples; these were compared to non-sterilized samples of the same soil without the addition of any inoculum. Since the standard *in situ* enzyme activity is higher at LSP 146 than at LSP 43 (*see Figure 8, Chapter 2, Section 2.3.2*), we hypothesized that the addition of LSP 146 microbes to non-sterilized LSP 43 will increase the enzyme activity of non-sterilized LSP 43; But that adding microbes from LSP 43 to non sterilized LSP 146 will have no effect on the phosphatase activity of LSP 146, and any effect will be a reduction in activity.

Contrary to our hypothesis, inoculating LSP 43 with LSP 146 microbes exhibits comparable activity to the sample of LSP 43 without inoculum (*see Figure 26, Section 3.4.3.2*). This suggests that the microbes of the inoculating soil (LSP 146) may not have been able to establish themselves in their new environment (non sterilized LSP 43). Furthermore, our hypothesis that the addition of LSP 43 microbes will have no effect when added to non-sterilized LSP 146 also did not hold. Rather, LSP 146 soil that was

inoculated with LSP 43 actually had slightly higher phosphatase activity than non-inoculated LSP 146 when averaged throughout the experimental period (*see Figure 26, Section 3.4.3.2*). While this implies that the addition of LSP 43 microbes may have improved the phosphatase activity of LSP 146, there's no straightforward method for attributing this behavior to either the native microbes of non-sterilized 146 or the inoculating microbes from LSP 43.

Overall, the experiments of this part were helpful in understanding the effects that microbes have on the enzyme activity at LSP. The magnitude and range in moisture levels increased gradually over time, with a majority of soil samples ranging between 20 and 25 %. While the non-inoculated soil samples exhibited lower moisture levels than inoculated soil samples over time, there was no difference in moisture between the source of the inoculum—inoculation with 43 or inoculation with 146. As mentioned early, a big concern of bioaugmentation is the behavior of the foreign microbes in its new environment—whether they will be able to establish themselves without competition form the native microbes? Or whether they will dominate and kill the microbes native to the contaminated soil? It will be interesting to characterize the microbial species at LSP to determine what is unique to each individual site.

3.5. Chapter 3 Summary

Recall from Chapter 2 (*see Figure 9 and 10, Chapter 2, Section 2.4*) that LSP has high levels of heavy metal concentration, and despite the findings of most published research[20, 39, 49, 8], high levels of phosphatase activity. This suggests that the microbes at LSP have undergone genetic changes that have allowed them to adapt to their

contaminated environment in order to produce enzymes. These resistive microbes are at the forefront of bioremediation, a more sustainable and affordable method for cleaning up contaminated sites. Bioremediation applies living organisms, such as a soil's microbial community, to degrade, detoxify or stabilize hazardous materials in the environment. The purpose of this entire chapter was to understand whether the microbes at LSP are really the drivers of LSP's surprising enzyme activity; and whether LSP can potentially become a source for resistive microbes used in the bioremediation of other contaminated sites.

A cross inoculation study, in which microbes from one LSP site are added to another LSP site, and vice versa, was conducted to understand two types of bioremediation strategies—biostimulation and bioaugmentation. Biostimulation, observed in Part 1 ("The Cross-Inoculation and Biostimulation of Microbes at LSP with Root Exudates") is the addition of root exudates, a mixture of complex organic compounds made up of oxygen, carbon, phosphorous and nitrogen, to excite or 'stimulate' the native microbes of the contaminated soil [18]. Part 2—"The Cross-Inoculation and Bioaugmentation of Microbes at LSP without Root Exudates"—observes another bioremediation strategy, bioaugmentation, in which a solution of metal resistive microbes, also known as an inoculum, is injected into the contaminated soil.

Only two sites at LSP—LSP 43 and LSP 146 are observed. Recall that LSP 146 has a higher heavy metal concentration and enzyme activity than LSP 43 (*see Figure 9 and 10, Chapter 2, Section 2.4*). For both Part 1 (*Section 3.3*) and Part 2 (*Section 3.4*), inoculation with LSP 146 showed greater activity than inoculation with LSP 43. This supported our hypothesis that the microbes of the inoculating soil had a strong influence on the enzyme activity. Furthermore, phosphatase activity was generally higher for the sterilized 43 soil

than for the sterilized 146 soil, regardless of the inoculum, in both Part 1 (*Section 3.3*) and Part 2 (*Section 3.4*). This suggests that the abiotic factors of LSP 146 do create a harsher environment than that of LSP 43, which makes it harder for microbes to establish themselves.

Furthermore, the results from both objectives collectively suggest that both sites at LSP have potential for use in the bioremediation of contaminated sites. Part 1 (*Section 3.3*) shows that the enzyme activity of microbes at both LSP 43 and LSP 146 can be stimulated because there was an increase in enzyme activity over time when root exudates where added to non-sterilized soil samples. In both parts of the chapter, whether root exudates were used or not, there was an increase in enzyme activity over time when LSP microbes were added to a sterilized site (*see Figure 17, section 3.3.3.2 and Figure 21, Section 3.4.3.2*). Since sterilized soil has been striped of its enzymes, both parts of this chapter suggest that LSP microbes can be used to improve the enzyme activity of contaminated soil that has very minimal enzyme activity.

However, part 2 further suggests that while LSP microbes can be used to improve the enzyme activity of soil with minimal enzyme activity (emulated by sterilizing the soil), it may not be effective in soil that already has relatively good enzyme activity (non-sterilized LSP soil). When LSP 146, which has significantly greater phosphatase activity than LSP 43, was added to the latter, the dramatic increase in phosphatase activity that was expected was not observed. This suggested that LSP 146 microbes might not have been able to establish itself among the native microbes of LSP 43, one of the main concerns of bioaugmentation.

In Part 1 (*Section 3.3*), where root exudates were used, none of the inoculation conditions allowed us to observe the competition between LSP 146 and LSP 43 microbes—observed in part 2, without root exudates—such that the microbes from one site is added to non sterilized soil of another site. It would be interesting to see if a source of additional nutrients, by the addition of root exudates, could have affected how well the new microbes would establish themselves in their new environment. Overall, both parts of this chapter provided profound data on the effect that LSP's microbial community has on their enzyme activity and strongly suggests that LSP's microbial community could be useful in the field of bioremediation.

4. Chapter 4: The Effect of Storage Conditions on Soil Enzyme Activity

4.1. Introduction

Throughout this thesis, enzyme activity measurements have been used to increase understanding of the microbes at LSP. Recall from both Chapter 1 and 2 that soil enzymes are great indicators of soil fertility and microbial function[5,9,10,30]. Measurement immediately after soil collection is the most accurate and preferred time to characterize the soil using this method. However, due to a variety of factors, including experimental limitations or magnitude of samples, this practice is sometimes impossible. Thus, in order to minimize inaccuracies in enzyme activity analysis, it is important to understand the best requirements for storage and time for the collected soil samples.[10,27,60,61,62] The purpose of this experiment was to understand how different storage temperatures affected the extracellular enzyme activity of LSP's soil biota over time.

A variety of studies that analyzed the effects of storage temperature measured the enzyme activity of various soil samples after just one month. Lee et. al.[62] reported that enzyme activity—β-glucosidase and acid phosphatase—was unaltered by storage at 4 °C and -20 °C after 28 days; however, at 80 °C cellulase activity increased by 20 % or 50 %, depending on the soil type. Similarly, Deforest[10] also reported that 4 °C or -20 °C storage had very minimal influence on the β-xylosidase, NAGase, phosphatase, and phenol oxidase enzyme activities, and that the direction of change was not consistent over the 21 day experimental period. More specifically, a 28-days study by Peoples and Koide[60], which also reported minimal effect on enzyme activity, stated that the maximum change was a 22 % decrease for a sample in -20 °C storage temperature after the first 14 days. In general, 4 °C and -20 °C were continuously reported as the ideal temperature to

store soil samples if enzyme analysis cannot be measured immediately after soil collection; and, there were no well-defined patterns or relationships between individual storage temperatures and the direction of change in enzyme activity.[60,10,61,62]

The objectives explored in this chapter were to determine:

1) The effect of storage time on the enzyme activity of soil samples from LSP at four different temperatures and,
2) How each of four unique storage temperatures influences the enzyme activity of soil samples from LSP.

In this experiment, both the conditions for storage time and temperature were broadened to include parameters rarely found in current literature. Samples were analyzed at sporadic time points over seven months, rather than the month-long observational period found in current literature[10,60,61,62]. In addition to the two temperatures (4 °C or -20 °C) more commonly reported, 22 °C and -80 °C were included. All together, they represented room (22 °C), fridge (4 °C), freezer (-20 °C) and deep freezer (-80 °C) temperature conditions. This chapter details the study of how different storage temperatures affect LSP's enzymatic activity over time.

4.2. Protocol

4.2.1. Study Sites

Only LSP 146 was observed in this study. *Please refer to Chapter 1, Section 1.5 for information regarding LSP.*

4.2.2. Soil Collection

Soil collection was completed on June 4, 2015 from LSP 146, the only site used in this study. Soil samples were the top 1-5 cm of a core; they were taken from only points

1, 3 and 5 of all three transects (*See "Layout of the Soil Site" Figure 4, Chapter 2, Section 2.2.2*), resulting in a total of 9 soil samples. Each soil sample was sieved separately through a 2 mm sieve. Then equivalent amounts from each of the three pins were amassed into one representative sample for each transect, resulting in three replicate samples (A, B, C). Then, each replicate was further divided into four equivalent samples and placed in the designated storage location (room temperature, fridge, freezer or deep freezer). Thus, there were three replicates, A, B and C, analyzed for each storage condition, for a total of 12 samples. Note that the initial enzyme activity was measured for each replicate before separating them into their storage location.

4.2.3. Experimental Design

The phosphate activity was measured for soil samples from LSP 146. Activity was measured sporadically over a seven-month period (Table 6) to determine the influence of time on the enzyme activity. Subsets of samples were stored at room (22 °C), fridge (4 °C), freezer (-20 °C) and deep freezer (-80 °C) temperature conditions to understand the effects of storage temperature. Approximately 3 sets of zip-lock bags (3 replicates), each containing 15 grams of soil, were stored in their designated temperature- location. On June 8, 2015, four days after soil collection, the initial activity was measured. This measurement (*initial*) is assumed to be the best measure of *in situ* enzyme activity and is used as a benchmark during analysis. The next measured activity, following the initial activity, was after 3 weeks, followed by weeks 4, 6, 7 and 8. The final enzyme activity assay was not measured until about four months later, during week 26.

Table 6: Experimental Chronology for The Effect of Storage Conditions on Enzyme Activity

Date	Activity	Approximate Time Course
6/04/2015	Soil collection	N/A
6/08/2015	Initial Activity measurement Storage allocation of soil samples	Week 0
6/30/2015	Activity measurement	Week 3
7/09/2015	Activity measurement	Week 4
7/21/2015	Activity measurement	Weeks 6
7/29/2015	Activity measurement	Week 7
8/04/2015	Activity measurement	Week 8
12/9/2015	Activity measurement	Week 26

4.2.4. Phosphatase Activity Assay

Phosphatase activity was measured for all three transects and all the storage conditions. *Please refer to Chapter 2, Section 2.2.3 for the procedure.*

4.3. Results

In this chapter, the relationships between storage temperature, time and enzyme activity were observed. Soil samples from LSP 146 were stored in four distinct temperature conditions: room (22 °C), fridge (4 °C), freezer (-20 °C) and deep freezer (-80 °C). Enzyme activity was initially measured four days following soil collection. (*See the experimental chronology in Table 6, Section 4.2.3).* During the first eight weeks following the initial measurement—beginning with week three—soil from each storage temperature was frequently measured (weeks 3,4,6,7,8). Activity was measured once more 26 weeks after the initial measurement. The alkaline phosphatase activity was

measured to observe the P nutrient cycling as affected by different storage temperatures (22 °C, 4 °C, -20 °C and -80 °C) over time.

Figure 27 shows that there is no definitive relationship between the storage time and the enzyme activity. Generally, activity decreased after the first four weeks; however, weeks 6 and 7 saw a slight overall increase in activity. Week 8 follows with a decrease, but by week 26, there was a slight increase in activity. Phosphatase Activity was relatively consistent over time for all temperature conditions with most activities equal to or below the benchmark.

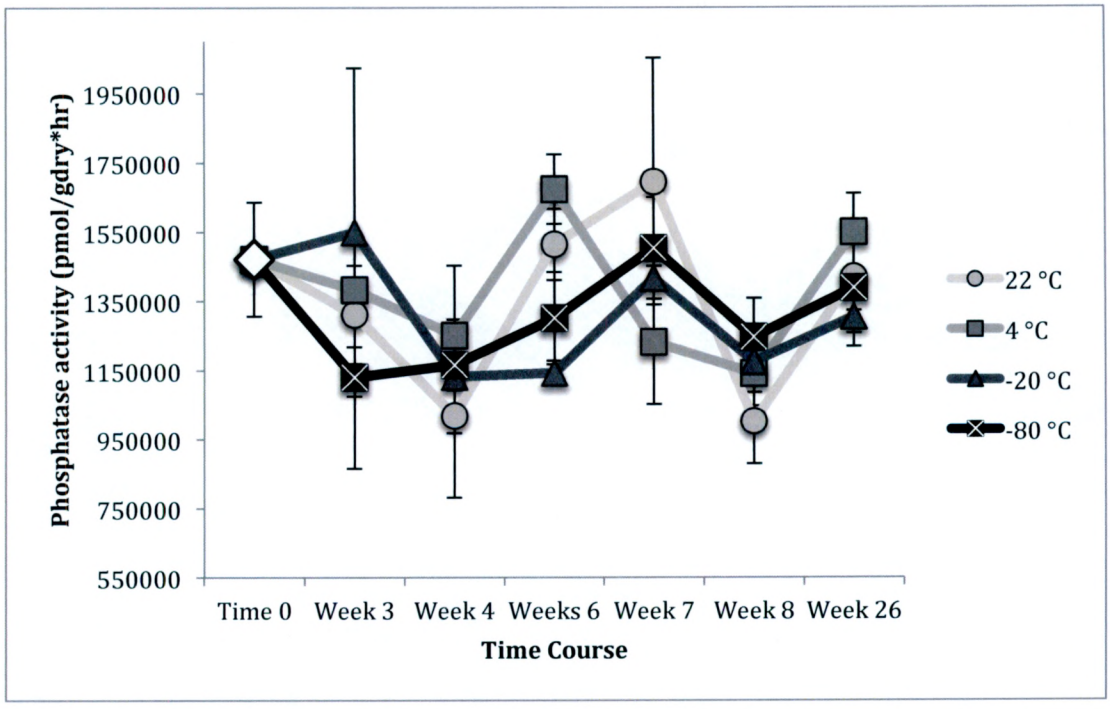


Figure 27. Time Course of the Average Phosphatase Activity at LSP 146 for All Temperature Conditions.

This shows the average phosphatase activity (n = 3) for all storage temperature conditions at each of the time points. Time 0 (white diamond) is the initial measurement taken four days after soil collection and represents the benchmark for activity.

A closer examination of Figure 27 shows that the highest activity above the benchmark activity occurred during week 6 by soil stored in 22 °C. On the contrary, the lowest activity below the benchmark occurred two weeks earlier in week 4, also by soil stored in 22 °C. The greatest range in activity is found in weeks 3 and 7 and the lowest in weeks 8 and 26. The phosphatase activity measured in both weeks 6 and 26 were centered, with minimal error, on the benchmark activity. There is no obvious relationship or consecutive pattern between the phosphatase activity and the time of storage for soil at LSP 146.

A bar graph of percent change (Figure 28) compares the initial benchmark activity to the activity measured for each temperature condition throughout the entire experiment. The overall deviation in phosphatase activity by soil stored at any of the storage temperatures, from the benchmark activity, was less than 50 %. Both the upper and lower maximum deviations from the benchmark activity occurred in soil stored at 22 °C, as was mentioned previously. The maximum increase and decrease was approximately 15 % and 20 % from the benchmark activity, respectively.

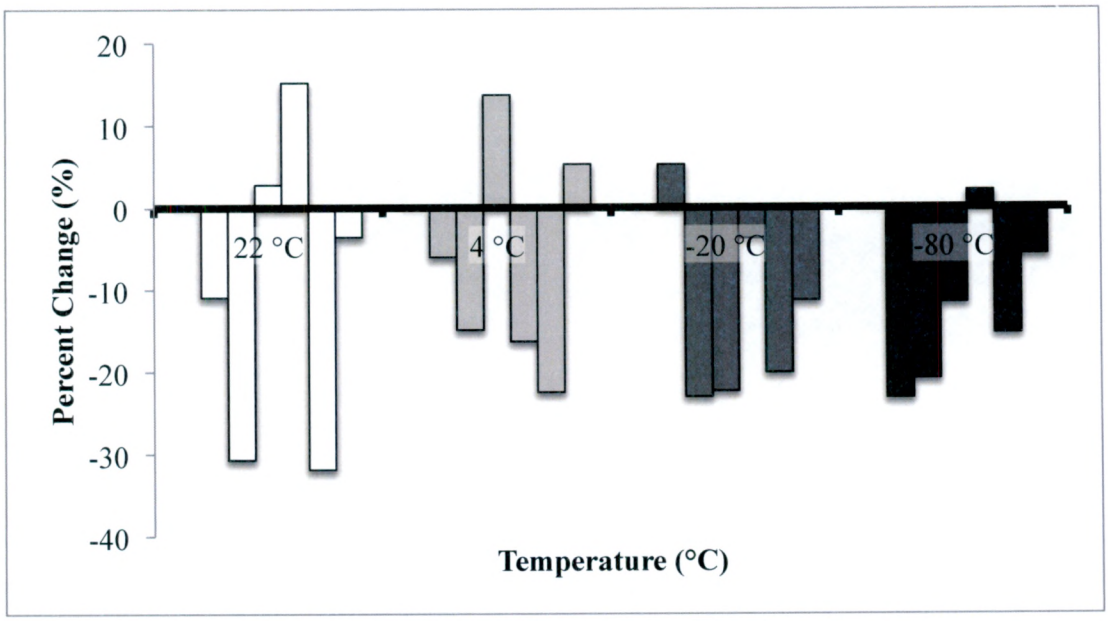


Figure 28. Percent Change in Average Phosphatase Activity of LSP 146 by Storage Temperature.

This shows the percent change among the average phosphatase activities *(values in Figure 27),* classified by different storage temperatures. Percent Change was calculated using formula [(X-Y)/Y] where Y is the initial benchmark value and X is any other activity measurement. The initial value is designated 0 % (black solid line) and the change in activity for each time point is the negative or positive bars. There are four sets of bars, each designating a storage temperature (22 °C *white*, 4 °C *light gray*, -20 °C *dark gray* and -80 °C *black*); and 6 bars within each set, consecutively representing the time of measurement (L to R: weeks 3,4,6,7,8, and 26).

Figure 28 also shows a weak directional pattern in the behavior of soils at each storage condition. For Both 22 °C (white) and 4 °C (light gray), the activity of soil decreases with time, although much more rapidly for soils stored in 22 °C. For these storage conditions, activity is closest to the benchmark during the first few weeks then increasingly deviates. However, for soils stored in – 20 °C and – 80 °C, activity is farthest from the benchmark during the first few weeks, but gets closer with time.

Converting the bars of Figure 28 into an area graph, shown in Figure 29, clearly shows that deviation from the benchmark decreases with decreasing temperature. The range of percent change decreases from approximately 45 % for soil stored at 22 °C to about 30 % for soil stored at –80 °C. The average activity of soils stored at negative temperatures was approximately 15 % below the benchmark with a range of about 30 %. Soils stored in above zero temperatures had an average activity that was within a 5 % decrease of the benchmark activity; but, their range of activity was slightly higher—35 % (4 °C) and 45 % (22 °°) approximate deviations from the benchmark. Even though the changes in activity caused by storage temperature were below 50 %, there were still observable changes in the effect on activity among each temperature condition.

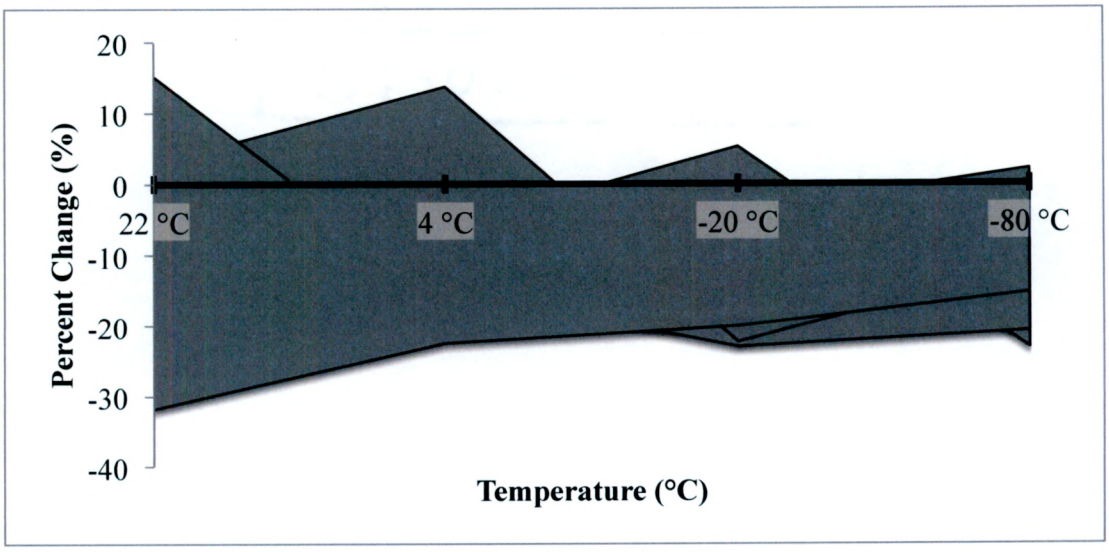


Figure 29: Phosphatase Activity Area Covered by LSP 146 for Each Storage Condition.

This graph is Figure 28 in an 'area graph' style to show the range of change in phosphatase activity found for soil samples stored at different temperatures. The purpose of the graph is to show how far activity deviates from the benchmark activity within each temperature condition so there is no special distinction for time.

4.4. Discussion

Recall from Chapter 1 and 2 that LSP 146 is unique because, despite its very high concentrations of heavy metals, it exhibits relatively high enzyme activity. In this chapter, enzyme activity assays (phosphatase) were measured to observe any relationships between storage time and temperature for LSP 146 soil. There are few publications[60,61,15,10] that observed the effect of storage conditions on microbial qualities. However, this study is unique because it extends beyond the usual 1-month time course, it observes four different temperature conditions and the site analyzed is a heavy metal contaminated site with unique properties. Subsets of soil samples from LSP 146 were stored in four different temperatures representing room temperature (22 °C), fridge (4 °C), freezer (-20 °C) and deep freezer (-80 °C). Phosphatase activity was measured sporadically over seven months (initial, weeks 3, 4, 6, 7, 8 and 26) for samples stored in each storage temperature.

Phosphatase Activity was relatively consistent over time for all temperature conditions with most activities equal to or below the benchmark. Similar to the study conducted by DeForest[10], there was no consistency in the direction of change for the activity of soil samples. Rather than a pattern of consecutive increase or decrease from the benchmark value over time, Figure 27 (*Section 4.3)* showed an up-and-down pattern above and below the benchmark activity as time progressed.

Analogous to the different studies mentioned in the introduction[60,61,62], this experiment confirmed minimal variation in the overall effect of storage temperature on the enzyme activity. The maximum deviation from the benchmark activity was an approximate decrease of 20 % (Figure 28, Section 4.3). While 4 °C and -20 °C were

reported as having minimal effect on the enzyme activity over time[60,61,62], this experiment showed that the lowest deviation from the benchmark activity was when soils were stored in -20 °C and -80 °C (Figure 29, Section 4.3). While the range of deviation was about 30 % for soil stored at these negative temperatures, it increased to 35 % for soil stored at 4 °C and 45 % for 22 °C.

Even though the overall range of deviation from the benchmark was lower for below zero temperatures than for above zero temperatures, above zero temperatures—especially 4 °C —are more reliable during the initial weeks of storage. Lee et. al.[62] reported that storage at 4 °C protects enzymes from the denaturation and physical disruption that occurs during the freezing and thawing cycle[62]. Figure 29 showed a maximum 15 % decrease in activity for soil stored in 4 °C within the first 4 weeks, but more than 25 % decrease from the benchmark was observed for soil stored at both below zero temperatures. Overall results suggest a sensitivity of LSP 146 soil to storage temperature.

4.5. Conclusion

The purpose of this chapter was to analyze the effects of storage time and temperature on the enzyme activity of soil from LSP 146. Although minimal, the data shows that the phosphate activity was more affected by the storage temperature, than the time. The worst temperature to retain enzyme activity for LSP 146 soils is room temperature (22 °C) because during the course of the experiment, this showed the greatest deviation from the initial benchmark activity. Enzyme activity should me measured as soon as possible but if storage is inevitable, the preferred temperature is 4 °C (fridge) because it had a minimal range of deviation from the benchmark and its average activity

was within 5% of the benchmark activity. In an active experiment, it is advisable to store a subset of soil samples in -20 °C or -80 °C immediately after collection that can be analyzed months or years later because for these temperatures, deviation from the benchmark decreased with time. This is useful if experiments need to be repeated during the peer-reviewing process of publishing findings. To avoid the denaturing effects of freezing and thawing, this sample should be preserved for when it is needed. The findings from this experiment coincide with the storage methods applied to the entire project because soil samples were stored in the fridge and analyzed promptly. The experiment further highlights the uniqueness of LSP 146 because even after storage outside of their natural environment for extended periods.

5. Chapter 5: Thesis Summary

Overall, this research provided invaluable insight into the effects that the microbes and heavy metals at LSP, have on its extracellular enzyme activity. There were four chapters: Chapter 1 (*Thesis Introduction*) detailed the importance of this research and provided necessary background for the thesis; Chapter 2 (*Enzyme Activity and Metal Concentrations at LSP and HMF*) provided preliminary research that quantified the metal concentration and enzyme activity at LSP, in comparison to a reference site, Hutcheson Memorial Forest (Franklin Township, NJ), with no history of heavy metal contamination; Chapter 3 (*Extracellular Enzyme Activity at LSP during Bioremediation*) contained two parts that used the microbes at LSP to improve the enzyme activity of contaminated poor-functioning soil; and, Chapter 4 (*The Effect of Storage Conditions on Enzyme Activity*) was a physical characterization study that explored the optimum storage condition to minimize changes in enzyme activity over time. Each of the four chapters detailed in this thesis contributed to two main goals.

The first goal was to understand any relationships between heavy metals and extracellular enzyme activity. Thus the heavy metals at LSP were quantified. The data showed that LSP has very high concentrations of heavy metals and enzyme activity. Both the phosphatase activity and metal concentration were higher at two sites at LSP, than at a reference site, HMF, which has no history of heavy metal contamination. The second goal was to understand the effect of microbes on extracellular enzyme activity during bioremediation. Two types of bioremediation strategies were analyzed, bioaugmentation—the introduction of microbes to contaminated soil to improve enzyme activity, and biostimulation—the introduction of a nutrient source (root exudates) to

stimulate the microbial population. Data from both parts suggested that LSP's microbes could be used to increase enzyme activity of poor functioning soil, and that the success of this was dependent on both the living and non-living contributors of soil environments.

In conclusion, this research improved our understanding of heavy metal contaminated soil and provided some data that supports the use of microbial communities to bring back extracellular enzyme activity and function to contaminated soil environments.

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Appendices

Appendix A

A-1. Inoculation Protocol for Study With Root Exudates

(This procedure was provided by Jay Singh, with no edits by Eleanor Ojinnaka).

Soil was collected from two sites (site 14/16 and site 43) at Liberty State park. Site 14/16 being a high metal contaminated soil while metal contamination level at site 43 was low. Soils from both the site were autoclaved twice before potting them in pots of size 3 inch round by 2.5 inches deep. A total of 36 pots were set up. We used a factorial design to set up the experiment testing the following factors – Sterilized or unsterilized, Soil origin, inoculation, and presence/absence of artificial root exudates. 10% soil slurry was used as inoculum to inoculate test pots. The control samples were watered with the same volume of sterile deionized water. In addition to inoculum, test pots were treated with artificial root exudates having a C/N ratio of 20.5. Initially, the test pots were treated with 1 μg C/g of soil for 14 days to help microbial community grow in these pots. In the following weeks, the concentration of artificial root exudates was increased to 100 μg C/g of soil. The control groups were treated with equal volume of sterile deionized water. The pots were kept in a climate controlled chamber with a 16-hour photoperiod. The temperature at night was kept at 19°C and 26°C during day with a relative humidity of 65% and 300 μmol/ m^2/ s. Repetitive sampling were carried out at the end of 1^{st}, 2^{nd} and 4^{th} week, and soil samples were tested for phosphatase activity.

A-2. Composition of Synthetic Root Exudates Used in this Thesis

(This procedure was provided by Jay Singh, with no edits by Eleanor Ojinnaka)

The root exudates were prepared using sterile deionized water, 3 carbohydrates, 3 carboxylic acids and 3 amino acids. The C/N ratio was kept at 20.5 and contained 18.4 mM glucose, 18.4 mM fructose, 9.2 mM sucrose, 9.2 mM citric acid, 18.4 mM lactic acid, 13.8 mM succinic acid, 9.2 mM alanine, 9.2 mM serine and 5.5 mM glutamic acid. The stock solution was appropriately diluted to have a final carbon concentration of either 1 or 100 μg C/g of soil.

Appendix B

B-1. Inoculation Protocol for Study Without Root Exudates

(This procedure was provided by Jay Singh, with no edits by Eleanor Ojinnaka).

Soil from site 14/16 and 43 were used for the current experiment. For our experiment we tested three factors: soil sterilized or unsterilized, soil origin and time after inoculation. Soils were steam sterilized twice before potting them in pot size of 1.5 inches square by 2.25 inches deep. A total of 240 pots were set up. 25 grams of soil were potted in each pot. Following pot set up, the soils were inoculated with soil slurry from site 14/16 and site 43. A soil slurry of 10% w/v was used to inoculate the test pots. 2 ml of inoculum was used to inoculate the test pots. The test pots were homogenized and re-inoculated the next day with 2 ml of 10% soil slurry. The controls were treated with 2 ml of sterile phosphate buffer. The pots were kept in a climate controlled chamber with a temperature of 23°C and a relative humidity of 65%. All pots were treated with 2 ml of water from Tuesday through Thursday, whereas 4 ml of water was added on Monday and Friday. Soils were destructively sampled every month for 6 months. The soil samples were tested for phosphatase activity and bacterial number using Epifluorescence microscopy.